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Evaluation of Analgesic Efficacy of Meloxicam and 2 Formulations of Buprenorphine after Laparotomy in Female Sprague–Dawley Rats

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Managing postoperative pain in rodents is an important part of any animal care and use program, and identifying an optimal analgesic plan for a surgical procedure is critical to providing for animal welfare. Opioids and NSAID are commonly used in rodents, but few studies have evaluated their efficacy in surgical models. The current study aimed to evaluate the therapeutic efficacy of clinically relevant doses of buprenorphine (2 formulations) or meloxicam used in combination with ketamine and xylazine anesthesia in a Sprague–Dawley rat ovariohysterectomy surgical model. Rats received either subcutaneous saline once daily for 3 d, low-dose (0.05 mg/kg SC) or high-dose (0.1 mg/kg SC) buprenorphine twice daily for 3 d, a single injection of sustained-release buprenorphine (1.2 mg/kg SC), or low-dose (1 mg/kg SC) or high-dose (2 mg/kg SC) meloxicam once daily for 3 d. Clinical analgesic efficacy was assessed over 8 d according to cageside observation scoring, body weight, and behavioral testing. Ovariohysterectomy was associated with 2 d of postoperative pain, and all 3 buprenorphine dosing strategies and both doses of meloxicam demonstrated varying amounts of analgesia. Given the results of the current study, we recommend 0.05 mg/kg SC buprenorphine at least twice daily or a single dose of 1.2 mg/kg SC of sustained-release buprenorphine for rats undergoing midline laparotomy with ovariohysterectomy. Alternatively, meloxicam at 1 to 2 mg/kg SC once daily could be used for this indication.

Abbreviations: HDB, high-dose buprenorphine; HDM, high-dose meloxicam; LDB, low-dose buprenorphine; LDM, low-dose meloxicam; RGS, rat grimace scale; SRB, sustained-release buprenorphine

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The research community has scientific, ethical, and regulatory responsibilities to provide analgesia to rodents used in research. Providing laboratory animals with appropriate analgesia is a basic component of an adequate veterinary care program.[14] Furthermore, the *Guide* also states that "guidelines for the selection and proper use of analgesic and anesthetic drugs should be developed, periodically reviewed, and updated."[14] The best method for evaluating pain and providing analgesia in rodents is unclear, and continued study is needed to provide investigators with appropriate guidelines for pain management.

Many rodent pain models have been used. The pain experienced by the animal and the clinical signs associated with it depend on the type of painful stimulus used as part of the model. In particular, the nociceptive tests traditionally used in neuroscience research of pain pathways do not always translate well to clinical applications.[2,3] For this reason, surgical models of pain, are often used to evaluate the potential effectiveness of analgesics for similar research procedures. In addition, surgical models of pain enable investigators to evaluate the practical efficacy of an analgesic in the postoperative period.

Laparotomy is a common procedure performed by researchers for various types of studies, and this procedure has been used by multiple researchers to assess the efficacy of analgesic drugs in rodents.[4,7,11,15,24,31,32,34,35,37,38,48] Animals undergoing laparotomy are most likely to demonstrate clinical signs of surgery-associated pain associated during the first 24 h after the procedure;[7] however, inconsistencies between studies highlight the difficulty of identifying a consistent analgesic regimen.[6,10,16,31] In addition, previous studies have often lacked important control groups (for example, analgesia without surgery), thus complicating interpretation and comparison between studies.[18,31,42]

In addition to the difficulty of choosing an appropriate pain model, generally accepted objective criteria for evaluating pain and analgesia in animals are unavailable.[17] Many physiologic parameters have been evaluated for assessing pain in rodents, including weight change,[5,24] heart rate, blood pressure,[4] and cortisol levels.[44,52] In addition, behavioral parameters including behavioral changes,[31,34,35] locomotor activity,[4,10,24,37] and facial grimace scoring[22,38] have been used to assess pain in rodents. For the current study, both physiologic (weight change) and behavioral methods (cageside scoring, activity level, vertical rises, and facial grimace scoring) were used to evaluate pain in the rat model of laparotomy surgical pain. We chose these methods given their ease of use and practicality in a research setting.

Opioid analgesics are widely used to control postoperative pain in rodents, by binding to opioid receptors and attenuating pain perception.[39] Buprenorphine is the most commonly used opioid in rodents, because of its efficacy and relatively long dosing interval.[39] Recently, a single, subcutaneous injection of 1.2 mg/kg sustained-release buprenorphine (SRB) provided 3 d of analgesia in the tibial defect model in male Sprague–Dawley rats; the pain relief of SRB was superior to single injections of buprenorphine HCl.[10] In addition, SRB demonstrated superi-

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ority in providing analgesia in an incisional pain model when compared with sustained-release meloxicam and carprofen gel in male Sprague–Dawley rats.[36]

NSAID are other commonly used group of analgesics in rodents. NSAID provide pain relief by inhibiting cyclooxygenase, which is the enzyme that catalyzes the first step in prostaglandin synthesis.[45,46] Advantages of NSAID for analgesia in the research setting are that they are effective for mild to moderate pain, and because they are not controlled substances, they do not require special licensure. In particular, meloxicam is a COX2-selective NSAID that only needs to be given once a day. Initial studies in rats suggest that meloxicam as a single-agent anesthetic may provide sufficient relief of postoperative pain.[4,5,34]

