

Mouse Behavior in the Open-field Test after Meloxicam Administration

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Several analgesics are suggested for pain management in mice. Nonsteroidal antiinflammatories (NSAIDs), such as meloxicam can be administered for the treatment of inflammation and acute pain; however, several side effects can occur which include gastrointestinal ulceration and renal and hepatic toxicity. We previously performed a pilot study to test the antinociceptive activity of meloxicam in mice, but we observed behavioral changes in unoperated control mice. These observations spurred further investigation. One hypothesis for the result was potential differences in formulation between commercial brands of meloxicam. Thus, this current study aimed to evaluate the effects of 3 different commercial brands of meloxicam (20 mg/kg) in the general activity of mice using the open field test. Our results showed that meloxicam had several effects on mouse behavior and caused the formation of skin lesions at the injection site, depending on the brand of the drug. The most significant adverse effect observed was decreased exploratory activity. Grooming frequency was reduced in all groups. These adverse effects might be related to the quality of the drugs because meloxicam formulations can contain crystal polymorphisms that affect drug quality and efficacy. This study points out the importance of drug quality variation that can affect the outcome of behavioral studies in mice.

Abbreviations: COX-2, cyclooxygenase 2

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Introduction

Pain management in laboratory animals is a matter of ethical, legal, and animal welfare concern. Untreated pain has negative effects in animals, such as hyperalgesia, allodynia, impairment of tissue healing process, changes in hormone secretion, and abnormal behavior, among others.⁹ Thus, pain should be managed by using analgesia protocols that minimize animal suffering and distress. In research animals, the analgesic regimen should consider not only the side effects of drugs but also the potential to affect scientific results and increase the variability of data.⁹

Meloxicam is an enolic acid-derived nonsteroidal antiinflammatory drug, a preferential COX-2 inhibitor, used for the treatment of rheumatoid arthritis, osteoarthritis, and for pain management in several species.^{16,17} In laboratory mice, this drug has been used to treat acute postoperative inflammation and pain.^{21,24,26} Similar to other NSAIDs, meloxicam causes several side effects depending on the dose, frequency, and duration of treatment, which include gastrointestinal ulceration and renal and hepatic toxicity.¹⁷ Also, acute overdose of NSAIDs causes central nervous system toxicity with sequelae such as ataxia, vertigo, dizziness, and disorientation.^{2,6} Thus, although meloxicam can be used to manage pain in laboratory animals, it also can potentially interfere with scientific results due to the side effects.

In a previous unpublished experiment in our laboratory, we aimed to determine the potential antinociceptive activity of the drug using a suggested dose by other authors to treat postoperative pain in mice.^{12,20,26} However, meloxicam significantly affected the behavior of unoperated controls which led us to investigate this effect. Mice demonstrated significant reductions in exploratory activity, rearing, and locomotion. One hypothesis for this reduction was that formulation variables of the drug could alter the results. Thus, this study aims to assess the effect of meloxicam on mouse open field activity. We compared 3 commercial brands of meloxicam to identify potential differences in the effects of different formulations.

Materials and Methods

This study used 55 C57BL/6J male mice from the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (FMVZ/USP), age 8 to 10 wk, weight 25.11 ± 2.997 g (mean \pm SD), free of specific pathogens according to the FELASA Guidelines.¹³ Mice were acclimatized for 2 wk before the experiments. Mice were housed in groups of up to 5 per cage in open-top polypropylene cages (28 \times 17 \times 12 cm) with autoclaved wood shavings bedding (Granja RG, Suzano, SP, Brazil) and paper towels for nesting material. The room was controlled for temperature at 22 ± 2 °C (71.6 ± 35.6 °F), air changes of 15 to 20/h, humidity $55 \pm 5\%$, and artificial light cycle 12/12h. Mice had unrestricted access to filtered and autoclaved water and autoclaved commercial pelleted AIN-93M rodent diet (Nuvilab, Quimtia, Paraná, Brazil).

