Casa abierta al tiempo

UNIVERSIDAD AUTÓNOMA METROPOLITANA

MAESTRÍA EN CIENCIAS AGROPECUARIAS

EVALUACIÓN DEL DOLOR MEDIANTE RESPUESTAS TERMOGRÁFICAS Y EXPRESIONES FACIALES EN RATAS DE LABORATORIO DURANTE LA APLICACIÓN DE DIFERENTES MÉTODOS DE EUTANASIA

T E S I S

(Idónea Comunicación de Resultados)

Que para obtener el grado de

Maestra en Ciencias Agropecuarias

PRESENTA

M.V.Z. Adriana Domínguez Oliva

COMITÉ TUTORAL:

Director:

Dr. Daniel Mota Rojas

Co-director:

Dr. Ismael Hernández Ávalos

Asesora:

Dr. Silvia Adriana Olmos Hernández

CIUDAD DE MÉXICO. Septiembre de 2023

AGRADECIMIENTOS

El presente trabajo fue realizado en el servicio de Bioterio y Cirugía Experimental del Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra (INR-LGII) de la Secretaría de Salud, bajo la dirección del Dr. Daniel Mota Rojas con adscripción a la línea de investigación “Neurofisiología, comportamiento y evaluación del bienestar animal” del Departamento de Producción Agrícola y Animal de la Universidad Autónoma Metropolitana y del Dr. Ismael Hernández Avalos del Departamento de Farmacología clínica y anestesia veterinaria de la Universidad Nacional Autónoma de México. El asesoramiento del presente trabajo estuvo a cargo de la Dra. Adriana Olmos Hernández.

El autor agradece al Consejo Nacional de Humanidades, Ciencia y Tecnología (CONAHCyT) por la beca otorgada para los estudios de maestría, con el número de registro 802181, que comprendió del periodo de 01 de Agosto de 2021 al 31 de Julio de 2023.

A mi comité tutoral

Agradezco enormemente al Dr. Mota, al Dr. Isma y a la Dra. Adriana por el apoyo y asesoramiento que recibí durante todo momento. Los tres son una gran inspiración para mí y me siento honrada de haber podido trabajar en este proyecto junto a ustedes. Creo que no siempre se tiene el privilegio de contar con personas que te ayuden a crecer tanto como ustedes me han ayudado, y eso es algo por lo que estaré agradecida toda la vida.

A mi familia y amigos

Mamá, papá, hermana, Kalani, las palabras no son suficientes para expresarles mi agradecimiento por siempre estar conmigo y apoyarme en todas las etapas de mi vida. Saben que sin ustedes nada de lo que he hecho sería posible y nada de lo que haga tendría sentido. Los quiero mucho y gracias por ser mi familia. A Viri, Karen y Verduzco, gracias por su apoyo y compañía. Los quiero mucho.

In memoriam

Farih

Junio 2010 – Octubre 2022

“If love could have saved you, you would have lived forever”

El jurado designado por La Comisión Académica de la Maestría en Ciencias Agropecuarias, de la Universidad Autónoma Metropolitana aprobó la tesis que presentó:

Adriana Domínguez Oliva

20 de septiembre de 2023.

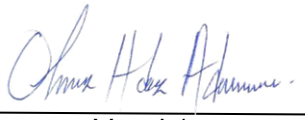
JURADO:

Presidente

Dra. Patricia Mora Medina (UNAM)

Secretario

M. en C. Salvador Carlos Flores Peinado (UNAM)



Vocal 1

Dra. Silvia Adriana Olmos Hernández (INR)

ÍNDICE GENERAL

ÍNDICE DE FIGURAS

ABREVIATURAS

ACTH: hormona adrenocorticotropa

ARRIVE: Animal Research: Reporting of In vivo Experiments

ASIC: canales iónicos sensibles al ácido

AVMA: Asociación Americana de Medicina Veterinaria para la Eutanasia de Animales de Laboratorio

BAT: tejido adiposo pardo

CINVESTAV: Centro de Investigación y de Estudios Avanzados

CO_2: dióxido de carbono

EEG: electroencefalograma

FAU: unidad de acción facial

INR-LGII: Instituto Nacional de Rehabilitación- Luis Guillermo Ibarra Ibarra

IP: intraperitoneal

IRT: termografía infrarroja

LORR: pérdida del reflejo de enderezamiento

NC3R: Centro Nacional para el Reemplazo, Refinamiento y Reducción de los Animales en la Investigación

NE: norepinefrina

RGS: Escala de Muecas de la Rata (Rat Grimace Scale)

SADER: Secretaría de Agricultura y Desarrollo Rural

SD: desviación estándar

SIH: hipertermia inducida por estrés

UPEAL: Unidad de Producción y Experimentación de Animales de Laboratorio

RESUMEN

El objetivo del presente estudio experimental prospectivo-comparativo es determinar la eficacia de distintos métodos de eutanasia en ratas de laboratorio, mediante la evaluación de la nocicepción y dolor a través de los cambios observados en la termografía infrarroja (IRT) y las expresiones faciales en la escala de muecas de rata (Rat Grimace Scale; RGS). Se emplearon sesenta ratas adultas de la cepa Wistar (*Rattus norvegicus*) (treinta machos y treinta hembras), con un peso promedio de 311 ± 62 g con 8–10 semanas de edad.

Los animales fueron asignados de manera aleatoria a seis grupos experimentales de acuerdo con el método de eutanasia. G_1: Administración intraperitoneal (IP) de pentobarbital a dosis de 400 mg/kg IP. G_2: Sobredosis de CO_2 dentro de una cámara de inducción personalizada para eutanasia (32.5 x 42 x 21 cm), con una tasa de flujo del 30% del volumen de la cámara por minuto (min). G_3: Decapitación empleando una guillotina para roedores. G_4: Inhalación de isoflurano mediante la técnica de la "gota abierta" ("open drop"), humedeciendo dos torundas de algodón, cada una con 2 ml de isoflurano. G_5: Sobredosis de ketamina + xilacina a dosis de 450 mg/kg IP y 45 mg/kg IP, respectivamente. G_6: Combinación de ketamina + CO_2 (exponiéndolos al CO_2 de 5 a 10 min después de la administración de ketamina). Dichos grupos se analizaron durante cinco tiempos: Basal: la evaluación se realizó 24 h antes del método de eutanasia; Ti_1: tres minutos antes de la aplicación de la eutanasia; Ti_2: durante la aplicación del método (p. ej., mientras el animal recibía la dosis IP de pentobarbital o mientras estaba dentro de la cámara de inducción o colocado en la guillotina); Ti_3: inmediatamente después de la aplicación del método de eutanasia hasta la pérdida del reflejo de enderezamiento (LORR) como signo de inconsciencia; y Ti_4: hasta el cese de la respiración y la frecuencia cardiaca. Las variables de estudio fueron la IRT y la RGS. La IRT fue evaluada con una cámara térmica en cuatro regiones anatómicas o ventanas térmicas: ocular ($T°_{ocu}$), auricular ($T°_{ear}$), interescapular ($T°_{dor}$), y la cola ($T°_{tai}$), capturando imágenes del lado derecho de los animales y reportando las temperaturas como máximas (T°max), mínimas (T°min) y promedios (T°media). La RGS se evaluó mediante una videograbación al rostro de las ratas (vista frontal y lateral) con cámaras de alta resolución para analizar cuatro unidades de acción facial (FAU): estrechamiento orbital, cambio de las orejas, aplanamiento de la nariz/mejillas, cambio en

las vibrisas. Empleando una escala del 0 al 2, se determinó el grado de dolor como no presente (0), moderadamente presente (1) y obviamente presente (2).

Los resultados de la IRT mostraron diferencias significativas ($P < 0.05$) en T°media en los grupos inhalatorios (G_2 y G_4). Para $T°_{ocu}$, G_2 (33.79 ±0.92°C) y G_4 (29.30 ±1.23°C) registraron las temperaturas más bajas en comparación con el resto de los grupos experimentales ($P = 0.0007$). Esto fue similar a la $T°_{ear}$ de ambos grupos, registrando un descenso significativo de la temperatura hasta 30.80 ± 2.83°C en G_2 y 29.0 ± 1.76°C en G_4 ($P = 0.0005$) desde Basal hasta Ti_4. En la $T°_{dor}$, G_4 mostró descensos significativos de hasta 3.56°C en Ti_2 ($P = 0.0001$), mientras que en $T°_{tail}$ todos los grupos mostraron disminuciones progresivas de temperatura desde Basal hasta Ti_4, pero con los valores más bajos en G2 (23.67 ± 1.90°C) y G_4 (25.77 ± 0.78°C). Respecto a la RGS, los hallazgos principales mostraron que, durante el Ti_2, el G_3 y G_5 tuvieron los puntajes más altos (0.6 ± 0.26 y 0.6 ± 0.16, respectivamente) ($P < 0.0001$), mientras que, en el Ti_3, G_2 (0.9 ± 0.18) y G_4 (1.2 ± 0.20) obtuvieron los puntajes más elevados ($P < 0.0001$).

De acuerdo con los resultados obtenidos en la IRT y la RGS, los métodos de eutanasia inhalatorios (CO_2 e isoflurano) generan marcadas alteraciones a nivel térmico y en la expresión facial, posiblemente asociado a una respuesta al estrés o al dolor que ambos agentes pueden ocasionar. De manera particular, la inhalación de isoflurano se podría considerar como un evento que puede generar distrés si se consideran las altas puntuaciones de la RGS y las marcadas alteraciones en la temperatura superficial de las ratas. No obstante, es importante considerar el efecto miorrelajante de los anestésicos inhalatorios y su influencia en la expresión facial. Asimismo, los puntajes de la RGS muestran que la decapitación y la ketamina + xilacina provocaron dolor agudo transitorio durante la aplicación del método de eutanasia, posiblemente debido al daño tisular causado por ambos métodos (inyección y guillotina). Por el contrario, la combinación de ketamina + CO_2 mostró minimizar el efecto de la exposición al CO_2, pudiendo considerarse como un método de refinamiento a la eutanasia con CO_2. Sin embargo, se requieren investigaciones adicionales para establecer un análisis integral del grado de dolor que los roedores pueden percibir durante la aplicación de métodos humanitarios de eutanasia.

Palabras clave: refinamiento de la eutanasia, bienestar, termografía infrarroja, expresión facial, exposición a CO2, isoflurano, decapitación, ketamina + xilacina.

ABSTRACT

The present prospective-comparative experimental study aimed to determine the efficacy of different euthanasia methods in laboratory rats, by evaluating pain through the changes observed with infrared thermography (IRT) and facial expressions using the Rat Grimace Scale (RGS). Sixty adult Wistar rats (*Rattus norvegicus*) (thirty males and thirty females) with an average weight of 311 ± 62 g at 8–10 weeks of age were used.

Animals were randomly assigned to six experimental groups according to the euthanasia method. G_1: Intraperitoneal (IP) administration of pentobarbital at a dose of 400 mg/kg. G_2: CO_2 overdose in a customized euthanasia induction chamber (32.5 x 42 x 21 cm), with a flow rateo f 30% of the camber volume per minute (min). G_3: Decapitation using a guillotine for rodents. G_4: Inhalation of isoflurane using the "open drop" technique, soaking two cotton swabs with 2 ml of isoflurane each. G_5: Ketamine + xylazine overdose at doses of 450 mg/kg IP and 45 mg/kg IP, respectively. G_6: Combination of ketamine + CO_2 (after 5–10 min of ketamine administration). These groups were analyzed five times: Baseline: the evaluation was carried out 24 h before the euthanasia method; Ti_1: three minutes before the application of euthanasia; Ti_2: during the application of the method (e.g., while the animal was receiving the IP dose of injectable agent or while it was inside the induction chamber or placed on the guillotine); Ti_3: immediately after the application of the euthanasia method until the loss of the righting reflex (LORR) as a sign of unconsciousness; and Ti_4: until the cessation of breathing and heart rate. The study variables were IRT and RGS. IRT was evaluated with a thermal camera in four anatomical regions or thermal windows: ocular ($T°_{ocu}$), auricular ($T°_{ear}$), interscapular ($T°_{dor}$), and tail ($T°_{tai}$), capturing images from the right side of the animals and reporting the temperatures as maximum (T°max), minimum (T°min), and averages (T°mean). RGS was assessed by videotaping the rats' faces (front and side view) with high-resolution cameras to analyze four facial action units (FAU): orbital narrowing, ear shifting, nose/cheek flattening, in the vibrissae Using a scale from 0 to 2, the degree of pain was determined as not present (0), moderately present (1) and obviously present (2).

The IRT results showed significant differences ($P < 0.05$) in T°mean in the inhalation groups (G_2 and G_4). For $T°_{ocu}$, G_2 (33.79 ±0.92°C) and G_4 (29.30 ±1.23°C) had the lowest temperatures compared to the rest of the experimental groups ($P = 0.0007$). This was similar to $T°_{ear}$ of both groups, recording a significant drop in temperature up to 30.80 ± 2.83°C in

G_2 and 29.0 ± 1.76°C in G_4 (P = 0.0005) from Basal to Ti_4. In $T°_{dor}$, G_4 showed significant decreases of up to 3.56°C in Ti_2 (P = 0.0001), while in $T°_{tail}$ all groups showed progressive decreases in temperature from Basal to Ti_4, but with the lowest values in G_2 (23.67 ± 1.90°C) and G_4 (25.77 ± 0.78°C). Regarding the RGS, the main findings showed that, during Ti_2, el G_3 and G_5 had the highest scores (0.6 ± 0.26 and 0.6 ± 0.16, respectively) (P < 0.0001), while, in Ti_3, G_2 (0.9 ± 0.18) and G_4 (1.2 ± 0.20) obtained the highest scores (P < 0.0001).

According to the results obtained in the IRT and the RGS, the inhalation euthanasia methods (CO_2 and isoflurano) marked alterations at the thermal level and in facial expression, possibly associated with the stress or pain response that both agents can cause. In particular, the inhalation of isoflurane could be considered an agent that might cause distress considering the high scores of the RGS and the marked alterations in the surface temperature of the rats. However, it is important to consider the muscle relaxant effect of inhalational anesthetics and their influence on facial expression. Likewise, the RGS scores show that decapitation and ketamine + xylazine caused transient acute pain during the application of the euthanasia method, possibly due to tissue damage caused by the injection and guillotine. On the contrary, the combination of ketamine + CO_2 minimized the effect of exposure to CO_2 alone and could be considered as a refinement method of CO_2. However, further research is required to establish a comprehensive analysis of the degree of pain that rodents may perceive during the application of humane euthanasia methods.

Keywords: euthanasia refinement, wellness, infrared thermography, facial expression, CO2 exposure, isoflurane, decapitation, ketamine + xylazine

1. INTRODUCCIÓN

El uso de animales de laboratorio constituye una pieza clave para la ciencia biomédica ya que no son *sólo* un método de investigación, sino son *el* medio para hacer ciencia (1,2). Actualmente, las ratas y ratones son las especies más utilizadas para la experimentación (3), representando entre un 87-98% del total mundial de los animales (4,5). No obstante, desde los inicios de la ciencia médica experimental, la investigación con ratas y otros animales es un tópico controversial que involucra desafíos científicos, éticos, legales, morales, sociales, culturales e inclusive religiosos. Esto es debido al potencial sufrimiento, distrés y dolor que los animales pueden percibir, no sólo durante la vida sino también durante el proceso de eutanasia, en donde el dolor se reconoce como aquella "experiencia sensorial y emocional desagradable, asociada con, o similar a la asociada con un potencial o real daño a tejidos", en la cual la incapacidad de comunicarlo verbalmente no niega la posibilidad de que se pueda experimentar (6).

Debido a ello, desde hace más de 50 años el Centro Nacional para el Reemplazo, Refinamiento y Reducción de los Animales en la Investigación (NC3Rs) ha promovido la iniciativa de Russel y Burch sobre las 3Rs con el fin de prevenir o minimizar el dolor, sufrimiento y angustia durante un diseño experimental (7,8). La aplicación de las 3Rs involucra aspectos asociados a la calidad de vida desde la adquisición de los animales, su transporte, alojamiento, mantenimiento y terapéutica (9). También considera los procedimientos de eutanasia o sacrificio humanitario para garantizar la calidad de muerte sin afectar la validez de los resultados (10).

De manera internacional, los métodos de eutanasia aprobados para roedores están descritos en la Guía de la Asociación Americana de Medicina Veterinaria para la Eutanasia de Animales de Laboratorio (AVMA) (11). Ésta, a su vez, sirve de base para regular la eutanasia en México bajo la NOM-062-ZOO-1999 sobre las Especificaciones técnicas para producción, cuidado y uso de los animales de laboratorio, creada por la Secretaría de Agricultura y Desarrollo Rural (SADER) (12). En ambos documentos se incluyen los métodos inyectables (barbitúricos y anestésicos generales), inhalatorios (CO_2 e isoflurano) y físicos (dislocación cervical y decapitación) aceptados o aceptados con condiciones para la eutanasia de roedores. Éstos deben ser técnicas de fácil administración, económicas, seguras, y generar una rápida e irreversible inconciencia, seguida de paro cardiorrespiratorio, la pérdida de la función cerebral y la muerte, con un grado de angustia

o dolor mínimo (13).

La eutanasia y el potencial dolor que las ratas de laboratorio pueden experimentar durante su aplicación es controversial debido a los resultados mostrados por diferentes autores, lo cual es uno de los puntos clave en torno a la experimentación animal y es uno de los principales temas de interés para la opinión pública que objeta su uso en la ciencia (14). Por ejemplo, agentes inyectables como el pentobarbital sódico y la ketamina tienen un pH básico o ácido, respectivamente, que puede causar dolor debido a la irritación tisular después de la administración IP (15). Por el contrario, otros estudios no han encontrado conductas relacionadas con el dolor (p. ej., contorsiones abdominales) después de la inyección IP de pentobarbital (16). Los métodos físicos como la decapitación se consideran estéticamente desagradables y la posibilidad de sentir dolor consciente está en debate (17). Varios autores han informado que la actividad cerebral se mantiene durante 2.7 a 40 segundos después de la decapitación (18) y que la presencia de actividad electroencefalográfica (EEG) rápida y de bajo voltaje es compatible con la percepción consciente de dolor (19). Sin embargo, debido al corto período de conciencia y a la posibilidad de que los índices EEG representen actividad de la corteza cerebral y no nocicepción, la decapitación todavía se considera un método de eutanasia humanitario (20,21). En cuanto a los métodos inhalados, el CO_2 es uno de los más empleados pero algunos estudios refieren el potencial dolor y activación de los canales iónicos sensibles al ácido (ASIC) debido a la formación de ácido carbónico cuando el CO_2 se combina con agua en la mucosa nasal u ocular (22). Además, aunque el isoflurano se considera una alternativa al CO_2, su pungencia moderada puede resultar aversiva y causar irritación a los roedores (15,23).

Las tendencias en torno al reconocimiento del dolor en medicina veterinaria han llevado al desarrollo y aplicación de tecnologías o herramientas no invasivas que permitan cuantificar el dolor en roedores y otros animales, o asociar ciertas alteraciones fisiológicas o de comportamiento a la percepción de un estímulo nocivo –resultando en distrés– (24). Una de éstas es la termografía infrarroja (IRT), la cual es una técnica basada en la detección de la temperatura superficial de la piel (25) que responde a los cambios en la microcirculación vascular bajo estados negativos como dolor, estrés, inflamación, miedo, y algunas patologías (26). La IRT se considera como una herramienta de utilidad en la investigación veterinaria ya que permite analizar las funciones fisiológicas de los animales e inclusive emplearse como técnica complementaria de diagnóstico temprano para detectar

procesos de hipotermia o hipertermia, endotoxemia, inflamación, isquemia e hipoperfusión sanguínea o eficacia analgésica (26,27). La literatura actual sugiere que la IRT es una herramienta complementaria para evaluar el bienestar de los animales (26,27). Sin embargo, aunque existen estudios acerca de la aplicación de la IRT durante el periodo *antemortem* de cerdos (28,29), a la fecha no existen estudios donde la IRT se haya empleado durante la eutanasia de especies de laboratorio.

Por otro lado, desde las publicaciones de Darwin en 1872 respecto a las emociones y la expresión facial de los animales, el estudio de estas ha llevado a estandarizar su uso mediante la codificación de movimientos musculares faciales y las denominadas "escalas de muecas". Diversos estudios han mostrado que, como los humanos, los animales poseen un sistema de comunicación no verbal con el que cambian su expresión facial dependiendo del tipo e intensidad del estímulo al que sean expuestos, en este caso el grado de dolor (30). En las ratas de laboratorio, la Escala de Muecas de la Rata o Rat Grimace Scale, en inglés (RGS) (31), es una escala validada que emplea cuatro Unidades de Acción Facial (FAU) (ajuste orbital, aplanamiento de la nariz y mejillas, posición de las orejas, y posición de las vibrisas) que representan movimientos específicos de grupos musculares cuyos cambios se asocian al dolor y a la severidad del mismo (32). En una escala de puntuación del 0 al 2, donde 0 es dolor ausente, 1 es moderado, y 2 es severo, la escala y la intensidad de la expresión facial en las ratas de laboratorio ha permitido identificar un puntaje de 0.67 como el umbral donde los roedores requieren analgesia de rescate durante un evento quirúrgico o potencialmente doloroso (33,34). El dolor que experimentan durante diversos procedimientos quirúrgicos como cirugías de columna, en modelos de dolor agudo, crónico o neuropático, y para analizar el bienestar durante procedimientos rutinarios como administración de fármacos o eutanasia (35).

La investigación con animales está comprometida a mantener altos estándares de cuidado y bienestar animal. Reconocer y prevenir el dolor es parte de las estrategias propuestas para minimizar la potencial alteración conductual, fisiológica e inmune que los roedores podrían experimentar durante un protocolo experimental y la eutanasia, disminuyendo también el efecto que el dolor podría tener en los resultados de la investigación. Por lo anterior, el objetivo de la presente tesis es determinar la eficacia de distintos métodos de eutanasia en ratas de laboratorio, mediante la evaluación del dolor a través de los cambios observados en la termografía infrarroja (IRT) y las expresiones faciales en la escala de muecas de rata (Rat Grimace Scale).

2. MARCO TEÓRICO

2.1. CAPÍTULO I

Artículo de revisión intitulado:

El bienestar en roedores con fines de investigación: Historia, aspectos controversiales e implicaciones éticas

Publicado en la revista de la Asociación Mexicana de Médicos Veterinarios Especialistas en Pequeñas Especies, en el volumen 33, número 3, páginas 70-79.

Domínguez-Oliva, A.; Mota-Rojas, D.; Hernández-Avalos, I.; Olmos-Hernández, Silvia A.; Martínez-Burnes, J. El bienestar en roedores con fines de investigación: Historia, aspectos controversiales e implicaciones éticas. AMMVEPE 2022, 33(3), 70–79. https://ammvepe.mx/revista008.html

ARTÍCULO DE INVESTIGACIÓN

AMMVEPE Vol. 33, No. 3 • Septiembre-Diciembre 2022
pp 70-79

El bienestar en roedores con fines de investigación: Historia, aspectos controversiales e implicaciones éticas

Welfare of rodents used in research: History, controversy and ethical implications

Adriana Domínguez-Oliva,* Daniel Mota-Rojas,** Ismael Hernández-Avalos,***
Silvia A. Olmos-Hernández,**** Julio Martínez-Burnes*****

RESUMEN

Los roedores, ratas y ratones, son la principal especie empleada en la ciencia biomédica. Su uso implica una serie de aspectos sociales, culturales, económicos, legales y científicos que rigen la ética del cuidado y bienestar de los animales de laboratorio. El objetivo de este trabajo es dar una visión histórica y global sobre el empleo de roedores para experimentación. Se abordará la importancia de esta especie en el avance del conocimiento científico desde distintos aspectos, así como las implicaciones éticas y legales, las controversias, y los procedimientos y estrategias que se han propuesto para evaluar y garantizar la calidad de vida y muerte de los roedores de laboratorio.

Palabras clave: Animales de laboratorio, ratas, bienestar, ética, 3Rs.

ABSTRACT

Rodents, rats and mice, are the main species used in biomedical science. Its use comprises a series of social, cultural, financial, legal, and scientific perspectives that dictate the ethics of the care and welfare of laboratory animals. The objective of this work is to give a historical and global vision of the use of laboratory animals. The importance of this species in the advancement of scientific knowledge, from different aspects, will be addressed. Likewise, the ethical, legal implications, and controversies will be discussed, as well as the procedures and strategies that have been implemented to evaluate and guarantee the quality of life and death of laboratory rodents.

Key words: *Laboratory animals, rats, welfare, ethics, 3Rs.*

INTRODUCCIÓN

El uso de animales de laboratorio se considera no sólo un método de investigación, sino el método para hacer ciencia.[1,2] Los roedores (ratas y ratones), constituyen la especie más empleada en experimentos a nivel mundial,[3] con alrededor de 111.5 millones de animales en Estados Unidos (E.U.).[4] Sus principales aportaciones incluyen la comprensión de funciones biológicas, la fisiopatología de enfermedades, y el desarrollo de técnicas de diagnóstico, tratamiento y prevención.[5,6] Asimismo, contribuyen al desarrollo de nuevos fármacos, protocolos de anestesia, técnicas quirúrgicas, y vacunas como la de la rabia, parvovirus, tétanos y leucemia felina.[2]

A pesar de su importancia biomédica, su uso constituye un tópico controversial en el que se involucran aspectos científicos, éticos, legales, morales y culturales.[3,7] Debido a ello, desde hace 50 años se ha

* Programa de Ciencias "Maestría en Ciencias Agropecuarias", Universidad Autónoma Metropolitana, Unidad Xochimilco (UAM-X).
** Neurofisiología del dolor y bienestar en animales domésticos y silvestres. DPAA. Universidad Autónoma Metropolitana, Unidad Xochimilco (UAM-X).
*** Farmacología Clínica y Anestesia Veterinaria. Departamento de Ciencias Biológicas. Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Cuautitlán (UNAM, FES-C).
**** División de Biotecnología-Bioterio y Cirugía Experimental. Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra (INR-LGII).
***** Grupo de Salud Animal. Universidad Autónoma de Tamaulipas, Facultad de Medicina Veterinaria y Zootecnia.

Sobretiros: MVZ Daniel Mota-Rojas
Correo electrónico: dmota100@yahoo.com.mx

promovido la iniciativa de las 3Rs (reemplazo, reducción y refinamiento) con el fin de minimizar el dolor, sufrimiento y angustia de los animales de laboratorio.[8]

El objetivo de este trabajo es dar una visión histórica y global sobre el empleo de roedores en la investigación. Se abordará la importancia de esta especie en el avance del conocimiento científico desde distintos aspectos, así como las implicaciones éticas y legales, las controversias en torno a la experimentación animal, y los procedimientos y estrategias que se han propuesto para evaluar y garantizar la calidad de vida y muerte de los roedores de laboratorio.

Antecedentes del uso de animales para la experimentación

El uso de animales para experimentación comenzó con vivisecciones en los siglos III y IV.[9] Aristóteles e Hipócrates fueron los primeros científicos que realizaron experimentos en animales vivos para conocer su anatomía, creando manuales de vivisección como el Historia *Animallium y Corpus Hippocraticum*.[7]

En esa época antropocéntrica, personajes como Aristóteles o René Descartes justificaban el sufrimiento de los animales no humanos, al considerarlos incapaces de sentir dolor.[10,11] En contraste, Agustín de Hipona y Thomas de Aquino consideraban un pecado la crueldad animal, y que el hombre tenía la obligación de prevenir el sufrimiento de las especies.[10,12] Por su parte, Kant, durante el Renacimiento, consideró a la vivisección como un acto de crueldad, pero primordial para el avance de la ciencia,[13] sobre todo en los años 1600, cuando incrementó el uso de ratas y ratones con fines de experimentación.[14]

En 1789, Bentham Jeremy fue el primer filósofo que desafió la experimentación animal, aseverando que los animales pueden sufrir. Su postura fue apoyada por Darwin y su estudio sobre las emociones de las especies en 1872, el cual sentó las bases para cuestionar de manera científica su bienestar,[15] y las consecuencias orgánicas que el dolor y sufrimiento podían generar.[7]

A la par del auge de la experimentación animal, surgieron los movimientos antivivisección y la primera sociedad de protección para los animales en 1824, en Inglaterra,[9,16] lo cual impulsó la primera legislación global para el uso y cuidado de animales destinados a la ciencia: el Acto de Crueldad a los Animales el 15 de agosto de 1876.[17]

En el siglo XIX, con el avance de ciencias como la farmacología, toxicología e inmunología, la investigación con animales se convirtió en un elemento imprescindible para el descubrimiento y prueba de fármacos y vacunas para erradicar enfermedades como la viruela, poliomielitis y el sarampión.[11] Claude Bernard, el padre de la fisiología y la medicina experimental, afirmó que "los experimentos en los animales son necesarios y conclusivos para la toxicología y la sanidad humana, ya que los efectos de las sustancias son los mismos en ambas especies, aunque se deben considerar algunas diferencias en cierto nivel".[18]

En 1999, el número de animales usados para la ciencia ascendía a 9.7 millones en la Unión Europea, de los cuales 5.3 millones eran ratones y 2.6 millones ratas.[5] En Japón, en 1998, de 5,626, 116 animales el 87% eran representados por ratas y ratones.[19] Mundialmente, en el 2005 se reportaban entre 82 a 154 millones de animales, 118 millones para el 2012, y un aproximado de 192.1 millones en el 2015, en donde México empleaba un estimado de 277,689 animales.[20] En el 2021, en EU, se ha reportado un aproximado de 12 y 24 millones de roedores,[21] aunque otros autores lo estiman hasta 111.5 millones.[4]

Aunque los roedores representan el mayor porcentaje de los animales empleados en la ciencia, los modelos vivos incluyen una diversidad de especies en función del objetivo e hipótesis del proyecto, la naturaleza, anatomía y fisiología del animal, así como a los requerimientos legales, financieros y de alojamiento.[9,11]

Especies empleadas dentro de la investigación biomédica

El término "modelo animal" proviene del latín *animae* (alma o espíritu) y de la palabra 'modelo' (imitar o ser similar).[11] De esta manera, los modelos animales son individuos que, por sus características biológicas, funcionales y genéticas, tienen similitud con el hombre y otros animales, y permiten comprender su fisiología. Estos no se limitan a mamíferos; por ejemplo, en las moscas de fruta (*Drosophila melanogaster*) se han estudiado síndromes epilépticos;[22] y en nemátodos (*Caenorhabditis elegans*)[23] y peces zebra (*Danio rerio*) se han probado opciones terapéuticas contra la obesidad y la diabetes.[24]

Los monos Rhesus (*Macaca mulata*) se consideran el modelo ideal para comprender la fisiopatología de enfermedades de alta prioridad como el SARS-CoV-2,[25] VIH, obesidad y disfunciones cognitivas. No obstante, su empleo conlleva controversias éticas por su proximidad al ser humano, al igual que los perros, aunque gracias a estos se han estudiado enfermedades degenerativas, congénitas, autoinmunes, y carcinogénesis,[26] ya que comparten hasta la mitad de 360 desórdenes genéticos.[27] Otras especies domésticas como los cerdos se emplean en la medicina de trasplantes,[28] mientras que hurones, conejos o cuyos contribuyen al estudio de dosis infectante de SARS-CoV-2,[29] en el desarrollo de vacunas contra VIH y mixomatosis,[30] o para determinar los daños histológicos derivados del ébola, respectivamente.[31]

Por su parte, los roedores, en particular las ratas, fueron el primer mamífero empleado en la ciencia y su uso ha aumentado desde el siglo XX.[32] En los E.U. se calculan anualmente entre 26 y 111.5 millones de roedores,[4] lo cual representa entre un 96 a 98% de todas las especies.[9] En el mundo, las tres principales cepas son la Wistar, Sprague-Dawley y Long Evans. Éstas se consideran el modelo ideal no sólo porque sus requerimientos de alojamiento y manejo son relativamente sencillos,[4] sino también porque su fisiología permite estudiar una gran cantidad de enfermedades (cardiovasculares, sepsis, obesidad, cáncer, úlceras gástricas, virales),[33] refinar procedimientos quirúrgicos (trasplante de órganos),[9] probar vacunas y otros fármacos.[34]

Roedores (*rattus norvegicus*) y su rol fundamental dentro del campo de la ciencia

La rata de laboratorio o *Rattus norvegicus* se considera el primer animal domesticado estrictamente con fines científicos,[35] representando entre un 87 a un 98% de todas las especies.[19] En México se consideran las más empleadas,[36] con un estimado anual de 277,689 ratas.[20]

A principios del siglo XIX las ratas blancas de laboratorio fueron introducidas en la ciencia con el objetivo de estudiar el funcionamiento de la glándula adrenal y procesos fisiológicos.[35] Más tarde se instauraron como modelos para accidentes cerebrovasculares, pruebas de nocicepción y hormonales, para estudiar el comportamiento,[27] o para patologías específicas como las hipertensivas en la línea Wistar Kyoto.[37]

Las ratas participan en estudios bioquímicos, enfermedades cardiovasculares, desórdenes metabólicos (metabolismo de lípidos y diabetes mellitus), desórdenes neurológicos (epilepsia y Parkinson), estudios de neurofisiología del comportamiento, trasplantes de órganos, enfermedades autoinmunes (artritis, encefalomielitis alérgica), cáncer y enfermedades renales.[38] De igual forma, contribuyen al desarrollo de nuevos fármacos y pruebas de toxicología, carcinogénesis o neurotoxicidad. Asimismo, son una pieza clave para el descubrimiento y prueba de vacunas como la de la polio,[39] o contra la COVID-19, demostrando que ratas gestantes no presentan efectos adversos y sus descendientes nacen con anticuerpos contra la enfermedad.[40]

Otros campos en los que las ratas destacan son en pruebas de dolor inducido, como el neuropático, el cual es un reto farmacológico en el 6.9% de la población humana, o en el mejoramiento de técnicas quirúrgicas mínimamente invasivas, como cirugías de remoción de gliomas intramedulares.[41] Igualmente, se asocian a la contención de armas biológicas como el anthrax (*Bacillus anthracis*) y la peste (*Yersinia pestis*), desarrollando vacunas con nanopartículas de bacteriófagos contra ambas enfermedades mortales.[42]

A pesar de las innumerables aportaciones de los roedores en la ciencia, su uso conlleva controversias en torno a diversos aspectos éticos, legales, sociales y culturales que deben ser considerados para emplear animales sin generarles dolor o sufrimiento.[43] Desde hace más de 50 años se ha promovido el concepto de las 3Rs (reemplazar, reducir, refinar) en la experimentación animal,[8] las cuales son la base del bienestar animal.

Implicaciones éticas y de bienestar animal en el uso de roedores (*Rattus norvegicus*) con fines de investigación

Trevor Poole mencionó que *"un buen bienestar equivale a una buena ciencia"*.[44] Esto significa que, si el animal se encuentra sano, física y mentalmente, libre de dolor, estrés y sufrimiento innecesario (es decir, en un buen bienestar),[45] los resultados obtenidos serán científicamente adecuados.

Donald Broom[46] refiere al bienestar animal como *"el estado de un individuo relacionado a la manera en la que hace frente a su ambiente"*, en la que se incluyen sus necesidades biológicas, así como su estado mental.[47] El estrés, por su parte, es la respuesta del organismo frente a factores externos e internos que alteren la homeostasis. Estos factores incluyen los fisiológicos (dolor, lesiones, cirugías, enfermedades, desnutrición, deshidratación), psicológicos (miedo, ansiedad, aburrimiento, soledad) y ambientales (manejo, ruidos, hábitat, temperatura, humedad).[48]

En torno a esto, el Centro Nacional para el Reemplazo, Refinamiento y Reducción de los Animales en la Investigación (NC3Rs), desde hace 50 años ha propuesto los principios de las 3Rs.[8] Estos principios se incluyen en la guía ARRIVE para la Investigación Animal y la manera de Reportar Experimentos *in vivo*, cuyo fin es preservar la calidad biológica, de bienestar, y científica dentro de la experimentación animal.[49]

Las tres R

En 1959, Russell y Burch describieron el principio de las 3Rs para el uso y regulación de animales de laboratorio.[50]

1. **Reemplazo.** Este implica sustituir a los roedores por métodos *in vitro*, cultivos celulares, simuladores, modelos matemáticos, material *"no sintiente"* o de origen humano.[51] Se requiere conocer la naturaleza del experimento y la biología del proceso a estudiar, ya que también incluye el desarrollo de nuevas tecnologías o de especies no convencionales como los insectos y nematodos.[52]
2. **Reducción.** Se refiere a asegurar que los resultados sean estadísticamente significativos, ocupando la menor cantidad posible de animales. La cantidad de individuos debe ser suficiente para evaluar el efecto deseado, obtener resultados replicables, y evitar el desaprovechamiento de estos.[45,51] Metodologías como el concepto de *"número necesario para tratar"* es un ejemplo.[49]
3. **Refinamiento.** Engloba todos los procedimientos cuyo fin es minimizar el estrés, miedo, ansiedad y dolor de las ratas de laboratorio, mientras se garantiza la validez y menor variabilidad de los resultados.[51] Esto implica personal altamente capacitado,[53] un micro y macroambiente propicio, y protocolos de anestesia, analgesia, técnicas mínimamente invasivas, y eutanasia.[2]

Adicionalmente, existen otras 3Rs enfocadas directamente a la calidad y valor científico:[54] la reproductibilidad, la replicabilidad y el rigor.[55] De esta manera, la ciencia de los animales de laboratorio involucra al bienestar desde sus necesidades biológicas, el refinamiento de procedimiento para minimizar el sufrimiento y dolor, y obtener resultados representativos que favorezcan la ética y normativa del cuidado de los animales.[11]

Normativa nacional e internacional para el empleo de animales de laboratorio

El uso de animales de laboratorio se encuentra estrictamente regulado por legislaciones internacionales y nacionales.[5] La Declaración de Helsinki en 1964 y la Declaración de Basel en 2011, son dos es-

Cuadro 1. Requerimientos de microambiente para las ratas de laboratorio *(R. norvegicus)*.

Parámetro (unidad)	Valor requerido			
Peso corporal nacimiento (g)	4.5 - 6			
Consumo diario alimento (g/100g Pe)	10 - 20			
Consumo diario agua (ml/100g Pe)	10 - 45			
Nutrientes	PC%	GC%	FC%	Cen%
	12.24	4-11	3-6	6-8
Jaula o caja	Área del piso (cm^2)		Altura (cm)	
< 100 g	110		17	
100-300	187		20	
300-400	258		20	
400-500	387		20	
> 500 g	452		20	
Amoniaco	> 25ppm			
Material de la caja	Plástico o acero inoxidable.			
Cama de la caja	Absorbente, libre de polvo, no tóxico, no costoso, esterilizable, libre de contaminantes.			

Pe: Peso corporal; PC: proteína cruda; GC: grasa cruda; FC: fibra cruda; Cen: cenizas.

Cuadro 2. Requerimientos de microambiente para las ratas de laboratorio *(R. norvegicus)*.

Rango (unidad)	
Temperatura	20-26 (°C)
Humedad	30-70 (%)
Limpieza	1 vez / semana
Ventilación	8-20 (recambios/h)
Iluminación	12 h
Intensidad luz	Entre 130-325 (lux)
Ruido	< 1000 Hz
Vibraciones	70-100 dB

tatutos relevantes para la experimentación en los cuales se incluye la necesidad de la experimentación animal de manera ética y humana.[56,57]

De manera general, las 3Rs son el fundamento de todo el marco legal.[58] En E.U., la política de Salud Pública en el Cuidado Humano y Uso de Animales de Laboratorio, la Guía para el Uso y Cuidado de los Animales de Laboratorio, y el Acta de Bienestar Animal (AWA) son las legislaciones que rigen los bioterios; no obstante, en la última, las ratas no se encuentran incluidas en la regulación.[58]

En México, la NOM 062-ZOO-1999 regula las Especificaciones técnicas para producción, cuidado y uso de los animales de laboratorio.[59] Su fundamento reside en cuatro guías: para el Cuidado y el Uso de Animales de Laboratorio, de la Asociación Americana de Medicina Veterinaria para la Eutanasia de Animales de Laboratorio (AVMA), de la Asociación Americana para la Ciencia de Animales de Laboratorio (AALAS), la del Cuidado de los Animales de Laboratorio del Consejo Canadiense para el Cuidado Animal, y por los lineamientos del NC3RS.[60] También se involucra el Código Sanitario para los Animales Terrestres, la NOM-051-ZOO-1995 sobre el transporte humanitario de animales, la NOM-087-ECOL-SSA1-2002 de protección ambiental y residuos biológico-infecciosos, la Ley General de Salud, la Ley Federal de Sanidad Animal y la Ley Federal de Bienestar Animal.[60]

La NOM 062, a diferencia de la normativa americana, incluye a roedores y es de aplicación obligatoria en todos los bioterios;[60] no obstante, no contempla alguna sanción de incumplimiento. Según Juárez-Portilla, *et al*.,[11] en el 2018 sólo 53 bioterios estaban registrados ante alguna institución oficial. El cumplimiento de la normativa lo realiza la Asociación Mexicana de la Ciencia de Animales de Laboratorio, el Comité Institucional para el Uso y Cuidado de Animales de Laboratorio (CICUAL) y el Comité de Bioética en la Investigación;[5] sin embargo, se menciona que alrededor del 30% de investigadores encuestados por Armas, *et al*.,[36] no cumple con los requerimientos señalados por la NOM-062 en cuanto a instalaciones, manejo o bienestar.

Requerimientos para el mantenimiento de roedores *(R. norvegicus)* en bioterios

Microambiente

En este se incluyen los factores mencionados en el *cuadro 1*,[5] con el fin de propiciar un ambiente estandarizado que propicie su bienestar y la calidad de los resultados.[61]

En algunos países se ha implementado el enriquecimiento ambiental adicionando bloques de madera, tubos de plástico o lugares para anidar. Su uso ha demostrado promover conductas naturales de la especie y mejorar sus capacidades de aprendizaje.[61] No obstante, se cuestiona el tener a los animales en condiciones "no convencionales" y su efecto en los resultados y replicabilidad.[27]

Macroambiente

Este incluye todos los factores mencionados en el *cuadro 2*.[5] La importancia de mantener dichos rangos se relaciona a la salud de las ratas. Por ejemplo, una correcta ventilación mantiene cantidades adecuadas de oxígeno, bajos niveles de dióxido de carbono y bajas concentraciones de amoniaco para evitar infecciones por micoplasmas,[27] mientras que los requerimientos de iluminación, sobre todo en ratas albinas, previenen daños a la retina o alteraciones reproductivas.[62]

Controversia sobre el uso de animales en experimentación

Como se ha mencionado, la controversia en torno al uso de animales en la investigación biomédica involucra criterios sociales, culturales, éticos y legales que influyen en el grado de aceptación o rechazo de algunas de estas prácticas.

Aspectos sociales

La opinión social respecto al uso de animales de laboratorio suele mostrar dos extremos. Por una parte, en 1989, el 75% de las personas reconocían que el uso de animales es necesario para el avance médico;[63] en el 2013, el 56% de los americanos lo consideraba moralmente aceptable ya que significa un avance en la medicina humana, aunque el 67% se manifestaba en contra de las pruebas de cosméticos con animales.[64] Estos porcentajes son cercanos a los reportados en España (66% a favor); sin embargo, sólo el 44% está de acuerdo que se empleen perros o primates no humanos.[57] En Japón, este tipo de posturas condujo a que el uso de perros y gatos en laboratorios se redujera un 65% de 1990 a 1998.[19] En el caso de los roedores, éstos sufren una des-animalización y des-especiación influenciada por el fenómeno de *"sintiencia social"*.[65] Éste se conoce como una idea antropocéntrica en el que las personas aceptan la experimentación en animales que consideran con un grado de *sintiencia* biológica inferior, mientras protegen a aquellos animales dentro de su contexto social.[1] En una encuesta realizada en E.U., la aceptación se redujo de 65 a 56% del año 2001 al 2013.[64] Entre los principales factores que influyen en ello se encuentra la sintiencia, alojamiento, manejo, eutanasia, y también los *"fracasos"* cuando los estudios con animales no pueden ser traspolados a humanos y eso conlleva al escepticismo en la ciencia.[2]

Los movimientos proteccionistas como la Fundación PETA (Personas para el Trato Ético de los Animales), creada en 1980, si bien no siempre son una referencia científica en el tema, su presencia en la sociedad contribuye a que el hombre se involucre e interese por el bienestar de todas las especies y, de manera indirecta, hace evidente la necesidad de estandarizar y refinar los procedimientos empleados en la experimentación, incluyendo la eutanasia.[66]

Aspectos culturales

La ética se interesa en lo que es moralmente correcto; sin embargo, la moral mantiene una estrecha relación con la cultura y sociedad. Por ejemplo, algunas personas aceptan fácilmente el uso de ratas en laboratorios, ya que han crecido en un ambiente que muestra antipatía hacia las mismas al asociarlas como vectores transmisores de enfermedades.[67] Davies,[55] menciona que la legislación y estándares sobre el uso y cuidado de animales de laboratorio depende mucho de la cultura y el país del que se hable, y a esto se le conoce como "la guerra cultural" en torno a la ciencia experimental. Haciendo una comparativa entre el Reino Unido (R.U.) y E.U., en el primero la experimentación es centralizada; es decir, el estado es el encargado de otorgar permisos; en contraste, en E.U. cada institución cuenta con un comité, lo que puede significar una eficiencia en tiempos, pero una carencia de completa regulación.[55] Asimismo, la "autoridad epistémica" (quien decide si se realiza un experimento o no) decide en función del criterio de la cultura de la persona y sus prioridades, si son de bienestar animal (R.U.) o de innovación científica y desempeño (E.U.).[55,68] El Acta de Bienestar Animal, publicada en 1966 en E.U., es una muestra de la influencia cultural en la ciencia. En ésta se excluye a los mamíferos del género *Rattus*, del género *Mus*, a las aves y animales de sangre fría, ya que no se consideran *"animales"* al ser especies reproducidas estrictamente para la experimentación, por lo cual su uso no está legislado.[69]

En contraste, cuando se desarrolla la cultura del cuidado de los animales de laboratorio, se consideran las expectativas sociales humanitarias de respeto, y se implementan estrategias que van más allá de seguir meramente lo que dicta la ley.[70]

Aspectos económicos

En la experimentación con animales existe un costo-beneficio cuando a éstos se les maneja de manera ética.[71] En este sentido, la ciencia tiene dos objetivos: ser reproducible (confirmatoria de lo que ya se sabe) y que contribuya a la acumulación de conocimiento (descubrimientos). En el mundo se reporta que se emplean más de 192 millones de animales en la ciencia médica. De estos, aproximadamente 25 millones son empleados en E.U. para llevar a cabo estudios clínicos o desarrollo de fármacos. El subsidio para estas prácticas se calcula hasta en 270 billones de dólares, de los cuales al menos de 13.3 a 23 billones se consideran como desperdicios cuando existen variables o resultados descontrolados.[72] Un diseño experimental que no está bien planeado significa un desaprovechamiento financiero sin significancia que pone en riesgo la salud humana o animal, además de generar sufrimiento innecesario y la pérdida de animales.[73]

En otros análisis sobre el costo de inversiones destinadas a investigaciones, Macleod y Mohan,[54] discuten que alrededor del 85% de los 300 billones de dólares anuales destinados a investigación se desperdician, y sólo 1 de cada 1000 investigadores reportan a detalle las características del diseño experimental.[54] Por otra parte, Van Norman,[74] calcula que el gasto total para las pruebas con animales en el 2018 fue de 7.4 billones para el descubrimiento de fármacos, 11.2 billones para ensayos preclínicos y de seguridad, 58.5 billones para el desarrollo clínico, y 2.3 billones para pruebas centrales en laboratorios.[74] En México, hay 67 laboratorios y bioterios registrados frente a la SADER; del 2000 al 2018 se calcula un total de 2,332,640 animales, del cual el 98% corresponde a roedores (en su mayoría ratas).[75] Si bien no se tiene un cálculo exacto del costo-beneficio de la experimentación, hay registros en los que se llega a invertir hasta 10 millones de pesos en adquirir y comenzar un solo proyecto.[76]

Aspectos religiosos

Aunque la religión no está directamente ligada al uso de animales con fines de investigación, en algunos aspectos puede dictar la manera de realizarse dependiendo del pensamiento. En el hinduismo, constituye una ofensa y delito el emplear animales altamente venerados como las vacas o los búfalos; no obstante, la experimentación con roedores es aceptada, y en estos se recomienda el desangramiento

como un método de eutanasia, algo que no está estipulado por la AVMA.[77] En otras religiones como el budismo y jainismo, consideran a los animales como seres sintientes con el mismo valor que los humanos, una ideología contraria al islamismo, en la que los humanos tienen derecho sobre los animales y ninguna obligación hacia ellos.[78] En la religión cristiana, en 1997 Haraway refirió a los roedores de laboratorio como símbolos de salvación, ya que representan el triunfo del bien sobre el mal (patologías).[67] En ésta se acepta la experimentación con "animales que no vivan dentro del ambiente emocional y doméstico de los humanos", pero se debe realizar de manera ética, evitando su sufrimiento.[79]

Aspectos científicos

Dentro de la misma comunidad científica se encuentra en discusión la validez y el valor predictivo de la experimentación con animales.[80] El fin de la mayoría de las investigaciones es trasladar a medicina humana el conocimiento obtenido. Sin embargo, se ha reportado que extrapolar resultados toxicológicos en animales a humanos llega a tener hasta un 90% de fracaso,[73] esto debido a las diferencias biológicas entre especies,[72] o los factores no controlados (como el estrés o dolor). Pese a ello, las pruebas de toxicidad en animales son un requisito obligatorio en la ciencia desde 1973 y contribuyen a la salud humana.[74]

Bienestar dentro de la experimentación con roedores

El dolor

Uno de los parámetros esenciales en la evaluación del bienestar, y punto clave para el debate sobre el uso de ratas y otros animales para experimentación, es el dolor.[81] Éste se define como *"una experiencia sensorial y emocional asociada con un daño actual o potencial a tejidos"*, en la cual la incapacidad de comunicarlo verbalmente no niega la posibilidad de que se perciba,[82] y es parte de los lineamientos internacionales para el uso y cuidado de los animales de laboratorio, procurando un mínimo dolor y sufrimiento.

En todas las especies empleadas para la ciencia, debe instaurarse una terapia analgésica y debe instaurarse siempre que sea posible,[83] ya que no aliviar el dolor se contrapone con la obligación moral de los investigadores de prevenir el sufrimiento en los animales de laboratorio, y también representa otro factor que puede alterar los biomarcadores o el comportamiento de las especies.[84]

Cuando se inicia una respuesta nociceptiva como parte de la metodología (e.g., cirugía), o como efecto secundario en alguno de los procesos de la investigación, se inicia una cascada de alteraciones orgánicas y bioquímicas, como la activación de los ejes simpático-adreno-medular o el hipotálamo-hipófisis-adrenal, con la consecuente secreción de glucocorticoides y catecolaminas que interrumpen la homeostasis de los animales.[85] La alteración en la función inmune, biológica, neurológica y fisiológica de los roedores puede repercutir en la interpretación de los resultados debido a que se alteran parámetros fisiológicos como la frecuencia cardiaca, frecuencia respiratoria y presión arterial, y biomarcadores como el lactato, citocinas, corticosterona, glucosa, entre otros, que pueden ser resultado del experimento en sí o respuestas adaptativas frente al dolor.[84] Pese a estas consecuencias, en México se reporta que solamente el 45% de los investigadores aplican un método de analgesia durante el experimento o durante la eutanasia.[36]

De esta manera, es esencia implementar diversos biomarcadores, herramientas y tecnologías para identificar de manera temprana y no invasiva los signos de dolor,[83] no solamente durante la vida, sino también con el fin de garantizar la calidad de muerte durante la eutanasia, proceso en el que los diferentes métodos existentes conllevan ventajas y desventajas respecto a la nocicepción.[81]

Eutanasia

La palabra *"eutanasia"* se deriva de las palabras griegas: '*eu'*, bueno; y, '*thanatos'*, muerte.[86] De acuerdo con las directrices internacionales y nacionales sobre la eutanasia en animales de laboratorio, el método ideal debe ser fácil, económico, seguro, y generar una pérdida rápida e irreversible de la conciencia, seguida de paro cardiaco, respiratorio y, finalmente, de una pérdida de la función cerebral con una angustia o dolor mínimo.[11]

Debido a que la eutanasia es el procedimiento más común, el bienestar animal se encuentra íntimamente ligado a dicho procedimiento, ya que las condiciones en las que se realice, influyen en gran medida en las respuestas que el organismo desencadena al término de su vida.[36] Por ello, la elección del método adecuado depende de los objetivos del estudio, la necesidad de minimizar el dolor y/o la angustia de los animales, las pautas y leyes aplicables, así como la capacitación y competencia del personal.[87]

- **Métodos inyectables:** En estos se incluyen la sobredosis con anestésicos, particularmente con pentobarbital.[88] Los barbitúricos son el método más empleado en México (29%), seguido del CO_2 (10%).[36] La vía de administración es intravenosa (IV) al triple de dosis habitual. Induce una transición suave a la inconsciencia y muerte por depresión del sistema nervioso central y los centros respiratorios.[88] La vía intraperitoneal (IP) está aceptada por la AVMA y se recomienda por encima de los anestésicos inhalados (por razones éticas, estéticas, y de eficacia).[3] Algunas desventajas de la IP incluyen el tiempo prolongado de acción, el riesgo de producir irritación y dolor, el costo, y los residuos en la canal del animal.[43]
- **Métodos inhalatorios:** Los principales son el CO_2 y los anestésicos inhalados. Ambos requieren de una cámara de inducción en la que el anestésico se administra en forma de gas o líquido.[43] El CO_2 es uno de los métodos más empleados en laboratorios. Sin embargo, debido a que genera muerte por hipoxia, se han reportado efectos adversos como vocalizaciones de baja frecuencia (asociadas a eventos estresantes) debido a un potencial miedo, ansiedad, dolor

y angustia antes de la inconciencia.[89] Los anestésicos inhalatorios, a pesar de ser una alternativa al CO_2, también presentan limitantes. El isoflurano, por ejemplo, ha mostrado generar aversión en roedores y alterar los procesos celulares en tejidos;[90] por ello, los métodos físicos suelen optarse.

- **Métodos físicos:** En estos se incluyen la decapitación y la dislocación cervical. En la primera, la guillotina interrumpe de manera inmediata el flujo sanguíneo al cerebro, generando anoxia y muerte.[91] Se considera el método de eutanasia más rápido y humano para roedores, sin generar alteraciones bioquímicas, pero se ha sugerido que la actividad cerebral puede preservar segundos después de la decapitación.[92] Por otra parte, la dislocación cervical es el método físico más empleado en México;[36] sin embargo, requiere de entrenamiento para llevarlo a cabo de manera correcta y no generar dolor.[93]

Perspectivas y oportunidades en torno al bienestar de roedores empleados en la investigación

Debido a la obligación moral, ética, cultural, social y científica que los investigadores tienen sobre los roedores de laboratorio, se han adoptado herramientas no invasivas para evaluar el dolor y el bienestar de los animales. En primera instancia, la termografía infrarroja (IRT) es una técnica que detecta la temperatura superficial corporal, derivada de la microcirculación dérmica,[94] y asocia sus cambios a emociones positivas inducidas por el juego, ejercicio o aumento en la actividad metabólica,[95] pero también a estados negativos como dolor, estrés, inflamación, miedo, o procesos de hipotermia o hipertermia, endotoxemia, inflamación, isquemia e hipoperfusión sanguínea, y eficacia analgésica.[96]

Por otro lado, la codificación de las expresiones faciales de los animales ha llevado a desarrollar las *«escalas de muecas"* en ratas (*Rat Grimace Scale*, RGS) y otras especies.[97] La RGS es un sistema de puntuación (del 0 al 2, donde 0 es dolor ausente, 1 es moderado, y 2 es severo) empleado para cuantificar el dolor empleando cuatro Unidades de Acción Facial: 1) Ajuste orbital; 2) Aplanamiento de la nariz y mejillas; 3) Posición de las orejas; y 4) Posición de bigotes.[98]

Otras técnicas no invasivas que se han implementado son cuantificar los cambios en el peso corporal, las concentraciones de metabolitos de cortisol fecal, la presentación de conductas naturales como el acicalamiento y el anidar, y aquellas encaminadas a promover estados positivos,[99,100] como las técnicas de cosquillas en las ratas que imitan el comportamiento de juego heteroespecífico y mejoran su estado mental. De igual manera, los registros de vocalizaciones ultrasónicas han permitido asociar las de alta (~50 kHz) y baja frecuencia (~22 kHz) a estímulos positivos como el juego, o a estímulos negativos como el dolor, respectivamente.[101]

El enriquecimiento ambiental aplicado en las ratas de laboratorio es otra estrategia que mejora la capacidad cognitiva y de aprendizaje; sin embargo, requiere estandarizarse para asegurar su valor científico.[102] Implementar estas nuevas alternativas promueve el bienestar de los animales, refina los procedimientos y, con ello, mejora la calidad de los resultados obtenidos.

CONCLUSIONES

Los roedores, en particular las ratas, son una especie que desde la antigüedad ha contribuido de manera esencial al avance médico mediante la comprensión de la fisiopatología, toxicología, desarrollo de fármacos y técnicas quirúrgicas. Debido a sus trascendentales aportaciones, el empleo de animales se debe realizar considerando factores sociales, culturales, éticos, económicos y científicos, con el fin de minimizar el sufrimiento, evitar el dolor innecesario y preservar la calidad de vida/muerte. Estos factores han contribuido al desarrollo de legislaciones, guías y nuevas estrategias de evaluación no invasiva (como la IRT o la RGS) que contribuyen a garantizar la aplicación de las 3Rs para promover un uso y cuidado apropiado de los roedores de laboratorio.

REFERENCIAS

1. Hobson-West P, Davies A. Societal sentience: constructions of the public in animal research policy and practice. *Sci Technol Human Values* 2018; 43(2): 671-93.
2. Andersen ML, Winter LM. Animal models in biological and biomedical research-experimental and ethical concerns. *Anais Acad Brasil Ci* 2019; 91(1): e20170238.
3. Laferriere CA, Pang DS. Review of intraperitoneal injection of sodium pentobarbital as a method of euthanasia in laboratory rodents. *J Am Assoc Lab Anim* 2020; 59(3): 254-63.
4. Carbone, L. Estimating mouse and rat use in American laboratories by extrapolation from animal welfare act-regulated species. *Sci Rep* 2021; 11(493): 1-6.
5. Kaliste E. The welfare of laboratory animal. United States: Springer; 2007.
6. Lee KH, Lee DW, Kang BC. The 'R' principles in laboratory animal experiments. *Lab Anim Res* 2020; 36(1): 1-3.
7. Franco NH. Animal experiments in biomedical research: A historical perspective. *Animals* 2013; 3(1): 238-73.
8. Marquardt N, Feja M, Hünigen H, Plendl J, Menken L, Fink H, Bert B. Euthanasia of laboratory mice: Are isoflurane and sevoflurane real alternatives to carbon dioxide? *PLoS ONE* 2018; 13(9): 1-29.
9. Fernandes MR, Pedroso AR. Animal experimentation: A look into ethics, welfare and alternative methods. *Rev Assoc Med Bras* 2017; 63: 923-8.
10. Arias-Ramírez DA. Entre el anti-especismo y el derecho de los animales (Aproximaciones para una fundamentación filosófica). *Principia Iuris* 2016; 13(25): 43-64.
11. Juárez-Portilla C, Zepeda-Hernández RC, Sánchez-Salcedo JA, Flores-Muñoz M, López-Franco O, Cortés-Sol A. El uso de los animales en la investigación y en la enseñanza: lineamientos y directrices para su manejo. *Rev Eduscientia* 2019; 2(4): 4-19.
12. Macdonald PA. Acknowledging animal rights: A thomistic perspective. *Am Cathol Philos Q* 2020; 95(1): 95-116.
13. Callanan JJ, Allais L. Kant and Animals. England: Oxford University Press; 2020.
14. Harding JD, Van Hoosier GL, Grieder FB. The contribution of laboratory animals to medical progress- Past, present, and future. En: Hau J, Schapiro SJ (Eds.). Handbook of Laboratory Animal Science. 3rd ed. United States: CRC Press; 2011.
15. Darwin C. La expresión de las emociones en los animales y en el hombre. España: Alianza Editorial; 1872.
16. Herzog HA. Dealing with the animal research controversy. En: Atkins CK, Panicker S, Cunningham CC (Eds.). Laboratory Animals

in Research and Teaching. United States: American Psychological Association; 2005: pp. 9-29.
17. Lyons D. The 1876 cruelty to animals act: protection for animals or animal researchers? En: The Politics of Animal Experimentation. United Kingdom: Palgrave Macmillan; 2013: pp. 113-48.
18. Hajar R. Animal testing and medicine. *Heart Views* 2011; 12(1): 42.
19. Matsuda Y. Recent trends in the number of laboratory animals used in Japan. *Altern Lab Anim* 2004; 32(1): 299-301.
20. Taylor K, Alvarez LR. An estimate of the number of animals used for scientific purposes worldwide in 2015. *Altern Lab Anim* 2019; 47(5-6): 196-213.
21. Speaking of Research (SR). Worldwide animal research statistics (19 de Agosto de 2021). https://speakingofresearch.com/facts/animal-research-statistics.
22. Lasko P, Lüthy K. Investigating rare and ultrarare epilepsy syndromes with Drosophila models. *Fac Rev* 2021; 10: 10.
23. Babac BD, Dulay RMR, Calpito RAS, Domingo MA, Macamos MGM, Mangabat AR, Zoberiaga NZL. In-vitro activity of ethanolic extract of Lentinus strigosus mycelia in N2 wild strain Caenorhabditis elegans–An animal model for obesity and its chemical composition. *J Appl Biol Biotechnol* 2021; 9(1): 41-6.
24. Zang L, Maddison LA, Chen W. Zebrafish as a model for obesity and diabetes. *Front Cell Dev Biol* 2018; 6: 91.
25. Munster VJ, Feldmann F, Williamson BN, van Doremalen N, Pérez-Pérez L, Schulz J, *et al*. Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. *Nature* 2020; 585(7824): 268-72.
26. Shaffer LG. Special issue on canine genetics: animal models for human disease and gene therapies, new discoveries for canine inherited diseases, and standards and guidelines for clinical genetic testing for domestic dogs. *Hum Genet* 2019: 138(5): 437-40.
27. Koolhaas JM. The laboratory rat. En: Hubrecht R, Kirkwood J (Eds.). The UFAW Handbook on the Care and Management of Laboratory and Other Research Animals. 8va ed. United States: Wiley-Blackwell; 2010: pp. 311-26.
28. Mariscal A, Caldarone L, Tikkanen J, Nakajima D, Chen M, Yeung J, *et al*. Pig lung transplant survival model. *Nat Protoc* 2018; 13(8): 1814-28.
29. Ryan KA, Bewley KR, Fotheringham SA, Brown P, Hall Y, Marriott AC, *et al*. Dose-dependent response to infection with SARS-CoV-2 in the ferret model: evidence of protection to re-challenge. *Nat Commun* 2020; 12(1): 81.
30. Esteves PJ, Abrantes J, Baldauf HM, BenMohamed L, Chen Y, Christensen N, *et al*. The wide utility of rabbits as models of human diseases. *Exp Mol Med* 2018; 50(5): 1-10.
31. Cooper TK, Huzella L, Johnson JC, Rojas O, Yellayi S, Sun MG, *et al*. Histology, immunohistochemistry, and in situ hybridization reveal overlooked Ebola virus target tissues in the Ebola virus disease Guinea pig model. *Sci Rep* 2018; 8(1): 1-12.
32. Homberg JR, Wöhr M, Alenina N. Comeback of the rat in biomedical research. *ACS Chem Neurosci* 2017; 8(5): 900-3.
33. Hu Z, Du X, Yang Y, Botchway BO, Fang M. Progesterone and fluoxetine treatments of postpartum depressive-like behavior in rat model. *Cell Biol Int* 2019; 43(5): 539-52.
34. Joyce C, Scallan CD, Mateo R, Belshe RB, Tucker SN, Moore AC. Orally administered adenoviral-based vaccine induces respiratory mucosal memory and protection against RSV infection in cotton rats. *Vaccine* 2018; 36(29): 4265-77.
35. Richter CP. The effects of domestication and selection on the behavior of the Norway rat. *J Natl Cancer Inst* 1954; 15(3): 725-38.
36. Armas LAC, Linares-Márquez P, Acosta-Hernández CC, Laiz BF. Bioética e testes em animais. Uma revisão da praxis científica no México. *Rev Colomb Bioet* 2018; 13(2): 26-35.
37. Wang Y, Zhang T, Zhang Y, Yu Y, Bai F, Zhang H, Chi Y. Effects of inverted photoperiods on the blood pressure and carotid artery of spontaneously hypertensive rats and Wistar-Kyoto rats. *J Hypertens* 2021; 39(5): 871-9.
38. Shull JD, Dennison KL, Check AC, Trentham-Dietz A. Rat models of 17β-estradiol-induced mammary cancer reveal novel insights into breast cancer etiology and prevention. *Physiol Genomics* 2018; 50(3): 215-34.
39. Van Steenis G, Van Wezel AL, Sekhuis VM. Potency testing of killed polio vaccine in rats. *Dev Biol Stand* 1981; 47: 119-28.
40. Bowman CJ, Bouressam M, Campion SN, Cappon GD, Catlin NR, Cutler MW, *et al*. Lack of effects on female fertility and prenatal and postnatal offspring development in rats with BNT162b2, a mRNA-based COVID-19 vaccine. *Reprod Toxicol* 2021; 103: 28-35.
41. Pennant WA, Sciubba DM, Noggle JC, Tyler BM, Tamargo RJ, Jallo GI. Microsurgical removal of intramedullary spinal cord gliomas in a rat spinal cord decreases onset to paresis, an animal model for intramedullary tumor treatment. *Childs Nerv Syst* 2008; 24(8): 901-7.
42. Tao P, Mahalingam M, Zhu J, Moayeri M, Sha J, Lawrence WS, *et al*. A bacteriophage T4 nanoparticle-based dual vaccine against anthrax and plague. *MBio* 2018; 9(5): e01926-18.
43. American Veterinary Medical Association. AVMA Guidelines for euthanasia of animals: 2020 edition. (18 de septiembre de 2021). https://www.avma.org/sites/default/files/2020-01/2020-Euthanasia-Final-1-17-20.pdf
44. Poole T. Happy animals make good science. *Lab Anim* 1997; 31, 116-24.
45. Prescott MJ, Lidster K. Improving quality of science through better animal welfare: the NC3Rs strategy. *Lab Anim* 2017; 46(4): 152-6.
46. Broom DM, Johnson KG. Stress and Animal Welfare: Key Issues in the Biology of Humans and Other Animals. 2nd ed. United Kingdom: *Springer Nature*; 2019.
47. Ward SJ, Hosey G. The need for a convergence of agricultural/laboratory and zoo-based approaches to animal welfare. *J Appl Anim Welf Sci* 2020; 23(4): 484-92.
48. Landoni M. Control of stress and distress. En: Rigalli A, di Loreto VE (Eds.). Experimental Surgical Models in the Laboratory Rat. United States: CRC Press; 2009.
49. Mendes D, Alves C, Batel-Marques F. Number needed to treat (NNT) in clinical literature: an appraisal. *BMC Med* 2017; 15(1): 1-13.
50. Robinson NB, Krieger K, Khan FM, Huffman W, Chang M, Naik A, *et al*. The current state of animal models in research: A review. *Int J Surg* 2019; 72: 9-13.
51. Clark JM. The 3Rs in research: a contemporary approach to replacement, reduction and refinement. *Br J Nutr* 2018; 120(1): 1-7.
52. Ashrafi K, Chang FY, Watts J, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. Genome-wide RNAi analysis of Caenorhabditis elegans fat regulatory genes. *Nature* 2003; 421: 268-72.
53. Brun LRM, di Loreto VE. Bioethics and animal care. En: Rigalli A, di Loreto VE (Eds.). Experimental Surgical Models in The Laboratory Rat. United States: CRC Press; 2009.
54. Macleod M, Mohan S. Reproducibility and rigor in animal-based research. *ILAR J* 2019; 60(1): 17-23.
55. Davies G. Locating the 'culture wars' in laboratory animal research: national constitutions and global competition. *Stud Hist Philos Sci* 2021; 89: 177-87.
56. Wahlman-Calderara T, Meurman JH. Ethics in Biomedical Research. En: Meurman JH (Ed.). Translational Oral Health Research. United States: Springer; 2018: 159-68.
57. Goñi-Balentziaga O, Vila S, Ortega-Saez I, Vegas O, Azkona G. Professional quality of life in research involving laboratory animals. *Animals* 2021(11): 2639.
58. Vasbinder MA, Locke P. Introduction: global laws, regulations, and standards for animals in research. *ILAR J* 2016; 57(3): 261-5.

59. SENASICA. NOM-062-ZOO-1999. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. (30 de septiembre de 2021). http://publico.senasica.gob.mx/?doc=743
60. Akimovna E, Rivera B, Carbone C, Hernández GR, Baamonde JM. Laboratory animal science legislation in Latin America. En: Guillén J (Ed.). Laboratory Animals. Regulations and Recommendations for Global Collaborative Research. United States: Elsevier; 2014: pp. 95-116.
61. Smith AL, Corrow DJ. Modifications to husbandry and housing conditions of laboratory rodents for improved well-being. *ILAR J* 2005; 46(2): 140-7.
62. Wasowicz M, Morice C, Ferrari P, Callebert J, Versaux-Botteri C. Long-term effects of light damage on the retina of albino and pigmented rats. *Invest Ophthalmol Vis Sci* 2002; 43(3): 813-20.
63. Herzog HA. The moral status of mice. *Am Psychol* 1988; 43: 473-4.
64. Newport F, Himelfarb I. In U.S., Record-High Say Gay, Lesbian Relations Morally OK. (19 de septiembre de 2021). https://news.gallup.com/poll/162689/record-high-say-gay-lesbian-relations-morally.aspx.
65. Shapiro K. A rodent for your thoughts: The social construction of animal models. En: Henninger-Voss M (Ed.) Animals in Human Histories. United States: University of Rochester Press; 2002: pp. 439-69.
66. Kong Q, Qin C. Laboratory animal science in China: current status and potential for the adoption of three R alternatives. *Alt Lab Anim* 2010; 38(1): 53-69.
67. Birke L. Who–or What–are the rats (and mice) in the laboratory. *Soc Anim* 2003; 11(3): 207-24.
68. Valverde M. Law's Dream of a Common Knowledge. United States: Princeton University Press, 2009.
69. Paulus-Jagric D. Of Birds, rats and mice: The protection of laboratory animals in US federal law. *Leg Inf Manag* 2006; 6(3): 181-7.
70. Davies G, Greenhough B, Hobson-West P, & Kirk RG. Science, culture, and care in laboratory animal research: Interdisciplinary perspectives on the history and future of the 3Rs. *Sci Tech Hum Values* 2018; 43(4): 1-19.
71. European Parlament. On Directive 86/609 on the protection of animals used for experimental and other scientific purposes (2001/2259(INI)). (10 de septiembre de 2021). https://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//TEXT+REPORT+A5-2002-0387+0+DOC+XML+V0//EN&language=ro
72. Keen J. Wasted money in United States biomedical and agricultural animal research. En: Herrmann K, Jayne K (Eds.). *Animal Experimentation: Working Toward a Paradigm Change.* Netherlands: Brill; 2019.
73. Kenehan S. The moral status of animal research subjects in industry: A stakeholder analysis. En: Herrmann K, Jayne K (Eds.). *Animal Experimentation: Working Towards a Paradigm Change.* Netherlands: Brill; 2010: pp. 209-23.
74. Van Norman GA. Limitations of animal studies for predicting toxicity in clinical trials: is it time to rethink our current approach? *J Am Coll Cardiol Basic Trans Science* 2019; 4(7): 845-54.
75. Gil G, Rodríguez X. Bioterios en México. 20 años de caos y riesgo. (30 de septiembre de 2021). https://www.connectas.org/especiales/bioterios-en-mexico/
76. Crail, A. La desgracia de los cuyos. 10 millones en animales para experimentación. (30 de septiembre de 2021). https://www.m-x.com.mx/al-dia/la-desgracia-de-los-cuyos-mexico-gasta-10-millones-en-animales-para-experimentacion
77. Qadri SS, Ramachandra SG. *Laws, regulations, and guidelines governing research animal Care and use in India.* En: Laboratory animals. United States: Academic Press; 2018: 237-61.
78. Ottuh P, Idjakpo OG. Animal Care and Ethics in Contemporary Religious Debates. *J Sosialisasi* 2021; 1(1): 128-41.
79. Massaro A. Understanding animal research in the light of Christian animal ethics. *Marb J Relig* 2020; 22(2): 1-11.
80. Singh VP, Pratap K, Sinha J, Desiraju K, Bahal D, Kukreti R. Critical evaluation of challenges and future use of animals in experimentation for biomedical research. *Int J Immunopathol Pharmacol* 2016; 29(4): 51-61.
81. Couto M, Cates C. Laboratory guidelines for animal care. En: Pelegri F (Ed). Vertebrate Embryogenesis. United States: Springer; 2019: 407-30.
82. Raja SN, Carr DB, Cohen M, Finnerup NB, Flor H, Gibson S, *et al*. The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. *Pain* 2020; 161(9): 1976-82.
83. Morton DB, Griffiths PH. Guidelines on the recognition of pain, distress and discomfort in experimental animals and a hypothesis for assessment. *Vet Rec* 1985; 116(16): 431-6.
84. Peterson NC, Nunamaker EA, Turner PV. To treat or not to treat: the effects of pain on experimental parameters. *Comp Med* 2017; 67(6): 469-82.
85. Hernández-Avalos I, Flores-Gasca E, Mota-Rojas D, Casas-Alvarado A, Miranda-Cortés AE, Domínguez-Oliva A. Neurobiology of anesthetic-surgical stress and induced behavioral changes in dogs and cats: A review. *Vet World* 2021; 14(2): 393.
86. Valentim AM, Guedes SR, Pereira AM, Antunes LM. Euthanasia using gaseous agents in laboratory rodents. *Lab Anim* 2016; 50(4): 241-53.
87. Shomer NH, Allen-Worthington KH, Hickman DL, Jonnalagadda M, Newsome JT, Slate AR, *et al*. Review of rodent euthanasia methods. *J Am Assoc Lab Anim Sci* 2020; 59(3): 242-53.
88. Shearer JK. Euthanasia of cattle: Practical considerations and application. Animals 2018; 8(4): 57.
89. Améndola L, Weary DM. Understanding rat emotional responses to CO_2. *Transl Psychiatry* 2020; 10(1): 1-12.
90. Bekhbat M, Merrill L, Kelly SD, Lee VK, Neigh GN. Brief anesthesia by isoflurane alters plasma corticosterone levels distinctly in male and female rats: Implications for tissue collection methods. *Behav Bran Res* 2016; 305: 122-5.
91. Pierozan P, Jernerén F, Ransome Y, Karlsson O. The choice of euthanasia method affects metabolic serum biomarkers. *Basci Clin Pharmacol Toxicol* 2017; 121(2): 113-8.
92. Rijn CMV, Krijnen H, Menting-Hermeling S, Coenen AM. Decapitation in rats: latency to unconsciousness and the 'wave of death'. *PloS One* 2011; 6(1): e16514.
93. Carbone L, Carbone ET, Yi EM, Bauer DB, Lindstrom KA, Parker JM, *et al*. Assessing cervical dislocation as a humane euthanasia method in mice. *J Am Assoc Lab Anim* 2012; 51(3): 352-6.
94. Casas-Alvarado A, Mota-Rojas D, Hernández-Avalos I, Mora-Medina P, Olmos-Hernández A, Verduzco-Mendoza A, Reyes-Sotelo B, Martínez-Burnes J. Advances in infrares thermography: Surgical aspects, vascular changes, and pain monitoring in veterinary medicine. *J Therm Biol* 2020; 92: 102664.
95. Weitkamp J. Effect of tickling and gentling on eye and tail temperature of laboratory rats during manual restraint, using infrared thermography (Master's thesis). Utrecht University, 2020.
96. Mota-Rojas D, Olmos-Hernández A, Verduzco-Mendoza A, Lecona-Butrón H, Martínez-Burnes J, Mora-Medina P, Orihuela A. Infrared thermal imaging associated with pain in laboratory animals. *Exp Anim* 2021; 70(1): 1-12.
97. Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, *et al*. The Rat Grimace Scale: A partially automated method for quantifying pain in the laboratory rat via facial expressions. *Mol Pain* 2011; 7: 55.

