JILMA MARÍA ALEMÁN LAPORTE

Ensuring animal welfare in biomedical research:

pain relief and better quality report in scientific publications

São Paulo

2021

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Thesis submitted to the Postgraduate Program in Experimental and Comparative Pathology of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo to obtain the Doctoral Degree in Sciences.

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Prof. Dr. Claudia Madalena Cabrera Mori, Ph.D.

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São Paulo, 17 de junho de 2021
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CERTIFICADO (Alteração do cadastro versão de 05/junho/2021)

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 05/junho/2021) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Solicita-se mudanÃ§a do tÃtulo para que fique mais adequado aos objetivos do projeto.".

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "Garantindo o Bem-Estar Animal na Pesquisa Biomédica: Alívio da dor e Melhor Qualidade da Informação em Publicações Científicas", protocolada sob o CEUA nº 3611111119 (ID 007582), sob a responsabilidade de **Claudia Madalena Cabrera Mori** *e equipe; Jilma María Alemán Laporte* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 18/03/2020.

We certify that the proposal "Ensuring Animal Welfare in Biomedical Research: Pain Relief and Better Quality Report in Scientific Publications.", utilizing 92 Heterogenics rats (92 males), protocol number CEUA 3611111119 (ID 007582), under the responsibility of **Claudia Madalena Cabrera Mori** *and team; Jilma María Alemán Laporte* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 03/18/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 01/2020 a 12/2020 Área: Patologia Experimental E Comparada

Origem:	Biotério Central do Instituto de Ciências Biomédicas da USP						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	8 a 12 semanas	N:	92
Linhagem:	Wistar Han			Peso:	250 a 350 g		

Local do experimento: Biotério do Departamento de Patologia da Faculdade de Medicina Veterinaria e Zootecnia, USP

São Paulo, 17 de junho de 2021

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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EVALUATION FORM

Author: ALEMÁN LAPORTE, Jilma María

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Date: _____/_____/_____

Committee Members

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

To my little angel in Heaven and to my little baby in my womb...

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To God. I want to thank you for all the opportunities you gave me to create and develop this project. Thank for all the beautiful and enriching experiences I have had in my life. I love you, God!

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God bless you all!

"When man learns to respect even the smallest being in creation, whether animal or vegetable, no one will need to teach him to love his fellowmen."

Albert Schweitzer

RESUMO

ALEMÁN LAPORTE, J.M. **Garantindo o bem-estar animal na pesquisa biomédica:** alívio da dor e melhor qualidade da informação em publicações científicas. N° 109 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

O princípio do refinamento, descrito por Rusell e Burch em 1959, refere-se à melhoria no bem-estar animal na investigação, por meio da aplicação de procedimentos que minimizem a dor ou distresse do animal. Portanto, a utilização de tratamentos analgésicos em procedimentos invasivos que antecipam a dor e o relato adequado de todos os procedimentos em publicações científicas são necessários para garantir o bem-estar animal e obter resultados de qualidade e reprodutíveis. Dois dos biomodelos mais usados em cirurgia experimental são o rato e o suíno. No caso do rato, uma das cirurgias mais praticadas no campo da neurociência é a cirurgia estereotáxica. Os suínos são usados principalmente para pesquisas de novas técnicas cirúrgicas. O objetivo deste estudo foi procurar o refinamento por meio do uso da analgesia avaliando seu efeito sobre o comportamento e sua eficácia em evitar a dor, bem como o relato correto de procedimentos cirúrgicos em dois biomodelos animais, a fim de garantir as melhores condições durante seu uso. Para tanto, o estudo foi dividido em três capítulos: No primeiro, uma bateria de diferentes testes comportamentais [campo aberto (CA), labirinto em cruz elevado (LCE) e o Grooming Transfer Test (GTT)] foi aplicada em ratos Wistar-Han naives que foram previamente injetados com diferentes analgésicos [dipirona (DIP), tramadol (TRA), meloxicam (MEL), dipirona+tramadol (DIP+TRA) e meloxicam+tramadol (MEL+TRA)] ou solução salina (SAL). O tratamento com DIP+TRA produziu às alterações mais significativas no comportamento dos ratos, reduzindo a locomoção, comportamento de levantar e grooming. Todos os grupos de tratamento que receberam TRA reduziram o comportamento de grooming no CA e um aumento da locomoção foi observado em ratos tratados com MEL. No segundo capítulo, quatro grupos de ratos foram submetidos à cirurgia de craniotomia e tratados com diferentes analgésicos (TRA, MEL, TRA+MEL) ou SAL e um grupo foi apenas anestesiado. Em seguida, foram submetidos a diferentes testes comportamentais (CA, GTT e uso de Enriquecimento Ambiental) e fisiológicos (perda de peso corporal, ingestão alimentar e hídrica) durante

o período pós-operatório (72 horas). Os grupos que receberam analgésicos apresentaram alterações comportamentais semelhantes às observadas no estudo referente ao primeiro capítulo. Todos os grupos operados também apresentaram alterações comportamentais evidenciadas nas primeiras 48 horas, indicando que a dor produzida pela craniotomia pode perdurar por esse período. Os resultados, dos testes comportamentais não permitiram concluir qual o melhor protocolo analgésico entre os fármacos utilizados, sendo necessário realizar estudos adicionais. Apesar do uso dos analgésicos do uso dos analgésicos modificar as respostas comportamentais no período pós-operatório, evitar a dor é um dever ético e essas alterações devem ser consideradas no planejamento do projeto experimental. No terceiro capítulo, 108 estudos foram analisados com base nos requisitos das diretrizes da guia ARRIVE. A maior parte da literatura carecia de informações importantes, como relato de protocolos anestésicos e analgésicos, cálculo do tamanho amostral e detalhes de alojamento e manejo. A falta de informação em publicações científicas pode prejudicar a reprodutibilidade dos estudos, por isso um bom relato científico é essencial para uma boa ciência e consequentemente garantir o bem-estar animal.

Palavras-chave: Animais de laboratório. Analgesia. Procedimentos invasivos. Dor. Reprodutibilidade.

ABSTRACT

ALEMÁN LAPORTE, J.M. **Ensuring animal welfare in biomedical research:** pain relief and better quality report in scientific publications. 2021. N° 109 p. Thesis (Doctoral Degree in Sciences) –Faculty of Veterinary Medicine and Zootechnics, University of São Paulo, São Paulo, 2021.

The principle of refinement, described by Rusell and Burch in 1959, refers to improvements to animal welfare in studies, applying husbandry or procedures that minimize pain or distress. Therefore, the use of analgesic treatments in invasive procedures that anticipate pain, and the adequate reporting of all the procedures in scientific publication is necessary to guarantee animal welfare and get quality and reproducible results. Two of the most used biomodels in experimental surgery are the rat and the swine. In the case of the rat, one of the most common surgeries practiced in the field of neuroscience is the stereotaxic surgery. Swine is mostly used for the investigations of new surgical techniques. The objective of this study was to seek refinement through the use of analgesia evaluating its effect on behavior and effectiveness in avoiding pain, as well as the correct reporting of surgical procedures in two animal biomodels in order to guarantee the best conditions during their use. With this purpose, the study was divided in three chapters: In the first one, a battery of different behavioral tests [Open field (OF), elevated plus maze (EPM) and grooming transfer test (GTT)] were applied in naïve Wistar-Han rats that were previously injected with different analgesics [Dipyrone (DIP), Tramadol (TRA), Meloxicam (MEL), Dipyrone+Tramadol (DIP+TRA) and Meloxicam+Tramadol (MEL+TRA)] or Saline (SAL). DIP+TRA treatment led to the most significant alterations in rats' behavior by reducing locomotion, rearing, and grooming. All treatment groups that received TRA reduced the grooming behavior in the open field and an increased locomotion was observed in rats with MEL treatment. In the second chapter, four groups of rats underwent craniotomy surgery and were treated with different analgesics (TRA, MEL, TRA+MEL) or SAL and one group was just anesthetized. After this, they were submitted to different behavioral (OF, GTT and use of Environmental Enrichment) and physiological tests (body weight lost, food and water intake) during the postoperative period (72 hours). Analgesics treated groups presented similar behavioral alterations to those occurred in the first study with the same analgesics. All groups that underwent

surgery also presented alterations in behavioral tests during the first 48 hours, indicating that pain for craniotomy can last this period of time. The results of the behavioral tests did not allow us to conclude which was the best analgesic protocol, so further studies are needed. Although the use of analgesics can modify behavioral responses in the postoperative period, avoiding pain is an ethical duty and these alterations must be considered during experimental design planning. In the third chapter, 108 studies were analyzed based on the ARRIVE guidelines requirements. The majority of literature lacked of important information such as reports of anesthetic and analgesic protocols, sample size calculation and housing and husbandry details. The lack of information in scientific publications can lead in a poor reproducibility, for this reason good scientific report is essential for good science and consequently ensure animal welfare.

Keywords: Laboratory Animals. Analgesia. Invasive procedures. Pain. Reproducibility

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SUMMARY

1 INTRODUCTION

The advance of science and medicine is strongly linked to the use of animals as biological models. Laboratory animals have been contributing to discover new strategies in prevention and treatment of diseases, and also for the development of new techniques for surgical treatment. (ANDRADE et al. 2002). The current knowledge improvement in biology, human medicine and veterinary, must include the responsibility to anticipate, minimize, and eliminate any procedure that can cause pain, distress, or animal discomfort. Thus, it implies the necessity of a constant update in techniques and procedures in animal research (NRC, 2011).

The subject of the animals' rights and their use in scientific experiments have been discussed for a long time worldwide. In 1959, two english scientists Rusell and Burch, managed to co-author with three words the Human Principle of Animal Experimentation: "Reduction, Replacement and Refinement" in the use of animals, which today is known as the Principle of the 3Rs, establishing the standards for the use of animals in experimentation (CERONI-CAZARIN CORRÊA; ZAMBRONE, 2004). The objective of this principle is to guarantee the best possible animal welfare, avoiding unnecessary suffering and considering that it is essential to maintain attitudes of respect for the animal in gratitude to the scientific contribution that they provide us (RUSSELL; BURCH, 1959).

This study is composed of three chapters written in article format. The first chapter consists in the evaluation of the effects caused by the administration of commonly used analgesics (tramadol, meloxicam and dipyrone) on the behavioral parameters, which was performed through a behavioral tests battery [Open field (OF), elevated plus maze (EPM) and grooming transfer test (GTT)] on naïve Wistar-Han rats. This article was submitted and is still under review in the journal Veterinary Behavior.

In the second chapter, analgesic efficacy of tramadol, meloxicam and their combination was evaluated in rats that underwent craniotomy surgery to establish a preemptive management of postoperative pain. These animals were submitted to different behavioral (OF, GTT and use of Environmental Enrichment) and physiological tests (body weight loss, and food and water intake) during the postoperative period (72 hours). This article will be further submitted to the journal Animal Welfare.

Finally, the third chapter consists of a systematic review of 108 studies in which swine was used as a surgical model. All these studies were analyzed based on the

ARRIVE guidelines requirements to determine the quality of their reports. This article was published in the journal Animals.

The aim of this study was to seek refinement through the use of analgesia evaluating its effect on behavior and effectiveness to avoid pain, as well as the correct reporting of surgical procedures in two animal biomodels in order to guarantee the best conditions during their use.

2 LITERATURE REVIEW

2.1 ANIMAL MODELS IN EXPERIMENTAL SURGERY

In the animal experimentation field, it is often necessary to carry out surgeries for experimental procedures and for create new surgical techniques. There is a wide range of biological models that can be used for this, considering their biological, anatomical, and physiological similarities to humans (DAMY et al. 2010).

One of the most used biological models is the rat due to its great genetic and biological resemblance to the human organism and also its size and great adaptation to new environments, being considered as a species of relatively simple and inexpensive care (MAURER AND QUIMBY, 2015). Rats are the most suitable models for training and development of studies in microsurgery; for organ or bone transplants; for the study of different metabolic, cardiovascular, autoimmune and kidney diseases; for neurological disorders, neural regeneration and studies in neuroscience (FABRÍCIO-BORGHESI; EBISUI; VALERO-LAPCHIK, 2017).

The pig as a research biomodel has been growing its utility since the 1980s. Due to its great physiological and anatomical similarities with humans, it has been a widely used specie for research of treatments, for pathophysiology studies, and new surgical techniques. Also, their size is favorable for teaching basic techniques and performing surgical training (ANDRADE et al. 2002).

2.2 PAIN IN LABORATORY ANIMALS

Pain, is defined, by the International Association for the Study of Pain (IASP), as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” Although this

experience, can serves as self-protection from harmful situations in the short term, it can become a detrimental condition in the long term (DEUIS et al. 2017).

Pain is considered as a physiological state which has nothing in common with nociception, a neural process of encoding noxious stimuli from the periphery to the higher parts of the brain where the activity ultimately may lead to the pain experience (HEDENQVIST; ROUGHAN; FLECKNELL, 2000)

During painful procedures like surgery, tissue damage causes physiological stress responses in the body, such as the activation of the Hypothalamic–Pituitary–Adrenal (HPA axis) that will increase glucocorticoid levels and release several humoral factors, such as interleukin (IL)-1 and tumor necrosis factor-alpha (TNF-α). These physiological alterations can affect the postoperative recovery of the animal, increasing the risk of complications and mortality (SUNDBOM, 2013).

Animal sentience refers to the capability of animals to feel and experience emotions such as joy, pleasure, fear, and pain. The knowledge of animal sentience is fundamental and imperative to preserve animal welfare in experimentation. (PROCTOR, 2013). Nowadays, it is recognized that accurate identification and pain treatment are essential for refining practices with laboratory animals that undergo painful procedures and improving the validity of translational research (TURNER; PANG; LOFGREN, 2019).

2.3 USE OF ANALGESIA DURING EXPERIMENTAL SURGERY PROCEDURES IN LABORATORY ANIMALS

The use of adequate anesthesia and analgesia in laboratory animals undergoing painful procedures is an ethical, legal, and scientific imperative. Administration of analgesic agents to animals in pain is also a form of refinement in the experimental procedures (CANNON et al. 2011).

Unfortunately, the use of effective regimens for mitigating pain remains underutilized or reported in laboratory animals. Factors contributing to the gap between the need for and the actual use of analgesia include: lack of sufficient evidence-based data on effective regimens; lack of appropriate training and experience in the detection of pain in experimental animals; refusal to change historical practices of not using analgesics; concerns about the potential impact of analgesics in study outcomes; and

beliefs that rodents recover quickly from invasive procedures and as such do not need the use of analgesics (FOLEY et al. 2019). Despite this, nowadays there is high pressure from the ethics committees and the scientific community which are concerned with animal welfare.

The analgesic protocol should include anticipation of pain in order to minimize sensitization, and individual animal evaluations to find a response to the therapy (FOLEY et al. 2019). The selection of an analgesic protocol for a particular procedure should include a test of the physiologic effects of the agents and potential complications to the objective of the research (KOHN, 2007). Also, it is necessary to consider dose, route, volume, duration, frequency, and species-specific variations in the reactions to the pharmacologic agents. The analgesic regime used should be appropriate for the level of tissue damage or surgical trauma involved, should take into account the environmental factors that may add to postoperative or post-traumatic pain, and should be based on a measurable assessment of both the adverse effects of pain and the effectiveness of the analgesia (SCHOFIELD; WILLIAMS, 2002).

2.3.1 Multimodal analgesia

Multimodal analgesia combines several analgesics with different action mechanisms into the treatment protocol, which often increases efficacy while using lower dosages of the individual agents (FOLEY, 2019). Usually, this protocol can be composed by the combination of opioids with nonsteroidal anti-inflammatory drugs (NSAIDs) and local or regional anesthesia.

Opioid analgesic drugs remain one of the powerful agents available for the treatment of moderate to severe pain. However, these drugs can cause many adverse effects such as respiratory depression, bradycardia, hypotension, dysphoria, abnormal behaviors, and constipation in animals. (TAVAKOLI; SHABANNIA; MOHAMMADYAR, 2012). On the other hand, NSAIDs are effective and long-acting agents, but their adverse side effects, such as gastric damage, at high doses limit their degree of usefulness (MORENO-ROCHA et al. 2012). The combinations of these two types of drugs are commonly used to control various pain statements because, together, they will improve antinociceptive effectiveness, due to antinociceptive synergism, with a reduction in dosage and consequently a significant decrease in the incidence of adverse effects (LÓPEZ-MUÑOZ et al. 2013).

2.3.2 Tramadol

Opioids, routinely used to treat pain in laboratory rodents, are potent analgesics that exhibit their pharmacological effects by binding and activating several specific receptors, which are widely distributed in the Central Nervous System (CNS) and gastrointestinal tract (JIRKOF, 2017).

Tramadol, a synthetic 4-phenyl-piperidine analog of codeine, is a weak opioid-agonist with analgesic properties that presents two action mechanisms. It acts on serotonergic and noradrenergic nociception, while its metabolite O-desmethyltramadol acts on the μ-opioid receptor. (DAYER; DESMEULES; COLLART, 1997)

Tramadol, like other opioids, selectively binds to different opiate receptors in the CNS. The liver enzyme, CYP2D6, converts tramadol to its active metabolite M1, which has a stronger affinity for the μ receptor compared to the inactive form. Nevertheless, this affinity for μ receptors remains low, being 6000 times lower than morphine. For this reason, it has a moderate analgesic effect compared to morphine. Moreover, the discovery of a monoaminergic activity demonstrated that tramadol also inhibits noradrenaline (norepinephrine) and serotonin (5-hydroxytryptamine; 5-HT) reuptake, making a significant contribution to the analgesic action by blocking nociceptive impulses at the spinal level. (DAYER; DESMEULES; COLLART, 1997; NATALINI, 2007; PEREZ et al. 2016).

Tramadol is considered a relatively safe analgesic and can also be administered concomitantly with other analgesics, particularly those with peripheral action enhancing its efficacy (DAYER; DESMEULES; COLLART, 1997). The main adverse reactions to tramadol therapy are constipation and respiratory depression (especially when combined with other CNS depressants) (TAKHTFOOLADI et al., 2014; TAYLOR et al., 2016). However, some studies demonstrated their neuroprotective, cardioprotective, and anti-inflammatory conditions (TAKHTFOOLADI et al., 2014).

2.3.3 Meloxicam

Meloxicam is an NSAID drug derived from enolic acid that exerts its analgesic effects by inhibiting cyclooxygenase enzyme (COX), which results in decreased production of prostaglandin that causes pain. Also, Meloxicam exhibits selectivity for cyclooxygenase (COX)-2, the dominant COX isoform in the spinal cord, and it is

associated with the recognition of pain by the CNS during inflammation (TAVAKOLI; SHABANNIA.; MOHAMMADYAR, 2012).

