

## RESEARCH ARTICLE

# Assessment of general activity and anxiety-like behavior in mice following tramadol and meloxicam administration for managing immediate post-operative pain

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## Abstract

After analgesic administration, we evaluated general activity in the Open-Field and anxiety-like behavior in the Elevated Plus Maze of vasectomized mice. We divided C57BL/6J male mice into eight groups: saline, three non-operated control groups treated with 10 mg/kg meloxicam, 20 mg/kg tramadol, or both intraperitoneally, and four vasectomized mice groups treated with the same analgesic protocol as the control groups. One group of vasectomized mice received both treatments and an additional 10 mg/kg lidocaine at the incision site. We conducted the vasectomy via scrotal approach under isoflurane inhalation anesthesia and performed behavioral tests after full anesthesia recovery. Mice treated with meloxicam demonstrated low ambulation, spontaneous activity, and rearing frequency. Mice treated with tramadol showed spontaneous behavior compared with the saline control. Due to behavior changes demonstrated by meloxicam controls, we were unable to identify whether meloxicam provided adequate analgesia. Vasectomized mice treated with tramadol showed general activity behavior similar to their control but displayed significantly less rearing, suggesting that they were under potential signs of pain or discomfort. In conclusion, the Open Field test and the Elevated Plus Maze can usefully pre-evaluate analgesic protocols to identify possible interference caused by adverse drug effects. For future directions, an appropriate regimen of meloxicam and tramadol for enhancing mice welfare post vasectomy should be better investigated.

**Keywords:** animal welfare, opioids, non-steroidal anti-inflammatory agents, multimodal treatments, adverse effects.

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## INTRODUCTION

Pain is responsible for important physio pathological effects, which undermine animal welfare and research results. Pain management after surgical procedures in laboratory animals should pursue legal and ethical principles. Also, replicable, and reliable results<sup>1</sup>. Therefore, it is necessary to implement effective analgesic protocols and appropriate methods to identify and evaluate pain in experimental animals<sup>2</sup>.

Thus, we can assess pain by observing clinical and physiological parameters, such as weight loss, food intake, body posture, corticosterone levels, and facial expressions<sup>3-6</sup>. Some researchers used the Open-Field test and the Elevated Plus Maze to evaluate the effects of anesthesia and analgesia protocols in mice after experimental procedures<sup>7,8</sup>. Those tests measure exploratory behavior, different types of motor parameters in an unknown environment and evaluate mice's anxiety-like behavior based on their natural aversion for open and elevated areas<sup>9-11</sup>.

Surgical procedures potentially promote pain and distress and are routinely used during in experimental and mouse-assisted reproduction protocols, commonly applied in animal facilities to develop transgenic animals, rederive infected strains, and cryopreserve embryos<sup>12-14</sup>. Vasectomy is one of surgical techniques applied for mouse-assisted reproduction purposes and some authors have already tested it in mice to assess pain, establishing analgesia protocols, and providing technical refinement<sup>15-17</sup>.

Among the analgesic drugs, tramadol and meloxicam demonstrated efficacy in mice<sup>18,19</sup>. Tramadol is a centrally-acting mu-opioid agonist for managing moderate to severe pain in several species<sup>20</sup>. The combination of tramadol, associated with other drugs, as nonsteroidal anti-inflammatories (NSAIDs), is recommended to maximize its antinociceptive activity<sup>21,22</sup>. Meloxicam is an enolic acid-derived NSAID that preferentially inhibits cyclooxygenase-2 (COX-2), thromboxane, and prostaglandin with excellent antipyretic and analgesic activity<sup>23</sup>.

This study aimed to assess the effects of tramadol and meloxicam treatment in mice as a preemptive form for post-vasectomy immediate pain management. We applied the Open Field test (OFT) for general activity and the Elevated Plus Maze (EPM) for anxiety-like behavior. Adequate pain management with different drugs treatment can promote refinement in laboratory animal science. We expected that animal behavior alterations caused by painful experimental procedures could be a tool to identify animal suffering. Moreover, we observed that most pain studies described the antinociceptive effect of analgesics but disregarded how these drugs affected animals' general behavior.