The objective of the current study was to evaluate the therapeutic efficacy of 2 formulations of buprenorphine or meloxicam used in combination with ketamine and xylazine anesthesia in a rat ovariohysterectomy model. The analgesics that we evaluated included buprenorphine (0.05 or 0.1 mg/kg SC), buprenorphine SR (1.2 mg/kg SC), or meloxicam (1 or 2 mg/kg SC). We evaluated the efficacy of these drugs by using practical, noninvasive parameters including cageside observations, body weight, behavior testing, and facial grimace scoring in rats.

Materials and Methods

Animals. Rats used during this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*[14] at the University of Illinois at Chicago (Chicago, IL), an AAALAC-accredited institution. All procedures were reviewed and approved by the University of Illinois at Chicago Animal Care Committee. Female Sprague–Dawley rats (Hsd: Sprague–Dawley SD; weight, 207.3 ± 7.7 g) were purchased from Envigo (Indianapolis, IN). The rats were not tested through the institutional sentinel program in light of the short duration of the study. According to the manufacturer's sentinel program, animals were presumed negative for the following agents: Hantaan virus, Kilham rat virus, lymphocytic choriomeningitis virus, mouse adenovirus types 1 and 2, pneumonia virus of mice, rat minute virus, rat parvovirus, rat Theiler virus, respiratory enteric virus III, Sendai virus, sialodacryoadenitis virus, Toolan H1 parvovirus, *Bordetella bronchiseptica*, cilia-associated respiratory bacillus, *Clostrium piliforme*, *Corynebacterium kutscheri*, *Helicobacter* spp., *Klebsiella* spp., *Mycoplasma pulmonis*, *Pasteurella* spp., *Pneumocystis carinii*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus aureus*, *Streptobacillus moniliformis*, and *Streptococcus* spp. In addition, manufacturer sentinels were free of helminths and external parasites. On arrival, rats were housed individually in static autoclaved polysulfone microisolation cages (Ancare, Bellmore, NY) with irradiated diet (no. 7912, Teklad, Madison, WI), a 14:10-h light:dark cycle, autoclaved municipal water in bottles, autoclaved corncob bedding (no. 7097, Envigo), and 3 or 4 wooden tongue depressors for enrichment. The room temperatures and humidity were maintained at 20 to 25 °C and 30% to 70% respectively. Rats were acclimated to the facility, handling, and video capture box for 1 wk prior to study onset. Animals were weighed on the morning of surgery and then again on study days 1, 2, 3, 5, and 8.

Experimental groups. By using a randomized block design, rats were randomly assigned to 1 of 13 groups (n = 10 per group (Figure 1). Animal groups included a naïve control, 6 anesthesia only with analgesic injections, and 6 anesthesia with surgery and analgesic injections. Anesthesia was administered as a single subcutaneous injection containing 80 mg/kg ketamine and 5 mg/kg xylazine. This combination of injectable agents provides both anesthesia and analgesia.[9,12,30,40,49-51] Subsequent analgesic injections were all given subcutaneously in the dorsal cervical region and included high- and low-dose buprenorphine, a single dose of SRB, a high and low dose of meloxicam, and saline, all injected subcutaneously. All analgesic doses follow current clinical use in our facility and supporting literature.[9] The first dose of the analgesic agent was given 1 h after anesthetic induction (day 0, Figure 2). Buprenorphine hydrochloride (Buprenex, Reckitt Benckiser Pharmaceuticals, Richmond, VA) was administered for 3 d at a low dose (LDB, 0.05 mg/kg twice daily on study days 0 through 2; Figure 2) or high dose (HDB, 0.1 mg/kg twice daily, days 0 through 2; Figure 2). SRB (3 mg/mL Buprenorphine SR, ZooPharm, Fort Collins, CO) was administered as a single injection of 1.2 mg/kg[6,10,16,36] on day 0. Meloxicam (Metacam, Boehringer Ingelheim Vetmedica, St Joseph, MO) was administered for 3 d at a low dose (LDM, 1 mg/kg once daily, days 0 through 2; Figure 2) or high dose (HDM, 2 mg/kg once daily, days 0 through 2; Figure 2). Saline (0.1 mL) was administered once daily for 3 d (days 0 through 2; Figure 2). The saline dose was similar in total volume to those of the buprenorphine and meloxicam doses.

Group	Anesthesia	Analgesia	Ovariohysterectomy
1	No	None	No
2	Yes	1.0 mg/kg M	No
3	Yes	2.0 mg/kg M	No
4	Yes	0.05 mg/kg B	No
5	Yes	0.1 mg/kg B	No
6	Yes	1.2 mg/kg SRB	No
7	Yes	0.1 mL saline	No
8	Yes	1.0 mg/kg M	Yes
9	Yes	2.0 mg/kg M	Yes
10	Yes	0.05 mg/kg B	Yes
11	Yes	0.1 mg/kg B	Yes
12	Yes	1.2 mg/kg SRB	Yes
13	Yes	0.1 mL saline	Yes

Figure 1. Experimental groups. Each group was specified by the use of an anesthetic, analgesic, and whether an ovariohysterectomy was performed. Anesthesia was induced with a single intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine. B, buprenorphine; M, meloxicam; SRB, sustained-release buprenorphine.

