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Concentration-dependent Toxicity after Subcutaneous Administration of Meloxicam to C57BL/6N Mice (*Mus musculus*)

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Studies using the Mouse Grimace Scale have shown that for many NSAID, including meloxicam, minimal doses of at least 20 mg/kg may be necessary to achieve adequate peri- and postoperative analgesia in mice. However, more data are needed to determine whether such NSAID doses exceed the threshold for gastrointestinal ulceration or induce other relevant pathology. We administered equal volumes of saline or injectable meloxicam (1 or 5 mg/mL) at a dose of 20 mg/kg SC to 20 young adult male and female C57BL/6N mice daily for 6 d and performed necropsies on all mice on the seventh day. Mice given 5 mg/mL meloxicam subcutaneously developed significantly more severe pathology at the injection site than saline controls. Pathology was characterized by full-thickness epidermal necrosis; cavitary lesions within subcutis, muscle, or fat; steatitis; and myositis. Mice that received 1 mg/mL meloxicam subcutaneously developed lesions that were qualitatively similar but far less severe than those after 5 mg/mL. However, no pathologic lesions typically associated with NSAID toxicity, such as gastric ulceration and liver and kidney lesions, were seen. These results demonstrate that although meloxicam injected subcutaneously causes concentration-dependent skin pathology at the injection site, a dose of 20 mg/kg can be safely administered subcutaneously at a concentration of 1 mg/mL for as long as 6 d.

Abbreviation: MGS, mouse grimace scale

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Veterinary and research personnel have an ethical obligation to minimize and alleviate pain in laboratory animals. The *Guide for the Care and Use of Laboratory Animals* states, "Pain is a stressor and, if not relieved, can lead to unacceptable levels of stress and distress in animals."[14] Of all laboratory animals, rodents are especially challenging patients when it comes to accurately assessing their pain. As prey species, rats and mice are likely to conceal behavioral indications of pain, and clinically relevant postoperative or chronic pain is particularly difficult to detect.[14,18,22,37] Traditional assays of rodent pain, including hypersensitivity testing and behavioral proxies (such as food consumption, locomotor activity), may not accurately measure clinically relevant spontaneous pain in rodents.[8,11,18,21,39] The development of the Mouse Grimace Scale (MGS) represents a refinement and advancement in detecting pain in rodents.[15] The MGS has high interobserver reliability and may capture the emotional state of the mouse associated with pain.[11,16,18,28] When applied retroactively on images, the MGS may capture pain that was missed during cageside clinical and behavioral assessment as well as cageside application of the MGS.[9,20]

The NSAID meloxicam is commonly used for postoperative pain in mice and rats in laboratory and private-practice settings. Recommended doses for meloxicam in mice range from 1 to 5 mg/kg either PO or SC[5,10,24] and between 1 to 10 mg/kg IP.[12] These dose ranges are inconsistently supported by behavioral proxy data, and far more such data are available for rats than for mice.[11,21,29-31,35]

Using the MGS and behavioral assays as indicators of pain, laboratory animal practitioners have recently reported new concerns about the efficacy of currently recommended dosages of common NSAID analgesics, including carprofen,[18] ketoprofen,[18] and meloxicam. Meloxicam at a dosage of 5 mg/kg SC postoperatively failed to provide adequate analgesia to CBA mice after vasectomy.[19] Likewise, CD1 mice needed a dosage of 20 mg/kg meloxicam SC for sufficient analgesia after vasectomy, as assessed by using the MGS and manual scoring of pain behaviors.[16] Another study showed that although providing meloxicam at 20 mg/kg reduces postoperative inflammation, this dose did not reduce postoperative pain in BALB/c mice as defined by both MGS score and automated scoring of activity.[27] These studies suggest that the widespread use of meloxicam at a maximal dose of 5 mg/kg in mice should be reevaluated, especially when meloxicam is used as the sole analgesic for surgical procedures.

A meloxicam dose of at least 20 mg/kg appears necessary for effective analgesia in mice.[16,28] However, this dose may exceed the threshold for gastrointestinal ulceration and renal or hepatic toxicity. Available toxicity data involving commonly used NSAID predominantly use the oral route of administration. Swiss mice displayed gastric ulceration after receiving 10 mg/kg meloxicam by gavage once daily for 5 d.[38] However, a single dose of 20 mg/kg meloxicam given by gavage to male C57BL/6J mice did not induce any NSAID-related toxicity in gastrointestinal, renal, or hepatic tissues.[13] In another study, mice displayed gastrointestinal ulceration at meloxicam doses of 17.5 to 35 mg/kg PO given once daily for 3 mo; no renal or

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hepatic toxicity was seen at these doses.[17] The LD_{50} threshold for oral meloxicam is 470 mg/kg.[16,17,28] These data suggest that when given orally, meloxicam at a dose of 20 mg/kg may be safe during the immediate perioperative period[18] but may cause gastric ulceration when 5 or more doses are given.