## MATERIAL AND METHODS

### Mice husbandry

We obtained C57BL/6J male mice aging 8-12 weeks and weighing 25-30g from the animal facility of the Department of Immunology, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The mice were specific pathogen-free following the Federation of European Laboratory Animal Science Associations (FELASA) recommendations<sup>24</sup>. We acclimatized the mice for two weeks before starting behavioral experiments in the animal facility of the Department of Pathology, School of the Veterinary Medicine and Animal Science of the University of Sao Paulo (FMVZ/USP) Brazil. Animals were housed in groups (five per cage) in open polypropylene cages (28 x 17 x 12 cm) with autoclaved corn cob (Granja RG, Suzano, SP, Brazil) as bedding and paper towels as nesting materials. Room conditions were controlled: temperature 22 + 2°C, air changes 15-20/hour, and humidity 55 + 5%. Artificial lighting was on in a 12/12-hour light/dark cycle (lights on at 7 am). Animals had unrestricted access to filtered and autoclaved water and commercial pellets formulated according to the AIN-93M rodent diet (Nuvilab, Quimtia, Parana, Brazil).

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo (CEUA/FMVZ-USP) approved the protocol number 3582200217

for the experimental study. The authors confirm that they have adhered to the Brazilian guidelines established by the National Council for the Control of Animal Experimentation (CONCEA), similar to those in the Guide for Care and Use of Laboratory Animals of the US National Research Council.

### Experimental groups and procedures

We divided sixty-four male mice into eight groups ( $n = 8$ ): saline control, three non-operated mice treated with analgesics, and four vasectomized mice treated with analgesics. Table 1 presents abbreviation and group treatments. We used the software G\*Power 3.1.9.3 to calculate sample size: One-Way ANOVA with eight groups,  $\alpha=0.05$ , minimum detectable difference=0.5,  $power=0.9$  (90%). Sample size ( $n$ )= 8 mice/group, number of groups=8, thus,  $N=8 \times 8 = 64$  mice.

Mice were randomly assigned to groups using GraphPad Prism calculator (<https://www.graphpad.com/quickcalcs/randomize1/>). We assigned 8 subjects to each of 8 groups single testing. There were mice from different groups in each cage.

Before the procedures, animals stayed in a quiet room for 30 minutes for acclimatization. We previously weighed mice on the morning of the experiments. The saline control group received 10 mL/kg of sterile saline injection via intraperitoneal. Non-operated treated groups, and vasectomized mice received 20 mg/kg tramadol hydrochloride (Tramadol<sup>®</sup> 5% injectable solution, Cristalia, Itapira, Sao Paulo, Brazil), 10 mg/kg meloxicam (Maxicam<sup>®</sup> 0.2% injectable solution, Ouro Fino, Cravinhos, Sao Paulo, Brazil), or both via intraperitoneal injection 30 minutes before the procedures.

### Vasectomy

Anesthesia was induced in an anesthetic chamber (Harvard Apparatus Anesthetic Vaporizer, Cambourne, UK) with 4% isoflurane (Isoforine<sup>®</sup> 100% solution, Cristalia, Itapira, Sao Paulo, Brazil) in oxygen (4 L/min) and maintained using a facemask with 2% oxygen (1 L/min). We prepared the skin using 1% povidone-iodine solution (Riodeine Degermante, Rioquimica, Sao Jose do Rio Preto, Sao Paulo, Brazil), and then, we shaved the scrotum with a razor. Surgery began after the loss of the pedal reflex. The VT20M10L group received 10 mg/kg lidocaine hydrochloride (Xylestesin<sup>®</sup> 2% injectable solution, Cristalia, Itapira, Sao Paulo, Brazil) by subcutaneous injection immediately before the incision in the scrotum skin. We performed vasectomy according to Nagy et al.<sup>25</sup> Briefly, we made a small

**Table 1.** Abbreviation used for each group following analgesic administration (intraperitoneally) and procedure along with the number of mice per group