98. Sperry MM, Yu YH, Welch RL, Granquist EJ, Winkelstein BA. Grading facial expression is a sensitive means to detect grimace differences in orofacial pain in a rat model. *Sci Rep* 2018; 8(1): 13894.
99. Abdelrahman A, Kumstel S, Zhang X, Liebig M, Wendt EHU, Eichberg J, *et al*. A novel multi-parametric analysis of non-invasive methods to assess animal distress during chronic pancreatitis. *Sci Rep* 2019; 9(1): 1-13.
100. Turner PV. Moving Beyond the Absence of Pain and Distress: Focusing on Positive Animal Welfare. *ILAR J* 2020; ilaa017: 1-7.
101. Coffey KR, Marx RG, Neumaier JF. DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations. *Neuropsychopharmacology* 2019; 44(5): 859-68.
102. Cortese GP, Olin A, O'Riordan K, Hullinger R, Burger C. Environmental enrichment improves hippocampal function in aged rats by enhancing learning and memory, LTP, and mGluR5-Homer1c activity. *Neurobiol Aging* 2018; 63: 1-11.

2.2. CAPÍTULO II

Artículo de revisión intitulado:
Importancia de los modelos animales en la investigación biomédica: conocimientos y aplicaciones actuales

Publicado en la revista Animals, misma que se encuentra indexada al JCR con un factor de impacto de 3, en el volumen 13, número 7, páginas 1223.

Domínguez-Oliva, A.; Hernández-Ávalos, I.; Martínez-Burnes, J.; Olmos-Hernández, A.; Verduzco-Mendoza, A.; Mota-Rojas, D. The Importance of Animal Models in Biomedical Research: Current Insights and Applications. *Animals* 2023, *13*(7), 1223. https://doi.org/10.3390/ani13071223. https://www.mdpi.com/2076-2615/13/7/1223

animals

MDPI

Review

The Importance of Animal Models in Biomedical Research: Current Insights and Applications

Adriana Domínguez-Oliva [1], Ismael Hernández-Ávalos [2], Julio Martínez-Burnes [3], Adriana Olmos-Hernández [4], Antonio Verduzco-Mendoza [4] and Daniel Mota-Rojas [5,*]

1 Master's Program in Agricultural and Livestock Sciences [Maestría en Ciencias Agropecuarias], Xochimilco Campus, Universidad Autónoma Metropolitana (UAM), Mexico City 04960, Mexico
2 Clinical Pharmacology and Veterinary Anesthesia, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México (UNAM), Cuautitlán 54714, Mexico
3 Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Tamaulipas, Victoria City 87000, Mexico
4 Division of Biotechnology—Bioterio and Experimental Surgery, Instituto Nacional de Rehabilitación-Luis, Guillermo Ibarra Ibarra (INR-LGII), Mexico City 14389, Mexico
5 Neurophysiology, Behavior and Animal Welfare Assessment, DPAA, Universidad Autónoma Metropolitana (UAM), Mexico City 04960, Mexico
* Correspondence: dmota100@yahoo.com.mx

Simple Summary: The present review highlights and examines the importance of animal models in relevant topics concerning current human and animal health. Over the past five years, different animal species have been used to study pandemics, such as the 2019 Coronavirus, diabetes, and obesity. Through murine, primate, porcine, and even aquatic models (e.g., zebrafish), several neurological, behavioral, cardiovascular, and oncological disorders are being understood while developing new therapeutic approaches. Nematodes and arthropods are some of the new alternatives for biomedical science; however, regardless of the species, many animal research studies show the vital role of animal models in advancing biomedical research.

Abstract: Animal research is considered a key element in advance of biomedical science. Although its use is controversial and raises ethical challenges, the contribution of animal models in medicine is essential for understanding the physiopathology and novel treatment alternatives for several animal and human diseases. Current pandemics' pathology, such as the 2019 Coronavirus disease, has been studied in primate, rodent, and porcine models to recognize infection routes and develop therapeutic protocols. Worldwide issues such as diabetes, obesity, neurological disorders, pain, rehabilitation medicine, and surgical techniques require studying the process in different animal species before testing them on humans. Due to their relevance, this article aims to discuss the importance of animal models in diverse lines of biomedical research by analyzing the contributions of the various species utilized in science over the past five years about key topics concerning human and animal health.

Keywords: translational research; animal research; laboratory animals; rodents; primates; pigs; zebrafish; nematodes

Citation: Domínguez-Oliva, A.; Hernández-Ávalos, I.; Martínez-Burnes, J.; Olmos-Hernández, A.; Verduzco-Mendoza, A.; Mota-Rojas, D. The Importance of Animal Models in Biomedical Research: Current Insights and Applications. *Animals* **2023**, *13*, 1223. https://doi.org/10.3390/ani13071223

Academic Editor: Garikoitz Azkona

Received: 21 February 2023
Revised: 19 March 2023
Accepted: 30 March 2023
Published: 31 March 2023

1. Introduction

The use of animals in scientific research is controversial [1]. However, the transformation of medicine from an art to a science can be mainly attributed to using a wide range of animal models [2], selected according to their functional and genetic characteristics for specific research lines [3]. Animal models contribute significantly to the advance of biomedical science through their meaningful contributions to our growing understanding of pathological and biological processes [4]. Moreover, they enable the development and testing of drugs, vaccines, and surgical techniques applicable to human and veterinary medicine [5].

The term "animal model" comes from the Latin *animae* (alma or spirit) and the word model, which means to imitate or be similar to [6]. Animal models are based on the principle of comparative medicine [7] as instruments that can replicate physiological and pathological processes [8]. The species is selected according to each project's objective and hypothesis [3] but also considers biological, anatomical, functional, and genetic similarities to humans or other animals [6]. Today, most of the species utilized in biomedical research are rodents [9], as they are deemed ideal models for studying pathologies that affect human populations due to their physiological homology [10], which allows them to be employed to further our understanding of such processes as sepsis, obesity, cancer, organ transplants, and biological development, among many others [11,12].

The species used in experimentation are not limited to small mammals. Rhesus monkeys (*Macaca mulata*) are utilized to study high-priority diseases such as the pandemic caused by the severe, acute respiratory syndrome type 2 coronavirus (SARS-CoV-2) [13]. Domestic pigs (*Sus scrofa*) are crucial for organ transplant medicine and immune therapies [14]. New species, including some invertebrates such as fruit flies (*Drosophila melanogaster*), are used to study neurological disorders such as epilepsy [15], nematodes such as *Caenorhabditis elegans* to study obesity [16], and aquatic models, such as the zebrafish (*Danio rerio*), to treat metabolic disorders, including diabetes [17].

The broad range of species used in research has brought exponential advances in medicine, especially with the introduction of genetically modified (transgenic) animals [18] and the implementation of supporting technologies such as nanotechnology and artificial intelligence [19]. In light of this, this article aims to discuss the importance of animal models in diverse lines of biomedical research by analyzing the contributions of the various species utilized in science over the past five years concerning key topics of human and animal health.

2. Search Methodology

The literature search was performed in the Web of Science, Scopus, and PubMed. Keywords related to the use of animal models applied to current research priorities were searched to select the relevant articles, for example, "emerging infectious disease", "diabetes and obesity", "neurodegenerative diseases", "pain therapies", "surgical techniques", "cancer models", and "alternative animal models". The search was limited to articles published in English in the last five years (2019–2023) and related to human and non-human medicine and therapeutics.

3. A Review of Animal Experimentation

Animal models are essential for several biomedical research fields such as cancer biology and therapeutics, neuroscience, pharmacology and toxicology, neurobiology of diseases, endocrinology, public health, palliative medicine, also, in studies in human and animal biology and for the discovery and testing of new drugs, vaccines, and other biologicals (e.g., antibodies, hormones) whose validation requires preclinical studies in animals [6,20]. Currently, these models address current research priorities, considered as those imposing major global threats to human and animal health. These include diseases that have afflicted humankind or increased exponentially in recent years such as SARS-CoV-2, different types of cancer and their therapy, cardiovascular diseases, metabolic and neurodegenerative disorders, and experimental refinement of surgical techniques to treat these issues [21]. The models may involve complete animals or only particular cells, tissues, organs, genes, or other agents that reproduce pathological processes (Figure 1) [8,22]. Species include rats, mice, guinea pigs, dogs, rabbits, birds, ruminants (cows, sheep), horses, fish, frogs, monkeys, cats, reptiles, squid, crabs, bees, chimpanzees, hamsters, sea slugs, pigs, nematodes (roundworm), fruit flies, and protozoans, among others [7].

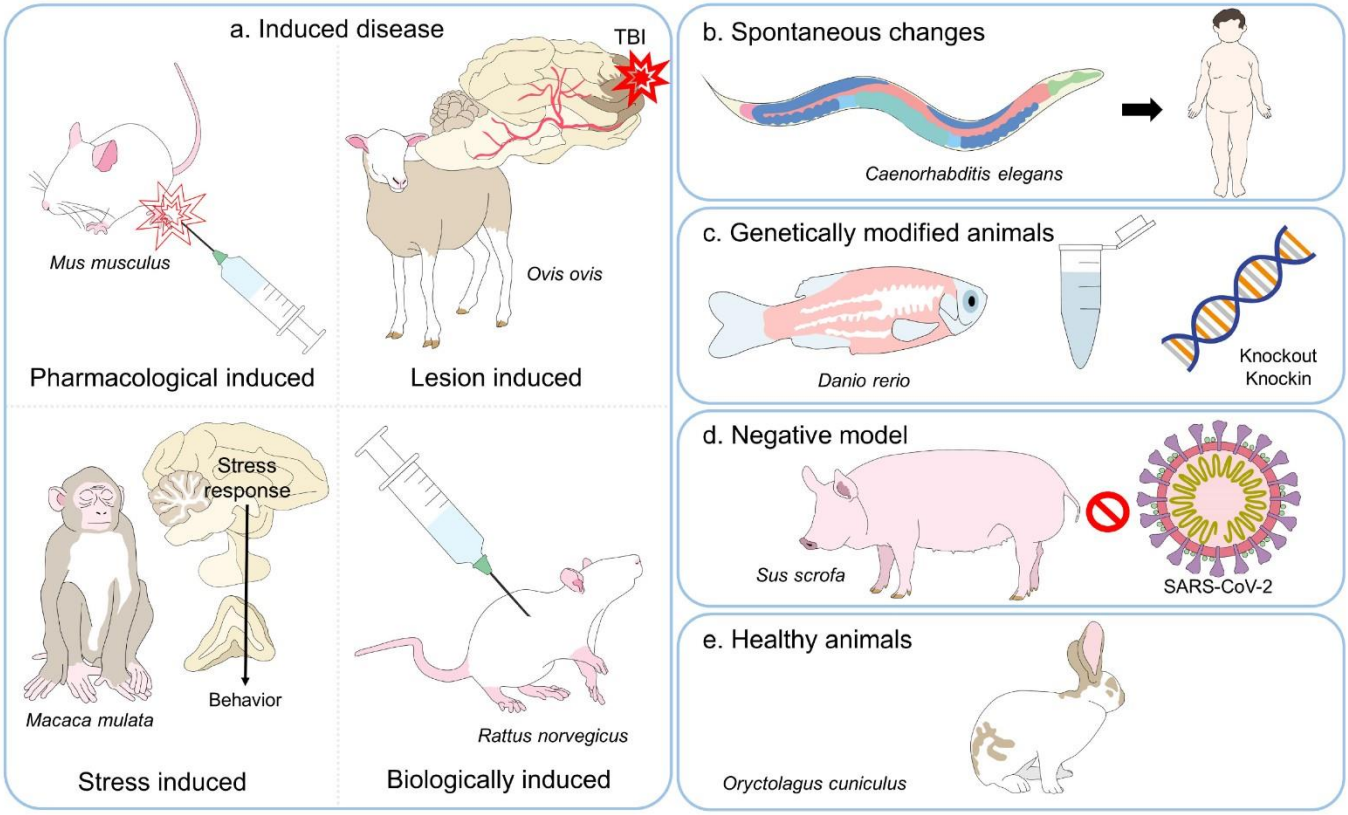


Figure 1. Classification of various animal models. The animals used in science can be divided into five broad types. (**a**) The main ones are models in which animals are induced to present a pathology similar to one that affects humans or other animals by administering drugs or other biologicals, inflicting injuries, or subjecting them to stress or other environmental conditions. In contrast, models based on spontaneous changes (**b**) include animals where the normal course of their life predisposes them to develop a specific disease. (**c**) Genetically-modified test subjects are animals with knockin or knockout genes or proteins. In contrast to using healthy animals (**e**), negative models (**d**) employ individuals that are not susceptible to certain diseases but serve to evaluate susceptibility to a specific pathology. TBI: traumatic brain injury.

The importance of animals in medical science is reflected, for example, in the percentage of Nobel Prizes studies in Physiology or Medicine using animal models (90%) [5]. From 1901 to 2020, two-thirds of those awards (186 of 222 projects [7]) employed animal models to understand pathogenic mechanisms, metabolic diseases, diagnostic and therapeutic procedures, develop vaccines, or test the efficacy of novel drugs [22]. At least 144 species used in those animal-based studies were mammals, and 42% were rodents [7]. Dogs were the first animal model used in metabolic research on gastric secretions [23] and for discovering insulin [24]. To date, rodents are the predominant species in research (Table 1) [9]. However, non-mammal species are trending, and the number of animals depends on the country and its legal regulation regarding the use and reporting of animals in research. Moreover, in some countries, there is no official annual report on animal research (e.g., South America), and not every country counts the same animals (e.g., the United States does not consider rats, mice, fish, birds, amphibians, reptiles, and cephalopods, they are not covered by the Animal Welfare Act). Although it might differ, Tables 1 and 2 show an overview of the use of animals according to species in some countries and a summary of the reported statistics worldwide.

Table 1. Overview of the number of animals used in research, according to the species.

Country	Year	Specie	Percentage (%)	Reference
European Union	2019	Rodents	61.9	[25]
		Fish	24.6	
		Birds	6.2	
		Amphibians	0.5	
		Cephalopods	0.2	
		Dogs	0.1	
		Non-human primates	0.07	
		Other mammals	6.5	
Canada	2020	Birds	50.0	[26]
		Rodents	24.5	
		Fish	11.7	
		Cattle	11.3	
		Amphibians	1.1	
		Pigs	0.4	
		Dogs	0.2	
		Non-human primates	0.1	
		Reptiles	0.1	
		Other animals	0.5	
United Kingdom [1]	2021	Mice	68.2	[27]
		Fish	12.9	
		Rats	6.5	
		Birds	8	
		Dogs	0.14	
		Non-human primates	0.09	
		Cats	0.01	
		Other animals	3.3	
United States [2]	2019	Guinea pigs	23	[28]
		Rabbits	18	
		Hamsters	12	
		Non-human primates	9	
		Dogs	7	
		Pigs	6	
		Cats	2	
		Sheep	2	
		Other species	21	
South Korea	2017	Rodents	91.8	[29]
		Fish	3.3	
		Birds	2.3	
		Rabbits	1	
		Non-human primates	0.08	
		Amphibians	0.07	
		Other species	1.21	
		Total	3,085,259	

[1] Excluding Northern Ireland; [2] Rats, mice, fish, birds, amphibians, reptiles, and cephalopods are not included.

Table 2. Approximate of the number of animals used in research worldwide between 2019–2020.

Country	Number of Animals	References
United States	20,000,000–24,000,000	[30–32]
China	16,000,000	
Japan	11,000,000	
European Union	9,400,000	
Australia	6,700,000	
Canada	5,067,778	
South Korea	4,141,433	
United Kingdom	3,300,000	
Norway	2,282,710	
Germany	2,151,805	
France	1,865,403	
Spain	761,012	
Mexico	685,315	
Switzerland	556,107	
Belgium	437,275	
New Zealand	240,000	

Several Nobel Prizes have been awarded for animal research, and the increasing number of animal models in different countries demonstrates these studies' importance for scientific advancement [7]. However, just as necessary, their use also entails ethical challenges that require surveillance through laws, norms, guides, and strict bioethical committees to monitor the use and care of laboratory animals based on the principles of the 3Rs [33]. In this regard, for 50 years, the National Center for the Replacement, Reduction, and Refinement of Animals in Research (NC3Rs) has promoted Russel and Burch's initiative of the 3Rs to reduce, replace, and refine procedures to improve the conditions of animals used in experimental protocols [34].

These norms differ from one nation to the next. However, one guide recognized internationally is ARRIVE (Animal Research: Reporting of in vivo Experiments), developed in 2010 to improve the in vivo experiments description to increase the reproducibility of results, refine the stages of study design, and clearly report the methods so they can be repeated and tested [35]. A second guide is PREPARE (Planning Research and Experimental Procedures on Animals), which seeks to determine and guarantee quality control in animal studies [36]. Today, for any experimental protocol requiring animals, proposals such as the Animal Study Registry (ASR) help researchers thoroughly plan their study design, methods, and statistical analyses to ensure transparency and reproducibility in their results [37]. Additionally, it is essential to mention that Ethic Committees must approve current experimental protocols within each institute to promote an appropriate use and care for animals in research.

Animal models certainly provide valuable information on the nature of diseases [38]. However, it is important to remember that inter-species limitations exist in anatomy, metabolism, physiology, and genetics [39], so a single preclinical model cannot represent all aspects of pathogenesis due to differences in resistance or susceptibility [38]. Currently, many animals used in biomedical studies undergo some genetic modification, such as transgenesis or the utilization of knockout or knockin genes, to visualize specific changes that would take years to develop under normal conditions [40]. Therefore, the selection of the animals depends on the specific research field; through their use, researchers develop scientific knowledge focused on human and veterinary medicine.

4. Animal Models and Their Application in Distinct Fields of Current Biomedical Science

4.1. Emerging Infectious Diseases

The SARS-CoV-2 virus is the etiologic agent of the coronavirus 2019 disease (COVID-19) [41]. This disease has claimed the lives of over 6.3 million people worldwide since

2019 [42,43]. The lack of knowledge of this virus and its rapid propagation at the onset of the pandemic made it essential to determine its physiopathology and identify therapeutic agents and vaccines that could mitigate its threatening consequences. These fundamental issues were solved using in vivo assays that replicated the virus in animals to untangle its pathogenesis, the immune response, and the adverse effects that might result from the vaccines and therapies proposed before testing in humans and their release to the public [41,44].

The choice of an animal model that would allow researchers to observe the histopathological, radiological, or immune changes that the virus caused required that the test animals be susceptible to lung tissue damage and capable of developing an inflammatory process [45]. Potential species included nonhuman primates, ferrets, rats, mice, Syrian hamsters, lagomorphs, minks, cats, camelids, and even zebrafish [46].

The transgenic mice can express the human angiotensin-converting enzyme II (hACE2), a functional receptor for the SARS-CoV-2 virus that mimics clinical signs observed in humans [47]. Sun et al.'s [48] research with 4.5–30-week-old transgenic mice successfully replicated the virus after intranasal and intragastric inoculation. It led to the discovery of viral loads in the lung, trachea, brain, and feces. Those authors also detected an immune and inflammatory response due to the presence of interleukins (IL). Adult mice showed more lesions in the alveolar epithelial cells, focal pulmonary hemorrhage, and more significant apoptosis of macrophages. Those findings concurred with human reports showing that COVID-19 affected older adults more severely, with the over-65 population representing 80% of all hospitalizations and a 23-fold greater risk of mortality. Reports emphasized clinical signs, such as respiratory distress and cytokine release syndromes [49]. Studies with Syrian hamsters found that while the virus is lung-tropic and infects the respiratory tract by binding to the ACE2 cell surface in the alveoli, causing pneumonia in 67% of the animals, the gastrointestinal signs reported in humans are due to viral replication and dissemination in enterocytes [50].

One animal model that shares multiple similarities with humans for the physiopathology of the SARS-CoV-2 virus is based on Rhesus macaques, African green monkeys (*Chlorocebus aethiops*), and crab-eating macaques (*Cynomolgus macaques*) [51]. The latter has been utilized to replicate the infection conditions in young (males and females of 3–9 years) and old-aged animals (23–29 years-old females). After intranasal and intratracheal viral inoculations, researchers found that nasal swabs (peak viral load of 10^6 copies/μL) had higher viral loads than pharynx and rectal ones (a maximum of 10^4 copies/μL). Additionally, viruses from nasal and pharynx samples were detected for longer periods in elderly monkeys [52]. This relation between age and disease mortality was also reported in Rhesus monkeys. Comparative studies of three nonhuman primates (three 3–5 years and two 15 years old macaques) infected intratracheally revealed that the viral replication detected by nasopharyngeal and anal swabs was persistently detected from 3 days post-infection (dpi) to 11 dpi in elderly animals. In older macaques, 104–107.5 copies/mL were also detected (while young individuals had approximately 104 copies/mL), often accompanied by the development of diffuse severe interstitial pneumonia [53].

The reinfection processes prevalent in human populations were replicated in studies with *C. aethiops*. Infection in six animals caused signs such as fever (50%), hypercapnia (66%), 2–7-fold increases in C-reactive protein concentrations (100%), and coagulopathy (100%) were recorded. That research proved that anal, oral, and nasal swabs could detect viral loads up to 15 dpi [44]. These findings are similar to those from other works with *M. mulata*, where viral RNA was found in swabs from the nose, pharynx, and anus, with amounts increasing up to 3 dpi (in an approximate range of 4–7 copies/mL) [53]. These nonhuman primate models undoubtedly contributed significantly to our understanding of the pathogenicity of COVID-19 and the physiological bases for implementing preventive and diagnostic measures and treatment.

Another important aspect of using animals is that they helped understand the transmission of the virus to other domestic species and showed that pets could acquire the

SARS-CoV-2 virus through contact with an infected human. However, there is no evidence of active pet-to-human transmission [54]. Studies with dogs, pigs, chickens, and ducks showed they were not susceptible to COVID-19 infection due to low viral replication [55]. Identifying susceptible species made it possible to choose appropriate models for developing and testing vaccines [55]. Ferrets, Syrian hamsters, rabbits, transgenic mice [47], and cats were all found to be susceptible, the latter even vulnerable to airborne transmission with the development of clinical signs such as hair loss and pulmonary alterations similar to those seen in humans [56,57]. Apart from domestic cats, wild felines (tigers, lions, pumas, snow leopards) [58] have been reported to show infections by this virus. Kang et al. [59], who reported the first Delta variant (SARS-CoV-2 Delta) case in three domestic cats with COVID-19-positive owners in China, insist that transmission to pets is a topic of concern due to their possible role as silent intermediate hosts.

4.2. Endocrinology and Metabolic Pathologies

Obesity is a public health problem affecting over 600 million people worldwide [60]. Obesity and its associated metabolic syndromes have consequences such as knee osteoarthritis, a disease prevalent in approximately 60% of the overweight population [61], but this is also associated with cancer, cardiovascular disease, hypertension, coronary artery disease, stroke, sleep apnea, asthma, gallstones, steatohepatitis, and dyslipidemia. Over one-third of the world's overweight or obese population is at risk of developing type 2 diabetes mellitus [23]. Using rodent models, researchers have determined that one element that promotes the development of type 2 diabetes mellitus is adipose tissue inflammation due to insulin resistance and excess fat mass [62]. The increase in the presentation of these comorbidities has led to the use of animal models to test new, improved strategies for reducing the incidence of this disease.

The role of the different types of adipose tissue in humans and animals is a crucial line of research that has developed with the use of rodents. For example, adipogenesis suppression and the browning of white adipose tissue (WAT) [63] have been suggested as strategies for preventing obesity [60]. The browning process creates a brown adipose-like tissue (BAT) that can participate in thermogenesis by transforming caloric intake into heat [64]. Since this is part of a central nervous system response to cold, certain medications and exercise can trigger browning as has been observed in obese and lean rats subjected to high-intensity training. In C57BL/6J mice, the transformation of beige adipocytes into WAT can be promoted with diets complemented with resveratrol for 16 weeks, as this induces a change in the intestinal microbiota in treated animals ($p < 0.01$) (increasing microorganisms of the genera *Bacteroides*, *Lachnospiraceae*, *Blautia*, *Lachnoclostridium*, and *Parabacteroides*, among others) that modulates lipid metabolism and has anti-inflammatory properties and anti-obesity effects [65].

The importance of physical activity in treating these conditions has been demonstrated in experiments with 48 Sprague-Dawley male rats, where aerobic exercise for 12 weeks combined with prebiotic fiber supplementation prevented knee joint damage, dyslipidemia, endotoxemia and normalized the effects of insulin resistance ($p < 0.001$) [61]. Studies with these supplements as part of a therapeutic protocol in Wistar rats, administered in presentations such as yogurt, have shown that supplementation with 5% of yogurt reduces levels of oxidative stress (significant decreases in NO levels, $p < 0.05$), and had fewer amounts of inflammatory cell infiltration and collagen deposits in the liver ($p < 0.05$) when compared to animals fed high-fat diets. According to these studies, this supplement could be a potential human therapeutic option [66].

Studies of the human genome have identified hundreds of genetic variants associated with obesity and opened the way to examining these genes in species such as *C. elegans*, a nematode capable of storing fat in the form of lipid droplets inside hypodermal and intestinal cells. *C. elegans* has 14 genes that promote diet-induced obesity and three that prevent it [67]. Those genes are now recognized as potential targets for anti-obesity treatment. Ke et al. [68] found that the knockdown of 23 fat-storing not only reduced excessive fat accumulation

but also improved the health and lifespan of this species ($p < 0.05$). The inhibitory effect of flavonoids such as butein on lipogenesis in *C. elegans* succeeded in reducing triglyceride levels by up to 27% without altering food intake or energy expenditure, an effect due to the downregulation of proteins involved in lipid metabolism [69]. Likewise, the appetite suppressant effect of administering vegetable extracts from the *Lentinus strigosus* mushroom (300 and 1000 μg/mL) to *C. elegans* functioned as a natural means of preventing obesity [70]. Studies of this kind allow researchers to address obesity as a complex pathology affected by diverse factors: diet, physical activity, developmental stage, age, genes, and environmental interaction [67].

Another animal species considered a promising model for studying metabolic syndromes is the zebrafish (*D. rerio*). This species has genetic homology with humans, so through genetic mutation, chemical induction, and changes in diet, they can be used to study hyperglycemia, obesity, diabetes, and hypertriglyceridemia [71]. Pigs, meanwhile, share similarities with humans in terms of organ size, lifespan, anatomy, physiology, and metabolic profile [40]. A study of obesity in Iberian pigs showed the pathogenesis of chronic kidney disease caused by overweight and obesity. Although the administration of high-fat diets did not generate diabetes in those pigs by day 100, analyses revealed hypercholesterolemia (142 ± 27 mg/dl), hypertriglyceridemia (75 ± 43), insulin resistance, and glomerular hyperfiltration [72]. These effects also occur in humans [73] and have been studied in obese male mice and ovariectomized females [74].

The domestic dog has been postulated as a valuable model for studying chronic morbidities brought on by environmental conditions since they share morbidity and mortality factors with humans. In this field, Hoffman et al. [75] reported that comorbidities behind chronic conditions such as obesity, arthritis, hypothyroidism, and diabetes reported in humans were also present in 73,835 canines and that those dogs showed a positive association between age and the number of morbidities ($p < 0.001$). Other studies have revealed that obesity in dogs (137/198) is closely linked to the alimentary habits of their owners, finding that the 79.8% of dogs from overweight owners (114 persons) were obese ($p < 0.001$) [76]. Therefore, studies of these animals could provide information on disease interaction.

4.3. Cancer in Biomedicine

According to the World Health Organization [77] and the National Cancer Institute [77,78], the most common types of cancer in humans in 2020 were breast (2.26 million cases), lung (2.21 million), colorectal (1.93 million), prostate (1.41 million), skin (1.20 million), and stomach (1.09 million). These cancers cause 10 million deaths per year. Projections for 2022 estimate that around 1,918,030 new cancer cases will be diagnosed in the United States, with 350 cancer-induced deaths per day, making this disease a primary cause of mortality [79]. The pathogeny of these cancers and testing new treatment options is another field that extensively uses animal models. Over 95% of studies use rats and mice to inject cancer cell lines subcutaneously, study the primary cancer lesion, and follow its growth before excising tumors [80,81]. However, one disadvantage of this subcutaneous tumor model, is that injections in athymic nude mice may not accurately represent the interaction among tumor cells, local stroma, and the tumor's microenvironment, depending on its precise location [82]. Contrarily, orthotopic murine models have been shown to replicate the tumor microenvironment –including metastasis– when inoculated in the original anatomical site of the tumor. In female BALB7c mice, inoculation of mammary cancer cell line 4T1 as a fat pad tumor model showed that 50% of the animals had metastasis to the ovaries, spleen, liver, and sternum. However, when compared to a heterotopic model, orthotopic tumors were smaller (1993.7 ± 197.15 mm^3 vs. 1078.4 ± 300.26 mm^3, $p < 0.05$) and had a significantly lower percentage of infiltrating cells ($p < 0.05$) [83]. Moreover, these orthotropic models, together with in vivo optical metabolic imaging, are proposed as an approach to studying how, for example, the fatty acid uptake by breast cancer cells increases accordingly to tumor aggressiveness and metastatic process ($p < 0.05$) [84] Attacking this complication

in tumor development is the principal objective of anticancer therapies, since most deaths from prostate cancer, for example, are due to metastasis into bone structures [80].

Koosha et al. [85] used diosmetin, an anti-tumorigenic, in colon cancer xenografts in 24 male nude mice. Results showed that tumor volume in the group treated with 100 mg/kg of diosmetin was significantly smaller than in the untreated group (264 ± 238.3 vs. 1428 ± 459.6 mm^3, $p < 0.01$). Promisingly, the drug did not produce toxicity even when administered at high doses. Studies of this kind show that laboratory animals allow researchers to test new drugs and better understand disease development but also aid in determining non-toxic doses that can be applied to humans or animals. Using these models as translational media for studying cancer has also revealed the importance of identifying the pain that animals may experience. Pain assessment is important in in human medicine and laboratory animal welfare. In this regard, recognizing degrees of cancer-induced bone pain has been studied by observing behavioral changes in rats and mice, where innate behaviors, such as burrowing, are reduced 9 days after inoculation when compared to control groups ($p < 0.05$) as a result of the nociception associated with the degree of severity of cancer due to reduced bone density [86].

The fact that the canine and human genomes share a high degree of similarity (75%) and that the risks of death due to neoplastic, congenital, and metabolic diseases are comparable means that the dog is an ideal translational model for studying human morbidity and mortality [75,87]. For example, the percentage of neoplasia is similar between dogs and humans (27.4 vs. 25.3%). However, because the types of cancer that affect each species correlate only marginally (Spearman rank $p = 0.661$) [75], dogs have been replaced in many preclinical studies by genetically-modified pigs [87].

Another novel anticancer strategy involves managing nerve-tumor interaction [88] since tumor-specific denervation can suppress neoplasia growth [89]. A study by Kamiya et al. [90] with female Balb/c-nu mice and the use of xenografts in Hras128 rats in a model of chemically-induced breast cancer showed that sympathetic stimulation of the nerves in tumors accelerated cancer growth but that parasympathetic stimulation reduced growth and downregulated the expression of programmed death. In contrast, in the case of late-stage colorectal cancer, parasympathetic denervation via vagotomy and atropine administration in 150 male Wistar rats reduced the incidence of tumors and their weight and volume after eight weeks, as well as cell proliferation, angiogenesis, and regulated expression of the nerve growth factor [89].

These neural anticancer therapies in humans and animals indicate that while sympathetic nerves show cancer-promoting effects in prostate and breast cancer, and melanoma cases, the parasympathetic/vagal nerves are believed to trigger both reactions. For example, vagal nerves can promote prostate, gastric, and colorectal cancers, but suppress breast and pancreatic cancers, due to β-adrenergic and muscarinic effects that modify the behavior of cancer cells, angiogenesis, tumor-associated macrophages, and antitumor immunity [88]. The axonogenesis process in species such as mice, linked to the development of metastasis in breast cancer, showed through immunofluorescence that nerve twigs tend to be sympathetic-like, with no expression of parasympathetic fibers [91].

In addition to the support of laboratory techniques such as immunofluorescence, non-invasive diagnostic methods are a priority in oncology. In immunocompetent genetically-engineered mouse models, Kirkpatrick et al. [92] utilized nanosensors with urine tests to detect protease activity in diverse types of cancer, including lung cancer, achieving 100% specificity and 81% sensitivity. In this way, monitoring with nanosensors and clinical assays in animals has demonstrated that this technique can be an option for conducting accurate, radiation-free diagnostic tests.

Nanoparticles and their application, together with in vivo imaging, can help to test novel luminescent particles and assess their tissue penetration to improve cancer therapy [93]. In vivo imaging enables us to understand tumor growth-related processes such as oxidative mitochondrial metabolism in mouse models with cell lung cancer [94]. Likewise, in a mouse model of brain tumor –glioblastoma– under general anesthesia, modified in vivo

optical imaging (Surface enhanced spatially offset Raman scattering) covers the inability of conventional techniques that rely on subcutaneous inoculation of cancerous cells because they cannot read deep tissues [95]. These techniques are the basis for imaging-guided phototherapies that are a current research field to find agents capable of inducing tumor cell apoptosis, such as photodynamic y and photothermal therapy [96].

4.4. Pharmacology and Therapeutics

Parallel to the advances in our knowledge of the physiopathology of diverse conditions, developing and testing new therapeutic options is another field destined for animal models. Algology is a science in constant actualization to provide new and efficient drugs to prevent the consequences of pain by reducing the number and severity of secondary effects in both human and veterinary patients [97,98]. Adequate models are needed to evaluate analgesic efficacy accurately. In the case of treatments for open wounds, Parra et al. [99] applied carprofen (5 mg/kg) and buprenorphine (0.1 mg/kg) to the left hind paw of Sprague Dawley rats of both sexes using a punch biopsy to assess analgesia in an open wound model. Using four behavioral tests associated with aspects of nociception, mechanical and thermal stimulation, guarding behavior, and the weight-bearing test, they found that carprofen promoted recovery of the thermal response to basal levels after just 2 h. The same rat species were utilized to test the renal and gastrointestinal safety of non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen by administering single and multiple oral doses to pediatric patients. Furthermore, the necropsies performed on pigs of different ages (8-week-old and 6-to-7-months-old) in the study by Millecam et al. [100] revealed no severe lesions in the stomach after multiple doses of ibuprofen at 5 mg/kg. However, significant histological score differences ($p < 0.025$) were observed in the duodenum (1.38 vs. 4) and jejunum (3.63 vs. 1.25) between the experimental and control group. Additionally, an increased clearance time for the drug after multiple doses was found, an effect similar to reports in human pediatric patients.

Due to the adverse effects that NSAIDs can generate, especially for treating chronic afflictions such as arthritis and cancer, opioids are another therapeutic option [101]. However, since the long-term use of these drugs is also associated with complications, research has begun to new concepts and explore directions. The opioid-free anesthesia technique was introduced to prevent tolerance and hyperalgesia and reduce the use of these drugs in the postoperative period. This method uses agents such as alpha-2-agonists, ketamine, and local analgesics with distinct action mechanisms in multimodal analgesia [102–104]. Other new opioid-based pharmacological options are transdermal patches impregnated with morphine-like compounds. In 6–12-week-old C57BL/6JJmsSlc mice, patches synthesized with two new opioids (new-opioids 1 and 2, N1 at 3 mg/kg; N2 at 10 mg/kg) showed the same analgesic efficacy as morphine at 3 mg/kg. The effect remained constant, even under repeated administration (in contrast to fentanyl), and the cutaneous trans-permeability rate was greater, at 1.71 ± 0.35 and 3.94 ± 1.36 µg/cm/h [105]. The administration of opioid nanoparticles has also been suggested to prevent opioid tolerance and reduce the severity of adverse effects. Leucine-enkephalin hydrochloride-based nanoparticles with a size of 100–200 nm have been tested in male Sprague Dawley rats by applying them intranasally, reaching the brain directly. After dosing, high concentrations were found in the olfactory bulb and cerebrum between the first 60 min (approximately 80 ng/g and 160 ng/g, respectively), while plasma concentrations were not detected at any evaluation time ($p < 0.0001$). This prevents the side effects of drug transit through peripheral pathways [106].

Techniques based on local anesthesia temporarily relieve pain by inhibiting nerve impulse transmission. However, when used to complement multimodal analgesia protocols, they can be associated with neurotoxicity in both human and veterinary patients [107]. Administration via polymer-based encapsulation is a new strategy designed to prevent toxicity and permit the prolonged release of the active ingredient to give a long-term analgesic effect for up to seven days [107]. A ketamine-polymer-based drug was applied transdermally to Wistar rats to determine its analgesic effects [98]. Results of the tail-flick

test and readings from an analgesiometer led them to determine a significant analgesic effect ($p < 0.01$) maintained for 24 h with a peak effect at 8 h and a response time on the test 5.72 s vs. a basal time of 2.44 s. The compound did not produce irritation when tested on rabbit skin. It prevented the secondary effects of intravenous, nasal, or oral administration, so it is a potential option for treating neuropathic pain [108].

4.5. Experimental Surgical Tecniques

In addition to developing novel drugs, advances in surgical technology and techniques have opened fields in microsurgery in human and animal medicine since the 1900s when Carrel and Guthrie performed the first transplants in dogs [109]. Later, in 1950–1960, Buncke and Schultz tested the first microsurgery techniques using models of digital amputations and reimplantation in Rhesus monkeys, performing vascular microsurgery to restore circulatory connections successfully [110]. Anastomosis of 1-mm blood vessels in the ears of adult rabbits by reimplantation was the first demonstration of microsurgery in reconstructive medicine [111].

Today, rodents are considered models for reimplanting extremities and restoring blood vessels because their vascularization is homologous to the human finger [112]. For example, developing heterotrophic osteomyocutaneus flap transplant protocols in Lewis rats furthered our understanding of the mechanisms and pathways involved in the immune response underlying tissue transplant rejection [113]. Likewise, in an experiment with five syngeneic mice and allografts—using a donor-supplied aorta and inferior vena cava—end-to-end anastomosis of those structures showed a 74% success rate as a technique for hind limb transplants [114]. In another study, Tee et al. [115] performed grafts of engineered cardiac muscle flaps in the epicardium of 8 rats. The flaps were transplanted by microsurgery to resolve one of the first limitations: failed vascular anastomosis. Those researchers performed successful end-to-end anastomosis of the carotid artery and jugular vein by placing the flap on the epicardium, achieving a survival rate of 75% during 4 weeks post-surgery, with viable cardiomyocytes and vascular connections between the flap and the epicardium by week 10 [115]. These techniques, tested first in animals, were later used with human patients with coronary artery disease caused by diseases such as squamous cell carcinoma, with a 96% survival rate of the flap in individuals subjected to neck and head surgery [116].

Another advance in biomedicine achieved thanks to experimental work with animal species such as pigs are based on animal-to-human organ transplants. On 7 January 2022, Bartley Griffith's team performed the first heart transplant from a genetically-modified pig to a 57-year-old human patient with terminal heart disease [117]. Although the patient's condition who received that xenotransplant deteriorated two months after surgery, and he died, the procedure set an important precedent. It showed the need to continue research on genetically-engineered animal organs and immunosuppressor drugs since the immune response and organ rejection are still the leading causes of transplant failure, especially when the organs come from other animal species [118].

Due to the physiological similarity between nonhuman primates and humans, procedures for organ transplants are often tested in those species. Over seven years, Lee et al. [119] performed 22 xenotransplants using hearts from transgenic pigs eliminating alpha-galactosidase transferase knockout or expression of the regulatory proteins CD46, CD39, or CD73 in Cynomolgus monkeys (*Macaca fascicularis*). Results showed that survival of the grafts was significantly higher in hearts with double or triple genetic manipulation (11.63 ± 11.29 days vs. 30.83 ± 20.34 days, $p = 0.03$). This is similar to the report by Cui et al. [120] on triple knockout cells from pigs (that do not express any of the three carbohydrate xenoantigens). The complement-dependent cytotoxicity response and the amount of anti-pig IgG/IgM immunoglobulins (Ig) were evaluated in serum from 72 specific pathogen-free (SPF) baboons and in human serum. Results for humans and old-world monkeys showed similar antibody binding, but the cytotoxicity measured in IgM and IgG was lower in the humans ($p < 0.05$ vs. $p < 0.01$).

Observations on the immunosuppressor response to compounds such as anti-thymocyte globulin (20 mg/kg) and rituximab (20 mg/kg) demonstrate that, in addition to the use of transgenic animals, a strict immunosuppressor regimen is a critical element in allotransplants [119]. In this regard, drugs injected in nanoparticles such as mycophenolate mofetil allow low-water soluble compounds to be combined with other compounds and administered as solid lipid nanoparticles to improve their absorption and release by as much as 68% in acid media [121].

In this field, sustained release options such as nanoparticle-anchoring hydrogel scaffolds of the immunosuppressant tacrolimus allowed the localized release of the drug with tissue regeneration in nude female mice or those of the BALB/c line that were given the drug in the hind limb. Those combinations allowed the sustained release of 77% of the drug, without toxicity, within 28 days at <100 ng/mL [122]. Thus, refining these drugs in the future will make it possible to reduce the cases of organ rejection due to the immune response. This finding is significant because their benefits are not accompanied by systemic toxicity, complications, or dose reduction without pharmacological efficacy [123].

4.6. Neurosciences

The field of neuroscience includes surgical and therapeutic procedures involving the central nervous system and conducts studies focused on specific diseases or pathologies of that system. With the discovery of neurological sequelae in COVID-19-infected patients, animal models have allowed researchers to observe the effects that the SARS-CoV-2 virus generates in sporadic cases, including epileptic seizures and encephalitis with a mortality rate of approximately 5.3% [124].

Estimates suggest that approximately 42 million people worldwide suffer brain injuries annually and that 80% of cases are classified as traumatic brain injury (TBI). Animal models based on rodent species are being used to improve our understanding of the physiopathology of TBI [125], though authors such as Vink [126] caution that neuroanatomical differences in the mouse's lissencephalic brain can generate biomechanical responses distinct from those in humans. Moreover, the replication of trauma may be greater in rodents since traumatisms in these animals tend to generate focal instead of diffuse lesions [127]. Grovola et al. [128] used male Yucatán miniature pigs to analyze neurological dysfunction in animals with mild traumatism 1-year postevent. They found a persistent neuroimmune response in animals with morphological changes to the microglia, with increased branches and junctions per cell ($p = 0.026$ and $p = 0.045$, respectively). In other research, models of medullar lesions are widely utilized with species such as rats, which are particularly important because between 236 and 1009 per million humans annually suffer a spinal cord injury [129]. Although this species is the one most often employed to replicate medullar damage, Filipp et al. [129] affirm that between-species differences (quadrupeds, bipeds) must be considered when evaluating the neuroplasticity of the spinal neurons.

Epilepsy is one of the most common neurological conditions, affecting over 50 million people worldwide [130] and 0.6–0.75% of the domestic canine population [131]. Recent studies of the physiopathology of this disorder and the testing of anti-seizure drugs have used fruit flies (*D. melanogaster*) because they manifest seizure-like behavior and share 70% of their genes with humans [15]. The use of the endocannabinoid anandamide (at 2, 20, and 200 µg/mL) in *Drosophilas* prevented induced seizures ($p < 0.0001$). This led to the discovery that the action mechanism of their metabolites is not linked to the cannabinoid receptors but, instead, to transient potential receptors (TRP). This makes the fruit fly a suitable medium for studying this type of drug [132].

Despite its nature and supposed organic simplicity, *Drosophila* has been used to understand the neurobiological bases of processes still considered mysteries by biology, such as sleep, plasticity, and memory [133]. After studying 12,000 exemplars of *D. melanogaster*, Toda et al. [134] reported the existence of the "nemuri" gene, a peptide with antimicrobial properties that favors sleep and helps these flies survive the infection. This suggests that its function could be linked to the immune competence of the sleep process in animals

and humans. The association of sleep with long-term memory, known as post-learning sleep, was studied by Lei et al. [135], who found a neural circuit that excites the mushroom body neurons and a connection to the fan-shaped ventral neurons that promotes post-learning sleep during courtship. This finding underlined the association between the longer learning experience and the reinforcement of long-term memory, mechanisms sometimes found in mammals.

Neuroscience techniques applied to species such as nonhuman primates and transgenic models of those species have recently been proposed as useful for studying human evolution and the cerebral functioning of people with autism disorders and neurodegenerative diseases such as Alzheimer's [136]. In humans, Alzheimer's disease is considered the most common neurodegenerative disease accounting for around 80% of cases of dementia worldwide [137]. It is widely recognized that mitochondrial dysfunction is an event that precedes the onset of Alzheimer's, and this has been studied in two lines of mice (APPswe/PSEN1 ΔE9 and C57BL/6J). There, the alteration of mitochondrial homeostasis and increased mitochondrial calcium levels caused damage and neuronal death ($p < 0.0001$) due to deposits of amyloid plaques. Recognition of this physiopathology helped scientists establish the goal of preventing this process as a novel therapeutic approach [138].

Another neurogenerative disease, Parkinson's, has been studied primarily with murine models [139]. Recently, however, researchers recognized that the zebrafish shares more neuroanatomical traits with humans and that mutations of the PARK7 gene in adult fish were associated with the development of Parkinson's in humans [140,141]. Exposure of zebrafish larvae to neurotoxins that act directly on the dopaminergic neurons constitutes a method to mimic the phenotype of Parkinson's disease. Specifically, the MPP+ neurotoxin affected the locomotor function (total distance and velocity) of fish, reducing its performance by 80% and 85%, respectively ($p < 0.001$). Furthermore, no systemic effects were observed, presenting a condition similar to Parkinson's [142].

Palliative treatments to control movement disorders such as dystonia, Huntington's, and Parkinson's disease have also been tested in zebrafish [143]. Treatment of Parkinsonian embryos with substances such as rosmarinic acid (RA) prevents the loss of dopaminergic neurons due to neurotoxicity. This acid has been proposed as a neuroprotector and antioxidant that reduces locomotor deficits measured, for example, by increasing the swimming distance in zebrafish treated with RA at concentrations of 10 or 100 μm (approximately 130 to 150 cm, $p < 0.01$) [144]. Similarly, it has been suggested that herbal medicines based on Tongtian oral liquid have neuroprotective and antioxidant properties. The administration of Tongtian to zebrafish prevented neurotoxicity and the degeneration of dopaminergic neurons ($p < 0.01$ when compared to non-treated fish) while reducing larval behavioral impairment measured as improvements in the total distance (peak distance around 180 cm) and velocity (peak values around 3.5 cm/s) ($p < 0.001$) [145].

Aquatic models are also utilized to study other neurodevelopmental problems, such as autism spectrum disorder in zebrafish and Medaka fish (*Oryzas celebensis*) [146]. Chen et al. [147] found that prenatal exposure to valproic acid (at 5 and 50 μM) in AB lines of zebrafish produced embryos and larvae with signs similar to those seen in autistic humans, including hyperactivity, manifested in a greater frequency of tail-bending, greater distances traveled after touching of the dorsal tail ($p < 0.001$, $p < 0.05$), increased swimming speed under both light and dark conditions, and deficient social interaction, anxiety, and macrocephaly, all as consequences of neuronal cerebral cell proliferation. In a separate study, when applied to 28 neonate rat pups, this acid generated oxidative stress in the cerebellar hemispheres and reduced the count and nuclear size of the Purkinje cells [148]. These findings appeared, as well, in the brains of children with this condition. In the case of rats, administering grape seed extract served as a neuroprotector thanks to its antioxidant effect.

Referring to neurodegenerative disorders, a key strategy is to improve symptomatology through physiotherapy and rehabilitation protocols, another line of research that has

increased in importance due to the prevalence of neurological conditions that can affect the quality of life of both humans and animals.

4.7. Physiotherapy and Rehabilitation

Because the number of neurodegenerative and traumatic diseases in humans and animals has been increasing in recent years, one of the main options for these cases is developing and implementing physiotherapy techniques. For example, stimulation of the lateral cerebellar nucleus with low-intensity ultrasound is a non-invasive therapy for reducing the consequences of cerebrovascular accidents in mice after induced ischemic stroke. In those test animals, functional asymmetry of the brain was restored, and pathological electrical cerebral delta activity was reduced, leading to improved performance on the beam-walking test [149].

In cases of osteoarthritis, for example, transcutaneous electrical nerve stimulation techniques (TENS) in physiotherapy protocols utilized in male Sprague Dawley rats with induced pain showed that when applied to the knee joint for 20 min a day for two weeks, TENS reduced the expression of c-fos ($p < 0.05$) (a biomarker of pain) on the day following the intervention (7302.80 $\pm$ 152.40% vs. 5074.50 $\pm$ 199.50%) in all the test animals that, in addition to TENS, did exercise on a treadmill (7333.40 $\pm$ 156.70% vs. 2790.00 $\pm$ 111.88%) [150]. In canine patients, functional neurorehabilitation after Hansen type I intervertebral disc surgery has been tested using a technique with bases similar to TENS called transcutaneous electrical spinal cord stimulation (TESCS). Combined with pharmacological treatment (4-AP) for 90 days, this approach restored ambulation in 88% of 16 animals thanks to the so-called multimodal neurorehabilitation protocol in a study by Martins et al. [151].

In human medicine, TENS has been used with patients with knee osteoarthritis. It improved performance on the stair-climbing test by 0.41 s [152] and reduced pain in individuals with head and neck cancer who had received radiation and developed oral mucositis with the pain. In those patients, 30 min of high-frequency TENS functioned as a non-pharmacological intervention that reduced pain levels at rest by approximately 3.0 from visit #1 to visit #3, as measured by the McGill Pain Questionnaire. However, this approach did not show results for controlling functional pain [153]. Pain reduction allowed the patients to exercise the limb and prevent the loss of mass, muscular strength, and joint instability with some cartilage recovery.

Electroacupuncture is a similar technique used to control chronic inflammatory pain. The action mechanisms of this technique have been studied in murine models after administering the complete Freund's adjuvant to the hind paw. In those animals, electroacupuncture produced analgesia by attenuating neuronal signaling in the dorsal ganglia of the spinal cord, the anterior cingulate cortex, and neurons of the somatosensorial cortex. This suggests that the analgesia generated affects cortical pain pathways and means that the somatosensorial and anterior cingulate cortices may be potential therapeutic targets for developing new options for pain management [154], one of the principal objectives of rehabilitative medicine in humans and animals.

5. New Models and Strategies Applied in Animal Research

The use of poorly developed or unconventional species is expanding to other areas of biomedicine. For example, the zebrafish is used to study anomalies in limbs and craniofacial regions [155]. In those fish, Bergen et al. [156] found 604 genes associated with processes of the formation, mineralization, and regeneration of scales, which demonstrated that those structures are reminiscent of bone. Mutations of these genes in humans generate bone mineralization disease. This suggests that scales could be a model for studying the pathogenesis of skeletal diseases, calcification, and matrix formation [156]. In another fish species –Medaka, the Japanese rice fish (*Oryzias latipes*)– researchers found that the electrocardiogram pattern was more similar to that of humans than those of rats and mice. This led authors such as Yonekura et al. [157] to use it as a model for testing cardiovascular therapies and the response of action potentials to verapamil, which causes bradycardia, an effect also seen in humans [158].

In addition to the use of mammals such as domesticated dogs as models for research on urinary pathologies due to their anatomical and physiological similarity to humans [159], the diverse species that have been incorporated into biomedical science include protozoans, platyhelminths, planarians, cnidarians, bivalve mollusks, gastropods, cephalopods, annelids such as the tardigrades, and arthropods such as hexapods, crustaceans, arachnids, and various insects in studies in broad fields of investigation [160]. In dermo-cosmetology, extraction of hyaluronic acid from mollusks such as *Mytilus galloprovincialis* and *Crassostrea gigas* to treat wounds in Wistar rats accelerated the processes of wound repair and re-epithelization, allowing lesions to heal completely within 15 days of treatment, in contrast to the results attained with commercial healing creams [161]. Another application of a cephalopod (*Octopus vulguris*) is in reconstructive medicine due to its capacity to regenerate nerves and adjacent tissues such as muscle and blood vessels. Despite these technical advances s in medical research, additional studies are required to determine markers, antibodies, and imaging techniques designed to take advantage of those species [162].

Non-animal alternatives such as cell cultures, 3D tissue cultures or organs-on-chips, mathematical models, stem cells, bioprinting, in silico tests, and advanced computer simulations have been increasing in recent years [163]. In leading research countries and regions such as the United States, United Kingdom, China, Germany, Japan, Canada, and Australia, among others [164], there has been a particular interest in replacing animal models with another methods. This is promoted by ethical pressures, the 3Rs initiative, and official instances such as the National Institute of Health [165]. An example of this is the new US law sponsored by the Food and Drug Administration (FDA), which states that drugs no longer require animal testing before human clinical trials [166]. Another example could be Canada and the statistics regarding the number of rats and fish used as animal models from 2019 to 2020. In 2019, rats and fish went from 3.9% and 19.9% to 2.6% and 11.7%, respectively [26,167].

When mentioning tissue engineering, the so-called "organoids"—transplantable tissues created by engineering—have raised expectations for replacing animals, resolving specific bioethical issues by making the study of pathologies and drug testing more specialized [168]. Protocols for head and neck squamous carcinoma have been published, using patient-derived organoids to study therapeutic agents and their drug sensitivity [169]. However, as materials that depend on in vitro handling and do not come from organisms that provide blood flow or the biochemical conditions of a live individual, their development and clinical application require further advances, not only in medicine but also in applicable biotechnologies [168]. Current trials aim to establish the vascularization of organoids, such as in human brains [170] or kidney organoids., In vitro culturing under millifluidic chips and endothelial cells is an alternative to creating vascular networks that need future studies but can be an option to research nephropathies [171]. Complex vascular networks made with mesodermal progenitor cells by Wörsdörfer et al. [172] replicated the ultrastructure of a blood vessel in tumor organoids with endothelial cell junctions, luminal caveolae, microvesicles, and antiangiogenic responsiveness to stimuli. Moreover, 3D bioprinting of organoids derived from stem cells (e.g., ectoderm, mesoderm, and endoderm) is another alternative to replicate developmental diseases in the brain, skin, kidney, heart, intestine, lung, and liver [173]. Those biotechnological advances include approaches in which animal models are accompanied by artificial intelligence [174].

The support that robotics and artificial intelligence provide to the advance of science has improved the technologies involved in techniques of robot-assisted, minimally-invasive surgery [175]. Recently, machine learning techniques have been used with animal models to help diagnose or identify specific behavioral or physiological changes in species. In this regard, models of Parkinson's disease in zebrafish have used video recording to teach the machine to differentiate between a movement disorder and a parkinsonian fish, a technique that may apply to cases of motor diseases in humans [140]. Deep learning algorithms, a type of machine learning, are another approach to the future of biomedical science, particularly in diagnosing a wide range of diseases. Based on CT images, it has been tested in hepato-

cellular carcinomas [176] and COVID-19 diagnosis, showing 85.2% accuracy a specificity and sensitivity of 88 and 87%, respectively [177]. A similar accuracy percentage (91%) was also obtained when testing deep learning to identify genetic syndromes according to facial features [178]. In veterinary medicine, automatizing facial recognition to assess pain is a current approach applied in cats, with an accuracy above 72% [179]. These applications suggest that new diagnostic tools might not require animal models. Nonetheless, implementing these technologies depends on their ability to simulate the physiology of a live organism, especially humans, to improve the replicability of results [180].

The replicability of animal models in preclinical protocols depends on their internal and external validity for transposing results to humans. However, the complexity of some human conditions and the physiological differences among species have led authors such as Pound and Ritskes-Hoitinga [181] to recommend focusing on techniques and technologies prioritizing human research. However, it is important to remember that experimentation with human subjects involves many serious ethical and legal controversies such as those surrounding experimentation with nonhuman primates [182]. One ethical way to deal with this topic consists in establishing and following norms and guidelines such as the 3R principles that promote the rational and humane use of laboratory species [33].

In summary, important advances in human and veterinary medicine have been mainly achieved thanks to animal species that allow us to improve our understanding of the etiology, pathology, physiology, and toxicology of diverse conditions that affect both humans and nonhuman animals [5]. However, using these species requires evaluating ethical considerations, existing limitations, the options available, earlier studies, and, above all, focusing on the welfare of laboratory species to fully recognize their enormous contributions to science.

6. Conclusions

Animal models—including a broad diversity of species of vertebrates and invertebrates—are a key element for experimental research aimed at replicating human and animal pathologies. Over the past five years, significant advances regarding worldwide priority diseases such as COVID-19, breast cancer, diabetes, obesity, and Parkinson, among others, were made in species such as nonhuman primates, rodents, lagomorphs, dogs, pigs, and even invertebrates such as zebrafish and nematodes. Moreover, before human clinical trials, novel therapeutic drugs, diagnostic techniques, and surgical procedures such as flaps or organ transplants have also been refined in animals.

These examples show the importance of using animals in biomedical research to study emerging or poorly understood human and animal diseases, and development of novel therapeutic options, including nanoparticles and in vivo techniques. Although animals will remain an essential element of science in the near future, due to their remarkable contributions, the ethical aspect of animal experimentation is significant.

The ethical pressure and the application of initiatives to reduce and replace the number of animals used in experimental protocols is leading to new strategies such as genetic engineering, artificial intelligence, organs-on-chips, mathematical models, bioprinting of organs, and advanced machine learning technologies. This multimodal approach is considered the best option for addressing the ethical dilemmas raised by using laboratory animals while emphasizing their valuable contributions to human and animal medicine.

Author Contributions: All the authors contributed to the conceptualization, writing, reading, and approval of the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Davies, G.; Gorman, R.; Greenhough, B.; Hobson-West, P.; Kirk, R.G.W.; Message, R.; Myelnikov, D.; Palmer, A.; Roe, E.; Ashall, V.; et al. Animal research nexus: A new approach to the connections between science, health and animal welfare. *Med. Humanit.* **2020**, *46*, 499–511. [CrossRef] [PubMed]
2. Hobson-West, P.; Davies, A. Societal Sentience: Constructions of the public in animal research policy and practice. *Sci. Technol. Hum. Values* **2018**, *43*, 671–693. [CrossRef] [PubMed]
3. Robinson, N.B.; Krieger, K.; Khan, F.M.; Huffman, W.; Chang, M.; Naik, A.; Yongle, R.; Hameed, I.; Krieger, K.; Girardi, L.N.; et al. The current state of animal models in research: A review. *Int. J. Surg.* **2019**, *72*, 9–13. [CrossRef]
4. Lee, K.H.; Lee, D.W.; Kang, B.C. The 'R' principles in laboratory animal experiments. *Lab. Anim. Res.* **2020**, *36*, 45. [CrossRef]
5. Andersen, M.L.; Winter, L.M.F. Animal models in biological and biomedical research—Experimental and ethical concerns. *An. Acad. Bras. Cienc.* **2019**, *91*, e20170238. [CrossRef]
6. Juárez-Portilla, C.; Zepeda-Hernández, R.C.; Sánchez-Salcedo, J.A.; Flores-Muñoz, M.; López-Franco, Ó.; Cortés-Sol, A. El uso de los animales en la investigación y en la enseñanza: Lineamientos y directrices para su manejo. *Rev. Eduscientia. Divulg. Cienc. Educ.* **2019**, *2*, 4–19.
7. Jota Baptista, C.V.; Faustino-Rocha, A.I.; Oliveira, P.A. Animal models in pharmacology: A brief history awarding the nobel prizes for physiology or medicine. *Pharmacology* **2021**, *106*, 356–368. [CrossRef] [PubMed]
8. Swearengen, J.R. Choosing the right animal model for infectious disease research. *Anim. Model. Exp. Med.* **2018**, *1*, 100–108. [CrossRef]
9. Makowska, I.J.; Weary, D.M. A good life for laboratory rodents? *ILAR J.* **2020**, *60*, 373–388. [CrossRef]
10. Carbone, L. Estimating mouse and rat use in American laboratories by extrapolation from Animal Welfare Act-regulated species. *Sci. Rep.* **2021**, *11*, 493. [CrossRef]
11. Smith, J.R.; Bolton, E.R.; Dwinell, M.R. The rat: A model used in biomedical research. In *Rat Genomics*; Hayman, T., Smith, J.R., Dwinell, M.R., Shimoyama, M., Eds.; Springer: Hertfordshire, UK, 2019; pp. 1–41.
12. Joyce, C.; Scallan, C.D.; Mateo, R.; Belshe, R.B.; Tucker, S.N.; Moore, A.C. Orally administered adenoviral-based vaccine induces respiratory mucosal memory and protection against RSV infection in cotton rats. *Vaccine* **2018**, *36*, 4265–4277. [CrossRef] [PubMed]
13. Munster, V.J.; Feldmann, F.; Williamson, B.N.; van Doremalen, N.; Pérez-Pérez, L.; Schulz, J.; Meade-White, K.; Okumura, A.; Callison, J.; Brumbaugh, B.; et al. Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. *Nature* **2020**, *585*, 268–272. [CrossRef] [PubMed]
14. Mariscal, A.; Caldarone, L.; Tikkanen, J.; Nakajima, D.; Chen, M.; Yeung, J.; Cypel, M.; Liu, M.; Keshavjee, S. Pig lung transplant survival model. *Nat. Protoc.* **2018**, *13*, 1814–1828. [CrossRef] [PubMed]
15. Lasko, P.; Lüthy, K. Investigating rare and ultrarare epilepsy syndromes with Drosophila models. *Fac. Rev.* **2021**, *10*, 10. [CrossRef]
16. Babac, B.D.; Milton Dulay, R.R.; Arwen Calpito, R.S.; Domingo, M.A.; Grace Macamos, M.M.; Mangabat, A.R.; Zhyra Zoberiaga, N.L. In-vitro activity of ethanolic extract of Lentinus strigosus mycelia in N2 wild strain Caenorhabditis elegans-An animal model for obesity and its chemical composition. *J. Appl. Biol. Biotech.* **2021**, *9*, 41–46. [CrossRef]
17. Zang, L.; Maddison, L.A.; Chen, W. Zebrafish as a Model for Obesity and Diabetes. *Front. Cell Dev. Biol.* **2018**, *6*, 91. [CrossRef]
18. Fernandes, M.R.; Pedroso, A.R. Animal experimentation: A look into ethics, welfare and alternative methods. *Rev. Assoc. Med. Bras.* **2017**, *63*, 923–928. [CrossRef]
19. Adir, O.; Poley, M.; Chen, G.; Froim, S.; Krinsky, N.; Shklover, J.; Shainsky-Roitman, J.; Lammers, T.; Schroeder, A. Integrating artificial intelligence and nanotechnology for precision cancer medicine. *Adv. Mater.* **2020**, *32*, 1901989. [CrossRef]
20. Bale, T.L.; Abel, T.; Akil, H.; Carlezon, W.A.; Moghaddam, B.; Nestler, E.J.; Ressler, K.J.; Thompson, S.M. The critical importance of basic animal research for neuropsychiatric disorders. *Neuropsychopharmacology* **2019**, *44*, 1349–1353. [CrossRef]
21. Zeggini, E.; Baumann, M.; Götz, M.; Herzig, S.; Hrabe de Angelis, M.; Tschöp, M.H. Biomedical research goes viral: Dangers and opportunities. *Cell* **2020**, *181*, 1189–1193. [CrossRef]
22. Okechukwu, I.B. Introductory Chapter: Animal models for human diseases, a major contributor to modern medicine. In *Experimental Animal Models of Human Diseases—An Effective Therapeutic Strategy*; Batholomew, I., Ed.; InTech: London, UK, 2018; pp. 3–10.
23. Kleinert, M.; Clemmensen, C.; Hofmann, S.M.; Moore, M.C.; Renner, S.; Woods, S.C.; Huypens, P.; Beckers, J.; de Angelis, M.H.; Schürmann, A.; et al. Animal models of obesity and diabetes mellitus. *Nat. Rev. Endocrinol.* **2018**, *14*, 140–162. [CrossRef]
24. Wright, J.R. Frederick Banting's actual great idea: The role of fetal bovine islets in the discovery of insulin. *Islets* **2021**, *13*, 121–133. [CrossRef]
25. Understanding Animal Research. EU-Wide Animal Research Statistics. Available online: https://www.understandinganimalresearch.org.uk/news/eu-wide-animal-research-statistics-2019 (accessed on 9 March 2023).
26. Canadian Council on Animal Care. CCAC Animal Data Report 2020. Available online: https://speakingofresearch.files.wordpress.com/2021/11/canada-2020.pdf (accessed on 9 March 2020).
27. Speaking of Research. UK Animal Research Statistics. Available online: https://speakingofresearch.com/facts/uk-statistics/ (accessed on 9 March 2023).
28. Speaking of Research. US animal Research Statistics. Available online: https://speakingofresearch.com/facts/statistics/ (accessed on 9 March 2023).