Anesthesia and surgery. Rats that underwent anesthesia only (groups 2 through 7, Figure 1) were anesthetized and placed in dorsal recumbency on a heating pad for the duration of anesthesia. Sterile lubricant was applied to the eyes to avoid corneal dehydration. At 60 min after the animal received the anesthetic injection, it received a subcutaneous injection of an analgesic, and a 4-mL IP injection of warmed saline was administered.

Rats that underwent anesthesia and surgery (groups 8 through 13, Figure 1) were anesthetized and placed in dorsal recumbency on a heating pad. Sterile lubricant was applied to the eyes to avoid corneal dehydration. The abdomen was shaved and prepared for surgery by using 3 alternating washes with povidone–iodine and 70% alcohol. After appropriate depth of anesthesia was confirmed through lack of response to a toe pinch, a single midline laparotomy incision was made. The ovaries and uterus were identified, ligated, and removed. The abdomen was closed in 2 layers by using 4-0 polydiaxanone suture (PDS, Ethicon, Somerville, NJ) in simple continuous and subcuticular patterns. Rats were placed on a heating pad to recover from anesthesia. At 60 min after the animal received the anesthetic injection, it received a subcutaneous injection of an analgesic, and a 4-mL IP injection of warmed saline were administered. All surgeries were performed in the morning, from 0800 to 1000.

	Day					
	0	1	2	3	5	8
Morning	Weight All ethograms Anesthesia ± surgery ± analgesia	Weight All ethograms Analgesia	Weight All ethograms Analgesia	Weight Cageside score Vertical rise Activity score	Weight Cageside score Vertical rise Activity score	Weight Cageside score Vertical rise Activity score
Evening	All ethograms LDB or HDB	Cageside score LDB or HDB	Cageside score LDB or HDB			

Figure 2. Procedures performed. 'All ethograms' includes cageside scoring, counting vertical rises, activity scoring, and RGS scoring (videorecorded and real-time assessment). Analgesia administered in the morning (0800 to 1000) included LDB, HDB, LDM, HDM, and saline. Analgesia administered in the afternoon (1600 to 1800) included LDB and HDB.

Ethograms. Because pain scoring has demonstrated utility in rodent postoperative and radiation models,[2,15,19,25,26,31,32] we used cageside observational scoring to assess rodent recovery in the current study. All rats were evaluated in their unopened cages in both the morning and afternoon on study days 0 through 2 (Figure 2), prior to analgesia administration. Subsequent observations on days 3, 5, and 8 were performed in the morning only. Although these observation time points were during the light phase and not when rats are expected to be most active, these time points correlated to when both veterinarians and investigators most likely would observe their animals; therefore we considered these time points to be the most clinically relevant times at which to perform these observations. Cages were not opened at any point during the scoring process. A subset of animals from all groups ($n = 73$) was observed by 2 independent observers to determine interobserver variability. All observers were female, trained on the scoring system, and familiar with its use prior to scoring postoperative animals. Observers performed all scoring within 30 min of initially entering the animal room. Animals received a score of 0 to 3 for each of the following criteria: posture, eye appearance, activity level, hair coat, and use of enrichment material (Figure 3). A total cumulative score of 0 to 15 was recorded for each animal at each time point. Although the analgesic regimen was not obvious to the observers scoring the animals, observers could not be blinded to the animal's surgical condition. This inability to blind observers to surgical condition applies to all ethograms used in the current study.

General activity was assessed for 1 min after opening the rat's home cage on a table top, as previously described.[10] Opened home cage activity was evaluated prior to surgery, in the afternoon after surgery (day 0), and then once daily in the morning on days 1, 2, 3, 5, and 8 (Figure 2). Activity was scored as follows: 0, no activity; 1, not as active as expected but some movement and exploration around cage; 2, normal activity level, with exploration of all 4 corners of the cage.

The number of vertical rises in a novel cage was assessed as previously described.[10,32,34] Total vertical rises were counted over a 2-min period. A full vertical rise was defined as standing on both hindlimbs, with both hind limbs supporting the entire body weight, the torso fully extended, and with front paws in the air or against the side of the cage. A partial vertical rise was defined as standing on both hindlimbs but without full extension of the torso; a partial rise was given half of the value of a full stand. Vertical rises were observed prior to surgery, and in the afternoon after surgery on day 0, and then once daily in the morning on days 1, 2, 3, 5, and 8 (Figure 2).