This drug acts peripherally blocking nociceptors with potent anti-inflammatory and analgesic activity together with low gastrointestinal toxicity in animal models (CHURCHILL et al. 1996; ENGELHARDT et al. 1996; PAIRET; ENGELHARDT, 1996). Meloxicam is usually used to reduce swelling and pain in inflamed joints and laparotomies, in experimental studies with rats (ROUGHAN; FLECKNELL, 2003; TAKAHASHI ET AL., 2005). Meloxicam is effective in both inflammatory and neurogenic pain but has limited analgesic abilities with visceral distension or thermal pain. Meloxicam shows moderate synergistic effects with opioids such as morphine (PINARDI; PRIETO; MIRANDA, 2005), allowing a reduction in dose for both drugs and improving the safety margin.

The therapeutic range of meloxicam in the rat, concerning inhibition of adjuvant arthritis, was several times greater than that of other NSAIDs. Meloxicam in therapeutic doses does not affect bleeding time or platelet aggregation in healthy volunteers. In clinical studies, meloxicam has shown reliable efficacy against rheumatoid arthritis, osteoarthritis, lumbago (low back pain), scapulohumeral periarthritis, and neck-shoulder-arm syndrome with low gastrointestinal toxicity (OGINO et al. 2002).

2.3.4 Dipyrone

Dipyrone, a pyrazolone derivative also known as metamizol, is a NSAIDs drug widely used as an analgesic in Europe and Latin America. As other analgesics of this group, dipyrone and its active metabolites 4-methylaminoantipyrine and 4-aminoantypirine decrease prostaglandin synthesis, mainly through cyclooxygenase-2 activity inhibition (HERNÁNDEZ-DELGADILLO; CRUZ, 2006).

Dipyrone is an effective analgesic and antipyretic agent. Additionally, dipyrone has other beneficial effects, such as its actions as a vascular smooth muscle relaxant, antiapoptotic agent, and anticonvulsant (LÓPEZ-MUÑOZ et al. 2008).

Several possible mechanisms have been proposed, including the involvement of 5-HT, of endogenous opioids and the arginine–nitric oxide–cGMP pathway, and it is effective at a variety of levels in the nociceptive route from the periaqueductal grey to the periphery. There are also suggestions that COX-2 is indeed the target, but through its peroxidase function, or that another variant of COX-1, located in the CNS, is the

crucial enzyme inhibited by paracetamol and dipyrone demonstrated that dipyrone could decrease prostaglandin synthesis by activating cyclooxygenase- 3. (LÓPEZ-MUÑOZ et al. 2008; REZENDE et al. 2008).

2.4 ADEQUATE SCIENTIFIC REPORT ON LABORATORY ANIMAL EXPERIMENTS

For scientific, ethical, and economic reasons, experiments involving animals should be appropriately designed, correctly analyzed, and transparently reported. This increases the scientific validity of the results and maximizes the knowledge gained from each experiment. Therefore, failure to describe research methods and to report results appropriately has potential scientific, ethical, and economic implications for the entire research process and its reputation.

Scientific publications must include a minimum amount of relevant information to guarantee that the methods and results of a study can be reviewed, analyzed, and repeated. Ideally, scientific publications should present sufficient information to 1. allow a knowledgeable reader to understand what was done, why, and how, 2. to assess the biological relevance of the study, the reliability, and validity of the findings (KILKENNY, 2009).

In order to improve scientific reports, The Animals in Research: Reporting in vivo Experiments (ARRIVE) Guidelines were published in 2010. The main objective of these guidelines was to maximize the availability and utility of information gained from every animal in every experiment, preventing unnecessary animal use, and to allow an accurate critical review of animal experiments, making results easier to compare among different research groups to validate and contextualize results to promote translational research to patients' benefit (GULIN; ROCCO; GARCÍA-BOURNISSEN, 2015).

The ARRIVE Guidelines consist of a checklist with different items that helps article authors to describe in detail all the minimum information that all scientific publications using animals should include, such as the number and specific characteristics of animals used; details of housing; husbandry and procedures; experimental design; and statistical and analytical methods (GULIN; ROCCO; GARCÍA-BOURNISSEN, 2015).

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CHAPTER 1

3. EFFECTS OF THE ANALGESIC TRAMADOL, MELOXICAM AND DIPYRONE ON THE BEHAVIOR OF LABORATORY RATS

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3.1. HIGHLIGHTS:

- -Dipyrone reduced general activity in rats.
- -Tramadol decreased grooming behavior in rats.
- -Meloxicam increased spontaneous locomotor activity in rats.
- -Combined administration of dipyrone and tramadol strongly inhibited behavioral display.
- -The experimental designs must consider the effects generated by the administration of analgesics to the animals.

3.2. ABSTRACT

Awareness about the ethical use of laboratory animals increased over the last decades. That favored the development of strict guidelines and legislation concerning their welfare in scientific research. In this regard, minimize animal suffering during experimentation is paramount yet sometimes neglected due to the presumable interference of analgesics in behavior. Therefore, it is vital comprehensive research with multiple pharmacological agents to include analgesics in experimental procedures. For that purpose, we aimed to evaluate the effect of different analgesics (meloxicam, dipyrone, tramadol, or the combination of tramadol with meloxicam or dipyrone) on behavioral parameters of laboratory rats. Forty-eight SPF male Wistar-Han rats were randomly divided into the following drug-treatment groups (n=8 each): saline (SAL), dipyrone (DIP), tramadol (TRA), meloxicam (MEL), dipyrone + tramadol

(DIP+TRA), meloxicam + tramadol (MEL+TRA). Animals received DIP: 178 mg/kg, TRA: 17.8 mg/kg, and MEL: 1.5 mg/kg by intraperitoneal injection. Thirty minutes after the injection, we submitted the animals to different behavioral tests (Open Field, Elevated Plus Maze, Grooming Transfer Test). DIP+TRA treatment led to the most significant alterations in rats' behavior by reducing locomotion, rearing, and grooming. DIP treatment also diminished exploratory behaviors. However, grooming was not remarkably affected, suggesting that DIP has a suppressive motor effect, possibly caused by its action on the endocannabinoid system and TRA can potentiate this outcome. All treatment groups that received TRA reduced the grooming behavior in the open field. We observed increased locomotion in this test with MEL treatment. Given that analgesia is an ethical duty, the experimental design should always consider the behavioral effects.

Keywords: Analgesia; behavioral test; grooming; locomotion; animal welfare.

3.3 INTRODUCTION

The research community has the ethical responsibility to prevent pain by refining experimental procedures and administrating analgesics since the pain has a profound effect on laboratory animal welfare. Additionally, pain management has important scientific and methodological implications for the design of experiments and the quality of the resulting data (Jirkof, 2017).

However, various systematic reviews showed that less than 50% of researchers described pain management in their publications (Alemán-Laporte et al., 2019; Coulter et al., 2017; Stokes et al., 2009). Some of the reasons that can explain the lack of analgesia use in laboratory animals are the clinical side-effects of analgesics, how their use could confound the results from experimental studies, and an apparent uncertainty about when and how to administer analgesia (Richardson and Flecknell, 2005).

Opioids and Nonsteroidal Anti-inflammatory Drugs (NSAID) are the main used analgesics in rodents. These drugs are used alone in procedures that inflict minimal pain. However, in procedures that generate moderate to severe pain, multimodal analgesia (the combination of two or more analgesics) is recommended because their different mechanisms of action can potentiate pain relief (Foley et al., 2019). Tramadol (TRA) is an opioid with a central action due to the high affinity for μ-opioid receptors

and both serotonin and norepinephrine reuptake inhibition (Taylor et al., 2016). NSAIDs, as meloxicam (MEL) and dipyrone (DIP), suppress the cyclooxygenase. It catalyzes the first step in prostaglandin synthesis, relieving pain (Nunamaker et al., 2018).

Understanding how each external variable affects animal behavior is crucial in studies that use behavioral tests. Therefore, it is essential to know the advantages, disadvantages, and complications of commonly used analgesics (Gaertner et al., 2008). The standard tools to evaluate activity, exploration, and emotionality in rodents are behavioral tests, such as the Open Field (OF) and the Elevated Plus Maze (PM). They are also routinely used to screen drugs for their psychopharmacological potential (Schmitt and Hiemke, 1998). The Grooming Transfer Test (GTT), developed by Oliver et al. (2018), allows indirect assessment of grooming behavior. It evaluates the elimination of fluorescent gel applied to the animal fur to estimate postoperative pain. Nonetheless, we decided to use this test to assess the pharmacological effect on grooming behavior of all the analgesic drugs along time (first 26 hours).

NSAIDs may have different adverse effects (like renal, cardiovascular, and gastrointestinal toxicity) on animal metabolisms (Jirkof, 2017). Some studies have also determined that tramadol can alter rats' behavior as sedation in high doses (Wolfe et al., 2015). Nevertheless, the effects of DIP, MEL, and their combination with TRA on the behavior of laboratory rats are poorly studied. For this reason, the aim of this study was to evaluate the effect of these analgesics and to point out the need that they might be considered in studies in which rat’s behavior must be evaluated

3.4 MATERIAL AND METHODS

3.4.1 Animal husbandry and treatment groups

Forty-eight male Wistar-Han rats (8 to 12 weeks old) were obtained from the Institute of Biomedical Sciences of the University of São Paulo. Animals were housed in the animal facilities of the Department of Pathology of the School of Veterinary Medicine and Animal Science, University of São Paulo. They were group-housed (4 per cage) in polypropylene cages (41 x 34 x 16 cm) filled with corncob bedding material (Granja R.G., SP, Brazil) and kept in a 12/12 h light/dark schedule (lights on at 6 a.m.), a temperature of 22°C (±2°C), and relative humidity of 55% (±10%). Irradiated diet

(Nuvilab CR1®-Quimtia, PR, Brazil) and water were provided ad libitum. Animals were free of endo and ectoparasites, Mycoplasma pulmonis, Pasteurella pneumotropica, Bordetella bronchiseptica, Helicobacter spp., Klebsiella oxytoca, Klebsiella pneumoniae, Pasteurella multocida, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus β-hemolytic spp., Streptococcus pneumoniae, Salmonella spp, Kilham Rat Virus, Pneumonia Virus of Rat, and Reovirus. Kraft paper was added as environmental enrichment. Behavioral assessments were performed between 6 a.m. and 12 p.m. and after one week of acclimatization to the new facilities. Animals were randomly distributed in one of the following drug-treatment groups (8 per group): saline (SAL), dipyrone (DIP), tramadol (TRA), meloxicam (MEL), dipyrone + tramadol (DIP+TRA), and meloxicam + tramadol (MEL+TRA).

3.4.2 Behavioral assessment

Thirty minutes after the drug administration by intraperitoneal route (IP), animals were placed in the center of an open-field (OF) arena and their behaviors were scored during 10 min. OF consisted of a circular, wooden chamber (90 cm diameter) covered with a black laminate-sheet and illuminated with white light (~106 lumens). Once the OF test was completed, animals were placed in the middle of an Elevated Plus Maze (EPM) for analyzing their reactivity to a new and more anxiogenic environment (~106 lumens). EPM consist of an elevated (40 cm height), black platform with four symmetrical arms (30 x 10 cm): two opposed arms were enclosed by walls (40 cm height) and connected to a central platform, whereas the other facing arms were perpendicularly attached to the central platform and had no walls. Animals were placed in the central platform facing the closed arm and they were allowed to explore for 5 min. For both tests, behaviors were video recorded for offline analyses. After testing each animal, the equipment was thoroughly cleaned with ethanol (5%), preventing any possible bias caused by odor cues left by previous rats.

3.4.2.1 Behavioral analysis

For the OF, locomotion was scored by the AnyMaze video tracking software (version 6.1, Stoelting Co., Wood Dale, IL, USA) and expressed in meters. The rearing frequency and the grooming duration were manually scored by trained observers using

Solomon Coder software (version 17.03.22; https://solomoncoder.com/download.php). Rearing consists in a bipedal posture (>45°) in which the animal extends upwards to explore its surroundings. This behavior could appear with the animal leaning against the apparatus walls or free-standing. Grooming is a set of self-directed movements including hand rubbing, face washing, unilateral and bilateral strokes over the head and ears, body-licking, head and body scratching, and tail licking. Grooming sequences were classified as previously described (Rojas-Carvajal and Brenes, 2020; Rojas-Carvajal et al., 2020). Briefly, we considered two variables: anatomical distribution and complexity. Anatomically, grooming was classified as cephalic (head- and forehand-directed sequences), caudal (directed to the body), and sequential (chained sequences of head and body grooming). Those sequences, including the use of the hind paws, were considered more complex and designated as variations of the standard form. As a result, six different subtypes were scored (Anatomical distribution*Complexity). Micro grooming (<1s) was also counted whereas discarding isolated scratching events. For the EPM, AnyMaze automatically graded the visiting frequency and duration to the open and closed arms. Trained observers manually scored frequency of rearing, stretch-attempt posture (SAP) and head-dipping (HD) using Solomon Coder. SAP consists in an exploratory behavior in which the animal stretches its body forward while keeping its hind paws still. HD comprises the animal projecting its whole head below the border of the open arms or central platform.

3.4.3 Grooming-transfer test (GTT)

Once the animals completed the EPM, they were separately placed in clean cages (41 x 34 x 16 cm) filled with bedding material and transported to an adjacent room. There, a drop of a gel fluorescent under UV-light (Glo Germ Oil, Glo Germ, Moab, UT) was applied on their foreheads. The gel presence was revealed by turning off the room light and exposing the animal to a UV-lamp at 15 cm height. A five-point scale was used to assess the absence/presence of the gel, in which an intense fluorescent signal was scored as 1 whereas the total absence of fluorescence was scored as 5 (Figure 1). Since the fluorescent gel is removed by animals' self-grooming, so its presence is inversely related to the grooming frequency. To assess the long-term effect of the drugs on this behavior, fluorescence was examined 2 h, 4 h, 8 h, 24 h and

26 h after the administration. Trained observers, blind to test conditions, assigned scores.

3.4.4 Drugs

Drugs were prepared, dissolved in saline (0.2 ml; 0.9%), and injected by intraperitoneal (IP) route daily. Doses were as follows: 178 mg/kg of DIP (Desalgina®-Galmedic, 50mg/ml, ASU, Paraguay), 17.8 mg/kg of TRA (Intradol®-Alcames, 50mg/ml, SJ, Costa Rica), and 1.5 mg/kg of MEL (Meloxic®-Provet, 5 mg/ml, Btá, Colombia). DIP+TRA and MEL+TRA groups received each corresponding dose. SAL animals received physiological saline (0.9%). Doses were based on previous reports (Moreno-Rocha et al., 2016; Nunamaker et al., 2018).

3.4.5. Statistical analysis

All analyzes were executed using IBM SPSS v21 software (IBM, USA). Kinetics of OF behaviors were analyzed by repeated measures ANOVAs (RM-ANOVA) using Minute (i.e., 1-10) as within-group factor and Analgesic (i.e., SAL, DIP, TRA, MEL, DIP+TRA, and MEL+TRA) as a between-group factor. SAL animals were compared to each one of the other analgesic-treated groups analyzing cumulative scores by planned contrasts ran using one-way ANOVAs. Grooming subtypes were analyzed by a mixed factorial ANOVA using Subtype (i.e., cephalic, cephalic with variations, caudal, caudal with variations, sequential, and sequential grooming with variations) and Analgesic as factors. When appropriated, independent analyses per group were run by one-way ANOVAs. Cumulative scores of EPM behaviors were analyzed by planned contrasts as described for the OF. Arms' preference was analyzed by a mixed factorial ANOVA using Arm (i.e., open, closed) and Analgesic as factors. Three animals from different groups (i.e., SAL, DIP, MEL+TRA) fell-off the EPM early on tests, so data were removed from the analyses, interfering with the degree of freedom among tests. GTT was analyzed by RM-ANOVA using Hour (i.e., 2h, 4h, 8h, 24h, 26h) and Analgesic as within-group factors. Independent analyzes for each group were run when appropriated. Group differences per hour were analyzed by planned contrasts as specified for previous tests. Bonferroni's adjustment was applied for pairwise comparison when appropriate. If Mauchly's Tests for Sphericity were $P<0.05$,

Greenhouse-Geisser correction was applied. The effect size was estimated with the partial eta-squared (ηp2) coefficient. Statistical significance was defined as P<0.05.

3.5. RESULTS

3.5.1 Open Field test

A minute-by-minute analysis of locomotion revealed a main effect of Analgesic ($F(5,42)=9.16$, $p<.001$, $\eta2p=.52$), Minute ($F(5.30,222.73)=77.94$, $p<.001$, $\eta2p=.65$), and Analgesic*Minute interaction($F(26.52, 222.73)=1.60$, $p<.05$, $\eta2p=.16$). Independent analyses revealed that animals treated with SAL, DIP, MEL, and DIP+TRA showed a progressive reduction over the minutes (all $P<.001$, $\eta2p=.72$-$.78$), with a substantial decrease from minute 1 to 5 (Bonferroni: $p<.001$ for all groups) and a non-significant decay afterward (Figure 2.A). TRA- ($F(2.62, 18.31)=7.43$, $p<.01$, $\eta2p=.51$) and MEL+TRA-treated animals ($F(5,63)=5.19$, $p<.01$, $\eta2p=.43$) showed a less stiff drop, with the first group showing a single reduction from minute 1 to 7 (Bonferroni: $p<.001$) and the second, a non-significant decrease through minutes (Bonferroni: all $P>.05$). Descriptively, MEL and MEL+TRA groups showed the highest average distance traveled per minute, whereas DIP+TRA animals showed the lowest scores compared to SAL-treated rats (Bonferroni: $P<.05$). Planned contrasts of cumulative scores revealed that DIP ($t(42)=-2.41$, $P<.05$) and DIP+TRA ($t(42)=-3.28$, $p<.01$) reduced locomotion compared to SAL-treated rats, whereas MEL and MEL+TRA descriptively increased it (Figure 2.B). The rearing frequency analysis revealed a main effect of Minute ($F(6.40,268.92)=6.69$, $P<.001$, $\eta2p=.14$), with animals in all groups showing a single reduction from minute 2 to 6 (Bonferroni: $P<.001$) (Figure 2.C). We also found a main effect of Analgesic ($F(5,42)=7.04$, $P<.001$, $\eta2p=.46$), with DIP and DIP+TRA animals representing the lowest average scores per minute compared to SAL-treated rats (Bonferroni: both $P<.05$). When analyzing cumulative rearing frequency, animals treated with DIP, ($t(42)=-3.32$, $p<.01$), DIP+TRA ($t(42)=-3.74$, $p<.001$) and MEL+TRA ($t(42)=-1.8$, $p=.08$) showed the lowest scores contrasted with SAL-treated animals ($F(5,47)=7.04$, $p<.001$) (Figure 2.D).