29. Speaking of Research. Rise in Animal Research in South Korea in 2017. Available online: https://speakingofresearch.com/2018/04/12/rise-in-animal-research-in-south-korea-in-2017/ (accessed on 9 March 2023).
30. Statista. Annual Number of Animals Used in Research and Testing in Selected Countries Worldwide as of 2020. Available online: https://www.statista.com/statistics/639954/animals-used-in-research-experiments-worldwide/ (accessed on 9 March 2023).
31. Speaking of Research. Worldwide Animal Research Statistics. Available online: https://speakingofresearch.com/facts/animal-research-statistics/ (accessed on 9 March 2023).
32. Gil, G.; Rodríguez, X. Experimentos con Animales en México: 20 Años de Caos y Riesgo. Available online: https://aristeguinoticias.com/2405/mexico/__trashed-25/ (accessed on 9 March 2023).
33. Hubrecht, R.C. The 3Rs and humane experimental technique: Implementing change. *Animals* **2019**, *9*, 754. [CrossRef] [PubMed]
34. Marquardt, N.; Feja, M.; Hünigen, H.; Plendl, J.; Menken, L.; Fink, H.; Bert, B. Euthanasia of laboratory mice: Are isoflurane and sevoflurane real alternatives to carbon dioxide? *PLoS ONE* **2018**, *13*, e0203793. [CrossRef] [PubMed]
35. Percie du Sert, N.; Hurst, V.; Ahluwalia, A.; Alam, S.; Avey, M.T.; Baker, M.; Browne, W.J.; Clark, A.; Cuthill, I.C.; Dirnagl, U.; et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *J. Cereb. Blood Flow Metab.* **2020**, *40*, 1769–1777. [CrossRef]
36. Smith, A.J.; Clutton, R.E.; Lilley, E.; Hansen, K.E.A.; Brattelid, T. PREPARE: Guidelines for planning animal research and testing. *Lab. Anim.* **2018**, *52*, 135–141. [CrossRef] [PubMed]
37. Bert, B.; Heinl, C.; Chmielewska, J.; Schwarz, F.; Grune, B.; Hensel, A.; Greiner, M.; Schönfelder, G. Refining animal research: The Animal Study Registry. *PLoS Biol.* **2019**, *17*, e3000463. [CrossRef]
38. Singh, A.; Gupta, U. Animal models of tuberculosis: Lesson learnt. *Indian J. Med. Res.* **2018**, *147*, 456. [CrossRef]
39. Kim, T.-W.; Che, J.-H.; Yun, J.-W. Use of stem cells as alternative methods to animal experimentation in predictive toxicology. *Regul. Toxicol. Pharmacol.* **2019**, *105*, 15–29. [CrossRef]
40. Yang, H.; Wu, Z. Genome editing of pigs for agriculture and biomedicine. *Front. Genet.* **2018**, *9*, 360. [CrossRef]
41. Muñoz-Fontela, C.; Dowling, W.E.; Funnell, S.G.P.; Gsell, P.-S.; Riveros-Balta, A.X.; Albrecht, R.A.; Andersen, H.; Baric, R.S.; Carroll, M.W.; Cavaleri, M.; et al. Animal models for COVID-19. *Nature* **2020**, *586*, 509–515. [CrossRef] [PubMed]
42. Dissanayake, H.A.; de Silva, N.L.; Sumanatilleke, M.; de Silva, S.D.N.; Gamage, K.K.K.; Dematapitiya, C.; Kuruppu, D.C.; Ranasinghe, P.; Pathmanathan, S.; Katulanda, P. Prognostic and therapeutic role of vitamin D in COVID-19: Systematic review and meta-analysis. *J. Clin. Endocrinol. Metab.* **2022**, *107*, 1484–1502. [CrossRef] [PubMed]
43. World Health Organization (WHO). WHO Coronavirus (COVID-19) Dashboard. Available online: https://covid19.who.int/ (accessed on 9 June 2022).
44. Woolsey, C.; Borisevich, V.; Prasad, A.N.; Agans, K.N.; Deer, D.J.; Dobias, N.S.; Heymann, J.C.; Foster, S.L.; Levine, C.B.; Medina, L.; et al. Establishment of an African green monkey model for COVID-19 and protection against re-infection. *Nat. Immunol.* **2021**, *22*, 86–98. [CrossRef] [PubMed]
45. Cleary, S.J.; Pitchford, S.C.; Amison, R.T.; Carrington, R.; Robaina Cabrera, C.L.; Magnen, M.; Looney, M.R.; Gray, E.; Page, C.P. Animal models of mechanisms of SARS-CoV-2 infection and COVID-19 pathology. *Br. J. Pharmacol.* **2020**, *177*, 4851–4865. [CrossRef]
46. Da Costa, C.B.P.; Cruz, A.C.D.M.; Penha, J.C.Q.; Castro, H.C.; Da Cunha, L.E.R.; Ratcliffe, N.A.; Cisne, R.; Martins, F.J. Using in vivo animal models for studying SARS-CoV-2. *Expert Opin. Drug Discov.* **2022**, *17*, 121–137. [CrossRef]
47. Younes, S.; Younes, N.; Shurrab, F.; Nasrallah, G.K. Severe acute respiratory syndrome coronavirus-2 natural animal reservoirs and experimental models: Systematic review. *Rev. Med. Virol.* **2021**, *31*, e2196. [CrossRef]
48. Sun, S.-H.; Chen, Q.; Gu, H.-J.; Yang, G.; Wang, Y.-X.; Huang, X.-Y.; Liu, S.-S.; Zhang, N.-N.; Li, X.-F.; Xiong, R.; et al. A mouse model of SARS-CoV-2 infection and pathogenesis. *Cell Host Microbe* **2020**, *28*, 124–133.e4. [CrossRef]
49. Mueller, A.L.; McNamara, M.S.; Sinclair, D.A. Why does COVID-19 disproportionately affect older people? *Aging* **2020**, *12*, 9959–9981. [CrossRef]
50. Devaux, C.A.; Lagier, J.-C.; Raoult, D. New insights into the physiopathology of COVID-19: SARS-CoV-2-associated gastrointestinal illness. *Front. Med.* **2021**, *8*, 640073. [CrossRef]
51. Bi, Z.; Hong, W.; Yang, J.; Lu, S.; Peng, X. Animal models for SARS-CoV-2 infection and pathology. *MedComm* **2021**, *2*, 548–568. [CrossRef]
52. Urano, E.; Okamura, T.; Ono, C.; Ueno, S.; Nagata, S.; Kamada, H.; Higuchi, M.; Furukawa, M.; Kamitani, W.; Matsuura, Y.; et al. COVID-19 cynomolgus macaque model reflecting human COVID-19 pathological conditions. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2104847118. [CrossRef] [PubMed]
53. Yu, P.; Qi, F.; Xu, Y.; Li, F.; Liu, P.; Liu, J.; Bao, L.; Deng, W.; Gao, H.; Xiang, Z.; et al. Age-related rhesus macaque models of COVID-19. *Anim. Model. Exp. Med.* **2020**, *3*, 93–97. [CrossRef] [PubMed]
54. Csiszar, A.; Jakab, F.; Valencak, T.G.; Lanszki, Z.; Tóth, G.E.; Kemenesi, G.; Tarantini, S.; Fazekas-Pongor, V.; Ungvari, Z. Companion animals likely do not spread COVID-19 but may get infected themselves. *GeroScience* **2020**, *42*, 1229–1236. [CrossRef] [PubMed]
55. Kumar, R.; Harilal, S.; Al-Sehemi, A.G.; Pannipara, M.; Behl, T.; Mathew, G.E.; Mathew, B. COVID-19 and domestic animals: Exploring the species barrier crossing, zoonotic and reverse zoonotic transmission of SARS-CoV-2. *Curr. Pharm. Des.* **2021**, *27*, 1194–1201. [CrossRef]

56. Shi, J.; Wen, Z.; Zhong, G.; Yang, H.; Wang, C.; Huang, B.; Liu, R.; He, X.; Shuai, L.; Sun, Z.; et al. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS–coronavirus 2. *Science* **2020**, *368*, 1016–1020. [CrossRef]
57. Hosie, M.J.; Hofmann-Lehmann, R.; Hartmann, K.; Egberink, H.; Truyen, U.; Addie, D.D.; Belák, S.; Boucraut-Baralon, C.; Frymus, T.; Lloret, A.; et al. Anthropogenic infection of cats during the 2020 COVID-19 pandemic. *Viruses* **2021**, *13*, 185. [CrossRef]
58. Pramod, R.K.; Nair, A.V.; Tambare, P.K.; Chauhan, K.; Kumar, T.V.; Rajan, R.A.; Mani, B.M.; Asaf, M.; Pandey, A.K. Reverse zoonosis of coronavirus disease-19: Present status and the control by one health approach. *Vet. World* **2021**, *14*, 2817–2826. [CrossRef]
59. Kang, K.; Chen, Q.; Gao, Y.; Yu, K. Detection of SARS-CoV-2 B.1.617.2 (Delta) variant in three cats owned by a confirmed COVID-19 patient in Harbin, China. *Vet. Med. Sci.* **2022**, *8*, 945–946. [CrossRef]
60. da Costa Rodrigues, K.C.; Pereira, R.M.; de Campos, T.D.P.; de Moura, R.F.; da Silva, A.S.R.; Cintra, D.E.; Ropelle, E.R.; Pauli, J.R.; de Araújo, M.B.; de Moura, L.P. The role of physical exercise to improve the browning of white adipose tissue via POMC neurons. *Front. Cell. Neurosci.* **2018**, *12*, 88. [CrossRef]
61. Rios, J.L.; Bomhof, M.R.; Reimer, R.A.; Hart, D.A.; Collins, K.H.; Herzog, W. Protective effect of prebiotic and exercise intervention on knee health in a rat model of diet-induced obesity. *Sci. Rep.* **2019**, *9*, 3893. [CrossRef]
62. Burhans, M.S.; Hagman, D.K.; Kuzma, J.N.; Schmidt, K.A.; Kratz, M. Contribution of adipose tissue inflammation to the development of type 2 diabetes mellitus. *Compr. Physiol.* **2018**, *9*, 1–58. [CrossRef] [PubMed]
63. Khalafi, M.; Mohebbi, H.; Symonds, M.E.; Karimi, P.; Akbari, A.; Tabari, E.; Faridnia, M.; Moghaddami, K. The impact of moderate-intensity continuous or high-intensity interval training on adipogenesis and browning of subcutaneous adipose tissue in obese male rats. *Nutrients* **2020**, *12*, 925. [CrossRef]
64. Lee, H.E.; Yang, G.; Han, S.-H.; Lee, J.-H.; An, T.-J.; Jang, J.-K.; Lee, J.Y. Anti-obesity potential of Glycyrrhiza uralensis and licochalcone A through induction of adipocyte browning. *Biochem. Biophys. Res. Commun.* **2018**, *503*, 2117–2123. [CrossRef]
65. Wang, P.; Li, D.; Ke, W.; Liang, D.; Hu, X.; Chen, F. Resveratrol-induced gut microbiota reduces obesity in high-fat diet-fed mice. *Int. J. Obes.* **2020**, *44*, 213–225. [CrossRef] [PubMed]
66. Lasker, S.; Rahman, M.M.; Parvez, F.; Zamila, M.; Miah, P.; Nahar, K.; Kabir, F.; Sharmin, S.B.; Subhan, N.; Ahsan, G.U.; et al. High-fat diet-induced metabolic syndrome and oxidative stress in obese rats are ameliorated by yogurt supplementation. *Sci. Rep.* **2019**, *9*, 20026. [CrossRef] [PubMed]
67. Bouyanfif, A.; Jayarathne, S.; Koboziev, I.; Moustaid-Moussa, N. The nematode Caenorhabditis elegans as a model organism to study metabolic effects of ω-3 polyunsaturated fatty acids in obesity. *Adv. Nutr.* **2019**, *10*, 165–178. [CrossRef] [PubMed]
68. Ke, W.; Reed, J.N.; Yang, C.; Higgason, N.; Rayyan, L.; Wählby, C.; Carpenter, A.E.; Civelek, M.; O'Rourke, E.J. Genes in human obesity loci are causal obesity genes in C. elegans. *PLoS Genet.* **2021**, *17*, e1009736. [CrossRef]
69. Farias-Pereira, R.; Zhang, Z.; Park, C.; Kim, D.; Kim, K.; Park, Y. Butein inhibits lipogenesis in *Caenorhabditis elegans*. *BioFactors* **2020**, *46*, 777–787. [CrossRef]
70. Yusela Kris, A.; Rich Milton, D.; Sofronio, K. Effect of Lentinus strigosus extract on the food intake and locomotion of N2 wild strain Caenorhabditis elegans as model for obesity. *J. Appl. Pharm. Sci.* **2020**, *10*, 23–28. [CrossRef]
71. Benchoula, K.; Khatib, A.; Jaffar, A.; Ahmed, Q.U.; Sulaiman, W.M.A.W.; Wahab, R.A.; El-Seedi, H.R. The promise of zebrafish as a model of metabolic syndrome. *Exp. Anim.* **2019**, *68*, 407–416. [CrossRef]
72. Rodríguez, R.R.; González-Bulnes, A.; Garcia-Contreras, C.; Elena Rodriguez-Rodriguez, A.; Astiz, S.; Vazquez-Gomez, M.; Luis Pesantez, J.; Isabel, B.; Salido-Ruiz, E.; González, J.; et al. The Iberian pig fed with high-fat diet: A model of renal disease in obesity and metabolic syndrome. *Int. J. Obes.* **2020**, *44*, 457–465. [CrossRef] [PubMed]
73. Chagnac, A.; Zingerman, B.; Rozen-Zvi, B.; Herman-Edelstein, M. Consequences of glomerular hyperfiltration: The role of physical forces in the pathogenesis of chronic kidney disease in diabetes and obesity. *Nephron* **2019**, *143*, 38–42. [CrossRef] [PubMed]
74. Rodríguez-Rodríguez, A.E.; Donate-Correa, J.; Luis-Lima, S.; Díaz-Martín, L.; Rodríguez-González, C.; Pérez-Pérez, J.A.; Acosta-González, N.G.; Fumero, C.; Navarro-Díaz, M.; López-Álvarez, D.; et al. Obesity and metabolic syndrome induce hyperfiltration, glomerulomegaly, and albuminuria in obese ovariectomized female mice and obese male mice. *Menopause* **2021**, *28*, 1296–1306. [CrossRef]
75. Hoffman, J.M.; Creevy, K.E.; Franks, A.; O'Neill, D.G.; Promislow, D.E.L. The companion dog as a model for human aging and mortality. *Aging Cell* **2018**, *17*, e12737. [CrossRef]
76. Suarez, L.; Bautista-Castaño, I.; Peña Romera, C.; Montoya-Alonso, J.A.; Corbera, J.A. Is dog owner obesity a risk factor for canine obesity? a "one-health" study on human–animal interaction in a region with a high prevalence of obesity. *Vet. Sci.* **2022**, *9*, 243. [CrossRef]
77. World Health Organization (WHO). Cancer. Available online: https://www.who.int/news-room/fact-sheets/detail/cancer (accessed on 9 March 2023).
78. National Cancer Institute. Common Cancer Types. Available online: https://www.cancer.gov/types/common-cancers (accessed on 9 March 2023).
79. Siegel, R.L.; Miller, K.D.; Fuchs, H.E.; Jemal, A. Cancer statistics, 2022. *CA Cancer J. Clin.* **2022**, *72*, 7–33. [CrossRef] [PubMed]
80. Berish, R.B.; Ali, A.N.; Telmer, P.G.; Ronald, J.A.; Leong, H.S. Translational models of prostate cancer bone metastasis. *Nat. Rev. Urol.* **2018**, *15*, 403–421. [CrossRef]

81. Onaciu, A.; Munteanu, R.; Munteanu, V.C.; Gulei, D.; Raduly, L.; Feder, R.-I.; Pirlog, R.; Atanasov, A.G.; Korban, S.S.; Irimie, A.; et al. Spontaneous and induced animal models for cancer research. *Diagnostics* **2020**, *10*, 660. [CrossRef]
82. Valcourt, D.M.; Kapadia, C.H.; Scully, M.A.; Dang, M.N.; Day, E.S. Best practices for preclinical in vivo testing of cancer nanomedicines. *Adv. Healthc. Mater.* **2020**, *9*, 2000110. [CrossRef]
83. Santana-Krímskaya, S.E.; Kawas, J.R.; Zarate-Triviño, D.G.; Ramos-Zayas, Y.; Rodríguez-Padilla, C.; Franco-Molina, M.A. Orthotopic and heterotopic triple negative breast cancer preclinical murine models: A tumor microenvironment comparative. *Res. Vet. Sci.* **2022**, *152*, 364–371. [CrossRef]
84. Madonna, M.C.; Duer, J.E.; Lee, J.V.; Williams, J.; Avsaroglu, B.; Zhu, C.; Deutsch, R.; Wang, R.; Crouch, B.T.; Hirschey, M.D.; et al. In vivo optical metabolic imaging of long-chain fatty acid uptake in orthotopic models of triple-negative breast cancer. *Cancers* **2021**, *13*, 148. [CrossRef] [PubMed]
85. Koosha, S.; Mohamed, Z.; Sinniah, A.; Alshawsh, M.A. Evaluation of anti-tumorigenic effects of diosmetin against human colon cancer xenografts in athymic nude mice. *Molecules* **2019**, *24*, 2522. [CrossRef]
86. Sliepen, S.H.J.; Diaz-del Castillo, M.; Korioth, J.; Olsen, R.B.; Appel, C.K.; Christoph, T.; Heegaard, A.-M.; Rutten, K. Cancer-induced bone pain impairs burrowing behaviour in mouse and rat. *In Vivo* **2019**, *33*, 1125–1132. [CrossRef] [PubMed]
87. Overgaard, N.H.; Fan, T.M.; Schachtschneider, K.M.; Principe, D.R.; Schook, L.B.; Jungersen, G. Of Mice, Dogs, Pigs, and Men: Choosing the Appropriate Model for Immuno-Oncology Research. *ILAR J.* **2018**, *59*, 247–262. [CrossRef] [PubMed]
88. Kamiya, A.; Hiyama, T.; Fujimura, A.; Yoshikawa, S. Sympathetic and parasympathetic innervation in cancer: Therapeutic implications. *Clin. Auton. Res.* **2021**, *31*, 165–178. [CrossRef]
89. Sadighparvar, S.; Darband, S.G.; Ghaderi-Pakdel, F.; Mihanfar, A.; Majidinia, M. Parasympathetic, but not sympathetic denervation, suppressed colorectal cancer progression. *Eur. J. Pharmacol.* **2021**, *913*, 174626. [CrossRef]
90. Kamiya, A.; Hayama, Y.; Kato, S.; Shimomura, A.; Shimomura, T.; Irie, K.; Kaneko, R.; Yanagawa, Y.; Kobayashi, K.; Ochiya, T. Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression. *Nat. Neurosci.* **2019**, *22*, 1289–1305. [CrossRef]
91. Grelet, S.; Fréreux, C.; Obellianne, C.; Noguchi, K.; Howley, B.V.; Dalton, A.C.; Howe, P.H. TGFβ-induced expression of long noncoding lincRNA Platr18 controls breast cancer axonogenesis. *Life Sci. Alliance* **2022**, *5*, e202101261. [CrossRef]
92. Kirkpatrick, J.D.; Warren, A.D.; Soleimany, A.P.; Westcott, P.M.K.; Voog, J.C.; Martin-Alonso, C.; Fleming, H.E.; Tammela, T.; Jacks, T.; Bhatia, S.N. Urinary detection of lung cancer in mice via noninvasive pulmonary protease profiling. *Sci. Transl. Med.* **2020**, *12*, eaaw0262. [CrossRef]
93. Xu, X.; An, H.; Zhang, D.; Tao, H.; Dou, Y.; Li, X.; Huang, J.; Zhang, J. A self-illuminating nanoparticle for inflammation imaging and cancer therapy. *Sci. Adv.* **2019**, *5*, eaat2953. [CrossRef]
94. Momcilovic, M.; Jones, A.; Bailey, S.T.; Waldmann, C.M.; Li, R.; Lee, J.T.; Abdelhady, G.; Gomez, A.; Holloway, T.; Schmid, E.; et al. In vivo imaging of mitochondrial membrane potential in non-small-cell lung cancer. *Nature* **2019**, *575*, 380–384. [CrossRef]
95. Nicolson, F.; Andreiuk, B.; Andreou, C.; Hsu, H.-T.; Rudder, S.; Kircher, M.F. Non-invasive in vivo imaging of cancer using surface-enhanced spatially offset Raman Spectroscopy (SESORS). *Theranostics* **2019**, *9*, 5899–5913. [CrossRef] [PubMed]
96. Chen, L.; Zuo, W.; Xiao, Z.; Jin, Q.; Liu, J.; Wu, L.; Liu, N.; Zhu, X. A carrier-free metal-coordinated dual-photosensitizers nanotheranostic with glutathione-depletion for fluorescence/photoacoustic imaging-guided tumor phototherapy. *J. Colloid Interface Sci.* **2021**, *600*, 243–255. [CrossRef]
97. Hernández-Avalos, I.; Mota-Rojas, D.; Mendoza-Flores, J.E.; Casas-Alvarado, A.; Flores-Padilla, K.; Miranda-Cortes, A.E.; Torres-Bernal, F.; Gómez-Prado, J.; Mora-Medina, P. Nociceptive pain and anxiety in equines: Physiological and behavioral alterations. *Vet. World* **2021**, *14*, 2984–2995. [CrossRef] [PubMed]
98. Singh, S.; Kumar, A.; Mittal, G. Ketamine-polymer based drug delivery system for prolonged analgesia: Recent advances, challenges and future prospects. *Expert Opin. Drug Deliv.* **2021**, *18*, 1117–1130. [CrossRef] [PubMed]
99. Parra, S.; Thanawala, V.J.; Rege, A.; Giles, H. A novel excisional wound pain model for evaluation of analgesics in rats. *Korean J. Pain* **2021**, *34*, 165–175. [CrossRef] [PubMed]
100. Millecam, J.; van Bergen, T.; Schauvliege, S.; Antonissen, G.; Martens, A.; Chiers, K.; Gehring, R.; Gasthuys, E.; Vande Walle, J.; Croubels, S.; et al. Developmental pharmacokinetics and safety of ibuprofen and its enantiomers in the conventional pig as potential pediatric animal model. *Front. Pharmacol.* **2019**, *10*, 505. [CrossRef]
101. Domínguez-Oliva, A.; Casas-Alvarado, A.; Miranda-Cortés, A.E.; Hernández-Avalos, I. Clinical pharmacology of tramadol and tapentadol, and their therapeutic efficacy in different models of acute and chronic pain in dogs and cats. *J. Adv. Vet. Anim. Res.* **2021**, *8*, 404. [CrossRef]
102. Mulier, J.P. Is opioid-free general anesthesia for breast and gynecological surgery a viable option? *Curr. Opin. Anaesthesiol.* **2019**, *32*, 257–262. [CrossRef]
103. Cicirelli, V.; Burgio, M.; Lacalandra, G.M.; Aiudi, G.G. Local and regional anaesthetic techniques in canine ovariectomy: A review of the literature and technique description. *Animals* **2022**, *12*, 1920. [CrossRef]
104. Miranda-Cortés, A.E.; Ruiz-García, A.G.; Olivera-Ayub, A.E.; Garza-Malacara, G.; Ruiz-Cervantes, J.G.; Toscano-Zapien, J.A.; Hernández-Avalos, I. Cardiorespiratory effects of epidurally administered ketamine or lidocaine in dogs undergoing ovariohysterectomy surgery: A comparative study. *Iran. J. Vet. Res.* **2020**, *21*, 92–96. [PubMed]

105. Komatsu, A.; Miyano, K.; Nakayama, D.; Mizobuchi, Y.; Uezono, E.; Ohshima, K.; Karasawa, Y.; Kuroda, Y.; Nonaka, M.; Yamaguchi, K.; et al. Novel opioid analgesics for the development of transdermal opioid patches that possess morphine-like pharmacological profiles rather than fentanyl: Possible opioid switching alternatives among patch formula. *Anesth. Analg.* **2022**, *134*, 1082–1093. [CrossRef]
106. Godfrey, L.; Iannitelli, A.; Garrett, N.L.; Moger, J.; Imbert, I.; King, T.; Porreca, F.; Soundararajan, R.; Lalatsa, A.; Schätzlein, A.G.; et al. Nanoparticulate peptide delivery exclusively to the brain produces tolerance free analgesia. *J. Control. Release* **2018**, *270*, 135–144. [CrossRef]
107. Wang, B.; Wang, S.; Zhang, Q.; Deng, Y.; Li, X.; Peng, L.; Zuo, X.; Piao, M.; Kuang, X.; Sheng, S.; et al. Recent advances in polymer-based drug delivery systems for local anesthetics. *Acta Biomater.* **2019**, *96*, 55–67. [CrossRef] [PubMed]
108. Wang, R.; Gan, J.; Li, R.; Duan, J.; Zhou, J.; Lv, M.; Qi, R. Controlled delivery of ketamine from reduced graphene oxide hydrogel for neuropathic pain: In vitro and in vivo studies. *J. Drug Deliv. Sci. Technol.* **2020**, *60*, 101964. [CrossRef]
109. Kania, K.; Chang, D.K.; Abu-Ghname, A.; Reece, E.M.; Chu, C.K.; Maricevich, M.; Buchanan, E.P.; Winocour, S. Microsurgery training in plastic surgery. *Plast. Reconstr. Surg.—Glob. Open* **2020**, *8*, e2898. [CrossRef]
110. Ozols, D.; Zarins, J.; Petersons, A. Novel technique for toe-to-hand transplantation: The fourth-toe as an alternative option for toe-to-hand transplantation for pediatric patients. *Tech. Hand Up. Extrem. Surg.* **2019**, *23*, 74–80. [CrossRef]
111. Dvořák, Z.; Stupka, I. Atypical replantation and reconstruction of frozen ear. *Medicine* **2020**, *99*, e20068. [CrossRef]
112. Khachatryan, A.; Tevosyan, A.; Novoselskiy, D.; Arakelyan, G.; Yushkevich, A.; Nazaretovich Nazarian, D. Experimental reimplantation models. In *Microsurgery Manual for Medical Students and Residents*; Springer International Publishing: Cham, Switzerland, 2021; pp. 139–144.
113. Goutard, M.; Randolph, M.A.; Taveau, C.B.; Lupon, E.; Lantieri, L.; Uygun, K.; Cetrulo, C.L.; Lellouch, A.G. Partial heterotopic hindlimb transplantation model in rats. *J. Vis. Exp.* **2021**, *172*, e62586. [CrossRef]
114. Vernon, R.; Wang, J.; Song, M.; Wilson, N.; Moris, D.; Cendales, L. Vascularized Composite Allotransplantation: A Functional Hind Limb Model in Mice. *J. Surg. Res.* **2020**, *250*, 119–124. [CrossRef]
115. Tee, R.; Morrison, W.A.; Dilley, R.J. A novel microsurgical rodent model for the transplantation of engineered cardiac muscle flap. *Microsurgery* **2018**, *38*, 544–552. [CrossRef]
116. Scaglioni, M.; Giovanoli, P.; Scaglioni, M.F.; Yang, J.C. Microsurgical head and neck reconstruction in patients with coronary artery disease: A perioperative assessment algorithm. *Microsurgery* **2019**, *39*, 290–296. [CrossRef] [PubMed]
117. Kotz, D. UM Medicine Performs Historic Xenotransplantation. Available online: https://www.umaryland.edu/news/archived-news/january-2022/um-medicine-performs-historic-xenotransplantation.php (accessed on 7 June 2022).
118. Wang, W.; He, W.; Ruan, Y.; Geng, Q. First pig-to-human heart transplantation. *Innovation* **2022**, *3*, 100223. [CrossRef]
119. Lee, S.J.; Kim, J.S.; Chee, H.K.; Yun, I.J.; Park, K.S.; Yang, H.S.; Park, J.H. Seven years of experiences of preclinical experiments of xeno-heart transplantation of pig to non-human primate (Cynomolgus Monkey). *Transplant. Proc.* **2018**, *50*, 1167–1171. [CrossRef]
120. Cui, Y.; Yamamoto, T.; Raza, S.S.; Morsi, M.; Nguyen, H.Q.; Ayares, D.; Cooper, D.K.C.; Hara, H. Evidence for GTKO/β4GalNT2KO pigs as the preferred organ-source for old world nonhuman primates as a preclinical model of xenotransplantation. *Transplant. Direct.* **2020**, *6*, e590. [CrossRef] [PubMed]
121. Iqbal, A.; Zaman, M.; Wahab Amjad, M.; Adnan, S.; Abdul Ghafoor Raja, M.; Haider Rizvi, S.F.; Mustafa, M.W.; Farooq, U.; Abbas, G.; Shah, S. Solid lipid nanoparticles of mycophenolate mofetil: An attempt to control the release of an immunosuppressant. *Int. J. Nanomed.* **2020**, *15*, 5603–5612. [CrossRef] [PubMed]
122. Li, R.; Liang, J.; He, Y.; Qin, J.; He, H.; Lee, S.; Pang, Z.; Wang, J. Sustained release of immunosuppressant by nanoparticle-anchoring hydrogel scaffold improved the survival of transplanted stem cells and tissue regeneration. *Theranostics* **2018**, *8*, 878–893. [CrossRef]
123. Xie, H.; Zhu, H.; Zhou, K.; Wan, J.; Zhang, L.; Yang, Z.; Zhou, L.; Chen, X.; Xu, X.; Zheng, S.; et al. Target-oriented delivery of self-assembled immunosuppressant cocktails prolongs allogeneic orthotopic liver transplant survival. *J. Control. Release* **2020**, *328*, 237–250. [CrossRef]
124. Natoli, S.; Oliveira, V.; Calabresi, P.; Maia, L.F.; Pisani, A. Does SARS-Cov-2 invade the brain? Translational lessons from animal models. *Eur. J. Neurol.* **2020**, *27*, 1764–1773. [CrossRef]
125. Badaut, J.; Adami, A.; Huang, L.; Obenaus, A. Noninvasive magnetic resonance imaging stratifies injury severity in a rodent model of male juvenile traumatic brain injury. *J. Neurosci. Res.* **2019**, *98*, 129–140. [CrossRef]
126. Vink, R. Large animal models of traumatic brain injury. *J. Neurosci. Res.* **2018**, *96*, 527–535. [CrossRef]
127. Ackermans, N.L.; Varghese, M.; Wicinski, B.; Torres, J.; De Gasperi, R.; Pryor, D.; Elder, G.A.; Gama Sosa, M.A.; Reidenberg, J.S.; Williams, T.M.; et al. Unconventional animal models for traumatic brain injury and chronic traumatic encephalopathy. *J. Neurosci. Res.* **2021**, *99*, 2463–2477. [CrossRef] [PubMed]
128. Grovola, M.R.; Paleologos, N.; Brown, D.P.; Tran, N.; Wofford, K.L.; Harris, J.P.; Browne, K.D.; Shewokis, P.A.; Wolf, J.A.; Cullen, D.K.; et al. Diverse changes in microglia morphology and axonal pathology during the course of 1 year after mild traumatic brain injury in pigs. *Brain Pathol.* **2021**, *31*, e12953. [CrossRef]
129. Filipp, M.; Travis, B.; Henry, S.; Idzikowski, E.; Magnuson, S.; Loh, M.; Hellenbrand, D.; Hanna, A. Differences in neuroplasticity after spinal cord injury in varying animal models and humans. *Neural Regen. Res.* **2019**, *14*, 7. [CrossRef]

130. Perucca, P.; Bahlo, M.; Berkovic, S.F. The genetics of epilepsy. *Annu. Rev. Genom. Hum. Genet.* **2020**, *21*, 205–230. [CrossRef] [PubMed]
131. Hobbs, S.L.; Law, T.H.; Volk, H.A.; Younis, C.; Casey, R.A.; Packer, R.M.A. Impact of canine epilepsy on judgement and attention biases. *Sci. Rep.* **2020**, *10*, 17719. [CrossRef] [PubMed]
132. Jacobs, J.A.; Sehgal, A. Anandamide metabolites protect against seizures through the TRP channel water witch in Drosophila melanogaster. *Cell Rep.* **2020**, *31*, 107710. [CrossRef]
133. Dissel, S. Drosophila as a model to study the relationship between sleep, plasticity, and memory. *Front. Physiol.* **2020**, *11*, 533. [CrossRef]
134. Toda, H.; Williams, J.A.; Gulledge, M.; Sehgal, A. A sleep-inducing gene, nemuri, links sleep and immune function in Drosophila. *Science* **2019**, *363*, 509–515. [CrossRef]
135. Lei, Z.; Henderson, K.; Keleman, K. A neural circuit linking learning and sleep in Drosophila long-term memory. *Nat. Commun.* **2022**, *13*, 609. [CrossRef]
136. Shi, L.; Su, B. A transgenic monkey model for the study of human brain evolution. *Zool. Res.* **2019**, *40*, 236–238. [CrossRef] [PubMed]
137. Hoffe, B.; Holahan, M.R. The use of pigs as a translational model for studying neurodegenerative diseases. *Front. Physiol.* **2019**, *10*, 838. [CrossRef]
138. Calvo-Rodriguez, M.; Hou, S.S.; Snyder, A.C.; Kharitonova, E.K.; Russ, A.N.; Das, S.; Fan, Z.; Muzikansky, A.; Garcia-Alloza, M.; Serrano-Pozo, A.; et al. Increased mitochondrial calcium levels associated with neuronal death in a mouse model of Alzheimer's disease. *Nat. Commun.* **2020**, *11*, 2146. [CrossRef]
139. Kin, K.; Yasuhara, T.; Kameda, M.; Date, I. Animal models for parkinson's disease research: Trends in the 2000s. *Int. J. Mol. Sci.* **2019**, *20*, 5402. [CrossRef] [PubMed]
140. Hughes, G.L.; Lones, M.A.; Bedder, M.; Currie, P.D.; Smith, S.L.; Pownall, M.E. Machine learning discriminates a movement disorder in a zebrafish model of Parkinson's disease. *Dis. Model. Mech.* **2020**, *13*, dmm045815. [CrossRef] [PubMed]
141. Barnhill, L.M.; Murata, H.; Bronstein, J.M. Studying the pathophysiology of Parkinson's disease using zebrafish. *Biomedicines* **2020**, *8*, 197. [CrossRef]
142. Kalyn, M.; Hua, K.; Mohd Noor, S.; Wong, C.E.D.; Ekker, M. Comprehensive analysis of neurotoxin-induced ablation of dopaminergic neurons in zebrafish larvae. *Biomedicines* **2019**, *8*, 1. [CrossRef]
143. Vaz, R.L.; Outeiro, T.F.; Ferreira, J.J. Zebrafish as an animal model for drug discovery in parkinson's disease and other movement disorders: A systematic review. *Front. Neurol.* **2018**, *9*, 347. [CrossRef]
144. Zhao, Y.; Han, Y.; Wang, Z.; Chen, T.; Qian, H.; He, J.; Li, J.; Han, B.; Wang, T. Rosmarinic acid protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurotoxicity in zebrafish embryos. *Toxicol. Vitr.* **2020**, *65*, 104823. [CrossRef]
145. Dongjie, S.; Rajendran, R.S.; Xia, Q.; She, G.; Tu, P.; Zhang, Y.; Liu, K. Neuroprotective effects of Tongtian oral liquid, a Traditional Chinese Medicine in the Parkinson's disease-induced zebrafish model. *Biomed. Pharmacother.* **2022**, *148*, 112706. [CrossRef]
146. Kodera, K.; Matsui, H. Zebrafish, medaka and turquoise killifish for understanding human neurodegenerative/neurodevelopmental disorders. *Int. J. Mol. Sci.* **2022**, *23*, 1399. [CrossRef] [PubMed]
147. Chen, J.; Lei, L.; Tian, L.; Hou, F.; Roper, C.; Ge, X.; Zhao, Y.; Chen, Y.; Dong, Q.; Tanguay, R.L.; et al. Developmental and behavioral alterations in zebrafish embryonically exposed to valproic acid (VPA): An aquatic model for autism. *Neurotoxicol. Teratol.* **2018**, *66*, 8–16. [CrossRef] [PubMed]
148. Arafat, E.A.; Shabaan, D.A. The possible neuroprotective role of grape seed extract on the histopathological changes of the cerebellar cortex of rats prenatally exposed to Valproic Acid: Animal model of autism. *Acta Histochem.* **2019**, *121*, 841–851. [CrossRef]
149. Baek, H.; Sariev, A.; Lee, S.; Dong, S.-Y.; Royer, S.; Kim, H. Deep cerebellar low-intensity focused ultrasound stimulation restores interhemispheric balance after ischemic stroke in mice. *IEEE Trans. Neural Syst. Rehabil. Eng.* **2020**, *28*, 2073–2079. [CrossRef]
150. Chen, C.-D.; Kim, S.-K.; HwangBo, G. Effect of Acute Phase Pain Control Using TENS on Pain Relief in Knee Osteoarthritis in a Rat Model. *J. Korean Soc. Phys. Med.* **2021**, *16*, 15–20. [CrossRef]
151. Martins, Â.; Gouveia, D.; Cardoso, A.; Carvalho, C.; Silva, C.; Coelho, T.; Gamboa, Ó.; Ferreira, A. Functional neurorehabilitation in dogs with an incomplete recovery 3 months following intervertebral disc surgery: A case series. *Animals* **2021**, *11*, 2442. [CrossRef] [PubMed]
152. Iijima, H.; Eguchi, R.; Shimoura, K.; Yamada, K.; Aoyama, T.; Takahashi, M. Transcutaneous electrical nerve stimulation improves stair climbing capacity in people with knee osteoarthritis. *Sci. Rep.* **2020**, *10*, 7294. [CrossRef]
153. Lee, J.E.; Anderson, C.M.; Perkhounkova, Y.; Sleeuwenhoek, B.M.; Louison, R.R. Transcutaneous electrical nerve stimulation reduces resting pain in head and neck cancer patients. *Cancer Nurs.* **2019**, *42*, 218–228. [CrossRef]
154. Hsiao, I.-H.; Liao, H.-Y.; Lin, Y. Optogenetic modulation of electroacupuncture analgesia in a mouse inflammatory pain model. *Sci. Rep.* **2022**, *12*, 9067. [CrossRef]
155. Truong, B.T.; Artinger, K.B. The power of zebrafish models for understanding the co-occurrence of craniofacial and limb disorders. *Genesis* **2021**, *59*, e23407. [CrossRef]

156. Bergen, D.J.M.; Tong, Q.; Shukla, A.; Newham, E.; Zethof, J.; Lundberg, M.; Ryan, R.; Youlten, S.E.; Frysz, M.; Croucher, P.I.; et al. Regenerating zebrafish scales express a subset of evolutionary conserved genes involved in human skeletal disease. *BMC Biol.* **2022**, *20*, 21. [CrossRef]
157. Yonekura, M.; Kondoh, N.; Han, C.; Toyama, Y.; Ohba, T.; Ono, K.; Itagaki, S.; Tomita, H.; Murakami, M. Medaka as a model for ECG analysis and the effect of verapamil. *J. Pharmacol. Sci.* **2018**, *137*, 55–60. [CrossRef]
158. Marzęda, P.; Drozd, M.; Tchórz, M.; Kisiel, K. Importance of early intervention in verapamil overdose—Case Report and antidotes review. *J. Pre-Clin. Clin. Res.* **2021**, *15*, 142–147. [CrossRef]
159. Ruetten, H.; Vezina, C.M. Relevance of dog as an animal model for urologic diseases. In *Progress in Molecular Biology and Translational Science*; Tao, Y.-X., Ed.; Academic Press: Oxford, UK, 2022; pp. 35–65.
160. Gajski, G.; Žegura, B.; Ladeira, C.; Pourrut, B.; Del Bo', C.; Novak, M.; Sramkova, M.; Milić, M.; Gutzkow, K.B.; Costa, S.; et al. The comet assay in animal models: From bugs to whales—(Part 1 Invertebrates). *Mutat. Res. Mutat. Res.* **2019**, *779*, 82–113. [CrossRef]
161. Bouriga, N.; Mili, S.; Bahri, W.R.; Jammeli, B.; Ben-Attia, M.; Quignard, J.-P.; Trabelsi, M. Skin Wound Healing Potential and Antioxidant Effect of Hyaluronic Acid Extracted from Mytilus galloprovincialis and Crassostrea gigas. *Pharm. Chem. J.* **2022**, *56*, 381–386. [CrossRef]
162. Imperadore, P.; Uckermann, O.; Galli, R.; Steiner, G.; Kirsch, M.; Fiorito, G. Nerve regeneration in the cephalopod mollusc Octopus vulgaris: Label-free multiphoton microscopy as a tool for investigation. *J. R. Soc. Interface* **2018**, *15*, 20170889. [CrossRef] [PubMed]
163. NIH When Are Alternatives to Animals Used in Research? Available online: https://grants.nih.gov/grants/policy/air/alternatives (accessed on 10 March 2023).
164. Conroy, G. These Are the 10 Best Countries for Life Sciences Research. Available online: https://www.nature.com/nature-index/news-blog/ten-best-countries-life-sciences-research-rankings (accessed on 10 March 2023).
165. Van Norman, G.A. Limitations of animal studies for predicting toxicity in clinical trials. *JACC Basic Transl. Sci.* **2019**, *4*, 845–854. [CrossRef]
166. Hernandez, J. The FDA No Longer Requires All Drugs to Be Tested on Animals before Human Trials. Available online: https://www.npr.org/2023/01/12/1148529799/fda-animal-testing-pharmaceuticals-drug-development (accessed on 10 March 2023).
167. Canadian Council on Animal Care. CCAC Animal Data Report 2019. Available online: https://speakingofresearch.files.wordpress.com/2021/08/canada-2019.pdf (accessed on 10 March 2023).
168. Xinaris, C. Organoids for replacement therapy: Expectations, limitations and reality. *Curr. Opin. Organ Transplant.* **2019**, *24*, 555–561. [CrossRef] [PubMed]
169. Driehuis, E.; Kretzschmar, K.; Clevers, H. Establishment of patient-derived cancer organoids for drug-screening applications. *Nat. Protoc.* **2020**, *15*, 3380–3409. [CrossRef]
170. Matsui, T.K.; Tsuru, Y.; Hasegawa, K.; Kuwako, K. Vascularization of human brain organoids. *Stem Cells* **2021**, *39*, 1017–1024. [CrossRef]
171. Homan, K.A.; Gupta, N.; Kroll, K.T.; Kolesky, D.B.; Skylar-Scott, M.; Miyoshi, T.; Mau, D.; Valerius, M.T.; Ferrante, T.; Bonventre, J.V.; et al. Flow-enhanced vascularization and maturation of kidney organoids in vitro. *Nat. Methods* **2019**, *16*, 255–262. [CrossRef] [PubMed]
172. Wörsdörfer, P.; Dalda, N.; Kern, A.; Krüger, S.; Wagner, N.; Kwok, C.K.; Henke, E.; Ergün, S. Generation of complex human organoid models including vascular networks by incorporation of mesodermal progenitor cells. *Sci. Rep.* **2019**, *9*, 15663. [CrossRef] [PubMed]
173. Rawal, P.; Tripathi, D.M.; Ramakrishna, S.; Kaur, S. Prospects for 3D bioprinting of organoids. *Bio-Des. Manuf.* **2021**, *4*, 627–640. [CrossRef]
174. Briceño, J. Artificial intelligence and organ transplantation: Challenges and expectations. *Curr. Opin. Organ Transpl.* **2020**, *25*, 393–398. [CrossRef]
175. Fattahi Sani, M.; Ascione, R.; Dogramadzi, S. Mapping surgeons hand/finger movements to surgical tool motion during conventional microsurgery using machine learning. *J. Med. Robot. Res.* **2021**, *6*, 2150004. [CrossRef]
176. Azer, S.A. Deep learning with convolutional neural networks for identification of liver masses and hepatocellular carcinoma: A systematic review. *World J. Gastrointest Oncol.* **2019**, *11*, 1218–1230. [CrossRef]
177. Wang, S.; Kang, B.; Ma, J.; Zeng, X.; Xiao, M.; Guo, J.; Cai, M.; Yang, J.; Li, Y.; Meng, X.; et al. A deep learning algorithm using CT images to screen for Corona virus disease (COVID-19). *Eur. Radiol.* **2021**, *31*, 6096–6104. [CrossRef]
178. Gurovich, Y.; Hanani, Y.; Bar, O.; Nadav, G.; Fleischer, N.; Gelbman, D.; Basel-Salmon, L.; Krawitz, P.M.; Kamphausen, S.B.; Zenker, M.; et al. Identifying facial phenotypes of genetic disorders using deep learning. *Nat. Med.* **2019**, *25*, 60–64. [CrossRef]
179. Feighelstein, M.; Shimshoni, I.; Finka, L.R.; Luna, S.P.L.; Mills, D.S.; Zamansky, A. Automated recognition of pain in cats. *Sci. Rep.* **2022**, *12*, 9575. [CrossRef]
180. Herrmann, K. Beyond the 3Rs: Expanding the use of human-relevant replacement methods in biomedical research. *ALTEX* **2019**, *36*, 343–352. [CrossRef]

181. Pound, P.; Ritskes-Hoitinga, M. Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail. *J. Transl. Med.* **2018**, *16*, 304. [CrossRef] [PubMed]
182. Carvalho, C.; Gaspar, A.; Knight, A.; Vicente, L. Ethical and scientific pitfalls concerning laboratory research with non-human primates, and possible solutions. *Animals* **2018**, *9*, 12. [CrossRef] [PubMed]

2.3. CAPÍTULO III

Artículo de revisión intitulado:
Neurobiología del dolor y movimientos faciales en roedores: Aplicaciones clínicas e investigación actual

Publicado en la revista Frontiers in Veterinary Science, misma que se encuentra indexada al JCR con un factor de impacto de 3.2, en el volumen 9, páginas 1016720.

Domínguez-Oliva, A.; Mota-Rojas, D.; Hernández-Avalos, I.; Mora-Medina, P.; Olmos-Hernández, A.; Verduzco-Mendoza, A.; Casas-Alvarado, A.; Whittaker, A. L. The neurobiology of pain and facial movements in rodents: Clinical applications and current research. *Frontiers in Veterinary Science 2022*, *9*, 1016720. https://doi.org/10.3389/fvets.2022.1016720.
https://www.frontiersin.org/articles/10.3389/fvets.2022.1016720/full

frontiers | Frontiers in Veterinary Science

TYPE Review
PUBLISHED 29 September 2022
DOI 10.3389/fvets.2022.1016720

OPEN ACCESS

EDITED BY
Petra Dmitrović,
University of Zagreb, Croatia

REVIEWED BY
Vincent Bombail, Scotland's Rural College,United Kingdom
Phillip R. Kramer,
Texas A&M University, United States

*CORRESPONDENCE
Daniel Mota-Rojas dmota@correo.xoc.uam.mx

SPECIALTY SECTION
This article was submitted to Veterinary Surgery and Anesthesiology,a section of the journal
Frontiers in Veterinary Science

RECEIVED 11 August 2022
ACCEPTED 12 September 2022
PUBLISHED 29 September 2022

CITATION
Domínguez-Oliva A, Mota-Rojas D, Hernández-Avalos I, Mora-Medina P,Olmos-Hernández A,
Verduzco-Mendoza A,
Casas-Alvarado A and Whittaker AL (2022) The neurobiology of pain and facial movements in rodents: Clinicalapplications and current research. *Front. Vet. Sci.* 9:1016720.
doi: 10.3389/fvets.2022.1016720

The neurobiology of pain and facial movements in rodents: Clinical applications and currentresearch

Adriana Domínguez-Oliva[1], Daniel Mota-Rojas[2]*, Ismael Hernández-Avalos[3], Patricia Mora-Medina[3], Adriana Olmos-Hernández[4], Antonio Verduzco-Mendoza[4],Alejandro Casas-Alvarado[2] and Alexandra L. Whittaker[5]

[1]Master in Science Program "Maestría en Ciencias Agropecuarias", Universidad Autónoma Metropolitana, Mexico City, Mexico, [2]Neurophysiology, Behavior and Animal Welfare Assesment, DPAA, Universidad Autónoma Metropolitana, Mexico City, Mexico, [3]Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Cuautitlán Izcalli, Mexico, [4]Division of Biotechnology-Bioterio and Experimental Surgery, Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra, Mexico City, Mexico, [5]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA, Australia

One of the most controversial aspects of the use of animals in science isthe production of pain. Pain is a central ethical concern. The activation of neural pathways involved in the pain response has physiological, endocrine,and behavioral consequences, that can affect both the health and welfare of the animals, as well as the validity of research. The strategy to prevent theseconsequences requires understanding of the nociception process, pain itself, and how assessment can be performed using validated, non-invasive methods.The study of facial expressions related to pain has undergone considerable study with the finding that certain movements of the facial muscles (called facial action units) are associated with the presence and intensity of pain. This review, focused on rodents, discusses the neurobiology of facial expressions,clinical applications, and current research designed to better understand painand the nociceptive pathway as a strategy for implementing refinement in biomedical research.

KEYWORDS

rodents, nociception, nociceptive pathway, Rat Grimace Scale, facial action units

in their use since the 20th century (6), this topic is of particular relevance to these species.

When animals experience pain during an experimental protocol, a cascade of physiological, hormonal, biochemical, and behavioral alterations are triggered, with the aim of protecting them from the harmful stimulus. However, when the duration or intensity of pain exceeds the animal's modulating capacity, homeostasis is interrupted, and persistent pain can cause consequences like hyperalgesia and sensitization (7). To avoid these adverse effects, the ideal approach is to prevent pain from occurring by providing pre-emptive analgesia. However, this is not always possible due to requirements of the animal model, or because analgesics only provide partial coverage. In these situations, opportunely identifying and evaluating pain *via* the use of appropriate tools, that then allow decision-making regarding analgesic treatments or humane endpoint implementation, is the next best scenario. In rodents the challenge of pain identification is madeeven greater since as prey species, behavioral signs of painare subtle, requiring keen observation and species-specific knowledge (8).

Current research into pain recognition in veterinary medicine has led to the development and application of non-invasive tools that can assist in quantifying pain in rodents. These tools include the use of pain scales and identification of pain-specific behaviors (9). Since Darwin's (10) pioneering publications on the linkage between emotions and facial expressions of animals, the study of these phenomena has evolved to standardize their use by codifying the facial expressions associated with pain using instruments called "grimace scales." The scales validated for laboratory mice and rats use four-five facial action units [orbital adjustment, cheek, and nose bulge (combined in rats), and the position of the ears and whiskers] that represent specific movements of muscle groups that are attributed to pain (11). These are scored on a scale from 0 to 2 based on their deviation from their basal position, to arrive at a combined score thought to indicate pain intensity (12).

The objective of this review is to discuss the theoretical bases and recent scientific advances related to the pain that laboratory rodents may perceive during research protocols. Due to the importance of pain in these settings the article addresseskey concepts and characteristics of the phenomenon of pain with a particular focus on new understanding, explains the nociceptive pathway and the organic consequences that derive from its perception. In a unique way, it elucidates the association of pain with changes in the facial expressions of rodents, with an approach on the muscular and nervous neurobiology of facial movements. It also analyzes facial movements relatedto pain responses and the usefulness of scales based on facial expressions for evaluating pain as a strategy for recognizing and ultimately refining experimental procedures to prevent pain in laboratory rodents.

Pain: Definition and function

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with, or similar to, that associated with potential or real tissue damage" (13), where the inability to expresspain verbally does not preclude the possibility that it may be experienced (2). This updated definition is important since it recognizes the growing scientific evidence that animals experience the emotional and cognitive aspects of pain, and respond to this through their facial expressions, behavior, and structured body language.

Functionally, pain (especially acute forms) serves as an alarm and protection system that informs the organism of potential damage (14) and triggers a series of physiological and behavioral responses to prevent or reduce damage, prevent its reappearance, and promote healing (7). Nociception is the process by which a harmful stimulus is transmitted along primary afferent nerve fibers (15, 16) to higher brain structures that convert pain into a conscious experience (17, 18). This process, called the nociceptive pathway, consists of five steps (Figure 1). Activation of the nociceptive pathway and the action of the autonomic nervous system (ANS) generate a cascade of physiological, endocrine, metabolic, and behavioral responses that act to reestablish homeostasis. When pain persists, it deteriorates the health and welfare of animals due to the involvement of the sensory afferent signals and affective brain circuits that make up the nociceptive pathway (19).

Recent findings in the study of the neurobiology of pain in rodents

Transduction

Transduction is the conversion of harmful stimuli (thermal, mechanical, or chemical) into electrical signals or action potentials in the peripheral nerve endings of nociceptors (18). Nociceptors being nerve fibers with free nerve endings that respond to high intensity, potentially harmful stimuli (20). The transformation to electrical signals proceeds throughactivation of receptors or ion channels that detect different kinds of stimuli (15). The transient receptor potentials (or TRP, TRPV1-4, TRPM8, TRPA1) (18)—especially the transient receptor potential vanilloid 1 channel (TRPV1)—are known to be especially important in mammals due to their function as molecular integrators and regulators of harmful stimuli and their participation in developing nocifensive behaviors and physiological responses to pain (21, 22).

The modulation, excitation, or inhibition of these receptors has been used to understand the nociceptive pathway and develop intervention strategies during the transduction of pain. Bereiter et al. (23), for example, demonstrated that

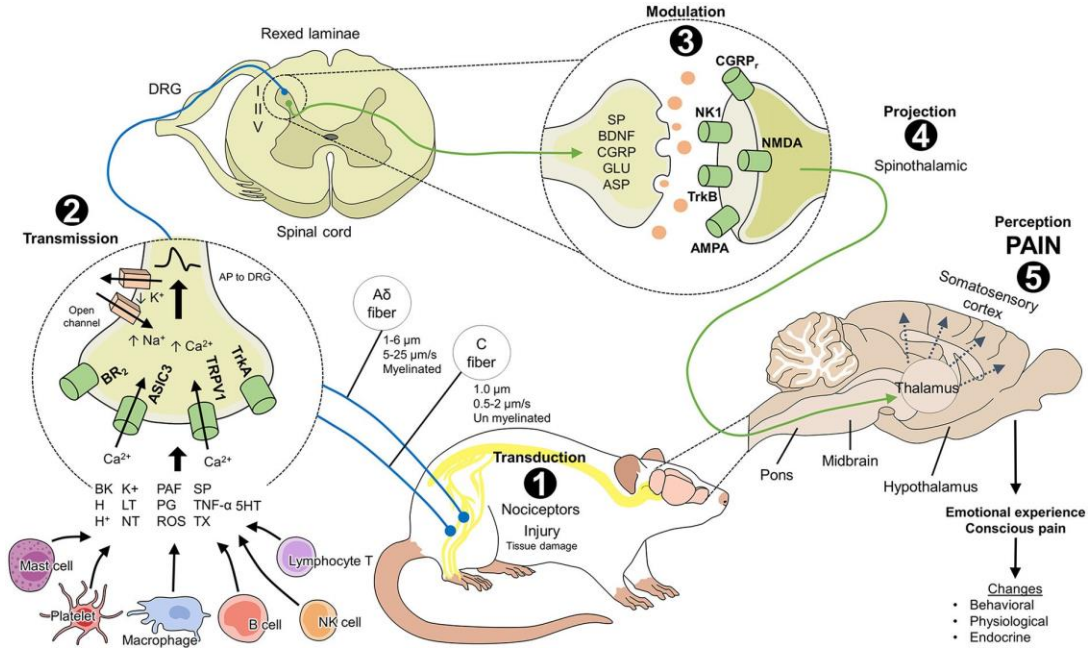


FIGURE 1
Neurobiology of pain in laboratory rats (*Rattus norvegicus*). The nociceptive pathway consists of 5 phases that are necessary to perceive pain. 1. Transduction: the transformation of a harmful stimulus into an electrical signal (or action potential) through the action of peripheral receptors (BR2, ASIC3, TRPV1, TrkA) that are activated by inflammatory mediators from mast cells, platelets, macrophages, and tissue damage. 2. Transmission: the action potentials are transmitted to the spinal cord laminae *via* first-order neurons (marked in blue) where they synapse with second-order neurons (marked in green). Laminae I, II, and V receive nociceptive input from Aδ and C fibers. 3. Modulation: the response to harmful stimuli in the inhibitory and excitatory interneurons of the spinal cord can increase or be inhibited depending on the participation of mediators such as SP, BDNF, CGRP, GLU, and ASP and their action on the membrane receptors of the postsynaptic neurons. 4. Projection: second-order neurons project the electrical signal to supraspinal centers through, for example, the spinothalamic tract, which is considered the most important for nociception. 5. Perception: once the signal reaches the higher brain centers, the thalamus connects with neurons in regions like the somatosensory cortex, where the conscious perception of pain and the consequent physiological, endocrine, and behavioral changes in rodents take place. AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ASIC3, acid-sensitive ion channel 3; ASP, aspartate; BDNF, brain-derived neurotrophic factor; BK, bradykinin; BR2, bradykinin receptor 2; CGRP, calcitonin gene-related peptide; CGRPr, calcitonin gene-related peptide receptor; DRG, dorsal root ganglion; GLU, glutamate; H+, hydrogen ions; H, histamine; IL, interleukins; LT, leukotrienes; NK1, neurokinin 1 receptor; NMDA, N-methyl-D-aspartate; NT, neurotrophins; PAF, platelet-activating factor; PG, prostaglandins; ROS, free radicals; SP, substance P; TNF, tumor necrosis factor; TrkA, tropomyosin receptor kinase A; TrkB, tropomyosin receptor-related kinase B; TRPV1; vanilloid receptor type 1 transient potential; TX, thromboxanes; 5HT, serotonin.

administering selective antagonists of TRPV1 reduce the expression of the receptor in rats with pain caused by severe dry eye disease induced by instilling a hypertonic saline solution and capsaicin. During processes of neuropathic pain in 3-week-old male Wistar rats, direct blocking of the TRPV1 by antagonists (AMG9810) reduced the effects of mechanical hyperalgesia in models of orofacial pain (21). This result was also observed in diabetes-mediated neuropathic pain in a study of 36 male Albino Wistar rats by Düzova et al. (24), where this type of pain increased the expression of TRPV1 in the DRG. Inhibition of transduction was achieved using antioxidants.

Antioxidants and other inflammatory mediators released by tissue damage [for example PG, nitric oxide (NO), and 5HT amongst others] (7), immune cells (e.g., IL-1β), TNF-α, nerve growth factor (NGF), neuropeptides [e.g., substance P (SP)], BDNF, and CGRP (25) go on to further activate peripheral receptors generating an enhanced response. Therefore, pathways related to these mediators represent a key target for painrelief intervention.

Inhibiting NO synthesis prevented thermal and mechanical hypersensitivity in a study of adult Wistar rats (26). Likewise, the regulation of mediators and their action on other receptors, such as TrkA/NGF, has been studied in Sprague-Dawley rat model of rectal hypersensitivity. In that work, electroacupuncture treatment reduced the expression of those channels (27). Activation of ASIC3 receptors has been shown to be associated with the pathogenesis of inflammatory bone pain (28). Studies of this kind have shown that information on harmful stimuli

is transduced by the peripheral receptors whose nerve endings transmit signals to spinal structures (20).

Transmission

The first-order nociceptive neurons, specialized in peripheral sensory activity, transmit the electrical signal from the site of a lesion to the dorsal horn of the spinal cord, passing through the dorsal ganglion to reach laminae I, II, and V (29).

Two types of nociceptors are recognized in animals: Aδ and C fibers. They are expressed in varying quantities in the dermis (12% Aδ, 30% polymodal C, and 20% mechanothermal C) (30). The myelinated, fast-conduction Aδ fibers (5–30 m/s) (20) are high-threshold mechano- and thermoreceptors (30) that have been studied as the main transmitters of pain induced by harmful cold (25–10°C) in rats of both sexes due to the latency of their action potentials (AP), of $\sim$221 ms (31). These fibers, which are activated in nociceptive tests like the tail flick test, are present from birth in rats. Their myelinization is completed as the days pass, but their number decreases after the three first weeks of life (32).

In studies using evoked potentials techniques, stimulating the Aδ fibers is deemed the most reliable method for evaluating nociception (33). Their activation is associated with a greater frequency of pain-like behaviors, such as the paw withdrawal threshold and paw licking in rats exposed to the complete Freund Adjuvant (34). Excitability of Aδ neurons has been demonstrated to occur as a result of Ach activation of nicotinic acetylcholine receptors (35). Rodents have a large number of nicotinic acetylcholine receptors, and they thus represent a useful pain modulating target.

The C fibers are small-diameter (0.02–1.5 μm), slow-conducting (0.5–2 meters/s), polymodal unmyelinated nerve endings (30) that act by transmitting the so-called "second pain" (20, 36). Unlike the Aδ fibers, 83% of the fibers activated by harmful heat are of the C type, which have an AP latency of $\sim$441 ms to a stimulus of 52°C (31). In newborn rats, these fibers complete their maturation within the first 3 weeks of life (37). This neuronal activation is tested in the nociceptive hot plate test commonly used in pain research (32).

The transmission of inflammatory and neuropathic pain in young female rats, and the behavioral response of spontaneous foot lifting, have been associated with models of spontaneous pain and the activation of C nociceptors due to cumulative neuroinflammation (38). The effect of pro-inflammatory substances like gamma interferon (γ) on spinal cord slices from rats has been shown to facilitate transmission from the C fibers in lamina I neurons through the action of the spinal microglia. This reaction can be attenuated by microglial inhibitors like minocycline (39). Further evidence for the role of microglia in neuropathic pain has been demonstrated using models of spinal nerve ligation in mice. These studies have shown that microglia in lamina II of the dorsal horn and the expression of receptors like P2Y12 participate in transmitting neuropathic pain. Consequently, their antagonism or complete absence in knockout mice decreases the presence of pain-related behaviors (40). Similarly, administering neuropeptides like oxytocin or orexin A and B participates in antinociception in lamina II (41).

The application of electroacupuncture to attenuate neuropathic pain in models of spared nerve injury has demonstrated that C fiber-evoked discharges are reduced by this technique and that mediators like BDNF, together with TrkB receptors, are involved in the signaling cascade of pain. They have also been suggested as mechanisms for controlling pain (42). The cumulative effect described is passed on to the ensuing phase of the nociceptive pathway, where the harmful signal is either inhibited or increased through modulation.

Modulation

This phase involves the mechanisms that inhibit or amplify the intensity of the stimuli that reach the spinal cord (43) *via* the excitatory or inhibitory interneurons that occupy ascendent (i.e., those that project signals fromthe spinal cord to the encephalon) (36) or descendent pathways (i.e., those that transmit inhibitory information from higher centers). The response is then projected to the brain to begin the conscious recognition of pain (44). The signals act upon laminae of the dorsal horn of the spinal cord known as the Rexed laminae (45). The principal modulatory mechanisms are the serotoninergic pathways from the periaqueductal gray (PAG), the noradrenergic pathways in the *locus coeruleus*, those involved in production of endogenous opioids (36, 46), the endocannabinoid systems (47), and gate theory as proposed by Melzack and Wall (48).

Evidence of modulation pathways has been demonstrated widely. For example, in inflammatory pain models in rats created by causing sciatic nerve lesions the use of spinally-applied drugs which act at serotoninergic receptors has been shown to attenuate mechanical and cold-generated hyperalgesia (49). In models of neuropathic diabetic pain in rodents, Jesus et al. (50) determined that cannabidiol exerts an anti-allodynic effect also *via* the serotoninergic system by increasing the concentrations of that neurotransmitter in the spinal cord. Moreover, the participation of the different types of 5-HT receptors in modulating nociceptive responses has been studied in rats, where findings show that the 5-HT1, 5-HT2, 5- HT3, and 5-HT7 receptors are principally involved due to their expression in afferent fibers and in laminae I and IIof the dorsal horn (51). In rodents with spinal cord injuries, administering agonists of 5-HT1 has also been associated with motor and postural control by inhibiting movements such as the monosynaptic stretch reflex (52). Antagonists like

tropisetron or granisetron 5-HT3 (53), cyanopindolol for 5-HT1A, ketanserin for 5-HT2 (54), and acetaminophen with its analgesic, antihyperalgesic, and antinociceptive action on the 5-HT1, 5-HT2, 5-HT3, and 5-HT7 receptors, are other examples (55) of the importance of serotonergic pathways in modulation. In addition, activation of gate theory—which results in the reduction of hyperalgesia and sensitization due to the participation of Aβ axons, opioids, 5-HT, and GABA—is achieved through transcutaneous electrical nerve stimulationor spinal cord stimulation in rats with joint inflammation and non-inflammatory muscle pain (56).

Excitatory neurotransmitters like ATP, SP, and Glu, and inhibitory ones such as gamma aminobutyric acid (GABA), endogenous opioids, and monoamines (5-HT, NE) (47), play a fundamental role in modulating pain by acting on the spinal receptors NMDA, AMPA, kainate (KA), and metabotropic glutamate receptors (mGluR) (57). Studies of rats have found sex-based differences in the expression of NMDA receptors and their isoforms in the dorsal horn. While the GluN2A and GluN2D subunits are found preferentially in males, GluN2B is found in the dorsal horn of females. These findings underscore the importance of differences in nociceptive circuits (58). Additionally, the use of neurotransmitters or central modulators can reduce sensitivity to pain (44). Neuropeptide modulators have a similar effect, with neuropeptide Y (NPY) being able to reduce nociception and pain-associated behaviors, as well as allodynia and hyperalgesia in rats with lesions in the hind paws (59).

The modulation of painful stimulus influences the degree of response observed in animals, particularly pain-related behaviors. For example, Glu receptors such as mGluR2 and mGluR3 inhibit the release of neurotransmitters, and therefore have antinociceptive properties during acute and chronic pain, altering the behavioral modulation (60). Likewise, the ascending- descending pathways that modulate the response through mesolimbic mechanism in an inflammatory model of rat pain can alter behavior in the mechanical paw withdrawal or open-field test, and depend of serotoninergic and noradrenergic activity (61).

In summary, amplification or inhibition of electrical signals occurs in the spinal cord, and that response is then projected to the brain to begin the conscious recognition of pain (44).

Projection

The projection of the nociceptive signal from the spinal neurons to supraspinal centers in the brain stem, thalamus, reticular formation, and PAG (47) occurs through the ascendent pathways in the white matter of the spinal cord. These pathways have been classified as: spinothalamic, spinoreticular, spinomesencephalic, trigeminothalamic, spinoparabrachial, and spinoparabrachial-hypothalamic (62). The spinothalamic tract is considered the primary nociceptive pathway (63). Its activation through tonic and/or burst electrical stimulation stimulatesthe brain areas that process the discriminative aspect of pain and the ones responsible for its cognitive, motivational, and emotional components (64). In rodents, spinal cord lesionsand injuries to this tract are associated with neuropathic pain in adult Sprague-Dawley rats (65) and with the effects of mechanical allodynia and motor deficiencies that can be reduced by administering neuroprotective substances like steroids (66). Alterations of the spinothalamic and cerebral projection neurons also generate maladaptation and hyperexcitability due to the absence of modulating pathways between the fibers of the dorsal horn and the thalamus (67). Finally, the neuroendocrine response that derives from activation of the spinothalamic tract after a lesion differs between females and males, causing variations between the central mechanisms that process emotions, pain, and the ensuing phase of nociception: perception (68).

Perception

In this final level, the primary somatosensory cortex (S1) receives projections from the thalamus (69). It is responsible for the processing, integration, and conscious experience of pain (36). EEG studies have demonstrated that when the hind paws of male Sprague-Dale rats are exposed to repetitive harmful stimuli, the S1 and anterior cingulate cortex register electrical activity during such induced painful and spontaneous pain-like events (70). As a result of this anatomical destination for projections, alterations to perception can be studied using the functional magnetic resonance technique (fMRI) due to remodeling of the somatosensory area as a result of induced neuroplasticity (71).

Since S1 neurocortical circuits participate in pain perception, dynamic neuronal oscillations (alpha, beta, and gamma) help to determine brain activity after noxious stimuli (72). Particularly, gamma oscillations are associated with acute and chronic pain, and the intensity or pain relief depends on its activation or blockage (73, 74). The induction of gamma oscillations in S1 has been shown to increase nociceptive sensitivity and the induction of aversive behaviors with the participation of the serotoninergic pathways (75). These results are similar to those reported by Peng et al. (76), who determined that gamma band oscillations are the only ones that correlate with such pain-related behaviors as flinching, withdrawal, and licking of the zone exposed to nociceptive stimuli in male rats.

Current techniques such as *in vivo* Ca2+ imagining of the anterior cingulate cortex (ACC), another structure involved in pain perception (77), are used in acute and chronic pain models in mice. In Zhao et al.'s work (78), noxious pressure stimulation evoked and enhanced the electrical activity of the ACC layer 5

neurons in mice with sciatic nerve injury. During acute pain, a rise in the somatic Ca2+ transients was reported. This response was associated with the intensity of the stimulus and induced the paw withdrawal reflex. In chronic pain states, the Ca2+ activity was 2-fold higher than in sham animals, and mice with nerve injury had mechanical allodynia. Allodynia is also present in mice models of dry eye disease. The modulation of nociceptors activity, such as TRPM8 in these cases can be evaluated using an electrophysiological multi-unit extracellular recording. Fakih et al. found that the topical administration of TRPM8 antagonist (M8-B) decreased the activity of the ciliary nerves, serving as a local antalgic agent for ocular pain (79).

The emotional and affective processing of pain can be evaluated through optogenetics. Fiber photometry-based Ca2+ imaging revealed that dopamine, an important neuromodulator of pain-related behavior in mammals, increases the activity of the media prefrontal cortex neurons in the ventrolateral PAG and modulates responses to neuropathic pain by descending pain pathways (80). Likewise, fiber photometry showed that pathways involving glutamatergic neurons in the basolateral amygdala, insular cortex, and the mediodorsal thalamic nucleus upon inflammatory pain activate and modulate aversive responses to pain in mice (81). MRI and neural connectivity with ultra-high-field in rat models of knee chronic pain showed connectivity between ACC and subcortical structures, as well as a suppression of burrowing, a behavior associated with the presence of pain (82).

During pain perception, the reciprocal relationship between this phase and other states of affect also influences degree response. For example, pain-induced depression is observed in rats with persistent neuropathic pain (and is also reported in humans). Rats with spinal nerve transection treated with antidepressants (rosiglitazone) ameliorate depressive- like behaviors by modulating neurotransmitter levels in the hippocampus (83). In depressive states in rat models of chronic postsurgical pain, the administration of ketamine (a compound known for its analgesic and antidepressant effects), decreased proinflammatory mediators (IL-1, IL-6, and TNFα) in the hippocampus and increased BDNF levels. This led to reduced depression-like behaviors without an effect on hyperalgesia (84). Anxiety is another comorbidity associated with chronic pain, including those of neuropathic and inflammatory origin (85). The importance of recognizing emotional pain is that it can regulate the intensity of physical pain, and is related to the levels of neurotransmitters that also participate in the modulation phase (86).

To summarize, perception of pain is the process through the which the brain recognizes this phenomenon as an unpleasant sensory and emotional experience associated with nociceptive transmission that culminates with the presentation of affective, behavioral, autonomous, and motor responses as mechanisms to confront the pain and impede additional damage (47).

Organic responses derived from pain in relation to pain assessment

At the onset of a nociceptive response, a cascade of organic and biochemical alterations activates the sympathetic-adrenal-medullary axis (SAM) or the hypothalamic-pituitary- adrenal axis (HPA), causing the secretion of glucocorticoids and catecholamines (87). These alterations in the animals' immune, biological, neurological, and physiological functions predispose them to pathologies due to immunosuppression that, in addition to affecting their health, may also have repercussions for results by altering physiological parameters such as heart rate, respiratory frequency, and blood pressure, as well asaltering behavior (88). For this reason, it is key that refinementin animal pain research is identified and controlled to the greatest extent possible. A discussion of some of these organic responses follows.

An objective pain assessment method does not currently exist, in spite of a wide variety of biomarkers and behavioral methods being available that may be suggestive of pain. Therefore, in general, pain assessment requires the integration of a number of changes in physiological, biochemical, endocrine, and behavioral parameters (89), to infer a painful state. It is however clear that these changes are not exclusive indicators of pain in any animal species (90, 91).

Physiological, endocrine, and metabolic responses

The integration and perception of pain in the CNS generates physiological responses that can include tachycardia, tachypnea, hypertension, mydriasis (92), and hyperthermia of $\sim$1.7°C (93). These responses result from participation of the hypothalamus, and activation of the SNS and its primary axes: HPA and SAM (3). The SAM axis increases circulating catecholamine concentrations while the HPA axis contributes to the secretion of glucocorticoids (92). As a result the main endocrine change is an increase in corticosterone, although there are reports of increases of other hormones, such as the adrenocorticotropic hormone (ACTH), oxytocin, and prolactin, as well as decreases in the growth hormone (GH) (94).

Corticosterone is however most commonly used as a biomarker, and can show substantial change. In rodents, corticosterone is the major output of the HPA axis (95), due to the lack of the CYP17α enzyme (96). For example, during acute pain in rats, levels increased as high as 385% compared with glucose at 30–195%, and prolactin up to 275% (93). It is important to note that corticosterone and some other markers are useful only in cases of acute pain (97). Because corticosterone regulates diverse activities, its increase signals alterations in

the metabolism of carbohydrates, proteins. and fats (98). The effect of corticosterone can be manifested as hyperglycemia and lipolysis, which can even put an organism into a diabetogenic state (99), and alter thermoregulating mechanisms (100) in both awake and surgical patients (101).

Other biomarkers that have been tested for their usefulness as indicators of pain, aside from glucose, include free fatty acids, lactic acid, ACTH, SP, beta-endorphins, and acute phase proteins. However, these biomarkers, just like corticosterone, are not pathognomonic of pain. As a result they are generally used as part of an assessment integrating a number of indices that allows us to infer that pain is being experienced by animals (92). Since the physiological response to pain is often non-specific, behavioral patterns represent an alternative avenue for the study of, and clinical recognition, of pain.

Behavioral responses

It has been widely suggested that in prey species suchas rodents, detecting pain-related behaviors from early manifestations is challenging because these animals tend to conceal signs to protect themselves (92). Weary et al. (102) consider that using these behaviors as a method for evaluating pain requires, first, distinguishing three types: (1) pain-like behaviors; (2) changes in the frequency of certain behaviors; and (3) preference behaviors.

Pain-like behaviors include vocalizations, flight responses, withdrawal of a body part, agitation, reduced mobility, repetitive grooming or licking of the injured area, back-arching, writhing, and twitching (103, 104). However, many of these responses may be unique to certain pain types, for example back arching, writhing and twitching have mainly been noted after abdominal surgery. Spontaneous pain behaviors in rats and mice, such as alteration in locomotor and gait activity, walking, stretching, or licking the injured area are considered pain indicators (9). In male rats, hind paw licking can be seen after injection of formalin (105). However, as Draxler et al. (106) state, the manifestation of the pain behavior depends on the pain model (inflammatory, postsurgical, cephalic, neuropathic, or chemotherapy-related), and analgesic therapy can reduce the frequency of behavioral changes.

A change in frequency of feeding is commonly used as a pain indicator in both acute and chronic pain (107), and can of course be reflected in body weight changes. A recent study has also investigated refeeding-induced analgesia in inflammatory pain, determining that the mechanism of this response *via* neural activities in the nucleus accumbens and anterior insula cortex may be a target target for chronic pain management (107). Measuring bodyweight is often the mainstay method used by researchers to assess pain in biomedical research as part of daily animal health checks. Talbot et al. (108) mention that a weight reduction of 20% or more is considered a humane endpoint in animal research.

Weight loss is commonly used to evaluate postoperative pain and the efficacy of analgesic drugs (e.g., meloxicam or buprenorphine). In Lewis male rats, Brennan et al. (109) determined that a reduction of more than 3% of daily weight gain could be an indicator of pain and a cut-off pointto reconsider the pharmacologic treatment. This was also observed in Sprague-Dawley and Dark Agouti rats undergoing laparotomy. Regardless of the analgesic drug, all animals lost weight and reduced their food and water intake on the first postsurgical day. Nonetheless, the weight reduction was lessened in animals receiving buprenorphine (110). In rat models of diabetic neuropathy, antihyperalgesic components such as rosemary extract significantly increased body weight at the end of the study (from 231.7 $\pm$ 4.326 to 241.2 $\pm$ 4.143 g), and also reduced the progress of diabetes-induced thermal hyperalgesia (111), showing the association between antinociception and weight maintenance. However, it is non-specific, since reduced feeding may also be triggered by malaise or nausea. This means that interventions specifically targeted at pain may fail. Moreover, the assessment of feeding behavior is generally impractical unless automated home cage monitoring is used, and it can take some time for changes in weight to be apparent, rendering the latter a relatively insensitive pain assessment method (112).

Nevertheless, in oro-facial disease models where tissue damage directly impacts eating function, assessment of eating behavior, is a common and reliable method. In rats, capsaicin-induced dental pain caused a reduction in food intake (113). Another example is the evaluation of meal duration, a measure that can be used as a non-invasive method to recognize nociception in rat models of induced temporomandibular pain, where joint inflammation impairs and slows their eating patterns (114). Restoring the normal meal duration can serve as an indicator of the pharmacologic efficacy of anti-inflammatory drugs such as dexamethasone (115), or capsaicin, a compound known to eliminate C-fibers that participate in the nociceptive pathway (114). Similarly, mice with temporomandibular joint pain had decreased eating duration and frequency, an effect that was consistent with cartilage degradation, making it a reliable method for pain recognition (116). Adequate dosage of multimodal analgesic treatments with opioids and non-steroidal anti-inflammatory drugs after a surgical procedure has been shown to preserve food intake in rats undergoing implantation of epidural electrodes (117). The neuronal pathway behind these changes has been evaluated by Hogri et al. (118) in male Sprague Dawley rats, who demonstrated that stimulation of neurons in the central nucleus of the amygdala, known for its role in nociceptive integration, decreases the presentation of nocifensive behaviors and promote food intake and appetite due to analgesia. A reduction in gnawing efficiency has been used to detect oral cancer pain through use of an instrument called a

dolognawmeter (119). Since gnawing is similar to chewing and uses the same masticatory muscles, this behavior is indicative of function-related pain that depends on its intensity, the sex of the animal, and analgesic therapies such as neutrophil-mediated analgesia (120).

Another pain-related behavior that is relevant in human-like diseases such as migraine is light aversion (121). Light-aversive behaviors were evaluated in rat models of induced migraine (122). In these animals, antinociceptive treatment with a multimodal neuropeptide agent reduced these behaviors and mechanical/thermal hyperalgesia (122). Events of hyperalgesia, allodynia, and photophobia, together with increased serum cortisol levels, were also observed in male Wistar rats, a response that was attenuated with the administration of ghrelin (123), a peptide that reduces the intensity of inflammatory pain through the secretion of anti-inflammatory cytokines (124). In mice, Shepherd et al. (125) reported that the color of the burrow tube influences burrowing performance, suggesting that light intensity in a lit room can induce aversion. This effect has a relation to the expression of CGRP in the medial nucleus of the cerebellum, where the stimulation of these neurons in female mice causes hypersensitivity to light (126).

There has been recent focus on the use of non-maintenance behaviors, sometimes named "luxury" behaviors, as an indicator more generally of affective state, which is usually impacted by pain. One example of a change in the frequency of a natural behavior in rats involves burrowing (17, 127). A lower frequency of burrowing has been associated with acute and chronic visceral pain (103), post-operative pain, osteoarthritis, and inflammatory (17) and neuropathic pain (128). This behavior has been also used to evaluate analgesic efficacy in rodents (129).

Nesting is another natural behavior in rodents, considered an activity of daily living or a luxury behavior (127, 130). The evaluation of the time spent in constructing the nest and its quality has been used as an indicator of pain in mice, including those in models of osteoarthritic pain. In the study by Dutta et al. (131), mice without pain and those treated with an analgesic compound (MCC22) formed more robust nests with no reduction in the functionalityof the animals. Similarly, in mice undergoing vasectomies or females undergoing sham embryo transfer, administration of local analgesics (lidocaine and bupivacaine) increased nest complexity in males and females between 12 and 24 h after the surgery (132). Therefore, the reduction in nest building is considered an indicator of diminished welfare during stressful conditions such as pain (133). However, authors such as Tappe-Theodor et al. (130) mention that the interruption of this type of behavior is not specific to identifying the pain since any stimulus that disrupts the wellbeing of rodents will generate an alteration in their behavior. Likewise, one must understand the natural propensityto burrow dependant on strain, sex species and individual characteristics (134).

In this context, the pain experience could differ within the same species and between individuals due to genetic, genomic, epigenetic, environmental, and psychological factors (135, 136). This involves differences in pain sensitivity, susceptibility to painful disorders, or efficacy of analgesic drugs (137). Traits such as temperament, sociability, or anxiety, among others, also play a role in pain-related behaviors. For example, a study of neuropathic pain in mice with low sociability and high anxiety phenotypes evoked neuronal activity in the amygdala and an enhanced hypersensitivity response to nerve injury (138). In female rats with strong fear extinction, models of acute and chronic pain (arthritis and neuropathic, respectively) had fewer vocalizations, since the emotional components of pain (such as fear) can alter the perception of pain and alter analgesic response (139).

The age and sex of rodents needs to be consideredin pain studies. Sex is a key contributor to differences in response to pain with now almost widespread acceptance thatthere are differences in pain thresholds between male and female rodents, with females having a lower pain threshold in response to a variety of nociceptive inputs (127). Inter-individual differences are a current area of research (135), where behavioral individuality could affect the intensity of response or the individual's pain threshold.

Likewise, as Mogil (140) states, there is a large interindividual variability in rodents due to genetic or heritability influences in the different strains used in biomedical research, such as the recombinant inbred CxBK mouse, the High Analgesia/Low analgesia, High Analgesic Response/Low analgesic Response, High Autotomy/Low Autototomy, and normotensive or hypertensive Wistar Kyoto rat (140). This interindividual difference has also been reported in rat models of neuropathic pain, where more than 40% of Sprague- Dawley rats do not respond to analgesic therapies such as electroacupuncture (141). Studies regarding this issue have shown that differences within the same strain can be attributed to the expression of anti-opioid peptide cholecystokininCCK-8, a component associated with individual sensitivity (136). In murine models of anxiety, differences between mice of different strains have also been documented (BALB/c –neophobic mouse strain–, C57BL/6, and 129S2). BALB/c animals are known to be highly neophobic but quickly adapt to their environment, so their response to the stressor may be diminished. Contrarily, 129S2 presents increased avoidance behavior and a higher physiological response to stress, assessed with increased avoidance behavior. therefore, display a greater amount of anxiety behaviors. These differences were attributed to a low expression of c-Fos, a marker for neural activity in the prelimbic cortex and lateral septum, areas involved in the emotional response of animals (142). Likewise, through genetic mapping in mice, it has been shown that protein expression of the subunit $\beta 3$ in the DRG contributes to pain sensitivity and strain differences between A/J and C57BL/6J (143). These few

examples are the tip of the iceberg; there is clear evidence across a range of rodent stocks and strains that individual epigenetic processes are strong influencers of physiological and behavioral responses in animal models.

Another approach to pain assessment which uses behavioral observations is the use of facial expression scoring. Studies of rodents, mainly rats and mice, have identified that the degree of muscular changes in the face is associated with the intensity of pain that the animal experiences, i.e., this method is thought to identify the affective component of pain. These changes involve the position of the ears and whiskers, orbital tightening, and flattening of the nose and cheeks (17).

Facial expressions, facial action units and the development of grimace scales

Facial expressions are the result of involuntary muscular responses to emotional stimuli creating changes in a group of facial muscles. These groups are called facial action units (FAU). Facial expressions play a role in communication that has been shown to be influenced by the social context of the emotion and the affective states of animals (144). Further, they function as a means of social exchange that allow one organism to respond to others (145). Ekman and Friesen (146) were the first to methodically study facial expressions. They developed the Facial Action Coding System (FACS) as a method for identifying the movements and positions of facial muscle groups in relation to universal emotions (147). The FACS system encompasses all anatomically possible facial movements and assigns a name to each one so they can be used in various fields, including veterinary medicine. FACS describes 44 FAU in humans, each one representing the activation of a muscle measured on a 5-point intensity scale (147, 148). Though described as an objective method of evaluation, FACS has the disadvantage that it considers only facial movements that are clearly visible, omitting more subtle changes and other facial phenomena—such as skin coloration—as well as tearing and sweating (149).

Darwin was the first author to attribute changes in behavior to emotions, including pain, to non-human animals, and to describe how facial expressions reflected them. These expressions were conceived as innate, adaptive, evolutionarily-conserved responses (10). Research on facial expressions in the past decade has analyzed observable movements of facial muscles in animals with the goal of associating them with specific events or emotions (150). Studies have focused on those FAU which are observed frequently in all animals, such asorbital tightening associated with pain (151). There has also been demonstration that facial changes are not only associated with negative emotions (described below), but also positive ones. As an example rats exposed to positive stimuli like gentle handling and tickling showed expression changes manifested as changes in the height and width of the eyes and the color (more pink) and more relaxed position of the ears (152). Likewise, facial movements associated with positive emotional states have been reported in behavioral tests such as the elevated plus-maze. Lecorps and Féron (153) found that ears were positioned in an upright and forward position in mice who openly explored their surroundings, when exposed to a novel odor, suggesting this was an indicator of emotional reactivity. In tests using palatable foods for rats, facial expression also varied suggesting an association with positive affective experiences (154). The advantage of facial expressions for evaluating pain in animals is that the method is simple and the response outwardly visible. This could then allow veterinarians and caregivers to rapidly assess painful state to allow mitigation through therapeutic administration or technique modification (151).

The neurophysiology of facial expressions and their relation to pain

Human facial expressions have been widely studied to assess the emotional and affective experience of pain. In the case of animals, the belief that facial expressions are not under voluntary control (except for non-human primates) suggests that certain movements of the facial muscles can indicate affective states like pain and emotions (155).

The manifestation of facial expressions begins with the perception of a stimulus. That stimulus does not depend only on an anatomical component, but is the result of the activity of a circuit that integrates subcortical and cortical areaslike the amygdala, primary motor cortex, ventrolateral motor cortex, and supplementary motor area, as well as two dorsal motor areas of the midcingulate cortex and the motor fibers that innervate the facial muscles (144, 145). In the case of a painful insult, the nociceptive pathway's third-order neurons that project the nociceptive stimulus to the somatosensorial cortex also connect to the amygdala (for emotional responses), hypothalamus (to generate autonomous responses), and motor areas of the cerebral cortex. The latter contain the final-order motor neurons that directly innervate the facial muscle fibers according to the signal sent from the circuit of cortical motor neurons (156, 157) (Figure 2).