The rat grimace scale (RGS) has previously been used to assess pain[38] and was used in the current study to evaluate postoperative pain. Animals were placed in an aquarium (5.75 × 12 × 7.5 in.). Rats were acclimated to the aquariums daily for 1 wk prior to videorecording. A digital videocamera was placed on either side of the cubicle, to maximize the likelihood of clear headshots. Each animal was videorecorded for 15 min by using high-definition digital video cameras (Sony High Definition Bloggie Camcorder, San Jose, CA). Rats were recorded before anesthesia (day 0), at 5 to 6 h after anesthetic induction, and in the morning on day 1 or 2 (Figure 2). The still images were obtained by extracting every 200th frame from the videorecording by using MATLAB (MathWorks, Natick, MA). We selected 2 to 6 representative frames according to capture of the rat's face and the clarity of the image. All images were copied into PowerPoint (Microsoft, Redmond, WA) and then cropped to remove the animal's body posture and any identification, to ensure that subsequent coding was performed blinded. Each slide contained all 2 to 6 images (3 × 3 in.) of a specific animal at a specific time point, and the slide order was randomized by using a PowerPoint macro.

Randomized and unlabeled slides were presented on an overhead projector, one at a time, and the scorer assigned a value of 0, 1, or 2 for each of 4 criteria (orbital tightening, nose or cheek flattening, ear changes, and whisker changes) for each individual image, as previously described.[38] Five veterinarians scored the images for all animals and time points. In addition, a single veterinarian scored the live animals in real time at same time as image collection for all animals. All veterinarians had been trained on the use of the RGS and were familiar with using the scoring system and identifying pain in rats. A score of 0 indicated high confidence of the scorer that the individual criterion was absent. A score of 1 indicated either high confidence of a moderate appearance of the criteria or equivocation regarding its presence or absence. A score of 2 indicated the detection of an obvious appearance of the criteria, with high confidence. All scores for each animal were normalized to baseline (day 0 morning) score, a process that can cause negative scores when the baseline score is not 0.

A detailed handout[38] that explained each feature and that provided prototypic photos for each intensity score (0 to 2) for each criterion was provided to scorers to reference during the scoring period. For orbital tightening, animals in pain display a narrowing of the orbital area, manifesting either as (partial or complete) eye closure or eye 'squeezing.' Regarding nose or cheek flattening, animals in pain display successively less bulging of the nose and cheek, with the eventual absence of the crease between the cheek and whisker pads. For ear changes, the ears of rats in pain tend to fold, curl, and angle forwards or outwards, resulting in a pointed shape. The space between the ears may appear wider. In the criterion of whisker change, the whiskers of animals in pain move forward (away from the face) from the baseline position and tend to bunch, giving the appearance of whiskers standing on end.

Fecal occult blood testing. All animals that received meloxicam and any rat that had dark stool were tested for the presence of blood in their stool. Feces were collected and tested on study

Score	Body posture	Activity level	Eye appearance	Coat appearance	Enrichment material
0	Normal posture	Moving around the cage normally and very active	<25% closed	Normal coat	>50% used
1	Mildly hunched	Slightly reduced activity or a mild gait abnormality	25% to 50% closed	Piloerection	25%–50% used
2	Moderately hunched	Moving slowly or a severe altered gait	50%–75% closed	Piloerection and unkempt	<25% used
3	Severely hunched	Moving reluctantly or not at all, taking no more than 3 or 4 steps	75%–100% closed	Severely unkempt ± porphyrin accumulation	Untouched

Figure 3. Rat ethogram criteria for cageside scoring. Rats received a score of 0 to 3 for each of the following criteria: body posture, activity level, eye appearance, coat appearance, and use of enrichment material. Enrichment use indicated the total approximate percentage of wooden tongue depressors gnawed by the rat. A total cumulative score of 0 through 15 was possible for each animal at each observation time point. During analysis, we discarded data for eye appearance and enrichment material, resulting in cumulative scores of 0 through 9.

days 1, 2, 3, 5, and 8 (Hemoccult assay, Beckman Coulter, Brea, CA).

Histopathology. Animals that had any skin reaction to the injection of an analgesic were collected at the time of euthanasia (that is, day 8) and fixed in 10% buffered formalin. Representative skin samples were submitted to a commercial reference lab (Charles River Laboratories Pathology Services, Durham, NC) for processing and evaluation.

Pharmacokinetics. Naïve rats and rats that did not receive surgery or buprenorphine (groups 1 through 3 and 7, Figure 1) were used to evaluate plasma concentrations of SRB after completion of the study. Awake rats received SRB at 1.2 mg/kg SC in the flank, and blood was collected as a terminal procedure at 1, 3, 4, 5, 7, or 9 d after injection. Animals were euthanized (5 mL IP, FatalPlus, Vortech Pharmaceuticals, Dearborn, MI), and 3 mL of blood was collected through cardiocentesis into 5-mL sodium heparin tubes from 3 rats at each time point. Tubes were placed immediately on ice after collection and centrifuged at 1000 × *g* for 10 min within 15 min of collection. The plasma was collected and stored at −80 °C until shipment on dry ice for analysis.