Group	Treatment	n
CTR	Saline control	8
T20	Non-operated treated with 20 mg/kg tramadol	8
M10	Non-operated treated with 10 mg/kg meloxicam	8
T20M10	Non-operated treated with 20 mg/kg tramadol + 10 mg/kg meloxicam	8
VM10	Vasectomized mice treated with 10 mg/kg meloxicam	8
VT20	Vasectomized mice treated with 20 mg/kg tramadol	8
VT20M10	Vasectomized mice treated with 20 mg/kg tramadol + 10 mg/kg meloxicam	8
VT20M10L	Vasectomized mice treated with 20 mg/kg tramadol + 10 mg/kg meloxicam + 10 mg/kg lidocaine	8
<b>Total</b>	-	<b>64</b>

incision (1 cm) in the scrotal sac and cauterized a section of approximately 2 mm of each vas deferens using heated fine forceps. We sutured the skin with nylon 4-0 (Brasuture Ind. Com. Imp. Exp. Ltda, Sao Sebastiao da Grama, Sao Paulo, Brazil). During surgery, we covered the eyes with sterile saline. The same veterinary surgeon performed all procedures and reported no intraoperative complications. The surgery lasted approximately 15 minutes per animal. Mice recovered from anesthesia in their

home cage with bedding material to minimize body temperature loss. Behavior tests underwent 30 minutes after recovery.

## Behavior test

The same operator performed the behavioral tests in a quiet room with indirect artificial light source (~108 lux) between 10:00 and 12:00. Mice from different groups were tested interspersed throughout the trials in sequence: Open-Field followed by the Elevated Plus Maze. The apparatuses were cleaned with a 5% alcohol/water solution before placing the animals and before subsequent tests to minimize odor cues left by the previous mouse. Testing was recorded by a high-definition video camera (JVC Everio HDD, JVCKenwood do Brasil Comercio de Eletronicos Ltda, Brazil) controlled by a remote device.

### *Open-Field test (OFT)*

The apparatus used consisted of a round white arena, boarded with an opaque metal wall and an open-top (40 cm diameter x 31 cm height). We placed one mouse at a time in the arena center for evaluation and recorded videos for five minutes. Later, we evaluated the videos using the Ethovision XT version 15.0.1416 video tracking system (Noldus Information Technology bv, The Netherlands) to measure distance traveled (cm), movement time, average speed (cm/s), and time spent in the periphery/center of the arena (periphery and center measured 50% each of the total area centered). A trained observer manually scored frequencies of rearing, grooming, number of fecal pellets, and urine puddles deposited in the arena.

### *Elevated Plus Maze test (EPM)*

For this test, we used an apparatus that consisted of a plus-shaped platform elevated 50 cm from the floor, with two open arms (30 x 5 cm) across from each other, and perpendicular to those, two walled arms referred to as closed arms (30 x 5 x 15 cm). We placed one mouse at a time in the maze center facing one of the closed arms. Behavior was scored manually by a trained observer for five minutes, including the number of entries into each arm, the time spent in each of them, frequencies of rearing and grooming. Figure 1 presents a diagram showing the steps of the procedures.

## Euthanasia

Mice were used only once and euthanized in a CO<sub>2</sub> euthanasia chamber (Red Industria e Comercio de Equipamentos Hospitalares e Laboratoriais, Caieiras, SP, Brazil) after completing behavior tests. Without pre-charging the chamber, animals were placed in it, and 100% carbon dioxide was introduced (fill rate of 30% of the chamber volume per minute). Cardiac and respiratory arrest confirmed the death.

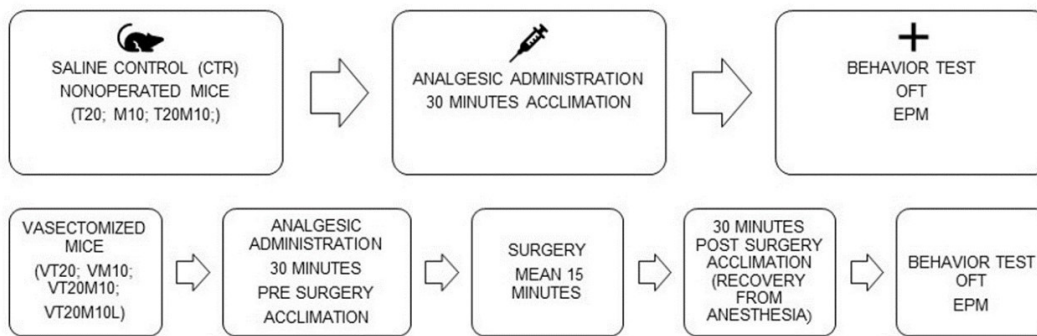
## Statistical analysis

We performed statistical analysis with GraphPad Prism version 9.0.0, GraphPad Software, San Diego, California, USA. Mean with SEM was calculated for all parameters. Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was employed to compare the control group with treated ones. Outliers were identified and removed by the ROUT method (Q = 1%). The results were significant at  $p < 0.05$ .