Based on the study of FAU and their relation to pain, researchers in veterinary medicine have developed grimace scales to score the diverse facial expressions associated with pain in various animal species (158). Langford et al. (11) were among the first to put such scales into practice using the Mouse Grimace Scale. That tool has been shown to have a precision of 72–81% for detecting signs of pain and can differentiate between sensory (abdominal contortions due to pain) and emotional responses reflected in facial expressions (11). This success has led to proposals of pain scales—grimace scales—for may domesticated species, including laboratory rats, horses and cats (155, 159).

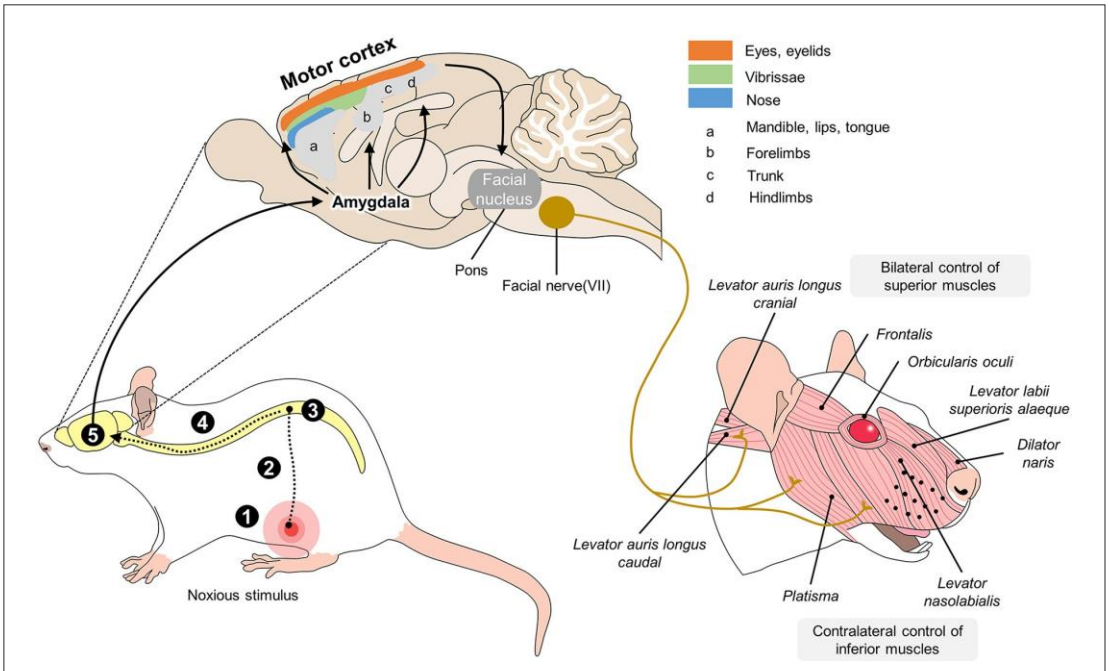


FIGURE 2
Neurobiology of facial expressions in R. norvegicus. The production of facial expressions associated with pain begins with the nociceptive pathway. When the nociceptive signal reaches the brain, the thalamus projects connections to the amygdala, the main center that initiates facial motor responses. From the amygdala, the motor cortex is activated in various areas responsible for controlling the eyes, vibrissae, nose, jaw, and pelvic and thoracic limbs. Once activated, areas of the motor cortex connect directly to the facial nucleus in the pons and, in turn, with cranial nerve VII, which innervates all the muscles involved in facial expressions. In rodents, the muscles described in the image are responsible for maintaining the position of the ears and whiskers and movements of the eyelids and nose, which are considered rat-specific facial action units (FAUs) for pain recognition. 1. transduction; 2. transmission; 3. modulation; 4. projection; 5. perception.

These scales enable observers to determine the absence/presence of pain and its severity, since this correlates with the intensity of the expression observed (11). In research with rats, these scales have been used to study pathologies, biological processes, and physiopathological mechanisms of pain that would be difficult to assess in humans or would raise serious ethical issues (17).

Rodent grimace scales

The pain scales developed and now applied in veterinary medicine began with rodents due to their importance in studies of diverse pathologies for biomedical research purposes(Figure 3), which involved both spontaneous or induced pain (11). They were also derived with clinical application in mind due to the challenges in evaluating negative states like pain in rodents (160). Their development has been based on taking images of animals with and without pain by video recording orusing still photos, with or without the use of software such as the "Rodent Face Finder," which automatically selects specific frames where the face of the rodent can be seen clearly (151).

The Rat Grimace Scale (RGS) was developed by Sotocinal (161) and validated by Oliver et al. (162). It was elaborated after its counterpart for mice (the MGS) by conducting three algesiometric assays (intraplantar injection of the Freud adjuvant, intraarticular injection of kaolin-carrageenan, and laparotomy) utilizing the conventional method of digital video recording for 30 min before the injections or surgery to capture images of the "absence of pain" and 30 min afterwards to evaluate responses, obtaining some 500 images. Though based on the MGS, there are differences in the FAU of these two species. In rats, the nose and cheek area are flattened when pain is felt, so only four FAU have been designated for this species. As Di Giminiani et al. (155) reported, an FAU is designated when the same movement occurs consistently in 25–50% of observations.

The FAU used with rats are controlled by the facial nerve that innervates two muscle groups: the superficial muscles and a set of three deep muscles. The ones associated with facial

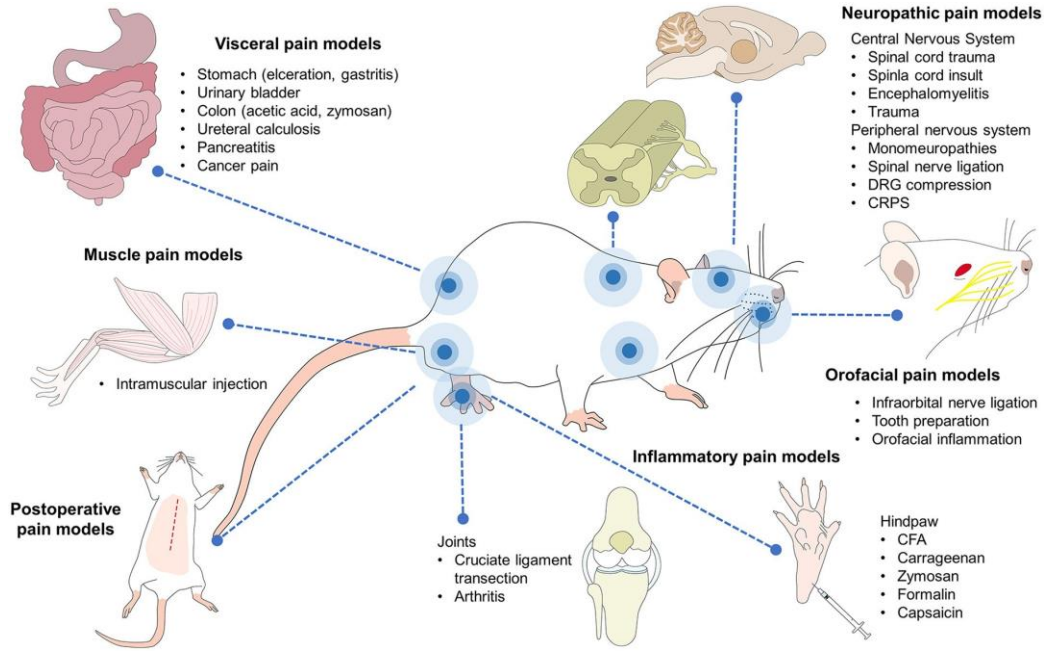


FIGURE 3
Pain models in rodents. Current research with rodent models includes several aspects of medicine and biological sciences, including pain. Several inflammatory, neuropathic, visceral, muscle-skeletal, and postoperative pain are currently studied in rodents. Orofacial, cancer, head, and burn pain are also part of the biomedical research where mice and rat play a key role in the comprehension of the physiopathology as well as the development of analgesic therapies.

expressions are the *nasolabialis* (including the *levator labii superioris* and *dilator daris* muscles), the *levator labii superioris* (superficial) and its fibers—which control the movement of the whiskers—together with the *dilator naris* (163). In mice, the motor control of the vibrissae is associated with pathways at the mesencephalic trigeminal nucleus (164). Regarding species, there are some differences that must be considered when assessing pain through facial expression. In the case of mice, naturally, there is no bulging of the nasal bridge and cheeks, a change that is noticeable when the animals experience severe pain (165). This difference influences the amount of FAU evaluated in each species (166). For example, while rats use four FAU the MGS employs five different muscular movements because bulging of the nose and cheeks is not always observed together (167).

Whilst it is recognized that there are study differences in the reported reliability of use of facial expressions to diagnose pain, and that reliability values differ based on whether a binary determination of the presence or absence of pain is sufficient, or if gradation of pain response is required. However, in spite of this variability the technique has been shown to have a validity of 81.6%, with no difference between the precision of photos taken from a frontal or profile angle, an exactitude of 76–87.5% for identifying facial expressions of pain, a sensitivity of 89.7%, and a specificity of 91.8%. Adequate training of the observers can increase the reliability of these recording to 90% (161), though some authors mention that using FACS adequately in humans requires as many as 100 h of training (148).

Pain is evaluated on a scale of 0–2 based on the degree of deviation of the FAU from expected (typical) position with FAUs being scored individually. The 0 means "not present," 1 indicates "partially or moderately present," and 2 denotes "markedly present." The final value is calculated by assimilating the scores of all the FAU either through averaging or summation (147). In some species intervention thresholds, at which it is considered necessary to administer rescue analgesia, have been described. Although these values likely need further investigation and validation (162, 168). In general most studies of grimace scales in rodents have employed models where pain was expected to be momentary or acute in nature (from a few minutes to several days) and this is where the strongest evidence for their utility exists (160). However, there is limited evidence of their utility in manifestations of chronic, neuropathic, and orthopedic pain which are expected to be more chronic in nature and that can

implicate pain-related stress (169). Figure 4 summarizes the four FAU used in rats as a representative species. Facial expressions have been studied using these FAU to determine degrees of pain in diverse research protocols. The FAU have been shown to be simple, non-invasive, real-time or retrospective tools for recognizing pain in rodents (11, 147, 158, 161, 163, 170).

Clinical applications of facial expressions in distinct experimental models of pain in laboratory rodents

From a clinical perspective, facial expressions have the potential to allow implementation of timely measures to minimize the suffering of laboratory rats and other animals (171) through administration of pain relief or implementation of humane endpoints. They have also been used to re-evaluate dosing regimens in laboratory animal medicine. There are however study differences in the sensitivity of the scales for detection of pain. This may be related to individual study characteristics, or the type of pain expected to be experienced.

Leung et al. (129) used changes in facial expressionsas a technique to refine simple methods of analgesia with buprenorphine and multimodal opioid analgesia with meloxicam and a control group (saline solution). After conducting an evaluation in real time using conventional videorecording, those authors concluded that both approaches can discriminate between the group that received analgesia and the one that did not, where the opioids reduce RGS scores compared to the control group and made it possible to identify pain early and quickly. In contrast, in models of chemotherapy-induced visceral pain, the RGS showed no significant differences in Dark Agouti rats compared to the disease activity index, and was not modified by use of opioid agents (172).

The MGS has been applied in craniotomy models to test the efficacy of post-surgical analgesics such as carprofen, meloxicam, and buprenorphine. In this study, MGS scores decreased in the first 24 h post-surgery ($p < 0.001$), with buprenorphine being the most effective drug in reducing scores at 8 h ($p = 0.046$) (173). During more common procedures, such as intraperitoneal administration of substances (e.g., CCl4 and oil as a control group), Erns et al. (174) reported that orbital narrowing was the most observed FAU in the MGS in mice after the intraperitoneal injection of CCl4, demonstrating that MGS can detect pain depending on the agent administered. Likewise, Heinsinger et al. (175) mentions that in murine models of cervical spinal cord injury, the nose and cheek bulge, orbital narrowing, and change of ear position are the most obvious FAU after 2 weeks of cervical contusion. This information does not only show the applicability of the grimace scales and FAU to pain recognition but is also an alternative to decide an analgesic approach for laboratory animals.

The sensitivity of RGS for communicating pain has been compared to other evaluation scales, such as the Composite Behavior Scale, which is based on body postures that denote pain; for example, writhing, arching the back, and staggering. In these cases, the scales were utilized to discriminate the analgesic effect of meloxicam and buprenorphine in a surgical model of laparotomy. Both scales showed higher scores during the 390 min of evaluation, but only the RGS scores descended when buprenorphine was administered, suggesting greater sensitivity for the study of facial expressions to distinguish between animals with or without pain (176). Those findings are similar to the report by Leung et al. (103), who compared these two scales with respect to the frequency with which the animals' behavior—in this case, burrowing activity—occurred in a model of colitis-induced acute and chronic inflammatory pain. The comparison of the scores obtained using facial expressions and the disease activity index showed that both increased during the acute and chronic phases, but that only burrowing decreased during the phase of acute pain. Their findings led the authors to conclude that the RGS can be used in cases of chronic pain, as was reported in a case of chronic pain caused by damage to the infraorbital nerve (177).

The facial expressions of rats have also been used to evaluate neuropathic pain caused by cervical radiculopathy, and have been validated for visceral, surgical, orthopedic, and inflammatory pain (178). Today, some automatic systems use computers to learn to recognize these facial changes. These systems can distinguish various facial expressions (179), but it is recommended that evaluators receive some type of training to detect changes in the FAU (180), since the study of pain and stressful events in animals used in science can be influenced by experience and the subjectivity of evaluations (181).

Areas of opportunity regarding pain assessment and the implementation of new techniques

There is a tendency in pain assessment of rodents to use a combination of methods which might include facial expression scoring, and use of other non-invasive techniques, such as quantifying bodyweight change (3). However, there has been less focus on the use of behaviors known to indicate positive states, such as allo-grooming, and nesting (182, 183). Whilst, these may be non-specific to pain, given the linkage between pain and emotion, for example depression as a co-morbidity in chronic pain which is prevalent in human populations, this may enhance validity and reproducibility rather than detract from the specific hypothesis testing goals. It is argued therefore, that there remains an opportunity to broaden current behavioral-based assessment techniques to consider assessment of the absence/presence of natural behaviors and vocalizations (184). Provision of resources that encourage these behaviors, and

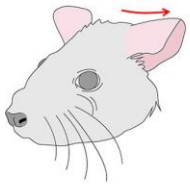
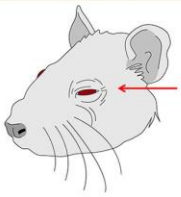
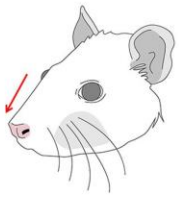
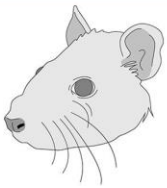
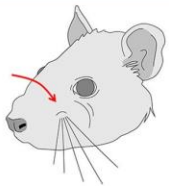
FAU	Movement	Muscles	Not present (0)	Moderately present (1)	Obviously present (2)
Ear changes	Ears curl inwards or are pulled apart. Pointed shape. Increases the space between ears (11,110)	*Auricularis anterior and auricularis posterior* (112)			
Orbital tightening	Narrowing of the orbital area or eyelids tightly closed (11)	*Levator palpebrae superioris, orbicularis oculi* y *frontalis* (107)			
Nose/ cheek flattening	In rats without pain, Contrarily to mice, when rats feel pain, the cheeks and nose become flattened, and the nose bridge looks elongated (11,110)	*Depresor septi nasi* y *levator labii superioris alaeque nasalis* depresses the tip of the nose. *Orbicularis oris* controls the shape of the lips, together with the dilator muscle and buccinador. Buccinador pulls on the angles of the mouth and cheeks (98).			
Whisker change	Whiskers lose their natural curvature and move forward. They may clump together and stiffen (110).	*Deflector nasi* and *nasolabialis* (118)			

FIGURE 4
Facial action units used to evaluate facial expression in rats and their muscles involved in its control.

training of staff in the value of them, may also be beneficial in avoiding or mitigating pain. For example, tickling techniques applied to laboratory rats imitate this species' heterospecific play behavior and have been shown to improve mental state. There does however need to be consideration of the practical implications and limitations of using some of these methods. For example, the uptake of tickling is thought to be low in facilities due to researchers' lack of time, personnel shortages, or limits imposed due to experimental design (185). Alternately, there are other assessment techniques that may, with some further development, be minimally labor intensive. For example, the analysis of ultrasonic vocalizations as an indicator of affective state is objective, sensitive to valence of affective state and can be done non-invasively. Studies report that high (~50 kHz) or low frequency (~22 kHz) vocalizations are associated to positive and negative experiences, respectively (186).

Employing environmental enrichment has also been associated with positive mental states and enhanced cognitive and learning capacities in rodents (187). Enrichment use has historically been controversial with some researchers stating that these practices need to be standardized to ensure that the studies that result are replicable and valid, and do not compromise findings or the potential for comparisons with earlier research (188). However, the proposition that standardization through reducing environmental variability is beneficial to research has been questioned (189). It may also have detrimental effects on the welfare of animals. Kentner et al. (190) argues that enrichment improves reproducibility, and Würbel and

Garner (191) suggest that it benefits welfare through reducing behavioral pathologies provided the enrichment caters to the biological needs of the species. They argue that in fact systematic environmental randomization could contribute to better science and could in fact be a refinement. The 3 Rs of Russell and Burch include refinement not only in the procedures but inthe housing, husbandry, health, and safety of the animals, understanding that endorsing better conditions for animals improves the quality of research (192).

In recent years, the search for objective evaluations ofpain has included proposals to use infrared thermography techniques (193), facial electromyography (149), recognition of facial expressions or behavior by means of sensors, automated recognition in production species like sheep (194), artificial vision technology using computers—suggested to recognize pain in horses (195)—and artificial intelligence where machines are taught to recognize certain FAU (196). As these cases reveal, the tendency is to develop precise, non-invasive methods that allow researchers to evaluate pain in laboratory rodents while causing the least stress possible by handling or the simple presence of the evaluator.

Conclusions

Pain is defined as an unpleasant sensory experience that entails the activation and integration of diverse neurobiological systems that are responsible for transducing and transmitting it and recognizing it consciously. Since laboratory rodents are most often utilized in biomedical science, the recognition of pain constitutes a fundamental step toward complying with existing norms for the use and care of laboratory animals, with the objective of preventing the physiological, endocrine, metabolic, and behavioral consequences described in this review.

The evaluation of pain in laboratory requires understanding the nociceptive pathway and the neurobiology associated with observable changes in facial expressions. Utilization of the FAU described (position of the ears and whiskers, ocular opening, and flattening of the nose or cheeks) has led to the adoption of facial expressions as a non-invasive method for determining degrees of pain (on a scale from 0 to 2) in diverse assays and models of acute, chronic, surgical, and neuropathic pain. The study of facial expressions allows researchers to recognize, objectively and integrally, the presence, degree, and intensity of pain that an animal may experience during its life or euthanasia processes. Thus, it constitutes a complementary tool for refining the use of rodents in research.

Author contributions

All authors contributed to the conceptualization, writing, reading, and approval of the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Russell WM, Burch RL. *The Principles of Humane Experimental Technique*. London: Methuen & Co (1959). p. 134–46.

2. Raja SN, Carr DB, Cohen M, Finnerup NB, Flor H, Gibson S,et al. The revised international association for the study of pain definition of pain: concepts, challenges, and compromises. *Pain.* (2020) 161:1976–82. doi: 10.1097/j.pain.0000000000001939

3. Kroll T, Kornadt-Beck N, Oskamp A, Elmenhorst D, Touma C, Palme R, et al. Additional assessment of fecal corticosterone metabolites improves visual rating in the evaluation of stress responses of laboratory rats. *Animals.* (2021) 11:710. doi: 10.3390/ani11030710

4. Matsuda Y. Recent trends in the number of laboratory animals used in Japan. *Altern Lab Anim.* (2004) 32:299–301. doi: 10.1177/026119290403201s48

5. National Association for Biomedical Research. *The Importance of Animal Testing in Biomedical Research.* (2021). Available online at: https://www.nabr.org/biomedical-research/importance-biomedical-research (accessed August 17, 2021).

6. Homberg JR, Wöhr M, Alenina N. Comeback of the rat in biomedical research. *ACS Chem Neurosci.* (2017) 8:900–3. doi: 10.1021/acschemneuro.6b00415

7. Hernández-Avalos I, Flores-Gasca E, Mota-Rojas D, Casas-Alvarado A, Miranda-Cortés AE, Domínguez-Oliva A. Neurobiology of anesthetic- surgical stress and induced behavioral changes in dogs and cats: a review. *Vet World.* (2021) 14:393–404. doi: 10.14202/vetworld.2021. 393-404

8. Turner PV, Pang DS, Lofgren JL. A review of pain assessment methods in laboratory rodents. *Comp Med.* (2019) 69:451– 67. doi: 10.30802/AALAS-CM-19-000042

9. Whittaker AL, Howartha G, Howarth GS. Use of spontaneous behaviour measures to assess pain in laboratory rats and mice: how are we progressing? *Appl Anim Behav Sci.* (2014) 151:1–12. doi: 10.1016/j.applanim.2013.11.001

10. Darwin C. *The Expression of the Emotions in Man and Animals*. London: John Murray (1872). 398 p.

11. Langford DDJ, Bailey AL AL, Chanda MLM, Clarke SE, Drummond TE, Echols S, et al. Coding of facial expressions of pain in the laboratory mouse. *Nat Methods.* (2010) 7:447–9. doi: 10.1038/ nmeth.1455

12. Sperry MM Yu Y-HH, Welch RL, Granquist EJ, Winkelstein BA. Grading facial expression is a sensitive means to detect grimace differences in orofacial pain in a rat model. *Sci Rep*. (2018) 8:13894. doi: 10.1038/s41598-018-32297-2

13. International Association for the Study of Pain (IASP). Terminology Background - IASP. *IASP Pain Terminol.* (1979). Available online at: https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=2051 (accessed July 2, 2021).

14. Bennett DL, Clark AJ, Huang J, Waxman SG, Dib-Hajj SD. The role of voltage-gated sodium channels in pain signaling. *Physiol Rev*. (2019) 99:1079–151. doi: 10.1152/physrev.00052.2017

15. Dai Y. TRPs and pain. *Semin Immunopathol.* (2016) 38:277–91. doi: 10.1007/s00281-015-0526-0

16. Bell A. The neurobiology of acute pain. *Vet J*. (2018) 237:55–62. doi: 10.1016/j.tvjl.2018.05.004

17. Deuis JR, Dvorakova LS, Vetter I. Methods used to evaluate pain behaviors in rodents. *Front Mol Neurosci.* (2017) 10:284. doi: 10.3389/fnmol.2017.00284

18. Ellison DL. Physiology of pain. *Crit Care Nurs Clin North Am*. (2017) 29:397–406. doi: 10.1016/j.cnc.2017.08.001

19. Baliki MN, Apkarian AV. Nociception, pain, negative moods, and behavior selection. *Neuron*. (2015) 87:474–91. doi: 10.1016/j.neuron.2015.06.005

20. Shimoji K, Kurokawa S. Anatomical physiology of pain. In: Shimoji K, Nader A, Wolfgang H, editors. *Chronic Pain Management in General and Hospital Practice*. Singapore: Springer Singapore (2021). p. 21–42.

21. Tamada M, Ohi Y, Kodama D, Miyazawa K, Goto S, Haji A. Modulation of excitatory synaptic transmissions by TRPV1 in the spinal trigeminal subnucleus caudalis neurons of neuropathic pain rats. *Eur J Pharmacol.* (2021) 913:174625. doi: 10.1016/j.ejphar.2021.174625

22. Akhilesh, Uniyal A, Gadepalli A, Tiwari V, Allani M, Chouhan D, et al. Unlocking the potential of TRPV1 based siRNA therapeutics for the treatment of chemotherapy-induced neuropathic pain. *Life Sci*. (2022) 288:120187. doi: 10.1016/j.lfs.2021.120187

23. Bereiter DA, Rahman M, Thompson R, Stephenson P, Saito H. TRPV1 and TRPM8 channels and nocifensive behavior in a rat model for dry eye. *Investig Opthalmol Vis Sci.* (2018) 59:3739. doi: 10.1167/iovs.18-24304

24. Düzova H, Naziroglu M, Çig B, Gürbüz P, Akatli AN. Noopept attenuates diabetes-mediated neuropathic pain and oxidative hippocampal neurotoxicity via inhibition of TRPV1 channel in rats. *Mol Neurobiol.* (2021) 58:5031–51. doi: 10.1007/s12035-021-02478-8

25. Duarte R, Argoff C. Classification of pain. In: Argoff C, Julie A, editors. *Pain Management Secrets*. Amsterdam: Elsevier (2009). p. 6–9.

26. Staunton CA, Barrett-Jolley R, Djouhri L, Thippeswamy T. Inducible nitric oxide synthase inhibition by 1400W limits pain hypersensitivity in a neuropathic pain rat model. *Exp Physiol.* (2018) 103:535–44. doi: 10.1113/EP086764

27. Chen Y, Cheng J, Zhang Y, Chen JD, Selaru FM. Electroacupuncture at ST36 relieves visceral hypersensitivity via the NGF/TrkA/TRPV1 peripheral afferent pathway in a rodent model of post-inflammation rectal hypersensitivity. *J Inflamm Res.* (2021) 14:325–39. doi: 10.2147/JIR.S285146

28. Morgan M, Thai J, Trinh P, Habib M, Effendi KN, Ivanusic JJ. ASIC3 inhibition modulates inflammation-induced changes in the activity and sensitivity of Aδ and C fiber sensory neurons that innervate bone. *Mol Pain*. (2020) 16:174480692097595. doi: 10.1177/1744806920975950

29. Khan A, Khan S, Kim YS. Insight into pain modulation: nociceptors sensitization and therapeutic targets. *Curr Drug Targets*. (2019) 20:775–88. doi: 10.2174/1389450120666190131114244

30. Sneddon LU. Comparative physiology of nociception and pain. *Physiology*. (2018) 33:63–73. doi: 10.1152/physiol.00022.2017

31. Gieré C, Melchior M, Dufour A, Poisbeau P. Spinal integration of hot and cold nociceptive stimuli by wide-dynamic-range neurons in anesthetized adult rats. *PAIN Rep*. (2021) 6:e983. doi: 10.1097/PR9.0000000000000983

32. Ströher R, Oliveira C, Costa Lopes B, Silva LS, Regner GG, Richardt Medeiros H, et al. Maternal deprivation alters nociceptive response in a gender-dependent manner in rats. *Int J Dev Neurosci.* (2019) 76:25–33. doi: 10.1016/j.ijdevneu.2019.05.001

33. Marshall A, Alam U, Themistocleous A, Calcutt N, Marshall A. Novel and emerging electrophysiological biomarkers of diabetic neuropathy and painful diabetic neuropathy. *Clin Ther*. (2021) 43:1441–56. doi: 10.1016/j.clinthera.2021.03.020

34. Tokunaga R, Takahashi Y, Touj S, Hotta H, Leblond H, Kato F, et al. Attenuation of widespread hypersensitivity to noxious mechanical stimuli by inhibition of GABAergic neurons of the right amygdala in a rat model of chronic back pain. *Eur J Pain*. (2022) 26:911–28. doi: 10.1002/ejp.1921

35. Shreckengost J, Halder M, Mena-Avila E, Garcia-Ramirez DL, Quevedo J, Hochman S. Nicotinic receptor modulation of primary afferent excitability with selective regulation of Aδ-mediated spinal actions. *J Neurophysiol.* (2021) 125:568–85. doi: 10.1152/jn.00228.2020

36. Cooley K. Physiology of pain. In: Goldberg M, Shaffran N, editors. *Pain Management for Veterinary Technicians and Nurses*. Iowa: Wiley Blackwell (2015). p. 30–41.

37. Mohtashami BF, Joushi S, Taheri ZZ, Sheibani V, Esmaeilpour K. Environmental enrichment and pain sensitivity; a study in maternally separated rats. *Int J Dev Neurosci.* (2020) 80:347–53. doi: 10.1002/jdn.10031

38. Djouhri L, Koutsikou S, Fang X, McMullan S, Lawson SN. Spontaneous pain, both neuropathic and inflammatory, is related to frequency of spontaneous firing in intact C-fiber nociceptors. *J Neurosci.* (2006) 26:1281–92. doi: 10.1523/JNEUROSCI.3388-05.2006

39. Reischer G, Heinke B, Sandkühler J. Interferon-γ facilitates the synaptic transmission between primary afferent C-fibres and lamina I neurons in the rat spinal dorsal horn via microglia activation. *Mol Pain*. (2020) 16:174480692091724. doi: 10.1177/1744806920917249

40. Yu T, Zhang X, Shi H, Tian J, Sun L, Hu X, et al. P2Y12 regulates microglia activation and excitatory synaptic transmission in spinal lamina II neurons during neuropathic pain in rodents. *Cell Death Dis.* (2019) 10:165. doi: 10.1038/s41419-019-1425-4

41. Kumamoto E. Cellular mechanisms for antinociception produced by oxytocin and orexins in the rat spinal lamina II—comparison with those of other endogenous pain modulators. *Pharmaceuticals*. (2019) 12:136. doi: 10.3390/ph12030136

42. Xue M, Sun Y-L, Xia Y-Y, Huang Z-H, Huang C, Xing G-G. Electroacupuncture modulates spinal bdnf/trkb signaling pathway and ameliorates the sensitization of dorsal horn wdr neurons in spared nerve injury rats. *Int J Mol Sci.* (2020) 21:6524. doi: 10.3390/ijms21186524

43. Tao Z-YY, Wang P-XX, Wei S-QQ, Traub RJ Li J-FF, Cao D-YY. The role of descending pain modulation in chronic primary pain: potential application of drugs targeting serotonergic system. *Neural Plast*. (2019) 2019:1–16. doi: 10.1155/2019/1389296

44. Moazen P, Taherianfard M, Ahmadi SM, Norozpor M. Synergistic effect of spexin and progesterone on pain sensitivity attenuation in ovariectomized rats. *Clin Exp Pharmacol Physiol.* (2018) 45:349–54. doi: 10.1111/1440-1681.12862

45. Hughes DI, Todd AJ. Central nervous system targets: inhibitory interneurons in the spinal cord. *Neurotherapeutics*. (2020) 17:874–85. doi: 10.1007/s13311-020-00936-0

46. Moriya S, Yamashita A, Nishi R, Ikoma Y, Yamanaka A, Kuwaki T. Acute nociceptive stimuli rapidly induce the activity of serotonin and noradrenalin neurons in the brain stem of awake mice. *IBRO Rep*. (2019) 7:1–9. doi: 10.1016/j.ibror.2019.05.005

47. Yam M, Loh Y, Tan C, Khadijah Adam S, Abdul Manan N, Basir R. General pathways of pain sensation and the major neurotransmitters involved in pain regulation. *Int J Mol Sci.* (2018) 19:2164. doi: 10.3390/ijms19082164

48. Melzack R, Wall PD. Pain mechanisms: a new theory. *Science (80-).* (1965) 150:971–9. doi: 10.1126/science.150.3699.971

49. Haranishi Y, Hara K, Terada T. Antihyperalgesic effects of intrathecal perospirone in a rat model of neuropathic pain. *Pharmacol Biochem Behav*. (2020) 195:172964. doi: 10.1016/j.pbb.2020.172964

50. Jesus CHA, Redivo DDB, Gasparin AT, Sotomaior BB, de Carvalho MC, Genaro K, et al. Cannabidiol attenuates mechanical allodynia in streptozotocin-induced diabetic rats via serotonergic system activation through 5-HT1A receptors. *Brain Res*. (2019) 1715:156–64. doi: 10.1016/j.brainres.2019.03.014

51. Cortés-Altamirano JL, Olmos-Hernández A, Jaime HB, Carrillo-Mora P, Bandala C, Reyes-Long S, et al. Review: 5-HT1, 5-HT2, 5-HT3 and 5-HT7 receptors and their role in the modulation of pain response in the central nervous system. *Curr Neuropharmacol.* (2018) 16:1–12. doi: 10.2174/1570159X15666170911021027

52. Lucas-Osma AM Li Y, Murray K, Lin S, Black S, Stephens MJ, Ahn AH, et al. 5-HT 1D receptors inhibit the monosynaptic stretch reflex by modulating C-fiber activity. *J Neurophysiol.* (2019) 121:1591–608. doi: 10.1152/jn.00805.2018

53. Giniatullin R. 5-hydroxytryptamine in migraine: the puzzling role of ionotropic 5-HT 3 receptor in the context of established therapeutic effect of metabotropic 5-HT 1 subtypes. *Br J Pharmacol*. (2022) 179:400–15. doi: 10.1111/bph.15710

54. Liu QQ, Yao XX, Gao SH Li R, Li BJ, Yang W, Cui RJ. Role of 5-HT receptors in neuropathic pain: potential therapeutic implications. *Pharmacol Res*. (2020) 159:104949. doi: 10.1016/j.phrs.2020.104949

55. Hoshijima H, Hunt M, Nagasaka H, Yaksh T. Systematic review of systemic and neuraxial effects of acetaminophen in preclinical models of nociceptive processing. *J Pain Res.* (2021) 14:3521–52. doi: 10.2147/JPR.S308028

56. Sato KL, Sanada LS, Silva MD da, Okubo R, Sluka KA. Transcutaneous electrical nerve stimulation, acupuncture, and spinal cord stimulation on neuropathic, inflammatory and, non-inflammatory pain in rat models. *Korean J Pain.* (2020) 33:121–30. doi: 10.3344/kjp.2020.33.2.121

57. Harte SE, Harris RE, Clauw DJ. The neurobiology of central sensitization. *J Appl Biobehav Res.* (2018) 23:e12137. doi: 10.1111/jabr.12137

58. Temi S, Rudyk C, Armstrong J, Landrigan JA, Dedek C, Salmaso N, et al. Differential expression of GluN2 NMDA receptor subunits in the dorsal horn of male and female rats. *Channels.* (2021) 15:179–92. doi: 10.1080/19336950.2020.1871205

59. Gupta S, Gautam M, Prasoon P, Kumar R, Ray SB, Kaler Jhajhria S. Involvement of neuropeptide Y in post-incisional nociception in rats. *Ann Neurosci.* (2018) 25:268–76. doi: 10.1159/000495130

60. Mazzitelli M, Palazzo E, Maione S, Neugebauer V. Group II Metabotropic glutamate receptors: role in pain mechanisms and pain modulation. *Front Mol Neurosci.* (2018) 11:383. doi: 10.3389/fnmol.2018.00383

61. Tobaldini G, Sardi NF, Guilhen VA, Fischer L. Pain Inhibits pain: an ascending-descending pain modulation pathway linking mesolimbic and classical descending mechanisms. *Mol Neurobiol.* (2019) 56:1000–13. doi: 10.1007/s12035-018-1116-7

62. Talbot K, Madden VJ, Jones SL, Moseley GL. The sensory and affective components of pain: are they differentially modifiable dimensions or inseparable aspects of a unitary experience? A systematic review. *Br J Anaesth.* (2019) 123:263–72. doi: 10.1016/j.bja.2019.03.033

63. Al-Chalabi M, Reddy V, Gupta S. *Neuroanatomy, Spinothalamic Tract*. Treasure Island, FL: StatPearls Publishing (2018). 1 p.

64. Heijmans L, Joosten EA. Mechanisms and mode of action of spinal cord stimulation in chronic neuropathic pain. *Postgrad Med.* (2020) 132:17–21. doi: 10.1080/00325481.2020.1769393

65. Jung HH, Koh CS, Park M, Kim JH, Woo H, Lee H, et al. Microglial deactivation by adeno-associated virus expressing small-hairpin GCH1 has protective effects against neuropathic pain development in a spinothalamic tract-lesion model. *CNS Neurosci Ther.* (2022) 28:36–45. doi: 10.1111/cns.13751

66. Alimoradian A, Abbaszadeh F, Jorjani M, Sadegh M. The behavioral and molecular effects of estradiol and progesterone on a rat model of spinothalamic tract lesion. *Physiol Pharmacol.* (2021) 25:314–27. doi: 10.52547/phypha.25.4.9

67. Zhang C, Ma Z, Huo B, Lu Y, Wu J, Hua X, et al. Diffusional plasticity induced by electroacupuncture intervention in rat model of peripheral nerve injury. *J Clin Neurosci.* (2019) 69:250–6. doi: 10.1016/j.jocn.2019.08.088

68. Samandari R, Hassanpour-Ezatti M, Fakhri S, Abbaszadeh F, Jorjani M. Sex differences and role of gonadal hormones on glutamate level, after spinal cord injury in rats: a microdialysis study. *Basic Clin Neurosci J.* (2018) 10:225–34. doi: 10.32598/bcn.9.10.260

69. Fregoso G, Wang A, Tseng K, Wang J. Transition from acute tto chronic pain: evaluating risk for chronic postsurgical pain. *Pain Physician.* (2019) 22:479–88.

70. Xiao Z, Martinez E, Kulkarni PM, Zhang Q, Hou Q, Rosenberg D, et al. Cortical pain processing in the rat anterior cingulate cortex and primary somatosensory cortex. *Front Cell Neurosci.* (2019) 13:165. doi: 10.3389/fncel.2019.00165

71. Wu J-J, Lu Y-C, Hua X-Y, Ma S-J, Shan C-L, Xu J-G. Cortical remodeling after electroacupuncture therapy in peripheral nerve repairing model. *Brain Res.* (2018) 1690:61–73. doi: 10.1016/j.brainres.2018.04.009

72. Iwamoto S, Tamura M, Sasaki A, Nawano M. Dynamics of neuronal oscillations underlying nociceptive response in the mouse primary somatosensory cortex. *Sci Rep.* (2021) 11:1667. doi: 10.1038/s41598-021-81067-0

73. Kim JA, Davis KD. Neural oscillations: understanding a neural code of pain. *Neurosci.* (2021) 27:544–70. doi: 10.1177/1073858420958629

74. Ploner M, Gross J. Gamma oscillations shape pain in animals and humans. *Trends Cogn Sci.* (2019) 23:450–1. doi: 10.1016/j.tics.2019.04.001

75. Tan LL, Oswald MJ, Heinl C, Retana RROA, Kaushalya SK, Monyer H, et al. Gamma oscillations in somatosensory cortex recruit prefrontal and descending serotonergic pathways in aversion and nociception. *Nat Commun.* (2019) 10:983. doi: 10.1038/s41467-019-08873-z

76. Peng W, Xia X, Yi M, Huang G, Zhang Z, Iannetti G, et al. Brain oscillations reflecting pain-related behavior in freely moving rats. *Pain.* (2018) 159:106–18. doi: 10.1097/j.pain.0000000000001069

77. Zhu D-Y, Cao T-T, Fan H-W, Zhang M-Z, Duan H-K, Li J, et al. The increased *in vivo* firing of pyramidal cells but not interneurons in the anterior cingulate cortex after neuropathic pain. *Mol Brain.* (2022) 15:12. doi: 10.1186/s13041-022-00897-9

78. Zhao R, Zhou H, Huang L, Xie Z, Wang J, Gan W-B, et al. Neuropathic pain causes pyramidal neuronal hyperactivity in the anterior cingulate cortex. *Front Cell Neurosci.* (2018) 12:107. doi: 10.3389/fncel.2018.00107

79. Fakih D, Baudouin C, Réaux-Le Goazigo A, Mélik Parsadaniantz S. TRPM8: a therapeutic target for neuroinflammatory symptoms induced by severe dry eye disease. *Int J Mol Sci.* (2020) 21:8756. doi: 10.3390/ijms21228756

80. Huang S, Zhang Z, Gambeta E, Xu SC, Thomas C, Godfrey N, et al. Dopamine inputs from the ventral tegmental area into the medial prefrontal cortex modulate neuropathic pain-associated behaviors in mice. *Cell Rep.* (2020) 31:107812. doi: 10.1016/j.celrep.2020.107812

81. Meng X, Yue L, Liu A, Tao W, Shi L, Zhao W, et al. Distinct basolateral amygdala excitatory inputs mediate the somatosensory and aversive-affective components of pain. *J Biol Chem.* (2022) 298:102207. doi: 10.1016/j.jbc.2022.102207

82. Morris LS, Sprenger C, Koda K. de la Mora DM, Yamada T, Mano H, et al. Anterior cingulate cortex connectivity is associated with suppression of behaviour in a rat model of chronic pain. *Brain Neurosci Adv.* (2018) 2:239821281877964. doi: 10.1177/2398212818779646

83. Zong J, Liao X, Ren B, Wang Z. The antidepressant effects of rosiglitazone on rats with depression induced by neuropathic pain. *Life Sci.* (2018) 203:315–22. doi: 10.1016/j.lfs.2018.04.057

84. Yang Y, Song Y, Zhang X, Zhao W, Ma T, Liu Y, et al. Ketamine relieves depression-like behaviors induced by chronic postsurgical pain in rats through anti-inflammatory, anti-oxidant effects and regulating BDNF expression. *Psychopharmacology.* (2020) 237:1657–69. doi: 10.1007/s00213-020-05490-3

85. Kremer M, Becker LJ, Barrot M, Yalcin I. How to study anxiety and depression in rodent models of chronic pain? *Eur J Neurosci.* (2021) 53:236–70. doi: 10.1111/ejn.14686

86. McMillan FD. A world of hurts—is pain special? *J Am Vet Med Assoc.* (2003) 223:183–6. doi: 10.2460/javma.2003.223.183

87. Hernández-Avalos I, Mota-Rojas D, Mendoza-Flores JE, Casas-Alvarado A, Flores-Padilla K, Miranda-Cortés AE, et al. Nociceptive pain and anxiety in equines: physiological and behavioral alterations. *Vet World.* (2021) 14:2984–95. doi: 10.14202/vetworld.2021.2984-2995

88. Peterson NC, Nunamaker EA, Turner P V. To treat or not to treat: the effects of pain on experimental parameters. *Comp Med.* (2017) 67:469–82.

89. Wideman TH, Edwards RR, Walton DM, Martel MO, Hudon A, Seminowicz DA. The multimodal assessment model of pain. *Clin J Pain.* (2019) 35:212–21. doi: 10.1097/AJP.0000000000000670

90. Steagall P V, Monteiro BP. Acute pain in cats: recent advances in clinical assessment. *J Feline Med Surg.* (2019) 21:25–34. doi: 10.1177/1098612X18808103

91. Hernández-Avalos I, Mota-Rojas D, Mora-Medina P, Martínez-Burnes J, Casas-Alvarado A, Verduzco-Mendoza A, et al. Review of different methods used for clinical recognition and assessment of pain in dogs and cats. *Int J Vet Sci Med.* (2019) 7:43–54. doi: 10.1080/23144599.2019.1680044

92. Gottlieb AJ. Pain management for the emergency and critical care patient. In: Norkus CL, editor. *Veterinary Technician's Manual for Small Animal Emergency and Critical Care*. Hoboken, NJ: John Wiley & Sons (2018). p. 479–503.

93. Balcombe JPJP, Barnard NDND, Sandusky C. Laboratory routines cause animal stress. *J Am Assoc Lab Anim Sci.* (2004) 43:42–51.

94. Douglas AJ, Brunton PJ, Bosch OJ, Russell JA, Neumann ID. Neuroendocrine responses to stress in mice: hyporesponsiveness in pregnancy and parturition. *Endocrinology.* (2003) 144:5268–76. doi: 10.1210/en.2003-0461

95. Dieterich A, Stech K, Srivastava P, Lee J, Sharif A, Samuels BA. Chronic corticosterone shifts effort-related choice behavior in male mice. *Psychopharmacology.* (2020) 237:2103–10. doi: 10.1007/s00213-020-05521-z

96. Ieko T, Sasaki H, Maeda N, Fujiki J, Iwano H, Yokota H. Analysis of corticosterone and testosterone synthesis in rat salivary gland homogenates. *Front Endocrinol.* (2019) 10:479. doi: 10.3389/fendo.2019.00479

97. MacRae AM, Joanna MI, Fraser D, Makowska I, Fraser D. Initial evaluation of facial expressions and behaviours of harbour seal pups (Phoca vitulina) in response to tagging and microchipping. *Appl Anim Behav Sci.* (2018) 205:167–74. doi: 10.1016/j.applanim.2018.05.001

98. Cimino I, Kim H, Tung YCL, Pedersen K, Rimmington D, Tadross JA, et al. Activation of the hypothalamic–pituitary–adrenal axis by exogenous and endogenous GDF15. *Proc Natl Acad Sci USA.* (2021) 118:e2106868118. doi: 10.1073/pnas.2106868118

99. Buckenmaier C, Mahoney PF. *Combat Anesthesia: The First 24 Hours*. Virginia: Office of the Surgeon General (2015). p. 1–547.

100. Mota-Rojas D, Titto CG, Orihuela A, Martínez-Burnes J, Gómez-Prado J, Torres-Bernal F, et al. Physiological and behavioral mechanisms of thermoregulation in mammals. *Animals*. (2021) 11:1733. doi: 10.3390/ani11061733

101. Seal SV, Turner JD. The 'jekyll and hyde' of gluconeogenesis: early life adversity, later life stress, and metabolic disturbances. *Int J Mol Sci*. (2021) 22:3344. doi: 10.3390/ijms22073344

102. Weary DM, Niel L, Flower FC, Fraser D. Identifying and preventing pain in animals. *Appl Anim Behav Sci*. (2006) 100:64–76. doi: 10.1016/j.applanim.2006.04.013

103. Leung VSY, Benoit-Biancamano M-O, Pang DSJ. Performance of behavioral assays: the rat grimace scale, burrowing activity and a composite behavior score to identify visceral pain in an acute and chronic colitis model. *PAIN Rep*. (2019) 4:e718. doi: 10.1097/PR9.0000000000000712

104. Baptista-de-Souza D, Rodrigues Tavares LR, Canto-de-Souza L, Nunes-de-Souza RL, Canto-de-Souza A. Behavioral, hormonal, and neural alterations induced by social contagion for pain in mice. *Neuropharmacology*. (2022) 203:108878. doi: 10.1016/j.neuropharm.2021.108878

105. Shi M, Wang J-Y, Luo F. Depression shows divergent effects on evoked and spontaneous pain behaviors in rats. *J Pain*. (2010) 11:219–29. doi: 10.1016/j.jpain.2009.07.002

106. Draxler P, Moen A, Galek K, Boghos A, Ramazanova D, Sandkühler J. Spontaneous, voluntary, and affective behaviours in rat models of pathological pain. *Front Pain Res*. (2021) 2:672711. doi: 10.3389/fpain.2021.672711

107. Lee GJ, Kim YJ, Shim SW, Lee K, Oh SB. Anterior insular-nucleus accumbens pathway controls refeeding-induced analgesia under chronic inflammatory pain condition. *Neuroscience*. (2022) 495:58–73. doi: 10.1016/j.neuroscience.2022.05.025

108. Talbot SR, Biernot S, Bleich A, van Dijk RM, Ernst L, Häger C, et al. Defining body-weight reduction as a humane endpoint: a critical appraisal. *Lab Anim*. (2020) 54:99–110. doi: 10.1177/0023677219883319

109. Brennan MP, Sinusas AJ, Horvath TL, Collins JG, Harding MJ. Correlation between body weight changes and postoperative pain in rats treated with meloxicam or buprenorphine. *Lab Anim*. (2009) 38:87–93. doi: 10.1038/laban0309-87

110. Jablonski P, Howden BO, Baxter K. Influence of bupronorphine analgesia on post-operative recovery in two strains of rats. *Lab Anim*. (2001) 35:213–22.

111. Rasoulian B, Hajializadeh Z, Esmaeili-Mahani S, Rashidipour M, Fatemi I, Kaeidi A. Neuroprotective and antinociceptive effects of rosemary (Rosmarinus officinalis L) extract in rats with painful diabetic neuropathy. *J Physiol Sci*. (2019) 69:57–64. doi: 10.1007/s12576-018-0620-x

112. Hasriadi D, Vajragupta O, Rojsitthisak P, Towiwat P. Automated home-cage monitoring as a potential measure of sickness behaviors and pain-like behaviors in LPS-treated mice. *PLoS ONE*. (2021) 16:e0256706. doi: 10.1371/journal.pone.0256706

113. Haghani J, Haghani F, Soleimani A, Abbasnejad M, Khodami M, Kooshki R, et al. Hydroalcoholic extracts of three Artemisia species attenuate dental pulp pain and pain-reñated abnormal feeding behavior of rats. *Iran J Vet Sci Technol*. (2022) 14:29–37. doi: 10.22067/ijvst.2022.72372.1075

114. Bellinger LL, Spears R, King CM, Dahm F, Hutchins B, Kerins CA, et al. Capsaicin sensitive neurons role in the inflamed TMJ acute nociceptive response of female and male rats. *Physiol Behav*. (2007) 90:782–9. doi: 10.1016/j.physbeh.2007.01.002

115. Kerins CA, Carlson DS, Hinton RJ, Hutchins B, Grogan DM, Marr K, et al. Specificity of meal pattern analysis as an animal model of determining temporomandibular joint inflammation/pain. *Int J Oral Maxillofac Surg*. (2005) 34:425–31. doi: 10.1016/j.ijom.2004.08.010

116. Li J, Ma K, Yi D, Oh C, Chen D. Nociceptive behavioural assessments in mouse models of temporomandibular joint disorders. *Int J Oral Sci*. (2020) 12:26. doi: 10.1038/s41368-020-00095-0

117. Schaap MWH, Uilenreef JJ, Mitsogiannis MD. van 't Klooster JG, Arndt SS, Hellebrekers LJ. Optimizing the dosing interval of buprenorphine in a multimodal postoperative analgesic strategy in the rat: minimizing side-effects without affecting weight gain and food intake. *Lab Anim*. (2012) 46:287–92. doi: 10.1258/la.2012.012058

118. Hogri R, Teuchmann HL, Heinke B, Holzinger R, Trofimova L, Sandkühler J. GABAergic CaMKIIα+ Amygdala output attenuates pain and modulates emotional-motivational behavior via parabrachial inhibition. *J Neurosci*. (2022) 42:5373–88. doi: 10.1523/JNEUROSCI.2067-21.2022

119. Guo W, Zou S, Mohammad Z, Wang S, Yang J, Li H, et al. Voluntary biting behavior as a functional measure of orofacial pain in mice. *Physiol Behav*. (2019) 204:129–39. doi: 10.1016/j.physbeh.2019.02.024

120. Scheff NN, Bhattacharya A, Dowse E, Dang RX, Dolan JC, Wang S, et al. Neutrophil-mediated endogenous analgesia contributes to sex differences in oral cancer pain. *Front Integr Neurosci*. (2018) 12:52. doi: 10.3389/fnint.2018.00052

121. Larson CM, Wilcox GL, Fairbanks CA. The study of pain in rats and mice. *Comp Med*. (2019) 69:555–70. doi: 10.30802/AALAS-CM-19-000062

122. Mahmoudi J, Mohaddes G, Erfani M, Sadigh-Eteghad S, Karimi P, Rajabi M, et al. Cerebrolysin attenuates hyperalgesia, photophobia, and neuroinflammation in a nitroglycerin-induced migraine model in rats. *Brain Res Bull*. (2018) 140:197–204. doi: 10.1016/j.brainresbull.2018.05.008

123. Farajdokht F, Babri S, Karimi P, Mohaddes G. Ghrelin attenuates hyperalgesia and light aversion-induced by nitroglycerin in male rats. *Neurosci Lett*. (2016) 630:30–7. doi: 10.1016/j.neulet.2016.07.026

124. Azizzadeh F, Mahmoodi J, Sadigh-Eteghad S, Farajdokht F, Mohaddes G. Ghrelin exerts analgesic effects through modulation of il-10 and tgf-β levels in a rat model of inflammatory pain. *Iran Biomed J*. (2017) 21:114–9. doi: 10.18869/acadpub.ibj.21.2.114

125. Shepherd AJ, Cloud ME, Cao Y-Q, Mohapatra DP. Deficits in burrowing behaviors are associated with mouse models of neuropathic but not inflammatory pain or migraine. *Front Behav Neurosci*. (2018) 12:124. doi: 10.3389/fnbeh.2018.00124

126. Wang M, Castonguay WC, Duong TL, Huebner MW, Flinn HC, Greenway AM, et al. Stimulation of CGRP-expressing neurons in the medial cerebellar nucleus induces light and touch sensitivity in mice. *Neurobiol Pain*. (2022) 12:100098. doi: 10.1016/j.ynpai.2022.100098

127. Chartier LC, Hebart ML, Howarth GS, Whittaker AL, Mashtoub S. Affective state determination in a mouse model of colitis-associated colorectal cancer. *PLoS ONE*. (2020) 15:e0228413. doi: 10.1371/journal.pone.0228413

128. Griffiths LA, Duggett NA, Pitcher AL, Flatters SJL. Evoked and ongoing pain-like behaviours in a rat model of paclitaxel-induced peripheral neuropathy. *Pain Res Manag*. (2018) 2018:1–11. doi: 10.1155/2018/8217613

129. Leung V, Zhang E, Pang DS. Real-time application of the Rat Grimace Scale as a welfare refinement in laboratory rats. *Sci Rep*. (2016) 6:1–12. doi: 10.1038/srep31667

130. Tappe-Theodor A, King T, Morgan MM. Pros and cons of clinically relevant methods to assess pain in rodents. *Neurosci Biobehav Rev*. (2019) 100:335–43. doi: 10.1016/j.neubiorev.2019.03.009

131. Dutta R, Lunzer MM, Auger JL, Akgün E, Portoghese PS, Binstadt BA, et al. bivalent compound targeting CCR5 and the mu opioid receptor treats inflammatory arthritis pain in mice without inducing pharmacologic tolerance. *Arthr Res Ther*. (2018) 20:154. doi: 10.1186/s13075-018-1661-5

132. Miller L, McPherson S. Age-dependent differences in pain-related depression of nesting in mice. *FASEB J*. (2019) 33:10918. doi: 10.1096/fasebj.2019.33.1_supplement.808.9

133. Whittaker AL, Barker TH. The impact of common recovery blood sampling methods, in mice (Mus Musculus), on well-being and sample quality: a systematic review. *Animals*. (2020) 10:989. doi: 10.3390/ani10060989

134. Neff EP. On the road to refinement. *Lab Anim*. (2021) 50:277–9. doi: 10.1038/s41684-021-00854-4

135. Demin KA, Lakstygal AM, Volgin AD, de Abreu MS, Genario R, Alpyshov ET, et al. Cross-species analyses of intra-species behavioral differences in mammals and fish. *Neuroscience*. (2020) 429:33–45. doi: 10.1016/j.neuroscience.2019.12.035

136. Kim SK, Moon HJ, Park JH, Lee G, Shin M-K, Hong M-C, et al. The maintenance of individual differences in the sensitivity of acute and neuropathic pain behaviors to electroacupuncture in rats. *Brain Res Bull*. (2007) 74:357–60. doi: 10.1016/j.brainresbull.2007.07.006

137. Mogil JS. Sources of individual differences in pain. *Annu Rev Neurosci*. (2021) 44:1–25. doi: 10.1146/annurev-neuro-092820-105941

138. Martínez-Navarro M, Lara-Mayorga IM, Negrete R, Bilecki W, Wawrzczak-Bargieła A, Gonçalves L, et al. Influence of behavioral traits in the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain. *Neuropharmacology*. (2019) 148:291–304. doi: 10.1016/j.neuropharm.2019.01.012

139. Presto P, Ji G, Junell R, Griffin Z, Neugebauer V. Fear extinction-based inter-individual and sex differences in pain-related vocalizations and anxiety-like behaviors but not nocifensive reflexes. *Brain Sci*. (2021) 11:1339. doi: 10.3390/brainsci11101339

140. Mogil JS. The genetic mediation of individual differences in sensitivity to pain and its inhibition. *Proc Natl Acad Sci USA*. (1999) 96:7744–51. doi: 10.1073/pnas.96.14.7744

141. Lee G, Rho S, Shin M, Hong M, Min B, Bae H. The association of cholecystokinin-A receptor expression with the responsiveness

of electroacupuncture analgesic effects in rat. *Neurosci Lett.* (2002) 325:17–20. doi: 10.1016/S0304-3940(02)00214-8

142. van der Goot MH, Keijsper M, Baars A, Drost L, Hendriks J, Kirchhoff S, et al. Inter-individual variability in habituation of anxiety-related responses within three mouse inbred strains. *Physiol Behav.* (2021) 239:113503. doi: 10.1016/j.physbeh.2021.113503

143. LaCroix-Fralish ML, Mo G, Smith SB, Sotocinal SG, Ritchie J, Austin J-S, et al. The β3 subunit of the Na+,K+-ATPase mediates variable nociceptive sensitivity in the formalin test. *Pain.* (2009) 144:294–302. doi: 10.1016/j.pain.2009.04.028

144. Erickson K, Schulkin J. Facial expressions of emotion: a cognitive neuroscience perspective. *Brain Cogn.* (2003) 52:52–60. doi: 10.1016/S0278-2626(03)00008-3

145. Gothard KM. The amygdalo-motor pathways and the control of facial expressions. *Front Neurosci.* (2014) 8:43. doi: 10.3389/fnins.2014.00043

146. Ekman P, Friesen W V. Measuring facial movement. *Environ Psychol Nonverbal Behav.* (1976) 1:56–75. doi: 10.1007/BF01115465

147. Clark EA, Kessinger J, Duncan SE, Bell MA, Lahne J, Gallagher DL, et al. The facial action coding system for characterization of human affective response to consumer product-based stimuli: a systematic review. *Front Psychol.* (2020) 11:920. doi: 10.3389/fpsyg.2020.00920

148. Kunz M, Meixner D, Lautenbacher S. Facial muscle movements encoding pain—a systematic review. *Pain.* (2019) 160:535–49. doi: 10.1097/j.pain.0000000000001424

149. Dawes TR, Eden-Green B, Rosten C, Giles J, Governo R, Marcelline F, et al. Objectively measuring pain using facial expression: is the technology finally ready? *Pain Manag.* (2018) 8:105–13. doi: 10.2217/pmt-2017-0049

150. Dolensek NN, Gehrlach DA, Klein AS, Gogolla N. Facial expressions of emotion states and their neuronal correlates in mice. *Science (80-).* (2020) 368:89–94. doi: 10.1126/science.aaz9468

151. McLennan KKM, Rebelo CCJB, Corke MMJ, Holmes MA, Leach MC, Constantino-Casas F. Development of a facial expression scale using footrot and mastitis as models of pain in sheep. *Appl Anim Behav Sci.* (2016) 176:19–26. doi: 10.1016/j.applanim.2016.01.007

152. Finlayson K, Lampe JFJ, Hintze S, Würbel H, Melotti L. Facial indicators of positive emotions in rats. *PLoS ONE.* (2016) 11:e0166446. doi: 10.1371/journal.pone.0166446

153. Lecorps B, Féron C. Correlates between ear postures and emotional reactivity in a wild type mouse species. *Behav Proc.* (2015) 120:25–9. doi: 10.1016/j.beproc.2015.08.002

154. Ebbesen CL, Froemke RC. Body language signals for rodent social communication. *Curr Opin Neurobiol.* (2021) 68:91–106. doi: 10.1016/j.conb.2021.01.008

155. Di Giminiani P, Brierley V, Scollo A. The assessment of facial expressions in piglets undergoing tail docking and castration: toward the development of the piglet grimace scale. *Front Vet Sci.* (2016) 14:100. doi: 10.3389/fvets.2016.00100

156. Freedman DJ, Ibos G. An integrative framework for sensory, motor, and cognitive functions of the posterior parietal cortex. *Neuron.* (2018) 97:1219–34. doi: 10.1016/j.neuron.2018.01.044

157. Ballotta D, Lui F, Porro CA, Nichelli PF, Benuzzi F. Modulation of neural circuits underlying temporal production by facial expressions of pain. *PLoS ONE.* (2018) 13:e0193100. doi: 10.1371/journal.pone.0193100

158. Descovich K. Facial expression: an under-utilised tool for the assessment of welfare in mammals. *ALTEX.* (2017) 34:409–29. doi: 10.14573/altex.1607161

159. Dalla Costa E, Minero M, Lebelt D, Stucke D, Canali E, Leach MC. Development of the horse grimace scale (HGS) as a pain assessment tool in horses undergoing routine castration. *PLoS ONE.* (2014) 9:e92281. doi: 10.1371/journal.pone.0092281

160. Whittaker AL, Liu Y, Barker TH. Methods used and application of the mouse grimace scale in biomedical research 10 years on: a scoping review. *Animals.* (2021) 11:673. doi: 10.3390/ani11030673

161. Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, et al. The rat grimace scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. *Mol Pain.* (2011) 7:55. doi: 10.1186/1744-8069-7-55

162. Oliver V, De Rantere D, Ritchie R, Chisholm J, Hecker KG, Pang DSJ. Psychometric assessment of the rat grimace scale and development of an analgesic intervention score. *PLoS ONE.* (2014) 9:e97882. doi: 10.1371/journal.pone.0097882

163. Watson CRR, Sakai S, Armstrong W. Organization of the facial nucleus in the rat. *Brain Behav Evol.* (1982) 20:19–28. doi: 10.1159/000121578

164. Severson KS, Xu D, Yang H, O'Connor DH. Coding of whisker motion across the mouse face. *Elife.* (2019) 8:e41535. doi: 10.7554/eLife.41535

165. Goldberg ME. How to be a pain management advocate for exotic and zoo animals. *Vet Nurse.* (2017) 8:389–97. doi: 10.12968/vetn.2017.8.7.389

166. Mota-Rojas D, Olmos-Hernández A, Verduzco-Mendoza A, Hernández E, Martínez-Burnes J, Whittaker AL. The utility of grimace scales for practical pain assessment in laboratory animals. *Animals.* (2020) 10:1838. doi: 10.3390/ani10101838

167. Hohlbaum K, Corte G, Humpenöder M. Reliability of the Mouse Grimace Scale in C57BL/6JRj mice. *Animals.* (2020) 10:1648. doi: 10.3390/ani10091648

168. McLennan KM, Miller AL, Dalla Costa E, Stucke D, Corke MJ, Broom DM, et al. Conceptual and methodological issues relating to pain assessment in mammals: the development and utilisation of pain facial expression scales. *Appl Anim Behav Sci.* (2019) 217:1–15. doi: 10.1016/j.applanim.2019.06.001

169. Lunde CE, Sieberg CB. Walking the tightrope: a proposed model of chronic pain and stress. *Front Neurosci.* (2020) 14:270. doi: 10.3389/fnins.2020.00270

170. Tereshenko V, Dotzauer DC, Maierhofer U, Festin C, Luft M, Laengle G, et al. Selective denervation of the facial dermato-muscular complex in the rat: experimental model and anatomical basis. *Front Neuroanat.* (2021) 15:650761. doi: 10.3389/fnana.2021.650761

171. Prescott MJ, Lidster K. Improving quality of science through better animal welfare: the NC3Rs strategy. *Lab Anim.* (2017) 46:152–6. doi: 10.1038/laban.1217

172. George RP, Howarth GS, Whittaker AL. Use of the Rat Grimace Scale to evaluate visceral pain in a model of chemotherapy-induced mucositis. *Animals.* (2019) 9:678. doi: 10.3390/ani9090678

173. Cho C, Michailidis V, Lecker I, Collymore C, Hanwell D, Loka M, et al. Evaluating analgesic efficacy and administration route following craniotomy in mice using the grimace scale. *Sci Rep.* (2019) 9:359. doi: 10.1038/s41598-018-36897-w

174. Ernst L, Bruch S, Kopaczka M, Merhof D, Bleich A, Tolba RH, et al. model-specific simplification of the mouse grimace scale based on the pain response of intraperitoneal CCl4 injections. *Sci Rep.* (2022) 12:10910. doi: 10.1038/s41598-022-14852-0

175. Heinsinger NM, Spagnuolo G, Allahyari RV, Galer S, Fox T, Jaffe DA, et al. Facial grimace testing as an assay of neuropathic pain-related behavior in a mouse model of cervical spinal cord injury. *Exp Neurol.* (2020) 334:113468. doi: 10.1016/j.expneurol.2020.113468

176. Klune CB, Larkin AE, Leung VSY, Pang D. Comparing the rat grimace scale and a composite behaviour score in rats. *PLoS ONE.* (2019) 14:e0209467. doi: 10.1371/JOURNAL.PONE.0209467

177. Akintola T, Raver C, Studlack P, Uddin O, Masri R, Keller A. The grimace scale reliably assesses chronic pain in a rodent model of trigeminal neuropathic pain. *Neurobiol Pain.* (2017) 2:13–7. doi: 10.1016/j.ynpai.2017.10.001

178. Philips BH, Weisshaar CL, Winkelstein BA. Use of the rat grimace scale to evauate neuropathic pain in a model of cervical radiculopathy. *Comp Med.* (2017) 67:34–42.

179. Xu X, Craig KD, Diaz D, Goodwin MS, Akcakaya M, Susam BT, et al. Automated pain detection in facial videos of children using human-assisted transfer learning. *Lect Notes Comput Sci.* (2018) 11326:162–80. doi: 10.1007/978-3-030-12738-1_12

180. Zhang EQ, Leung VSY, Pang DSJ. Influence of rater training on inter- And intrarater reliability when using the rat grimace scale. *J Am Assoc Lab Anim Sci.* (2019) 58:178–83. doi: 10.30802/AALAS-JAALAS-18-000044

181. Manthey T, Nagel-Riedasch S, Dülsner A. The interactive stress assessment in basic animal science training. *Animals.* (2021) 11:2145. doi: 10.3390/ani11072145

182. Abdelrahman A, Kumstel S, Zhang X, Liebig M, Wendt EHU, Eichberg J, et al. novel multi-parametric analysis of non-invasive methods to assess animal distress during chronic pancreatitis. *Sci Rep.* (2019) 9:14084. doi: 10.1038/s41598-019-50682-3

183. Turner PV. Moving beyond the absence of pain and distress: focusing on positive animal welfare. *ILAR J.* (2019) 60:366–72. doi: 10.1093/ilar/ilaa017

184. Whittaker AL, Latimer-Marsh L. The role of behavioural assessment in determining "positive" affective states in animals. *CAB Rev Perspect Agric Vet Sci Nutr Nat Resour.* (2019) 14:1–13. doi: 10.1079/PAVSNNR201914010

185. LaFollette MR, Cloutier S, Brady C, Gaskill BN. O'Haire ME. Laboratory animal welfare and human attitudes: A cross-sectional survey on heterospecific play or "rat tickling". *PLoS ONE.* (2019) 14:e0220580. doi: 10.1371/journal.pone.0220580

186. Coffey KR, Marx RG, Neumaier JF. DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations. *Neuropsychopharmacology*. (2019) 44:859–68. doi: 10.1038/s41386-018-0303-6

187. Cortese GP, Olin A, O'Riordan K, Hullinger R, Burger C. Environmental enrichment improves hippocampal function in aged rats by enhancing learning and memory, LTP, and mGluR5-Homer1c activity. *Neurobiol Aging*. (2018) 63:1–11. doi: 10.1016/j.neurobiolaging.2017.11.004

188. André V, Gau C, Scheideler A, Aguilar-Pimentel JA, Amarie O V, Becker L, et al. Laboratory mouse housing conditions can be improved using common environmental enrichment without compromising data. *PLoS Biol*. (2018) 16:e2005019. doi: 10.1371/journal.pbio.2005019

189. Voelkl B, Würbel H, Krzywinski M, Altman N. The standardization fallacy. *Nat Methods*. (2021) 18:5–7. doi: 10.1038/s41592-020-01036-9

190. Kentner AC, Speno A V, Doucette J, Roderick RC. The contribution of environmental enrichment to phenotypic variation in mice and rats. *eneuro*. (2021) 8:1–10. doi: 10.1523/ENEURO.0539-20.2021

191. Würbel H, Garner JP. Refinement of rodent research through environmental enrichment and systematic randomization. *NC3Rs*. (2007) 9:1–9.

192. Smith AJ, Lilley E. The role of the three rs in improving the planning and reproducibility of animal experiments. *Animals*. (2019) 9:975. doi: 10.3390/ani9110975

193. Verduzco-Mendoza A, Bueno-Nava A, Wang D, Martínez-Burnes J, Olmos-Hernández A, Casas A, et al. Experimental applications and factors involved in validating thermal windows using infrared thermography to assess the health and thermostability of laboratory animals. *Animals*. (2021) 11:3448. doi: 10.3390/ani11123448

194. McLennan K, Mahmoud M. Development of an automated pain facial expression detection system for sheep (Ovis Aries). *Animals*. (2019) 9:196. doi: 10.3390/ani9040196

195. Dalla Costa E, Pascuzzo R, Leach MC Dai F, Lebelt D, Vantini S, Minero M. Can grimace scales estimate the pain status in horses and mice? A statistical approach to identify a classifier. *PLoS ONE*. (2018) 13:e0200339. doi: 10.1371/journal.pone.0200339

196. Blumrosen G, Hawellek D, Pesaran B. Towards automated recognition of facial expressions in animal models. *2017 IEEE International Conference on Computer Vision Workshops (ICCVW)*. Venice (2017). p. 2810–9.

3. JUSTIFICACIÓN

El uso de animales con fines de investigación plantea diversos desafíos científicos, éticos y legales, los cuales requieren la aplicación de los principios de las 3Rs al promover el refinamiento de las técnicas empleadas con el fin de prevenir y minimizar el dolor y sufrimiento de las especies (36). Esto no sólo debe procurarse durante la vida del animal sino durante el periodo de eutanasia.

Actualmente existe controversia debido al potencial dolor que los animales pueden percibir por la aplicación de agentes inyectables, inhalatorios y físicos durante los procesos de eutanasia (3,37,38). Aunque existen normas y guías nacionales e internacionales que clasifican a estos métodos como aceptados (39), el nivel de dolor que puedan percibir los animales de laboratorio es un campo de investigación.

De esta manera, la aplicación de técnicas no invasivas como la IRT y la RGS pueden contribuir al estudio de la nocicepción y dolor, evaluando los componentes fisiológicos y emocionales del mismo, sin generar estrés adicional (26,40,41). La implementación de estas herramientas podría ayudar a valorar la calidad de la eutanasia, su eficacia y su relación con el bienestar animal.

4. PREGUNTAS DE INVESTIGACIÓN

1. ¿Cuáles son los cambios en la microcirculación asociados al dolor, durante la aplicación de distintos métodos inyectables, inhalatorios y físicos de eutanasia en ratas de laboratorio?

2. ¿Cuáles son las expresiones faciales que se modifican por efecto de la nocicepción y dolor durante diferentes métodos inyectables, inhalatorios y físicos de eutanasia en ratas de laboratorio?

5. HIPÓTESIS

1. El uso de la termografía infrarroja durante distintos métodos de eutanasia para ratas de laboratorio (*Rattus norvegicus*) permitirá reconocer cambios fisiológicos en la microcirculación, resultado de emociones negativas (nocicepción, dolor, ansiedad) relacionadas al método empleado.

2. La escala de muecas RGS ayudará a determinar el grado de nocicepción, dolor y bienestar animal de ratas de laboratorio (*R. norvegicus*) sometidas a diferentes métodos inyectables, inhalatorios y físicos de eutanasia.

3. El método de eutanasia por sobredosis con anestésico en combinación con la exposición a CO_2 evitará el sufrimiento prolongado.

6. OBJETIVO GENERAL

Determinar la eficacia de distintos métodos de eutanasia en ratas de laboratorio, mediante la evaluación de la nocicepción y dolor a través de los cambios observados en la termografía infrarroja y las expresiones faciales en la escala de muecas de rata (Rat Grimace Scale).

7. OBJETIVOS ESPECÍFICOS

1. Valorar los cambios termográficos y las expresiones faciales durante la eutanasia de ratas bajo diferentes métodos inyectables (sobredosis de pentobarbital, ketamina + xilacina), inhalatorios (CO_2, isoflurano), físicos (decapitación) y combinaciones con anestesia parenteral (ketamina + CO_2).

2. Determinar cuál método de eutanasia evita o disminuye el sufrimiento prolongado durante la eutanasia en ratas.

8. MATERIAL Y MÉTODOS

8.1. Tipo de estudio

Este fue un estudio experimental comparativo-prospectivo. Todas las mediciones fueron realizadas por un solo evaluador entrenado en un ensayo no ciego.

8.2. Localización y aprobación ética

El presente estudio se realizó en el área de laboratorios del Servicio de Bioterio y Cirugía Experimental del Instituto Nacional de Rehabilitación- Luis Guillermo Ibarra Ibarra (INR-LGII), de la Secretaría de Salud (Figura 1), ubicado en Calzada México-Xochimilco 289, Coapa, Guadalupe Tlalpan, Tlalpan, 14389 Ciudad de México, CDMX. Las coordenadas de ubicación son: 19.289667592615135, -99.14886385396737.

Figura 1. Área de laboratorios y quirófanos del servicio de bioterio del Instituto Nacional de Rehabilitación. Espacio físico donde se realizaron los diferentes métodos de eutanasia.

Todos los procedimientos fueron aprobados por el Comité para el Cuidado y Uso de Animales de Laboratorio (INRLGII/CICUAL/014/2021). El manejo y cuidado de los animales

de laboratorio fue con estricto apego a la Norma Mexicana NOM-062-ZOO-1999 (42), publicada por la Secretaría de Agricultura y Desarrollo Rural (SADER),la cual describe las especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. Los cadáveres de todos los animales fueron dispuestos mediante incineración, de acuerdo con la NOM-062-ZOO-1999.

8.3. Sujetos de estudio

Sesenta ratas adultas de la cepa Wistar (*R. norvegicus*), treinta machos y treinta hembras, fueron obtenidas de la Unidad de Producción y Experimentación de Animales de Laboratorio (UPEAL) del Centro de Investigación y de Estudios Avanzados (CINVESTAV) del Instituto Politécnico Nacional (IPN), ubicado en Avenida Instituto Politécnico Nacional 2508, San Pedro Zacatenco, Gustavo A. Madero, 07360 Ciudad de México, CDMX, México. Coordenadas 19°30′33″N 99°07′46″O.

Los animales tuvieron un peso promedio de 311 ± 62 g y 8-10 semanas de edad (en pubertad). Todas las ratas fueron obtenidas con un Certificado de Salud Animal en el cual se declararon como libres de patógenos infecciosos (bacterias, virus y parásitos). El tamaño de muestra fue calculado con el software G*Power 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). El tamaño total de muestras fue de 48 animales, considerando una probabilidad de error α de 0.05, nivel de confianza del 95%, poder (probabilidad de error 1-β) de 0.90, y una corrección entre medidas repetidas de 0.5 por los seis grupos experimentales con cinco tiempos de evaluación.

8.3.1. Criterios de inclusión

De acuerdo con los principios de las 3Rs (43), la reducción fue aplicada al número total de animales mediante el reúso de ratas provenientes de protocolos concluidos de etología (p.ej., pruebas de laberinto o de viga de equilibrio). Los animales seleccionados fueron parte de los grupos control para evitar la inclusión de ratas que hayan sido sometidas a procedimientos invasivos o que tuvieran residuos de fármacos. A través de un examen físico general las ratas fueron clasificadas como saludables sin signos de enfermedad, estrés o comportamientos asociados a estrés. El examen físico consideró el peso corporal, postura, nivel de conciencia, secreciones, color de las mucosas, estornudos, y repertorio conductual especie-específico.

8.3.2. Criterios de exclusión

Se eliminaron a todas las ratas que presentaron signos de enfermedad, lesiones o dolor al realizar el examen físico general, además de hembras gestantes.

8.4. Alojamiento de los animales

Las ratas fueron alojadas en cuartos diferentes de acuerdo con el sexo. Fueron colocados cinco animales por jaula, en cajas convencionales para roedores de policarbonato (47 x 36 x 21 cm) con cama de virutas de madera aspen esterilizable (Aspen, Nepco, USA) y sin enriquecimiento. Las ratas fueron mantenidas en un ciclo de luz-obscuridad de 12 horas/día, con las luces encendidas de las 0500 h a las 1700 h. La temperatura dentro de los cuartos de alojamiento y de procedimientos se mantuvo en un promedio de 23.2 ± 0.5°C y 22.9 ± 0.5°C (rango aceptado de 18-26°C), respectivamente, con una humedad relativa de 48% y 52% (rango aceptado de 40-70%), respectivamente, y una ventilación con 15 a 18 recambios de aire por hora, valores recomendados por la NOM-062-ZOO-1999 (Figura 2) (12).