All plasma samples were analyzed (Center for Human Toxicology, University of Utah, Salt Lake City, UT) by using a validated liquid chromatography–electrospray ionization–tandem mass spectrometry method described previously.[13] This method allowed for simultaneous detection of buprenorphine and its metabolites norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide. Briefly, 1-mL aliquots of plasma were extracted with methanol over a C18 solid-phase extraction column, reconstituted to 75 µL, centrifuged, and transferred to an autosampler vial. The autosampler (Surveyor, Thermo-Finnigan, San Jose, CA) injected the sample into a column (YMC 50 × 2 mm; catalog no. 3S ODS-AQ, Waters, Milford, MA) for analysis by the triple-stage quandrupole mass spectrometer (TSQ-Quantum, Thermo-Finnigan). Calibration curves with known peak:area ratios were used to determine the concentration of all analytes in each sample. The lower limit of quantitation of the assay for all analytes was 0.1 ng/mL for a 1-mL sample of plasma.[13] This quantitative assay has been validated at the University of Utah and used in both human and veterinary studies.[1,13,27,28,43]

Statistical analysis. Statistical analysis was performed by a statistician using SAS version 9.3 (SAS Institute, Cary, NC). Because of the longitudinal nature of the experimental design and the repeated observations of individual animals over the course of the study, a mixed-model method was used to model the patterns of the outcome measures over time. Such methods make the appropriate adjustment for the correlations of the measurements within a subject so that appropriate statistical tests of treatment effects can be obtained. By using mixed-model analyses, curves for individual animals were estimated by the inclusion of a random subject effect. Therefore, the model provided information about how the measures of a subject changed from its baseline values, instead of using a constructed measure like percentage change. For all data, both fixed (anesthesia, surgery, and specific analgesic or saline) and random effects were evaluated with the mixed-model method for statistical significance (defined as a P value less than 0.05), with differences being measured between entire curves. All data, except body weight, are presented as model-fitted curves.

By using a random number generator, a single independent observation was selected for each of the animals, to evaluate interobserver variability of cageside scoring. The agreement between observer scores was evaluated by calculating the κ coefficient, which estimates the proportion of concordant measurements and omits those that agree because of chance.[20,21] The strength of the agreement was interpreted as follows: poor, κ less than 0.2; fair, 0.21 to 0.40; moderate, 0.41 to 0.6; substantial, 0.61 to 0.8; and almost perfect, greater than 0.81.[21]

Results

Body weight. Although anesthesia and surgery affected body weight, average percentage changes were lower than 6.2% across all groups. Naïve animals had a steady increase in body weight. Rats that were anesthetized and received saline had an average percentage weight loss of 1.2% (range, 0% to 4.6%; n = 10) between days 0 through 2 and began to gain weight on day 3. In the animals that received surgery and saline, the average percentage weight loss was 5.4% (range, 3% to 10.7%; n = 10), which occurred during days 1 and 2, after which point animals began to gain weight, returning to baseline weight by day 5 (Figure 4 A).

Rats that were anesthetized and received either formulation of buprenorphine gained weight initially and then exhibited a delayed weight loss (Figure 4 B and C). Animals that received LDB had an average percentage weight loss of 2.6% (range, 0% to 9.1%; n = 10) on day 5, and those that received HDB had an average of 3.6% (range, 1.5% to 6.1%; n = 10) on day 5. In the rats that received surgery and LDB, the average percentage loss in body weight was 4.1% (range, 1.4% to 9.3%; n = 10), which occurred on day 5. Similarly, in animals that received surgery and HDB, the average percentage loss was 6.2% (range, 3.1% to 9.5%; n = 10), which occurred on day 5. Rats that received SRB lost an average of 0.9% (range, 0% to 6.8%, n = 10) on day 3. In animals that received surgery and SRB, the average percent-