## RESULTS

### **Open-Field test (OFT)**

CTR, T20, and VT20 moved longer distances (Figure 2a,  $F(7, 56) = 10.98, p < 0.0001$ ) and presented higher speeds (Figure 2b,  $F(7, 56) = 10.99, p < 0.0001$ ) compared to the other groups. We did not verify differences concerning the time spent in the periphery and center zones of the arena between groups (Figures 2c,  $F(7, 56) = 1.660, p = 0.1380$ , and 2d,  $F(7, 56) = 1.658, p = 0.1384$ ). Animals spent most of the time exploring the periphery (wall-following behavior) of the Open-Field arena. Regarding time



**Figure 1.** Diagram showing the steps of the experimental procedures. CTR = saline control, M10 = non-operated meloxicam 10 mg/kg; T20 = non-operated tramadol 20 mg/kg; T20M10 = non-operated tramadol 20 mg/kg + meloxicam 10 mg/kg; VM10 = vasectomized mice meloxicam 10 mg/kg; VT20 = vasectomized mice tramadol 20 mg/kg; VT20M10 = vasectomized mice tramadol 20 mg/kg + meloxicam 10 mg/kg; VT20M10L = vasectomized mice tramadol 20 mg/kg + meloxicam 10 mg/kg + lidocaine 10 mg/kg; OFT = Open-Field test; EPM = Elevated Plus Maze.

moving, CTR, T20, and VT20 explored the arena for a longer time in comparison to the other groups (Figure 2e,  $F(7, 56) = 8.498, p < 0.0001$ ). We noticed that control treated with meloxicam (M10) and surgery groups displayed fewer rearing frequencies than control saline and control-treated with tramadol (Figure 2f,  $F(7, 56) = 46.51, p < 0.0001$ ). Regarding grooming frequency, CTR presented the highest frequency (Figure 2g,  $F(7, 56) = 2.372, p = 0.0339$ ) between groups. The mean number of fecal pellets and urine puddles was not significant. Overall, we observed that animals treated with tramadol (T20 and VT20) showed similar locomotion behavior as the saline control group. On the other hand, meloxicam, alone or associated, significantly reduced locomotion and exploration.