Few data exist regarding the safety of meloxicam given subcutaneously, which is likely a more precise route of administration than voluntary ingestion from drinking water or gel cups. In one study, C57BL/6 mice exhibited weight loss and C3H/HeNCrl mice displayed reduced mobility after receiving meloxicam 20 mg/kg SC.[32] Safety data specific for mice are needed for potentially therapeutic doses of meloxicam of at least 20 mg/kg SC.[18]

We administered meloxicam to C57BL/6N mice at 20 mg/kg SC once daily for 6 d. On day 7, full gross necropsies, histopathology, CBC, and serum chemistry analyses were performed. We hypothesized that this treatment would not cause detectable pathology, but if present, pathology would include gastric or duodenal ulceration, liver and kidney toxicity, or signs of hemorrhage.

Materials and Methods

Animals. A total of 20 (6 female, 14 male) C57BL/6N mice (age, 10 wk; Charles River, Wilmington, MA) were enrolled in this study. We chose C57BL/6 mice because they are the most commonly used strain in our institution and, among popular inbred mouse strains, may be the most sensitive to nociception.[23] Initially, we had planned to use 8 male and 8 female mice (4 of each sex receiving meloxicam and 4 receiving saline) to test meloxicam at 5 mg/mL. The male mice were tested first, and because of the severity of the skin lesions present in all animals that received meloxicam (see Results), female mice were not tested at this concentration. These data were then used to estimate power for a follow-up study. This power analysis revealed that using 3 female and 3 male mice to test meloxicam at 1 mg/mL and 3 female and 3 male mice as saline controls would be sufficient to attain 88% power to determine a difference in pathology rate of 10% (saline) compared with 90% (meloxicam). In both studies, mice were randomly assigned to treatment groups. We selected 10-wk-old mice to minimize the occurrence of background pathology, which is more likely to occur in older mice.

Experimental procedures were approved by the Yale University IACUC and were in accordance with all federal policies and guidelines governing the use of vertebrate animals. According to recent vendor housing room health reports, all mice were free of epizootic diarrhea of infant mice virus, lymphocytic choriomeningitis virus, ectromelia virus, mouse hepatitis virus, Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, murine parvovirus, murine norovirus, Theiler encephalomyelitis virus, reovirus, mouse adenovirus, mouse cytomegalovirus, murine pneumotropic virus, mouse polyomavirus, hantavirus, mouse thymic virus, lactate dehydrogenase elevating virus, *Bordetella bronchiseptica*, *Citrobacter rodentium*, cilia-associated respiratory bacillus, *Corynebacterium kutscheri*, *Helicobacter* species, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, *Pasturella multocida*, *Pasteurella aeruginosa*, *Salmonella* species, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, β-*Streptococcus* spp., *Clostridium piliforme*, ectoparasites, helminths, *Giardia* spp., *Spironucleus* spp., and *Encephalitozoon cuniculi*. Mice were singly housed in autoclaved IVC (Tecniplast, West Chester, PA) with autoclaved corncob bedding (1/8 in., catalog no. 7092, Harlan, South Easton, MA) and nesting material (Nestlets, Ancare, Bellmore, NY) and on a 12:12-h light:dark cycle. Autoclaved rodent chow (diet no. 2018S, EnvigoTeklad, Huntingdon, United Kingdom) and hyperchlorinated water (8 to 10 ppm) were available without restriction. Room temperature and relative humidity were maintained at 22.2 ± 1.1 °C (72 ± 2 °F) and 50% ± 10%, respectively.

Meloxicam administration. After arrival at the facility, mice were allowed 4 d to acclimate before any handling or injections was performed. At our institution, postoperative analgesia is required for a minimum of 48 h (2 d), so we chose 3 times this duration (6 d) as the duration of administration, in accordance with FDA guidelines regarding toxicology testing of veterinary pharmaceuticals.[6] After the acclimation period, mice and food hoppers were weighed, and all mice received a physical exam including body condition score daily for 6 d. Examinations and injections were performed by a single veterinarian, who has 7 y of experience in handling mice (AES). Meloxicam (Metacam, Boehringer Ingelheim, Duluth, GA) was administered in the interscapular region at a dose of 20 mg/kg SC by using a fresh 25-gauge needle (BD PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ). Meloxicam was provided in 2 concentrations: 5 mg/mL (0.1 mL) or 1 mg/mL (0.5 mL). Meloxicam was diluted to a 1-mg/mL concentration by diluting 0.1 mL meloxicam (5 mg/mL) in a 1-mL syringe (Tuberculin Slip Tip, Becton Dickinson) with 0.4 mL sterile saline (Hospira, Lake Forest, IL). Control mice received an equal volume (0.1 or 0.5 mL) of sterile saline subcutaneously.