The minute-by-minute analysis of total grooming duration revealed a main effect of Analgesic ($F(5,42)=4.48$, $P<.01$, $\eta2p=.35$), with DIP+TRA and MEL+TRA animals showing the lowest average scores per minute compared with SAL-treated rats

(Bonferroni: both P<.05). There was a marginal main effect of Minute (P=.06), with grooming descriptively increasing through the test (Figure 2.E). Considering cumulative scores, treatment with TRA alone (t(12.07)=-2.31, p<.01), as well as the combination of MEL+TRA (t(12.25)=-2.62, p<.05) and DIP+TRA (t(7.07)=-4.52, p<.01) significantly reduced grooming duration compared with SAL-treated animals (F(5,47)=4.48, p<.01). Cumulative grooming per subtype also revealed a main effect of Analgesic (F(5,252)=3.93, P<.01, η2p=.07), Subtype (F(5,252)=20.10, P<.001, η2p=.28) and Analgesic*Subtype interaction (F(25,252)=2.82, P<.001, η2p=.22). Independent analyses showed that the most time-consuming sequence was sequential grooming in SAL- (F(5,42)=13.62, P<.001, η2p=.62) and MEL-treated rats (F(5,42)=13.77, P<.001, η2p=.62) compared to all other subtypes (Bonferroni: all P<.001). Contrarily, the duration of grooming per subtype did not differ in all other groups (Figure 2.F).

3.5.2. Elevated Plus Maze

Analyzing EPM arms entries, we found that MEL+TRA (t(39)=2.12, p<.05) and MEL rats (P=.08) showed the highest number of open arms visits(F(5,44)=4.13, p<.01), whereas the DIP- (t(39)=-2.25, p<.05) and DIP+TRA-treated rats (t(39)=-3.25, p<.01) showed the lowest number of entrances into the closed arms compared with SAL-treated ones (F(5,44)=6.77, p<.001) (Figure 3.A-D). Animals in all groups spent a similar amount of time in the open arms, but DIP rats (p=.07) stayed descriptively less time in the closed arms compared to SAL-treated rats (F(5,44)=3.14, p<.05). Nevertheless, animals in all groups showed a preference for the closed arms reflected by its higher visitation (F(1,78)=28.18, P<.001, η2p=.26) and permanence (F(1,78)=110.51, P<.001, η2p=.59). The analysis revealed that the administration of DIP (P=.05) and DIP+TRA (t(39)=-3.55, p<.001) reduced the rearing frequency (F(5,44)=7.01, p<.001) (Figure 3.E, left panel). Similarly, the administration of TRA (t(39)=-2.25, p<.05) and DIP+TRA (t(39)=-5.82, p<.001)induced a reduction of SAP contrasting with SAL animals (F(5,44)=4.20, p<.01) (Figure 3.E, middle panel) (Figure 3.E, middle panel). No differences were found when analyzing HD (Figure 3.E, right panel).

3.5.3. Grooming transfer test

Self-grooming was analyzed by an indirect factor: the presence of fluorescent gel on animals' fur in the following hours after the analgesic administration. Therefore, we found the main effect of Hour ($F(2.42,101.72)=132.95$, $P<.001$, $\eta2p=.76$), Analgesic ($F(5,42)=10.96$, $P<.001$, $\eta2p=.57$) and a Hour*Analgesic interaction ($F(12.11, 101.72)=8.35$, $P<.001$, $\eta2p=.50$). Two hours after the IP injection, animals treated with SAL, TRA, and MEL showed low fur fluorescence that tended to decrease in the measurement carried out 8 hours post-injection (Bonferroni: all $P>.05$) (all $P<.05$, $\eta2p=.50$-$.55$). When TRA and MEL were combined, moderate levels of fur fluorescence were noticed 2 hours post-injection, with scores showing a single reduction when assessed after 4 hours (Bonferroni: $P<.05$) ($F(4,28)=24.67$, $P<.001$, $\eta2p=.78$). Similarly, DIP-treated animals showed intermediate fluorescence levels when measured 2 hours following the injection; still, a steadier decrease occurred from 2 to 4 hours and from 4 to 24 hours post-injection (Bonferroni: both $P<.05$) ($F(4,28)=34.29$, $P<.001$, $\eta2p=.83$). In DIP+TRA animals, the fluorescent gel was entirely present when assessed 2 hours from the injection, with levels showing a progressive reduction from 2 to 4 hours, 4 to 8 hours post-injection, and 8 to 24 hours post-injection (Bonferroni: all $P<.05$) ($F(4,28)=91.35$, $P<.001$, $\eta2p=.93$). However, this group showed a complete absence of fluorescent fur within 26 hours after analgesic administration. As a result, animals treated with DIP+TRA exhibited higher fluorescence levels h ($t(42)=7.26$, $p<.001$), 4 h ($t(42)=4.88$, $p<.001$), 8 h ($t(42)=3.39$, $p<.01$), and 24 h post-injection ($t(42)=2.65$, $p<.01$) versus SAL animals (all $P<.05$). DIP-treated individuals also showed higher scores when measured after 2 h ($t(42)=3.15$, $p<.01$) and the 4 h ($t(42)=2.09$, $p<.05$), whereas MEL+TRA animals only presented high fluorescence levels during their first assessment compared with SAL rats ($t(42)=2.52$, $p<.05$). There were no other differences among the other groups (Figure 4).

3.6 DISCUSSION

The goal for any analgesic therapy is to minimize pain without compromising the animal's well-being (NRC, 2009). However, analgesic drugs usually produce different effects on animal's metabolism that can alter the physiological and behavioral

parameters (Jirkof, 2017). Therefore, the information obtained only using analgesic drugs should eliminate the effect created by scientific procedures and point out the most appropriate decisions to determine the best experimental design. The characteristics and possible physiological or behavioral alterations of each drug, used alone or combined, must be determined to minimize their interference in the results.

According to the results obtained in all the behavioral tests, the combination of DIP+TRA caused a generalized behavioral inhibition in the treated rats, reducing locomotion, rearing, and grooming (of all subtypes) in the OF and locomotion and rearing in the PM. The GTT revealed that rats of this group maintained a reduction in grooming during some hours because they removed less gel leastwise until the first 8 hours. On the other hand, rats treated only with DIP showed a sharp reduction in locomotion (in the OF) and rearing (in the OF and PM). However, it did not affect grooming behavior as much, showing a high expression of cephalic grooming.

The exact action mechanisms of DIP remain unclear (Schlosburg et al., 2012). DIP is an NSAID drug with few anti-inflammatory and antinociception effects due to its action in peripheral tissue and the central nervous system (Dos Santos et al., 2014). This drug acts mainly on COX-3 with little effect on COX-2 and COX-1. However, there is evidence that it also functions on the opioid system, the nitric oxide (NO)/cGMP/KATP signaling pathway, and the endocannabinoid system through CB1 and CB2 receptors (Dos Santos et al., 2014; Rezende et al., 2008).

Some studies have described locomotor suppressive effects in mice (Crunfli et al., 2015; Schlosburg et al., 2012) after administering DIP, especially in elevated doses. Crunfli et al. (2015) related these alterations in behavior to a possible action of DIP on the endocannabinoid system because they observed similar effects with a CB1 agonist (WIN 55,212-2). Evidence demonstrates that pharmacological treatments using CB1 receptor agonists can produce biphasic effects. Meaning, low doses induce motor activity, and high doses suppress motor activity or induce catalepsy (Crunfli et al., 2015).

A broad range of DIP doses (from 50 mg/kg to 450 mg/kg) is found for rats (EMEA, 2003). The dose used (178 mg/kg) was chosen based on an antinociception study in rats (Moreno-Rocha et al., 2012), in which they did not describe behavioral data. Although we did not use a high dose, rats treated with DIP, and DIP+TRA, presented the high dose side-effect. DIP and TRA combination compared to DIP alone revealed a more severe suppression of the motor activity caused by the first. It

suggests there is a synergism between these two drugs on rat's behavior. Moreno-Rocha (2012) previously described that combining both drugs can potentiate their antinociceptive effects. This synergism could be explain because the combination of opioid drugs and some NSAIDs activates the serotonergic system (Sandrini et al., 1998), the opioidergic system (Maves et al., 1994), prostanoid and opioid receptors, and the Nitric Oxide-cGMP (Moreno-Rocha et al., 2012).

All groups treated with TRA (alone or in combination) decreased grooming behavior. Contrary to TRA+DIP animals, TRA and MEL+TRA animals showed exploration behavior in the OF and the PM similar or higher than the control group. TRA has a multimodal mechanism of action: activates μ-opioid receptors and acts on the serotonergic system, inhibiting serotonin reuptake into the presynaptic nerve terminals (Raffa et al., 1992). These mechanisms are crucial in the analgesic effect of TRA, but they can also elevate serotonin leading to agitation, increased reflexes, and tremors which distract the rat from grooming its full body (Kolawole Balogun et al., 2020). Some authors (Xie et al., 2008) had suggested that serotonin 5-HT2A receptors are involved in the analgesic effect of tramadol. That could be related to the reduction of grooming because the blockage of these receptors in some brain regions in BTBR mouse (used as a model of autism spectrum disorder) also reduced grooming (Amodeo et al., 2017). The combination of DIP+TRA highly decreases this behavior for at least 8 hours, meaning the inhibitory effect of DIP potentiated TRA effect.

The most frequent grooming subtypes in all groups were cephalic and sequential. Rats display the cephalic subtype of grooming at the beginning of testing when exploratory and risk assessment behaviors are prominent. Later, they exhibit long and complex sequences when exploration behavior has decreased (Rojas-Carvajal et al., 2020). The cephalic subtype was the most observed among animals, probably because the behavioral recording in the OF was relatively short (10 minutes) and needed more time to observe the remaining grooming subtypes.

All rats treated with MEL (alone or in combination) showed an increment in spontaneous locomotor activity. Previous studies have suggested that COX2 selective inhibitors like MEL can considerably increase horizontal and vertical movements in the OF in depressive rats (Huang et al., 2019) because this drug can inhibit the expression of COX2 in the brain. However, more studies in naïve rats must be conducted to explain this effect.

3.7 CONCLUSIONS

The combination of DIP+TRA and DIP alone, in these specific doses and routes, are not suitable for behavioral studies because they suppress general activity in rats. All treatments that included TRA had a reduction in grooming behavior. On the other hand, the treatments that included MEL had an increase in locomotion. We observed all these behavioral alterations in naïve animals, so they should be considered under certain experimental conditions; however, they should be evaluated under surgical conditions to determine if they cause further behavioral disturbances.

There is little information about the effects of analgesics on rodent's behavior. That may be one of the reasons why many researchers are reluctant to use these drugs. Therefore, further study is required. Analgesia is needed to prevent pain in painful experimental procedures and to guarantee animal welfare, So, it is essential to consider its effects when designing the experiments; thus, it does not interfere with the results.

3.8 AKNOWLEDGEMENT

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3.9 ETHICAL STATEMENT

All procedures were conducted following the guidelines of the Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (No. 3611111119).

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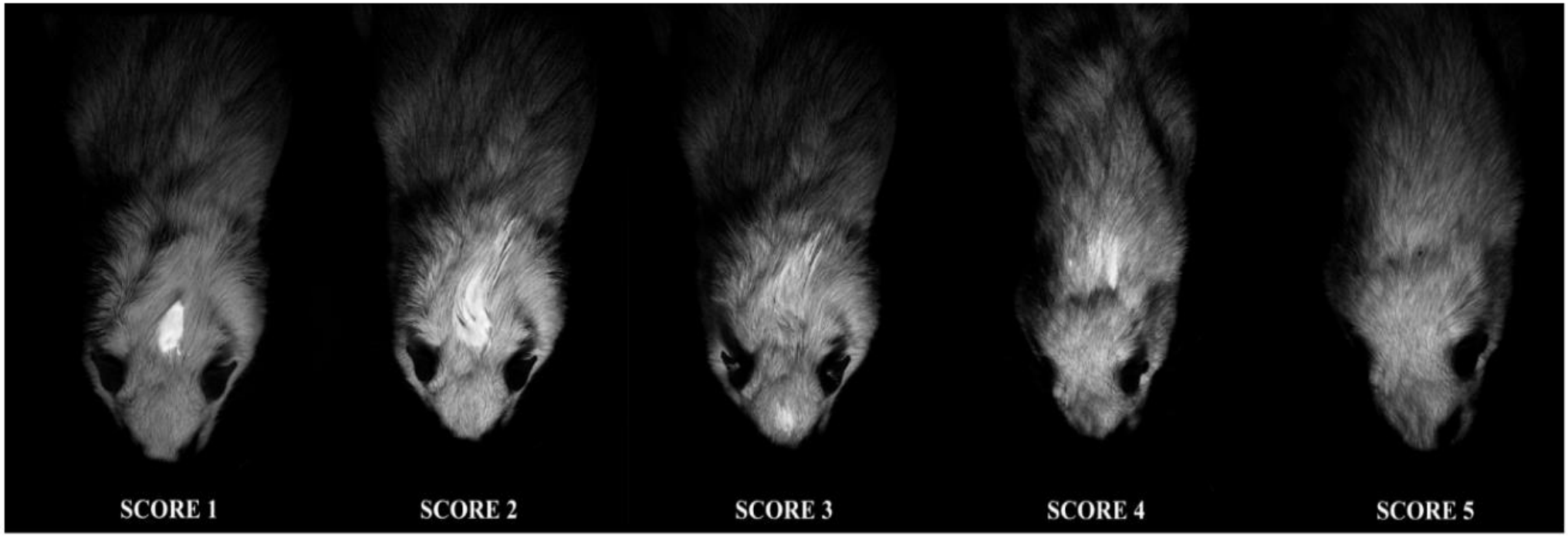

Figure 1. Grooming transfer test scoring. Fluorescence score was based on Oliver et al. 2018 methodology. Score 1: Strong fluorescent signal at the application site between the ears. Score 2: Fluorescent signal at the application area and signs that the gel was spread on the rat's back. Score 3: Fluorescence on the head, ears and back. Score 4. Florescent signal is almost absent but remains amounts at the application site. Score 5. Fluorescence is no longer detected.

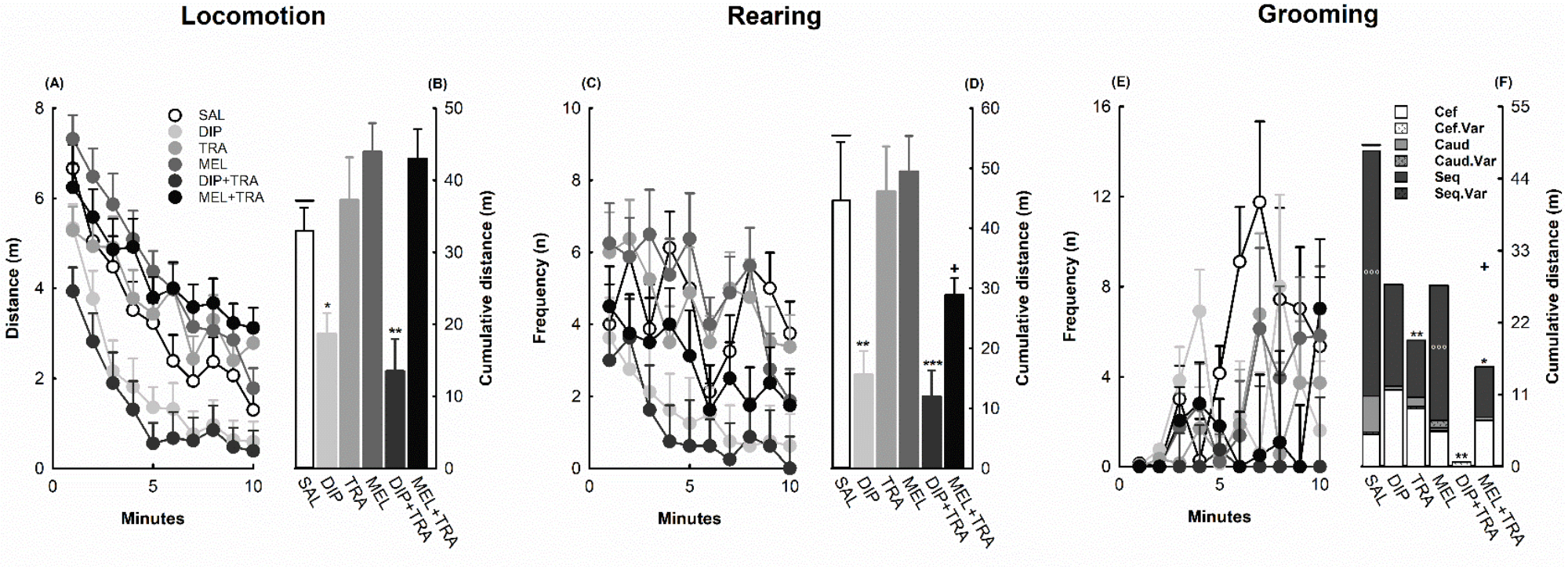

Figure 2. Behavioral kinetics and cumulative scores of OF behaviors. Ceph: Cephalic grooming; Ceph.var: Cephalic grooming with variations; Caud: Caudal grooming; Cuad.var: Caudal grooming with variations; Seq.: Sequential grooming; Seq.var: Sequential grooming with variations. Horizontal lines highlight the specific contrasts between SAL and analgesic-treated groups: +P<.08; *P<.05; **P<.01; ***P<.001. Subtypes comparison within groups: Sequential grooming compares to all other subtypes: °°°P<.001.