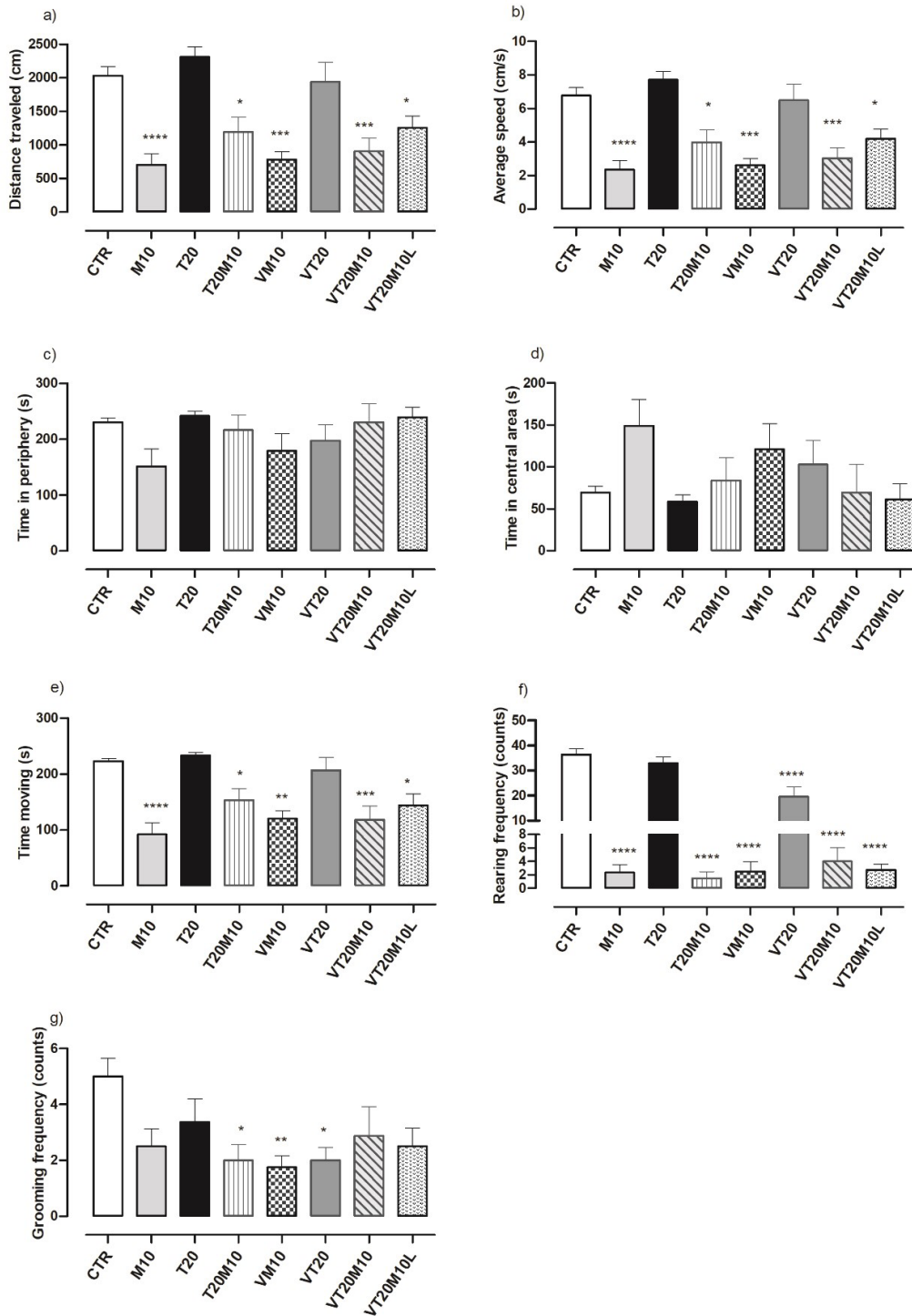
### Elevated Plus Maze (EPM)

Time spent in the closed arms presented significant differences between CTR and T20 in comparison to the other groups (Figure 3a,  $F(7, 55) = 6.687, p < 0.0001$ ); in the open arms, T20M10, VM10, and VT20M10 spent less time than CTR, M10, T20, VT20, and VT20M10L (Figure 3a,  $F(7, 55) = 7.384, p < 0.0001$ ). The number of entries into the closed arms presented: no differences between CTR and T20; a significant reduction related to M10, T20M10, VM10, VT20, VT20M10, and VT20M10L groups (Figure 3b,  $F(7, 56) = 14.71, p < 0.0001$ ). On the other hand, the treated groups showed significant differences in the number of entries into the open arms (Figure 3b,  $F(7, 56) = 9.854, p < 0.0001$ ); these animals presented a smaller number than CTR. Regarding rearing frequency, we observed a significant reduction in M10, T20M10, VM10, VT20, VT20M10, and VT20M10L groups in comparison to CTR and T20 (Figure 3c,  $F(7, 56) = 16.46, p < 0.0001$ ). We did not identify differences in the grooming frequency among groups (Figure 3d,  $F(7, 56) = 2.946, p = 0.0107$ ). In summary, all treated animals demonstrated a lower frequency of activity and preferably spent more time in closed arms except for the T20 group. The meloxicam-treated groups showed low rearing frequency.