The protocol for the experimental study was approved by the Institutional Animal Care and Use Committee, number 3582200217 – FMVZ/USP. We followed the Brazilian guidelines for animal experimentation which are similar to those in the *Guide for the Care and Use of Laboratory Animals*.⁷

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Meloxicam solution for parenteral injection was obtained from 3 different commercial suppliers: Maxicam 0.2% injectable solution (lot number 003/18, Ouro Fino Saúde Animal Ltda, São Paulo, Brazil), Flamavet 0.2% injectable solution, (lot number 1852340, União Química Farmacêutica Nacional, São Paulo, Brazil), and meloxicam 1% injectable solution (lot number 264330, Eurofarma Laboratórios SA, São Paulo, Brazil). These 3 products were encoded as M1, M2, and M3, respectively. Samples M1 and M2 were injected at 2 mg/mL (concentration from the manufacturer). M3 was diluted at the moment of the injections to 2 mg/mL in sterile saline 0.9% (Equiplex, Goiás, Brazil). Formulations M1 and M2 were intended for animal use, and M3 for human use. The pH of the formulations was measured right before the experiment using a pH meter (Gehaka PG 1800, São Paulo, Brazil).

Mice were weighed on the morning of the experiments and randomly assigned into 7 different groups. The dose of 20 mg/kg meloxicam was injected intraperitoneally in groups M1_{ip}, M2_{ip}, and M3_{ip} groups. A dose of 10 mL/kg of sterile 0.9% saline was intraperitoneally injected into mice of the control group (Ctr). The same dose of meloxicam (20 mg/kg) was injected subcutaneously between the scapulae of the M1_{sc}, M2_{sc}, and M3_{sc} groups.

Mice were acclimatized for 30 min in their home cage in a quiet room after injections. Behavioral testing was performed between 1000 h and noon by the same observer. The open field arena (40 cm diameter × 31 cm height, white background) was placed in a soundproof room with an indirect artificial light source. The arena was cleaned thoroughly with a 5% alcohol/water solution between each mouse to minimize odor cues. Mice from different groups were tested interspersed throughout the trials. One mouse at a time was placed in the center of the arena and spontaneous behavior was recorded for 5 min (high-definition video camera, JVC Everio HDD, JVC Kenwood do Brasil Comércio de Eletrônicos Ltda, Brazil). Later, the videos were evaluated by using Ethovision XT version 15.0.1416 video tracking system (Noldus Information Technology, the Netherlands) to measure the distance moved (cm), average speed (cm/s), time moving (s), and time spent in the periphery/center of the arena (s). Rearing, grooming, and fecal pellet frequencies were scored manually by a single individual. After the trials, mice were returned to their home cage and observed once during the following 24 hours to check overall activity (inactive, isolated), posture (hunched), body appearance (not grooming), and some facial expressions (orbital tightening, nose bulge, and ear position). Subsequently, mice were euthanized in a CO₂ gas euthanasia induction chamber (Red Indústria e Comércio de Equipamentos Hospitalares e Laboratoriais, Caieiras, Brazil) according to the American Veterinary Medical Association guidelines for the euthanasia of animals.¹ Mice were then submitted for post-mortem macroscopic examination. The overall appearance of fur, skin, and peritoneal cavity was checked. The subcutaneous tissues at the injection site were qualitatively checked for gross alterations, and potential lesions were identified based on distribution, texture, and color.

Statistical analysis was performed by using GraphPad Prism 9.0.0 software (GraphPad Software.). D'Agostino and Pearson was used for normality test, $\alpha = 0.05$. One-way analysis of variance followed by Tukey's multiple comparison test was applied to evaluate differences between treatments. The results were considered significant at $P < 0.05$. Data are presented as mean with SEM.

Results

Significant differences were detected in distance moved ($F(6, 48) = 145.4, P < 0.0001$) and average speed ($F(6, 48) = 147.0,$

$P < 0.0001$) between M1_{ip} and M1_{sc} and all other groups (Figures 1A and 1B). Significant differences were found between groups in time spent in the periphery of the arena ($F(6, 48) = 46.75, P < 0.0001$, Figure 1C) and time spent in the center ($F(6, 48) = 46.76, P < 0.0001$, Figure 1D). M1_{ip} and M1_{sc} had the lowest time moving ($F(6, 45) = 39.91, P < \text{less than } 0.0001$) and frequency of rearing ($F(6, 48) = 31.69, P < 0.0001$, Figures 1E and 1F). The control presented the highest mean grooming frequency as compared with the other groups ($F(6, 48) = 15.83, P < 0.0001$, Figure 1G.) Overall, M1_{ip} and M1_{sc} groups moved less and had lower average speed than the other groups. M1_{ip} and M1_{sc} spent most of their time in the same place they were initially positioned in the center of the arena. The mean number of fecal pellets was not significantly different between groups (data not shown).