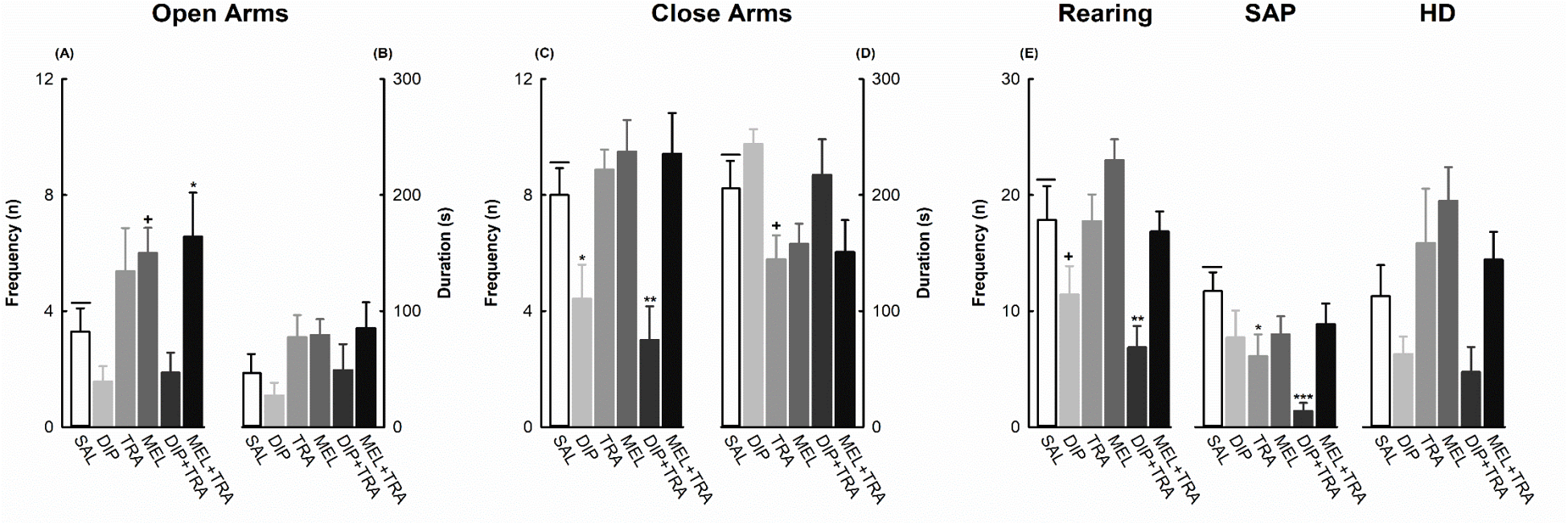

Figure 3. Cumulative scores of EPM behaviors. The horizontal line highlights the specific contrasts among SAL and analgesic-treated groups. SAP: stretch-attempt posture HD: head-dipping. +P<.07; *P<.05; **P<.01; ***P<.001.

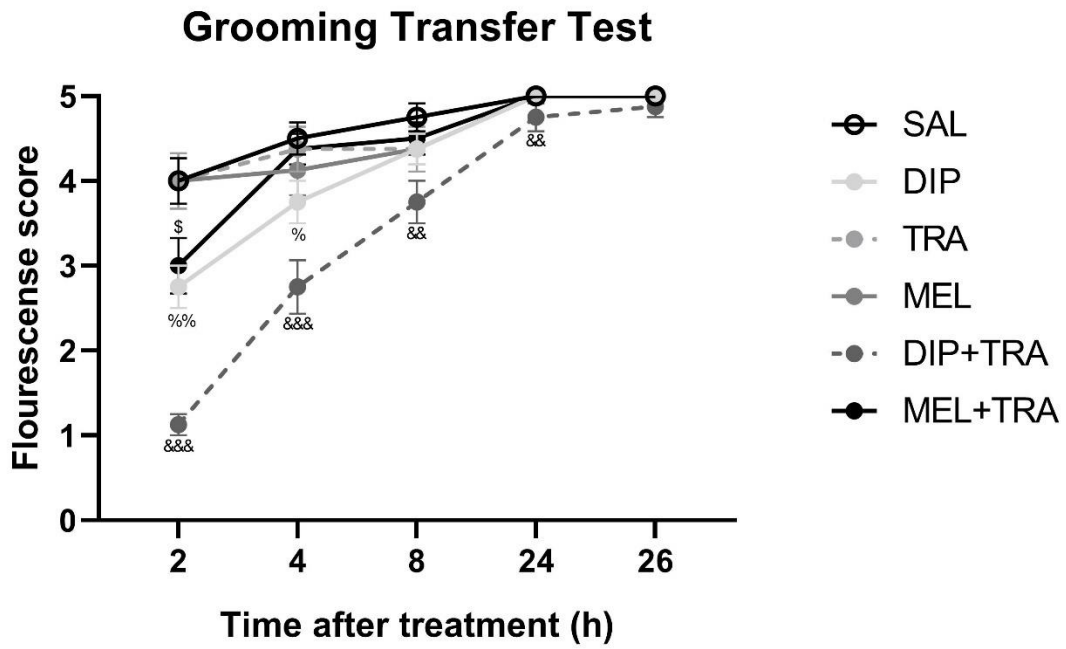

Figure 4. Grooming transfer test scores. MEL+TRA vs SAL: $P<.05; DIP vs SAL: %P<.05, %%P<.01; DIP+TRA: &&P<.01, &&&P<.001.

This paper will be submitted to Animal Welfare Journal

CHAPTER 2

4. Effects of meloxicam, tramadol and their combined administration on postoperative pain in rats undergoing craniotomy

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Running title: Effects of tramadol and meloxicam in rats

4.1 ABSTRACT

One of the most common surgical procedures in neuroscience is a stereotaxic surgery. However, there are no well-stablished analgesic protocols to prevent pain in this type of surgery. Therefore, our study aimed to evaluate the effects of tramadol and meloxicam alone or in combination, to avoid pain after a craniotomy in rats. Forty Wistar-Han rats were randomly divided into the following groups: Saline+Anesthesia (SAL+ANE), Saline+Surgery (SAL+SUR), Tramadol+Surgery (TRA+SUR), Meloxicam+Surgery (MEL+SUR), and Meloxicam/Tramadol+Surgery (MEL/TRA+SUR). Animals received SAL:0.2ml, TRA:17.8 mg/kg, and MEL:1.5 mg/kg by subcutaneous injection every 12h during 72h. Thirty minutes after the first injection, animals were submitted to anesthesia or surgery. Once rats were totally recovered, different behavioral tests (open field (OF), grooming transfer test (GTT) and nesting behavior (NB)) and physiological parameters (bodyweight loss and food/water intake) were evaluated during the postoperative time. All rats that underwent surgery had behavioral changes during the first 48h of evaluation. SAL+SUR and MEL+SUR showed an increase of locomotion and rearing in the OF. SAL+SUR, TRA+SUR and TRA/MEL+SUR rats also showed a reduction in the duration of grooming in this test. TRA/MEL+SUR and SAL+SUR presented the lowest GTT scores during the first 48h

and TRA/MEL+SUR group showed a reduction of NB during the first 24h. The results obtained showed that craniotomy can produce mild pain that can last at least 48 hours. Although we could not define the best analgesic treatment, the administration of analgesia and the refinement of the surgical technique are mandatory in this type of procedure to preserve animal welfare.

Keywords: Animal welfare, behavioral test, craniotomy, laboratory rats, pain management, postoperative care

4.2 INTRODUCTION

Stereotaxic surgery is one of the most common invasive experimental procedures practiced in neuroscience. This surgery consists of removal of a skull section to access the brain (Fornari et al 2012, Cho et al 2019). This procedure is used for cannulas or electrodes implantation into specific brain regions to evaluate the effect of local manipulation of neurotransmitters; for injecting different drugs to see their effect on the brain function; or for signaling pathways in awake animals (Fornari et al 2012).

Many scientists neglected pain following stereotaxic surgery because of the common belief that this surgery generates minimal pain (Chowdhury et al 2017). Additionally, there is a lack of standardized pharmacological regimes or evidence-based recommendations for the relief of craniotomy pain in rodents (Cho et al 2019). Carbone and Austin (2016) showed that only 8% of the studies (2 of 25) that performed craniotomy reported postoperative analgesia. Therefore, considering that inadequate pain management can produce many negative alterations in animal's physiology and behavior that can affect animal welfare and the validity of the scientific data, the administration of analgesics must be prioritized and properly evaluated (Miller & Leach 2015).

The goal of the pain management is to eliminate pain using analgesic drugs (alone or in combination) and non-pharmacological analgesia (refinement of invasive techniques, nursing care, etc.) (NRC 2009). For this purpose, analgesia should be

administrated, anticipating pain (before surgery), to minimize sensitization, and it must be maintained during the postoperative period, especially during the first hours, when most of the signs of pain occur (Foley et al 2019). Thus, pain management will increase survival rate and improve the general condition of the animals after surgery (Fornari et al 2012).

Pain evaluation is challenging, many studies commonly try to indirectly assess pain by stimulus-evoked pain-like behaviors such as mechanical, heat or cold stimuli. However, these assays are impractical for assessing pain from surgical procedures in the head (Cho et al 2019). A variety of preclinical models of headache include spontaneous behaviors (such as locomotor activity, rearing, nesting or even food and water intake) and nociceptive behaviors (such as grooming) to more accurately understand the degree of pain and assess analgesic efficacy (Vuralli et al 2019, Oliver et al 2018).

Thus, the present study aimed to evaluate the effects of two analgesics drugs alone or in combination: tramadol (a synthetic, centrally acting opioid) and meloxicam (a non-steroidal anti-inflammatory analgesic that inhibits COX-2) to avoid pain after a craniotomy in rats through different behavioral tests.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Forty male Wistar-Han rats (*Rattus norvegicus*), aged from 8 to 12 weeks old were obtained from the Institute of Biomedical Sciences, University of São Paulo. Animals were group-housed (4 per cage) in polypropylene cages (41 x 34 x 16 cm) filled with pine bedding (Granja R.G., SP, Brazil), and kept in a 12:12-h light/dark schedule (lights on at 6:00 h), at a temperature of 22°C (±2°C), and relative humidity of 55% (±10%). Irradiated diet (Nuvilab CR1®-Quimtia, PR, Brazil) and water were provided *ad libitum*. Animals were free of endo and ectoparasites. Complete health reports, especially the microbiological status based on the Federation of European Laboratory Animal Science Associations (FELASA) recommendations (Mähler et al

2014), were certified by the vendor. Paper towel was added as environmental enrichment. Behavioral assessments were performed between 6:00–12:00h after one week of acclimatization to the new facility. All procedures were done following the guidelines of the Ethics Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil (No. 3611111119).

4.3.2 Experimental groups

Animals were distributed using a block randomization technique (randomizing participants within blocks such that an equal number were assigned to each treatment.) in one of the following groups (n=8): SAL+ANE: Saline + Anesthesia; SAL+SUR: Saline + Surgery; TRA+SUR: Tramadol + Surgery; MEL+SUR: Meloxicam + Surgery; TRA/MEL+SUR: Tramadol/Meloxicam + Surgery. Drugs were daily prepared, dissolved in saline (1:5; 0.9%) in the following doses: Tramadol (Intradol®-Alcames, 50mg/ml, SJ, Costa Rica): 17.8 mg/kg, and Meloxicam (Meloxic®-Provet, 5 mg/ml, Btá, Colombia): 1.5 mg/kg TRA/MEL+SUR group received the corresponded doses for each drug. SAL+ANE and SAL+SUR animals were treated with 0.5 ml physiological saline (0.9%). Doses were based in previous reports (Moreno-Rocha et al 2016; Nunamaker et al 2018). SAL+ANE, TRA+SUR, MEL+SUR, and TRA/MEL+SUR groups received an injection of 0.2 ml of lidocaine in the incision area (Xylestesin, Cristália, 20 mg/ml, SP, Brazil).

4.3.3 Preoperative preparation and anesthesia

Thirty minutes before the induction of the anesthesia, the rats were injected with the corresponding treatment by subcutaneous (SC) route. The person who injected the animals did not know which treatment was applied. After this, each rat was placed in an induction chamber and was anesthetized with 5% isoflurane (Isoflurine, Cristalia, 1ml/ml, SP, Brazil) carried with 100% oxygen at a flow rate of 1 L/min. After losing the righting reflex, rats were laid in ventral recumbency on a preheated thermal blanket to minimize the loss of body temperature (between 35°C-37°C) and 1.5% to 2%

isoflurane was administrated with a nose mask for anesthesia maintenance. Sterile ocular lubricant (Vidisic® Gel-Bauch+Lomb, 2 mg/kg, SP, Brazil) was administered to both eyes. Rat's head was positioned in the stereotaxic frame and hair shaving, aseptically cleaning and surgical draping were performed in the surgical area. An injection of 0.2 ml lidocaine (SC) was applied on the incision area to the animals of all groups, except for the SAL+SUR group. SAL+ANE group was anesthetized for 30 minutes (average duration of surgery), while the other groups underwent craniotomy. The physiologic parameters and reflexes were measured every 5 minutes until the righting reflex returned.

4.3.4 Surgical procedure

Five minutes after the application of lidocaine, an incision of about 2.5 cm on the midline of the scalp of the animal (from between the eyes until the back of the ears) was made. The skin was held with four Bulldog clamps to keep the incision open, and the subcutaneous tissue was removed with a sterile cotton swab, exposing the skull surface. Then, a 0.9 mm OD hole was made with a sterile hand drill (5 mm caudal from the bregma and 3 mm lateral to the sagittal suture in both parietal bones). A 1x2 mm screw was placed and covered with dental acrylic resin (Dencrilay, Dencri, 25 g, SP, Brazil). After this, a simple suture with 0.3 nylon was made to close the incision. Finally, a clostebol and neomycin spray (Neowell, Wellcopharma, 30 g, GUA, Guatemala) was applied on the wound.

4.3.5 Postoperative analgesia and care

When rats recovered the righting reflex, they were placed alone in a cage with bedding material. A lit infrared lamp was placed one meter away from the cage to maintain a warm environment to avoid hypothermia during recovery.

The administration of each treatment was applied every 12 hours for 3 days by SC route. MEL+SUR animals were injected with saline in the second injection of each day, because this drug has a duration of action of 24 hours. The rat's health condition was evaluated by a veterinarian during 72 hours after surgery.

4.3.6 Behavioral analysis

One hour and half after surgery, when the animals were totally recovered from anesthesia, a battery of behavioral tests was started. Each rat was placed in the center of an open-field (OF) arena and its behavior was scored during 10 min. The arena consisted of a circular, wooden chamber (90 cm diameter) covered with a black laminate-sheet and illuminated with white light (~106 lumens over the OF arena).

Twenty-four hours after surgery, OF was repeated. Behaviors were video recorded for offline analyses. The equipment was thoroughly cleaned with ethanol (5%), after testing each animal to prevent any possible bias caused by odor cues left by the previous rat. Locomotion was scored by the Ethovision XT video tracking software (version 15.0.1416, Noldus Information Technology bv, The Netherlands) and expressed in meters. Rearing frequency and the grooming duration were manually scored by trained observers using Solomon Coder software (version 17.03.22; https://solomoncoder.com/download.php). Grooming is a set of self-directed movements including hand rubbing, face washing, unilateral and bilateral strokes over the head and ears, body-licking, head and body scratching, and tail licking. The grooming sequences were classified as previously described (Rojas-Carvajal and Brenes 2020; Rojas-Carvajal et al 2020). Briefly, we considered two variables: anatomical distribution and complexity. Anatomically, grooming was classified as cephalic (head- and forehand-directed sequences), caudal (directed to the body), and sequential (chained sequences of head and body grooming). Those sequences, including the use of the hind paws, were considered more complex and designated as variations of the standard form. As a result, six different subtypes were scored. Micro grooming (<1s) was also counted whereas discarding isolated scratching events.

4.3.7 Grooming transfer test (GTT)

Once the OF was completed, animals were separately placed in clean cages (41 x 34 x 16 cm) filled with bedding material and transported to an adjacent room. Then, a drop of a gel fluorescent under UV-light (Glo Germ Oil, Glo Germ, Moab, UT) was applied on their foreheads. The gel presence was revealed by turning off the room light and exposing the animal to a UV-lamp at 15 cm height. A five-point scale was used to assess the absence/presence of the gel, in which an intense fluorescent signal was scored as 1, whereas the total absence of fluorescence was scored as 5 (Figure 1). Since the fluorescent gel was removed by animals' self-grooming, its presence was inversely related to the grooming frequency. Therefore, to assess the long-term effect of the drugs on this behavior, fluorescence was examined 4 h, 6 h, 8 h,10 h and 24 h after the administration. Trained observers, blind to test conditions, assigned scores. To assess the effect of the drugs during the postoperative period, the gel was applied every morning after the administration of the treatment and the examination was made every day for a total of 72 hours.

4.3.8 Assessment of body weight, food/water intake and nesting behavior (NB)

Each rat was weighted daily during the postoperative time. Food and water intake were measured until 72 hours after surgery. NB was evaluated using a five-points scale: 5 to indicate that the animal uses the material (for nesting or totally destroying it) and 1 to indicated that the animal did not use it at all (Figure 2). For this, two sheets of towel paper with a dimension of 36.5 cm x 28 cm were placed in the left front side of the cage every day as well.

4.3.9 Endpoints and euthanasia

Animals with severe dyspnea, severe hypothermia (<35°C) during anesthesia, or with severe pain signs during the postoperative time must be euthanized. However, no animals presented these clinical signs.

At the end of the experiment all animals were euthanized in a CO_2 gas euthanasia induction chamber (Red Industria e Comercio de Equipamentos Hospitalares e

Laboratoriais, Caieiras, SP, Brazil). Death was confirmed by ascertaining cardiac and respiratory arrest.

4.3.10 Statistical analysis

Each group was compared to SAL+ANE and SAL+SUR, analyzing cumulative scores by planned contrasts ran using one-way ANOVA. OF results of 24h and 48h were compared to each other analyzing cumulative scores using two-way ANOVA using Hours (1.5h and 24h) and Treatment Groups (SAL+ANE, SAL+SUR, TRA+SUR, MEL+SUR, and TRA/MEL+SUR) as factors. Grooming subtypes were analyzed by a two-way ANOVA using Subtype (i.e., cephalic, cephalic with variations, caudal, caudal with variations, sequential, and sequential grooming with variations) and Treatment Groups as factors. When appropriated, independent analyses per group were run by one-way ANOVA. GTT and NB were analyzed by two-way ANOVA, using Hour (4h, 6h, 8h, 10h and 24h) and Treatment Groups as factors. Body weight, food/water intake and NB were analyzed by two-way ANOVA using Hour (24h, 48h and 72h) and Treatment Groups as within-group factors. Dunnett's multiple comparison tests were applied for pairwise comparison when appropriate. Statistical significance was set as $P<0.05$. All analyzes were executed using GraphPad Prism 8.2.1 software (GraphPad Software, Inc., 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA).