Necropsy and histology. On day 7 (that is, 24 h after the final injection), mice were euthanized by CO_2 asphyxiation, and gross necropsies were performed. Blood was submitted to a reference laboratory (AnTech Diagnostics, Lake Success, NY) for CBC and serum chemistry analysis. The overall appearance of the skin and fur, muscle, trachea, heart, lungs, liver, kidney, and gastrointestinal tract was noted. The stomach and proximal duodenum were inspected for gross ulceration. All tissues underwent routine fixation and paraffin processing followed by sectioning at 5 µm and staining with hematoxylin and eosin (Yale Mouse Research Pathology Core; http:/mrp.yale.edu).

Histopathology. Histology slides were examined by a board-certified veterinary pathologist (CJZ), who was blind to treatment. For each mouse, semiquantitative scoring of pathology at the injection site was performed, and the presence (1) or absence (0) of the following individual lesions was recorded: full thickness skin necrosis, steatitis, myositis, cavitary lesions in muscle or fat, necrosis in subcutis, muscle or fat, and dermal subcuticular inflammation. Values for each mouse were summed to obtain a severity score (7-stage scale; 0 [normal] to 6 [most severe]) for each animal. Histologic findings in remaining tissues were described qualitatively for each animal.

Statistical analysis. Power analysis to determine appropriate animal numbers was performed by using the Fisher Exact test prior to beginning experiments and again when initial results were obtained (see Results section). Statistical analysis of the presence of significant pathology was performed also by using a Fisher Exact test. A Student *t* test was performed to compare body weight between days 1 and 6. For all tests, statistical significance was defined as a P value of less than 0.05.

We performed a single statistical test of each substantive question considered. The primary question was whether meloxicam at 20 mg/kg SC results in pathology. This question was addressed by using the Fisher Exact test to compare the incidence of pathology between groups. This test was selected as our primary analysis method prior to performing the experiment. In addition to addressing this primary question, we wanted to describe the type of pathology in greater detail. To

Table 1. Summary of injection-site pathology and severity scores according to treatment group

	Injection volume			
	0.1 mL (n = 4 male mice per group)		0.5 mL (n = 3 male and 3 female mice per group)	
	Meloxicam (5 mg/mL)	Saline	Meloxicam (1 mg/mL)	Saline
Full-thickness skin necrosis	2	0	0	0
Steatitis	4	2	2/1	1/1
Myositis	4	1	0/2	0/1
Cavitary lesion	3	0	0	0
Subcuticular/fat/muscle necrosis	4	0	0	0
Sparse dermal subcuticular inflammation	4	1	0/1	0
Average (range) severity score	5.3 (5–6)	1 (1)	1.0 (1–3)	0.6 (0–2)

For the 0.1-mL volume, data shown are the total number of affected mice in each group; all mice were male. For the 0.5-mL groups, data are given as the number of male mice affected / number of female mice affected.

Table 2. Summary of background lesions according to treatment group

	Injection volume			
	0.1 mL (n = 4 per group)		0.5 mL (n = 6 per group)	
	Meloxicam (5 mg/mL)	Saline	Meloxicam (1 mg/mL)	Saline
Gastritis	1	2	0	4
Sternal costochondral degeneration and fracture	2	0	3	3
Right ventricular subepicardial fibrosis	0	0	2	2

Data reported are the number of affected mice in each group.

this end, we planned 2 additional tests to be performed only if we found evidence of overall pathology. These tests examined the presence of injection site pathology and NSAID-related pathology. In addition, we present descriptive tables outlining the specific pathologies observed (Tables 1 and 2). All analyses were performed by using SAS version 9.4 (SAS Institute, Cary, NC).

Results

Pathology at injection site. Mice given meloxicam were more likely (P = 0.005) to exhibit injection site pathology than were mice that received saline. Mice treated with 5 mg/mL SC developed the most severe local pathology, characterized by at least 5 of the individual lesions scored (Table 1). Specifically, 2 mice developed full-thickness epidermal necrosis that was apparent grossly by the fourth dose in one mouse and the fifth dose in the other (Figure 1) and subsequently on histopathology (Figure 2). Three mice developed cavitary lesions lined by macrophages and fibroblasts (and presumably harbored injected material) within the subcutis, muscle, or fat, and robust steatitis or myositis was evident in all 4 mice (Figure 2). The development of moderate to severe pathology at the injection site was significantly (P = 0.005) associated with subcutaneous administration of 5 mg/mL meloxicam (4 of 4 mice with severe pathology) compared with saline (0 of 4 mice with severe pathology).