Clinically, we observed alopecia and lesions due to scratching at the injection site of all mice (7 of 7) in the M1_{sc} group (Figures 2A–D). We did not observe skin lesions at the injection site in M2_{sc} and M3_{sc} groups. Concentration, pH, and composition of meloxicam preparations are shown in Table 1.

Discussion

This study showed meloxicam administration had different effects on mouse behavior and lesions at the injection site, depending on the brand of the drug. We used 2 brands of meloxicam that were approved for veterinary use in dogs and cats (M1 and M2) and one brand approved for human use (M3). All 3 brands affected behavior to some degree, but M1 caused the greatest alterations. The most significant behavioral effect was decreased exploratory activity and lethargy that resembled heavy sedation. Grooming frequency was reduced in all groups. In rodents, grooming behavior is sensitive to stress, experimental manipulation, and the use of drugs.^{10,11}

Meloxicam is an enolic acid drug derived from oxicam, characterized by low solubility in water and slight solubility in inorganic acids. Meloxicam has 5 crystalline forms (I, II, III, IV, and V) that are associated with solubility and dissolution rate.⁸ The crystal form I is indicated for the preparation of pharmaceutical products; however, polymorphic contamination and interconversion between forms can occur in raw materials. Therefore, the occurrence of polymorphism in active pharmaceutical ingredients may compromise their bioavailability and therapeutic efficacy. In a previous study, a mixture of forms I and III that was identified in a commercially available raw material meloxicam affected the solubility, intrinsic dissolution, and rate of dissolution of meloxicam tablets.⁸ In another study, the authors found 2 polymorphs of meloxicam in samples from different compounding pharmacies.¹⁹ That study also reported more antiinflammatory efficacy of polymorph I than polymorph III in a model of paw edema induced by carrageenan.¹⁹

The effects on behavior observed in our study were not described in other studies using similar or higher doses of meloxicam.^{3,11,20,26} Studies that assessed mouse behavior after meloxicam administration did not report significant differences in rearing and locomotion.^{12,20,26} In a study that used a higher dose (60 mg/kg), meloxicam treatment attenuated anhedonia (that is, diminished response to pleasure) in mice after splenectomy.⁴ We hypothesized that behavioral changes observed in our study might be related to pain or discomfort, as these drugs are not clinically approved for pain treatment in mice. Considerable variation in response could occur in mice due to variables such as strain, phenotype^{18,27} and sex.^{14,15,23} Such effects could be