Figura 2. Área de estancias para ratas. Zona del bioterio donde se alojan los animales, separados por especies y por sexos.

Las ratas tuvieron acceso *ad libitum* a alimento pelletizado y esterilizado para roedores (LabDiet 5010, LabDiet, USA). Los comederos serán rellenados en la mañana o en la tarde, dependiendo del consumo durante el transcurso del día. El agua purificada *ad libitum* se brindará en bebederos para roedores de 500 ml; el cambio del agua se realizará diariamente durante las mañanas.

Antes de ser incluidas en el presente estudio, las ratas pasarán por un periodo de habituación al evaluador. Este periodo constará de 15 días en los que el evaluador estará en constante acercamiento con los animales (durante 15 minutos en las mañanas, a medio día y en la tarde) para reducir el estrés al que los roedores son propensos a experimentar por el manejo (44).

8.5. Materiales e insumos físicos

8.5.1. Material físico

8.5.1.1. Material de laboratorio

- Jeringa insulínica estéril de 1 mL con aguja desmontable calibre 27G X 13 mm (Ambiderm ®, México)
- Guantes de látex estériles para exploración (Ambiderm ®, México)
- Cubrebocas quirúrgico de tres pliegos con filtro antibacterial (Ambiderm ®, México)
- Bolsa amarilla de residuos peligrosos sólidos biológico-infecciosos (Magry, México)
- Bolsa roja de residuos biológico-infecciosos (Mary, México)
- Algodón estéril
- Pinza de disección
- Campos quirúrgicos
- Ropa quirúrgica
- Papel de estraza
- Tapetes de corcho
- Cinta adhesiva

8.5.1.2. Equipo de laboratorio

- Guillotina (51330, Senna, México)
- Cámara de inducción para CO_2 (diseñada por los autores).
- Cámara de inducción a la anestesia para inhalación de isoflurano (diseñada por los autores).
- Balanza portátil (CS2000, Ohaus ®, México)
- Cámara termográfica (FLIR Thermal TM E60, FLIR Systems ®, USA)
- Cámara de video (Ixy 550, Cannon ®, Japón)

8.5.1.3. Formatos de registro

- Formato de monitorización para la IRT y la RGS

8.5.2. Material químico

- Ketamina (Anesket, Pisa Agropecuaria ®, México)
- Isoflurano (Fluriso, VET ONE ®, India)
- Pentobarbital (Pentobarbital, Aranda ®, México)

- Xilacina (Procin, Pisa Pisa Agropecuaria ®, México)
- Alcohol etílico al 70%

8.6. Diseño experimental

Todos los protocolos experimentales se llevarán a cabo con estricto apego a la norma NOM-062-ZOO-1999 para el uso y cuidado de los animales de laboratorio (12), y bajo las directrices ARRIVE (Animal Research: Reporting of In vivo Experiments) para el uso ético de los animales (45).

Una vez seleccionados a los sujetos de estudio, los animales pasaron por un periodo de habituación al manejo experimental, a la cámara de inducción de anestesia y a la presencia del evaluador. Los animales fueron divididos de manera aleatoria por generación de números (Microsoft Excel; Microsoft 365). 10 ratas fueron asignadas en cada grupo experimental, con cinco machos y cinco hembras, como se muestra en la Figura 3.

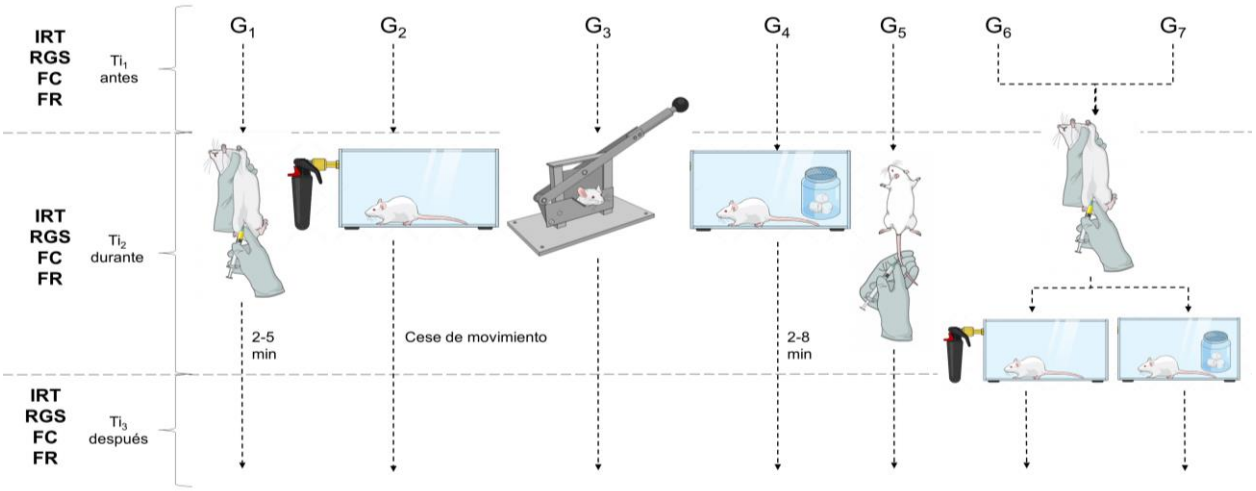


Figura 3. Diseño experimental. Diagrama que describe los grupos experimentales del presente estudio, los tiempos a evaluar y las variables de respuesta para cada individuo. Grupos (G1: pentobarbital; G2: CO2; G3: decapitación; G4: isoflurano; G5: Propofol; G6: ketamina + CO2; G7: ketamina + isoflurano). Tiempos (Ti_1: antes del procedimiento; Ti_2: durante el procedimiento; Ti_3: después del procedimiento). Variables de respuesta (FC: frecuencia cardiaca; FR: frecuencia respiratoria; IRT: termografía infrarroja; RGS: escala de muecas de rata).

8.6.1. Eutanasia por sobredosis de pentobarbital (G_1; n=10)

Se administró una sobredosis de 400 mg/kg IP de pentobarbital (Pentobarbital, Aranda ®, Mexico) con una jeringa estéril de 3 ml (Ambiderm ®, México), siguiendo el procedimiento de Lofgren et al. (46) y Villano y Sharp (47). La dosis fue calculada realizando un estudio piloto (información no publicada), empleando las dosis máximas y mínimas que aparecen en el estudio de Reimer et al. (48). La dosis seleccionada resultó en una rápida pérdida de la conciencia, depresión cardiorrespiratoria sin excitación, pérdida de reflejos y muerte clínica (Figura 4).

Figura 4. Administración de fármacos por vía intraperitoneal. Para esta vía de administración, se introdujo la aguja de la jeringa en el cuadrante abdominal inferior derecho. Se retrajo el émbolo para asegurar que la aguja se encontrara en cavidad abdominal y no se haya punzado algún órgano o vaso sanguíneo.

8.6.2. Eutanasia por exposición a CO_2 (G_2; n=10)

La sobredosis de CO_2 se realizó dentro de una cámara de inducción para eutanasia. La cámara de acrílico (Acrifactory, Mexico) (32.5 x 42 x 21 cm) fue personalizada y diseñada con cinco compuertas herméticas empleando neodimios para evitar la fuga de gas (Figura 5). Cada compuerta fue hecha al tamaño del lente de la cámara térmica (10.5 x 13 cm) para

permitir la toma de la IRT (Figura 6). La cámara fue conectada a un cilindro de gas comprimido de CO_2 y la administración del gas se realizó a una concentración mínima de 70%, con una tasa de flujo del 30% del volumen total de la cámara por minuto (49).

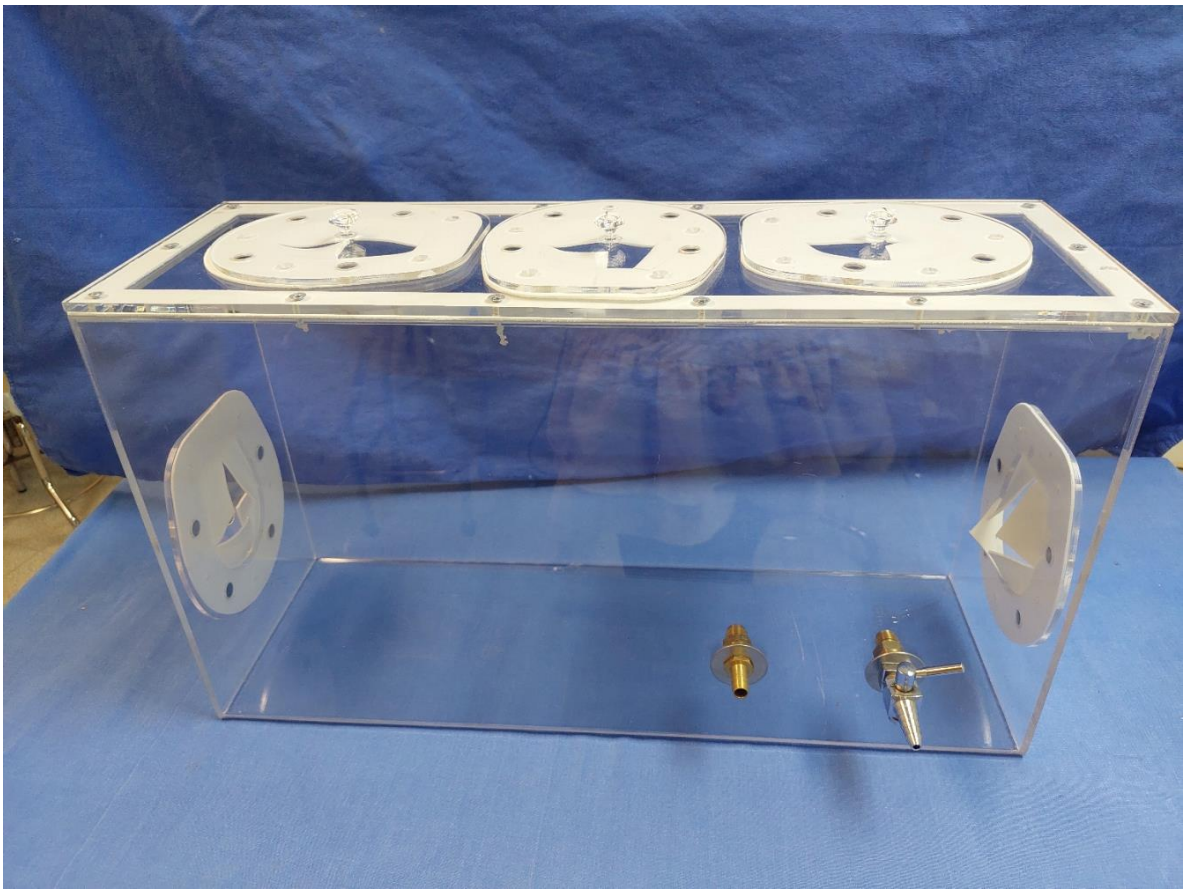


Figura 5. Cámara de inducción para eutanasia. Con el fin de permitir la evaluación térmica de los métodos inhalatorios, se personalizó una cámara de acrílico con cinco compuertas herméticas, al tamaño de la lente de la cámara térmica (10.5 x 13 cm), selladas con neodimios.

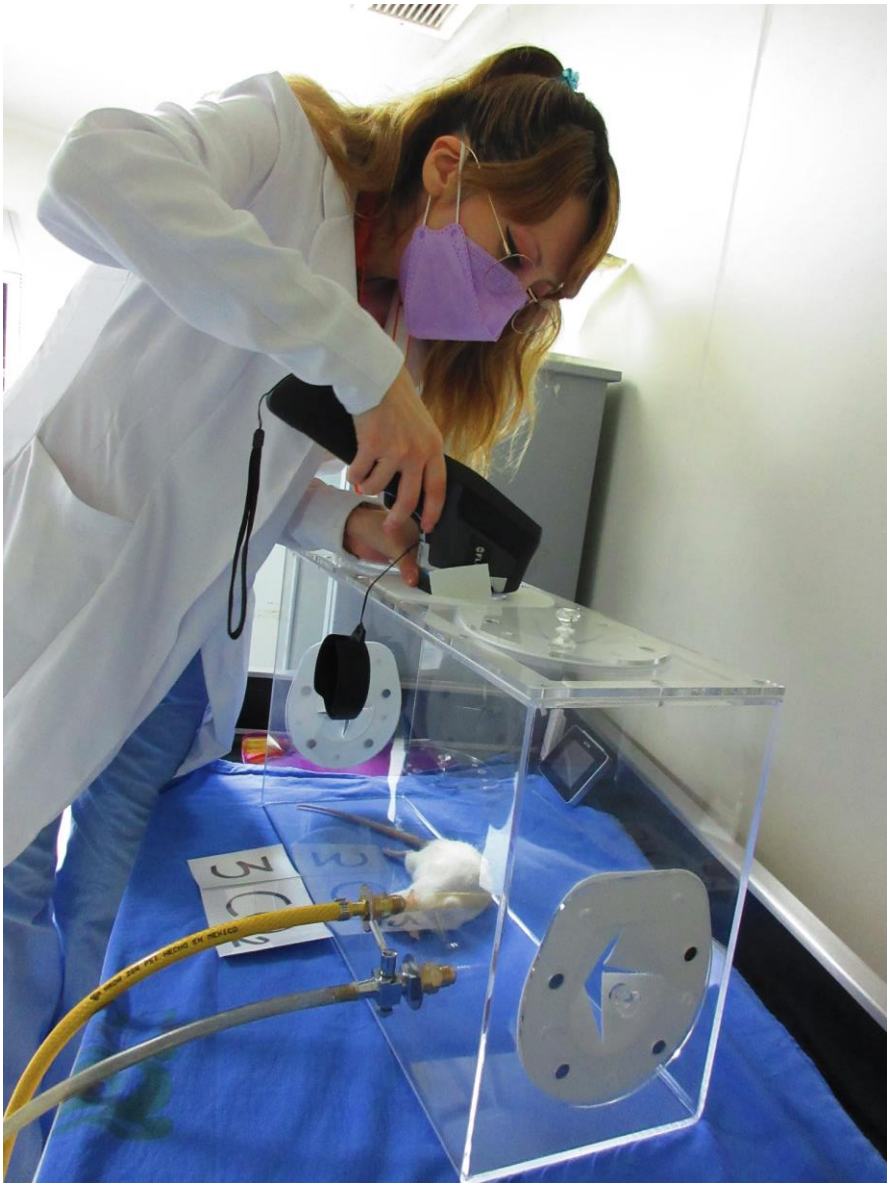


Figura 6. Colocación y monitoreo termográfico en la cámara de inducción de eutanasia para métodos inhalatorios (CO_2 e isoflurano).

8.6.3. Eutanasia por decapitación (G_3; n=10)

La decapitación se realizó empleando una guillotina para roedores (51330, Senna, México), con el fin de separar la cabeza del tronco a nivel de las vértebras cervicales (46) (Figura 7).

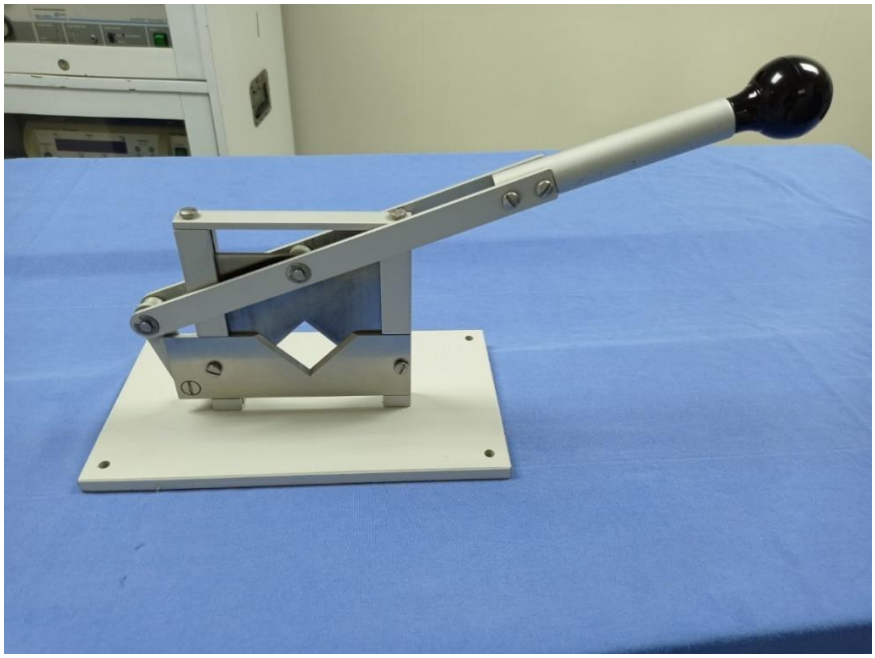


Figura 7. Guillotina para decapitación de ratas.

8.6.4. Eutanasia por exposición inhalatoria en circuito abierto con isoflurano (G_4; n=10)

Para la eutanasia con isoflurano (Fluriso, VET ONE ®, India) se empleó el método de “gota abierta”, humedeciendo dos torundas de algodón con 2 ml de isoflurano cada una (Figura 8). La dosis fue calcula tomando como base los estudios de Risling et al. (50) y de Brito (51). Las torundas fueron colocadas en un lugar donde los animales no tuvieran contacto directo con el anestésico.

Figura 8. Cámara de inducción para anestesia/eutanasia de ratas con isoflurano.

8.6.5. Eutanasia por sobredosis de ketamina + xilacina (G_5; n=10)

La sobredosis de ketamina (Anesket, Pisa Agropecuaria ®, México) + xilacina (Procin, Pisa Agropecuaria ®, Mexico) se realizó administrando vía IP 450 mg/kg IP y 45 mg/kg, respectivamente (39).

8.6.6. Eutanasia con ketamina + CO_2 (G_6; n=10)

Combinación de ketamina (100 mg/kg IP) + CO_2. Después de la administración de ketamina, los animales se mantuvieron en las cajas de policarbonato con cama de virutas de madera hasta 5-10 minutos post-inyección–considerando el tiempo promedio para lograr su máxima concentración (52)–. Posteriormente, los animales fueron introducidos en la cámara de inducción para CO_2 a una concentración mínima de 70% y flujo del 30% del volumen total de aire por minuto (53) (Figura 9).

Figura 9. Combinación de ketamina + CO_2 como un método de eutanasia. Metodología seguida para el G_6, en la que los animales permanecieron 5-10 min en las cajas de policarbonato antes de ser expuestos al CO_2.

En todos los grupos se verificó la muerte de los animales mediante la evaluación visual del cese de la respiración y del latido cardiaco con un estetoscopio (3M™ Littmann® Classic III™, USA). Asimismo, se evaluó la ausencia de reflejos palpebral, interdigital y de enderezamiento para confirmar la muerte de los sujetos.

8.7. Tiempos de evaluación

Las ratas en cada grupo experimental fueron evaluadas en cinco momentos: Basal: las mediciones fueron realizadas 24 h antes de realizar el método de eutanasia, dentro del cuarto de alojamiento. Ti_1: tres minutos antes de la aplicación del método de eutanasia, dentro del cuarto de procedimientos. En el día de experimentación, las ratas fueron trasladadas desde el cuarto del alojamiento hacia el cuarto de procedimiento (para evitar realizar la eutanasia cerca del resto de los grupos experimentales). A los animales se les permitieron 30 minutos de descanso y aclimatación al cuarto de procedimientos antes de empezar con el proceso experimental. Ti_2: durante la aplicación del método de eutanasia. Por ejemplo, mientras la rata está recibiendo la dosis IP de pentobarbital, ketamina + xilocaína, o mientras está dentro de la cámara de inducción o siendo colocada en la guillotina. Ti_3: inmediatamente después de aplicado el método de eutanasia hasta pérdida del reflejo de enderezamiento (LORR) como un signo de inconsciencia. Ti_4: desde LORR

hasta el cese de la frecuencia respiratoria y cardiaca, siendo evaluadas mediante observación y con un estetoscopio. La ausencia de reflejos (palpebral e interdigital) también fue empleada para confirmar la muerte de los animales. Es importante mencionar que todos los grupos, excepto en G_3 (decapitación), contaron con cinco tiempos de evaluación; sin embargo, en G_3, la separación de la cabeza e inconsciencia se consideraron como el mismo evento. Por ello, este grupo únicamente tuvo cuatro periodos de evaluación (Basal, Ti_1, Ti_2 y Ti_3).

De igual manera, para registrar la duración de cada método de eutanasia, a partir de Ti_2 se anotó el tiempo de muerte, tiempo para LORR, así como los tiempos para el cese de la respiración y del latido cardiaco. Los resultados fueron expresados en segundos y el promedio ± desviación estándar (SD) de los 10 animales por grupo fue procesado en una hoja de evaluación.

8.8. Descripción de las variables de estudio para la fase I. Termografía infrarroja

La captura de imágenes térmicas fue realizada mediante una cámara térmica FLIR™ E60 (FLIR Systems, USA) a 1 m de los animales, manteniendo un ángulo perpendicular al animal (90°). Las imágenes radiométricas fueron tomadas con una emisividad de 0.95, resolución IR de 320 x 240 pixeles, sensibilidad térmica de < 0.05°C, y precisión de ± 2%. Las imágenes infrarrojas fueron tomadas a la misma hora del día en todos los grupos experimentales (entre 0800 h y 1500 h), y del mismo lado de los animales (derecho). Previo al inicio de los métodos de eutanasia, las cajas de policarbonato, mesas, superficies y paredes donde se realicen las técnicas de eutanasia serán cubiertas con papel de estraza, tapetes de corcho, o con campos quirúrgicos con el objetivo de evitar que este tipo de superficies reflectantes interfieran con la lectura termográfica (Figura 10). Durante la sujeción de los animales para la administración IP de fármacos, los manejadores usaron en todo momento guantes de látex.

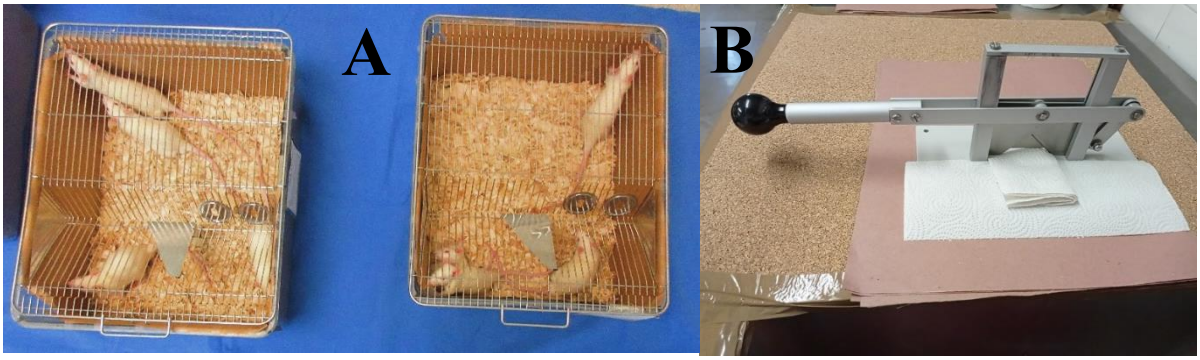


Figura 10. Superficies adaptadas para el uso de la cámara termográfica. A. Las cajas en donde las ratas fueron evaluadas se curbieron de papel de estraza para evitar el reflejo de la radiación. B. Las superficies de mesas y equipos se cubrieron con tapetes de corcho para evitar la reflectancia.

Durante los cinco tiempos de evaluación, se evaluaron cuatro regiones anatómicas –o ventanas térmicas–, las cuales fueron: superficie ocular ($T°_{ocu}$), región auricular ($T°_{ear}$), región interescapular ($T°_{dor}$), y en la cola ($T°_{tai}$). La delimitación de estas regiones de interés (ROI) se muestra en la Figura 11. Las imágenes térmicas fueron procesadas con el software FLIR Tools (Versión 6.4.17317.1002, FLIR Systems, USA) para obtener las temperaturas (°C) máximas, mínimas y promedio para $T°_{ocu}$, $T°_{ear}$ y $T°_{dor}$. Para $T°_{tai}$, únicamente se obtuvo la temperatura promedio en la parte proximal, media y distal de la cola. Esto es debido a que la delimitación del ROI en el software mediante un “spot” o “foco fijo” registra únicamente la temperatura promedio.

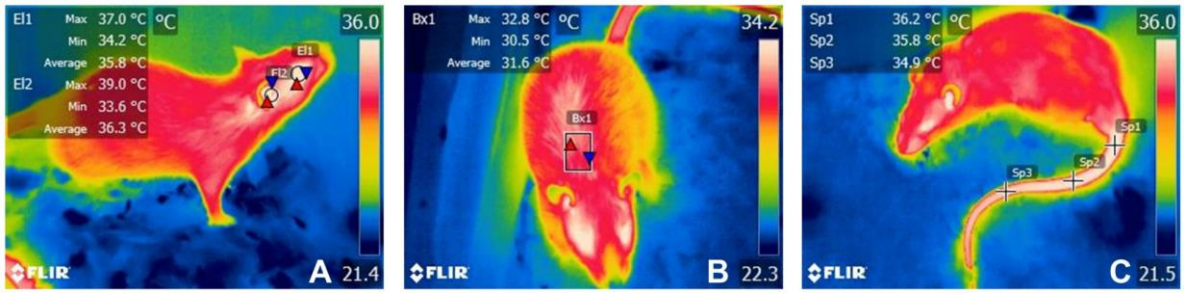


Figura 11. Representación de las cuatro ventanas térmicas evaluadas. A. $T°_{ocu}$. Se delimitó con un círculo (El1) que cubre por completo la región o el globo ocular, sin incluir el párpado superior o inferior. $T°_{ear}$ fue evaluado mediante un círculo (El2) en el canal auditivo externo

para evaluar la temperatura irradiada de la membra timpánica y el oído interno. B. $T°_{dor}$. Un rectángulo (Bx1) fue colocado en el área dorsal, justo en el espacio interescapular de las ratas. C. $T°_{tai}$. Tres puntos focales (Sp1, Sp2, y Sp3) fueron colocados en los segmentos proximal, medial y distal de la cola.

8.9. Descripción de las variables de estudio para la fase II. Expresión facial

Para obtener el puntaje de la RGS, se realizó una grabación continua con dos cámaras digitales de alta resolución (1920 x 1080) (Ixy 650, Cannon ®, Japón) durante los tiempos de evaluación. Las cámaras se colocaron a ambos lados del animal (frontal y lateral) para capturar fotografías de las ratas antes, durante y después de cada tratamiento de eutanasia. Las cámaras se montaron en trípodes a aproximadamente 20 cm de distancia de las ratas. A diferencia de la IRT, la videograbación para evaluar la RGS se realizó únicamente en los tiempos Basal, Ti_1, Ti_2 y Ti_3, debido a que después de la pérdida de la consciencia no se pueden observar cambios en la expresión facial.

Los videos se guardaron como archivos en formato MP4 para ser analizados por un codificador ciego a los métodos de eutanasia en un software de edición de video (Adobe Premiere Pro, Adobe, EE. UU.). Al observar los videos a una velocidad de 0.5x, se tomaron 10 imágenes fijas de los rostros de las ratas en cada tiempo de evaluación (54), obteniendo 40 imágenes por rata cuando se observó una clara vista frontal o lateral de la cabeza. La puntuación de las imágenes se realizó de acuerdo con el estudio de Sotocinal (31) utilizando cuatro FAU, en las cuales:

- Estrechamiento orbital: las ratas con dolor muestran estrechamiento del área orbital que se manifiesta como un cierre ocular parcial o completo.
- Cambios en la posición de las orejas: las orejas de las ratas con dolor tienden a curvarse e inclinarse hacia adelante o hacia afuera, dando una forma puntiaguda. El espacio entre las orejas puede parecer más amplio por el cambio de posición.
- Aplanamiento de nariz / mejilla: las ratas con dolor muestran un menor abultamiento dorsal de la nariz y mejillas, con ausencia ocasional del pliegue entre la mejilla y las vibrisas.

- Cambio en las vibrisas: las ratas con dolor desplazan hacia adelante estas estructuras (alejándose de la cara) desde su posición de referencia, por lo que tienden a agruparse dando la apariencia de bigotes de punta (Figura 12).

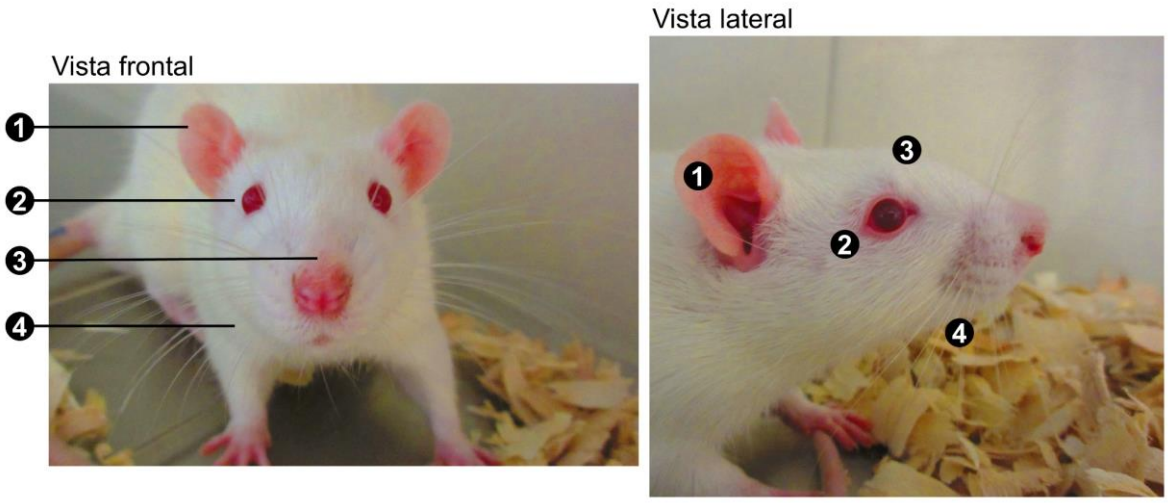


Figura 12. Unidades de acción facial empleadas en ratas. 1. Cambio de orejas; 2. Estrechamiento ocular: 3. Aplanamiento de nariz/mejillas; y 4. Cambio de vibrisas.

Usando una escala de puntuación de 0 a 2, donde 0 = no presente, 1 = moderadamente presente; y 2 = obviamente presente, la puntuación máxima obtenible fue 8. Para determinar la puntuación final, se sumaron las 10 imágenes por evento de evaluación en cada FAU para obtener un valor medio. Para cada rata se sumaron los valores medios de las cuatro FAU y se usó una puntuación total media en el análisis.

8.10. Descripción de procedimientos

Después de los 15 días de habituación y la asignación aleatoria de las ratas a los grupos experimentales, los procedimientos generales para ambas fases experimentales están esquematizados en la Figura 13.

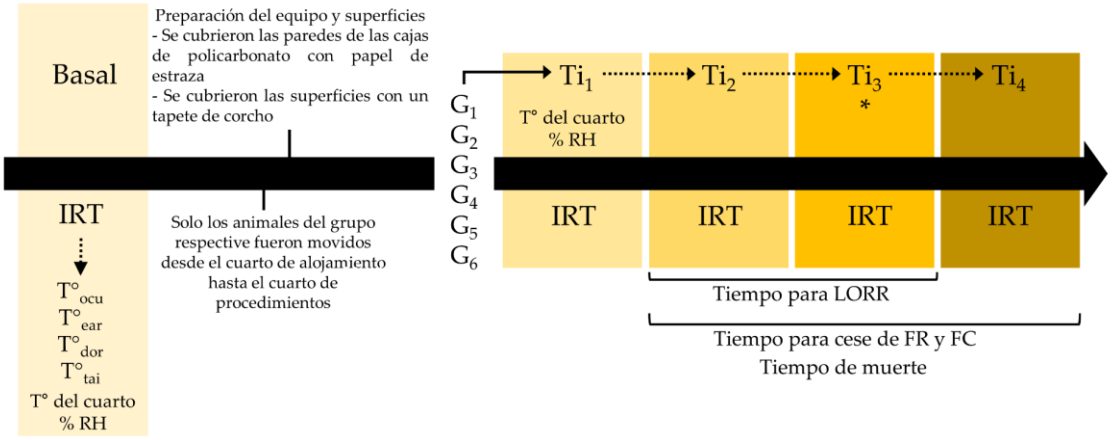


Figura 13. Esquematización del orden de procedimientos para la obtención de la IRT y RGS. FC: frecuencia cardiaca; FR: frecuencia respiratoria. *para G_3, Ti_3 incluye LORR y cese de la función cardiorrespiratoria.

Durante el periodo Basal, la IRT y la RGS se evaluaron por cinco minutos dentro del cuarto de alojamiento de los animales. Para la videograbación, las cámaras se colocaron en tripiés apuntando a dos lados de las cajas de policarbonato. Para llevar a cabo las técnicas de eutanasia, se realizó la movilización de los animales desde el cuarto de alojamiento hacia uno de los cuartos de procedimientos para separar a los individuos del grupo designado del resto. Se estableció un periodo de 30 minutos para que las ratas se aclimaten al cuarto y para evitar el estrés por transporte (55). Durante este tiempo, las cámaras para videograbación fueron montadas en tripiés en el cuarto de procedimientos, en diferentes ubicaciones de acuerdo con el método de eutanasia (p. ej., si fue un método inhalatorio, las cámaras se colocaron en dos lados de la cámara de acrílico). Transcurridos los 30 minutos, se inició con el Ti_1 del primer individuo del grupo en cuestión, capturando ambas variables de estudio por tres minutos. Se tomó la IRT de $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$ y $T°_{tai}$ y se videograbó la región rostral del animal. Posteriormente, ese animal se llevó a una sala separada donde exclusivamente se realizó el sacrificio humanitario de esa rata, con el fin de evitar que estímulos visuales, auditivos u olfatorios generen un efecto negativo en el resto de los sujetos de estudio. En el Ti_2, la IRT y videograbación para la RGS se registraron mientras se administró el fármaco vía IP, o en los primeros segundos de dejar a la rata dentro de las cámaras de inducción de isoflurano y CO_2. En el Ti_3, se valoraron las mismas variables de respuesta desde el momento en el que el fármaco fue administrado por

completo o desde que se inició con la entrada del agente inhalatorio a las cámaras de inducción (G_2, G_4 y G_6) hasta comprobado el cese de la frecuencia respiratoria y cardiaca (Ti_4). Para la RGS, a partir de Ti_2 y durante Ti_3 se realizó grabación continua del rostro de los animales hasta LORR. Debido a la diferencia de tiempo para alcanzar LORR de acuerdo con el método de eutanasia, la videograbación durante estos dos tiempos difirió.

La información obtenida de las tres variables en los tres tiempos y en los seis grupos fue posteriormente recopilada en bases de datos de Excel (Microsoft Office ®, USA) para su análisis estadístico.

8.11. Análisis estadístico

Todos los análisis se realizaron con el paquete estadístico GraphPad Prism 10.0.2 (California, EE. UU.).

8.11.1. Análisis estadístico de la fase I

Se realizó la prueba de Shapiro-Wilk para establecer la normalidad de los datos en el conjunto de datos recopilados de $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$ y $T°_{tai}$. Se obtuvieron estadísticos descriptivos, expresando los resultados como media ± desviación estándar (SD). Se utilizó un modelo mixto lineal para medidas repetidas para evaluar el efecto de los seis métodos de eutanasia (G_1, G_2, G_3, G_4, G_5 y G_6), en los cinco eventos (Basal, Ti_1, Ti_2, Ti3 y Ti_4) para cada una de las cuatro ventanas térmicas. La comparación múltiple de medias se realizó con la prueba post-hoc de Tukey. En todos los casos, el nivel de significancia se fijó en $P < 0.05$. Para establecer la correlación entre las ventanas térmicas se calcularon los coeficientes de correlación de Pearson. Todos los valores con $P < 0.05$ se consideraron significativos.

8.11.2. Análisis estadístico de la fase II

Se realizó la prueba de Kolmogorov-Smirnov para establecer la normalidad de los datos en el conjunto de datos recopilados de la UAF. Se obtuvo la estadística descriptiva, expresando los resultados como media ± SD. Se utilizó un modelo mixto lineal para medidas repetidas para evaluar el efecto de los seis métodos de eutanasia (G_1, G_2, G_3, G_4, G_5 y G_6), en los cuatro tiempos de evaluación (Basal, Ti_1, Ti_2 y Ti_3) para la puntuación total de RGS. La

comparación múltiple de medias se realizó con la prueba post-hoc de Tukey. Las correlaciones entre las cuatro FAU y los métodos de eutanasia se calcularon utilizando los coeficientes de correlación de Spearman. Todos los valores con $P < 0.05$ se consideraron estadísticamente significativos.

El tiempo de muerte, el tiempo hasta LORR, el tiempo hasta el cese de la respiración (evaluación visual) y el latido cardíaco (auscultación torácica) se expresaron como media ± SD.

9. RESULTADOS

9.1. CAPÍTULO IV

Resultados de la fase I. Termografía infrarroja

Artículo experimental intitulado:
Respuesta térmica de ratas de laboratorio (*Rattus norvegicus*) durante la aplicación de seis métodos de eutanasia evaluados a través de la termografía infrarroja

Publicado en la revista Animals, misma que se encuentra indexada al JCR con un factor de impacto de 3, en el volumen 13, 2820. https://doi.org/10.3390/ani13182820

Domínguez-Oliva, A.; Hernández-Avalos, I.; Olmos-Hernández, A.; Villegas-Juache, J.; Verduzco-Mendoza, A.; Mota-Rojas, D. Thermal response of laboratory rats (*Rattus norvegicus*) during the application of six methods of euthanasia assessed by infrared thermography. *Animals 2023*, *13*, en proceso de publicación.

animals

MDPI

Article

Thermal Response of Laboratory Rats (*Rattus norvegicus*) during the Application of Six Methods of Euthanasia Assessed by Infrared Thermography

Adriana Domínguez-Oliva [1,2], Ismael Hernández-Ávalos [3], Adriana Olmos-Hernández [4], Juan Villegas-Juache [5], Antonio Verduzco-Mendoza [4] and Daniel Mota-Rojas [2,*]

[1] Master in Science Program "Maestría en Ciencias Agropecuarias", Xochimilco Campus, Universidad Autónoma Metropolitana, Mexico City 04960, Mexico
[2] Neurophysiology of Pain, Behavior and Assessment of Welfare in Domestic Animals, DPAA, Xochimilco Campus, Universidad Autónoma Metropolitana (UAM), Mexico City 04960, Mexico
[3] Clinical Pharmacology and Veterinary Anesthesia, Biological Sciences Department, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Cuautitlán Izcalli 54714, Mexico
[4] Division of Biotechnology—Bioterio and Experimental Surgery, Instituto Nacional de Rehabilitación-Luis Guillermo Ibarra Ibarra (INR-LGII), Mexico City 14389, Mexico
[5] Bioterio and Experimental Surgery, Instituto Nacional de Cardiología Ignacio Chávez, Mexico City 14080, Mexico
* Correspondence: dmota100@yahoo.com.mx

Simple Summary: The present study aimed to assess the infrared thermal response of laboratory rats (*Rattus norvegicus*) during the application of six euthanasia methods (injectable, inhalational, and physical) to determine the method that prevents or diminishes the stress response. The surface temperature was assessed in four thermal windows: ocular ($T°_{ocu}$), auricular ($T°_{ear}$), interscapular ($T°_{dor}$), and caudal ($T°_{tai}$). The results showed that inhalant methods (CO_2 and isoflurane) had temperature alterations that could be suggestive of a marked stress response, in contrast to other methods such as pentobarbital, decapitation, and xylazine + ketamine. In conclusion, according to the thermal response of the rats, it is suggested that CO_2 and isoflurane might cause distress and this needs to be considered when selecting these techniques as the method of euthanasia for laboratory rats.

Abstract: Refinement is one of the principles aiming to promote welfare in research animals. The techniques used during an experimental protocol, including euthanasia selection, must prevent and minimize suffering. Although the current euthanasia methods applied to laboratory rodents are accepted, the controversial findings regarding the potential stress/distress they can cause is a field of research. The objective was to assess the thermal response of Wistar rats during various euthanasia methods using infrared thermography (IRT) to determine the method that prevents or diminishes the stress response and prolonged suffering. Pentobarbital (G_1), CO_2 (G_2), decapitation (G_3), isoflurane (G_4), ketamine + xylazine (G_5), and ketamine + CO_2 (G_6) were evaluated at five evaluation times with IRT to identify changes in the surface temperature of four anatomical regions: ocular ($T°_{ocu}$), auricular ($T°_{ear}$), interscapular ($T°_{dor}$), and caudal ($T°_{tai}$). Significant differences ($p < 0.05$) were found in G_2 and G_4, registering temperature increases from the administration of the drug to the cessation of respiratory rate and heart rate. Particularly, isoflurane showed a marked thermal response in $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$, and $T°_{tai}$, suggesting that, in general, inhalant euthanasia methods induce stress in rats and that isoflurane might potentially cause distress, an effect that must be considered when deciding humane euthanasia methods in laboratory rodents.

Keywords: rodents; infrared thermography; pentobarbital; decapitation; CO_2; isoflurane; euthanasia; refinement; welfare

check for updates

Citation: Domínguez-Oliva, A.; Hernández-Ávalos, I.; Olmos-Hernández, A.; Villegas-Juache, J.; Verduzco-Mendoza, A.; Mota-Rojas, D. Thermal Response of Laboratory Rats (*Rattus norvegicus*) during the Application of Six Methods of Euthanasia Assessed by Infrared Thermography. *Animals* **2023**, *13*, 2820. https://doi.org/10.3390/ani13182820

Academic Editors: Pietro Asproni, Míriam Marcet-Rius, Elbert Lambooij and Guido Rocchigiani

Received: 26 July 2023
Revised: 28 August 2023
Accepted: 1 September 2023
Published: 5 September 2023

1. Introduction

The use of animals is a key element for improvements in biomedical science [1,2], where rats and mice represent 87–98% of the total of species used in the scientific community [3,4]. The potential pain and stress that laboratory animals might experience is highly controversial [5]. Ethical animal research necessitates the selection of suitable euthanasia methods to minimize pain and distress, as proposed by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research [6], not only to provide welfare but also to ensure the quality of results. These initiatives need be applied not only during the life of research animals, but also during the application of euthanasia methods with the aim of providing humane endpoints [7].

Currently, there is a debate around the euthanasia methods that are approved by the American Veterinary Medicine Association (AVMA). Injectable drugs (e.g., barbiturates and general anesthetics), inhalant agents (e.g., CO_2 and isoflurane), and physical methods (e.g., decapitation and cervical dislocation) are recognized as acceptable techniques to induce a humane death without suffering [8]. However, some methods are under discussion since studies have shown potential adverse effects during their application. For example, inhalation of CO_2 is aversive for rats [9] and induces bradycardia and potential anxiety due to hypoxia before loss of consciousness [10,11]. Moreover, CO_2 forms carbonic acid and induces the activation of pain receptors [12]. Nonetheless, systematic reviews have contrasting results regarding the suitability of CO_2 and its potential distress [13]. Isoflurane is considered an alternative to CO_2 euthanasia. However, it is known as an aversive agent to rats for its mild pungency [14,15].

On the other hand, the administration of injectable pentobarbital has been associated with pain-related behaviors (e.g., writhing and back arching) due to intraperitoneal (IP) irritation [16–18], while the combination of xylazine and ketamine, although a common anesthetic protocol, demonstrates limited action as a euthanasic agent. However, high Mouse Grimace Scale scores and anxiety-related behaviors were found after repeated doses of the combination [19]. Moreover, Wellington et al. [20] found that IP administration of ketamine + xylazine to rats caused acute muscle and tissue necrosis, poor tolerance, and pain/discomfort behavioral reactions. In the case of decapitation, this procedure leads to the question of whether brain activity is present immediately after the procedure or not, as well as whether changes in the electroencephalogram (EEG) are associated with nociception during the first 15 s (s) following decapitation [21], as determined in rats by Derr [22] who reported that EEGs during the first 2.7 s after decapitation might indicate conscious awareness of pain and distress.

The refinement of procedures performed in research animals includes the implementation of non-invasive tools to assess their welfare without causing additional stress. Infrared thermography (IRT) is a technique that detects surface temperature changes as a neuroendocrine response of the Sympathetic Nervous System (SNS) after stressful/distressful and painful events [23,24]. Stress—known as the reaction of the organism when its homeostasis or psychological well-being is perturbated—and distress—a negative and aversive state when the organism cannot adapt or return to homeostasis [25]—activates two main systems: the hypothalamic–pituitary–adrenal (HPA) and the *locus coeruleus* sympathetic adrenomedullary (SAM) axes [26]. Both axes lead to the release of glucocorticoids and catecholamines, as well as the physiological changes required to adjust homeostasis [27], including alterations in body temperature and microcirculation. Therefore, temperature variations have been used as a stress-related marker in animals, as stress may cause central hyperthermia and peripheral reduction of the temperature due to vasoconstriction [28].

IRT detects these vasomotor changes as a difference in the amount of dissipated heat in different anatomical regions, where heat exchange is facilitated through the arteriovenous anastomosis and peripheral blood vessels, also called thermal windows [29]. In laboratory rodents, thermal windows such as the ocular, auricular, dorsal or interscapular, and tail region have been used to assess stress [28,30] or pain [31]. For example, Lecorps et al. [32] found that eye temperature increased in mice undergoing an elevated plus maze test,

while tail temperature diminished as a physiological response to stress (a result that was associated with anxiety-related behaviors). The ocular surface has great vascular sensitivity because the two main arteries responsible for its irrigation (the *arteria supraorbitalis* and *angularis occuli*), as well as the innervation through the facial nerve, rapidly respond to autonomous tone changes and endogenous catecholamines [29]. Likewise, Zevgolis et al. [33] reported that ocular IRT increased during the experimental handling of wild mice due to stress-induced hyperthermia (SIH). A similar response is observed in the auricular window, as shown by Wokke [34] in mice. In this study, restraining methods to administer IP drugs increased ear temperature and corticosterone levels. These temperature variations are mediated by sympathetic activity and vasodilation in the main blood vessels supplying irrigation (external jugular vein, external carotid artery, and its branches into marginal ear arteries) [29,35]. Hutu et al. [36] determined that IRT measured in the ear is positively correlated with rectal temperature in rabbits. Therefore, considering that SIH also causes changes in the amount of dissipated heat in thermal windows, ear temperature could be a way to assess acute stress.

For laboratory rodents, a thermal window that is closely related to sympathetic activation and norepinephrine (NE) release after the activation of the SAM axis is the interscapular region. In this zone, small mammals have large deposits of brown adipose tissue (BAT), a thermogenic structure whose activity depends on NE binding to β_3-adrenoreceptors located in BAT [37]. The increased thermogenic activity of this tissue has been associated with corticosterone secretion and with the administration of β_3-adrenoreceptor agonists and NE [38,39]. Furthermore, SIH is also related to BAT thermogenesis in rats and humans after excessive stress [40].

Lastly, the tail of rats is considered an important thermal window because it contributes to up to 25% of heat dissipation (by vasoconstriction) due to arteriovenous anastomosis (from the coccygeal artery) [29,41]. Vasoconstriction of peripheral regions such as the tail and paws is mediated by the sympathetic redistribution of blood flow to key organs (e.g., the heart and brain). In mice exposed to acute stressors, the superficial tail temperature decreased during different handling procedures, while the surface temperature of the body (assessed in the dorsal region of the mice) increased as a response to SIH [42]. Gjendal et al. [30] also reported a decrease in tail temperature (up to 3.5 °C) in mice exposed to three stressors (a maze test, IP injection, and isoflurane anesthesia) as a result of vasoconstriction of tail blood vessels.

The current literature suggests IRT as a non-invasive complementary tool to assess well-being in animals, including stress-related responses [24,43]. Although there are some studies regarding IRT and the pre-slaughter or antemortem period in domestic species such as pigs [44,45], there is no study up to now where IRT has been used to evaluate the effect of euthanasia methods on laboratory species. Therefore, the present study aimed to assess the infrared thermal response of laboratory rats (*Rattus norvegicus*) during the application of six euthanasia methods to determine the method that prevents or diminishes the stress response and prolonged suffering. Injectable drugs (pentobarbital, ketamine + xylazine), inhalant agents (CO_2, isoflurane), physical methods (decapitation), and the combination of inhaled and injectable anesthetics (CO_2 + ketamine) were evaluated with IRT to identify changes in the surface temperature of four anatomical regions (ocular, auricular, interscapular, and caudal). Also, differences by sex according to the thermal window and the euthanasia method will be studied. The hypotheses of the study were as follows: (i) the use of IRT during different euthanasia methods will help to recognize changes in the surface temperature of laboratory rats (*R. norvegicus*) in response to stress perception related to the method and (ii) the combination of an injectable anesthetic overdose (ketamine) with CO_2 exposure as the euthanasia method will reduce thermal alterations associated with stress.

2. Materials and Methods

2.1. Location and Ethical Statement

The present study was conducted at the Animal Facility and Experimental Surgery Facility from the Biotechnological Research Sub-Department of the Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra, Mexico City, Mexico. All procedures were approved by the Committee for the Care and Use of Laboratory Animals (INRLGII/CICUAL/014/2021) at the National Institute of Rehabilitation Luis Guillermo Ibarra-Ibarra.

The handling and care of the laboratory animals was in accordance with the Mexican norm for laboratory animals NOM-062-ZOO-1999, published by the Department of Agriculture, Rural Development, Fisheries and Alimentation [46]. All dead animals were disposed of by incineration following NOM-062-ZOO-1999.

2.2. Animals and Housing Conditions

A total of 60 adult Wistar rats (*R. norvegicus*), 30 male and 30 female, were purchased from the Center for Research and Advanced Studies at the National Polytechnic Institute (CINVESTAV-IPN). The animals had an average weight of 311 ± 62 g at 8–10 weeks old (in puberty) and were obtained with an animal health certificate to ensure they were free of infectious pathogens (bacteria, viruses, and parasites). The sample size was calculated using G*Power 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The total sample size was 48 animals, considering an α error probability of 0.05, confidence level of 95%, power (1-β error probability) of 0.90, and correction among repeated measures of 0.5 for six experimental groups with five measurements.

According to the principles of the 3Rs [47], reduction was applied to the total number of animals used by reusing animals from finished protocols related to behavioral tests (e.g., balance beam or maze tests). Rats that were part of the control groups were selected to avoid the inclusion of animals undergoing invasive procedures or those with residual drug levels. Through a general physical examination, the animals were classified as healthy without signs of disease, stress, or pain-related behaviors. The physical exam considered body weight, posture, level of consciousness, secretions, the color of the mucosa, sneezing, and a species-specific behavioral repertoire. Rats showing signs of disease, injury, or pain were eliminated. Pregnant females were excluded.

Rats were housed in separate rooms according to sex. They were placed in groups of five animals per cage in standard polycarbonate cages for rats (47 × 36 × 21 cm) with wood shavings as bedding (Aspen, Nepco, Riverside, RI, USA) and without enrichment. The rats were maintained under a 12 h day–night cycle with lights on between 0500 h and 1700 h. The controlled temperature inside the housing room and the testing room was set at an average of 23.2 ± 0.5 °C and 22.9 ± 0.5 °C, respectively, with respective humidity levels of 48% and 52%. The rats had ad libitum access to food (LabDiet 5010, LabDiet, St. Louis, MI, USA) and purified water (in 500 mL drinking water bottles), and the cages were cleaned once a week. Visual health inspection was performed twice daily.

2.3. Experimental Design

This was an experimental prospective–comparative study. All measurements were performed by a single trained and unblinded evaluator. Once the rats were selected for the study, they underwent habituation for 15 days to the customized euthanasia chamber, handling, and the evaluator's presence. The animals were randomly divided into six groups by number generation (Microsoft Excel; Microsoft 365). A total of 10 rats were assigned in each group (5 males and 5 females) as follows:

G_1: Pentobarbital (Pentobarbital, Aranda®, Mexico City, Mexico) overdose at 400 mg/kg performed via IP injection with a 3 mL sterile syringe (Ambiderm®, Baja California, Mexico) following Lofgren et al. [48]'s procedure. The dose was calculated through a pilot study (no published data) using the minimal and maximal doses that appear in Reimer et al. [17]'s study. The selected dose resulted in rapid loss of consciousness, cardiorespiratory depression without excitation, loss of reflexes, and clinical death. G_2:

CO_2 overdose administered inside a customized acrylic euthanasia chamber (Acrifactory, Mexico City, Mexico) (32.5 × 42 × 21 cm). The chamber had five gates with hermetically sealed doors that used neodymium magnets to avoid gas leaks. Each door was fitted to the size of the thermal camera lens to allow for thermal imaging (Figure 1). According to the AVMA [8], the flow rate was set at 30% of the chamber volume/min. G_3: Decapitation using a rodent guillotine (51330, Senna, Mexico) [48]. G_4: Inhalation of isoflurane (Fluriso, VET ONE®, Delhi, India) using the open-drop exposure method (two cotton swabs soaked with 2 mL of isoflurane each). The dose was calculated using the studies of Risling et al. [49] and de Brito [50] as a basis. The cotton swabs were placed where animals could not have direct contact with the inhalant anesthetic drug. G_5: Ketamine (Ketamin-Pet, Aranda®, Mexico) + xylazine (Procin, Pisa Agropecuaria®, Nuevo México, Mexico) overdose administered at doses of 450 mg/kg IP and 45 mg/kg IP, respectively [8]. G_6: Combination of ketamine (100 mg/kg IP) + CO_2 (after 5–10 min of ketamine administration) [51].

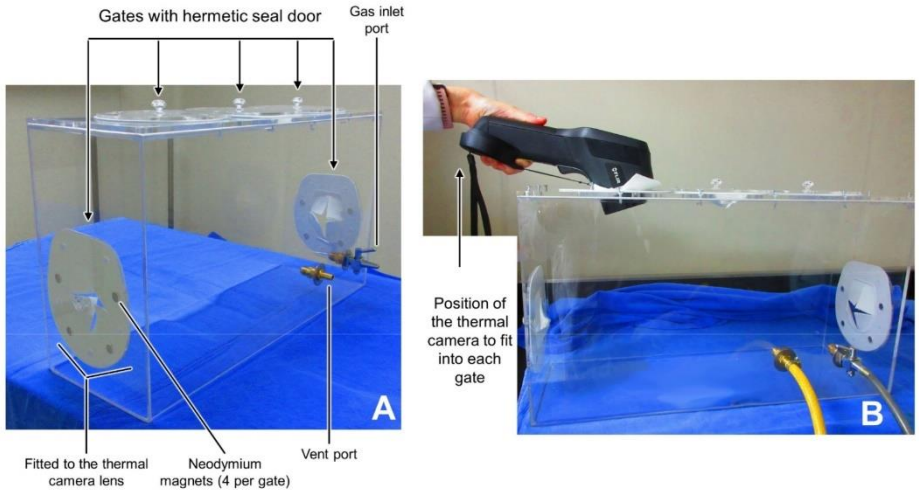


Figure 1. Customized acrylic euthanasia chamber for thermal imaging. (**A**) shows the components of the chamber, with the five hermetic seal doors and the respective gas inlet and vent port. (**B**) shows the position of the thermal camera that ensured the lens fit into each gate during the evaluation of inhalant euthanasia.

Rats from all groups were assessed at five evaluation times. The basal time point represents assessment that was performed 24 h before the euthanasia method inside the housing room, and Ti_1 represents three minutes before the application of the euthanasia in the test room. On the trial day, the rats were moved from the housing room to the test room, allowing for 30 min of rest and room acclimatization before starting the trial at Ti_2, the time during the application of the method (e.g., while the animal received the IP dose of pentobarbital, ketamine + xylazine, or while it was inside the induction chamber or placed in the guillotine). Ti_3 represents the time immediately after the application of the euthanasia method until loss of the righting reflex (LORR) as a sign of unconsciousness, and Ti_4 represents the time until the cessation of breathing and heartbeat by visual assessment and assessment using a stethoscope (3M™ Littmann® Classic III™, 3M, Saint Paul, MN, USA). The absence of palpebral, interdigital, and righting reflex was also used to confirm the euthanasia method. It is noteworthy to mention that all groups, except G_3 (decapitation), had the same five evaluation times. In G_3, the separation of the head from the body and

unconsciousness was considered the same event; therefore, only four evaluation times were considered for this group (basal, Ti_1, Ti_2, and Ti_3).

2.4. Assessed Parameters

2.4.1. Infrared Thermography (IRT)

Thermal imaging was performed using an FLIR™ E60 camera (FLIR Systems, Orlando, FL, USA) positioned 1 m from the rats while maintaining a perpendicular angle to the subject (90°). Radiometric images were taken with an emissivity of 0.95, an IR resolution of 320 × 240 pixels, thermal sensitivity of <0.05 °C, and accuracy of ±2%. To prevent reflective heat affecting the acrylic cages placed in the housing room and the test room, the walls were covered with kraft paper. The handler used latex gloves when restraining the rats for IP injection and decapitation. Moreover, thermal imaging was performed at the same time of the day in all experimental groups (between 0800 h and 1500 h).

During basal, Ti_1, Ti_2, Ti_3, and Ti_4, the four evaluated body regions (or thermal windows) were the ocular ($T°_{ocu}$), auricular ($T°_{ear}$), interscapular ($T°_{dor}$), and tail ($T°_{tai}$) regions. Thermal imaging for $T°_{ocu}$ and $T°_{ear}$ was taken from the right side of the animals. The delimitation of these regions of interest (ROIs) is shown in Figure 2. The thermal images were processed with FLIR Tools software (FLIR Systems, USA) to obtain the maximum, minimum, and average temperatures for $T°_{ocu}$, $T°_{ear}$, and $T°_{dor}$. For $T°_{tai}$, only the average temperature in the proximal, medial, and distal parts of the tail was recorded. This is due to the delimitation of the ROI with a spot, which only provides the average value.

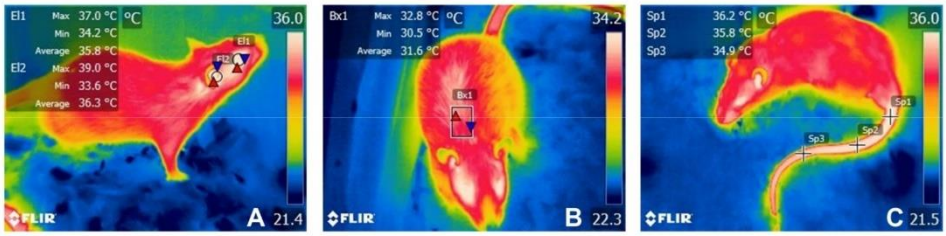


Figure 2. Representation of the four evaluated thermal windows. (**A**). $T°_{ocu}$ was delimited by a circle (El1) covering the entire ocular region or ocular globe, without including the upper or lower eyelid. $T°_{ear}$ was evaluated using a circle (El2) in the external ear canal to assess the irradiated temperature of the tympanic membrane and inner ear. (**B**). For $T°_{dor}$, a rectangle (Bx1) was placed in the dorsal area over the interscapular space. (**C**) For $T°_{tai}$, three spots (Sp1, Sp2, y Sp3) were placed at the proximal (T°prox), medial (T°medial), and distal (T°distal) segments of the tail.

2.4.2. Time to Death

To record the duration of each euthanasia method, after Ti_2, the evaluator started a timer to register the time of death, the time to LORR, and the time to the cessation of breathing (visual assessment) and heartbeat (thoracic auscultation). The results were expressed as seconds, and the average value ± standard deviation (SD) of the 10 animals per group was recorded on an evaluation sheet.

2.5. Procedures

After the 15-day habituation period and the random assignment of the rats into the six experimental groups, the procedures were performed as shown in Figure 3.

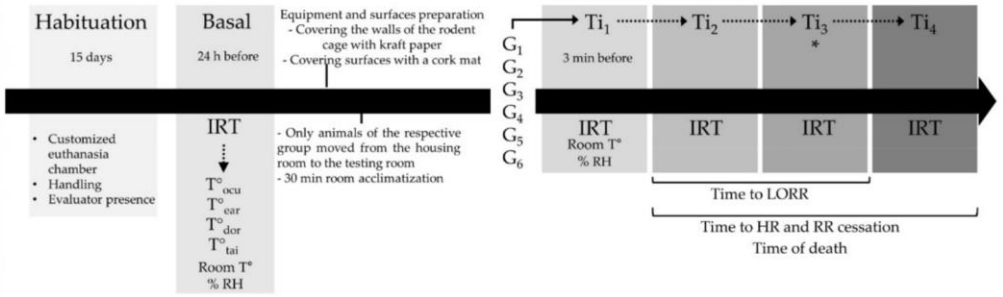


Figure 3. Experimental timeline for the euthanasia methods applied in rats. HR: heart rate; RR: respiratory frequency. * for G_3, Ti_3 includes LORR and HR/RR cessation.

During the basal time point, IRT, as well as room temperature and relative humidity (% RH), was recorded inside the housing room of the selected experimental group with a wireless indoor and outdoor weather station with a hygrometer (Taylor®, Oak Brook, IL, USA). The equipment and surfaces were conditioned to IRT readings 24 h after by covering the walls of the polycarbonate cages with kraft paper and the use of wood shavings as bedding. The surfaces to place the guillotine, cages, and induction chamber were also covered by either sterile drapes or cork pads to avoid reflective heat. The rats from the corresponding group were moved from the housing room to the testing room so that euthanasia was not performed where the rest of the animals were housed. A period of 30 min was given to the rats to acclimatize them to the controlled temperature in the testing room and avoid stress related to transportation. Following this 30 min, the euthanasia method started. Room temperature, % RH, IRT, and time of death were recorded for each individual in all experimental groups.

2.6. Statistical Analyses

All analyses were performed using the GraphPad Prism 10.0.0 (San Jose, Ca, USA) statistical package. The Shapiro–Wilk test was performed to establish data normality in the data set collected from $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$, and $T°_{tai}$. Descriptive statistics were obtained and results were expressed as mean ± standard deviation (SD). A linear mixed model for repeated measures was used to evaluate the effect of the six euthanasia methods (treatments G_1, G_2, G_3, G_4, G_5, and G_6) at the five time points (basal, Ti_1, Ti_2, Ti_3, and Ti_4) for each of the four thermal windows. Multiple comparison of means was performed with the post-hoc Tukey test. In every case, the significance level was set at $p < 0.05$. The following statistical model was used:

$$Yijk = \mu + \tau i + \tau j + \tau i\tau j + \beta k + eij$$

where the symbols indicate the following:

Y = variable response (IRT);
τi = fixed effect (G_1, G_2, G_3, G_4, G_5, G_6);
τj = evaluation times (basal, Ti_1, Ti_2, Ti_3, and Ti_4);
β = aleatory effect (rat);
μ = general mean;
e = error.

To determine if there were differences between males and females from each experimental group, repeated measure ANOVA was performed with a Greenhouse–Geisser correction and a post-hoc Tukey test for multiple comparisons. Time of death, time to LORR, and time to the cessation of breathing (visual assessment) and heartbeat (thoracic auscultation) were expressed as mean ± SD. To establish the correlation between the ther-

mal windows, Pearson correlation coefficients were calculated. All values with $p < 0.05$ were considered significant.

3. Results

Differences in the thermal response of the rats grouped in different experimental groups were obtained according to the thermal window, assessing their maximum (T°max), minimum (T°min), and mean temperature (T°mean). In general, G_2 and G_4 registered significant differences between evaluation times and between groups in three of the four thermal windows. Additionally, G_4 individuals showed a progressive increase in temperature in all thermal windows, in contrast to the other experimental groups.

3.1. Ocular Surface Temperature ($T°_{Ocu}$)

Table 1 shows the mean and standard deviation (SD) values for the temperature of $T°_{ocu}$. For T°max, T°min, and T°mean, differences among evaluation times were recorded in G_2 ($p = 0.0097$) and G_4 ($p = 0.0001$). For G_2, a decrease in T°mean of up to 2.15 °C at Ti_4 was observed when compared to basal values. Similarly, T°mean in G_4 decreased by up to 5.82 °C at Ti_2. Regarding differences between groups, a progressive temperature decline was registered in all euthanasia methods. However, differences between the groups were observed during Ti_2 ($p = 0.0007$), Ti_3 ($p = 0.0002$), and Ti_4 ($p = 0.0019$), with the lowest T°mean values in G_2 (33.79 ± 0.92 °C) and G_4 (29.30 ± 1.23 °C) registered at Ti_2.

Table 1. Effect of the six euthanasia methods, assessed at five evaluation times, on the maximum, minimum, and mean surface temperature (mean ± standard deviation, SD) of $T°_{ocu}$ (°C) in Wistar rats.

	Groups	Basal	Ti_1	Ti_2	Ti_3	Ti_4	*p*-Value
T°max	G_1 ($n = 10$)	36.62 ± 0.56 ^{a,1}	35.85 ± 0.84 ^{a,1}	35.88 ± 0.83 ^{a,1}	36.44 ± 0.36 ^{a,2,3}	36.25 ± 0.38 ^{a,3}	$p = 0.97$
	G_2 ($n = 10$)	36.03 ± 0.76 ^{b,1}	35.93 ± 0.65 ^{b,1}	34.10 ± 1.65 ^{a,b,2}	34.90 ± 0.91 ^{a,b,2}	33.78 ± 1.18 ^{a,2}	**$p = 0.007$**
	G_3 ($n = 10$)	36.62 ± 0.39 ^{a,1}	36.26 ± 0.56 ^{a,1}	35.60 ± 1.25 ^{a,1}	35.97 ± 0.52 ^{a,2,3}	ND	$p = 0.98$
	G_4 ($n = 10$)	36.46 ± 0.39 ^{b,1}	35.82 ± 0.48 ^{b,1}	29.76 ± 1.61 ^{a,2}	29.9 ± 1.34 ^{a,1}	30.41 ± 0.96 ^{a,1}	**$p = 0.0001$**
	G_5 ($n = 10$)	36.52 ± 0.39 ^{a,1}	36.66 ± 1.08 ^{a,1}	36.50 ± 1.16 ^{a,1}	36.83 ± 0.81 ^{a,3}	36.54 ± 0.52 ^{a,3}	$p = 0.99$
	G_6 ($n = 10$)	36.88 ± 0.69 ^{a,1}	36.18 ± 1.07 ^{a,1}	36.19 ± 1.03 ^{a,1}	36.80 ± 0.10 ^{a,3}	35.85 ± 0.90 ^{a,3}	$p = 0.11$
	p-value	$p = 0.89$	$p = 0.88$	**$p = 0.0005$**	**$p = 0.0006$**	**$p = 0.0001$**	
T°min	G_1 ($n = 10$)	34.04 ± 0.90 ^{a,2}	33.28 ± 0.88 ^{a,1}	33.42 ± 0.56 ^{a,3}	34.26 ± 0.59 ^{a,3}	34.23 ± 0.47 ^{a,3}	$p = 0.49$
	G_2 ($n = 10$)	32.91 ± 0.77 ^{a,1,2}	33.33 ± 0.47 ^{c,1}	30.78 ± 1.22 ^{a,1,2}	32.34 ± 0.97 ^{b,c,2}	31.24 ± 1.24 ^{a,b,2}	**$p = 0.0042$**
	G_3 ($n = 10$)	33.94 ± 0.55 ^{a,1,2}	33.55 ± 0.68 ^{a,1}	32.05 ± 1.06 ^{a,2,3}	32.88 ± 1.01 ^{a,2,3}	ND	$p = 0.12$
	G_4 ($n = 10$)	32.73 ± 0.94 ^{b,1,2}	31.93 ± 0.93 ^{b,1}	28.35 ± 1.30 ^{a,1}	28.50 ± 1.10 ^{a,1}	28.87 ± 0.83 ^{a,1}	**$p = 0.0001$**
	G_5 ($n = 10$)	32.49 ± 0.93 ^{a,1}	32.45 ± 0.77 ^{a,1}	32.66 ± 1.21 ^{a,2,3}	32.70 ± 1.07 ^{a,2,3}	32.47 ± 0.89 ^{a,2}	$p = 0.99$
	G_6 ($n = 10$)	34.48 ± 0.97 ^{a,1,2}	33.64 ± 1.35 ^{a,1}	34.09 ± 1.18 ^{a,3}	34.69 ± 1.00 ^{a,3}	33.78 ± 1.59 ^{a,2}	$p = 0.99$
	p-value	**$p = 0.0023$**	$p = 0.99$	**$p = 0.0002$**	**$p = 0.0007$**	**$p = 0.0010$**	
T°mean	G_1 ($n = 10$)	35.58 ± 0.61 ^{a,1}	34.75 ± 0.96 ^{a,1}	34.88 ± 0.73 ^{a,2,3}	35.48 ± 0.31 ^{a,3,4}	35.36 ±0.38 ^{a,3}	$p = 0.99$
	G_2 ($n = 10$)	34.88 ± 0.71 ^{a,b,1}	34.90 ± 0.47 ^{b,1}	32.89 ± 1.44 ^{a,b,1,2}	33.79 ± 0.92 ^{a,b,2}	32.73 ±1.21 ^{a,2}	**$p = 0.0097$**
	G_3 ($n = 10$)	35.51 ± 0.40 ^{a,1}	35.10 ± 0.49 ^{a,1}	34.15 ± 1.40 ^{a,2,3}	34.88 ± 0.49 ^{a,2,3}	ND	$p = 0.98$
	G_4 ($n = 10$)	34.96 ± 0.62 ^{a,1}	34.21 ± 0.54 ^{a,1}	29.14 ± 1.46 ^{b,1}	29.30 ± 1.23 ^{b,1}	29.77 ± 0.88 ^{b,1}	**$p = 0.0001$**
	G_5 ($n = 10$)	34.84 ± 0.46 ^{a,1}	34.79 ± 0.94 ^{a,1}	34.94 ± 1.20 ^{a,2,3}	35.10 ± 0.86 ^{a,3,4}	34.89 ± 0.66 ^{a,2,3}	$p = 0.99$
	G_6 ($n = 10$)	35.90 ± 0.77 ^{a,1}	35.05 ± 1.25 ^{a,1}	35.38 ± 1.18 ^{a,3}	35.96 ± 0.47 ^{a,4}	34.98 ± 1.23 ^{a,3}	$p = 0.67$
	p-value	$p = 0.20$	$p = 0.24$	**$p = 0.0007$**	**$p = 0.0002$**	**$p = 0.0019$**	

^{a,b,c} different literals indicate significant differences ($p < 0.05$) between events (basal, Ti_1, Ti_2, Ti_3, Ti_4). ^{1,2,3,4} different numerals indicate significant differences ($p < 0.05$) between treatments (G_1, G_2, G_3, G_4, G_5, G_6). Bold *p*-values represent statistically significant differences between events and treatments. ND = not determined due to the experimental group. Treatments (G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2). Evaluation times (basal: 24 h before the procedure; Ti_1: three minutes before the procedure; Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until loss of righting reflex (LORR); Ti_4: from LORR to cessation of heartbeat and breathing).

3.2. Auricular Surface Temperature ($T°_{ear}$)

Differences in T°mean between evaluation times were observed in G_4 ($p = 0.0011$) (Table 2). When comparing Basal with Ti_2, Ti_3, and Ti_4, a decrease in $T°_{ear}$ by 5.82 °C, 5.04 °C and 4.77 °C, respectively, was reported. Between groups, G_2 and G_4 individuals

had the lowest T°mean (30.80 ± 2.83 °C and 29.0 ± 1.76 °C, respectively) and differed from the other groups at Ti_2 (0.0005), Ti_3 ($p = 0.001$), and Ti_4 ($p = 0.04$).

Table 2. Mean ± standard deviation (SD) of $T°_{ear}$ (°C) values of the six euthanasia methods, assessed in five evaluation times, registering the maximum, minimum and mean surface temperatures.

	Groups	Basal	Ti_1	Ti_2	Ti_3	Ti_4	*p*-Value
T°max	G_1 ($n = 10$)	38.42 ± 0.57 ^{a,1}	37.70 ± 0.37 ^{a,1}	37.41 ± 0.96 ^{a,2}	38.47 ± 0.37 ^{a,3}	38.35 ± 0.81 ^{a,2}	$p = 0.09$
	G_2 ($n = 10$)	36.06 ± 0.89 ^{a,2,4}	36.63 ± 0.70 ^{a,1}	33.80 ± 3.95 ^{a,1,2}	34.72 ± 1.80 ^{a,2}	32.64 ± 4.40 ^{a,1,2}	$p = 0.68$
	G_3 ($n = 10$)	36.68 ± 0.64 ^{a,2,3}	37.26 ± 0.66 ^{a,1}	36.03 ± 2.16 ^{a,2}	36.44 ± 1.64 ^{a,2,3}	ND	$p = 0.95$
	G_4 ($n = 10$)	36.99 ± 1.16 ^{b,1,3,4}	36.87 ± 0.97 ^{b,1}	29.76 ± 2.10 ^{a,1}	30.54 ± 2.08 ^{a,1}	30.90 ± 1.21 ^{a,1}	**$p = 0.0003$**
	G_5 ($n = 10$)	37.47 ± 0.70 ^{a,1,3}	37.13 ± 1.37 ^{a,1}	35.37 ± 2.37 ^{a,1,2}	37.73 ± 1.41 ^{a,2,3}	38.04 ± 0.89 ^{a,2}	$p = 0.20$
	G_6 ($n = 10$)	38.15 ± 0.71 ^{a,1}	37.70 ± 0.58 ^{a,1}	37.89 ± 0.87 ^{a,2}	38.67 ± 0.48 ^{a,3}	37.44 ± 1.38 ^{a,2}	$p = 0.13$
p-value		**$p = 0.0119$**	$p = 0.1226$	**$p = 0.0003$**	**$p = 0.0001$**	**$p = 0.0003$**	
T°min	G_1 ($n = 10$)	32.25 ± 1.27 ^{a,1}	31.36 ± 0.91 ^{a,1}	31.97 ± 1.57 ^{a,2}	32.53 ± 0.88 ^{a,3}	31.92 ± 1.04 ^{a,3}	$p = 0.92$
	G_2 ($n = 10$)	30.24 ± 0.94 ^{b,2}	29.78 ± 1.15 ^{a,b,1}	28.89 ± 3.58 ^{a,b,1,2}	27.67 ± 1.43 ^{a,b,1}	27.53 ± 1.30 ^{a,1}	**$p = 0.03$**
	G_3 ($n = 10$)	30.19 ± 0.94 ^{a,2}	30.22 ± 1.09 ^{a,1}	29.77 ± 1.71 ^{a,1,2}	31.17 ± 1.2 ^{a,2,3}	ND	$p = 0.87$
	G_4 ($n = 10$)	31.95 ± 0.99 ^{b,1}	31.22 ± 0.75 ^{b,1}	27.81 ± 1.15 ^{a,1}	28.31 ± 1.50 ^{a,1,2}	28.88 ± 1.06 ^{a,1,2}	**$p = 0.0023$**
	G_5 ($n = 10$)	32.06 ± 0.83 ^{a,1}	31.57 ± 1.73 ^{a,1}	31.90 ± 1.97 ^{a,1,2}	31.44 ± 1.81 ^{a,2,3}	32.61 ± 1.57 ^{a,3}	$p = 0.82$
	G_6 ($n = 10$)	32.56 ± 1.22 ^{a,1}	31.61 ± 0.97 ^{a,1}	32.80 ± 1.34 ^{a,2}	32.35 ± 0.74 ^{a,3}	30.99 ± 1.98 ^{a,2,3}	$p = 0.59$
p-value		**$p = 0.0004$**	$p = 0.4742$	**$p = 0.0005$**	**$p = 0.0010$**	**$p = 0.0018$**	
T°mean	G_1 ($n = 10$)	35.85 ± 0.84 ^{a,1}	35.0 ± 0.65 ^{a,1,2}	34.70 ± 1.09 ^{a,2}	36.24 ± 0.63 ^{a,2}	35.77 ± 0.80 ^{a,3}	$p = 0.08$
	G_2 ($n = 10$)	33.60 ± 0.92 ^{a,1}	33.78 ± 0.69 ^{a,1}	30.80 ± 2.83 ^{a,1,2}	31.60 ± 1.61 ^{a,1}	31.49 ± 2.42 ^{a,1,2}	$p = 0.25$
	G_3 ($n = 10$)	34.08 ± 0.94 ^{a,1}	34.32 ± 0.82 ^{a,1,2}	33.55 ± 1.15 ^{a,2}	34.40 ± 1.64 ^{a,2}	ND	$p = 0.91$
	G_4 ($n = 10$)	34.82 ± 0.54 ^{a,1}	34.47 ± 0.92 ^{a,1,2}	29.0 ± 1.76 ^{b,1}	29.78 ± 1.90 ^{b,1}	30.05 ± 1.20 ^{b,1}	**$p = 0.0011$**
	G_5 ($n = 10$)	35.27 ± 0.75 ^{a,1}	34.85 ± 1.45 ^{a,1,2}	33.78 ± 2.23 ^{a,2}	35.21 ± 1.67 ^{a,2}	36.02 ± 1.22 ^{a,1,2}	$p = 0.43$
	G_6 ($n = 10$)	35.88 ± 0.99 ^{a,1}	35.11 ± 0.62 ^{a,2}	35.06 ± 2.26 ^{a,2}	35.58 ± 2.04 ^{a,2}	34.57 ± 1.85 ^{a,2,3}	$p = 0.98$
p-value		$p = 0.06$	**$p = 0.02$**	**$p = 0.0005$**	**$p = 0.001$**	**$p = 0.04$**	

^{a,b,c} different literals indicate significant differences ($p < 0.05$) between events (Basal, Ti_1, Ti_2, Ti_3, Ti_4). ^{1,2,3,4} different numerals indicate significant differences ($p < 0.05$) between treatments (G_1, G_2, G_3, G_4, G_5, G_6). Bold *p*-values represent statistically significant differences between events and treatments. ND= not determined due to the experimental group. Treatments (G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2). Evaluation times (basal: 24 h before the procedure; Ti_1: three minutes before the procedure; Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until loss of righting reflex (LORR); Ti_4: from LORR to cessation of heartbeat and breathing).