Mice given 1 mg/mL meloxicam subcutaneously developed lesions that were qualitatively similar but subjectively less severe than those of mice that received 5 mg/mL meloxicam (Figure 3). Epidermal necrosis did not occur in any of the mice treated with 1 mg/mL meloxicam. Cavitary lesions were present in 2 mice but were smaller and associated with less severe inflammation than those after 5 mg/mL. Except for a single male mouse in which quite marked steatitis and myositis were evident, saline-injected mice were devoid of pathology or developed only minimal localized inflammation in the subcutis, fat, or muscle. The presence of pathology at the injection site in these groups was not associated with treatment status (P = 0.56, 4 of 6 mice given meloxicam with pathology compared with 3 of 6 saline-treated mice with pathology).

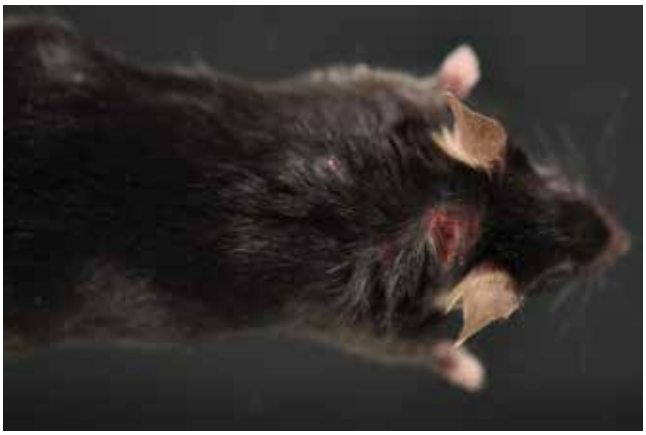

Figure 1. Gross skin ulceration at the injection site in a mouse that received 5 mg/mL meloxicam subcutaneously.

Pectus excavatum, sternal fracture and cardiac pathology. On physical exam, 5 mice appeared to have pectus excavatum. On histopathology of the sternum, 3 of these 5 mice and 8 of 20 mice in total exhibited sternal costochondral degeneration resulting in fracture (Figure 4 A and B). Right ventricular subepicardial fibrosis was evident in 4 of 20 mice. These lesions did not segregate with treatment status (Table 2).

Gastritis. In total, 7 mice displayed mixed deep proprial gastritis (Figure 4 C and D) toward the gastroduodenal junction, ranging from mild (n = 6) to moderate (n = 1). Mice that received saline were significantly (P = 0.01) more likely to develop gastritis (6 of 10 with gastritis) than mice that received meloxicam