4.4 RESULTS

4.4.1 Open field test

At 24h the highest average of distance traveled was observed in SAL+SUR rats ($F (4, 35) = 2.533$, $p= 0.0398$) and MEL+SUR rats ($F (4, 35) = 2.533$, $P=0.0274$) compared to SAL+ANE rats (Figure 3A). Also, SAL+SUR rats ($F (4, 35) = 2.533$, $P= 0.0398$) and MEL+SUR rats ($F (4, 35) = 2.533$, $P=0.0275$) showed the highest velocity compare to SAL+ANE at 24h (Figure 3B). MEL+SUR rats ($F (4, 35) = 2.533$, $P= 0.0488$) showed less movement than SAL+ANE at 1.5h; however, SAL+SUR rats (F

(4, 35) = 2.428, P= 0.038) and MEL+SUR rats (F (4, 35) = 2.428, P=0.0392) showed an increase movement than SAL+ANE at 24h after surgery (Figure 3C). In addition, MEL+SUR group showed an increase of the distance (F (4, 70) = 2.291, P=0.0212), velocity (F (4, 70) = 2.292, P=0.0212) and movement (F (4, 70) = 3.963, P=0.0005) between 1.5 hours and 24 hours after surgery (Figure 3A, 3B, 3C).

Although no differences were observed among groups in rearing frequency at 1.5h., all groups that underwent surgery presented less frequency than SAL+ANE (Figure 3D). SAL+SUR (F (4, 35) = 3.446, P=0.0465), TRA+SUR (F (4, 35) = 3.446, P=0.0085), and TRA/MEL+SUR (F (4, 35) = 3.446, P=0.0148) groups showed significantly less rearing duration compared to SAL+ANE at 1.5h (Figure 3E). SAL+SUR group (Frequency: F (4, 70) = 2.778, P= 0.0047; Duration: F (4, 70) = 2.010, P= 0.0018) and MEL+SUR group (Frequency: F (4, 70) = 2.778, P= 0.0005; Duration: F (4, 70) = 2.010, P= 0.0048) increased the frequency and duration of rearing between 1.5h and 24h (Figure 3D and 3E).

There were no differences in the frequency of grooming in both OF tests (Figure 4A). MEL+SUR group presented the highest duration of grooming at 1.5h and it was significantly different in comparison with SAL+ANE (F (4, 35) = 7.032, P= 0.0027) and SAL+SUR group (F (4, 35) = 7.032, P=0.0011), while SAL+ANE group (F (4, 35) = 3.337, P= 0.0399) presented a higher duration of grooming at 24h compared to SAL+SUR group (F (4, 70) = 5.138, P= 0.0399), TRA+SUR (F (4, 70) = 5.138, P= 0.0193) and TRA/MEL+SUR (F (4, 70) = 5.138, P= 0.0099) (Figure 4B). MEL+SUR group (F (4, 70) = 5.138, P= 0.0028) presented a significant reduction in grooming duration between 1.5h and 24h (Figure 4B).

MEL+SUR group developed sequential grooming subtype with greater frequency and longer duration than other the rest of the groups, with a significant difference in frequency (F (24, 245) = 1.838, P= 0.0001) compared to SAL+SUR group and in duration (F (24, 245) = 4.153, P=<0.0001) in comparison to SAL+ANE and SAL+SUR groups at 1.5h (Figure 4C and 4D). On the other hand, TRA+SUR was the group that developed this grooming subtype with less frequency and shorter duration in relation to all groups. This group also had a significant difference in frequency (F (24, 245) = 1.838, P=0.0398) and duration (F (24, 245) = 1.838, P=0.0398) compared to SAL+ANE group at 1.5h (Figure 4C and 4D). TRA/MEL+SUR group presented a significantly lower duration of sequential grooming (F (24, 245) = 1.838, P= 0.0459) in comparison to SAL+ANE group (Figure 4D), and this group was the only one that

developed scratching grooming, with a significant difference in frequency (F (24, 245) = 1.838, P=0.0398) in relation to SAL+SUR group at 1.5h (Figure 4C).

SAL+ANE presented the highest frequency (F (24, 245) = 1,590, P=0.0042) and duration (F (24, 245) = 4.074, P=<0.0001) of sequential grooming subtype in comparison to SAL+SUR group at 24h. All groups that underwent surgery showed significantly lower frequency (TRA+SUR: F (24, 245) = 1.590, P=0.004, MEL+SUR: F (24, 245) = 1.590, P=<0.0001 and TRA/MEL+SUR: F (24, 245) = 1.590, P=0.0459) and duration (SAL+SUR: F (24, 245) = 4.074, P=<0.0001, TRA+SUR: F (24, 245) = 4.074, P=<0.0001, MEL+SUR: F (24, 245) = 4.074, P=<0.0001 and TRA/MEL+SUR: F (24, 245) = 4.074, P= <0.0001) of sequential grooming in comparison to SUR+ANE group at 24h (Figure 4E and 4F).

4.4.2 Grooming Transfer Test

During the first 24h, SAL+ANE developed more grooming behavior than the other groups, showing a significant difference (F (16, 175) = 0.7647, P=0.0316) with the SAL+SUR at 10h. TRA/MEL+SUR was the group that showed the lowest scores of grooming at 6h (F (16, 175) = 0.7647, P=0.0088), 8h (F (16, 175) = 0.7647, P=0.0004) and 10h (F (16, 175) = 0.7647, P=<0.0001) in comparison to SAL+ANE group. MEL+SUR group also presented significant lower scores at 8h (F (16, 175) = 0.7647, P=0.0088) and 10h (F (16, 175) = 0.7647, P=0.0316) in comparison to SAL+ANE group (Figure 5A).

After 48 hours, SAL+ANE was the group that presented the highest grooming behavior scores. MEL+SUR group showed the lowest grooming score at the first evaluation at 4h (F (16, 175) = 0.3656, P=0.0254) on this day. SAL+SUR (F (16, 175) = 0.3656, P=0.0254), TRA+SUR (F (16, 175) = 0.3656, P=) and TRA/MEL+SUR (F (16, 175) = 0.3656, P=0.0254) were the groups with lowest scores at 6h of evaluation. Then, SAL+SUR (F (16, 175) = 0.3656, P=0.0254) and TRA+SUR (F (16, 175) = 0.3656, P=0.0254) continued having the lowest scores of grooming at the last hours of evaluation in the second day (Figure 5B). Finally, on the third day of evaluation (72h) there were not differences among all the groups (Figure 5C).

4.4.3 Body weight, food/water intake and nesting behavior (NB).

No differences in body weight were observed among groups in the postoperative period. Only the MEL+SUR group showed a body weight increase between 24h and 48h (Figure 6A). There were no differences among groups in water intake in the postoperative period (Figure 6B). Regarding food intake, TRA/MEL+SUR group presented lower consumption at 24h and 48h after surgery compared to SAL+ANE (24h:F (8, 105)= 0.3288, P=0.0391; and 48h: F (8, 105)= 0.3288, P=0.0035) and during the three days after surgery compared to SAL+SUR group (24h:F (8, 105)= 0.3288, P=0.0335; 48h: F (8, 105)= 0.3288, P=0.0107; and 72h: F (8, 105)= 0.3288, P=0.0148). TRA+SUR group also presented a significant reduction in food consumption at 48h (F (8, 105) = 0.3288, P=0.05) compared to SAL+ANE group and at 72h (F (8, 105) = 0.3288, P=0.0148compared to SAL+SUR group (Figure 6C).

SAL+ANE was the group with the highest NB score and showed a significant difference compared to SAL+SUR group (F (16, 175) = 0.4233, P=0.0194) at 8h during the first day of evaluation. On the first day of evaluation, all groups treated with analgesics showed lower NB scores, with significant differences at 6h (TRA+SUR: F (16, 175) = 0.4233, P=0.0067; MEL+SUR: F (16, 175) = 0.4233, P=0.0006; and TRA/MEL+SUR: F (16, 175) = 0.4233, P=0.0021), 8h (TRA+SUR: F (16, 175) = 0.4233, P=0.0002; MEL+SUR: F (16, 175) = 0.4233, P=0.0002; and TRA/MEL+SUR: F (16, 175) = 0.4233, P=<0.0001), and 10h (TRA+SUR: F (16, 175) = 0.4233, P=0.0021; MEL+SUR: F (16, 175) = 0.4233, P=0.0006; and TRA/MEL+SUR: F (16, 175) = 0.4233, P=<0.0001) compared to SAL+ANE group. TRA/MEL+SUR group (TRA/MEL+SUR: F (16, 175) = 0.4233, P=0.0006) maintained the lowest NB score until the last evaluation of the first postoperative day compared to SAL+ANE group (Figure 7A).

After the second day (48h) of evaluation all groups presented similar NB scores (Figure 7B and 7C).

4.5 DISCUSSION

Pain is an important health problem that must be avoided under all circumstances, especially during invasive experimental procedures to preserve animal welfare. Inadequate pain management can affect results, especially in those

experiments in which animal behavior must be analyzed. In that regard, detection of craniotomy-related pain and its prevention is critically important in neuroscience studies (Cho et al 2019).

SAL+ANE group was only anesthetized to have a control group without any intervention that could produce pain to be compared to the other groups. This group showed very similar exploratory behavior at 1.5h and 24h after surgery in the OF, but grooming significantly increased at 24h. Locomotion allows the rat to have a spatial and sensorimotor representation of the place being explored (Blanchard & Blanchard 2008) and rearing allows it to monitor the environment to identify possible sources of danger (Brenes et al 2006; Blanchard & Blanchard 2008). Usually, when rats are subjected to OF over and over, they can develop habituation, because the environment is no longer a novelty and they remember that it had no harmful consequences on them (Rojas-Carvajal et al 2018). For this reason, grooming started to be gradually displayed more frequently and with more duration. In addition, this result allows us to demonstrate that anesthesia (using isoflurane) did not greatly interfere with the animal behavior during the first 24 hours in this test.

SAL+SUR and MEL+SUR were the groups with more exploration changes in the OF at 24h, with a high expression of the locomotion and rearing behavior. We hypothesized that rats from the SAL+SUR group could have the increase of exploration due to pain. In various studies in which abdominal surgeries were performed (Liles & Flecknell 1993a,b, Liles et al 1998), authors reported a reduction of locomotion and rearing as an indicator of postsurgical pain. However, in this case, the surgery was performed on rats' skulls, so the procedure may not interfere in the movement of the animal. Measuring pain in animals is difficult and often relies on reflex measures of external limbs, but in the case of headache as a consequence of craniotomy, it can be more challenging. Depending on the measures, these experiences range from assessment of pain-stimulated behaviors to pain-depressed behaviors (Larson et al. 2019). However, OF has only been studied for a limited number of painful conditions in rodents, and differences in the type of pain or surgical procedure can produce different reactions, so this could limit the utility of this test for clinical assessment of pain (Turner et al 2019).

On the other hand, the increase in locomotion of MEL+SUR is complex to interpret because if we compare this reaction with that of the SAL+SUR group, we might think that the analgesic treatment was not as effective in eliminating pain. However, in a previous study (unpublished data) we demonstrated that MEL can produce an increase of locomotion in naïve rats in the OF.

In the OF was observed a reduction of grooming in all groups submitted to surgery. However, the MEL+SUR group, had an increment in frequency and duration of grooming at 1.5h. Some studies related active movements and reduced grooming to an analgesic benefit (Flecknell and Liles 1991, Liles and Flecknell 1993b), but the high frequency and duration of grooming showed by MEL+SUR rats at the first 1.5h prevents us from associating the effect of MEL with the reduction of pain.

SAL+SUR, TRA+SUR and TRA/MEL+SUR rats also showed a reduction in grooming duration, especially in the sequential grooming subtype. The GTT revealed that the reduction of grooming behavior was prolonged until the first 48 hours in some of the rats that underwent surgery. The most affected groups were TRA/MEL+SUR and SAL+SUR who presented the lowest GTT scores during this period. Grooming is a complex patterned behavior, which generally proceeds in a cephalocaudal direction, in which the sequential subtype starts with licking the paws, followed by washing the nose, face and head and continuing to clean the body (Smolinsky et al 2009).

Considering that craniotomy causes pain, rats could reduce the contact to the injured area, with a consequent reduction of grooming. Thus, pain could affect SAL+SUR rats for the absence of analgesics in the postoperative time. In the case of TRA+SUR and TRA/MEL+SUR, the reduction can be related to the effect of the TRA. In previous results (unpublished data) we could demonstrate that rats treated with TRA presented a reduction in grooming in the OF. We connected this effect to different factors related to the mechanism of action of TRA, such as increasing of serotonin levels that can lead unrest and distract the rat from grooming all its body (Kolawale et al 2020); and the blockage of the serotonin 5-HT_{2A} receptors (that are involved in the analgesic effect of tramadol) that can produce a reduction in grooming (Amodeo et al 2017).

Injection site reaction was an unexpected side effect of TRA and TRA/MEL drugs. The majority of the animals injected with these treatments developed ulcerative

skin injuries, although these treatments were diluted in a 1:5 proportion with saline. This could be the reason for the high frequency of scratching grooming behavior in TRA/MEL at 1.5h. Cannon et al. 2010, also reported the appearance of ulcerative-type lesions in rats that were injected with TRA, but in these cases, these lesions occurred in animals injected with higher doses of tramadol (25 to 50 mg / kg). These alterations could be related to the TRA formulation used. Although it is for veterinary use and can be used subcutaneously, was designed for other species.

Only TRA/MEL+SUR and TRA+SUR groups reduced food intake during postoperative time. However, this reduction did not cause body weight loss in these animals, because no significant difference was evident in this parameter. This finding is likely to have arisen due to appetite suppression as a result of excess opioid activity, as a consequence of an increased sedation, or a nauseating effect (Whittaker et al 2016).

The reduction of NB was more evident in the animals treated with analgesics, especially in the TRA/MEL group. Changes on general behavior might indicate commitment in wellbeing (Hess et al 2008). Considering that reduction of NB could be pain-related, the data might suggest that the analgesics used in this study did not provide a satisfactory level of analgesia. However, SAL+SUR showed higher scores of NB than the other groups that had analgesic treatment during the first 24h, so it is difficult to say that pain can be a cause of this reaction. Studies report that TRA can produce sedation in rats (Cannon et al. 2010, Nakhaee et al. 2021). This would explain the low scores of NB in the groups that were treated with this drug, but there are no reports about the effect of MEL over the NB. Therefore, specific studies should be made to clarify these results.

All surgeries were performed trying to avoid the animal suffering by refining the surgical technique as much as possible. For this, we administered the analgesics 30 minutes before surgery, we applied local lidocaine, we used a dental resin that does not achieve temperatures more than 40°C, as well as we used completely sterile equipment at all times. These actions possibly reduced postoperative pain (evidenced by the lack of difference in many of the results between SAL+SUR and SAL+ANE groups) and promoted a rapid recovery of the animals.

4.6 CONCLUSIONS

The data taken together, suggest that craniotomy produced mild pain in the animals, as the results obtained in most of the tests did not differ significantly from the group without analgesia. For this reason, the use of analgesic is required at least during the first 48 hours post-surgery, a period in which the greater changes in the behavior of animals that underwent surgery were observed and refine the surgical technique to reduce pain. It is important to consider that analgesics can produce different effects on animal behavior, so these variables must be considered in the experimental design and planning, or to perform behavioral tests 72 hours after surgery when analgesic treatment has finished.

4.7 ANIMAL WELFARE IMPLICATIONS

In this study we demonstrate that craniotomy can produce pain at least during the first 48 hours, so the administration of analgesics and the refinement of the surgical technique is mandatory. Despite analgesics can generate physiological and behavioral changes in the rats, it is important to consider these effects, to reduce influence on the results.

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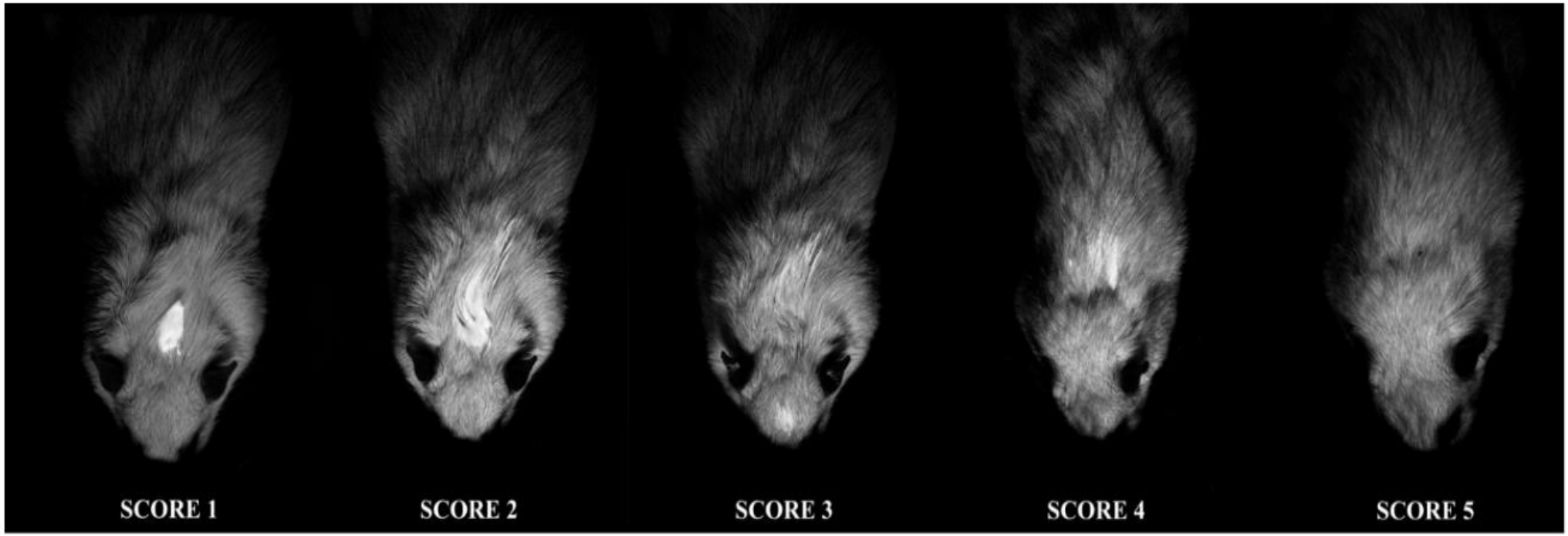

Figure 1. Grooming transfer test scoring. Fluorescence score was based on Oliver et al. 2018 methodology. Score 1: Strong fluorescent signal at the application site between the ears. Score 2: Fluorescent signal at the application area and signs that the gel was spread on the rat's back. Score 3: Fluorescence on the head, ears and back. Score 4. Florescent signal is almost absent but remains amounts at the application site. Score 5. Fluorescence is no longer detected.