3.3. Interscapular Surface Temperature $T°_{Dor}$

Regarding T°mean of the interscapular region, differences between evaluation times were present in G_1 ($p = 0.007$), G_2 ($p = 0.009$), G_4 ($p = 0.001$) and G_6 ($p = 0.004$) (Table 3). G1 showed a difference of 1.4 °C when comparing Basal (32.46 ± 0.75 °C) vs. Ti_1 (31.06 ± 0.82 °C), while a higher difference of 2.15 °C was obtained for G_6 in Basal (32.21 ± 0.66 °C) vs. Ti_4 (30.06 ± 0.67 °C). The inhalational agents recorded the lowest temperatures during Ti_2 for G_2 (29.08 ± 1.05 °C) and G_4 (28.59 ± 1.28 °C), with temperature drops of 2.75 °C and 3.56 °C, respectively. Regarding differences by group, G_2 and G_4 significantly differed from the other four experimental groups at Ti_2 ($p = 0.0001$), Ti_3 ($p = 0.001$), and Ti_4 ($p = 0.0009$). Particularly, G_4 registered lower T°mean $T°_{dor}$ than G_2 in the mentioned evaluation times (28.59 ± 1.28, 28.78 ± 0.91 and 29.10 ± 0.72 °C, respectively).

3.4. Tail Surface Temperature ($T°_{Tai}$)

Table 4 shows the $T°_{tai}$ at the proximal (T°prox), medial (T°medial) and distal segment (T°distal). In general, all groups showed a progressive decrease in the temperature, starting at Ti_2. In the T°prox, G_1 Ti_3 and Ti_4 significantly differed ($p = 0.005$) from Ti_1 and Ti_2, having a minimum temperature of 27.99 ± 1.23 °C at Ti_4. Ti_2 and Ti_3 of G_3 showed significant differences ($p = 0.004$) from Ti_1, while G_4 significantly differed ($p = 0.002$) at Ti_2, Ti_3 and Ti_4. In the T°medial of G1, all events differed from Basal values ($p = 0.001$), while Ti_2 and Ti_3 of G_3 were statistical different from Basal and Ti1 ($p = 0.002$). Similarly to the T°prox, T°medial ($p = 0.02$), and T°distal ($p = 0.02$) of G_4 differed at Ti_2, Ti_3 and Ti_4, recording the lowest values at Ti_2 for both tail segments (25.70 ± 0.51 °C and 25.27 ± 0.50 °C, respectively).

Table 3. Mean ± standard deviation (SD) of $T°_{dor}$ (°C) maximum, minimum and mean values of the six euthanasia methods, assessed in five evaluation times.

	Groups	Basal	Ti_1	Ti_2	Ti_3	Ti_4	*p*-Value
T°max	G_1 (*n* = 10)	33.28 ± 0.78 ^{a,1}	32.04 ± 1.14 ^{a,1}	33.07 ± 0.94 ^{a,2}	32.57 ± 0.67 ^{a,2}	33.07 ± 0.87 ^{a,2}	*p* = 0.89
	G_2 (*n* = 10)	32.88 ± 1.10 ^{b,1}	31.47 ± 0.91 ^{a,1}	30.41 ± 0.98 ^{a,1}	31.37 ± 1.39 ^{a,2}	31.20 ± 0.75 ^{a,1}	***p* = 0.01**
	G_3 (*n* = 10)	33.58 ± 0.84 ^{a,b,1}	32.09 ± 1.57 ^{a,1}	34.78 ± 0.82 ^{b,2}	34.76 ± 1.14 ^{b,3}	ND	***p* = 0.001**
	G_4 (*n* = 10)	32.97 ± 0.94 ^{b,1}	30.91 ± 0.78 ^{a,1}	29.04 ± 1.21 ^{a,1}	29.32 ± 1.01 ^{a,1}	29.62 ± 0.66 ^{a,1}	***p* = 0.0004**
	G_5 (*n* = 10)	32.55 ± 0.62 ^{a,1}	31.79 ± 1.22 ^{a,1}	33.44 ± 0.76 ^{a,2}	33.04 ± 1.05 ^{a,2,3}	33.08 ± 0.51 ^{a,2}	*p* = 0.09
	G_6 (*n* = 10)	32.91 ± 0.72 ^{b,1}	31.77 ± 0.55 ^{a,1}	33.01 ± 1.30 ^{a,2}	32.73 ± 1.02 ^{a,b,2,3}	31.51 ± 1.06 ^{a,1,2}	***p* = 0.06**
p-value		*p* = 0.16	*p* = 0.47	***p* = 0.005**	***p* = 0.002**	***p* = 0.008**	
T°min	G_1 (*n* = 10)	31.51 ± 0.59 ^{a,1}	30.32 ± 0.81 ^{a,1}	30.35 ± 0.53 ^{a,2}	30.75 ± 0.48 ^{a,3}	31.23 ± 0.73 ^{a,3}	*p* = 0.15
	G_2 (*n* = 10)	30.98 ± 0.96 ^{b,1}	29.54 ± 0.62 ^{a,1}	28.89 ± 3.37 ^{b,1}	28.13 ± 0.88 ^{b,1}	28.39 ± 1.51 ^{b,1}	***p* = 0.01**
	G_3 (*n* = 10)	31.80 ± 0.56 ^{a,1}	30.20 ± 0.65 ^{a,1}	30.15 ± 1.97 ^{a,2}	29.97 ± 2.43 ^{a,2}	ND	*p* = 0.07
	G_4 (*n* = 10)	31.27 ± 1.22 ^{b,1}	29.55 ± 0.90 ^{a,1}	28.17 ± 1.34 ^{a,1}	28.30 ± 0.88 ^{a,1,2}	28.64 ± 0.78 ^{a,1}	***p* = 0.008**
	G_5 (*n* = 10)	31.18 ± 0.49 ^{a,1}	29.89 ± 1.51 ^{a,1}	31.38 ± 1.36 ^{a,3}	30.88 ± 1.16 ^{a,3}	30.72 ± 0.50 ^{a,2}	*p* = 0.50
	G_6 (*n* = 10)	31.54 ± 0.61 ^{c,1}	30.20 ± 0.74 ^{b,1}	30.88 ± 0.70 ^{b,2,3}	30.63 ± 1.37 ^{b,2,3}	28.77 ± 0.73 ^{a,1}	***p* = 0.007**
p-value		*p* = 0.73	*p* = 0.33	***p* = 0.01**	***p* = 0.02**	***p* = 0.003**	
T°mean	G_1 (*n* = 10)	32.46 ± 0.75 ^{c,1}	31.06 ± 0.82 ^{a,1}	31.58 ± 0.73 ^{a,b,2}	31.62 ± 0.41 ^{a,b,c,3}	32.12 ± 0.75 ^{b,c,2}	***p* = 0.007**
	G_2 (*n* = 10)	31.83 ± 1.01 ^{b,1}	30.58 ± 0.73 ^{a,1}	29.08 ± 1.05 ^{a,1}	29.35 ± 0.91 ^{a,1,2}	29.24 ± 0.68 ^{a,1}	***p* = 0.009**
	G_3 (*n* = 10)	32.58 ± 0.62 ^{a,1}	31.25 ± 1.03 ^{a,1}	32.48 ± 0.93 ^{a,2}	32.27 ± 1.05 ^{a,3}	ND	*p* = 0.22
	G_4 (*n* = 10)	32.15 ± 1.01 ^{b,1}	30.21 ± 0.83 ^{a,b,1}	28.59 ± 1.28 ^{a,1}	28.78 ± 0.91 ^{a,1}	29.10 ± 0.72 ^{a,1}	***p* = 0.001**
	G_5 (*n* = 10)	31.84 ± 0.53 ^{a,1}	30.95 ± 1.27 ^{a,1}	32.43 ± 1.05 ^{a,2}	31.85 ± 0.97 ^{a,3}	31.78 ± 0.41 ^{a,2}	*p* = 0.07
	G_6 (*n* = 10)	32.21 ± 0.66 ^{c,1}	31.0 ± 0.61 ^{a,b,1}	31.97 ± 0.64 ^{b,c,2}	31.24 ± 1.06 ^{a,b,c,2,3}	30.06 ± 0.67 ^{a,1}	***p* = 0.004**
p-value		*p* = 0.57	*p* = 0.99	***p* = 0.0001**	***p* = 0.001**	***p* = 0.0009**	

^{a,b,c} different literals indicate significant differences ($p < 0.05$) between events (Basal, Ti_1, Ti_2, Ti_3, Ti_4). ^{1,2,3,} different numerals indicate significant differences ($p < 0.05$) between treatments (G_1, G_2, G_3, G_4, G_5, G_6). Bold *p*-values represent statistically significant differences between events and treatments. ND= not determined due to the experimental group. Treatments (G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2). Evaluation times (basal: 24 h before the procedure; Ti_1: three minutes before the procedure; Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until loss of righting reflex (LORR); Ti_4: from LORR to cessation of heartbeat and breathing).

Between groups, significant differences were reported in Basal values of the three segments (p = 0.002, 0.001 and 0.005). Particularly, during Ti_2 of the T°prox segment, differences were observed in G_2 and G_4 (p = 0.05), with the lowest $T°_{tai}$ of 26.14 ± 2.02 °C and 26.42 ± 0.51 °C, respectively.

Correlations between the four thermal windows and the experimental groups were obtained and are shown according to the experimental group in Supplementary Tables S1–S6. In all groups, significant ($p < 0.001$) and strong correlations ($r \geq 0.96$) were found between thermal windows.

Table 5 summarizes descriptive analysis of the recorded times (in seconds) for each group. Considering the total time of death, the longest duration was observed in G_6, followed by G_4 (294.2 ± 74.3) and G_2 (390.2 ± 171.4). Similarly, cessation of RR and HR was longer in G_6 and G_4. The groups that reached LORR faster were G_5 and G_2 (67 ± 10.3 and 78 ± 29.0 s, respectively).

3.5. Effect of Sex on the Thermal Response of the Rats

Figure 4 illustrates the impact of sex on the temperatures of each thermal window according to the euthanasia methods. In general, no marked effect by sex was found in the present study. Only four statistically significant differences were registered for $T°_{ocu}$, $T°_{ear}$, and $T°_{dor}$, while no effect was found on $T°_{tai}$. For $T°_{ocu}$ (Figure 4A). A significant difference (p = 0.04) was found in the Tmean of G_6, where males had the highest temperatures (36.0 °C) in comparison to females (34.9 °C). However, a tendency to show a difference was observed in Tmax (p = 0.08) and Tmin (0.07). For $T°_{ear}$ (Figure 4B), temperatures between males and females significantly differed in terms of Tmin and Tmean for G_5 (p = 0.002 and p = 0.007, respectively), finding the highest temperatures in males rather than females (33.0 and 35.9 °C vs. 30.8 and 34.2 °C, respectively). Figure 4C shows a statistical significance between sexes in terms of Tmax for G_1 ($p < 0.0001$) for $T°_{dor}$. G_1 males registered a Tmax $T°_{dor}$ of 33.3 °C, while females recorded 32.2 °C.

Table 4. Mean ± standard deviation of $T°_{tai}$ (°C) values at the proximal (T°prox), medial (T°medial), and distal (T°distal) segments of the tail, assessed on the six euthanasia methods at five evaluation times.

	Groups	Basal	Ti_1	Ti_2	Ti_3	Ti_4	*p*-Value
T°prox	G_1 (n = 10)	33.42 ± 1.76 ^{b,1}	28.81 ± 3.2 ^{a,1,2}	29.69 ± 2.52 ^{a,1,2}	28.80 ± 1.97 ^{b,1}	27.99 ± 1.23 ^{b,1}	**p = 0.005**
	G_2 (n = 10)	28.09 ± 2.31 ^{a,b,2}	28.59 ± 1.92 ^{a,2}	26.14 ± 2.02 ^{a,2}	26.35 ± 1.88 ^{a,1}	25.71 ± 1.62 ^{a,1}	p = 0.07
	G_3 (n = 10)	31.30 ± 1.60 ^{a,b,2}	31.61 ± 0.75 ^{b,1}	29.18 ± 0.85 ^{a,1,2}	28.82 ± 0.98 ^{a,1}	ND	**p = 0.004**
	G_4 (n = 10)	31.70 ± 1.64 ^{b,2}	30.74 ± 1.50 ^{b,1,2}	26.42 ± 0.51 ^{a,2}	26.75 ± 0.94 ^{a,1}	27.36 ± 0.84 ^{a,1}	**p = 0.002**
	G_5 (n = 10)	29.99 ± 0.65 ^{a,2}	29.62 ± 1.28 ^{a,1,2}	30.22 ± 2.01 ^{a,1}	29.75 ± 1.62 ^{a,1}	29.23 ± 1.50 ^{a,1}	p = 0.53
	G_6 (n = 10)	30.31 ± 1.28 ^{a,2}	29.97 ± 2.11 ^{a,1,2}	30.01 ± 2.25 ^{a,1,2}	29.17 ± 2.31 ^{a,1}	27.82 ± 3.28 ^{a,1}	p = 0.79
p-value		**p = 0.002**	**p = 0.03**	**p = 0.05**	p = 0.15	p = 0.06	
T°medial	G_1 (n = 10)	33.49 ± 1.79 ^{a,3}	27.09 ± 3.84 ^{b,1}	28.82 ± 2.63 ^{b1}	27.19 ± 2.04 ^{b,1}	26.21 ± 1.48 ^{b,1}	**p = 0.001**
	G_2 (n = 10)	26.75 ± 2.78 ^{a,1}	27.88 ± 2.29 ^{a,1}	25.80 ± 2.81 ^{a,1}	25.11 ± 2.02 ^{a,1}	24.56 ± 2.15 ^{a,1}	p = 0.98
	G_3 (n = 10)	30.48 ± 2.07 ^{b,2}	30.65 ± 1.36 ^{b,1}	27.03 ± 1.37 ^{a,1}	26.83 ± 1.28 ^{a,1}	ND	**p = 0.002**
	G_4 (n = 10)	30.65 ± 2.07 ^{b,2}	29.54 ± 1.89 ^{b,1}	25.70 ± 0.51 ^{a,1}	25.92 ± 1.10 ^{a,1}	26.06 ± 0.82 ^{a,1}	**p = 0.02**
	G_5 (n = 10)	28.77 ± 0.91 ^{a,2}	28.18 ± 1.75 ^{a,1}	28.95 ± 3.15 ^{a,1}	28.18 ± 2.56 ^{a,1}	27.59 ± 2.30 ^{a,1}	p = 0.78
	G_6 (n = 10)	29.24 ± 1.95 ^{a,2}	28.37 ± 2.38 ^{a,1}	28.54 ± 2.47 ^{a,1}	27.64 ± 1.48 ^{a,1}	25.20 ± 2.05 ^{a,1}	p = 0.09
p-value		**p = 0.001**	p = 0.23	p = 0.37	p = 0.34	p = 0.41	
T°distal	G_1 (n = 10)	33.34 ± 2.08 ^{c,1}	26.50 ± 3.77 ^{b,1}	28.13 ± 2.68 ^{b,1}	26.54 ± 2.10 ^{b,1}	25.83 ± 1.61 ^{b,1}	**p = 0.003**
	G_2 (n = 10)	26.29 ± 2.55 ^{a,b,3}	27.08 ± 2.77 ^{a,1}	24.41 ± 2.34 ^{a,1}	24.30 ± 2.23 ^{a,1}	23.67 ± 1.90 ^{a,1}	p = 0.20
	G_3 (n = 10)	29.23 ± 2.63 ^{b,1,2}	29.32 ± 1.73 ^{b,1}	25.28 ± 1.56 ^{a,1}	25.14 ± 1.48 ^{a,1}	ND	**p = 0.002**
	G_4 (n = 10)	30.23 ± 2.14 ^{c,1,2}	28.65 ± 1.90 ^{b,1}	25.27 ± 0.50 ^{a,1}	25.67 ± 1.26 ^{a,1}	25.77 ± 0.78 ^{a,1}	**p = 0.02**
	G_5 (n = 10)	28.26 ± 1.13 ^{a,b,2}	27.35 ± 1.79 ^{a,1}	28.04 ± 3.08 ^{a,1}	27.22 ± 2.62 ^{a,1}	26.86 ± 2.43 ^{a,1}	p = 0.47
	G_6 (n = 10)	28.32 ± 2.32 ^{a,b,2}	27.48 ± 2.28 ^{a,1}	27.61 ± 2.60 ^{a,1}	26.37 ± 1.84 ^{a,1}	23.89 ± 1.80 ^{a,1}	p = 0.10
p-value		**p = 0.005**	p = 0.74	p = 0.33	p = 0.65	p = 0.19	

^{a,b,c} different literals indicate significant differences ($p < 0.05$) between events (basal, Ti_1, Ti_2, Ti_3, Ti_4). ^{1,2,3,} different numerals indicate significant differences ($p < 0.05$) between treatments (G_1, G_2, G_3, G_4, G_5, G_6). Bold *p*-values represent statistically significant differences between events and treatments. ND = not determined due to the experimental group. Treatments—G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2. Evaluation times—basal: 24 h before the procedure; Ti_1: three minutes before the procedure; Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until loss of righting reflex (LORR); Ti_4: from LORR to cessation of heartbeat and breathing.

Table 5. Comparison between recorded times (in seconds) according to the experimental group (mean ± SD).

Group	Time of Death (s)	Time of LORR (s)	Time of RR Cessation (s)	Time of HR Cessation (s)
G_1	230.1 ± 42.4	94.2 ± 19.8	193.8 ± 28.8	230.1 ± 42.2
G_2	294.2 ± 74.3	78 ± 29.0	211.8 ± 64.1	305.5 ± 76.3
G_3	6.2 ± 4.0	ND	ND	ND
G_4	390.2 ± 171.4	97.8 ± 49.5	288 ± 15.7	390.2 ± 17.1
G_5	257.9 ± 30.1	67 ± 10.3	172.3 ± 25.6	257.9 ± 30.1
G_6	420.3 ± 47.3	122.7 ± 21.8	291.8 ± 31.7	420.3 ± 47.3

G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2; HR: heart rate; LORR: loss of righting reflex; ND: not determined; RR: respiratory rate; s: seconds; SD: standard deviation.

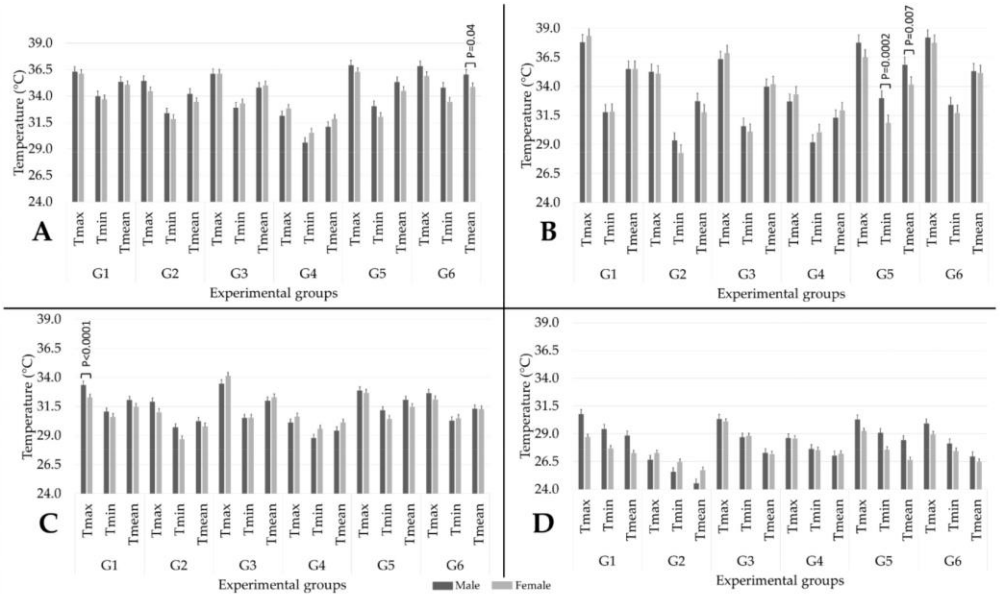


Figure 4. Effect of sex (5 males and 5 females in each group) on the maximum (max), minimum (min), and mean temperature of each thermal window in the six experimental groups: (**A**) $T°_{ocu}$; (**B**) $T°_{ear}$; (**C**) $T°_{dor}$; (**D**) $T°_{tai}$. G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2.

4. Discussion

Based on the findings of this study, in contrast to injectable, physical, and combined euthanasia methods, inhalant agents (CO_2 and isoflurane) resulted in substantial alterations in the $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$, and $T°_{tai}$ of Wistar rats. To date, there are no studies where IRT was used to evaluate different euthanasia methods or their effect on the thermal response of rats. However, studies addressing the mechanism of action of CO_2 and isoflurane, as well as its overdose to induce euthanasia, have shown that both drugs trigger physiological stress and cardiovascular alterations in laboratory rodents [26]. Although both are considered safe, inexpensive, and effective methods to induce unconsciousness, the results obtained agree with previous findings suggesting that gases with anesthetic properties cause high levels of aversion and stress in mice and rats [15,52].

Before discussing the IRT results according to the thermal windows, the current literature regarding endocrine and behavioral changes after CO_2 and isoflurane euthanasia in rats and mice suggests that the thermal response due to stress/distress could be associated with both methods. CO_2 inhalation used in G_2 rats induces hypercapnia, acidosis, and suppression of the synaptic potentials [52–54] and activates the stress-mediated sympathetic HPA and SAM axes [55]. This was corroborated by Borovsky et al. [56], who reported that exposure of rats to CO_2 increased their blood pressure (50–60 mmHg) and NE concentration up to ten times in response to hypoxia. CO_2 euthanasia at 10% is also related to distress due to a high exhibition of anxiety behavior in rats [11]. Moreover, aversion to high concentrations of CO_2 (more than 40% of the induction chamber volume) is potentially associated with carbonic acid formation in the mucous membranes, causing irritation and discomfort behaviors such as spinning and pawing in rodents [57]. Acute exposure of mice to CO_2, increased NE and adrenocorticotropic hormone (ACTH) levels [55], and studies

in outbred mice and rats have shown that CO_2 inhalation increases total serum protein levels, a biomarker associated with stress [58]. In contrast, Hackbarth et al. [59] found no behavioral signs of distress or endocrine alterations (ACTH and corticosterone) in rats undergoing CO_2 euthanasia.

Studies comparing the effect of both CO2 and isoflurane euthanasia have also shown different results. Isoflurane administered in G_4 rats is a volatile anesthetic that causes depression in the cardiorespiratory centers, leading to hypoxemia and death [26,60]. Although isoflurane has been considered as a method of CO_2 euthanasia refinement [14,15], in comparison with CO_2 and other volatile anesthetics, isoflurane has mild pungency [61], causing more aversion responses in laboratory rodents that is possibly due to airway irritation [52,62], air hunger, and dyspnea [52].

Powell et al. [57] found that the use of isoflurane during euthanasia increased anxiety-related behaviors, agitation scores, and corticosterone concentrations in mice compared to the low CO_2 flow rate (30%), the same one used in the present research. Boivin et al. [63] compared isoflurane anesthesia followed by CO_2, CO_2, and barbiturates administration as euthanasia methods in mice. The authors found that, according to ACTH concentrations, barbiturates were less stressful than the other two methods. Nonetheless, cardiovascular alterations and pain/stress-related responses did not differ in the three methods, suggesting that isoflurane does not provide benefits above CO_2 euthanasia. In this sense, Valentine et al. [64] reported that a combination of isoflurane and CO_2 caused more signs of distress in mice and that CO_2 alone has less evidence of stress in the animals.

In contrast to what was mentioned, Makowska and Weary's [15] study reported that CO_2 and inhalant agents are aversive to rodents, though the aversion is lower for isoflurane. Likewise, exposure to high concentrations of CO_2 increased adrenaline and noradrenaline concentrations compared to isoflurane euthanasia. This could be indicative of a stress response, but since no stress-related behaviors (grooming, audible vocalizations) were reported in the CO_2 group, CO_2 could not be considered as more stressful than isoflurane [6]. In rats, Zardooz et al. [65] found that plasma corticosterone and insulin levels increased in rats exposed to CO_2, while isoflurane caused a contrary reaction. In Hickman et al. [55]'s research, ACTH, corticosterone, and noradrenaline levels were detected in rats anesthetized with isoflurane; however, the increase was not as significant as with CO_2.

The present study did not assess behavioral or endocrine parameters to associate the thermal response of rats to the different euthanasia methods. However, the literature shows that both methods trigger stress-related responses that have physiological consequences for the organism, which can be associated with temperature variations according to the thermal window.

4.1. $T°_{ocu}$

A significant increase in the T°mean of $T°_{ocu}$ from Ti_2 to Ti_3 and from Ti_2 to Ti_4 in G_2 and G_4, respectively, was observed in the rats. The stress-mediated thermoregulatory impairment that CO_2 and isoflurane cause on thermosensitive neurons due to the acidosis and hypoxic effect [66,67] could explain the increase in $T°_{ocu}$.

Several studies have shown that epinephrine, NE, ACTH, and corticosterone levels increase after CO_2 and isoflurane exposure due to the potential stress that both drugs cause [6,68–70]. Although the present study did not consider these biomarkers for evaluation, their release modifies the vasomotor reaction of the microvasculature, inducing vasodilation in key organs (e.g., the eye) and an increased amount of dissipated heat, registered as higher IRT temperatures [29,71] like the ones observed in $T°_{ocu}$ for G_2 and G_4 rats.

Ocular surface temperature in animals has been used as a thermal window to assess acute stress and pain, indicated by a recorded increase in both cases [72,73]. To the authors' knowledge, there are only two studies combining IRT and the effect of isoflurane as an anesthetic [30,74], though these studies did not compare CO_2 and isoflurane as a euthanasic. Gjendal et al. [30] determined that, from three different types of stimulus, isoflurane

anesthesia in mice had a marked stress response due to the alterations in ocular temperature. Similarly, Vogel et al. [74] used isoflurane anesthesia and found that ocular temperature changed according to the isoflurane concentration and that this temperature also reflects rectal temperature in rodents. Nonetheless, no association was made with stress.

Conversely, while there is no published evidence on euthanasia and ocular IRT, an increase in ocular temperature was reported in wild rodents (*Apodemus mystacinus*) as a reflection of SIH during the manipulation of individuals [33] during a fear-conditioned test in rats (increasing the eye temperature by up to 1.5 °C) [75], while in mice SIH and active behaviors were positively correlated [76]. Furthermore, in guinea pigs, the ocular temperature increased in relation to negative human interaction (petting) [77]. Similarly, Wongsaengchan et al. [78] used eye temperature to assess acute exposure to a stressor (small cage, handling, and restraint cone). The authors found significant increases in the left ocular temperature of females during restraint, together with corticosterone increases.

The data suggest that the peripheral vasomotor changes might respond to the flight–fight response when exposed to a stressor. Increases in $T°_{ocu}$ from Ti_2 in all experimental groups suggest that rats perceived stress regardless of the euthanasia method. Nonetheless, knowing that CO_2 and isoflurane inhalation might trigger stress-mediated pathways, this could explain the significant changes observed only in G_2 and G_4 from the application of the drug to LORR, probably due to an increased stress response. Finally, although both groups showed significant increases in $T°_{ocu}$, G_2 and G_4 maintained overall lower temperatures than the rest of the groups, possibly due to heat loss facilitation due to the vasodilator properties of both drugs [53]. A similar result was obtained in Gjendal et al. [30]'s study, where isoflurane anesthesia in mice led to a reduction in T°max due to the hypothermia caused by general anesthetics.

4.2. $T°_{ear}$

Comparable to $T°_{ocu}$, significant increases in the T°mean of $T°_{ear}$ from Ti_2 were observed in both inhalant groups (G_2 and G_4). This pattern was expected because ear temperature assessed at the external ear canal is associated with the carotid artery and hypothalamic temperature, the main structure involved in central and peripheral thermoregulatory adaptations [79], particularly when exposed to stressors. Studies conclude that CO_2 and isoflurane exert acute stress in rodents [52,55,58].

In animals, auricular temperature was associated with stress due to the administration of intraperitoneal drugs and restraining techniques in Wokke [34]'s study, as well as in rabbits during handling [80]. In rats, increases ranging between 0.8 and 1.5 °C were observed during conditioned fear reactions [75]. In the present study, in all experimental groups, an increase in the T°mean of $T°_{ear}$ was observed. However, only CO_2 and isoflurane caused significant increases. This response and its association with previously reported behavioral and endocrine responses with CO_2 and isoflurane euthanasia/anesthesia might cause SIH. Since authors such as Hutu et al. [36] have concluded that superficial ear temperature is correlated to core temperature (around 37.1 ± 0.2 °C), the increase in $T°_{ear}$ could be the reflection of SIH in G_2 and G_4.

In contrast to the reported findings, some studies have not found significant changes or decreases in the ear temperature of mice and rats. This might be because of the lack of arteriovenous anastomosis present in other species, such as rabbits [81]. Additionally, conflicting results can be derived from the thermal window delimitation used by other authors (e.g., external ear canal or auricular pavilion).

4.3. $T°_{dor}$

An expected increase in T°mean values recorded for $T°_{dor}$ after the administration of the euthanasia method was found in all experimental groups. Particularly, significant differences were reported in G_2 and G_4, maybe due to the induced acute stress that CO_2 and halogenated anesthetics induce in rats. In the anatomical region where $T°_{dor}$ was evaluated, large amounts of BAT can be found [37]. This thermogenic tissue responds to

window can also differ depending on the sex, as shown in a study where, according to IRT, females were prone to show an exacerbated stress response to restraint [78].

A possible explanation for the lack of significant differences between males and females in the present study could be due to the short period of evaluation used for each euthanasia method. Euthanasia times are (and must be) short so as to avoid high levels of stress. Although, as shown in Table 5, G_4 (294.2 ± 74.3 s) and G_2 (390.2 ± 171.4) were two of the three euthanasia methods with longer time of death, this time might not have permitted the finding of differences according to sex. Powell et al. [57] mention that rodents require at least two minutes of stressor exposure to increase corticosterone values in response to stress. Nonetheless, since IRT has not been previously evaluated during euthanasia methods considering both sexes, future research needs to consider these factors.

4.6. Time of Death and Additional Findings

Regarding the time of death, time of LORR, and cessation of RR and HR, the times obtained in the six experimental groups are in accordance with previous studies evaluating time of death with pentobarbital [18], CO_2 [86], decapitation [87], isoflurane [6], and ketamine + xylazine [8].

Lastly, a distinct pattern and pronounced difference between G_4 and the rest of the experimental groups should be noted. $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$, and $T°_{tai}$ for G_4 rats showed a progressive increase in the surface temperature from Ti_2 to the death of the animals, apart from recording the lowest temperatures from Ti_2 to Ti_4 when compared to the other five groups. In contrast, animals from the other groups, including G_2, presented a temperature increase from Ti_2 to Ti_3 and a subsequent decrease in all thermal windows. This suggests that the anesthetic stress and physiological response triggered by isoflurane is more marked than that induced by other inhalant, injectable, and physical methods of euthanasia. The present results are in agreement with what other authors have stated regarding isoflurane as a refinement method for CO_2 [52,62] and affirm that precautions should be taken when deciding to use isoflurane as a sole method for the humane killing of research animals.

4.7. Limitations and Future Recommendations

The main limitation of the current study, and a field for complementary research using IRT aimed to evaluate euthanasia methods, is the lack of monitoring using physiological markers such as NE, ACTH, corticosterone, glucose, and other parameters (e.g., rectal temperature) that have been reported to increase their concentration during the application of different types of euthanasia [26,55]. Moreover, histological analyses could also help to identify the possible tissular changes associated with an inflammatory response to different drugs, providing additional information according to the euthanasia method. Additionally, analyzing the time of death, IRT response, and other biomarkers could help to understand the influence of the application speed and the thermal response of rodents. In the present study, the novel conception of an anesthesia induction chamber designed to allow for IRT readings during inhalant euthanasia is a valuable tool that might serve to further assess how euthanasia drugs, in combination with other physiological, endocrinal, and behavioral parameters, can contribute to the refinement of animal research.

In this sense, an important finding of the present study that can be considered for future research as a refinement in euthanasia procedures in Wistar rats is the combination of injectable agents and CO_2. As the results showed, contrary to the use of CO_2 alone, the combination administered in G_6 diminished the thermal alterations observed in G_2. This could be due to the sedative properties of ketamine before CO_2 exposure, antagonizing NMDA receptors, modulating neuronal activity, and reducing the discomfort sensation with CO_2 [88,89]. This could prevent physiological responses due to induced hypoxia, acidosis, and stress-related changes [12].

Regarding the non-significant effect of sex in the thermal response of the subjects, the contrasting information between the present results and the published literature shows the complexity of using IRT as a tool to evaluate stress. For example, this variable and the other

factors mentioned by Wongsaengchan et al. [78] (e.g., period of evaluation, sex, left/right side for the taking of thermal image) are important elements that need to be considered in further studies where IRT is intended to be used as a tool to improve the welfare of laboratory rodents. Similarly, the weight of rodents should also be considered when using IRT because the thermal response of animals might differ according to their energy reserves and metabolic activity (e.g., obesity in mammals is associated with increased depots of adipose tissue) [90]. Moreover, studies have shown that external traits such as coat color or type of fur can affect the amount of radiated heat [91]. In the present study we only used Wistar rats (white coat); however, when using IRT in other strains or species, these traits need to be addressed to objectively interpret thermal imaging.

Considering that IRT serves as a non-invasive method to assess the thermal response that can be associated with vasomotor changes due to sympathetic activation, IRT could be implemented as a complementary tool to evaluate stress under other conditions (e.g., heat stress). Likewise, pain assessment and even disease detection can be other fields where thermal imaging could be applied together with biomarkers and other technologies with the aim of improving laboratory animal welfare [24,29–31,73].

5. Conclusions

Based on the results obtained, it can be concluded that CO_2 and isoflurane elicit stress-mediated thermal responses during rat euthanasia. In particular, isoflurane exposure might be a euthanasia method that causes potential distress, and this must be considered when deciding to use this drug as part of a euthanasic protocol. Refinement techniques such as the combination of ketamine + CO_2 were shown to minimize the alterations observed with the sole use of CO_2, but further research is required to perform a comprehensive evaluation of this alternative. Furthermore, the present study shows the usefulness of IRT as a non-invasive tool for the evaluation of euthanasia techniques and the thermal response of laboratory rodents. In this way, thermal imaging could be recommended together with other physiological, endocrinal, and behavioral parameters to assess and improve the welfare of research animals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13182820/s1, Table S1: Correlations "Pentobarbital" (G_1); Table S2: Correlations "CO_2 overdose" (G_2); Table S3: Correlation "Decapitation" (G_3); Table S4: Correlations "Inhalation of isoflurane" (G_4); Table S5: Correlation "Ketamine" (G_5); Table S6: Correlations "Combination of ketamine + CO_2" (G_6).

Author Contributions: A.D.-O., I.H.-Á., A.O.-H., J.V.-J., A.V.-M. and D.M.-R. contributed to conceptualization, writing, and proofreading. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All procedures were approved by the Committee for the Care and Use of Laboratory Animals (INRLGII/CICUAL/014/2021) at the National Institute of Rehabilitation Luis Guillermo Ibarra Ibarra. The handling and care of the laboratory animals that were used to obtain the thermograms of this article were conducted in accordance with the recommendations of Mexican norm NOM-062-ZOO-1999 for laboratory animals, published by the Department of Agriculture, Rural Development, Fisheries and Alimentation. All dead animals were disposed of by incineration, following NOM-062-ZOO-1999.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are contained within the article.

Acknowledgments: Adriana Domínguez-Oliva extends her gratitude to the National Council for Science and Technology (CONACYT) in Mexico for the Scholarship No. 1143774 awarded to her to pursue her master's studies in Agricultural Sciences at Universidad Autónoma Metropolitana.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Hobson-West, P.; Davies, A. Societal Sentience: Constructions of the public in animal research policy and practice. *Sci. Technol. Hum. Values* **2018**, *43*, 671–693. [CrossRef] [PubMed]
2. Domínguez-Oliva, A.; Hernández-Ávalos, I.; Martínez-Burnes, J.; Olmos-Hernández, A.; Verduzco-Mendoza, A.; Mota-Rojas, D. The Importance of Animal Models in Biomedical Research: Current Insights and Applications. *Animals* **2023**, *13*, 1223. [CrossRef] [PubMed]
3. Matsuda, Y. Recent Trends in the Number of Laboratory Animals Used in Japan. *Altern. Lab. Anim.* **2004**, *32*, 299–301. [CrossRef] [PubMed]
4. National Association for Biomedical Research. The Importance of Animal Testing in Biomedical Research. Available online: https://www.nabr.org/biomedical-research/importance-biomedical-research (accessed on 17 August 2021).
5. Franco, N.H. Animal Experiments in Biomedical Research: A Historical Perspective. *Animals* **2013**, *3*, 238. [CrossRef]
6. Marquardt, N.; Feja, M.; Hünigen, H.; Plendl, J.; Menken, L.; Fink, H.; Bert, B. Euthanasia of laboratory mice: Are isoflurane and sevoflurane real alternatives to carbon dioxide? *PLoS ONE* **2018**, *13*, e0203793. [CrossRef]
7. Baumans, V. Use of animals in experimental research: An ethical dilemma? *Gene Ther.* **2004**, *11*, S64–S66. [CrossRef]
8. American Veterinary Medical Association (AVMA). *AVMA Guidelines for the Euthanasia of Animals*; AVMA: Schaumburg, IL, USA, 2020.
9. Makowska, I.J.; Weary, D.M. Using Rat Behavior to Assess Aversion to Euthanasia Agents. *ALTEX* **2012**, *1*, 465–467.
10. Chisholm, J.M.; Pang, D.S.J. Assessment of Carbon Dioxide, Carbon Dioxide/Oxygen, Isoflurane and Pentobarbital Killing Methods in Adult Female Sprague-Dawley Rats. *PLoS ONE* **2016**, *11*, e0162639. [CrossRef]
11. Hickman, D.; Fitz, S.; Bernabe, C.; Caliman, I.; Haulcomb, M.; Federici, L.; Shekhar, A.; Johnson, P. Evaluation of Low versus High Volume per Minute Displacement CO_2 Methods of Euthanasia in the Induction and Duration of Panic-Associated Behavior and Physiology. *Animals* **2016**, *6*, 45. [CrossRef]
12. Boivin, G.P.; Hickman, D.L.; Creamer-Hente, M.A.; Pritchett-Corning, K.R.; Bratcher, N.A. Review of CO_2 as a Euthanasia Agent for Laboratory Rats and Mice. *J. Am. Assoc. Lab. Anim. Sci.* **2017**, *56*, 491–499.
13. Turner, P.V.; Hickman, D.L.; van Luijk, J.; Ritskes-Hoitinga, M.; Sargeant, J.M.; Kurosawa, T.M.; Agui, T.; Baumans, V.; Choi, W.S.; Choi, Y.-K.; et al. Welfare Impact of Carbon Dioxide Euthanasia on Laboratory Mice and Rats: A Systematic Review. *Front. Vet. Sci.* **2020**, *7*, 411. [CrossRef] [PubMed]
14. De la Torre, C.; Jiménez, P.; Ramos, M.M.; De la Torre, G. Anestesia para colonoscopia: Anestesia inhalatoria con sevoflurano frente a anestesia intravenosa con propofol. *Sanid. Mil.* **2012**, *68*, 27–32. [CrossRef]
15. Makowska, I.J.; Weary, D.M. Rat aversion to induction with inhalant anaesthetics. *Appl. Anim. Behav. Sci.* **2009**, *119*, 229–235. [CrossRef]
16. Flecknell, P. *Laboratory Animal Anaesthesia*, 4th ed.; Elsevier: Kidlington, UK, 2016; p. 350.
17. Reimer, J.N.; Schuster, C.J.; Knight, C.G.; Pang, D.S.J.; Leung, V.S.Y. Intraperitoneal injection of sodium pentobarbital has the potential to elicit pain in adult rats (*Rattus norvegicus*). *PLoS ONE* **2020**, *15*, e0238123. [CrossRef]
18. Zatroch, K.K.; Knight, C.G.; Reimer, J.N.; Pang, D.S.J. Refinement of intraperitoneal injection of sodium pentobarbital for euthanasia in laboratory rats (*Rattus norvegicus*). *BMC Vet. Res.* **2016**, *13*, 60. [CrossRef]
19. Hohlbaum, K.; Bert, B.; Dietze, S.; Palme, R.; Fink, H.; Thöne-Reineke, C. Impact of repeated anesthesia with ketamine and xylazine on the well-being of C57BL/6JRj mice. *PLoS ONE* **2018**, *13*, e0203559. [CrossRef]
20. Wellington, D.; Mikaelian, I.; Singer, L. Comparison of ketamine-xylazine and ketamine-dexmedetomidine anesthesia and intraperitoneal tolerance in rats. *J. Am. Assoc. Lab. Anim. Sci.* **2013**, *52*, 481–487.
21. Kongara, K.; McIlhone, A.; Kells, N.; Johnson, C. Electroencephalographic evaluation of decapitation of the anaesthetized rat. *Lab. Anim.* **2014**, *48*, 15–19. [CrossRef]
22. Derr, R. Pain perception in decapitated rat brain. *Life Sci.* **1991**, *49*, 1399–1402. [CrossRef]
23. Huggins, J.; Rakobowchuk, M. Utility of lacrimal caruncle infrared thermography when monitoring alterations in autonomic activity in healthy humans. *Eur. J. Appl. Physiol.* **2019**, *119*, 531–538. [CrossRef]
24. Mota-Rojas, D.; Olmos-Hernández, A.; Verduzco-Mendoza, A.; Lecona-Butrón, H.; Martínez-Burnes, J.; Mora-Medina, P.; Gómez-Prado, J.; Orihuela, A. Infrared thermal imaging associated with pain in laboratory animals. *Exp. Anim.* **2021**, *70*, 1–12. [CrossRef] [PubMed]
25. National Research Council. National Association for Biomedical Research Recognition and Alleviation of Pain in Laboratory Animals. Available online: https://www.ncbi.nlm.nih.gov/books/NBK32654/ (accessed on 1 September 2022).
26. Nichols, K.E.; Holliday-White, K.L.; Bogie, H.M.; Swearingen, K.M.; Fine, M.S.; Doyle, J.; Tiesma, S.R. Cardiovascular and Metabolic Responses to Carbon Dioxide Euthanasia in Conscious and Anesthetized Rats. *J. Am. Assoc. Lab. Anim. Sci.* **2020**, *59*, 742–749. [CrossRef] [PubMed]
27. López-Moraga, A.; Beckers, T.; Luyten, L. The effects of stress on avoidance in rodents: An unresolved matter. *Front. Behav. Neurosci.* **2022**, *16*, 983026. [CrossRef] [PubMed]
28. Faraji, J.; Metz, G.A.S. Infrared Thermography Reveals Sex-Specific Responses to Stress in Mice. *Front. Behav. Neurosci.* **2020**, *14*, 79. [CrossRef]

29. Verduzco-Mendoza, A.; Bueno-Nava, A.; Wang, D.; Martínez-Burnes, J.; Olmos-Hernández, A.; Casas, A.; Domínguez, A.; Mota-Rojas, D. Experimental Applications and Factors Involved in Validating Thermal Windows Using Infrared Thermography to Assess the Health and Thermostability of Laboratory Animals. *Animals* **2021**, *11*, 3448. [CrossRef]
30. Gjendal, K.; Franco, N.H.; Ottesen, J.L.; Sørensen, D.B.; Olsson, I.A.S. Eye, body or tail? Thermography as a measure of stress in mice. *Physiol. Behav.* **2018**, *196*, 135–143. [CrossRef]
31. Whittaker, A.L.; Muns, R.; Wang, D.; Martínez-Burnes, J.; Hernández-Ávalos, I.; Casas-Alvarado, A.; Domínguez-Oliva, A.; Mota-Rojas, D. Assessment of Pain and Inflammation in Domestic Animals Using Infrared Thermography: A Narrative Review. *Animals* **2023**, *13*, 2065. [CrossRef]
32. Lecorps, B.; Rödel, H.G.; Féron, C. Assessment of anxiety in open field and elevated plus maze using infrared thermography. *Physiol. Behav.* **2016**, *157*, 209–216. [CrossRef]
33. Zevgolis, Y.G.; Zannetos, S.P.; Akriotis, T. Physiological response of a wild rodent to experimental manipulations in its natural environment using infrared thermography. *Hystrix It. J. Mamm.* **2022**, *33*, 80–91. [CrossRef]
34. Wokke, E.S. Refinement: Evaluating Stress and Accuracy of Different Intraperitoneal Techniques in Mice. Master's Thesis, University of Utrecht, Utrecht, The Netherlands, February 2017.
35. International Committee on Veterinary Gross Anatomical Nomenclature. *Nomina Anatomica Veterinaria*; World Association of Veterinary Anatomist: Hanover, Germany, 2017; pp. 1–178.
36. Hutu, I.; Paras, I.; Gherghel, D.; Lungu, B.; Mircu, C. *Application of Infrared Thermography in Rabbit Orthopaedic Models*; Universitatea de Științe Agricole și Medicină Veterinară: Iași, Romania, 2018.
37. Lezama-García, K.; Mota-Rojas, D.; Martínez-Burnes, J.; Villanueva-García, D.; Domínguez-Oliva, A.; Gómez-Prado, J.; Mora-Medina, P.; Casas-Alvarado, A.; Olmos-Hernández, A.; Soto, P.; et al. Strategies for Hypothermia Compensation in Altricial and Precocial Newborn Mammals and Their Monitoring by Infrared Thermography. *Vet. Sci.* **2022**, *9*, 246. [CrossRef]
38. Mousovich-Neto, F.; Matos, M.S.; Costa, A.C.R.; Melo Reis, R.A.; Atella, G.C.; Miranda-Alves, L.; Carvalho, D.P.; Ketzer, L.A.; Corrêa da Costa, V.M. Brown adipose tissue remodelling induced by corticosterone in male Wistar rats. *Exp. Physiol.* **2019**, *104*, 514–528. [CrossRef]
39. Jackson, D.; Hambly, C.; Trayhurn, P.; Speakman, J. Can non-shivering thermogenesis in brown adipose tissue following NA injection be quantified by changes in overlying surface temperatures using infrared thermography? *J. Therm. Biol.* **2001**, *26*, 85–93. [CrossRef] [PubMed]
40. Kataoka, N.; Shima, Y.; Nakajima, K.; Nakamura, K. A central master driver of psychosocial stress responses in the rat. *Science* **2020**, *367*, 1105–1112. [CrossRef] [PubMed]
41. Owens, N.C.; Ootsuka, Y.; Kanosue, K.; McAllen, R.M. Thermoregulatory Control of Sympathetic Fibres Supplying the Rat's Tail. *J. Physiol.* **2002**, *543*, 849–858. [CrossRef] [PubMed]
42. Blenkuš, U.; Gerós, A.F.; Carpinteiro, C.; de Aguiar, P.C.; Olsson, I.A.S.; Franco, N.H. Non-Invasive Assessment of Mild Stress-Induced Hyperthermia by Infrared Thermography in Laboratory Mice. *Animals* **2022**, *12*, 177. [CrossRef] [PubMed]
43. Redaelli, V.; Papa, S.; Marsella, G.; Grignaschi, G.; Bosi, A.; Ludwig, N.; Luzi, F.; Vismara, I.; Rimondo, S.; Veglianese, P.; et al. A refinement approach in a mouse model of rehabilitation research. Analgesia strategy, reduction approach and infrared thermography in spinal cord injury. *PLoS ONE* **2019**, *14*, e0224337. [CrossRef]
44. Gariepy, C.; Amiot, J.; Nadai, S. Ante-mortem detection of PSE and DFD by infrared thermography of pigs before stunning. *Meat Sci.* **1989**, *25*, 37–41. [CrossRef]
45. Flores-Peinado, S.; Mota-Rojas, D.; Guerrero-Legarreta, I.; Mora-Medina, P.; Cruz-Monterrosa, R.; Gómez-Prado, J.; Guadalupe Hernández, M.; Cruz-Playas, J.; Martínez-Burnes, J. Physiological responses of pigs to preslaughter handling: Infrared and thermal imaging applications. *Int. J. Vet. Sci. Med.* **2020**, *8*, 71–84. [CrossRef]
46. Diario Oficial de la Federación Normal Oficial Mexicana (NOM-062-ZOO-1999). Especificaciones Técnicas para la Producción, Cuidado y Uso de los Animales de Laboratorio. Available online: https://www.gob.mx/cms/uploads/attachment/file/203498/NOM-062-ZOO-1999_220801.pdf (accessed on 20 June 2023).
47. Kovalcsik, R.; Devlin, T.; Loux, S.; Martinek, M.; May, J.; Pickering, T.; Tapp, R.; Wilson, S.; Serota, D. Animal reuse: Balancing scientific integrity and animal welfare. *Lab. Anim.* **2006**, *35*, 49–53. [CrossRef]
48. Lofgren, J.L.; Foley, P.L.; Golledge, H.D.R. Anesthesia, analgesia, and euthanasia. In *The Laboratory Rat*; Suckow, M.A., Hankenson, R., Wilson, R., Foley, P.L., Eds.; Academic Press: London, UK, 2020; pp. 699–745.
49. Risling, T.E.; Caulkett, N.A.; Florence, D. Open-drop anesthesia for small laboratory animals. *Can. Vet. J. Rev. Vet. Can.* **2012**, *53*, 299–302.
50. de Brito, C.F.; Evangelista, A.A.; Felippe, R.M.; Cascabulho, C.; Fragoso, V.M.; de Oliveira, G.M. Laboratory Mice Euthanasia: Speed Death and Animal Welfare. *Am. J. Biomed. Sci. Res.* **2020**, *8*, 341–355. [CrossRef]
51. Ko, M.J.; Mulia, G.E.; van Rijn, R.M. Commonly Used Anesthesia/Euthanasia Methods for Brain Collection Differentially Impact MAPK Activity in Male and Female C57BL/6 Mice. *Front. Cell. Neurosci.* **2019**, *13*, 96. [CrossRef] [PubMed]
52. Kulkarni, S.; Hickman, D. Isoflurane and Carbon Dioxide Elicit Similar Behavioral Responses in Rats. *Animals* **2020**, *10*, 1431. [CrossRef] [PubMed]
53. Xu, F.; Uh, J.; Brier, M.R.; Hart, J.; Yezhuvath, U.S.; Gu, H.; Yang, Y.; Lu, H. The Influence of Carbon Dioxide on Brain Activity and Metabolism in Conscious Humans. *J. Cereb. Blood Flow Metab.* **2011**, *31*, 58–67. [CrossRef]

54. Leach, M.C.; Bowell, V.A.; Allan, T.F.; Morton, D.B. Degrees of aversion shown by rats and mice to different concentrations of inhalational anaesthetics. *Vet. Rec.* **2002**, *150*, 808–815. [CrossRef] [PubMed]
55. Hickman, D.L. Interpreting Neuroendocrine Hormones, Corticosterone, and Blood Glucose to Assess the Wellbeing of Anesthetized Rats during Euthanasia. *J. Am. Assoc. Lab. Anim. Sci.* **2018**, *57*, 725–728. [CrossRef]
56. Borovsky, V.; Herman, M.; Dunphy, G.; Caplea, A.; Ely, D. CO_2 asphyxia increases plasma norepinephrine in rats via sympathetic nerves. *Am. J. Physiol. Integr. Comp. Physiol.* **1998**, *274*, R19–R22. [CrossRef]
57. Powell, K.; Ethun, K.; Taylor, D.K. The effect of light level, CO_2 flow rate, and anesthesia on the stress response of mice during CO_2 euthanasia. *Lab. Anim.* **2016**, *45*, 386–395. [CrossRef]
58. Khokhlova, O.N.; Borozdina, N.A.; Sadovnikova, E.S.; Pakhomova, I.A.; Rudenko, P.A.; Korolkova, Y.V.; Kozlov, S.A.; Dyachenko, I.A. Comparative Study of the Aftereffect of CO_2 Inhalation or Tiletamine–Zolazepam–Xylazine Anesthesia on Laboratory Outbred Rats and Mice. *Biomedicines* **2022**, *10*, 512. [CrossRef]
59. Hackbarth, H.; Küppers, N.; Bohnet, W. Euthanasia of rats with carbon dioxide-animal welfare aspects. *Lab. Anim.* **2000**, *34*, 91–96. [CrossRef]
60. Hampton, A. Euthanizing agents. In *Veterinary Pharmacology & Therapeutics*; Riviere, J., Papich, M., Eds.; Wiley-Blackwell: Hoboken, NJ, USA, 2018; pp. 387–394.
61. Sio, L.C.O.; Varbanova, M.; Bautista, A. Isoflurane: Mechanisms and applications. In *Treatments, Mechanisms, and Adverse Reactions of Anesthetics and Analgesics*; Rajendram, R., Patel, V.B., Preedy, V.R., Martin, C.R., Eds.; Elsevier: London, UK, 2022; pp. 101–108.
62. Frost, K.; Shah, M.; Leung, V.S.Y.; Pang, D.S.J. Aversion to Desflurane and Isoflurane in Sprague-Dawley Rats (*Rattus norvegicus*). *Animals* **2020**, *10*, 950. [CrossRef] [PubMed]
63. Boivin, G.P.; Bottomley, M.A.; Schiml, P.A.; Goss, L.; Grobe, N. Physiologic, behavioral, and histologic responses to various euthanasia methods in C57BL76NTac male mice. *J. Am. Assoc. Lab. Anim. Sci.* **2017**, *56*, 69–78. [PubMed]
64. Valentine, H.; Williams, W.O.; Maurer, K.J. Sedation or inhalant anesthesia before euthanasia with CO_2 does not reduce behavioral or physiologic signs of pain and stress in mice. *J. Am. Assoc. Lab. Anim. Sci.* **2012**, *51*, 50–57. [PubMed]
65. Zardooz, H.; Rostamkhani, F.; Zaringhalam, J.; Faraji, S. Plasma corticosterone, insulin and glucose changes induced by brief exposure to isoflurane, diethyl ether and CO_2 in male rats. *Physiol. Res.* **2010**, *59*, 973–978. [CrossRef]
66. Angilletta, M.J.; Youngblood, J.P.; Neel, L.K.; VandenBrooks, J.M. The neuroscience of adaptive thermoregulation. *Neurosci. Lett.* **2019**, *692*, 127–136. [CrossRef]
67. Wright, C.L.; Boulant, J.A. Carbon dioxide and pH effects on temperature-sensitive and -insensitive hypothalamic neurons. *J. Appl. Physiol.* **2007**, *102*, 1357–1366. [CrossRef]
68. Creamer-Hente, M.A.; Lao, F.K.; Dragos, Z.P.; Waterman, L.L. Sex- and Strain-related Differences in the Stress Response of Mice to CO_2 Euthanasia. *J. Am. Assoc. Lab. Anim. Sci.* **2018**, *57*, 513–519. [CrossRef]
69. Moffitt, A.D.; Brignolo, L.L.; Ardeshir, A.; Creamer-Hente, M.A. The Role of Emotional Contagion in the Distress Exhibited by Grouped Mice Exposed to CO_2. *J. Am. Assoc. Lab. Anim. Sci.* **2019**, *58*, 430–437. [CrossRef]
70. Altholtz, L.Y.; Fowler, K.A.; Badura, L.L.; Kovacs, M.S. Comparison of the stress response in rats to repeated isoflurane or CO_2:O_2 anesthesia used for restraint during serial blood collection via the jugular vein. *J. Am. Assoc. Lab. Anim. Sci.* **2006**, *45*, 17–22.
71. Nazari, S.; Kourosh-Arami, M.; Komaki, A.; Hajizadeh, S. Relative contribution of central and peripheral factors in superficial blood flow regulation following cold exposure. *Physiol. Pharmacol.* **2020**, *24*, 89–100. [CrossRef]
72. Sutherland, M.A.; Worth, G.M.; Dowling, S.K.; Lowe, G.L.; Cave, V.M.; Stewart, M. Evaluation of infrared thermography as a non-invasive method of measuring the autonomic nervous response in sheep. *PLoS ONE* **2020**, *15*, e0233558. [CrossRef] [PubMed]
73. Casas-Alvarado, A.; Mota-Rojas, D.; Hernández-Avalos, I.; Martínez-Burnes, J.; Rosas, M.; Miranda-Cortés, A.E.; Domínguez-Oliva, A.; Mora-Medina, P. Assessment of thermal response, cardiorespiratory parameters and postoperative analgesia in dogs undergoing ovariohysterectomy with different combinations of epidural anesthesia and isoflurane. *J. Anim. Behav. Biometeorol.* **2023**, *11*, e2023009. [CrossRef]
74. Vogel, B.; Wagner, H.; Gmoser, J.; Wörner, A.; Löschberger, A.; Peters, L.; Frey, A.; Hofmann, U.; Frantz, S. Touch-free measurement of body temperature using close-up thermography of the ocular surface. *MethodsX* **2016**, *3*, 407–416. [CrossRef] [PubMed]
75. Vianna, D.M.L.; Carrive, P. Changes in cutaneous and body temperature during and after conditioned fear to context in the rat. *Eur. J. Neurosci.* **2005**, *21*, 2505–2512. [CrossRef]
76. Nijland, N. The Influence of Different Types of Behaviour on the Eye Temperature of Mice using Infrared Thermograohy. Master's Thesis, Utrecht University, Utrecht, The Netherlands, 2021.
77. Wirth, S.; Gebhardt-Henrich, S.; Riemer, S.; Hattendorf, J.; Zinsstag, J.; Hediger, K. The influence of human interaction on guinea pigs: Behavioral and thermographic changes during animal-assisted therapy. *Physiol. Behav.* **2020**, *225*, 113076. [CrossRef]
78. Wongsaengchan, C.; McCafferty, D.J.; Evans, N.P.; McKeegan, D.E.F.; Nager, R.G. Body surface temperature of rats reveals both magnitude and sex differences in the acute stress response. *Physiol. Behav.* **2023**, *264*, 114138. [CrossRef]
79. Insler, S.R.; Sessler, D.I. Perioperative Thermoregulation and Temperature Monitoring. *Anesthesiol. Clin. N. Am.* **2006**, *24*, 823–837. [CrossRef]
80. Jaén-Téllez, J.A.; Sánchez-Guerrero, M.J.; López-Campos, J.I.; Valera, M.; González-Redondo, P. Acute stress assessment using infrared thermography in fattening rabbits reacting to handling under winter and summer conditions. *Span. J. Agric. Res.* **2020**, *18*, e0502. [CrossRef]

81. Xu, Z.; Agbigbe, O.; Nigro, N.; Yakobi, G.; Shapiro, J.; Ginosar, Y. Use of high-resolution thermography as a validation measure to confirm epidural anesthesia in mice: A cross-over study. *Int. J. Obstet. Anesth.* **2021**, *46*, 102981. [CrossRef]
82. Miyazono, S.; Hasegawa, K.; Miyazaki, S.; Sakakima, H.; Konno, S.; Meguro, S.; Sasajima, H.; Noguchi, T.; Osada, K.; Kashiwayanagi, M. Etizolam attenuates the reduction in cutaneous temperature induced in mice by exposure to synthetic predator odor. *Eur. J. Pharmacol.* **2018**, *824*, 157–162. [CrossRef]
83. Weitkamp, J. Effect of Tickling and Gentling on Eye and Tail Temperature of Laboratory Rats during Manual Restraint, using Infrared Thermography. Master's Thesis, Utrecht University, Utrecht, The Netherlands, 2020.
84. Munting, L.P.; Derieppe, M.P.P.; Suidgeest, E.; Denis de Senneville, B.; Wells, J.A.; Weerd, L. Influence of different isoflurane anesthesia protocols on murine cerebral hemodynamics measured with pseudo-continuous arterial spin labeling. *NMR Biomed.* **2019**, *32*, e4105. [CrossRef] [PubMed]
85. Faraji, J.; Ambeskovic, M.; Sauter, N.; Toly, J.; Whitten, K.; Lopes, N.A.; Olson, D.M.; Metz, G.A.S. Sex-specific stress and biobehavioral responses to human experimenters in rats. *Front. Neurosci.* **2022**, *16*, 965500. [CrossRef] [PubMed]
86. Hickman, D.L. Minimal Exposure Times for Irreversible Euthanasia with Carbon Dioxide in Mice and Rats. *J. Am. Assoc. Lab. Anim. Sci.* **2022**, *61*, 283–286. [CrossRef]
87. Van Rijn, C.M.; Krijnen, H.; Menting-Hermeling, S.; Coenen, A.M.L. Decapitation in Rats: Latency to Unconsciousness and the 'Wave of Death'. *PLoS ONE* **2011**, *6*, e16514. [CrossRef] [PubMed]
88. Hudetz, J.A.; Pagel, P.S. Neuroprotection by Ketamine: A Review of the Experimental and Clinical Evidence. *J. Cardiothorac. Vasc. Anesth.* **2010**, *24*, 131–142. [CrossRef]
89. Miranda-Cortés, A.E.; Ruiz-García, A.G.; Olivera-Ayub, A.E.; Garza-Malacara, G.; Ruiz-Cervantes, J.G.; Toscano-Zapien, J.A.; Hernández-Avalos, I. Cardiorespiratory effects of epidurally administered ketamine or lidocaine in dogs undergoing ovariohysterectomy surgery: A comparative study. *Iran. J. Vet. Res.* **2020**, *21*, 92–96. [PubMed]
90. Gómez-Hernández, A.; Beneit, N.; Díaz-Castroverde, S.; Escribano, Ó. Differential Role of Adipose Tissues in Obesity and Related Metabolic and Vascular Complications. *Int. J. Endocrinol.* **2016**, *2016*, 1216783. [CrossRef] [PubMed]
91. Mota-Rojas, D.; Titto, C.G.; de Mira Geraldo, A.; Martínez-Burnes, J.; Gómez, J.; Hernández-Ávalos, I.; Casas, A.; Domínguez, A.; José, N.; Bertoni, A.; et al. Efficacy and Function of Feathers, Hair, and Glabrous Skin in the Thermoregulation Strategies of Domestic Animals. *Animals* **2021**, *11*, 3472. [CrossRef]

9.2. CAPÍTULO V

Resultados de la fase II. Escala de muecas

Artículo experimental intitulado:
Aplicación de la Escala de Muecas de Rata para evaluar seis métodos de eutanasia en ratas Wistar

En proceso de revisión, enviado a la revista Animals, misma que se encuentra indexada al JCR con un factor de impacto de 3.

Domínguez-Oliva, A.; Olmos-Hernández, A.; Hernández-Avalos, I.; Lecona-Butrón, H.; Mota-Rojas, D. Rat Grimace Scale as a method to evaluate nociception and facial expression during euthanasia of Wistar rats. *Animals 2023*, *13*, en proceso de revisión.

animals

MDPI

Article

Rat Grimace Scale as a method to evaluate nociception and facial expression during euthanasia of Wistar rats

Adriana Domínguez-Oliva [1,2], Adriana Olmos-Hernández [2], Ismael Hernández-Avalos [3], Hugo Lecona-Butrón[2], and Daniel Mota-Rojas [2,*]

[1] Master in Science Program "Maestría en Ciencias Agropecuarias", Universidad Autónoma Metropolitana, Xochimilco Campus, Mexico City, Mexico; mvz.freena@gmail.com (A.D.-O.)
[2] Neurophysiology of Pain, Behavior and Assessment of Welfare in Domestic Animals, DPAA. Universidad Autónoma Metropolitana (UAM), Mexico City, Mexico; dmota100@yahoo.com.mx (D.M.-R.)
[3] Division of Biotechnology—Bioterio and Experimental Surgery, Instituto Nacional de Rehabilitación-Luis Guillermo Ibarra Ibarra (INR-LGII), Tlalpan, Mexico City 14389, Mexico; solmos@inr.gob.mx (A.O.-H.)
[4] Clinical Pharmacology and Veterinary Anesthesia. Biological Sciences Department. Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Cuautitlán, Cuautitlán Izcalli 54714, Mexico; mvziha@hotmail.com (I.H.-Á.)

* Corresponding author: author: dmota100@yahoo.com.mx (D.M.-R).

Simple Summary: This study aimed to evaluate pain associated with six methods of euthanasia (injectable, inhalational, and physical) in Wistar rats, applying the Rat Grimace Scale (RGS) to compare the scores and determine the method with the highest score that might indicate pain for laboratory rodents. According to the results, during the application of the euthanasia method, the intraperitoneal administration of ketamine + xylazine and decapitation caused the highest RGS scores (0.6 ± 0.26 and 0.6 ± 0.16, respectively) ($P < 0.0001$), while after the application of the euthanasia methods, CO_2 and isoflurane recorded the highest scores ($P < 0.0001$) (0.9 ± 0.18 and 1.2 ± 0.20, respectively). The results might indicate that injection and guillotine use could cause short-term pain in rodents, while high isoflurane scores could be associated with nociception/pain or to the myorelaxant properties of the drug. Further research is needed to establish a comprehensive study of pain during euthanasia, where RGS could be used minding the limitations that anesthetics might have on facial expression.

Abstract: Refinement of experimental procedures in animal research has the objective of preventing and minimizing pain/distress in animals, including the euthanasia period. This study aimed to evaluate pain associated with six methods of euthanasia in Wistar rats (injectable, inhalational, and physical), applying the Rat Grimace Scale (RGS) to compare the scores and determine the method with the highest score that might indicate pain for laboratory rodents. Sixty adult male and female Wistar rats were used and assigned to six treatments: G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine + CO_2. Videorecording to assess RGS scores was performed in four events: Basal: 24 h before the procedure; Ti_1: three minutes before the procedure; Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until LORR. The main findings of the study showed that, during Ti_2, G_3 and G_5 had the highest scores (0.6 ± 0.26 and 0.6 ± 0.16, respectively) ($P < 0.0001$), while at Ti_3, G_2 (0.9 ± 0.18) and G_4 (1.2 ± 0.20) recorded the highest scores ($P < 0.0001$). According to the present results, decapitation, and ketamine + xylazine elicited short-term acute pain possibly due to the tissular damage caused by both methods (injection and guillotine). In contrast, isoflurane's RGS scores recorded during Ti_3 might be associated with nociception/pain due to the pungency of the drug, or to the pharmacological muscle relaxant effect of isoflurane. Further research is needed to establish a comprehensive study of pain during euthanasia, where RGS could be used minding the limitations that anesthetics might have on facial expression.

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

Keywords: facial expression; decapitation; CO_2 exposure; isoflurane; ketamine + xylazine

1. Introduction

Refinement is a principle proposed by the National Center for the Replacement, Refinement, and Reduction (NC3Rs) to prevent or minimize pain, suffering, or distress in research animals [1,2]. Currently, one of the most controversial topics regarding the use of laboratory rodents is the potential pain that animals might experience due to the application of injectable/inhalational drugs and physical methods that can also be used for euthanasia [3,4]. Therefore, refinement does not only apply during animals' life but also needs to be extended to the euthanasia period to prevent pain.

Nociception, known as the "neural processes of encoding and processing noxious stimulus" is the first step necessary to transmit nervous signaling from peripheral nociceptors to spinal and cerebral centers [5]. Pain is referred to as an "unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage", in which definition, the verbal inability to communicate pain does not negate that non-human animals might experience it [6]. Therefore, pain is the conscious perception of nociceptive signaling [5]. When an animal perceives pain, a cascade of physiological, endocrine, behavioral, and immune responses are triggered, irrupting its homeostasis [7]. Preventing pain in laboratory rodents is not only a scientific, legal, moral, and ethical duty but can also help to minimize variability and low reproducibility of results due to the mentioned alterations [8].

During the application of euthanasia, pain might be present due to the method or the properties of the drug. For example, injectable agents such as sodium pentobarbital and ketamine have basic or acid pH, respectively, that can cause pain due to tissular irritation after intraperitoneal (IP) administration [9]. Contrarily, other studies have not found pain-related behaviors (e.g., abdominal writhing) after IP injection of pentobarbital [10]. Physical methods such as decapitation are considered aesthetically unpleasant and the possibility of feeling conscious pain is under debate [11]. Several authors have reported that the cerebral activity is maintained for 2.7–40 seconds after decapitation [12] and that the presence of low voltage, fast electroencephalographic (EEG) activity is compatible with conscious awareness of pain [13]. Nonetheless, due to the short period of consciousness and the possibility that EEG indices represent cerebral cortex activity and not nociception, decapitation is still considered a humane euthanasia method [14,15]. Regarding inhalant methods, CO_2 is a common euthanasia method but some studies refer to the potential pain and activation of acid-sensitive channels (ASIC) due to carbonic acid formation when CO_2 is combined with water in nasal or ocular mucosa [16]. Moreover, although isoflurane is considered an alternative to CO_2, its moderate pungency might be aversive and can cause discomfort to rodents [9,17].

Due to the potential nociceptive activation and/or pain that laboratory rodents might perceive during an experimental protocol, implementing tools for pain recognition is one of the essential steps to refine procedures. One of the most used methods is evaluating pain-related behaviors such as writhing, back arching, twitching, stagger-fall, or lack of grooming and burrowing, among others [18]. Currently, studying pain facial expressions in domestic and wildlife species is a trend derived from Darwin's studies regarding emotion and its association with facial expressions [19]. This has led to the development of "grimace scales", scoring systems that categorize movements of facial muscles –called Facial Action Units (FAU)– related pain [20–23]. In rats, Sotocinal et al. [22] developed the Rat Grimace Scale (RGS), using as a basis the Mouse Grimace Scale (MGS), a validated tool that uses four FAU to determine the pain level: 1. Ear change; 2. Orbital tightening; 3. Nose/cheek flattening; and 4. Whisker change.

On a scale of zero to two, 0 = not present; 1 = moderately present; and 2 = obviously present, the RGS has been used to non-invasively evaluate pain surgical, visceral, orthopedic, and inflammatory pain [24]. It has been applied as a method to refine analgesic protocols or the efficacy of the analgesic drug [25,26], as well as in animal models of acute/chronic/neuropathic pain, and to assess the effect of routine practices such as drug administration or euthanasia procedures [25,27,28].

Animal research is committed to maintaining high standards of animal care and welfare. Recognizing and preventing pain is part of the proposed strategies to minimize the potential behavioral, physiological, and immune alteration that rodents might experience during an experimental protocol and euthanasia, also decreasing the confounding effect that pain might have on the research. The authors hypothesize that, regardless of the euthanasia method, the RGS will be able to distinguish changes in rats FAU due to the application of the method. Therefore, this study aimed to evaluate pain associated with six methods of euthanasia in Wistar rats (injectable, inhalational, and physical), applying the RGS to compare the scores. Additionally, we studied the correlation between each euthanasia method with a specific FAU, to determine the method with the highest score that might indicate pain for laboratory rodents.

2. Material and Methods

2.1. Study animals and housing standards

The present study was performed in the Animal Facility and Experimental Surgery service from the Biotechnological Research Sub-department of the Instituto Nacional de Rehabilitación- Luis Guillermo Ibarra Ibarra, Mexico City, Mexico.

Sixty adult Wistar rats (*R. norvegicus*), 30 male and 30 female were obtained from the Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV-IPN) (average weight of 311 ± 62 g at 8-10 weeks old). The sample size was calculated using G*Power 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The sample size considered an α error probability of 0.05, confidence level of 95%, 1-β error probability of 0.90, and correction among repeated measures of 0.5 for the six treatments at five events.

In accordance with the 3Rs principles [29], reduction in the use of laboratory animals was encouraged by reusing rats coming from control groups of concluded behavioral tests (e.g., balance beam or maze tests). A physical exam considering body weight, posture, level of consciousness, secretions, the color of the mucosa, sneezing, and species-specific behavioral repertoire was performed to select healthy animals. Rats that underwent invasive procedures, had residual drugs, or signs of disease, stress, and pain, or were pregnant were excluded.

Rats were placed in groups of five animals per cage. Standard polycarbonate cages for rats were used (47 x 36 x 21 cm), with wood shavings as bedding (Aspen, Nepco, USA), and without enrichment. A controlled temperature inside the housing room was set at an average of 23.2 ± 0.5°C and 48% of relative humidity, maintaining a 12-hour-day-night cycle (lights on between 0500 h and 1700 h). The rats had *ad libitum* access to food (LabDiet 5010, LabDiet, USA) and purified water (in 500 ml drinking water bottles). Visual health inspection was performed twice daily, and cleaning of the cages was performed once a week.

2.2. Treatments

This was an experimental prospective-comparative study. All measurements were taken by a single trained and unblinded evaluator. Before starting the experimental phase, rats underwent a habituation period of 15 days to the evaluator's presence and animal handling. The animals were randomly divided into six treatments by number generation (Microsoft Excel; Microsoft 365). 10 rats were assigned to each treatment (five males and five females), according to the euthanasia method:

G_1- Pentobarbital (Pentobarbital, Aranda ®, Mexico) overdose at 400 mg/kg IP with a 3 ml sterile syringe (Ambiderm ®, México) [30]. The dose was calculated through a pilot study (no published data), using the minimal and maximal doses that appear in Reimer et al. [31]'s study. G_2- CO_2 overdose inside an acrylic euthanasia chamber (Acrifactory, Mexico) (32.5 x 42 x 21 cm). The flow rate was set at 30% of the chamber volume/min [32]. G_3- Decapitation using a rodent guillotine (51330, Senna, Mexico) [30]. G_4- Inhalation of isoflurane (Fluriso, VET ONE ®, India) using the open-drop exposure method (two cotton swabs soaked with 2 mL of isoflurane each). The dose was calculated using Risling et al. [33] and de Brito [34] study as a basis. The cotton swabs were placed where animals could not have direct contact with the inhalant anesthetic drug. G_5- Ketamine (Ketamin-Pet, Aranda ®, Mexico) + Xylazine (Procin, Pisa Agropecuaria ®, Mexico) overdose, at doses of 450 mg/kg IP and 45 mg/kg IP, respectively [32]. G_6- Combination of ketamine (100 mg/kg IP) + CO_2 (after 5-10 minutes of ketamine administration) [35].