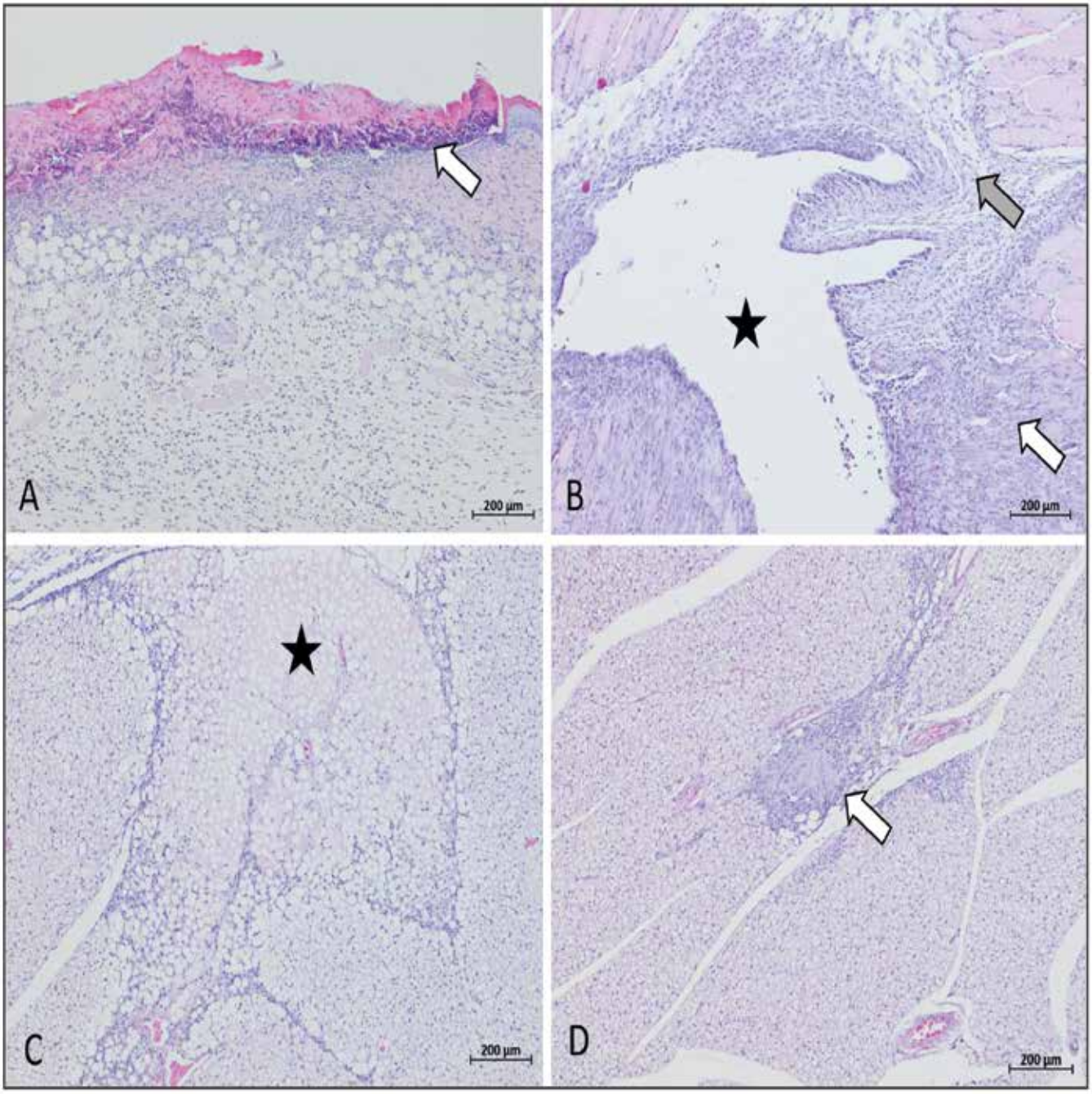

Figure 2. Representative histopathology in (A through C) mice that received 5 mg/mL meloxicam subcutaneously and (D) saline-control mice. Lesions in mice treated subcutaneously with meloxicam included (A) full-thickness epidermal necrosis (arrow), (B) cavitary spaces lined by inflammatory cells in muscle or fat (star), associated with myositis and cellulitis (white and gray arrows respectively), and (C) focal fat necrosis (star). (D) A small focal region of steatitis (white arrow) is evident in a control animal that received saline. Hematoxylin and eosin stain; bar, 200 µm.

of either concentration (1 of 10 with gastritis). Only one mouse that received meloxicam (at 5 mg/mL) demonstrated gastritis. No other gross abnormalities or pathology were seen, including ulceration of the gastric lining or proximal duodenum. There was no evidence of hemorrhage in any of the mice.

CBC and serum chemistry analyses. CBC were performed for 18 of the 20 mice and were within normal limits (data not shown).[33,41] Serum chemistry was performed for all mice. Serum chemistry values were within normal limits for all mice, except for elevated creatine phosphokinase levels (739, 819, 861, and 1131 IU/L) in 4 mice, 2 of which received 5-mg/mL meloxicam and 2 received saline.

Body weight. Body weight did not change between days 1 and 6 in any treatment group (data not shown). All mice had a body condition score of 3 (on a scale of 5) throughout the daily observation period.[36] Daily weights of food hoppers daily showed that most mice ate 3 to 4 g of food daily.

Discussion

Subcutaneous administration of meloxicam at its standard concentration (5 mg/mL) caused marked necrosis of skin, subcutaneous fat, and muscle at the injection site in all mice to which the drug was administered. This result appeared to be concentration-dependent, given the lack of difference in injection site pathology between the 6 mice that received 1 mg/mL