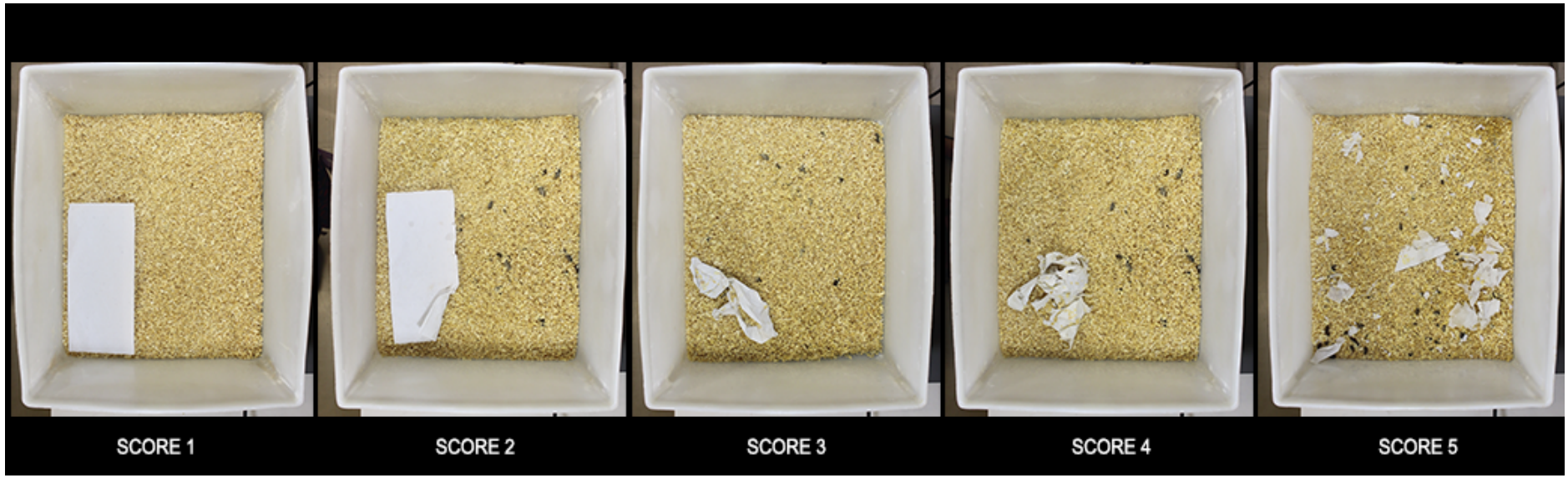

Figure 2. Nesting behavior scoring. Score 1: Nesting material is intact and in the same position as it was initially placed. Score 2: Nesting material poorly handled, with some signs of biting (more than 90% is intact). Score 3: 25%-50% of nesting material is shredded. Score 4. 50%-90% of nesting material is shredded but remains in the same position as it was initially placed. Score 5. The towel papers are totally destroyed and scattered in the cage.

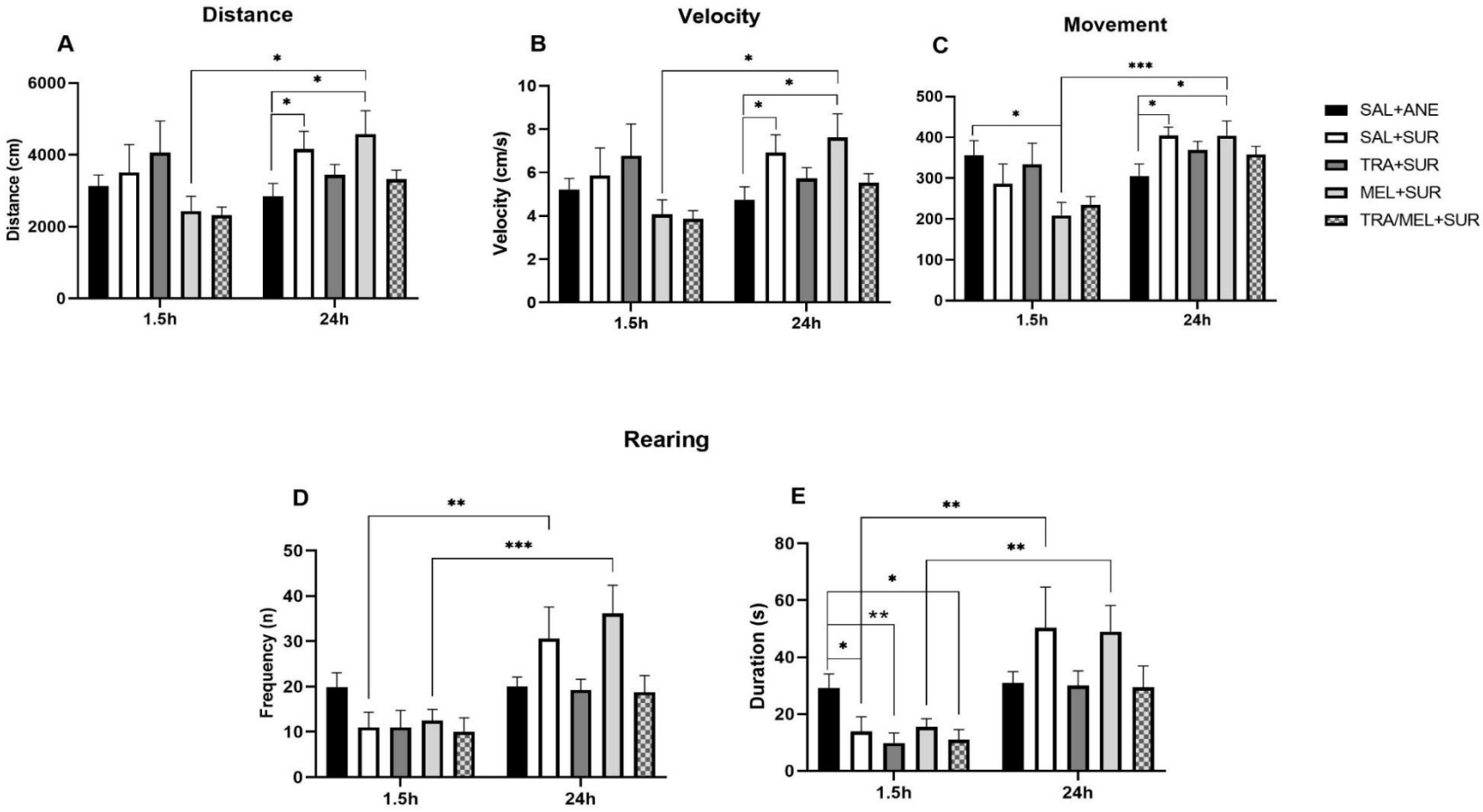

Figure 3. Exploratory behavior of rats in the Open Field at 1.5 hours and 24 hours after anesthesia or surgery. **P*<0.05; ***P*<0.01; ****P*<0.001. SAL: Saline, ANE: Anesthesia, SUR: Surgery, TRA: Tramadol, MEL: Meloxicam.

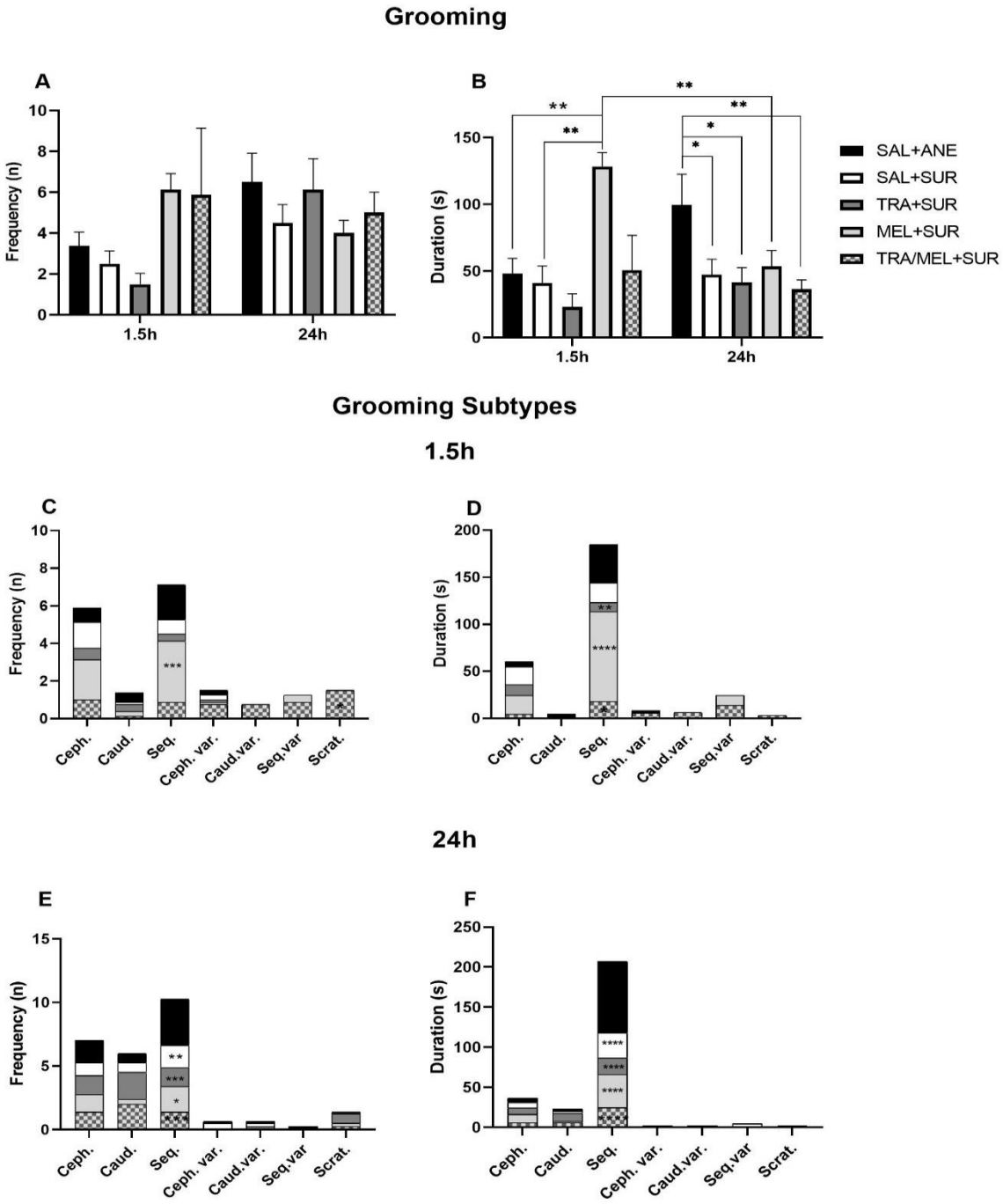

Figure 4. Grooming behavior of rats in the Open Field at 1.5 hours and 24 hours after anesthesia or surgery. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. SAL: Saline, ANE: Anesthesia, SUR: Surgery, TRA: Tramadol, MEL: Meloxicam, Ceph: Cephalic grooming; Ceph.var: Cephalic grooming with variations; Caud: Caudal grooming; Cuad.var: Caudal grooming with variations; Seq: Sequential grooming; Seq.var: Sequential grooming with variations.

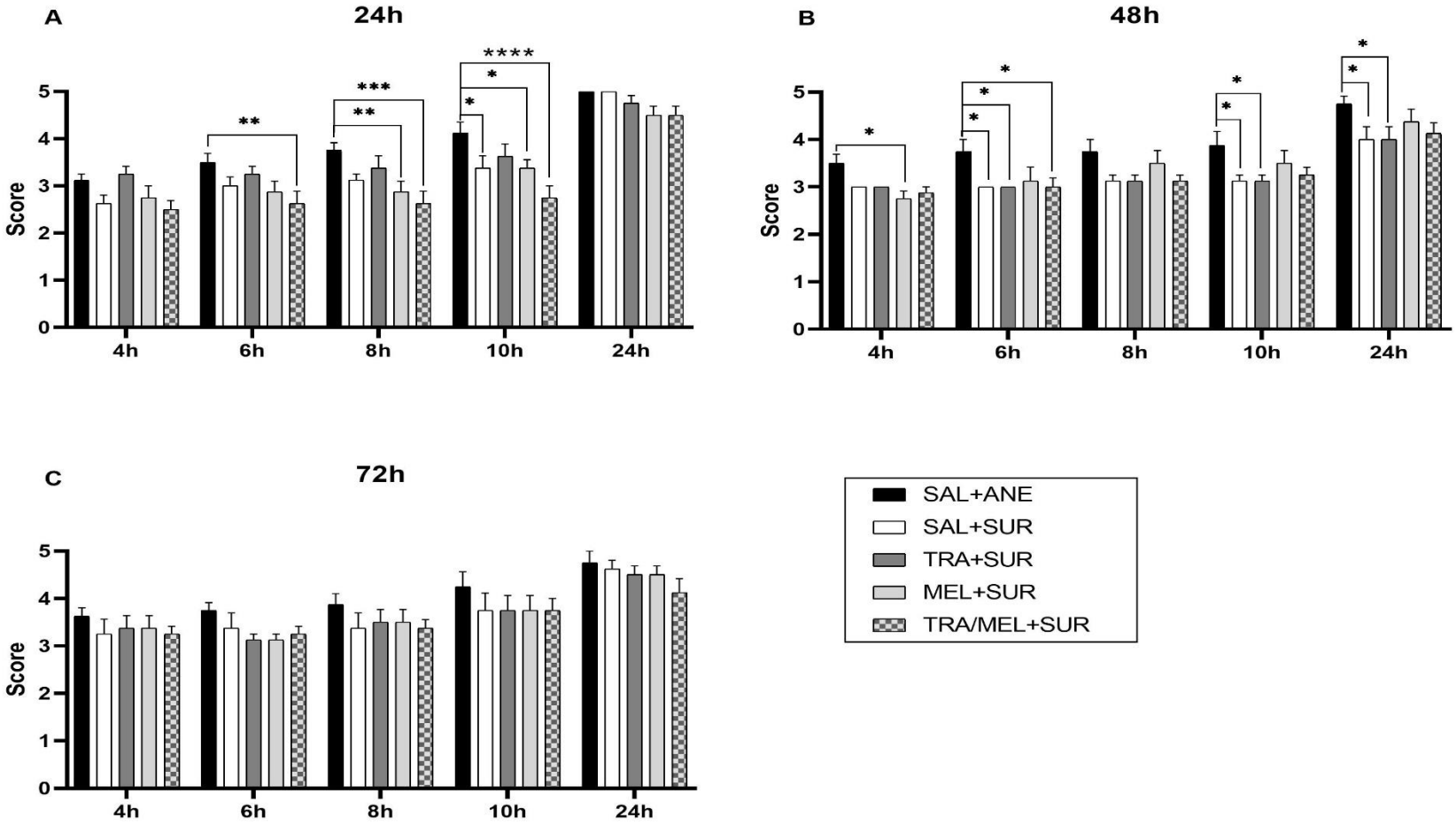

Figure 5. Grooming Transfer Test scores during the first 72 hours of the postoperative time. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. SAL: Saline, ANE: Anesthesia, SUR: Surgery, TRA: Tramadol, MEL: Meloxicam.

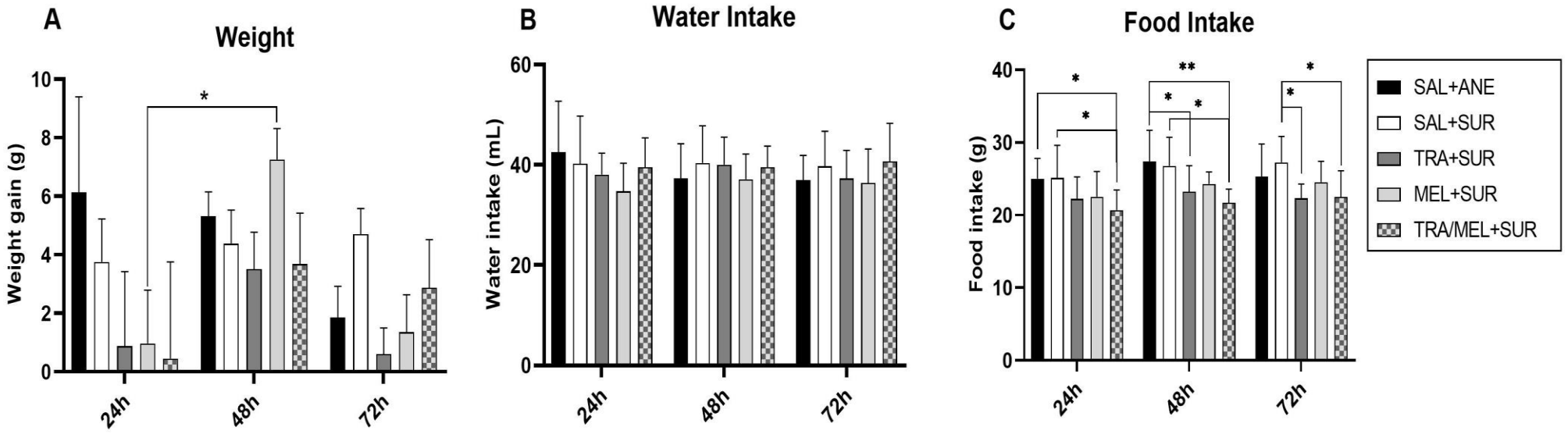

Figure 6. Body Weight Gain and Water and Food/ intake evaluation during the first 72 hours of the postoperative time. *P<0.05; **P<0.01. SAL: Saline, ANE: Anesthesia, SUR: Surgery, TRA: Tramadol, MEL: Meloxicam.