2.3. Evaluation events

Four events were recorded. Basal: video recording for five minutes, 24 h before the euthanasia method, inside the housing room; Ti_1: three minutes of recording before the application of the euthanasia in the test room. On the trial day, the rats were moved from the housing room to the test room (average temperature of 22.9 ± 0.5°C and 52% humidity), allowing rest and room acclimatization for 30 minutes before starting the trial; Ti_2: during the application of the method (e.g., while the animal received the IP dose of pentobarbital, ketamine + xylazine, or while it was inside the induction chamber or placed in the guillotine); Ti_3: immediately after the application of the euthanasia method until loss of the righting reflex (LORR) as a sign of unconsciousness. The absence of palpebral, interdigital, and righting reflexes was also used to confirm the euthanasia method. Cessation of breathing and heart rate was also used to confirm the death of the rats.

2.4. Rat Grimace Scale recording and scoring

To obtain the RGS score, continuous recording with two high-resolution (1920 x 1080) digital cameras (Ixy 650, Cannon ®, Japan) was performed during each evaluation event. The cameras were placed on both sides of the animal (front and side) to capture headshots of the rats before, during, and after each euthanasia treatment. Cameras were mounted on tripods at approximately 20 cm of distance from the rats.

The videos were saved as MP4 format files to be analyzed by a blinded coder in a video editing software (Adobe Premiere Pro, Adobe, USA). By watching the videos at a speed of 0.5x, 10 still images of the rats' faces were taken at each evaluation event (Basal, Ti_1, Ti_2, and Ti_3) [36], obtaining 40 images per rat when a clear front or lateral view of the head was observed. Scoring of the images was performed according to Sotocinal [22]'s study using four FAU: orbital tightening, nose/cheek flattening, ear change, and whisker change (**Figure 1**). Using a score scale from 0 to 2, where 0 = not present, 1 = moderately present; and 2 = obviously present, the maximum score obtainable was 8. To obtain the final score, the 10 images per evaluation event in each FAU were summed to obtain a mean value. For each rat, the mean values of the four FAU were summed, and a mean total score was used in the analysis.

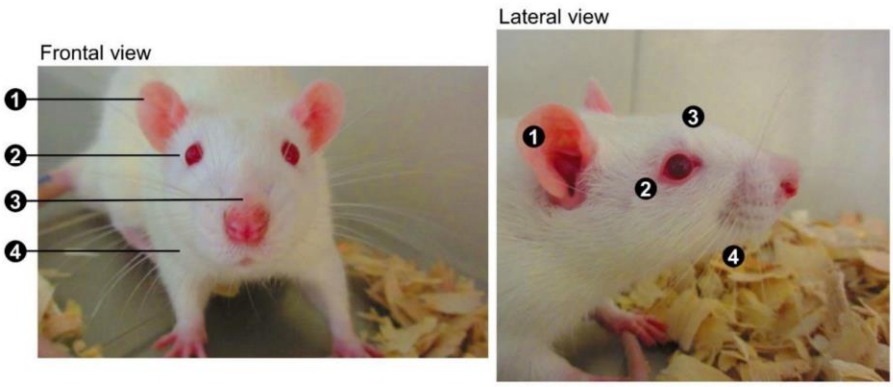


Figure 1. Example of the FAU evaluated in the sixty rats and the assigned score. 1. Ear change; 2. Orbital tightening; 3. Nose/cheek flattening; 4. Whisker change.

2.5. Procedures

During Basal, recording for RGS was performed for five minutes inside the housing room. The cameras were placed in tripods on two sides of the polycarbonate cages. 24 h after, the rats from the corresponding treatment were moved from the housing room to a testing room to perform euthanasia away from the rest of the housed animals. A period of 30 minutes was established so the rats could acclimatize to the testing room and avoid transport stress. During this time, the cameras were mounted on tripods in the testing room, in different locations according to the treatment (e.g., if it was an inhalant method, the cameras were placed on both sides of the euthanasia chamber). After the preparation of the room and acclimatization of the animals, before the application of the euthanasia method, three min recording was performed. After this and during Ti_2 and Ti_3, a continuous recording was taken until LORR, therefore, the length of the video in these events differed according to the treatment.

2.6. Statistical analyzes

GraphPad Prism 10.0.2 (California, USA) statistical package was used to analyze the data. The Kolmogorov-Smirnov test was performed to establish data normality in the data set collected from the FAU. Descriptive statistics were obtained, expressing the results as mean ± standard deviation (SD). A linear mixed model for repeated measures was used to evaluate the effect of the six treatments (G_1, G_2, G_3, G_4, G_5, and G_6), in the four events (basal, Ti_1, Ti_2, and Ti_3,) for the total RGS score. Multiple comparison of means was performed with the post-hoc Tukey test. Correlations between the four FAU and the treatments was calculated using Spearman correlation coefficients. All values with $p < 0.05$ were considered statistically significant.

2.7. Ethical statement

The present study was approved by the Committee for the Care and Use of Laboratory Animals (INRLGII/CICUAL/014/2021) of National Institute of Rehabilitation Luis Guillermo Ibarra-Ibarra.

The handling and care of the laboratory animals was in accordance with the Mexican Norm NOM-062-ZOO-1999 for Laboratory Animals [37]. All dead animals were disposed by incineration, following the NOM-062-ZOO-1999[38].

3. Results

A total of 758.58 min of recording and 2400 images taken from the videos were evaluated. Table 1 shows the total RGS score (expressed as mean ± SD) of the six euthanasia methods during the four events. When making comparisons between treatments, significant differences were found at Ti_2 ($P < 0.0001$) in rats undergoing euthanasia by decapitation (G_3) and a combination of ketamine + xylazine (G_6), obtaining scores of 0.6 ± 0.26 and 0.6 ± 0.16, respectively. In contrast, at Ti_3, significant differences ($P < 0.0001$) were recorded for CO_2 (G_2) and isoflurane (G_4), obtaining the highest scores of 0.9 ± 0.18 and 1.2 ± 0.20, respectively. The analysis between events shows an expected significant increase in RGS score in all treatments from Basal/Ti_1 to Ti_2 and Ti_3 ($P < 0.05$). However, the biggest increase in scores was found in G_3 during Ti_2 ($P = 0.0052$) and in G_4 during Ti_3 ($P < 0.0001$) (Figure 2).

Table 1. RGS scores (mean ± standard deviation) of the six euthanasia methods during the four events.

Treatments	Basal	Ti_1	Ti_2	Ti_3	*P*-value
G_1	$0.0 \pm 0.0^{a,1}$	$0.0 \pm 0.0^{a,1}$	$0.1 \pm 0.07^{a,b,1}$	$0.2 \pm 0.14^{b,1}$	***P* = 0.0294**
G_2	$0.0 \pm 0.0^{a,1}$	$0.0 \pm 0.0^{a,1}$	$0.2 \pm 0.11^{b,1,2}$	$0.9 \pm 0.18^{c,2}$	***P* < 0.0001**
G_3	$0.0 \pm 0.0^{a,1}$	$0.0 \pm 0.0^{a,1}$	$0.6 \pm 0.26^{b,3}$	$0.3 \pm 0.24^{a,b,1}$	***P* = 0.0052**
G_4	$0.0 \pm 0.0^{a,1}$	$0.0 \pm 0.0^{a,1}$	$0.3 \pm 0.16^{b,1,2,3}$	$1.2 \pm 0.20^{c,2}$	***P* < 0.0001**
G_5	$0.0 \pm 0.0^{a,1}$	$0.0 \pm 0.0^{a,1}$	$0.6 \pm 0.16^{b,3}$	$0.3 \pm 0.12^{c,1}$	***P* < 0.0001**
G_6	$0.0 \pm 0.0^{a,1}$	$0.0 \pm 0.0^{a,1}$	$0.4 \pm 0.15^{b,2,3}$	$0.5 \pm 0.10^{b,1}$	***P* < 0.0001**
P-value	$P > 0.05$	$P > 0.05$	***P* < 0.0001**	***P* < 0.0001**	

^{a,b,c} Different letters represent statistically significant differences between events.
^{1,2,3} Different numbers represent statistically significant differences between treatments.
G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine+CO_2. Basal: 24 h before the procedure; Ti_1: three minutes before the procedure; Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until LORR.

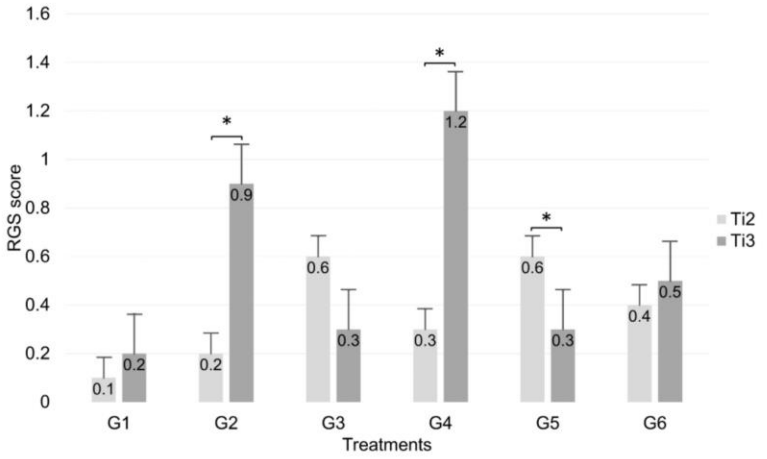


Figure 2. Mean ± SD score of the RGS in the six treatments during Ti_2 (light gray) and Ti_3 (dark gray). G_1: pentobarbital; G_2: CO_2; G_3: decapitation; G_4: isoflurane; G_5: ketamine + xylazine; G_6: ketamine+CO_2. Ti_2: during the application of the euthanasia method; Ti_3: immediately after the application until LORR. *: statistically significant differences between events.

Correlations between treatments according to the FAU were obtained. **Table 2** shows the correlation coefficients for "Ear change", where a statistically significant ($p < 0.0001$) moderate correlation ($r = 0.685$) was found between rats receiving CO_2 (G_2) and isoflurane (G_4).

Table 2. Correlation matrix for the FAU "Ear change".

	G_1	G_2	G_3	G_4	G_5	G_6
G_1	1					
G_2	0.354 $P < 0.001$	1				
G_3	-0.070 $P = 0.326$	-0.086 $P = 0.226$	1			
G_4	0.391 $P < 0.001$	0.685 $P < 0.001$	0.001 $P = 0.990$	1		
G_5	0.155 $P = 0.028$	-0.053 $P = 0.456$	0.151 $P = 0.033$	-0.079 $P = 0.269$	1	
G_6	0.345 $P < 0.001$	0.259 $P < 0.001$	0.172 $P = 0.015$	0.295 $P < 0.001$	0.450 $P < 0.001$	1

Similarly, to "Ear change", "Orbital tightening" had significant moderate correlations between G_2 and G_4 ($r = 0.540$) ($P < 0.0001$), while G_4 rats had a negative moderate correlation with rats receiving ketamine + xylazine (G_5) ($r = -0.465$) ($P < 0.0001$). A statistically significant correlation was also found between G_3 and G_6 treatments ($r = 0.466$) ($P < 0.0001$) (**Table 3**).

Table 3. Correlation matrix for the FAU "Orbital tightening"

	G_1	G_2	G_3	G_4	G_5	G_6
G_1	1					
G_2	0.236 $P < 0.001$	1				
G_3	-0.038 $P = 0.593$	-0.275 $P < 0.001$	1			
G_4	0.239 $P < 0.001$	0.540 $P < 0.001$	0.140 $P = 0.049$	1		
G_5	0.141 $P = 0.047$	-0.343 $P < 0.001$	0.395 $P < 0.001$	-0.465 $P < 0.001$	1	
G_6	0.070 $P = 0.322$	-0.254 $P < 0.001$	0.466 $P < 0.001$	-0.389 $P < 0.001$	0.622 $P < 0.001$	1

Regarding "Nose/cheek flattening", statistically significant ($P < 0.0001$) moderate correlations ($r = 0.670$) were found between G_2 and G_4 (**Table 4**), a similar case as the one observed in **Table 5** for "Whisker change", where G_2 and G_4 had a moderate correlation ($r = 610$) ($P < 0.0001$). In the same FAU, G_6 was moderately correlated with both G_2 ($r = 577$) ($P < 0.0001$) and G_4 ($r = 0.493$) ($P < 0.0001$).

Table 4. Correlation matrix for the FAU "Nose/cheek flattening".

	G_1	G_2	G_3	G_4	G_5	G_6
G_1	1					
G_2	0.164 $P = 0.020$	1				
G_3	-0.034 $P = 0.633$	-0.118 $P = 0.097$	1			
G_4	0.266 $P < 0.001$	0.670 $P < 0.001$	-0.154 $P = 0.029$	1		
G_5	0.041 $P = 0.568$	0.177 $P = 0.012$	0.087 $P = 0.221$	0.107 $P = 0.13$	1	
G_6	0.339 $P < 0.001$	0.387 $P < 0.001$	-0.250 $P < 0.001$	0.441 $P < 0.001$	0.242 $P = 0.001$	1

Table 5. Correlation matrix for the FAT "Whisker change".

	G_1	G_2	G_3	G_4	G_5	G_6
G_1	1					
G_2	0.369 $P < 0.001$	1				
G_3	-0.221 $P = 0.001$	-0.341 $P < 0.001$	1			
G_4	0.206 $P = 0.003$	0.610 $P < 0.001$	-0.014 $P = 0.840$	1		
G_5	0.163 $P = 0.021$	0.180 $P = 0.011$	0.102 $P = 0.149$	0.252 $P < 0.001$	1	
G_6	0.415 $P < 0.001$	0.577 $P < 0.001$	-0.212 $P = 0.003$	0.493 $P < 0.001$	0.398 $P < 0.001$	1

4. Discussion

In laboratory rodents, it is known that regardless of the euthanasia method, handling, and the procedure per se (e.g., IP injection) might imply distress and pain for the animals. This was observed in the present study in all euthanasia methods as an expected increase in the RGS score from Basal/Ti_1 to Ti_2 and Ti_3. At Ti_2, the highest scores were reported in G_3, and G_5, followed by G_6, while G_4 and G_2 registered the highest values at Ti_3. It is known that an average score of 0.67 in the RGS is considered as the intervention threshold to administer analgesic drugs during a painful event [39,40]. Therefore, the facial changes observed during and after the application of the euthanasia methods could be related to the nociceptive pathway and its motor responses.

In rats, the movements of the four FAU used in the RGS are controlled by the facial nerve that innervates facial and superficial mimetic muscles. The muscles associated with facial expression are the *levator auris longus cranial, levator auris longus caudal, frontalis, orbicularis oculi, levator nasolabialis* including the *levator labii superioris,* and the *dilator naris* which is related to whisker follicle movement [21,41]. Relaxation or contraction of said muscles, when animals perceive pain, is triggered by the projection of third-order neurons (from the thalamus) to the somatosensory cortex, amygdala, hypothalamus, and motor cortex [42]. From the ventrolateral side of the frontal cortex, motor neurons directly innervate superior and inferior mimetic muscles [43]. Due to this connection, as seen in humans, pain can elicit changes in the facial expression of species such as laboratory rodents

[44]. By using the RGS, several routine practices, handling techniques, surgical procedures, and analgesic protocols can be evaluated and improved accordingly procedures [25,27,28].

Considering that all treatments increased their RGS score from Basal/Ti_1 to Ti_2 and Ti_3, some studies have reported the RGS score related to the effect of the euthanasia method. For example, Reimer et al. [31] compared pentobarbital, saline, and vehicle control groups in Sprague Dawley and Wistar rats to determine the level of pain in both strains. It was found that RGS scores were above 0.67 only in the saline and vehicle groups, also having a higher frequency of writhing and back arching. Similarly, Khoo et al. [27] researched the influence that sodium pentobarbital euthanasia had on RGS and behavioral responses such as abdominal writhing and ultrasonic vocalizations. The authors found that a combination with local anesthetics (lidocaine) reduced the frequency of writhing but did not influence the RGS, where low values were registered between 0.44 ± 0.14 and 0.37 ± 0.11. The obtained scores by Khoo et al. [27] were higher than the ones reported in the present study (0.1 ± 0.07 at Ti_2 and 0.2 ± 0.14 at Ti_3. However, both findings suggest that IP administration of pentobarbital might cause mild pain –together with potential stress and discomfort–, as reported by Svendsen et al. [45] in rats, in whom an increase in c-fos-expressing neurons in the spinal cord is related to nociception.

As mentioned by Laferriere and Pang [46], although pentobarbital is a killing method recommended over inhalational agents (e.g., CO_2 and isoflurane), barbiturates are weak acids that are changed into soluble basic solutions for their IP administration, transforming pentobarbital into a highly alkaline solution with a pH between 11–12. Hence, IP injection might cause pain and irritation of the peritoneal cavity and viscera because the tissular nonirritating pH is around 4.5–8.0 [9,47]. Pain-related behaviors reported after pentobarbital administration are writhing, vocalization, increase in locomotion, or flinching [46], and the incidence of said behaviors can range between 36%–46% with low (200 mg/kg) and high doses (800 mg/kg), respectively. Nonetheless, no changes or no statistical differences have also been reported in mice receiving IP pentobarbital, implying that barbiturates might cause peritoneal inflammation but fast action of the euthanasia method does not elicit signs of pain [10]. Likewise, Boivin et al. [48] determined that IP administration of pentobarbital-phenytoin caused less stress to mice, according to the ACTH concentrations, than inhalant agents (CO_2, and isoflurane anesthesia followed by CO_2 inhalation), as could also be assumed in the present research, where G_1 rats had the lowest RGS scores during and after the administration of pentobarbital.

A similar case is observed in G_5, animals who received a commonly administered combination of ketamine plus xylazine as euthanasia. At Ti_2, the G_5 rats' RGS score was 0.6 ± 0.16, one of the two highest values during this event (together with G_3). These findings might be due to IP injection pain, and to ketamine chemical properties. It is known that ketamine has a low pH (3.5 a 4.1) to facilitate solubilization [49]. However, after parenteral administration, ketamine has been associated with tissular irritation, pain, and muscle damage at the injection site [50]. In this sense, repeated IP anesthesia with ketamine + xylazine in mice increased the MGS score (approximately between 0.60–1.20) and caused short-term anxiety-like behaviors [51], while anesthesia using the same combination caused a more severe muscle and tissue necrosis in Wistar Han rats than those receiving ketamine + dexmedetomidine. Although histopathologic changes were not assessed in the present study, the high scores observed in Ti_2 in both groups receiving IP ketamine (G_5 and G_6) could be associated with ketamine's properties. Additionally, Schoell et al. [52] compared the same euthanasic combination using two routes of administration: retroorbital and intravenous, finding that retroorbital administration was the most efficient euthanasia method, when compared with CO_2 and pentobarbital, reducing the time to death to 5 s, a technique that also permitted post-mortem sample collection without affecting lung tissue.

During Ti_2, the other treatment that recorded the highest RGS scores was decapitation (0.6 ± 0.26). Decapitation is a highly controversial method, not only because it might

be aesthetically unpleasant, but because of the potential pain that animals might perceive [11,53]. Decapitation of small rodents is an acceptable method because several authors have reported a short period of consciousness after head detachment from the spinal cord (between 3–15 s) [14]. Through electroencephalography (EEG), Gagea-Iurascu and Craig [12] and Mikesha and Klemm [54] mention that cerebral activity is present between 2.7 to 40 s after decapitation, while Cartner et al. [55] recorded a decrease in cortical function and visual evoked potentials of mice within 15–20 s and 10–15 s, respectively. These results show that decapitation leads to a rapid dysfunction of cerebral activity. Moreover, the immediate loss of blood flow to the brain hastens hypoxia, anoxia, and subsequent death [56]. Nonetheless, other studies have associated decapitation with nociception due to the presence of low voltage and fast EEG activity –a sign of conscious perception of pain– [13]. Likewise, an increase in median frequency (F50) and spectral edge frequency (F95) within the first 15 s after decapitation have been reported [15], an EEG change that indicates nociception in rats [57].

The possible short-term presence of nociception, conscious pain awareness, and the high RGS score obtained in the present research would need further study combining behavioral, endocrine, physiological, and EEG assessment. However, a possible explanation for why nociception/pain might be present could be due to the activation of peripheral nociceptors located in the skin and muscles of the cervical region of rodents. Although the loss of consciousness is fast after decapitation, before head detachment, tissular damage to said structures would trigger the nociceptive pathway (e.g., transduction, transmission, modulation, projection, and perception) culminating in pain perception for a couple of seconds before cessation of nervous signaling [7,21,58]. It is known that after decapitation there is an anatomical disconnection between the mesencephalon and the cardiorespiratory centers –leading to death– and that the electric signaling present post-decapitation cannot solely be attributed to nociception since it has been found in healthy anesthetized animals or during REM sleep [14,32]. Thus, while animals might not be able to perceive pain within 3 s after decapitation, making it a humane killing method, there could be a debate about the degree of the short-term pain or nociceptive activation that laboratory rodents might perceive due to tissular damage before reaching death, requiring future comprehensive studies about this topic.

Regarding the inhalational methods (G_2 and G_4), an interesting result was observed during Ti_3. At Ti_2, both methods obtained a score of 0.2 and 0.3, respectively, being lower than G_3, G_5, and G_6. In contrast, at Ti_3, the highest RGS scores were found in G_2 (0.9 ± 0.18) and G_4 (1.2 ± 0.20). In this sense, regarding CO_2, there are no studies where RGS was used during the euthanasia or anesthesia of rats; however, some studies in rodents have found that animals perform active and passive defense behaviors when exposed to CO_2 and that physiological responses such as bradycardia and corticosterone increases are present [59]. Similarly, Marquardt et al. [2] concluded that glucose, adrenaline, and noradrenaline increased in mice exposed to high concentrations of CO_2 (60% and 100%), in contrast to isoflurane and sevoflurane inhalation. When assessing cortical EEG changes due to CO_2 euthanasia, Thurauf et al. [60] reported that different concentrations of CO_2 evoked a negative mucosal potential due to painful stimulation. Apart from pain responses, CO_2 is aversive to rodents, causing behavioral alterations such as escape behaviors, vocalizations, freezing, and the activation of fear/anxiety brain regions (e.g., amygdala, dorsomedial region of the hypothalamus, hypothalamus, and bed nucleus of the stria terminalis) is prevalent with CO_2 [59].

The suggestion that CO_2 exposure elicits pain in rodents has been translated from studies in humans, in whom concentrations between 50%–100% were regarded as highly unpleasant and painful [53,61]. The possible association between pain and CO_2 has been related to carbonic anhydrase and the formation of carbonic acid within mucosal surfaces when CO_2 interacts with water [16,62]. Leach et al. [63] mention that rodents exposed to CO_2 increase stress-induced grooming as a potential indicator of mucosa irritation, particularly with high concentrations and pre-filled chambers, whereas Golledge et al. [64]

mention that CO_2 at 100% induces loss of cerebral cortical activity within 39 s; nonetheless, this could be associated with nociception or pain. Furthermore, CO_2 inhalation decreases the pH (< 7.2), resulting in tissue acidosis and the activation of nociceptors such as ASIC [16], or polymodal-nociceptive neurons –also known as wide dynamic range nociceptive neurons– with CO_2 concentrations up to 40% [65,66].

On the other hand, isoflurane euthanasia significantly increased the RGS score to 1.2 ± 0.20 during Ti_3, recording the highest score in all treatments during all events. Isoflurane is known to be a moderate pungent agent, more than halothane and enflurane, that causes high aversion to rodents [9,17]. There are no studies assessing isoflurane and RGS during euthanasia; nonetheless, the present findings regarding isoflurane agree with Miller et al. [67,68] studies in rats and mice, where the authors reported the influence of the anesthetic on the RGS. The authors found that isoflurane increased RGS scores after 12 min of anesthesia [67], while in DBA/2 mice, the MGS scores increased animals receiving the halogenated drug [68], results that might be associated with the residual effect of isoflurane and its pharmacological properties. Likewise, single and repeated exposure to isoflurane anesthesia in female mice resulted in higher RGS scores in the first 30 min after anesthesia (approximately 0.8 and 1.2), and reduced burrowing behavior and food intake [69]. Furthermore, Wong et al. [70] determined that CO_2 is more aversive than isoflurane, and that isoflurane sedation before CO_2 euthanasia is a refinement. Even recent studies have proposed the isoflurane "drop method" to anesthetize rodents before CO_2 euthanasia in mice [71].

Considering the available literature regarding isoflurane anesthesia and the application of the RGS, additional studies are necessary to determine if, during euthanasia, the high RGS score found in G_4 rats could be completely associated with pain or might be a pharmacological effect of isoflurane. In this sense, isoflurane inhibits acetylcholine receptors, causing dose-dependent muscle relaxation of both skeletal and smooth muscles [72]. This property could influence the innervation of mimetic muscles used to evaluate facial expression in rodents, as presented in this research. For example, ocular muscles such as *the orbicularis oculi* and the *levator palpebrae superioris* muscle are structures that control the opening/closing of the eyelids through the innervation of the facial and oculomotor nerve [73,74]. Since muscle functionality is necessary to maintain the eyes open, the muscle relaxant properties of isoflurane might influence the FAUs evaluated during the immediate period following isoflurane. A period of 30 min post laparotomy or 1 hour after surgical anesthesia with isoflurane has been used by authors such as Klune et al. [26] and Leung et al. [25] to evaluate the RGS without the cofounding factor of isoflurane residual effects.

The findings regarding both inhalational agents can also be observed in the correlations obtained in the present study and the potential noxious and pharmacological effects of CO_2 and isoflurane. For all FAUs, G_2 and G_4 were moderately and positively correlated, meaning that the aforementioned activation of nociceptors and pungency level could influence RGS scores. Likewise, the negative moderate correlation found between G_4 and G_5 could explain the effect that the drug can have on the facial response of animals. In this case, G_4 animals differed from G_5 treatment possibly due to the ketamine-induced catalepsy [75].

Refinement of current euthanasia methods for laboratory rodents requires recognizing that distress, nociception, and pain might be present during the process. This also helps to advocate for alternatives, such as the combination of local anesthetics (e.g., lidocaine, bupivacaine) with IP drugs, resulting in less frequency of abdominal writhing and a low incidence of ultrasonic vocalizations related to pain [27]. In the present study, a combination of ketamine + CO_2 increased the RGS during Ti_2 (possibly due to the IP injection) but only by an average of 0.2, while in Ti_3, the RGS score decreased. In contrast, Valentine et al. [76] reported that rodents receiving CO_2 without premedication caused few signs of stress and pain (e.g., lower cerebral expression of c-fos) than its combination with acepromazine, midazolam, or isoflurane. A reason why ketamine in combination with CO_2 didn't have a high RGS score as CO_2 used alone might be due to the inhibitory

properties of ketamine in NMDA receptors, inducing amnesia, analgesia, and prevention of central sensitization [9]. Ketamine's mechanism of action prevents neural signaling from second-order neurons to supraspinal structures, decreasing thalamocortical, limbic, and reticular activity (regions necessary for conscious pain recognition) [77,78].

4.1. Limitations and recommendations for future research

Nociception and pain have a multidimensional nature with sensory/discriminative, affective/motivational, and cognitive components [79]. Due to this, nociception and pain assessment and recognition in animals require the comprehensive study of several parameters that could help to evaluate the different dimensions of pain. In this sense, the main limitation of the present study is the lack of behavioral assessment using other scales (e.g., composite behavioral scale), technologies (e.g., EEG or electrophysiological techniques to measure nociceptor activity), or comparison with pain-related biomarkers in rodents such as corticosterone, adrenaline, noradrenaline, c-fos, or ultrasonic vocalizations. A recent study used infrared thermography as a non-invasive technique to evaluate the stress/distress caused during the euthanasia of Wistar rats [80]. The authors found a significant effect of inhalational agents on the thermal response of animals, which might be associated with higher distress. Therefore, an overall evaluation of pain is necessary, particularly in research animals.

According to the obtained results, it is suggested that during the application of the euthanasia method, RGS scores might increase due to tissular damage and potential activation of peripheral nociceptors, eliciting short-term pain when using injectable and physical methods. Some authors have proposed oral administration of premedication before the actual euthanasia method to refine euthanasia. For example, Rodríguez-Sánchez et al. [81] recommended the use of oral tiletamine + zolazepam before euthanasia to induce sedation and decrease aversion. Nonetheless, Dudley and Boivin [82] found in mice that orally ingested pentobarbital solution in cookie dough did not induce LORR, unconsciousness, or death. Hence, evaluating through the RGS, and other pain-related markers, the application of novel strategies with current euthanasia methods could help to perform a complete and objective evaluation. Nonetheless, it must be acknowledged the effect that anesthetics (e.g., pentobarbital and isoflurane) might have on facial expression and mimetic muscle control, which could limit the behavioral assessment of euthanasic drugs [31,67].

In the present study, only Wistar rats were assessed. However, some reports have shown differences in behavioral responses associated with pain between different strains of rats (e.g., Sprague Dawley and Wistar) [31]. Winter et al. [83] found significant differences between Sprague Dawley, Wistar, Long Evans, and Wistar-Kyoto rats exposed to CO_2. Freezing was prevalent in Long Evans and Wistar/Kyoto rats, and behavioral responses such as rearing and grooming also depended on the strain, as well as the expression of serotonergic, noradrenergic, and dopaminergic neurons. Thus, these results show that nociception and pain evaluation require considering the strain and the possible implications when selecting the euthanasia method, not only from a physiological and endocrine perspective but as a thorough assessment.

5. Conclusion

According to the high RGS scores observed during the application of injectable (pentobarbital, ketamine + xylazine) and physical methods (decapitation), the findings of the present study suggest that Wistar rats can perceive short-term nociception when compared to inhalational agents. In contrast, during the first minutes after performing euthanasia, inhalant agents record the highest RGS scores, particularly isoflurane. While this could be associated with the activation of peripheral nociceptors, pungency, or mucosal

irritation –variables that were not assessed in the present research–, the pharmacological effect of isoflurane and other anesthetics as muscle relaxants need to be considered to objectively interpret changes in the facial expression of rats.

Therefore, the RGS could be applied as a method to evaluate and reconsider some of the euthanasia methods. However, as pain is multidimensional, behavioral, physiological, and electrophysiological assessment must be used together with facial expression rating to measure the level of pain that current euthanasia procedures evoke in research animal models.

Authors contribution: All authors contributed to the conceptualization, writing, and reading. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All procedures were approved both the Committee for the Care and Use of Laboratory Animals (INRLGII/CICUAL/014/2021) of National Institute of Rehabilitation Luis Guillermo Ibarra-Ibarra. The handling and care of the laboratory animals that were used to obtain the videos of this article were conducted in accordance with the recommendations of the Mexican Norm NOM-062-ZOO-1999 for Laboratory Animals, published by the Department of Agriculture, Rural Development, Fisheries and Alimentation. All dead animals were disposed by incineration, following the NOM-062-ZOO-1999.

Data Availability Statement: The data presented in this study is contained within the article and in [insert article or supplementary material here].

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Prescott, M.J.; Lidster, K. Improving quality of science through better animal welfare: the NC3Rs strategy. *Lab Anim. 2017 464* **2017**, *46*, 152–156, doi:10.1038/laban.1217.
2. Marquardt, N.; Feja, M.; Hünigen, H.; Plendl, J.; Menken, L.; Fink, H.; Bert, B. Euthanasia of laboratory mice: Are isoflurane and sevoflurane real alternatives to carbon dioxide? *PLoS One* **2018**, *13*, 1–29, doi:10.1371/journal.pone.0203793.
3. Kroll, T.; Kornadt-Beck, N.; Oskamp, A.; Elmenhorst, D.; Touma, C.; Palme, R.; Bauer, A. Additional Assessment of Fecal Corticosterone Metabolites Improves Visual Rating in the Evaluation of Stress Responses of Laboratory Rats. *Animals* **2021**, *11*, 710, doi:10.3390/ani11030710.
4. Baumans, V. Use of animals in experimental research: an ethical dilemma? *Gene Ther.* **2004**, *11*, S64–S66, doi:10.1038/sj.gt.3302371.
5. Hirose, M. Nociception during surgery. In *Features and Assessments of Pain, Anaesthesia, and Analgesia*; Rajendram, R., Patel, V.B., Preedy, V.R., Martin, C.R., Eds.; Academic Press: London, UK, 2022; pp. 235–245.
6. Raja, S.N.; Carr, D.B.; Cohen, M.; Finnerup, N.B.; Flor, H.; Gibson, S.; Keefe, F.J.; Mogil, J.S.; Ringkamp, M.; Sluka, K.A.; et al. The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. *Pain* **2020**, *161*, 1976–1982, doi:10.1097/j.pain.0000000000001939.
7. Hernández-Avalos, I.; Flores-Gasca, E.; Mota-Rojas, D.; Casas-Alvarado, A.; Miranda-Cortés, A.E.; Domínguez-Oliva, A. Neurobiology of anesthetic-surgical stress and induced behavioral changes in dogs and cats: A review. *Vet. World* **2021**, *14*, 393–404, doi:10.14202/vetworld.2021.393-404.
8. Descovich, K. Facial expression: An under-utilised tool for the assessment of welfare in mammals. *ALTEX* **2017**, *34*, 409–29, doi:10.14573/altex.1607161.
9. Flecknell, P. *Laboratory Animal Anaesthesia*; 4th ed.; Elsevier: Kidlington, England, 2016.
10. Dutton, J.W.; Artwohl, J.E.; Huang, X.; Fortman, J.D. Assessment of Pain Associated with the Injection of Sodium Pentobarbital in Laboratory Mice (Mus musculus). *J. Am. Assoc. Lab. Anim. Sci.* **2019**, *58*, 373–379, doi:10.30802/AALAS-JAALAS-18-000094.

11. Turner, M.D. "The Most Gentle of Lethal Methods": The Question of Retained Consciousness Following Decapitation. *Cureus* **2023**, *15*, e33830, doi:10.7759/cureus.33830.
12. Gagea-Iurascu, M.; Craig, S. Euthanasia and Necropsy. In *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents*; Suckow, M.A., Stevens, K.A., Wilson, R.P., Eds.; Academic Press: San Diego, USA, 2012; pp. 117–139.
13. Derr, R. Pain perception in decapitated rat brain. *Life Sci.* **1991**, *49*, 1399–1402, doi:10.1016/0024-3205(91)90391-N.
14. Holson, R.R. Euthanasia by decapitation: Evidence that this technique produces prompt, painless unconsciousness in laboratory rodents. *Neurotoxicol. Teratol.* **1992**, *14*, 253–257, doi:10.1016/0892-0362(92)90004-T.
15. Kongara, K.; McIlhone, A.; Kells, N.; Johnson, C. Electroencephalographic evaluation of decapitation of the anaesthetized rat. *Lab. Anim.* **2014**, *48*, 15–19, doi:10.1177/0023677213502016.
16. Boivin, G.P.; Hickman, D.L.; Creamer-Hente, M.A.; Pritchett-Corning, K.R.; Bratcher, N.A. Review of CO_2 as a Euthanasia Agent for Laboratory Rats and Mice. *J. Am. Assoc. Lab. Anim. Sci.* **2017**, *56*, 491–499.
17. De la Torre, C.; De la Torre, C.; Jiménez, P.; Ramos, M.M.; De la Torre, G.. Anestesia para colonoscopia: anestesia inhalatoria con sevoflurano frente a anestesia intravenosa con propofol. *Sanid. Mil.* **2012**, *68*, 27–32.
18. Whittaker, A.L.; Howartha, G.; Howarth, G.S. Use of spontaneous behaviour measures to assess pain in laboratory rats and mice: How are we progressing? *Appl. Anim. Behav. Sci.* **2014**, *151*, 1–12, doi:10.1016/j.applanim.2013.11.001.
19. Darwin, C. *The Expressions of the Emotions in Man and Animals*; Oxford Uni.; D Appleton and Company: New Yorrk, 1872;
20. Deuis, J.R.; Dvorakova, L.S.; Vetter, I. Methods Used to Evaluate Pain Behaviors in Rodents. *Front. Mol. Neurosci.* **2017**, *10*, 284, doi:10.3389/fnmol.2017.00284.
21. Domínguez-Oliva, A.; Mota-Rojas, D.; Hernández-Avalos, I.; Mora-Medina, P.; Olmos-Hernández, A.; Verduzco-Mendoza, A.; Casas-Alvarado, A.; Whittaker, A.L. The neurobiology of pain and facial movements in rodents: Clinical applications and current research. *Front. Vet. Sci.* **2022**, *9*, 1016720, doi:10.3389/fvets.2022.1016720.
22. Sotocinal, S.G.; Sorge, R.E.; Zaloum, A.; Tuttle, A.H.; Martin, L.J.; Wieskopf, J.S.; Mapplebeck, J.C.; Wei, P.; Zhan, S.; Zhang, S.; et al. The Rat Grimace Scale: A Partially Automated Method for Quantifying Pain in the Laboratory Rat via Facial Expressions. *Mol. Pain* **2011**, *7*, 55, doi:10.1186/1744-8069-7-55.
23. Whittaker, A.L.; Liu, Y.; Barker, T.H. Methods Used and Application of the Mouse Grimace Scale in Biomedical Research 10 Years on: A Scoping Review. *Animals* **2021**, *11*, 673, doi:10.3390/ani11030673.
24. Philips, B.H.; Weisshaar, C.L.; Winkelstein, B.A. Use of the Rat Grimace Scale to evauate neuropathic pain in a model of cervical radiculopathy. *Comp. Med.* **2017**, *67*, 34–42.
25. Leung, V.; Zhang, E.; Pang, D.S. Real-time application of the Rat Grimace Scale as a welfare refinement in laboratory rats. *Sci. Reports 2016 61* **2016**, *6*, 1–12, doi:10.1038/srep31667.
26. Klune, C.B.; Larkin, A.E.; Leung, V.S.Y.; Pang, D. Comparing the Rat Grimace Scale and a composite behaviour score in rats. *PLoS One* **2019**, *14*, e0209467, doi:10.1371/JOURNAL.PONE.0209467.
27. Khoo, S.Y.-S.; Lay, B.P.P.; Joya, J.; McNally, G.P. Local anaesthetic refinement of pentobarbital euthanasia reduces abdominal writhing without affecting immunohistochemical endpoints in rats. *Lab. Anim.* **2018**, *52*, 152–162, doi:10.1177/0023677217721260.
28. Akintola, T.; Raver, C.; Studlack, P.; Uddin, O.; Masri, R.; Keller, A. The grimace scale reliably assesses chronic pain in a rodent model of trigeminal neuropathic pain. *Neurobiol. Pain* **2017**, *2*, 13–17, doi:10.1016/j.ynpai.2017.10.001.
29. Kovalcsik, R.; Devlin, T.; Loux, S.; Martinek, M.; May, J.; Pickering, T.; Tapp, R.; Wilson, S.; Serota, D. Animal reuse: balancing scientific integrity and animal welfare. *Lab Anim. (NY).* **2006**, *35*, 49–53, doi:10.1038/laban1006-49.
30. Lofgren, J.L.; Foley, P.L.; Golledge, H.D.R. Anesthesia, analgesia, and euthanasia. In *The Laboratory Rat*; Suckow, M.A., Hankenson, R.., Wilson, R.., Foley, P.L., Eds.; Academic Press: London, UK, 2020; pp. 699–745 ISBN 9780128143384.
31. Reimer, J.N.; Schuster, C.J.; Knight, C.G.; Pang, D.S.J.; Leung, V.S.Y. Intraperitoneal injection of sodium pentobarbital has the

potential to elicit pain in adult rats (Rattus norvegicus). *PLoS One* **2020**, *15*, e0238123, doi:10.1371/JOURNAL.PONE.0238123.

32. American Veterinary Medical Association (AVMA) *AVMA Guidelines for the Euthanasia of Animals*; AVMA: Schaumburg, IL, 2020; ISBN 9781882691548.
33. Risling, T.E.; Caulkett, N.A.; Florence, D. Open-drop anesthesia for small laboratory animals. *Can. Vet. J. = La Rev. Vet. Can.* **2012**, *53*, 299–302.
34. de Brito, C.F.; Evangelista, A.A.; Felippe, R.M.; Cascabulho, C.; Fragoso, V.M.; de Oliveira, G.M. Laboratory Mice Euthanasia: Speed Death and Animal Welfare. *Am. J. Biomed. Sci. Res.* **2020**, *8*, 341–355, doi:10.34297/AJBSR.2020.08.001300.
35. Ko, M.J.; Mulia, G.E.; van Rijn, R.M. Commonly Used Anesthesia/Euthanasia Methods for Brain Collection Differentially Impact MAPK Activity in Male and Female C57BL/6 Mice. *Front. Cell. Neurosci.* **2019**, *13*, doi:10.3389/fncel.2019.00096.
36. Whittaker, A.L.; Leach, M.C.; Preston, F.L.; Lymn, K.A.; Howarth, G.S. Effects of acute chemotherapy-induced mucositis on spontaneous behaviour and the grimace scale in laboratory rats. *Lab. Anim.* **2016**, *50*, 108–118, doi:10.1177/0023677215595554.
37. Diario Oficial de la Federación Normal Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio Available online: https://www.gob.mx/cms/uploads/attachment/file/203498/NOM-062-ZOO-1999_220801.pdf.
38. NOM-062-ZOO-1999, S. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio Available online: http://publico.senasica.gob.mx/?doc=743 (accessed on Sep 30, 2022).
39. Oliver, V.; De Rantere, D.; Ritchie, R.; Chisholm, J.; Hecker, K.G.; Pang, D.S.J. Psychometric Assessment of the Rat Grimace Scale and Development of an Analgesic Intervention Score. *PLoS One* **2014**, *9*, e97882, doi:10.1371/journal.pone.0097882.
40. McLennan, K.M.; Miller, A.L.; Dalla Costa, E.; Stucke, D.; Corke, M.J.; Broom, D.M.; Leach, M.C. Conceptual and methodological issues relating to pain assessment in mammals: The development and utilisation of pain facial expression scales. *Appl. Anim. Behav. Sci.* **2019**, *217*, 1–15, doi:10.1016/j.applanim.2019.06.001.
41. Watson, C.R.R.; Sakai, S.; Armstrong, W. Organization of the Facial Nucleus in the Rat. *Brain. Behav. Evol.* **1982**, *20*, 19–28, doi:10.1159/000121578.
42. Ballotta, D.; Lui, F.; Porro, C.A.; Nichelli, P.F.; Benuzzi, F. Modulation of neural circuits underlying temporal production by facial expressions of pain. *PLoS One* **2018**, *13*, e0193100, doi:10.1371/journal.pone.0193100.
43. Freedman, D.J.; Ibos, G. An Integrative Framework for Sensory, Motor, and Cognitive Functions of the Posterior Parietal Cortex. *Neuron* **2018**, *97*, 1219–1234, doi:10.1016/j.neuron.2018.01.044.
44. Gothard, K.M. The amygdalo-motor pathways and the control of facial expressions. *Front. Neurosci.* **2014**, *8*, 43, doi:10.3389/fnins.2014.00043.
45. Svendsen, O.; Kok, L.; Lauritzenã, B. Nociception after intraperitoneal injection of a sodium pentobarbitone formulation with and without lidocaine in rats quantified by expression of neuronal c-fos in the spinal cord – a preliminary study. *Lab. Anim.* **2007**, *41*, 197–203, doi:10.1258/002367707780378140.
46. Laferriere, C.A.; Pang, D.S. Review of Intraperitoneal Injection of Sodium Pentobarbital as a Method of Euthanasia in Laboratory Rodents. *J. Am. Assoc. Lab. Anim. Sci.* **2020**, *59*, 254–263, doi:10.30802/AALAS-JAALAS-19-000081.
47. Shimizu, S. Routes of administration. In *The Laboratory Mouse*; Hedrich, H.J., Bullock, G., Eds.; Elsevier: London, UK, 2004; pp. 527–541.
48. Boivin, G.P.; Bottomley, M.A.; Schiml, P.A.; Goss, L.; Grobe, N. Physiologic, behavioral, and histologic responses to various euthanasia methods in C57BL76NTac male mice. *J. Am. Assoc. Lab. Anim. Sci.* **2017**, *56*, 69–78.
49. Mion, G.; Villevieille, T. Ketamine Pharmacology: An Update (Pharmacodynamics and Molecular Aspects, Recent Findings). *CNS Neurosci. Ther.* **2013**, *19*, 370–380, doi:10.1111/cns.12099.
50. Sun, F.; Wright, D.; Pinson, D.M. Comparison of ketamine versus combination of ketamine and medetomidine in injectable anesthetic protocols: Chemical immobilization in macaques and tissue reaction in rats. *J. Am. Assoc. Lab. Anim. Sci.* **2003**, *42*,

32–37.

51. Hohlbaum, K.; Bert, B.; Dietze, S.; Palme, R.; Fink, H.; Thöne-Reineke, C. Impact of repeated anesthesia with ketamine and xylazine on the well-being of C57BL/6JRj mice. *PLoS One* **2018**, *13*, e0203559, doi:10.1371/journal.pone.0203559.
52. Schoell, A.R.; Heyde, B.R.; Weir, D.E.; Chiang, P.-C.; Hu, Y.; Tung, D.K. Euthanasia method for mice in rapid time-course pulmonary pharmacokinetic studies. *J. Am. Assoc. Lab. Anim. Sci.* **2009**, *48*, 506–11.
53. Clarkson, J.M.; Martin, J.E.; McKeegan, D.E.F. A review of methods used to kill laboratory rodents: issues and opportunities. *Lab. Anim.* **2022**, *56*, 419–436, doi:10.1177/00236772221097472.
54. Mikeska, J.A.; Klemm, W.R. EEG evaluation of humaneness of asphyxia and decapitation euthanasia of the laboratory rat. *Lab. Anim. Sci.* **1975**, *25*, 175–179.
55. Cartner, S.C.; Barlow, S.C.; Ness, T.J. Loss of cortical function in mice after decapitation, cervical dislocation, potassium chloride injection, and CO2 inhalation. *Comp. Med.* **2007**, *57*, 570–3.
56. Pierozan, P.; Jernerén, F.; Ransome, Y.; Karlsson, O. The Choice of Euthanasia Method Affects Metabolic Serum Biomarkers. *Basic Clin. Pharmacol. Toxicol.* **2017**, *121*, 113–118, doi:10.1111/BCPT.12774.
57. Singh, P.; Kongara, K.; Harding, D.; Ward, N.; Dukkipati, V.S.R.; Johnson, C.; Chambers, P. Comparison of electroencephalographic changes in response to acute electrical and thermal stimuli with the tail flick and hot plate test in rats administered with opiorphin. *BMC Neurol.* **2018**, *18*, 43, doi:10.1186/s12883-018-1047-y.
58. Mota-Rojas, D.; Olmos-Hernández, A.; Verduzco-Mendoza, A. The utility of grimace escales for practical pain assessment in laboratory animals. *Animals* **2020**, *10*, 1–17.
59. Améndola, L.; Weary, D.M. Understanding rat emotional responses to CO2. *Transl. Psychiatry* **2020**, *10*, 253, doi:10.1038/s41398-020-00936-w.
60. Thürauf, N.; Friedel, I.; Hummel, C.; Kobal, G. The mucosal potential elicited by noxious chemical stimuli with CO2 in rats: Is it a peripheral nociceptive event? *Neurosci. Lett.* **1991**, *128*, 297–300, doi:10.1016/0304-3940(91)90283-Y.
61. Conlee, K.M.; Stephens, M.L.; Rowan, A.N.; King, L.A. Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats. *Lab. Anim.* **2005**, *39*, 137–161, doi:10.1258/0023677053739747.
62. Turner, P. V.; Hickman, D.L.; van Luijk, J.; Ritskes-Hoitinga, M.; Sargeant, J.M.; Kurosawa, T.M.; Agui, T.; Baumans, V.; Choi, W.S.; Choi, Y.-K.; et al. Welfare Impact of Carbon Dioxide Euthanasia on Laboratory Mice and Rats: A Systematic Review. *Front. Vet. Sci.* **2020**, *7*, 411, doi:10.3389/fvets.2020.00411.
63. Leach, M.C.; Bowell, V.A.; Allan, T.F.; Morton, D.B. Degrees of aversion shown by rats and mice to different concentrations of inhalational anaesthetics. *Vet. Rec.* **2002**, *150*, 808–815, doi:10.1136/vr.150.26.808.
64. Golledge, H.; Roughan, J.; Niel, L.; Richardson, C.; Wright-Williamson, S.; Flecknell, P. Carbon dioxide euthanasia in rats - Behavioural and autonomic system responses to exposure. In Proceedings of the SECAL-ESLAV International Congress; Elche, Spain, 2005.
65. Burkholder, T.H.; Niel, L.; Weed, J.L.; Brinster, L.R.; Bacher, J.D.; Foltz, C.J. Comparison of carbon dioxide and argon euthanasia: effects on behavior, heart rate, and respiratory lesions in rats. *J. Am. Assoc. Lab. Anim. Sci.* **2010**, *49*, 448–53.
66. Valentim, A.M.; Guedes, S.R.; Pereira, A.M.; Antunes, L.M. Euthanasia using gaseous agents in laboratory rodents. *Lab. Anim.* **2016**, *50*, 241–253, doi:10.1177/0023677215618618.
67. Miller, A.; Golledge, H.; Leach, M. The influence of isoflurane anaesthesia on the rat grimace scale. *PLoS One* **2016**, *11*, e0166652.
68. Miller, A.; Kitson, G.; Skalkoyannis, B.; Leach, M. The effect of isoflurane anaesthesia and buprenorphine on the mouse grimace scale and behaviour in CBA and DBA/2 mice. *Appl. Anim. Behav. Sci.* **2015**, *172*, 58–62, doi:10.1016/j.applanim.2015.08.038.
69. Hohlbaum, K.; Bert, B.; Dietze, S.; Palme, R.; Fink, H.; Thöne-Reineke, C. Severity classification of repeated isoflurane

anesthesia in C57BL/6JRj mice—Assessing the degree of distress. *PLoS One* **2017**, *12*, e0179588, doi:10.1371/journal.pone.0179588.

70. Wong, D.; Makowska, I.J.; Weary, D.M. Rat aversion to isoflurane versus carbon dioxide. *Biol. Lett.* **2013**, *9*, 20121000, doi:10.1098/rsbl.2012.1000.
71. Bodnar, M.J.; Ratuski, A.S.; Weary, D.M. Mouse isoflurane anesthesia using the drop method. *Lab. Anim.* **2023**, *0*, 1–8, doi:10.1177/00236772231169550.
72. Sio, L.C.O.; Varbanova, M.; Bautista, A. Isoflurane: Mechanisms and applications. In *Treatments, Mechanisms, and Adverse Reactions of Anesthetics and Analgesics*; Rajendram, R., Patel, V.B., Preedy, V.R., Martin, C.R., Eds.; Academic Press: London, UK, 2022; pp. 101–108.
73. Tong, J.; Lopez, M.J.; Patel, B.C. *Anatomy, head and neck, eye orbicularis oculi muscle*; StatPearls Publishing: Florida, USA, 2023;
74. Knight, B.; Lopez, M.J.; Patel, B.C. *Anatomy, Head and Neck: Eye Levator Palpebrae Superioris Muscles*; StatPearls Publishing: Florida, USA, 2023.
75. Pai, A.; Heining, M. Ketamine. *Contin. Educ. Anaesth. Crit. Care Pain* **2007**, *7*, 59–63, doi:10.1093/bjaceaccp/mkm008.
76. Valentine, H.; Williams, W.O.; Maurer, K.J. Sedation or inhalant anesthesia before euthanasia with CO2 does not reduce behavioral or physiologic signs of pain and stress in mice. *J. Am. Assoc. Lab. Anim. Sci.* **2012**, *51*, 50–57.
77. Zhang, Y.; Ye, F.; Zhang, T.; Lv, S.; Zhou, L.; Du, D.; Lin, H.; Guo, F.; Luo, C.; Zhu, S. Structural basis of ketamine action on human NMDA receptors. *Nature* **2021**, *596*, 301–305, doi:10.1038/s41586-021-03769-9.
78. Hashimoto, K. Rapid-acting antidepressant ketamine, its metabolites and other candidates: A historical overview and future perspective. *Psychiatry Clin. Neurosci.* **2019**, *73*, 613–627, doi:10.1111/pcn.12902.
79. Larson, C.M.; Wilcox, G.L.; Fairbanks, C.A. The Study of Pain in Rats and Mice. *Comp. Med.* 2019, *69*, 555–570.
80. Domínguez-Oliva, A.; Hernández-Ávalos, I.; Olmos-Hernández, A.; Villegas-Juache, J.; Verduzco-Mendoza, A.; Mota-Rojas, D. Thermal response of laboratory rats (*Rattus norvegicus*) during the application of six methods of euthanasia assessed by infrared thermography. *Animals* **2023**, *13*, Accepted.
81. Rodriguez-Sanchez, R.; Barnaby, E.; Améndola, L.; Hea, S.-Y.; Smith, B.; Webster, J.; Zobel, G. Voluntary Oral Ingestion of a Sedative Prior to Euthanasia with CO2: Behavioural Responses of Mice. *Animals* **2021**, *11*, 2879, doi:10.3390/ani11102879.
82. Dudley, E.S.; Boivin, G.P. Evaluation of a Commercially Available Euthanasia Solution as a Voluntarily Ingested Euthanasia Agent in Laboratory Mice. *J. Am. Assoc. Lab. Anim. Sci.* **2018**, *57*, 30–34.
83. Winter, A.; Ahlbrand, R.; Naik, D.; Sah, R. Differential behavioral sensitivity to carbon dioxide (CO2) inhalation in rats. *Neuroscience* **2017**, *346*, 423–433, doi:10.1016/j.neuroscience.2017.01.003.

10. DISCUSIÓN

La eutanasia se reconoce como un proceso en el cual los animales pueden estar expuestos a distrés y dolor debido al manejo, a las características del fármaco, y al procedimiento *per se* (p. ej., inyección IP). Basado en los hallazgos de este estudio, los agentes inhalatorios (CO_2 e isoflurano) dieron como resultado alteraciones sustanciales en la respuesta térmica de las cuatro ventanas térmicas ($T°_{ocu}$, $T°_{ear}$, $T°_{dor}$, y $T°_{tai}$) de ratas Wistar durante el Ti_2, Ti_3 y Ti_4. De igual forma, los métodos inhalatorios generaron cambios significativos en la expresión facial durante el Ti_3, mientras que, durante el Ti_2, los puntajes más altos de la RGS se registraron en G_3, G_5 y G_6, sugiriendo que durante la aplicación de los métodos de eutanasia se observan alteraciones térmicas y motoras debido al distrés, nocicepción o dolor.

A la fecha no existe algún estudio donde se emplee la IRT para evaluar diferentes métodos de eutanasia y su efecto sobre la respuesta térmica de roedores de laboratorio. En contraste, pocos estudios han sido publicados en donde la RGS ha sido evaluada durante la aplicación de un fármaco eutanásico. A pesar de la falta de evidencia científica, los estudios que abordan el mecanismo de acción del CO_2 e isoflurano, así como su sobredosis para inducir eutanasia, han demostrado que ambas drogas desencadenan un estado de estrés fisiológico con las consecuentes alteraciones cardiovasculares y los cambios en la expresión facial (56–58). Aunque ambos métodos se consideran seguros, económicos y efectivos para inducir inconsciencia, los resultados obtenidos coinciden con hallazgos de otros autores, en donde los cambios endocrinos y de comportamiento observados en ratas y ratones durante la inhalación de CO_2 e isoflurano sugieren que la respuesta térmica y de expresión facial podrían asociarse a estrés/distrés, nocicepción o dolor (59,60).

En primera instancia, la inhalación de CO_2, utilizada en ratas del grupo G_2, induce hipercapnia, acidosis, supresión de los potenciales sinápticos (59,61,62) y activa ejes asociados al estrés (p. ej., el hipotálamo-hipófisis-adrenal y el simpático-adreno-medular) (Figura 14) (63). Esto fue corroborado por Borovsky et al. (64), quienes reportaron que la inhalación de CO_2 aumenta la presión arterial (50-60 mmHg) y la concentración de norepinefrina (NE) hasta diez veces en ratas, en respuesta a la hipoxia. Asimismo, la exposición aguda de ratones a CO_2, provocó el aumento de norepinefrina (NE) y de la hormona adrenocorticotrópica (ACTH) (63), mientras que otros estudios en ratones y ratas

han demostrado que la inhalación de CO_2 aumenta los niveles de proteínas séricas totales y de biomarcadores asociados al estrés (p. ej., corticosterona) (65). Además de las alteraciones endocrinas, se han reportado comportamientos de ansiedad y angustia también en ratas expuestas a eutanasia con CO_2 al 10% (66). La aversión de los roedores a las altas concentraciones de CO_2 (más del 40% del volumen de la cámara de inducción) está asociada con la anhidrasa carbónica y la formación de ácido carbónico dentro de las superficies mucosas (ocular y nasal) cuando el CO_2 interactúa con el agua, causando irritación y comportamientos de incomodidad, como acicalamiento excesivo y estornudos (22,67,68). En contraste, Hackbarth et al. (69) no encontraron conductas asociadas a angustia o alteraciones endocrinas (ACTH y corticosterona) en ratas bajo eutanasia con CO_2.

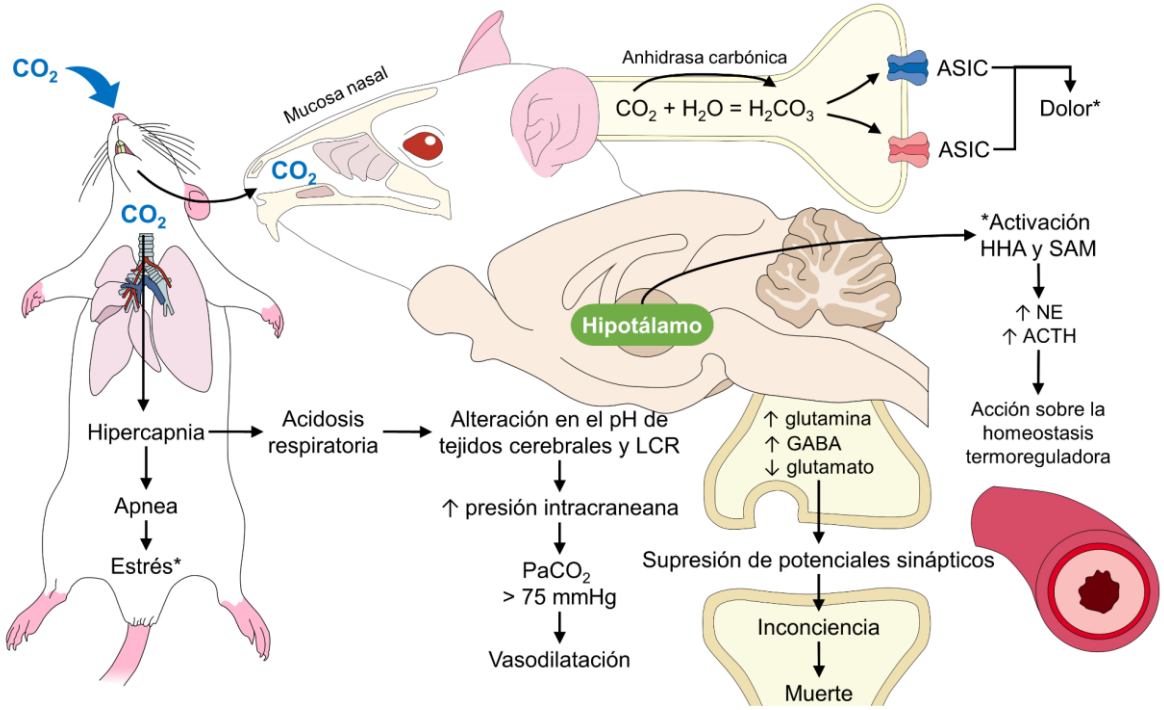


Figura 14. Mecanismo de acción del CO_2 y la respuesta fisiológica asociada a su uso durante la eutanasia. La inhalación de CO_2 genera la muerte por efecto de la hipercapnia, acidosis respiratoria y supresión de potenciales sinápticos. Sin embargo, su uso ocasiona otros efectos como un potencial estrés/distrés por el estado de apnea, y dolor por la activación de los ASIC en la mucosa nasal. Ambos eventos pueden activar los dos principales ejes asociados al estrés (HHA y SAM) y la consecuente respuesta fisiológica, endocrina, térmica y conductual. ACTH: hormona adrenocorticótropa; ASIC: receptores

sensibles al ácido; GABA: ácido gama aminobutírico; H_2CO_3: ácido carbónico; HHA: hipotálamo-hipófisis-adrenal; LCR: líquido cefalorraquídeo; NE: norepinefrina; $PaCO_2$: presión parcial de CO_2; SAM. Simpático-adreno-medular.

Por otro lado, en cuanto a la inhalación de isoflurano, método empleado en ratas del G_4, se reconoce que este agente es un anestésico volátil que causa depresión en los centros cardiorrespiratorios, generando hipoxemia y muerte (56,70) (Figura 15). Aunque el isoflurano se ha considerado como un método de refinamiento para el CO_2 (23,60), su nivel de pungencia es moderado, en comparación con el del CO_2 y otros anestésicos volátiles (71), lo que causa más respuestas de aversión en los roedores de laboratorio posiblemente debido a la irritación de las vías respiratorias y disnea (59,72).

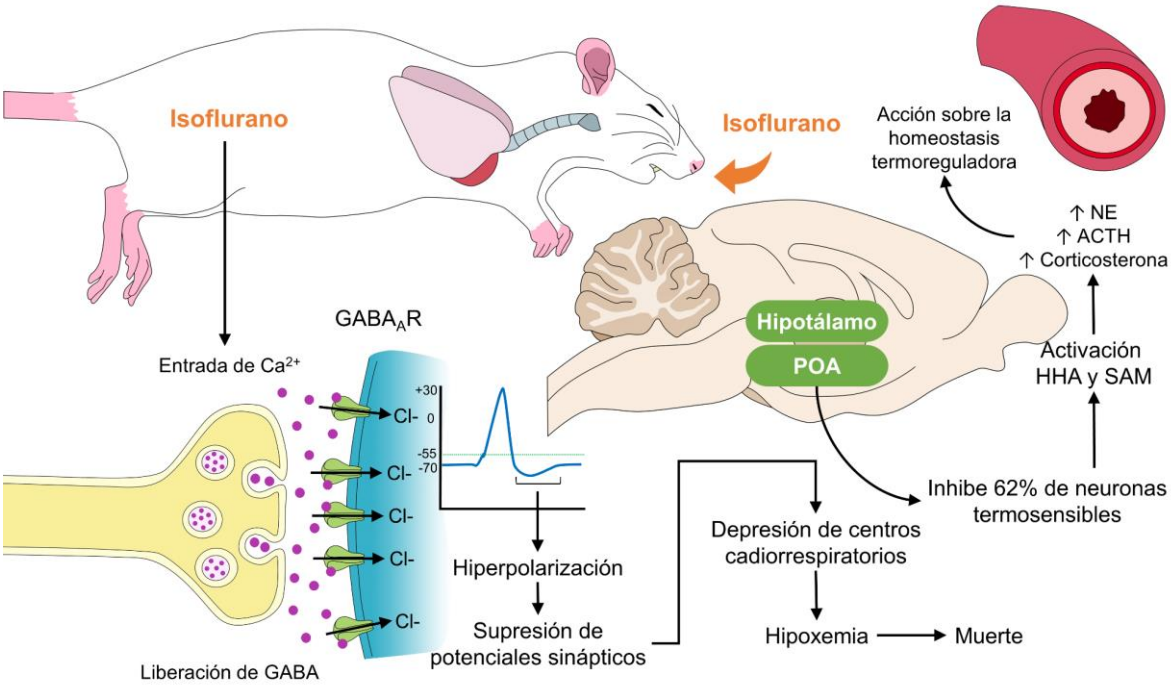


Figura 15. Mecanismo de acción del isoflurano y la respuesta fisiológica asociada a su uso durante la eutanasia. El isoflurano, por su mecanismo de acción, actúa sobre los receptores $GABA_AR$ de la membrana postsináptica. Estos generan la hiperpolarización de la membrana y, con ello, la supresión de potenciales sinápticos, lo cual afecta a los centros cardiorrespiratorios y a centros termorreguladores como lo es el hipotálamo. El grado de

pungencia moderado también es el responsable de activar el HHA y el SAM, generando la secreción de catecolaminas y glucocorticoides, alterando la respuesta térmica a nivel central y periférico. ACTH: hormona adrenocorticótropa; $GABA_AR$: receptor de ácido gama aminobutírico A; HHA: hipotálamo-hipófisis-adrenal; NE: norepinefrina; POA: área preóptica del hipotálamo; SAM. Simpático-adreno-medular.

Powell et al. (67) encontraron que el uso de isoflurano durante la eutanasia aumentó la ansiedad, puntuaciones de agitación y concentraciones de corticosterona en ratones, en contraste con animales expuestos a una tasa de flujo baja de CO2 (30%), la misma tasa empleada en la presente investigación. En este sentido, Boivin et al. (73) compararon la anestesia con isoflurano seguida de la administración de CO_2 y barbitúricos como métodos de eutanasia en ratones. Los autores descubrieron que, según las concentraciones de ACTH, los barbitúricos fueron menos estresantes que los otros dos métodos. No obstante, las alteraciones cardiovasculares y las respuestas relacionadas con el dolor/estrés no difirieron en los tres métodos, lo que sugiere que el isoflurano no proporcionó beneficios por encima de la eutanasia con CO_2. Por el contrario, Valentine et al. (74) señalaron que la combinación de isoflurano y CO_2 causó más signos de angustia y estrés en ratones que el uso de CO_2.

En contraste con lo mencionado, el estudio de Makowska y Weary (60) mostró que el CO_2 y los agentes inhalatorios son aversivos para los roedores, pero la aversión es menor con el isoflurano. En otro estudio se encontró que altas concentraciones de CO_2 aumentaron las concentraciones de adrenalina y noradrenalina, a diferencia de la eutanasia con isoflurano. Esto podría ser indicativo de una respuesta al estrés; sin embargo, debido a que no se registraron comportamientos relacionados al estrés (p. ej., acicalamiento o vocalizaciones audibles) en el grupo de CO_2, éste no podría considerarse más estresante que el isoflurano (7). Zardooz et al. (75) hallaron que los niveles plasmáticos de corticosterona e insulina aumentaron en ratas expuestas al CO_2, mientras que el isoflurano provocó la reacción contraria. Asimismo, el estudio de Hickman et al. (63) detectó incrementos en las concentraciones de ACTH, corticosterona y noradrenalina en ratas anestesiadas con isoflurano; no obstante, el aumento no fue tan significativo como con el CO_2.

La presente investigación no evaluó parámetros conductuales ni endocrinos para asociar la respuesta térmica y de expresión facial murina a los diferentes métodos de eutanasia. Sin embargo, la literatura muestra que ambos métodos desencadenan respuestas relacionadas al estrés, cuyas consecuencias fisiológicas pueden estar asociadas con cambios en la temperatura superficial y en la expresión facial. A continuación, se realizará la discusión de la IRT por ventana térmica, para posteriormente discutir los hallazgos en cuanto a la expresión facial.

10.1. $T°_{ocu}$

En las ratas del presente estudio se observó un aumento significativo en la T°media de $T°_{ocu}$ de Ti_2 a Ti_3 y de Ti_2 a Ti_4 en G_2 y G_4, respectivamente. Este aumento en la temperatura superficial podría estar mediado por los cambios termorreguladores que el estrés por exposición a CO_2 e isoflurano genera a nivel cerebral, alterando las neuronas termosensibles debido a la acidosis e hipoxia (76,77).

Diversos estudios han mostrado que los niveles de epinefrina, NE, ACTH y corticosterona aumentan después de la exposición al CO_2 y al isoflurano debido a que fungen como un potencial estresor (Figura 16) (7,78–80). Aunque el presente estudio no evaluó biomarcadores, su liberación modifica la respuesta vasomotora de la microvasculatura dérmica, induciendo vasodilatación en órganos clave (p. ej., el globo ocular) y una mayor cantidad de calor disipado, registrado como valores IRT más altos (40,81), como los observados en $T°_{ocu}$ del G_2 y G_4.

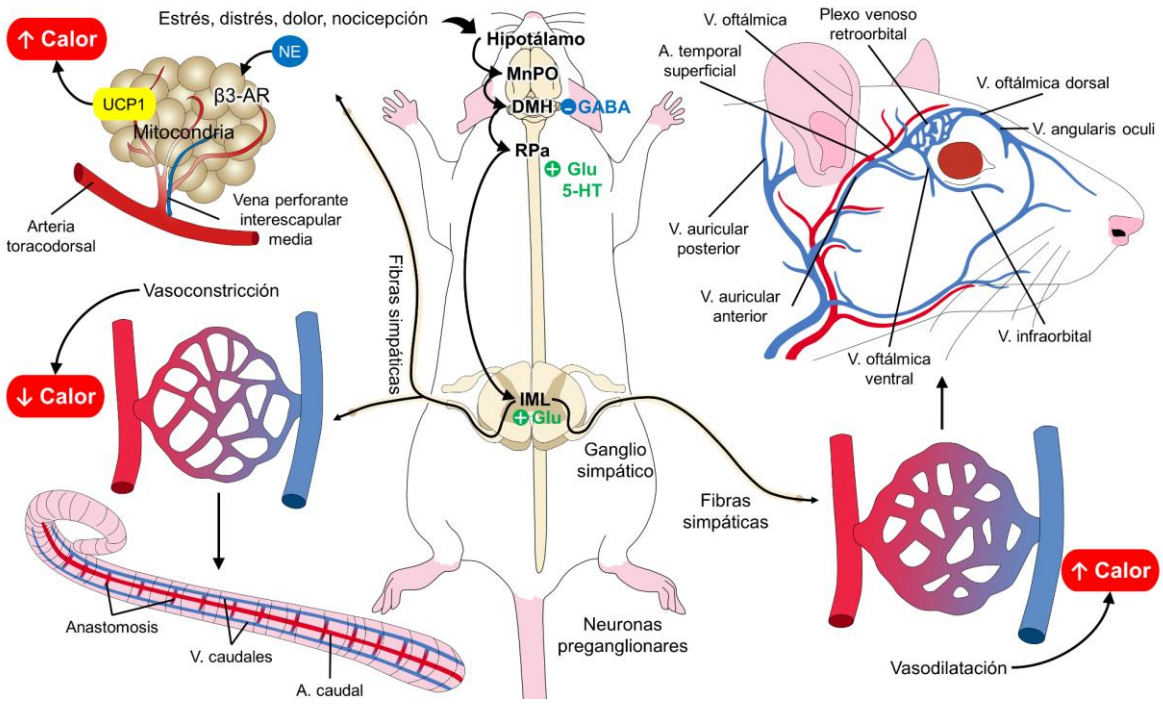


Figura 16. Estrés y modulación térmica. La percepción de eventos nocivos como el estrés/distrés, nocicepción o dolor, activa al hipotálamo, el centro termorregulador simpático del organismo. Una vez que estos eventos son detectados, una cascada de comunicación entre el MnPO, DMH, RPa y las IML generan vasodilatación o vasoconstricción dérmica. En el caso de la superficie ocular y auricular, la temperatura superficial incrementa debido a la vasodilatación periférica, mientras que el efecto contrario se observa en regiones como la cola y las extremidades, en donde existe una vasoconstricción para redirigir la sangre a órganos clave. Además de esta respuesta térmica, tejidos termógenicos como el BAT se activan por la liberación de NE por el eje SAM, generando calor y aumentando la cantidad de éste que se disipa al medio. A: arteria; β3-AR: receptor adrenérgico β3; DMH: hipotálamo dorsomedial; GABA: ácido gama aminobutírico; GLU: glutamato; IML: lámina intermediolateral; MnPO: área preóptica mediana; NE: norepinefrina; RPa: rafe rostral pálido; UCP1: proteína desacoplante 1; V: vena.

La temperatura de la superficie ocular en animales se ha utilizado como ventana térmica para evaluar el estrés y el dolor agudo, registrándose un aumento en ambos casos (82,83). Actualmente, sólo hay dos estudios que combinan la IRT y el efecto del isoflurano como anestésico (84,85), pero no existe alguno que haya aplicado la IRT para evaluar al CO_2 e isoflurano como eutanásicos. Gjendal et al. (85) determinaron que, a partir de tres tipos

diferentes de estímulos, la anestesia de ratones con isoflurano alteró significativamente la temperatura ocular. De manera similar, Vogel et al. (84) utilizaron anestesia con isoflurano y descubrieron que la temperatura ocular cambiaba según la concentración de isoflurano, reflejando de manera precisa la temperatura rectal en roedores. Sin embargo, no se estableció alguna asociación con el estrés.

Por el contrario, si bien no hay evidencia publicada sobre la eutanasia y la IRT ocular, el aumento de la temperatura ocular se ha observado en roedores salvajes (*Apodemus mystacinus*) como un reflejo de la hipertermia inducida por estrés (SIH) durante la manipulación de los animales (86), durante una prueba de miedo condicionado en ratas (aumentando la temperatura ocular hasta 1.5°C) (87), y en ratones, donde SIH y comportamientos activos se correlacionaron positivamente (88). Además, en los conejillos de indias la temperatura ocular aumentó en relación con la interacción humana negativa (caricias) (89). De manera similar, Wongsaengchan et al. (90) utilizaron la temperatura ocular de ratones para evaluar la exposición aguda a un factor estresante (jaula pequeña, manipulación y cono de sujeción). Los autores encontraron aumentos significativos en la temperatura ocular izquierda de las hembras durante la sujeción, una respuesta que se acompañó con aumentos de corticosterona.

Dichos resultados sugieren que los cambios vasomotores periféricos podrían estar relacionados a la respuesta de huida-lucha cuando los animales enfrentan un estresor. Los aumentos en $T°_{ocu}$ de Ti_2 en todos los grupos experimentales sugieren que las ratas percibieron estrés independientemente del método de eutanasia –respuesta que también se vio reflejada en la expresión facial y será discutida posteriormente–. No obstante, debido a que se observaron únicamente cambios significativos en G_2 y G_4 desde la aplicación del método hasta LORR, esto podría sugerir que ambos métodos inhalatorios generaron una mayor respuesta al estrés. Finalmente, aunque ambos grupos mostraron aumentos significativos en el $T°_{ocu}$ al comparar entre eventos, de manera general, G_2 y G_4 mantuvieron temperaturas más bajas que el resto de los grupos, posiblemente debido a la propiedad vasodilatadora de ambos fármacos, lo cual facilita la pérdida de calor (61). Un resultado similar fue obtenido por Gjendal et al. (85), donde la anestesia con isoflurano en ratones redujo la T°max ocular debido a la hipotermia causada por los anestésicos generales.

10.2. $T°_{ear}$

De manera similar a $T°_{ocu}$, la T°media de $T°_{ear}$ de Ti_2 registró aumentos significativos en ambos G_2 y G_4. Este patrón de temperatura era esperado debido a que la temperatura del canal auditivo externo está asociada con la arteria carótida y la temperatura hipotalámica, la estructura principal involucrada en las adaptaciones termorreguladoras centrales y periféricas (91).

En animales, la temperatura auricular se ha asociado a estrés por la administración de fármacos IP y técnicas de sujeción en el estudio de Wokke (92), así como durante el manejo de conejos (93). En ratas, aumentos entre 0.8 y 1.5 °C se han observado durante eventos de miedo condicionado (87). En el presente estudio, todos los grupos experimentales mostraron un aumento de la T°mean de la $T°_{ear}$. Sin embargo, sólo el CO_2 e isoflurano provocaron aumentos significativos. Esta respuesta podría estar asociada a lo que menciona Hutu et al. (94), quienes concluyeron que la temperatura superficial del oído está correlacionada con la temperatura central (alrededor de 37.1 ± 0.2°C). Considerando que el uso de CO_2 y eutanasia/anestesia con isoflurano podrían causar SIH, el aumento en $T°_{ear}$ podría ser el reflejo de SIH en G_2 y G_4, como una respuesta vasodilatadora compensatoria del organismo para disipar calor.