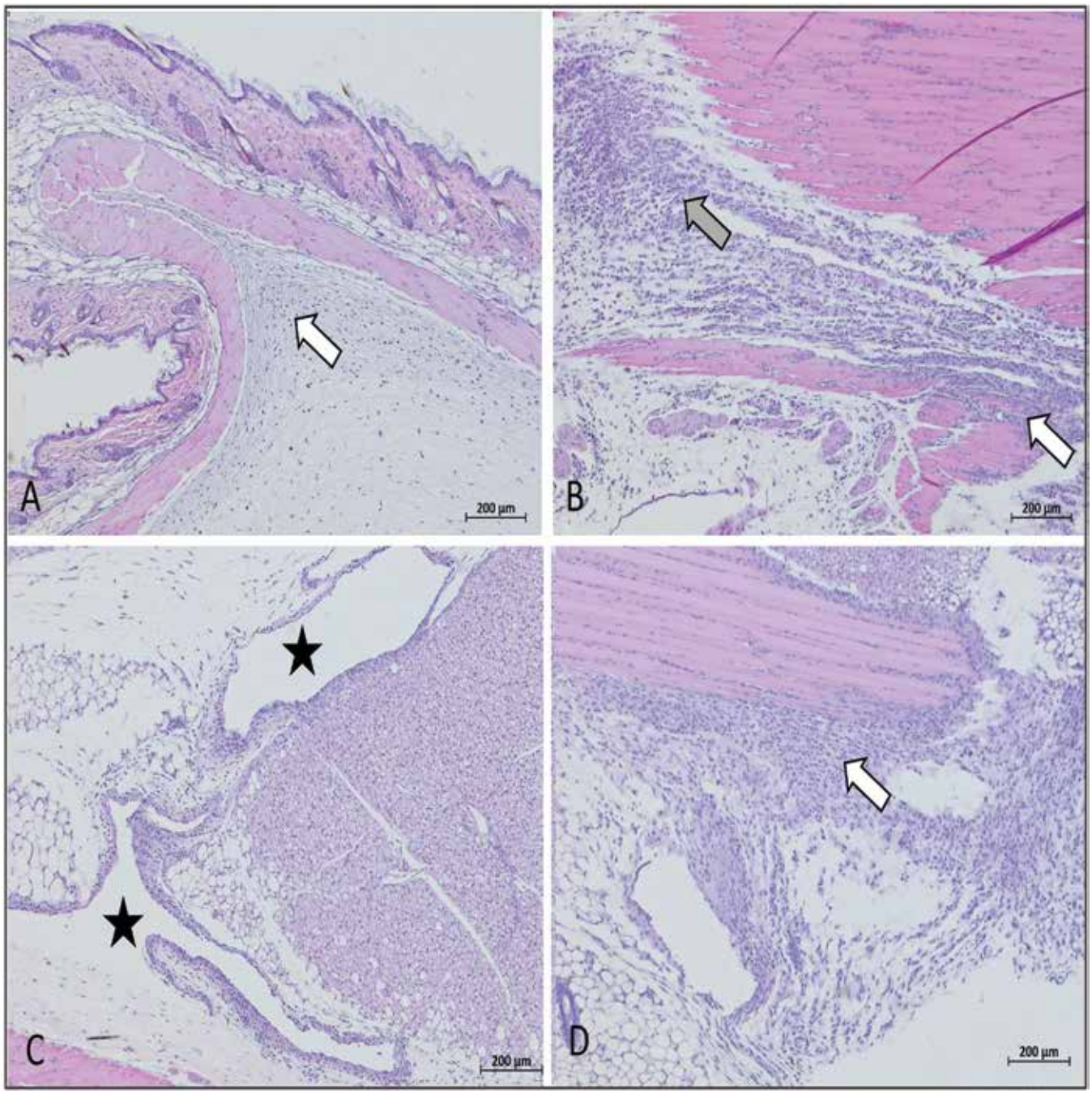

Figure 3. Representative histopathology in (A through C) mice treated subcutaneously with 1 mg/mL meloxicam and (D) saline-control mice. Lesions in mice given 1 mg/mL meloxicam subcutaneously included (A) mild subcuticular inflammation and edema (arrow), (B) myositis and cellulitis (white and gray arrows, respectively), and cavitary spaces lined by inflammatory cells in fat (star). (D) Saline-treated mice typically had no to minimal inflammation in the subcutis, although one mouse had marked steatitis and myositis (white arrow). Hematoxylin and eosin stain; bar, 200 µm.

SC meloxicam and those that received saline subcutaneously. We conclude that meloxicam diluted to 1 mg/mL and given subcutaneously at a dose of 20 mg/kg is safe in C56BL/6N mice for a maximum of 6 once-daily doses.

To our knowledge, this report is the first description of epidermal necrosis, myositis, and steatitis in mice after subcaneous injection of 5 mg/mL meloxicam. Ulcerative dermatitis has been reported after 3 once-daily subcutaneous doses of either 1 or 2 mg/kg of rats.[26] This study[26] and the current study used the same brand of meloxicam, which is formulated at a concentration of 5 mg/mL. Another similarity between the two studies is the daily repetition of injections: the rats received 3 daily injections, whereas the mice in the current study received 6. Other reports of injection site pathology after meloxicam injection bear less similarity to the current report. Sustained-release meloxicam at a concentration of 10 mg/mL has been reported to cause injection site erythema, necrosis, and abscessation in cynomolgus macaques, although the authors believe this outcome was due to a specific matrix that is not present in the standard-release meloxicam formulation.[2] Injection site pathology was not noted in other studies in which mice received a single dose of 20 mg/kg SC meloxicam.[15,27] Dogs given meloxicam subcutaneously at the labeled dose of 0.2 mg/kg only rarely demonstrate pain or pruritus at the injection site.[3] Cutaneous adverse reactions to