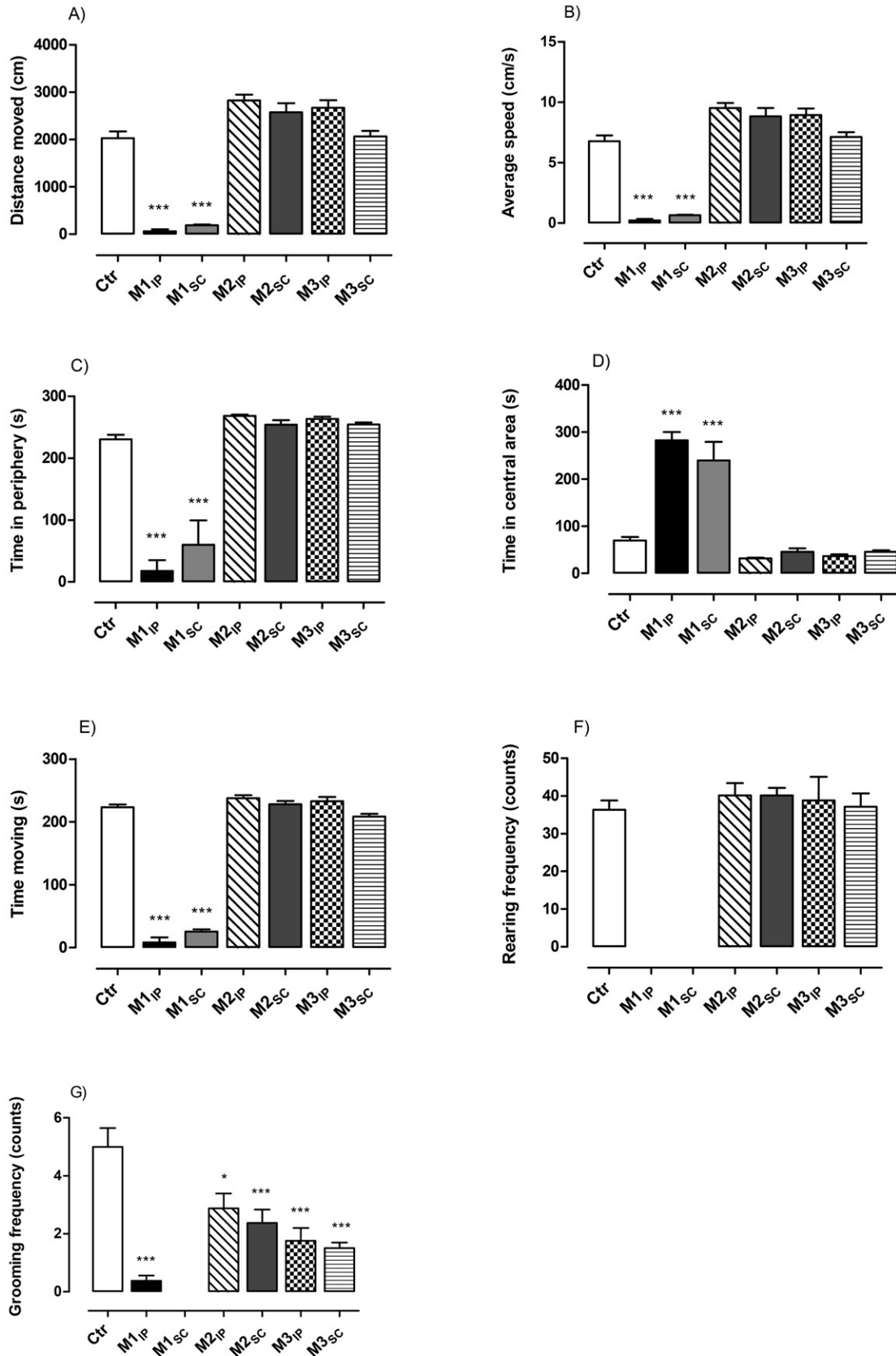


Figure 1. Assessment of general activity of C57BL/6J mice in the open field test (OFT) after meloxicam administration. Distance traveled (cm) (A); average speed (cm/s) (B); time spent in the peripheral zone of the arena (C); time spent in the center zone of the arena (s) (D); time moving (s) (E); rearing frequency (counts) (F); grooming frequency (counts) (G). Data are presented as the means ± SEM. ANOVA followed by Tukey's multiple comparisons test was used to compare differences between groups. M1_{SC} (n = 7); CTR, M1_{IP}, M2_{SC}, M2_{IP}, M3_{SC}, and M3_{IP} (n = 8/group). IP, intraperitoneal; SC, subcutaneous. ***P < 0.001 (extremely significant).