A diferencia de los hallazgos reportados en el presente estudio, otros autores no han encontrado cambios o disminuciones significativas en la temperatura del oído de ratones y ratas. Esto podría deberse a la falta de anastomosis arteriovenosas presente en otras especies como los conejos (95). Además, se podrían derivar resultados contradictorios debido a la delimitación de la ventana térmica utilizada por otros autores (p. ej., canal auditivo externo o pabellón auricular).

10.3. $T°_{dor}$

En todos los grupos experimentales se registró un aumento esperado en la T°media de $T°_{dor}$ después de la administración del método de eutanasia. En particular, se reportaron diferencias significativas en G_2 y G_4, probablemente debido al estrés agudo inducido por el CO_2 y los anestésicos halogenados. En la región anatómica donde se evaluó la $T°_{dor}$ se pueden encontrar grandes cantidades de tejido adiposo pardo (BAT) (96). Este tejido

termogénico responde a la liberación de NE. Borovsky et al. (64) y Hickman (63) reportaron aumentos de NE después de la exposición de ratas y ratones al CO_2 y al isoflurano.

Debido a estos elementos, en la presente investigación se utilizó la región interescapular para determinar el efecto de los diferentes métodos de eutanasia, encontrando que G_2 y G_4 tuvieron aumentos significativos en la actividad del BAT. De manera similar, un estudio de Blenkuš (97) registró temperaturas superficiales dorsales más altas en ratones expuestos a factores estresantes (manipulación diaria) y pruebas de comportamiento (interacción voluntaria y laberinto elevado). Miyazono et al. (98) encontraron que la temperatura de la superficie corporal (evaluada en la región dorsal de ratones) aumentó después de estrés agudo (p. ej., olor de depredador), mientras que la SIH también está relacionada con la termogénesis a través del BAT en ratas y humanos después de estrés excesivo (99). La percepción del dolor en modelos murinos de lesión espinal también ha mostrado aumentos en la temperatura interescapular, un efecto que puede disminuir con la administración de fármacos analgésicos (27), y el cual también puede tener asociación son el puntaje de la RGS observada en el Ti_2 y Ti_3 de todos los grupos experimentales.

Por lo tanto, los hallazgos sugieren que la hipertermia local detectada en la $T°_{dor}$ de los sujetos G_2 y G_4 podría traducirse a una respuesta frente a un estímulo negativo.

10.4. $T°_{tai}$

Al contrario de las otras ventanas térmicas, se observó una reducción progresiva de $T°_{tai}$ en $T°_{prox}$, $T°_{medial}$ y $T°_{distal}$, independientemente del grupo experimental. Esto podría relacionarse con el efecto vasoconstrictor que las catecolaminas –biomarcador de estrés– ejercen sobre la microcirculación de regiones periféricas como la cola y las patas, y la posterior reducción del calor irradiado detectado por la IRT (40,100).

En diferentes estudios la temperatura superficial de la cola ha sido utilizada para evaluar el estrés y las respuestas emocionales negativas de roedores de laboratorio, registrando disminuciones luego de la exposición al estresor, como se encontró en la presente investigación. Se ha mostrado que la exposición a factores estresantes como el manejo y la sujeción reduce la temperatura de la cola en ratas (101). Asimismo, ratas bajo miedo condicionado presentaron una disminución gradual en la temperatura de la superficie de la cola de hasta 5.3°C (87), mientras que la temperatura en la cola también disminuyó en

ratones durante una prueba de laberinto elevado como resultado del estrés y ansiedad (102). Por otro lado, Blenkuš et al. (97) informaron disminuciones en la temperatura de la cola después de 30 a 60 segundos de exposición a un factor estresante (manipulación diaria, prueba de interacción voluntaria y prueba de laberinto elevado). Además, Weitkamp (101) ha mencionado que $T°_{tai}$ no sólo sirve para identificar factores estresantes agudos sino que también refleja la intensidad de los mismos. Esto es relevante y consistente con los presentes resultados porque, aunque todos los métodos de eutanasia resultaron en cambios térmicos asociados al estrés, solo los agentes inhalatorios causaron efectos significativos en $T°_{tai}$ y todas las ventanas térmicas.

Un hallazgo por resaltar es que mientras se observó una reducción progresiva de $T°_{tai}$ en todos los métodos de eutanasia (lo que sugiere que la eutanasia provoca cambios relacionados con el estrés, independientemente del método), en G_2 y G_4 se observaron disminuciones significativas con las temperaturas más bajas de $T°_{tai}$, en comparación con los otros grupos. Esto podría deberse a las potentes propiedades vasodilatadoras del CO_2 e isoflurano (61,103), desencadenando un cambio circulatorio para restringir la circulación periférica –$T°_{tai}$– y redirigir el flujo sanguíneo a sitios centrales –$T°_{ocu}$, $T°_{ear}$ y $T°_{dor}$–.

10.5. RGS

De manera similar a los cambios significativos que se registraron en las ventanas térmicas, los resultados de la RGS muestran que todos los métodos de eutanasia registraron aumentos en el puntaje de Basal/Ti_1 a Ti_2 y Ti_3. De manera particular, durante el Ti_2 los métodos inyectables obtuvieron los mayores puntajes, mientras que, durante el Ti_3, G_2 y G_4 tuvieron puntajes elevados, difiriendo de manera significativa con los agentes inyectables y físicos. El aumento en los puntajes de la RGS tiene relación con los resultados de la IRT debido a que el aumento de la temperatura de $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$ y la disminución de $T°_{tai}$, así como el aumento en los puntajes de la RGS en todos los grupos experimentales, sugiere que todos los métodos de eutanasia generan estrés y dolor. No obstante, esta respuesta y el nivel de dolor presente puede depender del método, el fármaco y el tiempo de evaluación.

En cuanto a la expresión facial y su asociación con el potencial dolor que las ratas pueden percibir durante la eutanasia, los movimientos de las cuatro FAU utilizadas en el RGS están controlados por el nervio facial que inerva los músculos faciales y miméticos superficiales.

Los músculos asociados con la expresión facial son el *levator auris longus cranial, levator auris longus caudal, frontalis, orbicularis oculi, levator nasolabialis* incluyendo el *levator labii superioris* y el *dilator naris*, los cuales están relacionados con el movimiento de los ojos, vibrisas, orejas y nariz (104,105) (Figura 17).

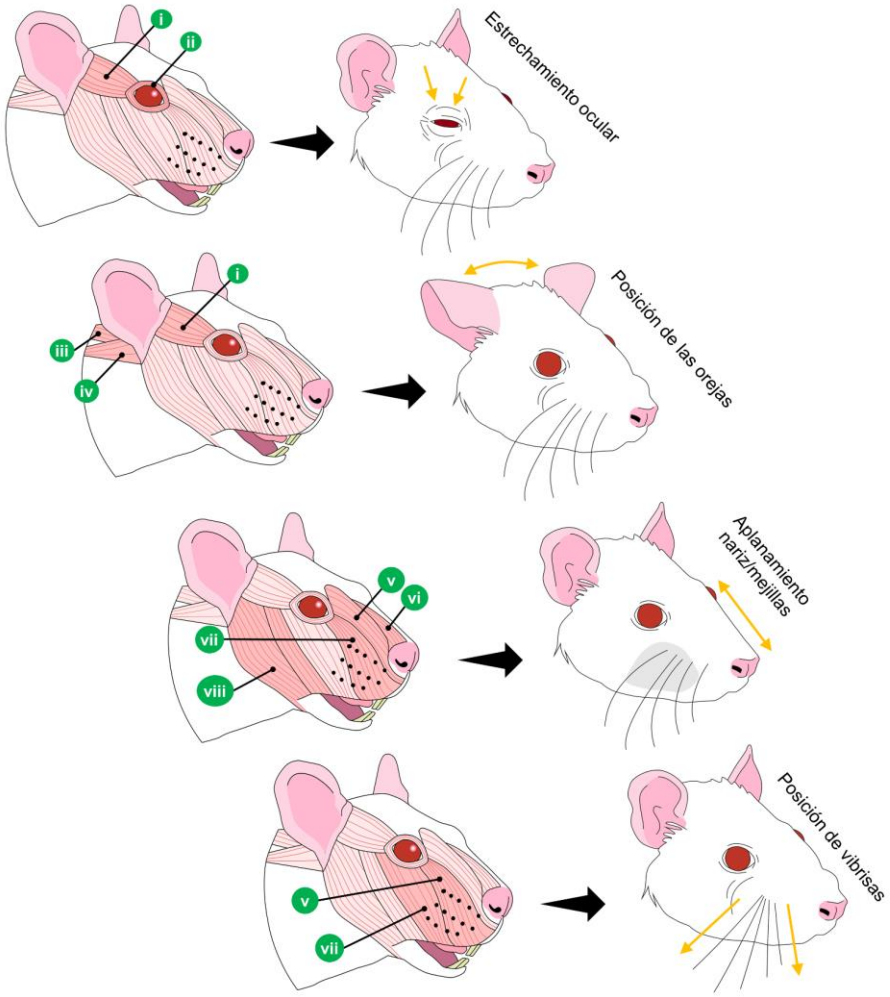


Figura 17. Aspectos anatómicos de la expresión facial en ratas y su asociación con las unidades de acción facial. Se esquematiza el grupo de músculos miméticos que generan cambios en cada una de las unidades de acción facial empleadas en ratas. i: frontalis; ii: orbicularis occuli; iii: levator auris longus cranial; iv: levator auris longus caudal; v: levator labii superioris alaeque; vi: dilator naris; vii: levator nasolabialis; viii: platisma.

La relajación o contracción de dichos músculos, cuando los animales perciben dolor, se desencadena por la proyección de neuronas de tercer orden (del tálamo) hacia la corteza

somatosensorial, amígdala, hipotálamo y corteza motora (106). Desde el lado ventrolateral de la corteza frontal, las neuronas motoras inervan directamente los músculos miméticos superiores e inferiores (107). Debido a esta conexión, como se observa en los humanos, el dolor puede provocar cambios en la expresión facial de especies como los roedores de laboratorio (Figura 18) (108). Por ello es que el estudio de la expresión facial a través de la RGS ha permitido evaluar prácticas de rutina, técnicas de manejo, procedimientos quirúrgicos y protocolos analgésicos en ratas (35,109,110).

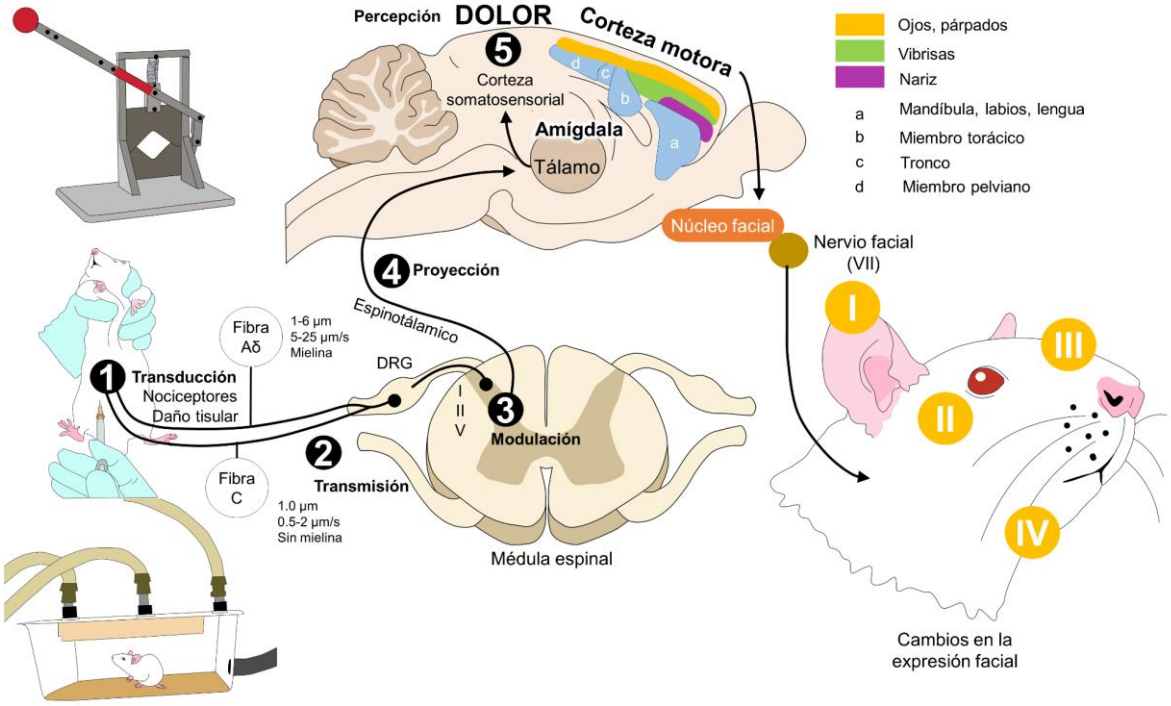


Figura 18. Arco nociceptivo y el control de la expresión facial durante la aplicación de métodos de eutanasia. La administración IP, guillotina y los métodos inhalatorios de eutanasia pueden generar un potencial dolor que activa el arco nociceptivo hasta llegar a centros supra espinales como el tálamo. En esta estructura, las proyecciones hacia la corteza somatosensorial generan el reconocimiento del dolor. Sin embargo, el tálamo también se comunica con la amígdala, una estructura que brinda el componente emocional del dolor y que también se encarga de activar neuronas en la corteza motora. A partir de esta corteza y a través del núcleo facial, los músculos miméticos son inervados por el par craneal VII, generando los movimientos o alteraciones en las cuatro FAU empleadas en la RGS. DRG: ganglio dorsal de la médula espinal; I: cambio de orejas; II: estrechamiento ocular; III: aplanamiento de nariz/mejillas; IV: cambio de vibrisas.

Considerando que todos los tratamientos aumentaron el puntaje de la RGS de Basal/Ti_1 a Ti_2 y Ti_3, algunos estudios han reportado el puntaje de la RGS relacionado con el efecto del método de eutanasia. Por ejemplo, Reimer et al. (48) compararon grupos de pentobarbital, solución salina y vehículo control en ratas Sprague Dawley y Wistar para determinar el nivel de dolor en ambas cepas. Se encontró que las puntuaciones de RGS estuvieron por encima de 0.67 sólo en los grupos de solución salina y vehículo, teniendo también una mayor frecuencia de contorsiones y arqueamiento de la espalda. De manera similar, Khoo et al. (109) investigaron la influencia que tuvo la eutanasia con pentobarbital sódico sobre la RGS y las respuestas conductuales como las contorsiones abdominales y las vocalizaciones ultrasónicas. Los autores encontraron que una combinación con anestésicos locales (lidocaína) redujo la frecuencia de las contorsiones, pero no influyó en la RGS, donde se registraron valores bajos entre 0.44 ± 0.14 y 0.37 ± 0.11. Las puntuaciones obtenidas por Khoo et al. (109) fueron superiores a las encontradas en el presente estudio (0.1 ± 0.07 en Ti_2 y 0.2 ± 0.14 en Ti_3); sin embargo, ambos hallazgos sugieren que la administración IP de pentobarbital podría causar nocicepción, como lo informaron Svendsen et al. (111) en ratas, en las que un aumento de expresión de c-fos en neuronas de la médula espinal está relacionado con la nocicepción.

Como lo mencionan Laferriere y Pang (3), aunque el pentobarbital es un método de eutanasia recomendado por encima de los agentes inhalatorios (p. ej., CO_2 e isoflurano), los barbitúricos se caracterizan por ser soluciones básicas solubles para su administración IP, transformando el pentobarbital en una sustancia altamente alcalina (pH entre 11 y 12). Por lo tanto, la inyección IP podría causar dolor e irritación de la cavidad peritoneal y las vísceras debido a que el pH tisular alrededor de 4.5 a 8.0 se considera como no irritante (15,112). Una de las respuestas que se ha relacionado a la irritación tisular es la observación de comportamiento como retorcerse, vocalizar, aumentar la locomoción o estremecerse después de la administración IP de pentobarbital (3), los cuales se presentan con una incidencia de 36 % y 46 % con dosis bajas (200 mg/kg) y altas (800 mg/kg), respectivamente. Sin embargo, otros autores no refieren cambios en ratones que recibieron pentobarbital IP, lo cual implica que los barbitúricos pueden causar inflamación peritoneal pero la acción rápida del método de eutanasia no provoca signos de dolor (16). Asimismo, Boivin et al. (73) determinaron que, de acuerdo a las concentraciones de ACTH, la

administración IP de pentobarbital-fenitoína genera menos estrés en ratones que los agentes inhalantes (CO_2 y anestesia con isoflurano seguida de inhalación de CO_2). Estos resultados son similares a los obtenidos en la presente investigación, donde las ratas del G_1 tuvieron las puntuaciones más bajas en la RGS durante y después de la administración de pentobarbital.

En contraste al G_1, durante Ti_2, animales del G_5, obtuvieron una de las puntuaciones más altas con 0.6 ± 0.16. Estos hallazgos podrían deberse al dolor de la inyección IP y a las propiedades químicas de la ketamina. Se sabe que la ketamina tiene un pH bajo (3.5 a 4.1) para facilitar la solubilización (113). Sin embargo, después de la administración parenteral, la ketamina se ha asociado con irritación tisular, dolor y daño muscular en el lugar de la inyección (114). En este sentido, la anestesia IP repetida con ketamina + xilacina en ratones aumentó la puntuación de la Mouse Grimace Scale (aproximadamente entre 0.60 y 1.20) y provocó comportamientos similares a la ansiedad (115), mientras que la anestesia con la misma combinación provocó un daño muscular y tisular más severo, así como necrosis en ratas Wistar Han, en comparación con animales que recibieron ketamina + dexmedetomidina (116).

Aunque los cambios histopatológicos no se evaluaron en el presente estudio, las altas puntuaciones observadas en Ti_2 en ambos grupos que recibieron ketamina IP (G_5 y G_6) podrían estar asociadas con las propiedades de la ketamina. Además, Schoell et al. (117) compararon la misma combinación eutanásica utilizando dos vías de administración: retroorbitaria e intravenosa, encontrando que la administración retroorbitaria fue el método de eutanasia más eficiente, al compararlo con el CO_2 y el pentobarbital, reduciendo el tiempo hasta la muerte a 5 s, técnica que también permitió la recolección de muestras post mortem sin afectar el tejido pulmonar.

Durante Ti_2, el otro tratamiento que registró mayores puntuaciones de RGS fue la decapitación (0.6 ± 026). La decapitación es un método muy controversial, no sólo porque puede resultar estéticamente desagradable, sino por el potencial dolor que los animales podrían percibir antes de llegar a inconsciencia (17,118). La decapitación de pequeños roedores es un método aceptable debido a que varios autores han informado de un breve período de conciencia después del desprendimiento de la cabeza de la médula espinal (entre 3 y 15 s) (20). Mediante electroencefalografía (EEG), Gagea-Iurascu y Craig (18) y Mikesha y Klemm (119) mencionan que la actividad cerebral está presente entre 2.7 a 40 s

después de la decapitación, mientras que Cartner et al. (120) reportaron una disminución en la función cortical y los potenciales evocados visuales de ratones en 15 a 20 s y 10 a 15 s, respectivamente. Estos resultados muestran que la decapitación conduce a una rápida disfunción de la actividad cerebral. Además, la pérdida inmediata del flujo sanguíneo al cerebro acelera la hipoxia, anoxia y la posterior muerte (121). Sin embargo, otros estudios han asociado la decapitación con la nocicepción debido a EEG de bajo voltaje y actividad rápida –un signo de percepción consciente del dolor– (19). Asimismo, se ha informado de un aumento en la frecuencia mediana (F50) y la frecuencia del borde espectral (F95) dentro de los primeros 15 s después de la decapitación (21), un cambio en el EEG que indica nocicepción en ratas (122).

La posible presencia de nocicepción a corto plazo, conciencia del dolor y la alta puntuación obtenida en la presente investigación necesitaría estudios adicionales que combinen evaluaciones conductuales, endocrinas, fisiológicas y EEG. Sin embargo, una posible explicación de por qué podría estar presente el dolor podría deberse a la activación de nociceptores periféricos ubicados en la piel y los músculos de la región cervical de los roedores. Aunque la pérdida de conciencia es rápida después de la decapitación, antes del desprendimiento de la cabeza, el daño tisular de dichas estructuras desencadenaría la vía nociceptiva (p. ej., transducción, transmisión, modulación, proyección y percepción) culminando en la percepción del dolor durante unos segundos antes del cese de la señalización nerviosa (24,41,105). No obstante, algunos autores mencionan que la decapitación produce una desconexión anatómica entre el mesencéfalo y los centros cardiorrespiratorios –llevando a la muerte–, por ello, la señalización eléctrica presente tras la decapitación no puede atribuirse únicamente a la nocicepción/dolor ya que la misma actividad EEG se ha encontrado en animales anestesiados sanos o durante el sueño REM (20,49). Por lo tanto, si bien los animales podrían no ser capaces de percibir el dolor dentro de los 3 segundos posteriores a la decapitación, lo que lo convierte en un método de eutanasia compasivo, podría haber un debate sobre el grado de dolor a corto plazo que los roedores de laboratorio podrían sentir debido al daño tisular antes de llegar a la muerte, requiriendo futuros estudios integrales sobre este tema.

En cuanto a los métodos de inhalación (G_2 y G_4), durante el Ti_3, se observó que ambos grupos fueron los únicos que mostraron diferencias significativas, guardando similitud con los cambios encontrados en la IRT. En Ti_2, ambos métodos obtuvieron una puntuación de

0.2 y 0.3, respectivamente, siendo inferior a G_3, G_5 y G_6. Por el contrario, en Ti_3, las puntuaciones de la RGS más altas se encontraron en G_2 (0.9 ± 0.18) y G_4 (1.2 ± 0.20). En este sentido, respecto al CO_2, no existen estudios donde se haya utilizado la RGS durante la eutanasia o anestesia de ratas; sin embargo, algunos estudios en roedores han encontrado que los animales realizan conductas de defensa activa y pasiva cuando se exponen al CO_2 y que están presentes respuestas fisiológicas como bradicardia y aumento de corticosterona (123).

De manera similar a los resultados referentes a la IRT, las alteraciones observadas en las ratas del G_2 pueden deberse a la aversión y dolor asociado con este gas (123). La sugerencia de que la exposición al CO_2 provoca dolor en roedores se ha trasladado de estudios en humanos, en quienes concentraciones entre 50% y 100% se consideraban muy desagradables y dolorosas (118,124). Asimismo, la formación del ácido carbónico se relaciona a irritación de las mucosas (22,68), en donde Leach et al. (62) mencionan que los roedores expuestos al CO_2 aumentan el acicalamiento inducido por estrés como un indicador potencial de irritación, particularmente con altas concentraciones y cámaras precargadas, mientras que Golledge et al. (125) mencionan que el CO_2 al 100% induce la pérdida de actividad cortical cerebral en 39 s; no obstante, esto podría estar asociado con el dolor. Además, la inhalación de CO_2 disminuye el pH (< 7,2), lo que produce acidosis tisular y activación de nociceptores como el ASIC (22), o neuronas nociceptivas polimodales –también conocidas como neuronas nociceptivas de amplio rango dinámico– con concentraciones de CO_2 de hasta el 40% (126,127). Dichos estudios permiten comprender las alteraciones que el efecto del estrés por la exposición al CO_2 y el potencial dolor que este genera en las ratas altera la respuesta térmica de los animales y su expresión facial, como se observó en el presente estudio.

Por otro lado, la eutanasia con isoflurano aumentó significativamente la puntuación de la RGS a 1.2 ± 0.20 durante Ti_3, registrando la puntuación más alta en todos los grupos experimentales durante todos los tiempos de evaluación. No existen estudios que evalúen el isoflurano y la RGS durante la eutanasia; no obstante, los presentes hallazgos con respecto al isoflurano concuerdan con los estudios de Miller et al. (57,58) en ratas y ratones, donde los autores informaron la influencia del anestésico en la RGS. Los autores encontraron que el isoflurano aumentó las puntuaciones de RGS después de 12 minutos de anestesia (57), mientras que en ratones DBA/2, las puntuaciones de MGS aumentaron

en los animales que recibieron el fármaco halogenado (58), resultados que podrían estar asociados con el efecto residual del isoflurano y sus propiedades farmacológicas. Del mismo modo, la exposición única y repetida a anestesia con isoflurano en ratones hembra dio lugar a puntuaciones de RGS más altas en los primeros 30 minutos después de la anestesia (aproximadamente 0.8 y 1.2) y redujo el comportamiento de excavación y la ingesta de alimentos (128). En contraste, Wong et al. (129) determinaron que el CO_2 es más aversivo que el isoflurano, y que la sedación con isoflurano antes de la eutanasia con CO_2 es un refinamiento.

Teniendo en cuenta la literatura disponible sobre la anestesia con isoflurano y la aplicación de la RGS, se necesitan estudios adicionales para determinar si, durante la eutanasia, la alta puntuación de RGS encontrada en ratas G_4 podría estar completamente asociada con el dolor y estrés –como lo sugieren los resultados de la IRT– o podría ser un efecto farmacológico del isoflurano. En este sentido, así como el isoflurano es un potente agente vasodilatador que influye en la respuesta térmica periférica de las ratas (p. ej., disminuye la $T°_{tail}$ por la vasoconstricción compensatoria del organismo), este anestésico también inhibe los receptores de acetilcolina, provocando una relajación muscular dosis-dependiente tanto del músculo esquelético como del liso (71). Esta propiedad podría influir en la inervación de los músculos miméticos utilizados para evaluar la expresión facial en roedores, como se presenta en esta investigación. Por ejemplo, los músculos oculares como el *orbicularis oculi* y el *levator palpebrae superioris* son estructuras que controlan la apertura/cierre de los párpados a través de la inervación del nervio facial y oculomotor (130,131). Dado que la funcionalidad muscular es necesaria para mantener los ojos abiertos, las propiedades miorrelajantes del isoflurano podrían influir en las FAU evaluadas durante el período inmediato posterior al isoflurano. De hecho, algunos autores como Klune et al. (132) y Leung et al. (35) mencionan un periodo de 30 min post laparotomía o 1 hora después de la anestesia quirúrgica con isoflurano para evaluar la RGS sin efectos residuales del isoflurano.

Los hallazgos sobre ambos agentes inhalatorios también se pueden observar en las correlaciones obtenidas en el presente estudio y los posibles efectos nocivos y farmacológicos del CO_2 y el isoflurano. Para todas las FAU, G_2 y G_4 tuvieron una correlación moderada y positiva, lo que significa que la activación de los nociceptores y el nivel de pungencia antes mencionados podrían influir en las puntuaciones de RGS. Asimismo, la correlación moderada negativa encontrada entre G_4 y G_5 podría explicar el efecto que puede

tener el fármaco en la respuesta facial de los animales. En este caso, los animales G_4 se diferenciaron del tratamiento G_5 posiblemente debido a la catalepsia inducida por la ketamina (133).

Por consiguiente, los resultados obtenidos en cuanto a la IRT y la RGS sugieren que los métodos de eutanasia inhalatorios (CO_2 e isoflurano) no sólo pueden generan un estrés y distrés marcado, sino que también podrían activar vías nociceptivas que culminen en la percepción del dolor.

10.6. Tiempo de muerte y hallazgos adicionales

En cuanto al tiempo de muerte, tiempo de LORR, cese de la frecuencia respiratoria y frecuencia cardiaca, los tiempos obtenidos en los seis grupos experimentales están dentro de los tiempos previamente descritos en estudios con pentobarbital (134), CO_2 (135), decapitación (136), isoflurano (7), y ketamina + xilacina (49).

Por último, cabe señalar el patrón distintivo y la diferencia pronunciada entre G_4 y el resto de los grupos experimentales, tanto a nivel térmico como de expresión facial. En primera instancia, $T°_{ocu}$, $T°_{ear}$, $T°_{dor}$ y $T°_{tai}$ de ratas en el G_4 mostró un aumento progresivo en la temperatura superficial desde Ti_2 hasta la muerte de los animales, además de registrar las temperaturas más bajas de Ti_2 a Ti_4, en comparación con los otros cinco grupos. En contraste, los animales de los otros grupos –incluido el G_2– presentaron un aumento de temperatura de Ti_2 a Ti_3 y una posterior disminución en todas las ventanas térmicas. Asimismo, los puntajes más altos en la RGS fueron registrados en el Ti_3 del G_4. En conjunto, esto sugiere que el estrés anestésico (p. ej., cambios bioquímicos y funcionales en respuesta a la pérdida de homeostasis por el fármaco), el potencial dolor percibido, y la respuesta fisiológica desencadenada por el isoflurano es más marcada que otros métodos de eutanasia inhalados, inyectables y físicos. Los resultados actuales coinciden con lo que otros autores han cuestionado con respecto al uso del isoflurano como método de refinamiento de CO_2 (59,72), ya que se deben tomar precauciones al decidir usar isoflurano como único método para generar la muerte de los animales con un mínimo dolor o incomodidad, considerando los efectos residuales del fármaco si se pretende evaluar la expresión facial o cambios conductuales.

10.7. Limitantes del presente estudio

La principal limitante del presente estudio –y un campo de investigación complementaria a la IRT y a la RGS para evaluar métodos de eutanasia– es la falta de monitorización mediante marcadores fisiológicos como NE, ACTH, corticosterona, glucosa y otros parámetros (p. ej., temperatura rectal) que se asocian al estrés y dolor. En este sentido, el dolor tiene una naturaleza multidimensional con componentes sensoriales/discriminativos, afectivos/motivacionales y cognitivos (137). Debido a ello, su evaluación y reconocimiento en animales requiere el estudio exhaustivo de varios parámetros que puedan ayudar a evaluar las diferentes dimensiones del dolor. Por ejemplo, emplear otras escalas de comportamiento (p. ej., escala conductual compuesta, vocalizaciones ultrasónicas) o tecnologías (p. ej., EEG o técnicas electrofisiológicas para medir la actividad de los nociceptores) (56,63). Además, los análisis histológicos también podrían ayudar a identificar los posibles cambios tisulares asociados a una respuesta inflamatoria a diferentes fármacos, aportando información adicional según el método de la eutanasia.

En el presente estudio, la novedosa concepción de la cámara de inducción de anestesia diseñada para permitir lecturas termográficas durante la eutanasia por inhalación es una herramienta valiosa que podría servir para realizar investigaciones futuras en torno a los fármacos de eutanasia, en combinación con otros parámetros fisiológicos, endocrinos y de comportamiento, para contribuir al refinamiento de la investigación con animales.

11. CONCLUSIONES

Con base en los resultados obtenidos tanto de la IRT como la RGS, se puede concluir que el CO_2 y el isoflurano provocan aversión y respuestas mediadas por el estrés y, potencialmente, por dolor durante la eutanasia en ratas.

Un método de eutanasia debe ser aquel que cause la muerte con mínimo dolor e incomodidad. Los hallazgos del presente estudio sugieren que las ratas Wistar pueden percibir dolor a corto plazo durante la aplicación de métodos inyectables (pentobarbital, ketamina + xilacina) y físicos (decapitación) debido a las puntuaciones en la RGS. En contraste, después de la administración de agentes inhalantes se observan marcadas alteraciones tanto a nivel térmico como en expresión facial, particularmente con el isoflurano.

De acuerdo con la IRT, la exposición al isoflurano podría ser un método de eutanasia que cause potencial distrés y dolor por la activación de nociceptores periféricos, pungencia o irritación de las mucosas –variables que no fueron evaluadas en la presente investigación–. Estos efectos deben tenerse en cuenta al decidir utilizar este fármaco como parte de un protocolo de eutanasia. Asimismo, es necesario considerar el efecto farmacológico del isoflurano y otros anestésicos con propiedades miorrelajantes para interpretar objetivamente los cambios en la expresión facial de ratas.

Técnicas de refinamiento como la combinación de ketamina + CO_2 demostraron disminuir las alteraciones térmicas y los puntajes obtenidos en la RGS observadas con el uso exclusivo de CO2, pero se requieren investigaciones adicionales para realizar una evaluación integral de esta alternativa.

Por tanto, la IRT y la RGS se sugieren como herramientas no invasivas para evaluar y reconsiderar algunos de los métodos de eutanasia. Sin embargo, como el dolor y distrés son eventos multidimensionales, la evaluación conductual, endocrina, fisiológica y electrofisiológica debe emplearse en conjunto con ambas técnicas con el fin de establecer los cambios negativos y el nivel de dolor que los procedimientos actuales de eutanasia evocan en modelos animales de investigación.

12. APLICACIONES PRÁCTICAS

Los hallazgos de la presente investigación en cuanto al efecto que los métodos de eutanasia generan en ratas Wistar pueden ser aplicados en bioterios y laboratorios a nivel nacional e internacional con el fin de disminuir el distrés, nocicepción y dolor durante el periodo de eutanasia.

Actualmente, el refinamiento de los procedimientos a los que son expuestos los animales empleados en la investigación requiere que prácticas rutinarias sean evaluadas y, a partir de los resultados, se propongan alternativas. Por ejemplo, los resultados de la IRT y la RGS mostraron que el isoflurano no se puede recomendar como método de refinamiento para la inhalación de CO_2. Asimismo, la combinación de ketamina + CO_2 mostró disminuir los puntajes de la RGS y minimizar las alteraciones térmicas de los animales que recibieron únicamente CO_2, pudiendo sugerirse como una alternativa. Estas observaciones podrían ser aplicadas en otros bioterios para mejorar la calidad de muerte de los roedores de laboratorio, considerando siempre el objetivo del protocolo experimental y el efecto que el método de eutanasia tendrá sobre los parámetros que se requieran evaluar.

Debido a que este es el primer trabajo que emplea la IRT como un método no invasivo para evaluar la respuesta térmica asociada a cambios vasomotores debido a la activación simpática, la IRT podría implementarse como una herramienta complementaria para evaluar el estrés/distrés en otras condiciones (p. ej., estrés por calor). Asimismo, la evaluación del dolor e incluso la detección de enfermedades pueden ser otros campos en los que se podrían aplicar las imágenes térmicas junto con la RGS, biomarcadores y otras tecnologías destinadas a mejorar el bienestar de los animales de laboratorio [24,29–31,73].

13. PERSPECTIVAS

El refinamiento de todas las prácticas empleadas en roedores de laboratorio, incluyendo la eutanasia, requiere reconocer que el distrés, nocicepción y dolor pueden estar presentes durante el proceso. En el presente estudio, la combinación de ketamina + CO_2 del G_6 disminuyó las alteraciones observadas con el uso del CO_2 solo (G_2), tanto a nivel térmico como en la expresión facial, lo cual puede ser considerado para investigaciones futuras. Una razón por la cual la adición de ketamina sugiere que es una ventaja puede deberse a las propiedades sedantes de la ketamina antes de la exposición al CO_2, que antagoniza los receptores NMDA, modula la actividad neuronal y reduce la sensación de malestar con el CO_2, previeniendo la sensibilización central (138,139). Esto podría disminuir las respuestas fisiológicas ocasionadas por hipoxia, acidosis y cambios relacionados con el estrés (15,22). De igual forma, el mecanismo de acción de la ketamina previene la señalización neuronal desde las neuronas de segundo orden a las estructuras supraespinales, disminuyendo la actividad talamocortical, límbica y reticular (regiones necesarias para el reconocimiento consciente del dolor) (140,141).

Adicionalmente, los resultados obtenidos sugieren que la aplicación del método de eutanasia podría estar asociado a dolor transitorio debido al daño tisular y posible activación de los nociceptores periféricos al utilizar métodos físicos e inyectables. Algunos autores han propuesto la administración oral de premedicación antes del método de eutanasia para refinar la técnica. Por ejemplo, Rodríguez-Sánchez et al. (142) recomendaron el uso oral de tiletamina + zolazepam antes de la eutanasia para inducir sedación y disminuir la aversión. No obstante, Dudley y Boivin (143) encontraron en ratones que una solución de pentobarbital ingerida por vía oral en masa para galletas no indujo LORR, pérdida del conocimiento o muerte. De ahí que, evaluando a través del RGS y otros marcadores relacionados con el dolor, la aplicación de estrategias novedosas con los métodos de eutanasia actuales podría ayudar a realizar una evaluación completa y objetiva. No obstante, hay que reconocer el efecto que los anestésicos (p. ej., pentobarbital e isoflurano) podrían tener sobre la expresión facial y el control de los músculos miméticos, lo que podría limitar la evaluación conductual de los fármacos durante la eutanasia (48,57).

Otro elemento que debe considerarse en futuras investigaciones es el peso de los roedores cuando se utiliza la IRT debido a que la respuesta térmica puede diferir según las reservas de energía y actividad metabólica (p. ej., la obesidad en los mamíferos se asocia con mayores depósitos de tejido adiposo) (144). Además, los estudios han demostrado que rasgos externos como el color del pelaje o el tipo de pelaje pueden afectar la cantidad de calor irradiado (145). En el presente estudio se utilizaron únicamente ratas Wistar de manto blanco; sin embargo, cuando se utiliza IRT en otras cepas o especies, es necesario abordar estos rasgos para interpretar objetivamente las imágenes térmicas.

Respecto a las diferentes cepas de ratas y roedores que actualmente se emplean en la ciencia, en el presente estudio sólo se emplearon ratas Wistar. No obstante, algunos estudios han mostrado diferencias en las respuestas conductuales asociadas con el dolor entre diferentes cepas de ratas (p. ej., Sprague Dawley y Wistar) (48). Winter et al. (146) encontraron diferencias significativas entre ratas Sprague Dawley, Wistar, Long Evans y Wistar-Kyoto expuestas al CO_2. El comportamiento de “freezing” o permanecer inmóvil frente a un estresor fue más frecuente en las ratas Long Evans y Wistar/Kyoto, mientras que las respuestas conductuales como la crianza y el aseo también dependieron de la cepa, así como el grado de expresión de neuronas serotoninérgicas, noradrenérgicas y dopaminérgicas. Así, estos resultados muestran que la evaluación del dolor y distrés requiere considerar la cepa y las posibles implicaciones al seleccionar el método de eutanasia, no sólo desde una perspectiva fisiológica y endocrina sino como parte de una evaluación integral.

14. REFERENCIAS

1. Hobson-West P, Davies A. Societal Sentience: constructions of the public in animal research policy and practice. *Sci Technol Hum Values* (2018) **43**:671–693. doi: 10.1177/0162243917736138

2. Domínguez-Oliva A, Hernández-Ávalos I, Martínez-Burnes J, Olmos-Hernández A, Verduzco-Mendoza A, Mota-Rojas D. The Importance of Animal Models in Biomedical Research: Current Insights and Applications. *Animals* (2023) **13**:1223. doi: 10.3390/ani13071223

3. Laferriere CA, Pang DS. Review of Intraperitoneal Injection of Sodium Pentobarbital as a Method of Euthanasia in Laboratory Rodents. *J Am Assoc Lab Anim Sci* (2020) **59**:254–263. doi: 10.30802/AALAS-JAALAS-19-000081

4. Matsuda Y. Recent Trends in the Number of Laboratory Animals Used in Japan. *Altern to Lab Anim* (2004) **32**:299–301. doi: 10.1177/026119290403201s48

5. National Association for Biomedical Research. The Importance of Animal Testing in Biomedical Research. (2021) https://www.nabr.org/biomedical-research/importance-biomedical-research [Accessed August 17, 2021]

6. Raja SN, Carr DB, Cohen M, Finnerup NB, Flor H, Gibson S, Keefe FJ, Mogil JS, Ringkamp M, Sluka KA, et al. The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. *Pain* (2020) **161**:1976–1982. doi: 10.1097/j.pain.0000000000001939

7. Marquardt N, Feja M, Hünigen H, Plendl J, Menken L, Fink H, Bert B. Euthanasia of laboratory mice: Are isoflurane and sevoflurane real alternatives to carbon dioxide? *PLoS One* (2018) **13**:1–29. doi: 10.1371/journal.pone.0203793

8. Franco NH. Animal Experiments in Biomedical Research: A Historical Perspective. *Anim an Open Access J from MDPI* (2013) **3**:238. doi: 10.3390/ANI3010238

9. Baumans V. Use of animals in experimental research: an ethical dilemma? *Gene Ther* (2004) **11**:S64–S66. doi: 10.1038/sj.gt.3302371

10. Descovich K. Facial expression: An under-utilised tool for the assessment of welfare

in mammals. *ALTEX* (2017) **34**:409–29. doi: 10.14573/altex.1607161

11. Akimovna E, Rivera B, Carbone C, Hernández GR, Baamonde JM. “Laboratory animal science legislation in Latin America.,” In: Guillén J, editor. *Laboratory Animals. Regulations and Recommendations for Global Collaborative Research*. Elsevier (2014). p. 91-116.

12. NOM-062-ZOO-1999 S. Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. (2001) http://publico.senasica.gob.mx/?doc=743 [Accessed September 30, 2022]

13. Juárez-Portilla C, Zepeda-Hernández RC, Sánchez-Salcedo JA, Flores-Muñoz M, López-Franco Ó, Cortés-Sol A. El uso de los animales en la investigación y en la enseñanza: lineamientos y directrices para su manejo. *Rev Eduscientia Divulg la Cienc Educ* (2019) **2**:4–19.

14. Andersen ML, Winter LMF. Animal models in biological and biomedical research - experimental and ethical concerns. *An Acad Bras Cienc* (2019) **91**:e20170238. doi: 10.1590/0001-3765201720170238

15. Flecknell P. *Laboratory Animal Anaesthesia*. 4th ed. Kidlington, England: Elsevier (2016). 1.

16. Dutton JW, Artwohl JE, Huang X, Fortman JD. Assessment of Pain Associated with the Injection of Sodium Pentobarbital in Laboratory Mice (Mus musculus). *J Am Assoc Lab Anim Sci* (2019) **58**:373–379. doi: 10.30802/AALAS-JAALAS-18-000094

17. Turner MD. “The Most Gentle of Lethal Methods”: The Question of Retained Consciousness Following Decapitation. *Cureus* (2023) **15**:e33830. doi: 10.7759/cureus.33830

18. Gagea-Iurascu M, Craig S. “Euthanasia and Necropsy.,” In: Suckow MA, Stevens KA, Wilson RP, editors. *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents*. San Diego, USA: Academic Press (2012). p. 117–139 doi: 10.1016/B978-0-12-380920-9.00004-3

19. Derr R. Pain perception in decapitated rat brain. *Life Sci* (1991) **49**:1399–1402. doi: 10.1016/0024-3205(91)90391-N

20. Holson RR. Euthanasia by decapitation: Evidence that this technique produces prompt, painless unconsciousness in laboratory rodents. *Neurotoxicol Teratol* (1992) **14**:253–257. doi: 10.1016/0892-0362(92)90004-T

21. Kongara K, McIlhone A, Kells N, Johnson C. Electroencephalographic evaluation of decapitation of the anaesthetized rat. *Lab Anim* (2014) **48**:15–19. doi: 10.1177/0023677213502016

22. Boivin GP, Hickman DL, Creamer-Hente MA, Pritchett-Corning KR, Bratcher NA. Review of CO_2 as a Euthanasia Agent for Laboratory Rats and Mice. *J Am Assoc Lab Anim Sci* (2017) **56**:491–499. http://www.ncbi.nlm.nih.gov/pubmed/28903819

23. De la Torre C., De la Torre C., Jiménez P., Ramos MM., De la Torre G. Anestesia para colonoscopia: anestesia inhalatoria con sevoflurano frente a anestesia intravenosa con propofol. *Sanid Mil* (2012) **68**:27–32.

24. Hernández-Avalos I, Flores-Gasca E, Mota-Rojas D, Casas-Alvarado A, Miranda-Cortés AE, Domínguez-Oliva A. Neurobiology of anesthetic-surgical stress and induced behavioral changes in dogs and cats: A review. *Vet World* (2021) **14**:393–404. doi: 10.14202/vetworld.2021.393-404

25. Huggins J, Rakobowchuk M. Utility of lacrimal caruncle infrared thermography when monitoring alterations in autonomic activity in healthy humans. *Eur J Appl Physiol* (2019) **119**:531–538. doi: 10.1007/s00421-018-4041-6

26. Mota-Rojas D, Olmos-Hernández A, Verduzco-Mendoza A, Lecona-Butrón H, Martínez-Burnes J, Mora-Medina P, Gómez-Prado J, Orihuela A. Infrared thermal imaging associated with pain in laboratory animals. *Exp Anim* (2021) **70**:1–12. doi: 10.1538/expanim.20-0052

27. Redaelli V, Papa S, Marsella G, Grignaschi G, Bosi A, Ludwig N, Luzi F, Vismara I, Rimondo S, Veglianese P, et al. A refinement approach in a mouse model of rehabilitation research. Analgesia strategy, reduction approach and infrared thermography in spinal cord injury. *PLoS One* (2019) **14**:e0224337. doi: 10.1371/journal.pone.0224337

28. Gariepy C, Amiot J, Nadai S. Ante-mortem detection of PSE and DFD by infrared thermography of pigs before stunning. *Meat Sci* (1989) **25**:37–41. doi:

10.1016/0309-1740(89)90064-8

29. Flores-Peinado S, Mota-Rojas D, Guerrero-Legarreta I, Mora-Medina P, Cruz-Monterrosa R, Gómez-Prado J, Guadalupe Hernández M, Cruz-Playas J, Martínez-Burnes J. Physiological responses of pigs to preslaughter handling: infrared and thermal imaging applications. *Int J Vet Sci Med* (2020) **8**:71–84. doi: 10.1080/23144599.2020.1821574

30. Deuis JR, Dvorakova LS, Vetter I. Methods Used to Evaluate Pain Behaviors in Rodents. *Front Mol Neurosci* (2017) **10**:284. doi: 10.3389/fnmol.2017.00284

31. Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, Mapplebeck JC, Wei P, Zhan S, Zhang S, et al. The Rat Grimace Scale: A Partially Automated Method for Quantifying Pain in the Laboratory Rat via Facial Expressions. *Mol Pain* (2011) **7**:55. doi: 10.1186/1744-8069-7-55

32. Sperry MM, Yu Y-HH, Welch RL, Granquist EJ, Winkelstein BA. Grading facial expression is a sensitive means to detect grimace differences in orofacial pain in a rat model. *Sci Rep* (2018) **8**:13894. doi: 10.1038/s41598-018-32297-2

33. Oliver V, De Rantere D, Ritchie R, Chisholm J, Hecker KG, Pang DSJ. Psychometric Assessment of the Rat Grimace Scale and Development of an Analgesic Intervention Score. *PLoS One* (2014) **9**:e97882. doi: 10.1371/journal.pone.0097882

34. McLennan KM, Miller AL, Dalla Costa E, Stucke D, Corke MJ, Broom DM, Leach MC. Conceptual and methodological issues relating to pain assessment in mammals: The development and utilisation of pain facial expression scales. *Appl Anim Behav Sci* (2019) **217**:1–15. doi: 10.1016/j.applanim.2019.06.001

35. Leung V, Zhang E, Pang DS. Real-time application of the Rat Grimace Scale as a welfare refinement in laboratory rats. *Sci Reports 2016 61* (2016) **6**:1–12. doi: 10.1038/srep31667

36. Hubrecht, Carter. The 3Rs and Humane Experimental Technique: Implementing Change. *Animals* (2019) **9**:754. doi: 10.3390/ani9100754

37. Carbone L. *Euthanasia and Laboratory Animal Welfare*. Elsevier (2013). 157–169 p. doi: 10.1016/B978-0-12-385103-1.00011-7

38. Shomer NH, Worthington KHA, Hickman DL, Jonnalagadda M, Newsome JT, Slate AR, Valentine H, Williams AM, Wilkinson M. Review of rodent euthanasia methods. *J Am Assoc Lab Anim Sci* (2020) **59**:242–253. doi: 10.30802/AALAS-JAALAS-19-000084

39. AVMA. *AVMA GUIDELINES FOR THE EUTHANASIA OF ANIMALS: 2020 EDITION*. (2020). 127–143 p. doi: 10.1163/9789004294530_006

40. Verduzco-Mendoza A, Bueno-Nava A, Wang D, Martínez-Burnes J, Olmos-Hernández A, Casas A, Domínguez A, Mota-Rojas D. Experimental Applications and Factors Involved in Validating Thermal Windows Using Infrared Thermography to Assess the Health and Thermostability of Laboratory Animals. *Animals* (2021) **11**:3448. doi: 10.3390/ani11123448

41. Mota-Rojas D, Olmos-Hernández A, Verduzco-Mendoza A. The utility of grimace escales for practical pain assessment in laboratory animals. *Animals* (2020) **10**:1–17.

42. Diario Oficial de la Federación. Normal Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. (2001) https://www.gob.mx/cms/uploads/attachment/file/203498/NOM-062-ZOO-1999_220801.pdf

43. Kovalcsik R, Devlin T, Loux S, Martinek M, May J, Pickering T, Tapp R, Wilson S, Serota D. Animal reuse: balancing scientific integrity and animal welfare. *Lab Anim (NY)* (2006) **35**:49–53. doi: 10.1038/laban1006-49

44. Marcotte M, Bernardo A, Linga N, Pérez-Romero CA, Guillou J-L, Sibille E, Prevot TD. Handling Techniques to Reduce Stress in Mice. *J Vis Exp* (2021) **175**:e62593. doi: 10.3791/62593

45. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research*. *J Cereb Blood Flow Metab* (2020) **40**:1769–1777. doi: 10.1177/0271678X20943823

46. Lofgren JL, Foley PL, Golledge HDR. “Anesthesia, analgesia, and euthanasia.,” In: Suckow MA, Hankenson R., Wilson R., Foley PL, editors. *The Laboratory Rat*.

London, UK: Academic Press (2020). p. 699–745 doi: 10.1016/C2017-0-01188-6

47. Villano J, Sharp PE. *The laboratory rat*. 2nd ed. Boca Raton, FL, USA: CRC Press (2013). 186–190 p.

48. Reimer JN, Schuster CJ, Knight CG, Pang DSJ, Leung VSY. Intraperitoneal injection of sodium pentobarbital has the potential to elicit pain in adult rats (Rattus norvegicus). *PLoS One* (2020) **15**:e0238123. doi: 10.1371/JOURNAL.PONE.0238123

49. American Veterinary Medical Association (AVMA). *AVMA Guidelines for the Euthanasia of Animals*. Schaumburg, IL: AVMA (2020). 1–121 p. doi: 10.1163/9789004294530_006

50. Risling TE, Caulkett NA, Florence D. Open-drop anesthesia for small laboratory animals. *Can Vet J = La Rev Vet Can* (2012) **53**:299–302. http://www.ncbi.nlm.nih.gov/pubmed/22942448

51. de Brito CF, Evangelista AA, Felippe RM, Cascabulho C, Fragoso VM, de Oliveira GM. Laboratory Mice Euthanasia: Speed Death and Animal Welfare. *Am J Biomed Sci Res* (2020) **8**:341–355. doi: 10.34297/AJBSR.2020.08.001300

52. McDougall SA, Park GI, Ramirez GI, Gomez V, Adame BC, Crawford CA. Sex-dependent changes in ketamine-induced locomotor activity and ketamine pharmacokinetics in preweanling, adolescent, and adult rats. *Eur Neuropsychopharmacol* (2019) **29**:740–755. doi: 10.1016/j.euroneuro.2019.03.013

53. Ko MJ, Mulia GE, van Rijn RM. Commonly Used Anesthesia/Euthanasia Methods for Brain Collection Differentially Impact MAPK Activity in Male and Female C57BL/6 Mice. *Front Cell Neurosci* (2019) **13**: doi: 10.3389/fncel.2019.00096

54. Whittaker AL, Leach MC, Preston FL, Lymn KA, Howarth GS. Effects of acute chemotherapy-induced mucositis on spontaneous behaviour and the grimace scale in laboratory rats. *Lab Anim* (2016) **50**:108–118. doi: 10.1177/0023677215595554

55. Grandin T. Assessment of stress during handling and transport. *J Anim Sci* (1997) **75**:249. doi: 10.2527/1997.751249x

56. Nichols KE, Holliday-White KL, Bogie HM, Swearingen KM, Fine MS, Doyle J,

Tiesma SR. Cardiovascular and Metabolic Responses to Carbon Dioxide Euthanasia in Conscious and Anesthetized Rats. *J Am Assoc Lab Anim Sci* (2020) **59**:742–749. doi: 10.30802/AALAS-JAALAS-19-000166

57. Miller A, Golledge H, Leach M. The influence of isoflurane anaesthesia on the rat grimace scale. *PLoS One* (2016) **11**:e0166652.

58. Miller A, Kitson G, Skalkoyannis B, Leach M. The effect of isoflurane anaesthesia and buprenorphine on the mouse grimace scale and behaviour in CBA and DBA/2 mice. *Appl Anim Behav Sci* (2015) **172**:58–62. doi: 10.1016/j.applanim.2015.08.038

59. Kulkarni S, Hickman D. Isoflurane and Carbon Dioxide Elicit Similar Behavioral Responses in Rats. *Animals* (2020) **10**:1431. doi: 10.3390/ani10081431

60. Makowska IJ, Weary DM. Rat aversion to induction with inhalant anaesthetics. *Appl Anim Behav Sci* (2009) **119**:229–235. doi: 10.1016/j.applanim.2009.04.003

61. Xu F, Uh J, Brier MR, Hart J, Yezhuvath US, Gu H, Yang Y, Lu H. The Influence of Carbon Dioxide on Brain Activity and Metabolism in Conscious Humans. *J Cereb Blood Flow Metab* (2011) **31**:58–67. doi: 10.1038/jcbfm.2010.153

62. Leach MC, Bowell VA, Allan TF, Morton DB. Degrees of aversion shown by rats and mice to different concentrations of inhalational anaesthetics. *Vet Rec* (2002) **150**:808–815. doi: 10.1136/vr.150.26.808

63. Hickman DL. Interpreting Neuroendocrine Hormones, Corticosterone, and Blood Glucose to Assess the Wellbeing of Anesthetized Rats during Euthanasia. *J Am Assoc Lab Anim Sci* (2018) **57**:725–728. doi: 10.30802/AALAS-JAALAS-17-000159

64. Borovsky V, Herman M, Dunphy G, Caplea A, Ely D. CO 2 asphyxia increases plasma norepinephrine in rats via sympathetic nerves. *Am J Physiol Integr Comp Physiol* (1998) **274**:R19–R22. doi: 10.1152/ajpregu.1998.274.1.R19

65. Khokhlova ON, Borozdina NA, Sadovnikova ES, Pakhomova IA, Rudenko PA, Korolkova Y V., Kozlov SA, Dyachenko IA. Comparative Study of the Aftereffect of CO2 Inhalation or Tiletamine–Zolazepam–Xylazine Anesthesia on Laboratory Outbred Rats and Mice. *Biomedicines* (2022) **10**:512. doi: 10.3390/biomedicines10020512

66. Hickman D, Fitz S, Bernabe C, Caliman I, Haulcomb M, Federici L, Shekhar A, Johnson P. Evaluation of Low versus High Volume per Minute Displacement CO2 Methods of Euthanasia in the Induction and Duration of Panic-Associated Behavior and Physiology. *Animals* (2016) **6**:45. doi: 10.3390/ani6080045

67. Powell K, Ethun K, Taylor DK. The effect of light level, CO2 flow rate, and anesthesia on the stress response of mice during CO2 euthanasia. *Lab Anim (NY)* (2016) **45**:386–395. doi: 10.1038/laban.1117

68. Turner P V., Hickman DL, van Luijk J, Ritskes-Hoitinga M, Sargeant JM, Kurosawa TM, Agui T, Baumans V, Choi WS, Choi Y-K, et al. Welfare Impact of Carbon Dioxide Euthanasia on Laboratory Mice and Rats: A Systematic Review. *Front Vet Sci* (2020) **7**:411. doi: 10.3389/fvets.2020.00411

69. Hackbarth H, Küppers N, Bohnet W. Euthanasia of rats with carbon dioxide-animal welfare aspects. *Lab Anim* (2000) **34**:91–96. doi: 10.1258/002367700780578055

70. Hampton A. “Euthanizing agents.,” In: Riviere J, Papich M, editors. *Veterinary Pharmacology & Therapeutics*. Hoboken, NJ, USA: Wiley-Blackwell (2018). p. 387–394

71. Sio LCO, Varbanova M, Bautista A. “Isoflurane: Mechanisms and applications.,” In: Rajendram R, Patel VB, Preedy VR, Martin CR, editors. *Treatments, Mechanisms, and Adverse Reactions of Anesthetics and Analgesics*. London, UK: Academic Press (2022). p. 101–108 doi: 10.1016/B978-0-12-820237-1.00011-9

72. Frost K, Shah M, Leung VSY, Pang DSJ. Aversion to Desflurane and Isoflurane in Sprague-Dawley Rats (Rattus norvegicus). *Animals* (2020) **10**:950. doi: 10.3390/ani10060950

73. Boivin GP, Bottomley MA, Schiml PA, Goss L, Grobe N. Physiologic, behavioral, and histologic responses to various euthanasia methods in C57BL76NTac male mice. *J Am Assoc Lab Anim Sci* (2017) **56**:69–78.

74. Valentine H, Williams WO, Maurer KJ. Sedation or inhalant anesthesia before euthanasia with CO2 does not reduce behavioral or physiologic signs of pain and stress in mice. *J Am Assoc Lab Anim Sci* (2012) **51**:50–7. http://www.ncbi.nlm.nih.gov/pubmed/22330868

75. Zardooz H, Rostamkhani F, Zaringhalam J, Faraji S. Plasma corticosterone, insulin and glucose changes induced by brief exposure to isoflurane, diethyl ether and CO2 in male rats. *Physiol Res* (2010) **59**:973–978.

76. Angilletta MJ, Youngblood JP, Neel LK, VandenBrooks JM. The neuroscience of adaptive thermoregulation. *Neurosci Lett* (2019) **692**:127–136. doi: 10.1016/j.neulet.2018.10.046

77. Wright CL, Boulant JA. Carbon dioxide and pH effects on temperature-sensitive and -insensitive hypothalamic neurons. *J Appl Physiol* (2007) **102**:1357–1366. doi: 10.1152/japplphysiol.00303.2006

78. Creamer-Hente MA, Lao FK, Dragos ZP, Waterman LL. Sex- and Strain-related Differences in the Stress Response of Mice to CO 2 Euthanasia. *J Am Assoc Lab Anim Sci* (2018) **57**:513–519. doi: 10.30802/AALAS-JAALAS-18-000005

79. Moffitt AD, Brignolo LL, Ardeshir A, Creamer-Hente MA. The Role of Emotional Contagion in the Distress Exhibited by Grouped Mice Exposed to CO 2. *J Am Assoc Lab Anim Sci* (2019) **58**:430–437. doi: 10.30802/AALAS-JAALAS-18-000098

80. Altholtz LY, Fowler KA, Badura LL, Kovacs MS. Comparison of the stress response in rats to repeated isoflurane or CO2:O2 anesthesia used for restraint during serial blood collection via the jugular vein. *J Am Assoc Lab Anim Sci* (2006) **45**:17–22.

81. Nazari S, Kourosh-Arami M, Komaki A, Hajizadeh S. Relative contribution of central and peripheral factors in superficial blood flow regulation following cold exposure. *Physiol Pharmacol* (2020) **24**:89–100. doi: 10.32598/ppj.24.2.50

82. Sutherland MA, Worth GM, Dowling SK, Lowe GL, Cave VM, Stewart M. Evaluation of infrared thermography as a non-invasive method of measuring the autonomic nervous response in sheep. *PLoS One* (2020) **15**:e0233558. doi: 10.1371/journal.pone.0233558

83. Casas-Alvarado A, Mota- Rojas D, Hernández- Avalos I, Martínez- Burnes J, Rosas M, Miranda-Cortés AE, Domínguez- Oliva A, Mora- Medina P. Assessment of thermal response, cardiorespiratory parameters and postoperative analgesia in dogs undergoing ovariohysterectomy with different combinations of epidural anesthesia and isoflurane. *J Anim Behav Biometeorol* (2023)

84. Vogel B, Wagner H, Gmoser J, Wörner A, Löschberger A, Peters L, Frey A, Hofmann U, Frantz S. Touch-free measurement of body temperature using close-up thermography of the ocular surface. *MethodsX* (2016) **3**:407–416. doi: 10.1016/j.mex.2016.05.002

85. Gjendal K, Franco NH, Ottesen JL, Sørensen DB, Olsson IAS. Eye, body or tail? Thermography as a measure of stress in mice. *Physiol Behav* (2018) **196**:135–143. doi: 10.1016/j.physbeh.2018.08.022

86. Zevgolis YG, Zannetos SP, Akriotis T. *Physiological response of a wild rodent to experimental manipulations in its natural environment using infrared thermography*. (2022). doi: 10.4404/hystrix-00478-2021

87. Vianna DML, Carrive P. Changes in cutaneous and body temperature during and after conditioned fear to context in the rat. *Eur J Neurosci* (2005) **21**:2505–2512. doi: 10.1111/j.1460-9568.2005.04073.x

88. Nijland N. The influence of different types of behaviour on the eye temperature of mice using infrared thermograohy. Utrecht University (2021). 1–25 p.

89. Wirth S, Gebhardt-Henrich S, Riemer S, Hattendorf J, Zinsstag J, Hediger K. The influence of human interaction on guinea pigs: Behavioral and thermographic changes during animal-assisted therapy. *Physiol Behav* (2020) **225**:113076. doi: 10.1016/j.physbeh.2020.113076

90. Wongsaengchan C, McCafferty DJ, Evans NP, McKeegan DEF, Nager RG. Body surface temperature of rats reveals both magnitude and sex differences in the acute stress response. *Physiol Behav* (2023) **264**:114138. doi: 10.1016/j.physbeh.2023.114138

91. Insler SR, Sessler DI. Perioperative Thermoregulation and Temperature Monitoring. *Anesthesiol Clin North America* (2006) **24**:823–837. doi: 10.1016/j.atc.2006.09.001

92. Wokke ES. Refinement: Evaluating stress and accuracy of different intraperitoneal techniques in mice. (2017).

93. Jaén-Téllez JA, Sánchez-Guerrero MJ, López-Campos JI, Valera M, González-Redondo P. Acute stress assessment using infrared thermography in fattening rabbits reacting to handling under winter and summer conditions. *Spanish J Agric*

Res (2020) **18**:e0502. doi: 10.5424/sjar/2020182-15706

94. Hutu I, Paras I, Gherghel D, Lungu B, Mircu C. Application of infrared thermography in rabbit orthopaedic models. Romania (2018). 9–14 p.

95. Xu Z, Agbigbe O, Nigro N, Yakobi G, Shapiro J, Ginosar Y. Use of high-resolution thermography as a validation measure to confirm epidural anesthesia in mice: a cross-over study. *Int J Obstet Anesth* (2021) **46**:102981. doi: 10.1016/j.ijoa.2021.102981

96. Lezama-García K, Mota-Rojas D, Martínez-Burnes J, Villanueva-García D, Domínguez-Oliva A, Gómez-Prado J, Mora-Medina P, Casas-Alvarado A, Olmos-Hernández A, Soto P, et al. Strategies for Hypothermia Compensation in Altricial and Precocial Newborn Mammals and Their Monitoring by Infrared Thermography. *Vet Sci* (2022) **9**:246. doi: 10.3390/vetsci9050246

97. Blenkuš U, Gerós AF, Carpinteiro C, Aguiar P de C, Olsson IAS, Franco NH. Non-Invasive Assessment of Mild Stress-Induced Hyperthermia by Infrared Thermography in Laboratory Mice. *Animals* (2022) **12**:177. doi: 10.3390/ani12020177

98. Miyazono S, Hasegawa K, Miyazaki S, Sakakima H, Konno S, Meguro S, Sasajima H, Noguchi T, Osada K, Kashiwayanagi M. Etizolam attenuates the reduction in cutaneous temperature induced in mice by exposure to synthetic predator odor. *Eur J Pharmacol* (2018) **824**:157–162. doi: 10.1016/j.ejphar.2018.02.015

99. Kataoka N, Shima Y, Nakajima K, Nakamura K. A central master driver of psychosocial stress responses in the rat. *Science (80-)* (2020) **367**:1105–1112. doi: 10.1126/science.aaz4639

100. Owens NC, Ootsuka Y, Kanosue K, McAllen RM. Thermoregulatory Control of Sympathetic Fibres Supplying the Rat's Tail. *J Physiol* (2002) **543**:849–858. doi: 10.1113/jphysiol.2002.023770

101. Weitkamp J. Effect of tickling and gentling on eye and tail temperature of laboratory rats during manual restraint, using infrared thermography. (2020) http://dspace.library.uu.nl/handle/1874/395860 [Accessed September 28, 2021]

102. Lecorps B, Rödel HG, Féron C. Assessment of anxiety in open field and elevated

plus maze using infrared thermography. *Physiol Behav* (2016) **157**:209–216. doi: 10.1016/j.physbeh.2016.02.014

103. Munting LP, Derieppe MPP, Suidgeest E, Denis de Senneville B, Wells JA, Weerd L. Influence of different isoflurane anesthesia protocols on murine cerebral hemodynamics measured with pseudo-continuous arterial spin labeling. *NMR Biomed* (2019) **32**:e4105. doi: 10.1002/nbm.4105

104. Watson CRR, Sakai S, Armstrong W. Organization of the Facial Nucleus in the Rat. *Brain Behav Evol* (1982) **20**:19–28. doi: 10.1159/000121578

105. Domínguez-Oliva A, Mota-Rojas D, Hernández-Avalos I, Mora-Medina P, Olmos-Hernández A, Verduzco-Mendoza A, Casas-Alvarado A, Whittaker AL. The neurobiology of pain and facial movements in rodents: Clinical applications and current research. *Front Vet Sci* (2022) **9**:1016720. doi: 10.3389/fvets.2022.1016720

106. Ballotta D, Lui F, Porro CA, Nichelli PF, Benuzzi F. Modulation of neural circuits underlying temporal production by facial expressions of pain. *PLoS One* (2018) **13**:e0193100. doi: 10.1371/journal.pone.0193100

107. Freedman DJ, Ibos G. An Integrative Framework for Sensory, Motor, and Cognitive Functions of the Posterior Parietal Cortex. *Neuron* (2018) **97**:1219–1234. doi: 10.1016/j.neuron.2018.01.044

108. Gothard KM. The amygdalo-motor pathways and the control of facial expressions. *Front Neurosci* (2014) **8**:43. doi: 10.3389/fnins.2014.00043

109. Khoo SY-S, Lay BPP, Joya J, McNally GP. Local anaesthetic refinement of pentobarbital euthanasia reduces abdominal writhing without affecting immunohistochemical endpoints in rats. *Lab Anim* (2018) **52**:152–162. doi: 10.1177/0023677217721260

110. Akintola T, Raver C, Studlack P, Uddin O, Masri R, Keller A. The grimace scale reliably assesses chronic pain in a rodent model of trigeminal neuropathic pain. *Neurobiol Pain* (2017) **2**:13–17. doi: 10.1016/j.ynpai.2017.10.001

111. Svendsen O, Kok L, Lauritzenã B. Nociception after intraperitoneal injection of a sodium pentobarbitone formulation with and without lidocaine in rats quantified by expression of neuronal c-fos in the spinal cord – a preliminary study. *Lab Anim*

(2007) **41**:197–203. doi: 10.1258/002367707780378140

112. Shimizu S. “Routes of administration.,” In: Hedrich HJ, Bullock G, editors. *The Laboratory Mouse*. London, UK: Elsevier (2004). p. 527–541

113. Mion G, Villevieille T. Ketamine Pharmacology: An Update (Pharmacodynamics and Molecular Aspects, Recent Findings). *CNS Neurosci Ther* (2013) **19**:370–380. doi: 10.1111/cns.12099

114. Sun F, Wright D, Pinson DM. Comparison of ketamine versus combination of ketamine and medetomidine in injectable anesthetic protocols: Chemical immobilization in macaques and tissue reaction in rats. *J Am Assoc Lab Anim Sci* (2003) **42**:32–37.

115. Hohlbaum K, Bert B, Dietze S, Palme R, Fink H, Thöne-Reineke C. Impact of repeated anesthesia with ketamine and xylazine on the well-being of C57BL/6JRj mice. *PLoS One* (2018) **13**:e0203559. doi: 10.1371/journal.pone.0203559

116. Wellington D, Mikaelian I, Singer L. Comparison of ketamine-xylazine and ketamine-dexmedetomidine anesthesia and intraperitoneal tolerance in rats. *J Am Assoc Lab Anim Sci* (2013) **52**:481–7. http://www.ncbi.nlm.nih.gov/pubmed/23849447

117. Schoell AR, Heyde BR, Weir DE, Chiang P-C, Hu Y, Tung DK. Euthanasia method for mice in rapid time-course pulmonary pharmacokinetic studies. *J Am Assoc Lab Anim Sci* (2009) **48**:506–11. http://www.ncbi.nlm.nih.gov/pubmed/19807971

118. Clarkson JM, Martin JE, McKeegan DEF. A review of methods used to kill laboratory rodents: issues and opportunities. *Lab Anim* (2022) **56**:419–436. doi: 10.1177/00236772221097472

119. Mikeska JA, Klemm WR. EEG evaluation of humaneness of asphyxia and decapitation euthanasia of the laboratory rat. *Lab Anim Sci* (1975) **25**:175–179.

120. Cartner SC, Barlow SC, Ness TJ. Loss of cortical function in mice after decapitation, cervical dislocation, potassium chloride injection, and CO2 inhalation. *Comp Med* (2007) **57**:570–3. http://www.ncbi.nlm.nih.gov/pubmed/18246869

121. Pierozan P, Jernerén F, Ransome Y, Karlsson O. The Choice of Euthanasia Method Affects Metabolic Serum Biomarkers. *Basic Clin Pharmacol Toxicol* (2017)

121:113–118. doi: 10.1111/BCPT.12774

122. Singh P, Kongara K, Harding D, Ward N, Dukkipati VSR, Johnson C, Chambers P. Comparison of electroencephalographic changes in response to acute electrical and thermal stimuli with the tail flick and hot plate test in rats administered with opiorphin. *BMC Neurol* (2018) **18**:43. doi: 10.1186/s12883-018-1047-y

123. Améndola L, Weary DM. Understanding rat emotional responses to CO2. *Transl Psychiatry* (2020) **10**:253. doi: 10.1038/s41398-020-00936-w

124. Conlee KM, Stephens ML, Rowan AN, King LA. Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats. *Lab Anim* (2005) **39**:137–161. doi: 10.1258/0023677053739747

125. Golledge H, Roughan J, Niel L, Richardson C, Wright-Williamson S, Flecknell P. Carbon dioxide euthanasia in rats - Behavioural and autonomic system responses to exposure. *SECAL-ESLAV International Congress*. Elche, Spain (2005)

126. Burkholder TH, Niel L, Weed JL, Brinster LR, Bacher JD, Foltz CJ. Comparison of carbon dioxide and argon euthanasia: effects on behavior, heart rate, and respiratory lesions in rats. *J Am Assoc Lab Anim Sci* (2010) **49**:448–53. http://www.ncbi.nlm.nih.gov/pubmed/20819391

127. Valentim AM, Guedes SR, Pereira AM, Antunes LM. Euthanasia using gaseous agents in laboratory rodents. *Lab Anim* (2016) **50**:241–253. doi: 10.1177/0023677215618618

128. Hohlbaum K, Bert B, Dietze S, Palme R, Fink H, Thöne-Reineke C. Severity classification of repeated isoflurane anesthesia in C57BL/6JRj mice—Assessing the degree of distress. *PLoS One* (2017) **12**:e0179588. doi: 10.1371/journal.pone.0179588

129. Wong D, Makowska IJ, Weary DM. Rat aversion to isoflurane versus carbon dioxide. *Biol Lett* (2013) **9**:20121000. doi: 10.1098/rsbl.2012.1000

130. Tong J, Lopez MJ, Patel BC. *Anatomy, head and neck, eye orbicularis oculi muscle*. Florida, USA: StatPearls Publishing (2023). 1 p.

131. Knight B, Lopez MJ, Patel BC. *Anatomy, Head and Neck: Eye Levator Palpebrae*

Superioris Muscles. Florida, USA: StatPearls Publishing (2023). 1 p.

132. Klune CB, Larkin AE, Leung VSY, Pang D. Comparing the Rat Grimace Scale and a composite behaviour score in rats. *PLoS One* (2019) **14**:e0209467. doi: 10.1371/JOURNAL.PONE.0209467

133. Pai A, Heining M. Ketamine. *Contin Educ Anaesth Crit Care Pain* (2007) **7**:59–63. doi: 10.1093/bjaceaccp/mkm008

134. Zatroch KK, Knight CG, Reimer JN, Pang DSJ. Refinement of intraperitoneal injection of sodium pentobarbital for euthanasia in laboratory rats (Rattus norvegicus). *BMC Vet Res* (2016) **13**:60. doi: 10.1186/s12917-017-0982-y

135. Hickman DL. Minimal Exposure Times for Irreversible Euthanasia with Carbon Dioxide in Mice and Rats. *J Am Assoc Lab Anim Sci* (2022) **61**:283–286. doi: 10.30802/AALAS-JAALAS-21-000113

136. Rijn CM van., Krijnen H, Menting-Hermeling S, Coenen AML. Decapitation in Rats: Latency to Unconsciousness and the 'Wave of Death.' *PLoS One* (2011) **6**:e16514. doi: 10.1371/journal.pone.0016514

137. Larson CM, Wilcox GL, Fairbanks CA. The Study of Pain in Rats and Mice. *Comp Med* (2019) **69**:555–570. doi: 10.30802/AALAS-CM-19-000062

138. Hudetz JA, Pagel PS. Neuroprotection by Ketamine: A Review of the Experimental and Clinical Evidence. *J Cardiothorac Vasc Anesth* (2010) **24**:131–142. doi: 10.1053/j.jvca.2009.05.008

139. Miranda-Cortés AE, Ruiz-García AG, Olivera-Ayub AE, Garza-Malacara G, Ruiz-Cervantes JG, Toscano-Zapien JA, Hernández-Avalos I. Cardiorespiratory effects of epidurally administered ketamine or lidocaine in dogs undergoing ovariohysterectomy surgery: a comparative study. *Iran J Vet Res* (2020) **21**:92–96.

140. Zhang Y, Ye F, Zhang T, Lv S, Zhou L, Du D, Lin H, Guo F, Luo C, Zhu S. Structural basis of ketamine action on human NMDA receptors. *Nature* (2021) **596**:301–305. doi: 10.1038/s41586-021-03769-9

141. Hashimoto K. Rapid-acting antidepressant ketamine, its metabolites and other candidates: A historical overview and future perspective. *Psychiatry Clin Neurosci*

(2019) **73**:613–627. doi: 10.1111/pcn.12902

142. Rodriguez-Sanchez R, Barnaby E, Améndola L, Hea S-Y, Smith B, Webster J, Zobel G. Voluntary Oral Ingestion of a Sedative Prior to Euthanasia with CO2: Behavioural Responses of Mice. *Animals* (2021) **11**:2879. doi: 10.3390/ani11102879

143. Dudley ES, Boivin GP. Evaluation of a Commercially Available Euthanasia Solution as a Voluntarily Ingested Euthanasia Agent in Laboratory Mice. *J Am Assoc Lab Anim Sci* (2018) **57**:30–34. http://www.ncbi.nlm.nih.gov/pubmed/29402349

144. Gómez-Hernández A, Beneit N, Díaz-Castroverde S, Escribano Ó. Differential Role of Adipose Tissues in Obesity and Related Metabolic and Vascular Complications. *Int J Endocrinol* (2016) **2016**:1–15. doi: 10.1155/2016/1216783

145. Mota-Rojas D, Titto CG, de Mira Geraldo A, Martínez-Burnes J, Gómez J, Hernández-Ávalos I, Casas A, Domínguez A, José N, Bertoni A, et al. Efficacy and Function of Feathers, Hair, and Glabrous Skin in the Thermoregulation Strategies of Domestic Animals. *Animals* (2021) **11**:3472. doi: 10.3390/ani11123472

146. Winter A, Ahlbrand R, Naik D, Sah R. Differential behavioral sensitivity to carbon dioxide (CO2) inhalation in rats. *Neuroscience* (2017) **346**:423–433. doi: 10.1016/j.neuroscience.2017.01.003