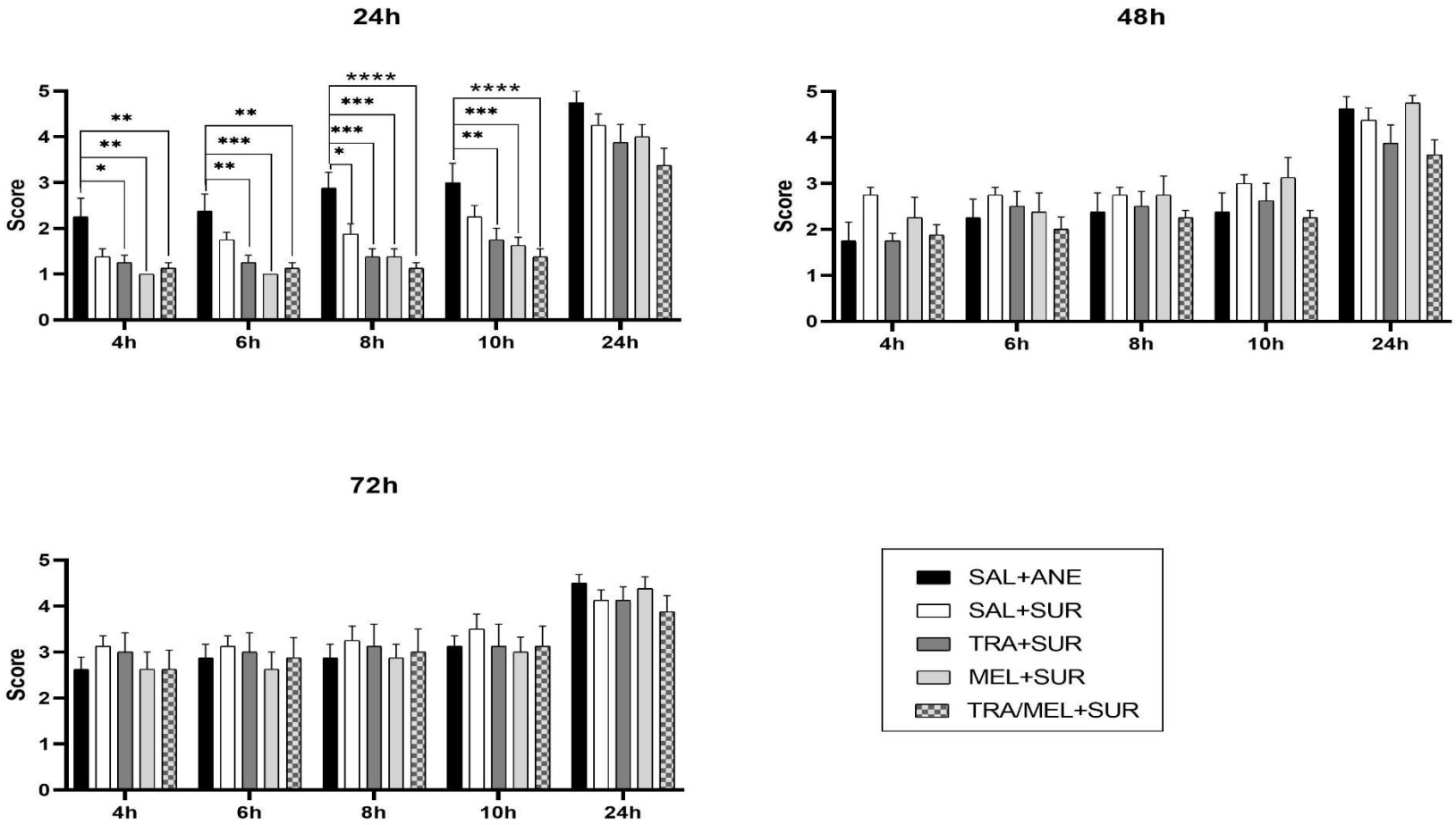

Figure 7. Nesting behavior scores during the first 72 hours of the postoperative time. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. SAL: Saline, ANE: Anesthesia, SUR: Surgery, TRA: Tramadol, MEL: Meloxicam.

SUPPLEMENTARY MATERIAL

4.9. MATERIALS AND METHODS

4.9.1 Elevated Plus Maze (EPM)

Once the OF test was completed, animals were submitted to Elevated Plus Maze (EPM) test for analyzing their reactivity to a new and more anxiogenic environment. EPM consists of an elevated (40 cm height) black platform with four symmetrical arms (30 x 10 cm): two opposed arms were enclosed by walls (40 cm height) and connected to a central platform, whereas the other facing arms were perpendicularly attached to the central platform and had no walls. Animals were placed in the central platform facing the closed arm, and they were allowed to explore for 5 min. After testing each animal, the equipment was thoroughly cleaned with ethanol (5%), preventing any possible bias caused by odor cues left by previous rats. Trained observers manually scored frequency and duration of open and closed arms visiting, rearing, stretch-attempt posture (SAP), and head-dipping (HD) using Solomon Coder (version 17.03.22; https://solomoncoder.com/download.php). SAP consists of an exploratory behavior in which the animal stretches its body forward while keeping its hind paws still. HD comprises the animal projecting its whole head below the border of the open arms or central platform.

4.9.2 Statistical analysis

Each group was compared to SAL+ANE and SAL+SUR, analyzing cumulative frequency and duration by planned contrasts ran using one-way ANOVAs. Outliers of each group were identified and removed using ROUT method (Q=1%). Six animals from different groups fell off the EPM early on tests, so data were removed from the analyses, interfering with the degree of freedom among tests. Dunnett’s multiple comparison tests were applied for pairwise comparison when appropriate. Statistical

significance was defined as $P<0.05$. All analyzes were executed using GraphPad Prism 8.2.1 software (GraphPad Software, Inc., 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA).

4.10 RESULTS

Only animals of the groups that underwent surgery fell off of the EPM. SAL+SUR and MEL+SUR were the groups with more falls (two rats), while TRA+SUR, TRA/MEL/SUR just had one animal that fell down.

Animals of all groups showed more frequency of entries and spent more time in the close arms (Figure S1.A). TRA+SUR was the group that spent more time in the open arms and was significantly higher compared to the SAL+ANE group ($F(4, 27)= 2.495$, $P=0.0437$) (Figure S1.B). SAP behavior was significantly reduced in the SAL+SUR group compared to SAL+ANE group ($F(4, 28)= 2.220$, $P=0.0356$).

4.11 DISCUSSION

Different from data reported by Walf and Frye (2007), who indicate that only 1% of the rats usually fell off from the open arms of the EPM, in our study 15% of rats fell. This percentage of animals corresponds only to the animals that underwent craniotomy surgery, excluding that the anesthesia could be the cause of this situation because any animal of SAL+ANE fell. This situation could be related to the fact that the surgery could produce some alteration in the balance of the animals or that the pain in the head could distract the animal and cause it to fall.

All groups revealed a special preference for the close arm. This is a normal behavior because rodents normally have an aversion to open and elevated areas to consider them as dangerous environments (Komada et al. 2008). The high frequency and duration of exploration of the open arms showed by the TRA+SUR group is typically interpreted as a low level of anxiety (Shoji and Miyakawa 2021). This can be associated

with an anxiolytic effect produced by TRA, that also has been described in other studies (Sadat-Shirazi et al. 2018, Nawrocka 2002). In the case of TRA/MEL+SUR the anxiolytic effect, was probably not evident due to the action of MEL that could counteract this effect.

Some authors have demonstrated that acute pain following surgery may lead to depression and anxiety (Cho et al. 2019). The reduction of the SAP in the SAL+SUR group can be related to an increase of anxiety, probably because of pain, once these animals did not receive any analgesic after surgery.

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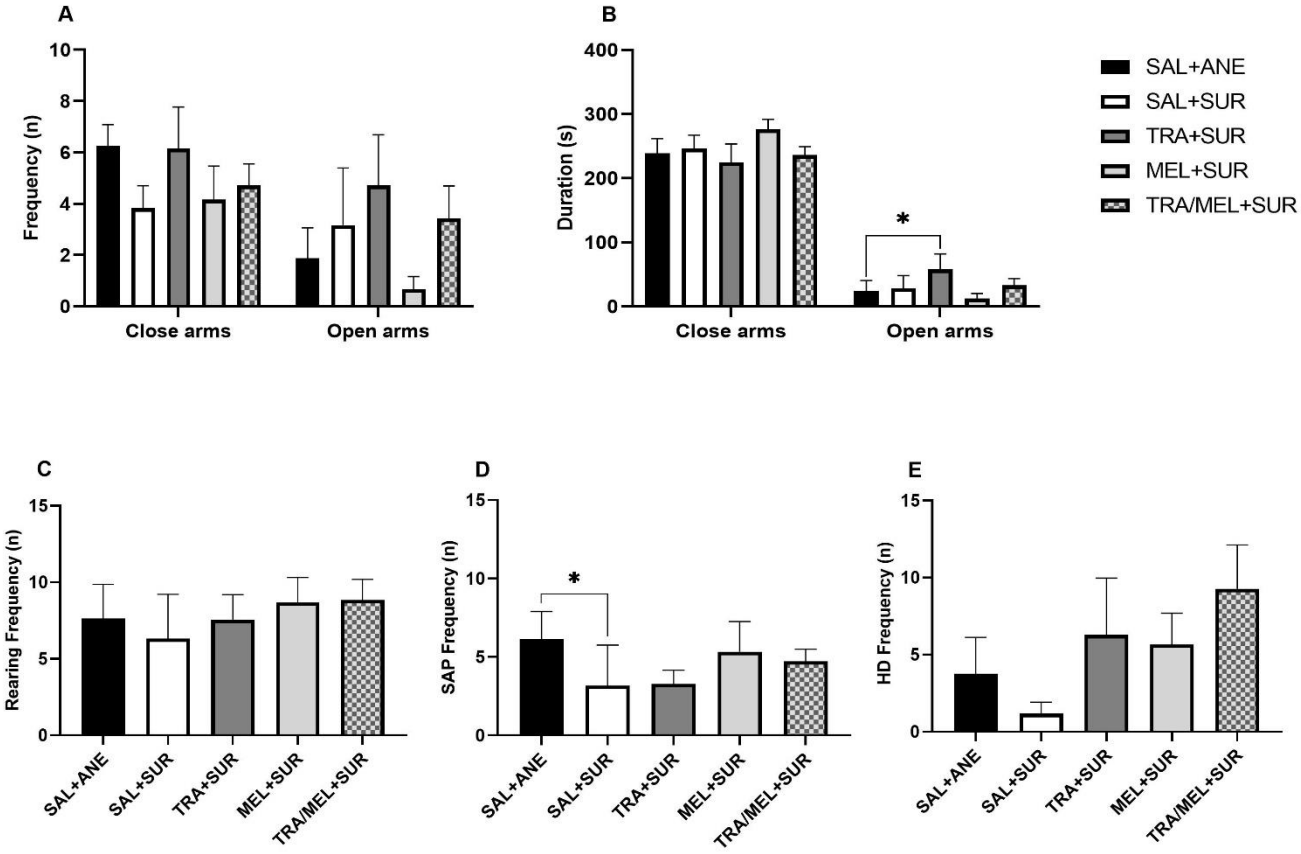

Figure S1. Cumulative scores in the Elevated Plus Maze behaviors. *P<0.05. SAL: Saline, ANE: Anesthesia, SUR: Surgery, TRA: Tramadol, MEL: Meloxicam

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CHAPTER 3

5 Quality of Reporting of the Material and Methods Section in Studies where Swine are used as Surgical Biomodels According to ARRIVE Guidelines: A Systematic Review (2013-2018)

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5.1 SIMPLE SUMMARY

Through a systematic review of reports where swine were used as animal biomodels for training or researching new surgical techniques, we seek to determine the quality of the report of the methodologies carried out based on ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) in a total of 108 studies, from 2013 to 2018. In a high percentage of the articles, the information presented in the methodology of the studies showed insufficient and incomplete data according to ARRIVE guidelines requirements for the use of animals. There was a strong focus on the data regarding the surgical technique; however, information about sample size calculation, description of maintenance conditions, animal handling, and anaesthetic and pain management protocols used were not very detailed. This could lead to poor reproducibility of experimental results. For this reason, we encourage authors to implement these guidelines to improve the quality of scientific reports to ensure animal welfare.

5.2 ABSTRACT

In recent years, pigs have become animal biomodels widely used for the investigation and practice of surgical techniques due to their great physiological and anatomical similarities. Even though many of these studies must be carried out later in humans, the description of basic information is limited, making results unable to be reproduced. In this review, 108 studies were considered from 2013 to 2018, to

determine the quality of the report of the methodologies performed based on ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).The majority of the literature lacked the quality based on ARRIVE guidelines in data directly related to the welfare of animals undergoing surgeries, as in the report of anaesthetic protocols and analgesics. Topics related to sample size calculation and animal housing and husbandry were also very limited. We believe that the ARRIVE guide is an excellent tool for reporting with good quality. We encourage scientists to mandatorily use it as a way to improve the quality of scientific reports and, consequently, ensure animal welfare.

Keywords: pigs; ARRIVE guidelines; surgery; analgesia; anaesthesia

5.3 INTRODUCTION

Swine have become popular animal models for preclinical trials for medical research because of their size and anatomical and physiological similarity to humans. For this reason, these animals are widely used for research on physiopathology and new surgical techniques. Worldwide, over the last 20 years, swine have replaced dogs as the general surgical model for both training and research [1].

A scientific and moral argument is that if pigs are used to ‘model’ human beings undergoing surgery, then they should receive the equivalent standards of perioperative care humans would; however, most bioscience journals provide little or no guidance on what information to report when describing animal research and many details are omitted [2]. Unfortunately, this might be the reason that researchers have been increasingly recognized for not replicating successful treatments in animal studies in the clinical trials followed [3]. Ideally, scientific publications should present enough information to allow a knowledgeable reader to understand what, why and how this was done, and to assess the reliability and validity of findings [4]. Omitting essential information might lead to scientific and ethical concerns, and does not allow reproducibility of results [5].

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), a UK government-sponsored scientific organization, has led an initiative to produce guidelines for reporting animal research. In 2010, the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines were

published [5] to address the growing concerns with poor experimental design and lack of transparent reporting of in vivo experiments in published literature. These guidelines consist of a checklist of 20 categories that provide all the information that researcher should include in scientific publications using animals [6].

Recent publications focuses on the quality of reporting revealed very little improvement in reporting standards since the guidelines were introduced. [7-8]. Research with pigs has also presented minimum information published on topics about perioperative care (anaesthetic protocols and pain alleviation) [2]. Bradbury et al. (2016) found that reporting postoperative pain management in studies was remarkably low, reflecting either under-reporting or under-use of it. For this reason, a systematic review was performed to evaluate the quality of the report on research in surgeries where swine were used as biomodels. We decided to analyse only the materials and methods section of each article because this is the section that describes procedures that have a direct impact on animal welfare, with a special emphasis on the given anaesthetic and analgesic procedures.

5.4 MATERIALS AND METHODS

5.4.1 Search strategy

A literature review was performed between journals published from January 2013 to December 2018. An internet search was performed using PubMed, Google Scholar, Scopus and Science Direct as electronic databases. The keywords used were: Swine OR Pig OR Minipig AND Surgery.

5.4.2 Inclusion and exclusion criteria

Articles included had these characteristics:

- Original articles published in English.
- Use of swine model in vivo.
- Studies that include surgery as an experimental procedure.
- Studies published between 2013 and 2018. This period was selected to sample the recent biomedical literature (for the last 5 years), considering a suitable period for the ARRIVE guidelines to be implemented.

- Studies that described painful experimental surgical procedures like skin incision, craniotomies, thoracotomies, laparotomies, laparoscopies, dental surgeries and orthopaedic surgeries [9].

Review articles, commentaries or communications were excluded. Studies without in vivo experiments were also excluded.

5.4.3 Evaluation of publication quality

We used the ARRIVE guidelines to analyze the articles focusing on the “Material and methods” section to evaluate the degree of compliance of publications with these guidelines.

We established a score based on 3 levels for evaluation of the categories of the guidelines. They were defined as follows: Score 0: not mentioned, total absence of any type of information, Score 1: unclear / not complete, items not mentioned completely in the category assess, Score 2: adequate / clear, complete information for all items corresponding to the category evaluated.

5.4.4 Statistics

Data was extracted from the articles, put into tables, and the information of each ARRIVE’s categories, subcategories and evaluated items were calculated and expressed as percentages.

Forty-three subcategories or items were evaluated as the levels of greater detail indicated for the ARRIVE guidelines. The frequency for each year was calculated and expressed in percentage according to the maximum and minimum percentage presented for each subcategory or item.

5.5 RESULTS

5.5.1 Study selection

From a total of 2775 articles, 145 articles were eligible by the analyses of the title and abstract. After this, each article was fully screened, and 37 articles were excluded for the following reasons: surgical procedures not included in the

aforementioned list (n=18), description of surgical procedure was insufficient or just cited (n=6), procedures performed on dead animals (n=6), non-original articles (n=4), non-surgical procedures performed in the studies (n=3) (Fig 1). Finally, a total of 108 publications (Supplementary information 1) of 81 different journals fulfilled the inclusion criteria required for this systematic review (Fig. 1).

5.5.2 Information analyzed in each article

In general, studies included some information related to the methodology used with animals. However, most of the literature lacked the quality based on ARRIVE guidelines, even though 27 of the journals consulted encouraged other publishers to use ARRIVE guidelines. All categories of reporting information in all articles were unclear or incomplete, except the category of experimental outcomes, in which most of the journals gave very little or no information (Table 1.)

Of all articles analyzed, those published in 2014 and 2015 provided more information about material and methods section (42% and 35% respectively, of the 43 subcategories/items presented maximum mean of report for the years considered). Articles published in 2013, 2016, 2017 and 2018 presented less information (all these years presented around 30% of the 43 subcategories/items presented minimum mean of report for the years considered) (Table 2).

The most reported subcategories/items were the following: surgical procedure (100%), total number of animals used (94%), and approval by an ethical committee (92%). Several subcategories/items were not mentioned in all the articles (e.g. time of the day, 0%) or little mentioned (e.g. choice of a specific anaesthetic, route and dose, 1%; bedding material, 3%; environmental enrichment, 3%; and an explanation of how the number of animals was determined, 3%) (Table 2).

5.5.3 Surgery and anaesthesia

Most reported surgical procedures in articles were laparotomies, orthopaedic surgeries and thoracotomies (Table 3).

5.5.4 Anaesthesia

The drug used for anaesthesia was reported in 81% of articles; however, reports of doses of each of these drugs were only recorded in 71% of the articles, and the route of administration in 68%. Most commonly used drugs were ketamine (58.3% of the articles), isoflurane (44.4% of the articles), xylazine (31.5% of the articles) and midazolam (25% of the articles). These drugs were used alone or in combination with other drugs, totalling up to 55 different anaesthetic protocols.

5.5.5 Analgesia

Intraoperative or postoperative analgesia was only reported in 41% of the articles. Doses of the analgesic drugs used were reported in 32% of the articles, and the route chosen for administration was reported in only 24% of the articles. Fentanyl (reported in 14.8% of the articles) and lidocaine (reported in 13.9% of the articles) were the drugs used for intraoperative analgesia in most studies. The most used drugs in postoperative analgesia were buprenorphine (reported in 12 articles) and meloxicam (reported in 8.3% articles).

5.6. DISCUSSION

The “Materials and methods” section of research papers should provide basic information about how research was performed. Comprehensive reporting is essential to understand how investigations were undertaken, to properly interpret findings properly [7], and to allow the reproduction of results if the same methodology is carried out in other studies. The information obtained in this systematic review showed that many of these studies would not be reproducible, considering that important details were omitted.