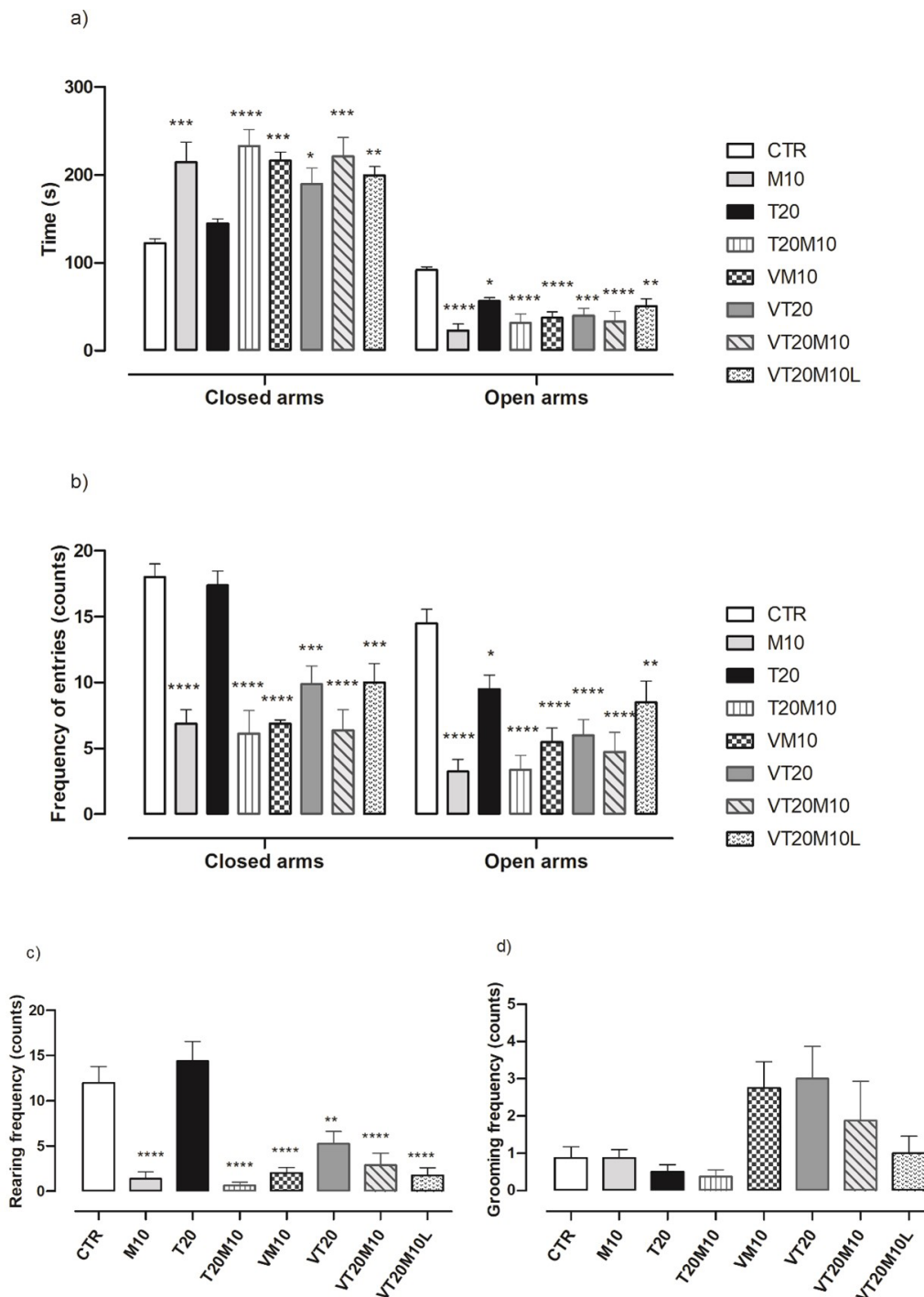
## DISCUSSION

We presented in this study the results of general activity and anxiety like-behavior of mice in the Open-Field Test (OFT) and Elevated Plus Maze (EPM) following tramadol and meloxicam administration for immediate post-operative pain management. The purpose of this study was to identify the potential effects of these drugs on mouse behavior and implement adequate pain management of vasectomized mice. Researchers must not neglect the impacts of unrelieved pain and analgesia on laboratory animals due to the potential of biased experimental outcomes and animal suffering<sup>26</sup>.

First, we evaluated potential behavior changes associated with administering tramadol and meloxicam without a surgical procedure. The tramadol-treated group (T20) presented similar general activity as the control group (CTR) in the OFT, characterized by active exploratory behavior and the tendency to primarily explore the peripheral zone of the arena (i.e., thigmotaxis). In the EPM, the