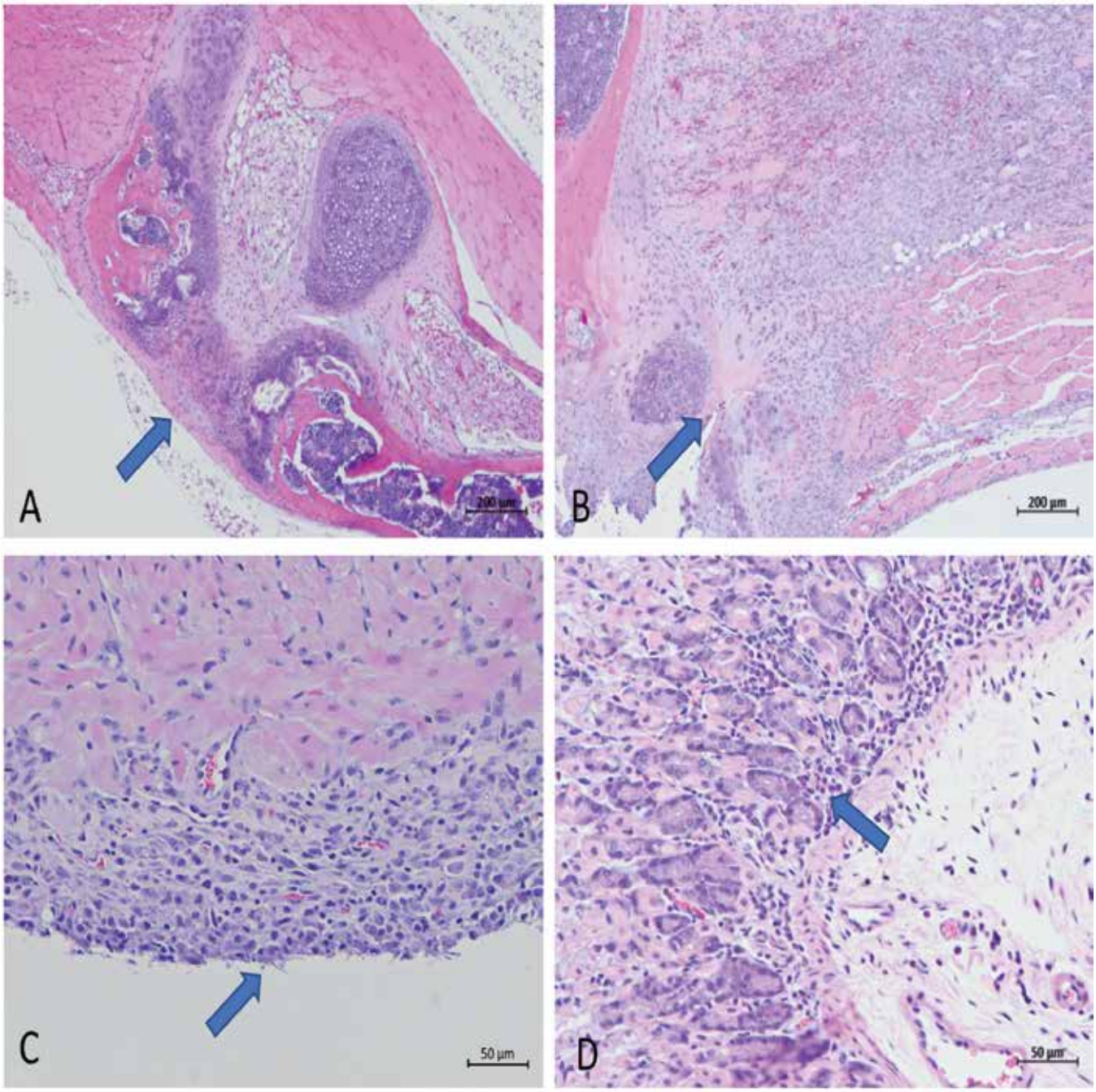

Figure 4. Background pathology in meloxicam- and saline-treated mice. Clinically noted pectus excavatum was associated with (A) sternal degeneration and (B) fracture. Some mice with sternal lesions also had (C) subepicardial nonsuppurative inflammation and fibroplasia of the right ventricle, and some had (D) minimal gastric mucosal inflammation, which was unrelated to meloxicam administration. Hematoxylin and eosin stain; bar: 200 µm (A and B); 50 µm (C and D).

oral meloxicam are rare but have been reported in both dogs[25] and humans.[40] Although meloxicam has been administered at a dose of 20 mg/kg in other stocks and strains of mice, the current study is the first published report of C57BL/6 mice that received meloxicam once daily at this dose and for this duration. Whether the injection-site pathology that we noted in the current study is related to the strain, meloxicam brand, volume administered, number of doses administered in the same location,[26] or another variable is unknown. However, the injection volume is unlikely to have contributed to pathology, because our maximal volume of 0.5 mL SC is much less than the 2 to 3 mL frequently recommended, [34] and rats have displayed similar pathology after receiving much smaller volume of 5 mg/mL meloxicam (0.04–0.08 mL).[26] Through histopathology, we verified that the injectate did not go intradermally instead of subcutaneously. However, our results indicate that the 5 mg/mL concentration contributed to the lesions since diluting the solution to 1 mg/mL reduced the incidence of adverse reactions at the injection site.