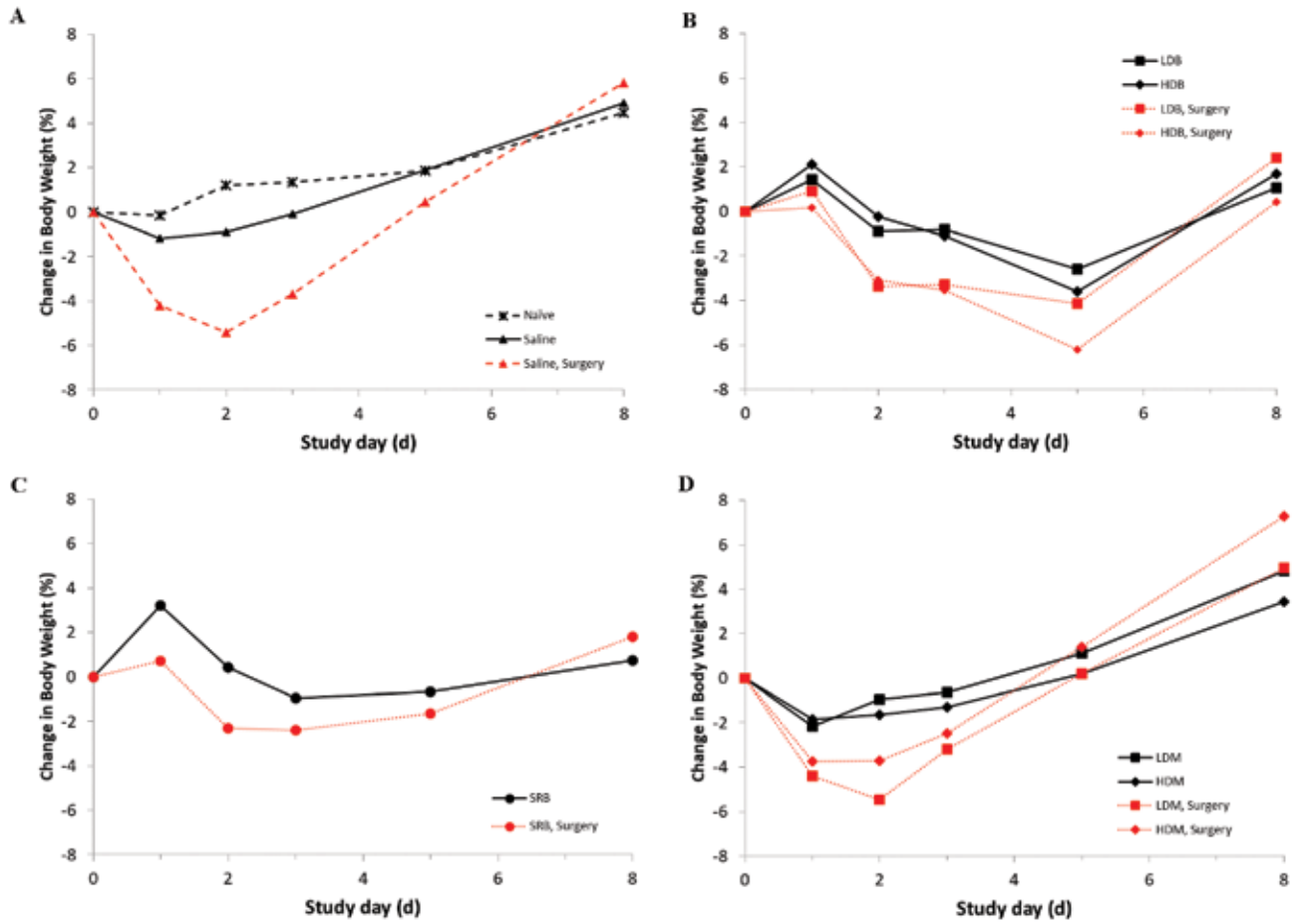

Figure 4. Average percentage change in body weight by group (n = 10 rats per group) throughout the entire study. All animals that received saline or analgesia without surgery were anesthetized in the same way as the animals that had surgery.(A) Curves for control rats, including naïve (group 1), saline only (group 7), and surgery with saline (group 13). (B) Curves for rats in that received either dose of buprenorphine with or without surgery (groups 2, 3, 8, and 9) (C) Curves for rats that received SRB only or SRB and surgery (groups 4, 10, and 13). (D) Curves for rats that received either dose of meloxicam with or without surgery (groups 5, 6, 11, and 12).

age weight loss was 2.4% (range, 0% to 5.8%; n = 10), which occurred on day 3.

Rats that were anesthetized and received either dose of meloxicam showed minimal effects on body weight (Figure 4 D). Animals that received either LDM or HDM had an average percentage body weight loss of 2.1% (range, % to 4.8%; n = 10) or 1.8%, (range, 0.5% to 4.9%; n = 10), respectively, on day 1. In animals that received surgery and LDM, the average percentage weight loss was 3.2% (range, 2.4% to 16.5%; n = 10) on day 3. Similarly, animals that received surgery and HDM, the average percentage loss was 3.7% (range, 1% to 6.5%; n = 10) on day 1.

Due to the variability of weight loss within groups and because even rats that were given only saline after surgery still had an average weight loss of only 5.4%, statistical analysis of this variable did not seem clinically relevant and is not presented.

Cageside scoring. In comparing control animals (naïve with saline and surgery with saline), we determined that only body posture, activity, and coat appearance provided any indication of pain. Neither eye appearance nor enrichment scores showed any significant difference between groups. Therefore, all data analysis is based on scores for body posture, activity, and coat only, with a range in total score of 0 through 9. In addition, the κ score for interobserver variability was 0.53, indicating moderate agreement between observers. Scores that did not agree showed a total difference of only 1 between observers.

Cageside score curves differed significantly ($P < 0.05$) between positive and negative control animals and between experimental groups. Baseline values (day 0, morning observation) were excluded from model fitting and are not displayed in the graphs because all rats, regardless of experimental group, scored 0. The rats that received anesthesia plus saline had significantly lower curves for cageside scoring than animals that received surgery plus saline ($P < 0.0001$, Figure 5). All scores for animals that received anesthesia only were below 2 in the afternoon after anesthesia (day 0) and were below 1 by day 2 (Figure 5 and Table 1).

Cageside score curves showed a statistical trend toward differing ($P = 0.0588$) between rats that received HDB compared with saline after surgery (Figure 5 A). Animals that received SRB or LDB after surgery had significantly lower curves for cageside scores than rats that received saline ($P < 0.0001$ and $P = 0.0006$) or HDB after surgery ($P = 0.0269$ and $P = 0.0010$, Figure 5 A).