**Figure 2.** Assessment of general activity of C57BL/6J mice in the Open-Field test (OFT) following meloxicam and tramadol administration. Distance traveled cm (a); average speed cm/s (b); time spent in the periphery zone of the arena (c); time spent in the center zone of the arena (d); time moving in seconds (e); rearing frequency (f); grooming frequency (g). N = 8/group. Data are presented as the means  $\pm$  SEM. ANOVA followed by Dunnett’s multiple comparisons test was employed to compare control group with treated ones. CTR = saline control, M10 = non-operated meloxicam 10 mg/kg; T20 = non-operated tramadol 20 mg/kg; T20M10 = non-operated tramadol 20 mg/kg + meloxicam 10 mg/kg; VM10 = vasectomized mice meloxicam 10 mg/kg; VT20 = vasectomized mice tramadol 20 mg/kg; VT20M10 = vasectomized mice tramadol 20 mg/kg + meloxicam 10 mg/kg; VT20M10L = vasectomized mice tramadol 20 mg/kg + meloxicam 10 mg/kg + lidocaine 10 mg/kg. \* $p < 0.05$  (significant), \*\* $p < 0.01$  (highly significant), \*\*\* $p < 0.001$  (extremely significant).



**Figure 3.** Assessment of anxiety-like behavior of C57BL/6J mice in the Elevated Plus Maze test (EPM) following meloxicam and tramadol administration. Time spent in the closed and open arms (a); number of entries into the closed and open arms (b); rearing frequency (c); grooming frequency (d). N= 8/group. Data are presented as the means ± SEM. Two-way ANOVA followed by Bonferroni post hoc test were used to evaluate differences between groups in frequency of entries and time spent in the closed and open arms. ANOVA followed by Bonferroni's post hoc test was employed to evaluate rearing and grooming differences between groups. CTR = saline control, M10 = non-operated meloxicam 10 mg/kg; T20 = non-operated tramadol 20 mg/kg; T20M10 = non-operated tramadol 20 mg/kg + meloxicam 10 mg/kg; VM10 = vasectomized mice meloxicam 10 mg/kg; VT20 = vasectomized mice tramadol 20 mg/kg; VT20M10 = vasectomized mice tramadol 20 mg/kg + meloxicam 10 mg/kg; VT20M10L = vasectomized mice tramadol 20 mg/kg + meloxicam 10 mg/kg + lidocaine 10 mg/kg. \*p<0.05 (significant); \*\*p<0.01 (highly significant); \*\*\*p<0.001 (extremely significant).

T20 group also presented behavior similar to CTR, preferably spending more time in closed arms and avoiding entries into the open ones. This behavior is natural in mice and might indicate open space-induced anxiety<sup>9</sup>. Thus, these evaluations indicated that 20 mg/kg tramadol did not interfere with animal behavior. Literature shows that tramadol has an anti-depressant-like effect in mice, likely mediated by the noradrenergic system and NMDA receptor signaling<sup>27,28</sup>.

On the other hand, meloxicam-treated animals (M10 and T20M10) showed apathy and reduced general activity, affecting other parameters in the OFT and the EPM. These adverse effects of meloxicam interfered in our analysis of the analgesic efficacy of the drug post vasectomy. We also observed similar events even when associated with tramadol. Some authors indicated higher doses of meloxicam for mouse pain management after surgery: 20 mg/kg for vasectomy and laparotomy and 60 mg/kg for splenectomy<sup>15,29-31</sup>. Higher doses of meloxicam might cause several known side-effects of most NSAIDs - gastrointestinal ulceration, renal and hepatic lesions<sup>32</sup>. The effects of NSAIDs result from reversible inhibition of cyclo-oxygenase enzymes (COX-1 and COX-2) and subsequent reduction of prostaglandins<sup>33</sup>. In our study, meloxicam 10 mg/kg caused behavior alterations. To our knowledge, this is the first report of meloxicam causing adverse effects on mouse behavior. We hypothesized that these adverse effects in mice might be related to variables in the meloxicam formulations<sup>34,35</sup>. Besides that, other variables might have interfered with, for example, phenotype differences between mouse strains<sup>36,37</sup>.