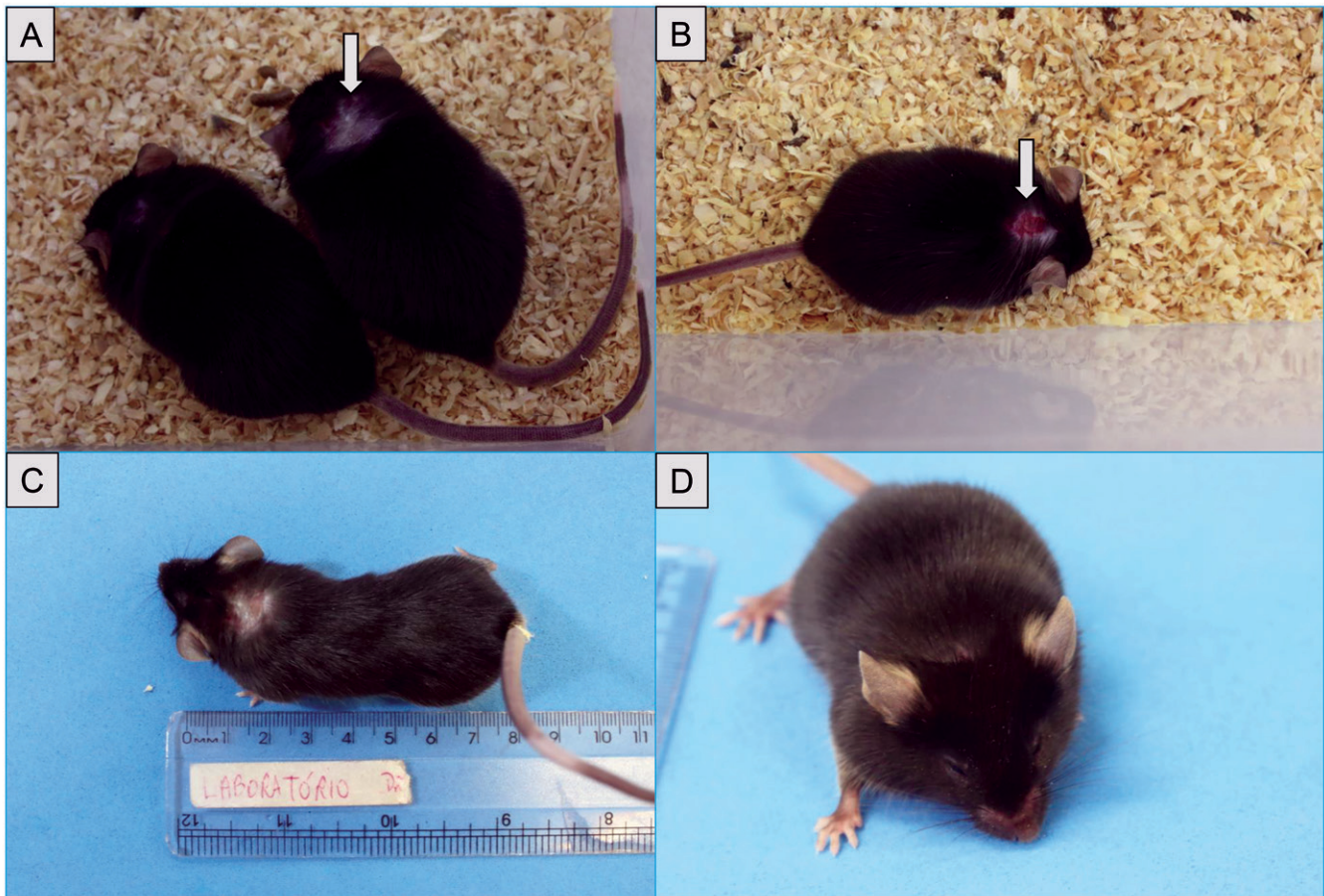


Figure 2. Photographs of mice after subcutaneous injection of M1_{sc} sample. Mice showed alopecia and scratching on the skin at the injection site after subcutaneous injection (arrows).

Table 1. Concentration, pH, and composition of meloxicam samples.

Sample	Concentration*	pH	Composition*
M1	0.2%	9.35	Meloxicam 200 mg; excipients 100.0 mL (details were not available from manufacturer)
M2	0.2%	8.53	Meloxicam 2.0 mg; excipients 1.0 mL (meglumine, glycine, ethylic alcohol, sodium chloride, edetate disodium dihydrate, benzyl alcohol, and water for injection)
M3	1%	7.91**	Meloxicam 10 mg; excipients 1.0 mL (meglumine, glycofurol, lutrol F68, sodium chloride, glycine, sodium hydroxide, and water for injection)

* According to the manufacturer; ** measured after diluted to 0.2% in sterile 0.9% saline.

interpreted as a confounding effect of the experimental design, rather than an effect of analgesia.

The high pH of the 2 veterinary products was an additional variable in our study. The acceptable range of pH for most administration routes is between 4.5 and 8.0.⁵ The intraperitoneal route is commonly used in mice because it is simple to execute, requires minimal restraint, and can be used to administer large volumes. However, substances with an irritating nonphysiological pH are not recommended for intraperitoneal administration due to the potential to cause pain, peritoneal irritation, and peritonitis.^{3,5,25} In our study, no gross alterations were observed in the peritoneal cavity. Thus, we could not infer whether the drug caused irritation and pain that could explain those behavior alterations.