Animals that received either HDM or LDM after surgery had significantly lower curves for cageside scoring than rats that received saline ($P = 0.0051$ and $P = 0.0006$, Figure 5 B). However, cageside scoring did not differ between rats that received HDM compared with LDM after surgery ($P = 0.7364$).

RGS. RGS scoring from still photographs did not differ between rats that had received saline after anesthesia only compared with after anesthesia and surgery. We interpreted this finding to mean that this parameter did not detect postsurgical

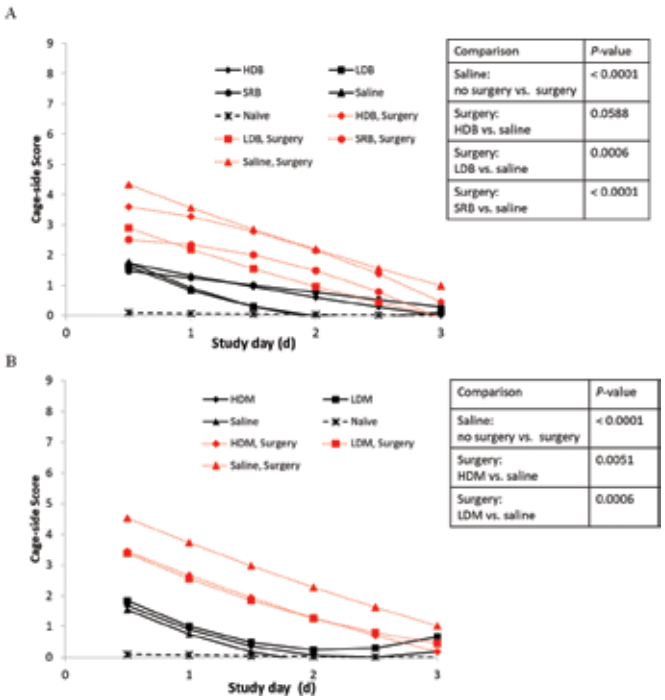

Figure 5. Model-fitted curves of the mean cageside score of rats (n = 10 per group) over days 0.5 through 3. P values are reported for each pairwise curve comparison. (A) Curves for rats in buprenorphine groups and saline controls (groups 1, 4 through 7, and 10 through13). (B) Curves for rats in meloxicam groups and saline controls (groups 1 through 3, 7 through 9, and 13).

pain in rats effectively in our hands. Therefore we did not pursue further statistical analysis, and results are not reported.

In contrast to postprocedural RGS scoring of still photographs, real-time RGS scoring was informative. Baseline values for real-time RGS were excluded from model fitting and are not displayed in the graphs because all animals, regardless of experimental group, scored 0. According to real-time RGS scoring, rats that received saline after anesthesia only had a significantly lower curve than animals that received saline after anesthesia and surgery ($P < 0.0001$, Figure 6 and Table 2). Regardless of dose, rats that received buprenorphine after surgery had lower curves for real-time RGS scoring than rats that received saline afterward (HDB, $P = 0.0012$; LDB, $P = 0.0002$; and SRB, $P < 0.0001$; Figure 6 A). Animals that received either HDM or LDM after surgery had lower real-time RGS curves than rats that received saline after surgery ($P = 0.0002$ and $P = 0.0044$, Figure 6 B). RGS curves did not differ between animals that received HDM compared with LDM after surgery or between the buprenorphine groups thereafter.

Vertical rises. Rats that received saline after anesthesia only had more vertical rises than rats that received saline after anesthesia followed by surgery ($P = 0.0013$, Figure 7 and Table 3) . The curves of rats that received buprenorphine (regardless of dose) after surgery did not differ from those that received saline afterward. The curves of rats that received meloxicam (either dose) after surgery were similar to those of their anesthesia-only controls (data not shown).

General activity in the home cage. Home cage activity did not differ between rats given saline after anesthesia only and rats that received saline after anesthesia plus surgery. We interpreted this finding to mean that this parameter did not detect postsurgical pain in rats effectively, and further statistical analysis was not pursued (data not shown).

Plasma SRB concentrations. A single injection of SRB resulted in detectable plasma concentrations of buprenorphine and its metabolites buprenorphine-3-glucuronide and norbuprenorphine-3-glucornide (Figure 8); the metabolite norbuprenorphine was not detectable at any time point. The plasma buprenorphine concentration remained quantifiable (that is, higher than 0.1 ng/mL) over the entire 9-d testing period in all animals. The average buprenorphine plasma concentration was 1.01 ± 0.01 ng/mL on day 1 and decreased to 0.6 ± 0.03, 0.43 ± 0.09, 0.53 ± 0.10, 0.64 ± 0.64, and 0.39 ± 0.02 ng/mL on days 3, 4, 5, 7, and 9 after injection, respectively.

Behavioral evidence of abdominal pain. Additional evidence of abdominal pain in the rats that underwent surgery was not captured in the ethograms but was recorded after daily observations of the animals. In particular, 9 of the 10 rats that received saline after surgery demonstrated other behavioral evidence of pain on study day 0 after surgery and on days 1 and 2, including writhing and tensing of the abdomen (7 of 10 rats) and vocalization with handling (3 of 10 rats). In addition, 1 rat from the surgery with LDB group that displayed writhing at the morning observation on day 1.