In contrast to previous reports,[38] NSAID-related pathology such as gastrointestinal ulceration or liver or kidney damage was not observed in our cohort. Gastric ulceration, gastritis, and inflammation of the liver was reported in Swiss mice given 10 mg/kg PO meloxicam for 5 d,[38] suggesting that subcutaneous administration of 20 mg/kg meloxicam may be safer than oral administration of the same dose.

Saline-injected mice did display some pathology, including mild localized inflammation at the site of injection. Although the histopathology at sites of subcutaneous injections is rarely reported, a small degree of local inflammation at any injection site is to be expected and should not be viewed as evidence of incorrect technique.

Background lesions were observed in both control and treatment groups. These lesions included gastritis, sternal abnormalities, and right ventricular inflammation and fibroplasia. Although we chose young (10 wk) mice for this study to minimize the occurrence of background lesions, they cannot be eliminated. Gastritis was observed in 7 of 20 mice and was significantly more frequent in mice that received saline than meloxicam. A possible explanation for the prevalence of gastritis among control mice is that it occurred as a background or stress-associated lesion in some mice. Our data are not sufficient to determine whether meloxicam treatment reduced this prevalence of this lesion in treated mice. However we can conclude that meloxicam did not increase gastric inflammation or cause ulcers. The majority of the gastritis seen in our current study was mild and if recognized previously may not have seemed remarkable enough to report in the literature.

On their arrival to our facility, 5 of 20 mice had gross pectus excavatum, and 8 of 20 mice had sternal fracture or focal sternal cartilage degeneration. Right ventricular subepicardial fibrosis was noted in 4 mice. All of these lesions are considered background pathology and have previously been described in C57BL/6N mice.[1] As reported, these 2 lesions displayed high cooccurrence (3 of the 4 mice with right ventricular subepicardial fibrosis also had sternal fracture).[1]

The only abnormality noted on CBC and serum chemistry analysis was elevated creatinine phosphokinase (greater than 700 IU/L)[33] in 4 mice: 2 given saline and 2 that received 5 mg/mL meloxicam. These abnormalities seem to be clinically insignificant and may have been consequences of handling and injection.

The current study was limited to the administration of meloxicam to clinically healthy, young C57BL/6N mice. Whether meloxicam at 20 mg/kg SC given once daily for 6 d induces pathology in mice that are geriatric or sick, receiving other analgesics, recovering from anesthesia and surgery, or of another strain or stock is unknown. In addition, whether C57BL/6 mice require more analgesia than outbred stocks is unknown; C57BL/6 mice have previously been reported to be particularly sensitive to many forms of nociception,[23] and some standard assays of mechanical and chemical nociception did increase MGS score in this strain.[15] Paradoxically, C57BL/6 reportedly have lower baseline MGS scores than C3H/He and CD1 mice, in the absence of a noxious stimulus.[20]

Although other veterinary species often receive meloxicam once every 24 h,[3] mice may require more frequent dosing. The half-life of meloxicam in rats permits once-daily dosing.[7] However, mice have been reported to clear meloxicam 10 times faster than rats.[4] At a dose of 1.6 mg/kg SC, meloxicam may need to be given every 12 h to exceed the COX2 inhibition constant in plasma.[7] Oral dosing of NSAID in mice may provide a higher, more consistent plasma drug level with less variability than subcutaneous administration, but the efficacy of analgesia provided by oral route has not been established.[13] As discussed previously, more safety data are needed. Meloxicam dosages lower than 20 mg/kg SC might be sufficient for analgesia if the drug was given more frequently than once every 24 h.

Our results show that meloxicam (1 mg/mL) can be administered safely at a dosage of 20 mg/kg SC to C56BL/6N mice for as long as 6 d. Previous studies have shown that 20 mg/kg meloxicam SC is necessary for analgesia after vasectomy in mice,[16,19] although this dose of meloxicam only decreased inflammation but not associated pain in a study that used laparotomy.[28] These studies suggest that at least 20 mg/kg SC of meloxicam is necessary for sufficient analgesia in clinical postoperative contexts, and our current data show that this same dosage at a concentration of 1 mg/ml results in minimal pathology. However, more data are needed to determine a safe and effective dose and route for postoperative meloxicam and other NSAID in mice.

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