Besides the behavioral changes, skin lesions at the site of injection could be identified after M1 administration. These lesions might be related to the potential irritating characteristic of M1 sample, although other variables might be involved as

well. In a previous study, a 20 mg/kg subcutaneous injection of meloxicam intended for animal use caused skin ulceration in C57BL/6N mice, possibly because of the drug concentration (5 mg/mL).²² The lesions were less severe if meloxicam was diluted to 1 mg/mL in sterile saline; nonetheless, the authors suggested more investigations to determine a safer dose and route of administration of meloxicam.

In summary, our study indicates that drug variation can influence the outcome of behavioral studies, pain assessment, and pain management in animal research due to the potential adverse side-effects of the drug formulations. Our data show clinical and behavioral differences between groups of mice that received distinct commercial brands of meloxicam. A complete assessment of the physicochemical characterization of meloxicam could identify potential polymorphic contamination of these formulations. Although meloxicam caused adverse effects in mice used in this study, it should not be rejected for pain management. Additional drug safety stud-

ies should be conducted to determine the optimal method of meloxicam use in mice.

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References

1. AVMA. [Internet]. 2020. Guidelines for the euthanasia of animals. Available at: https://www.avma.org/sites/default/files/2020-01/2020_Euthanasia_Final_1-15-20.pdf
2. Auriel E, Regev K, Korczyn AD. 2014. Nonsteroidal anti-inflammatory drugs exposure and the central nervous system, p 577–584. In: Biller J, Ferro, JM, editors. Handbook of Clinical Neurology, vol 119. Elsevier. <https://doi.org/10.1016/B978-0-7020-4086-3.00038-2>
3. Davis JN, Courtney CL, Superak H, Taylor DK. 2014. Behavioral, clinical and pathological effects of multiple daily intraperitoneal injections on female mice. *Lab Anim (NY)* **43**:131–139. <https://doi.org/10.1038/labani.433>.
4. Haile M, Boutajangout A, Chung K, Chan J, Stolper T, Vincent N, Batchan M, D'Urso J, Lin Y, Kline R, Yaghoor F, Jahfal S, Kamal R, Aljohani W, Blanck T, Bekker A, Wisniewski T. 2016. The Cox-2 inhibitor meloxicam ameliorates neuroinflammation and depressive behavior in adult mice after splenectomy. *J Neurophysiol Neurol Disord* **3**:101.
5. Hirota J, Shimizu S. 2012. Routes of administration, p 709–725. In: Hedrich, HH, editor. The laboratory mouse, second ed. Amsterdam: Elsevier. <https://doi.org/10.1016/B978-0-12-382008-2.00030-1>
6. Hunter LJ, Wood DM, Dargan PI. 2011. The patterns of toxicity and management of acute nonsteroidal anti-inflammatory drug (NSAID) overdose. *Open Access Emerg Med* **3**:39–48. <https://doi.org/10.2147/OAEM.S22795>.
7. Institute for Laboratory Animal Research. 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
8. Jacon Freitas JT, Santos Viana OMM, Bonfilio R, Doriguetto AC, de Araújo MB. 2017. Analysis of polymorphic contamination in meloxicam raw materials and its effects on the physicochemical quality of drug product. *Eur J Pharm Sci* **109**:347–358. <https://doi.org/10.1016/j.ejps.2017.08.029>.
9. Jirkof P. 2017. Side effects of pain and analgesia in animal experimentation. *Lab Anim (NY)* **46**:123–128. <https://doi.org/10.1038/labani.1216>.
10. Kalueff AV, Tuohimaa P. 2005. Mouse grooming microstructure is a reliable anxiety marker bidirectionally sensitive to GABAergic drugs. *Eur J Pharmacol* **508**:147–153. <https://doi.org/10.1016/j.ejphar.2004.11.054>.
11. Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM, Fentress JC. 2015. Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nat Rev Neurosci* **17**:45–59. <https://doi.org/10.1038/nrn.2015.8>.