After examining articles from 2013 (a year after creation and implementation of this guide), it is interesting to see that the report of all data that described the methodology according to the ARRIVE guidelines did not improve through the years, even though 27 of the 81 journals consulted requested the authors to use these guidelines. The outcomes we obtained resemble what Barker [11] et al. concluded in 2014; they made an analysis of papers published in PLOS and Nature journals and

they realized that very little improvement in reporting standards were reported since publication of the ARRIVE guidelines in 2010. In fact, it was observed in this review a better report of the methods in the years 2014 and 2015 (although they were incomplete in most cases), but then the quality of report decreases again, with 2017 the year with the least reported data, followed by 2013 and 2018. The reproducibility of these studies and the possibility of guaranteeing the welfare of animals can be affected in similar studies to those described in these journals [2-12] .

The high percentage of reports of ethical review permissions is an indication that projects were previously reviewed to ensure the welfare of the animals by ethical institutional committees; however, many articles did not indicate the protocol number or guides on which they were based to carry out their protocols. These data should allow tracking the approved protocols and the guides that justify the care provided to the animals used in each investigation [13].

The report of study design had a relative increase in number of subcategories of experimental and control groups and experimental units; however, the detailed methods description to avoid bias was undermentioned (only in 27% of the journals). The lack of randomization and blinding can affect scientific validity because biased, conscious or unconscious, factors with no relation to biological action can influence results, so mentioning the way it was done is a critical point [14].

Considering experimental procedure, journals omitted important data such as the time in which experiments were performed in all the articles. Probably, due to the scope of these studies focused on the surgical procedures´ description, this factor may not have been considered. Circadian cycle might change animal physiology, leading to different results, which makes it important to describe the time of day experiments were done to ensure experiment reproducibility [15]. Another poorly described data was explaining the choice of medications, doses and administration routes of drugs used in pigs undergoing surgery. According to its pharmacokinetics and pharmacodynamics, each medication presents a different effect on animal physiology, which may cause results to vary, so justifying their use is essential. Similarly, different routes of administration may differ on the absorption levels, and the effect generated may produce differences in results generated, so their choice should be well justified as well [16].

The least mentioned data in the category of experimental animals were the source, sex and race of animals. These data should be mandatory in any report of

experimental procedure using animals because genetic and/or hormonal characteristics of animals might influence results. Most articles also poorly mentioned poor information about housing and husbandry. Although studies are not specific to animal welfare issues, these data must be mentioned so other researchers can reproduce the same conditions. Experimental animals should not be unnecessarily stressed and should be kept under appropriately controlled conditions. Poor animal welfare is likely to result in poor science [7].

Most of the studies reported the number of animals; however, almost none reported the statistical method used to calculate this number. Determining sample size by power size or simple calculations help to design an animal research with an appropriate number of animals to detect biologically important effects. Omission in reporting means potentially flawed research [17].

The statistical methods were not mentioned in 24% of the studies. This section should not be omitted because it shows the way data was analysed. Likewise, much of this information reported in journals was not written in detail and raises doubts if statistical methods were correct or enough to evaluate results. While focussing on technically challenging science and generating innovative science, many journals fail to ensure adherence to that basic standard of experimental design and data analysis are adhered to. One solution to this problem is to have additional statistical review of submitted manuscripts (as is often done by journals in the health sciences). In addition, learned societies might suggest methods of analysis of standard outcomes and data reporting to their members [11].

All the surgical procedures generate pain in different intensities according to the degree of invasion and amount of tissue damage degree [18]. In this review, surgeries that were most reported are those which can generate moderate to severe pain. Thoracotomy and orthopaedic surgeries were the most invasive. On the contrary, dentistry was considered slightly less invasive and skin incisions and craniotomies the least invasive procedures [9]. That is why the use of effective anaesthetic and analgesic protocols is essential to ensure welfare of animals, decreasing pain and stress.

In this review, we were able to confirm a high level of general report (81%) of the drugs used to anaesthetize animals. The great variety of anaesthetic protocols and administered doses showed the absence of standardization on these protocols. This may be due to the type of procedure developed and the local availability of products.

However, it was reported that ketamine, which is classified as a N-methyl D-Aspartate (NMDA) antagonist that causes a dissociative anaesthesia, [19], was the most used drug in combination with other medications for both induction and anaesthetic maintenance. Isoflurane was the second anaesthetic administered alone or in combination with other drugs probably because it allows the maintenance of the animal's unconsciousness in a simple and long-lasting manner.

On the other hand, the under reporting of analgesia is a concern because it reveals an inadequate management of pain in pigs that have not being improved over the years (2014 was the year in which the drugs used were most reported and only occurred in 56% of the articles). These results are like those of the Bradbury study, in which they also conducted a review of pain management in pigs in articles from 2012 to 2014. The need to control pain, particularly in animals used in research, is not only for ethical reasons, but also because pain side effects may occur on the used animal models [9]. Along with surgical stress, pain leads to an endocrine response, which could generate massive physiological changes that could alter the quality of results [20]. In addition, all cases were biomedical studies that sought to extrapolate results to humans. Conditions should be the same as those used in a human patient; otherwise, results could not be compared. Coulter and colleagues [21] found that papers reporting ethical approval were also more likely to report systemic analgesic administration than those that did not. Furthermore, standards of ethical review differ widely between countries. The lack of reporting data may be the result of an omission because the objectives of these articles are not related to animal pain. Failure to provide adequate postoperative analgesia undermines 3 Rs principles: not complying with refinement - which seeks to improve experimental procedures to avoid suffering and pain of the animals, replacement - individual animals cannot be replaced by alternative methods, or reduction - on their numbers cannot be done by the use of more powerful study design [2].

Another factor is that the selected drugs were the best choice for a good pain management in the experiments. The few articles that reported analgesics in this review looked for adequate options for pain management. The fentanyl (intraoperative analgesic most reported in this review) is a strong opioid that can generate excellent analgesia, if properly applied. Lidocaine, on the other hand, is a widely local anaesthetic used to generate local anaesthesia in dental and orthopaedic surgeries. For postoperative analgesia, buprenorphine (an opioid agonist drug that has been

clinically shown to have a longer duration of action compared to other opioid drugs in pigs) [22] was the most commonly used drug, and meloxicam (a nonsteroidal anti-inflammatory drug (NSAID,) that has a preferential inhibitor of cyclooxygenase-2, and has demonstrated potent analgesic and anti-inflammatory activity) was the second most used drug. However, few articles mentioned how postoperative pain was evaluated, which does not allow the reader to verify if the protocol used was the most appropriate for the type of surgery to be performed. Development of a pain-scoring system in pigs, together with the mandatory description of pain management in submitted articles, would contribute to improved laboratory pig welfare [2].

5.7 CONCLUSIONS

Our review revealed a poor report in studies of surgical procedures in swine that have worsened over the years, especially in data directly related to the welfare of animals undergoing surgery, as in the report of anaesthetic protocols and analgesics. We believe that the ARRIVE guidelines is an excellent tool for reporting with good higher quality. However, its current underutilization may be due to a lack of commitment on the part of many authors to use this guide, and because many journals have no mandatory instruction to follow this guide, especially by those whose main objective is not animal welfare. It is a fact that increasing methodology details might lead to increasing articles length, whereas many journals have a limited maximum word count. However, details can be added as supplementary material [17]. That is why we encourage authors and journals to continue making use of this guide mandatory to improve scientific reporting quality and, consequently, ensure animal welfare.

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5.10 AUTHOR CONTRIBUTIONS

Jilma Alemán-Laporte and Claudia Mori conceived the idea of the paper, Jilma Alemán and Gilbert Alvarado wrote and made the analyses of the information, Mariana AS Garcia-Gomes, Ana Tada Fonseca and Marco Zuñiga-Montero, reviewed and edited the manuscript.

5.11 CONFLICTS OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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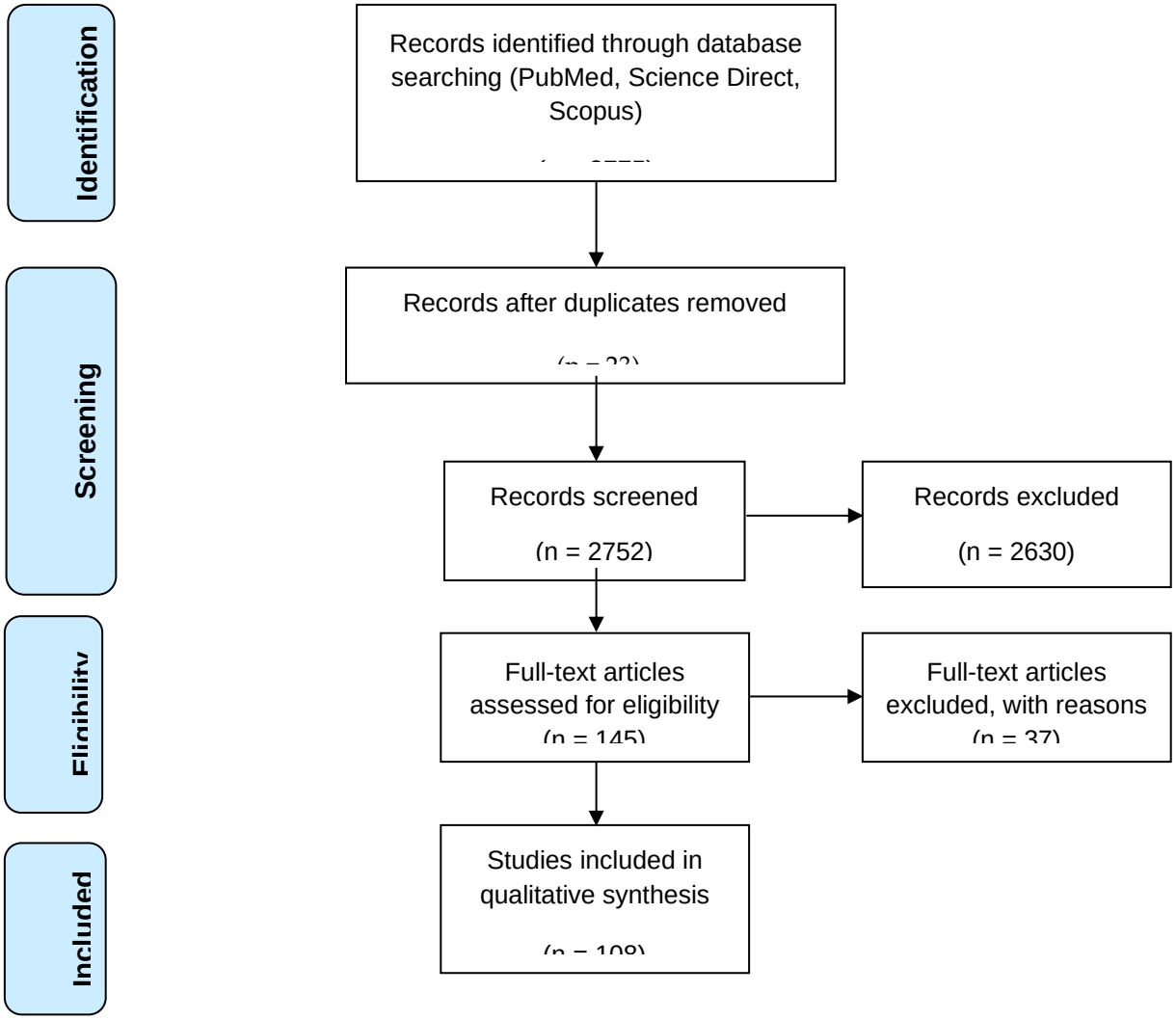

Figure 1. Flow diagram summarizing search strategy (PRISMA guidelines is used to design this search strategy)[10]

Table 1. Scores used to assess quality of the reported methods in selected articles with surgical procedures in pigs (based on the ARRIVE guidelines). The data were expressed as a percentage.

Category	0	1	2
1. Ethical Statement	6%	80%	14%
2. Study Design	21%	53%	26%
3. Experimental procedures	0%	100%	0%
4. Experimental animals	3%	90%	7%
5. Housing and Husbandry	43%	57%	0%
6. Sample size	7%	90%	3%
7. Allocating animals to experimental groups	19%	74%	7%
8. Experimental outcomes	95%	NA	5%
9. Statistical methods	37%	49%	14%

0: No mentioned 1: Unclear /Not complete 2: Adequate/Clear.NA: Does not apply

Table 2. Information obtained from revision of categories, subcategories and evaluated items, based on ARRIVE guidelines. The data were expressed as a percentage (total mean of reports described in the totality of the articles, and averages of the maximum and the minimum of reports corresponding to the year of publication).

Category	Subcategory	Items	Mean (%)	Max. (%, Year)	Min. (%, Year)
1. Ethical Statement	1.1. Ethical review permissions	1.1.1. Refers to guidelines	52	69 (2014)	35 (2017)
		1.1.2. Approved by ethical committee	92	100 (2016)	81 (2014)
		1.1.3. Protocol number	20	57(2018)	17 (2013)
2. Study Design	2.1. Number of experimental and control groups		71	88 (2014)	57 (2018)
	2.2. Steps taken to minimize the effects of subjective bias		27	40 (2016)	11 (2013)
	2.3. The experimental unit		79	94 (2014)	57 (2018)
3. Experimental procedures	3.1. How	3.1.1. Anaesthesia drugs	81	94 (201)5	71 (2017)
		3.1.2. Dose of anaesthesia	71	83 (2015)	57 (2018)
		3.1.3. Route	68	78 (2015)	60 (2016)
		3.1.4. Monitoring during anaesthesia	30	48 (2016)	21 (2018)
		3.1.5. Analgesia drugs	41	56 (2014)	28 (2016)
		3.1.6. Dose of analgesia	32	39 (2015)	21 (2018)
		3.1.7. Route	24	33 (2015)	7 (2018)
		3.1.8. Surgical procedure	100	100 (All)	0 (None)

		3.1.9. Method of euthanasia	32	43 (2018)	22 (2013)
	3.2. When	3.2.1. Time of the day	0	0 (None)	0 (All)
	3.3. Where	3.3.1. Home cage	13	22 (2015)	6 (2014, 2017)
	3.4. Why	3.4.1. Choice of a specific anaesthetic, route and dose	1	6 (2014)	0 (All except 2014)
4. Experimental animals	4.1. Details of animals	4.1.1. Breed	69	83 (2015)	59 (2017)
		4.1.2. Sex	58	88 (2014)	40 (2016)
		4.1.3. Age/Weight	86	100 (2014)	78 (2013)
	4.2. Further information	4.2. Source of animals	48	64 (2018)	22 (2013)
5. Housing and Husbandry	5.1. Housing	5.1.1. Type of facility	6	14 (2018)	0 (2 015, 2016)
		5.1.2. Type of cage	19	33 (2015)	6 (2017)
		5.1.3. Bedding material	3	11 (2015)	0 (2013, 2014, 2016, 2017)
		5.1.4. Number of cage companions	13	31 (2014)	0 (2018)
	5.2. Husbandry conditions	5.2.1. Light/Dark cycle	9	19 (2014)	4 (2016)
		5.2.2. Temperature	10	19 (2014)	4 (2016)
		5.2.3. Humidity	6	13 (2014)	0 (2017)
		5.2.4. Type of food	36	63 (2014)	22 (2013)
		5.2.5. Access to water or food	31	56 (2014)	22 (2013)

		5.2.6. Environmental enrichment	3	11 (2015)	0 (2013, 2017, 2018)
		5.2.7. Adaptation	12	18 (2017)	4 (2016)
	5.3. Welfare related assessment	5.3.1. Welfare intervention	24	44 (2017)	12 (2015)
	6.1. Total number of animals used		94	100 (all except 2017)	76 (2017)
6. Sample size	6.2. Explanation how the number of animals was arrived at		3	6 (2015, 2016)	0 (2013, 2014, 2018)
	6.3. Indicate the number of independent replications of each experiment		11	18 (2016)	0 (2013, 2014)
7. Allocating animals to experimental groups	7.1. Details of how animals where allocated		24	38 (2014)	6 (2013)
	7.2. Order of experimental treatment		48	68 (2016)	18 (2017)
8. Experimental outcomes	8.1. Primary and secondary experimental outcomes assessed		8	13 (2014)	0 (2018)
9. Statistical methods	9.1. Details of statistical methods		76	88 (2014)	65 (2017)
	9.2. Unit of analysis for each dataset		59	72 (2015)	41 (2016)
	9.3. Methods used to assess whether the data met assumptions of the statistical approach		49	56 (2015)	41 (2016)

Table 3. Surgeries reported in the reviewed articles. The data were expressed as a percentage.

Type of surgery	N° of journal	% of journals
Craniotomies	3	2.8%
Dental surgeries	15	13.9%
Laparoscopies	28	25.9%
Laparoscopy + Thoracotomy	1	0.9%
Laparotomies	12	11.1%
Orthopaedic surgeries	22	20.4%
Skin incisions	11	10.2%
Thoracotomies	16	14.8%

5.13 SUPPLEMENTARY MATERIAL

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6. FINAL CONSIDERATIONS

The principle of refinement must be implemented whenever it is necessary to carry out invasive procedures that can cause pain or suffering to the animals in experimentation, such as the use of analgesic protocols to prevent pain. However, the refinement also includes improving the reporting of data in scientific publications in order to guarantee the reproducibility of the results. This was evidenced during the development of this project in which the following conclusions could be obtained:

In chapter 1. all the analgesic treatment administrated to the rats produced different behavioral responses in the animals. DIP+TRA, and DIP produced a severe reduction of the locomotion and rearing of the animals. All protocols that included TRA also produced a reduction of grooming behavior. Finally, the treatments with MEL caused an increase of locomotion. Because nowadays the use of analgesics is an ethical duty, it is very important to know these effects in order to consider them during the experimental design planning.

In the Chapter 2. all operated animals presented physiological and behavioral changes during the first 48 hours of the postoperative period. Many of these alterations were related to the effects of the analgesics described in Chapter 1. The fact that SAL+SUR did not present great differences with the analgesic treated groups can be related to the refinement of the surgical technique, which helped to reduce postoperative pain.

Finally in Chapter 3. the systematic review revealed a poor report in studies of surgical procedures in swine in important topics such as sample size calculation, housing and husbandry information and anesthetic and analgesics protocols. This situation has worsened over the years despite the creation of guidelines such as ARRIVE that were created to standardize the scientific report information. For this reason, it is important that authors and journals continue making use of this guide to improve scientific reporting quality and consequently ensure animal welfare.

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