The vasectomized VM10, VT20M10, and VT20M10L presented lower general activity through shorter distances with lower average speed than the CTR group, except for the VT20 group that demonstrated high locomotion and exploration behavior, similar to the controls. We observed a preference for thigmotaxis in the OFT and reduced time spent in closed arms in the EPM. The vasectomized groups exhibited significantly reduced rearing behavior. Only VM10 and VT20 showed reduced grooming behavior which was a limiting finding. Thus, considering those measures could indicate pain or discomfort after the surgical procedure<sup>16,17</sup>. However, our study presented a limitation caused by the adverse effects of meloxicam. We chose it because of its anti-inflammatory and analgesic properties<sup>32</sup>. Also, meloxicam pharmacokinetics demonstrated an elimination half-life of 8-12 hours<sup>38-40</sup>. Tramadol has a half-life shorter than 2 hours; therefore, repeated, and stressful injections are needed to maintain adequate active plasma levels<sup>41</sup>. Repeatedly administering tramadol produced antinociceptive tolerance in Swiss mice<sup>42</sup>. Also, meloxicam and tramadol present potential dose-dependent antinociceptive effects synergism, reducing doses of both drugs and minimizing the side effects of higher doses<sup>22</sup>. In this manner, the combination of meloxicam and tramadol could provide beneficial effects on mice.

Several studies in the literature reported distinct conclusions about tramadol efficacy in laboratory animals. For example, analgesic protocols using tramadol alone presented efficacy in a rat model for ureteral calculus and a rabbit for post-gastrotomy<sup>43,44</sup>. Associating tramadol with other analgesic drugs - as N-palmitoylethanolamide (an endogenous fatty acid amide) and paracetamol - enhanced its antinociceptive efficacy in mice after embryo transfer, formalin, and tail-flick test<sup>21,45,46</sup>. Also, tramadol was considered ineffective in C57BL/6 male mice post laparotomy<sup>47</sup>. Our results indicated no analgesic efficacy of tramadol treatment in post-operated animals. VT20 presented low rearing and grooming frequencies in the OFT and anxiety-like behavior in the EPM, demonstrated by closed arms preference. In our understanding, these behavior alterations might be associated with pain or discomfort caused by the procedure. Thus, a multimodal therapy would be indicated for post-vasectomy pain management in C57BL/6 mice.

Although meloxicam was the source of bias in our experiments due to its potential for spontaneous behavioral changes, other methods of evaluation would have been less influenced by behavior changes. Leach et al.<sup>15</sup> suggested that the Mouse Grimace Scale (MGS) could be applied to assess pain after vasectomy, and the efficacy of 20 mg/kg meloxicam or 5 mg/kg bupivacaine treatment in mice. These authors identified high MGS scores in saline controls after surgery that were positively correlated with some pain-related behaviors, wound lick, and groom. Both treatments reduced pain scores post-surgery. Other authors also reported increase in MGS scores after laparotomy in mice, but meloxicam at 1, 5, or 20 mg/kg was not effective to reduce pain-related changes<sup>30</sup>.

Finally, it is noteworthy to mention that our initial proposal to the Institutional Animal Care and Use Committee included a vasectomized control group with no analgesic treatment. Though it



seemed necessary, we decided to eliminate this group for humane reasons. In the literature, some authors using surgery controls with no analgesia have already inferred pain-related behavior in mice caused by the procedure. Similar vasectomy models described differences such as time moving, abnormal walking, rearing, posture, grooming, and facial pain score in animals following surgery without analgesia<sup>5,15,17,29</sup>. Moreover, we decided not to perform an isoflurane control group to reduce the number of animals. As mentioned before, we based our decision on literature that anesthesia by isoflurane had no significant effects on animal's behavior that could compromise our methods for pain assessment<sup>5,7,31,48</sup>. The results presented by Wright-Williams et al.<sup>29</sup> indicated that vasectomy was the primary factor responsible for the endocrine stress response and not a reaction caused by anesthesia or mice handling.

## CONCLUSIONS

In conclusion, behavioral tests, such as OFT and EPM, can have a practical application for the pre-evaluation of analgesic protocols to identify possible interferences caused by adverse drug effects. To our knowledge, the present study was the first to report changes in animal behavior caused by 10 mg/kg meloxicam. This effect interfered in our evaluation of its analgesic effects and pointed out further investigation. 20 mg/kg tramadol did not affect mice's behavior, but this dose was not adequate for this model either. Thus, researchers should investigate an appropriate regimen of meloxicam and tramadol for enhancing mice welfare post vasectomy.

## IDENTIFICATION OF DISSERTATION-ORIGINATED MATERIAL

This material is part of an academic thesis developed at the School of Veterinary Medicine and Animal Science of the University of Sao Paulo, Sao Paulo, Brazil.

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