12. Leach MC, Klaus K, Miller AL, Scotto di Perrotolo M, Sotocinal SG, Flecknell PA. 2012. The assessment of post-vasectomy pain in mice using behaviour and the mouse grimace scale. *PLoS One* **7**:e35656. <https://doi.org/10.1371/journal.pone.0035656>.
13. Mähler Covenor M, Berar M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, Raspa M. 2014. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim* **48**:178–192. <https://doi.org/10.1177/0023677213516312>.
14. Miller LR, Marks C, Becker JB, Hurn PD, Chen W, Woodruff T, McCarthy MM, Sohrabji F, Schiebinger L, Wetherington CL, Makris S, Arnold AP, Einstein G, Miller VM, Sandberg K, Maier S, Cornelison TL, Clayton JA. 2016. Considering sex as a biological variable in preclinical research. *FASEB J* **31**:29–34. <https://doi.org/10.1096/fj.201600781r>.
15. Mogil JS, Bailey AL. Sex and gender differences in pain and analgesia. 2010. *Prog Brain Res* **186**:141–157. <https://doi.org/10.1016/B978-0-444-53630-3.00009-9>
16. Noble S, Balfour JA. 1996. Meloxicam. *Drugs* **51**:424–430. Available at: <https://doi.org/10.2165/00003495-199651030-00007>.
17. Papich MG, Messenger K. 2017. Non-steroidal anti-inflammatory drugs, p227–243. In: Grimm KA, Lamont, LA, Tranquilli WJ, Greene SA, Robertson SA, editors. Veterinary anesthesia and analgesia. Chichester (UK): John Wiley & Sons, Ltd. <https://doi.org/10.1002/9781119421375.ch12>
18. Rogers AB. 2019. Stress of strains: inbred mice in liver research. *Gene Expr* **19**:61–67. <https://doi.org/10.3727/105221618X15337408678723>.
19. Romani LFA, Yoshida MI, Gomes ECL, Machado RR, Rodrigues FF, Coelho MM, Oliveira MA, Freitas-Marques MB, San Gil RAS, Mussel WN. 2018. Physicochemical characterization, the Hirshfeld surface, and biological evaluation of two meloxicam compounding pharmacy samples. *J Pharm Anal* **8**:103–108. <https://doi.org/10.1016/j.jpha.2017.12.006>.
20. Roughan JV, Wright-Williams SL, Flecknell PA. 2009. Automated analysis of postoperative behaviour: assessment of HomeCageScan as a novel method to rapidly identify pain and analgesic effects in mice. *Lab Anim* **43**:17–26. <https://doi.org/10.1258/la.2008.007156>.
21. Roughan JV, Bertrand HGMJ, Isles HM. 2015. Meloxicam prevents COX-2-mediated post-surgical inflammation but not pain following laparotomy in mice. *Eur J Pain* **20**:231–240. <https://doi.org/10.1002/ejp.712>.
22. Sarfaty AE, Zeiss CJ, Willis AD, Harris JM, Smith PC. 2019. Concentration-dependent toxicity after subcutaneous administration of meloxicam to C57BL/6N mice (*Mus musculus*). *J Am Assoc Lab Anim Sci* **58**:802–809. <https://doi.org/10.30802/AALAS-JAALAS-19-000037>.
23. Smith JC. 2019. A review of strain and sex differences in response to pain and analgesia in mice. *Comp Med* **69**:490–500. <https://doi.org/10.30802/AALAS-CM-19-000066>.
24. Tubbs JT, Kissling GE, Travlos GS, Goulding DR, Clark JA, King-Herbert AP, Blankenship-Paris TL. 2011. Effects of buprenorphine, meloxicam, and flunixin meglumine as postoperative analgesia in mice. *J Am Assoc Lab Anim Sci* **50**:185–191.
25. Turner PV, Brabb T, Pekow C, Vasbinder MA. 2011. Administration of substances to laboratory animals: routes of administration and factors to consider. *J Am Assoc Lab Anim Sci* **50**:600–613.
26. Wright-Williams SL, Courade J-P, Richardson CA, Roughan JV, Flecknell PA. 2007. Effects of vasectomy surgery and meloxicam treatment on faecal corticosterone levels and behaviour in two strains of laboratory mouse. *Pain* **130**:108–118. <https://doi.org/10.1016/j.pain.2006.11.003>.
27. Zurita E, Chagoyen M, Cantero M, Alonso R, González-Neira A, López-Jiménez A, López-Moreno JA, Landel CP, Benítez J, Pazos F, Montoliu L. 2011. Genetic polymorphisms among C57BL/6 mouse inbred strains. *Transgenic Res* **20**:481–489. <https://doi.org/10.1007/s11248-010-9403-8>.