Fecal occult blood. A total of 3 of the 45 animals tested positive for blood by using the Hemoccult assay (Beckman Counter). One animal in the LDM group tested positive once on day 1, and another animal from the same group tested positive twice, on days 2 and 3. A single animal from the saline plus anesthesia-only group tested positive once on day 2.

Adverse effects. Buprenorphine and SRB were associated with both pica and injection-site reactions. All rats that received LDB, HDB, or SRB with or without surgery were observed to ingest corncob bedding on days 0 and 1. Injection-site reactions were associated with SRB administration and with both doses of meloxicam. Specifically, 4 of the 20 rats that were injected with SRB in the dorsal cervical region developed a 1- to 3-mm erythematous plaque that was consistent with a mild mixed dermatitis on histopathology. Approximately 80% (14 of 18) of the rats that received flank injections while awake for the pharmacokinetic study had injection-site reactions, ranging from erythematous plaques to 0.5-cm cutaneous ulcers with scabbing and with or without purulent debris. In these cases, histology revealed ulcerative dermatitis with secondary bacterial infection.

Injection-site reactions occurred in 25 (62.5%) of rats that were injected with meloxicam. Specifically, 11 of the 20 rats that received LDM and 14 of those that received HDM developed 0.3- to 1-cm cutaneous ulcers with an overlying scab. Ulcerative dermatitis was identified on histopathology. Samples taken from the meloxicam bottle failed to support aerobic growth, and the injection-site reactions continued to occur even after a new, second bottle of meloxicam was used for injections.

Discussion

We evaluated multiple parameters to detect postoperative pain and analgesia in rats after laparotomy and ovariohyterectomy. The control groups (naïve rats, rats that received saline after anesthesia only or after anesthesia and surgery) were evaluated to determine the utility of the parameters for detecting pain in rats. According to the investigated parameters that we found useful, the data collectively indicate that pain can be assessed and quantified behaviorally in different ways. Assessing adequate analgesia by using practical behavioral tests that can be widely applied to rodents postoperatively in a research setting remains challenging. No analgesic tested in the current study consistently provided complete pain relief according to all evaluated parameters. However, 'complete pain relief' is an

Table 1. Cageside scores (mean ± 1 SD; n = 10 per group)

	Day 0		Day 1		Day 2		Day 3
Group	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Morning
1	0.8 ± 0.8	3.2 ± 0.4	2.5 ± 0.7	2.3 ± 0.9	1.9 ± 1.0	1.6 ± 0.9	1.6 ± 0.8
2	1.0 ± 1.1	4.9 ± 1.9	3.1 ± 1.1	3.0 ± 0.7	2.3 ± 0.9	1.9 ± 0.6	1.1 ± 0.9
3	1.2 ± 0.8	5.0 ± 1.4	3.5 ± 0.8	2.9 ± 0.7	2.4 ± 1.2	2.1 ± 1.0	2.0 ± 1.0
4	0.9 ± 0.9	4.5 ± 1.0	2.7 ± 2.5	2.3 ± 2.7	1.1 ± 2.0	1.1 ± 2.0	0.4 ± 0.5
5	1.0 ± 1.2	4.6 ± 1.3	3.1 ± 1.4	2.5 ± 1.5	0.6 ± 1.1	0.7 ± 1.1	0.6 ± 1.2
6	0.8 ± 1.0	4.7 ± 1.3	2.8 ± 2.4	2.5 ± 3.1	1.4 ± 2.7	1.2 ± 2.5	0.3 ± 0.7
7	0.7 ± 0.8	4.5 ± 0.7	2.7 ± 0.5	2.3 ± 0.5	2.1 ± 0.4	1.9 ± 0.6	1.1 ± 0.9
8	0.8 ± 0.9	6.4 ± 1.2	5.1 ± 1.7	4.6 ± 2.4	3.0 ± 4.6	3.1 ± 4.1	2.3 ± 4.3
9	1.6 ± 1.0	6.3 ± 1.3	4.3 ± 1.4	4.5 ± 1.8	2.4 ± 1.5	2.6 ± 1.8	1.7 ± 1.3
10	0.9 ± 1.0	5.8 ± 1.2	4.3 ± 2.3	3.3 ± 1.3	1.6 ± 1.4	1.0 ± 1.2	0.9 ± 0.9
11	1.0 ± 0.9	6.4 ± 1.7	5.8 ± 3.6	4.6 ± 2.6	2.8 ± 2.4	2.0 ± 2.3	1.6 ± 1.6
12	1.2 ± 0.4	5.1 ± 1.9	4.6 ± 3.5	2.8 ± 2.2	1.0 ± 0.8	0.9 ± 1.5	0.2 ± 0.4
13	1.0 ± 0.8	7.5 ± 1.5	6.2 ± 1.9	5.9 ± 2.0	4.0 ± 2.8	2.9 ± 3.1	1.7 ± 2.2

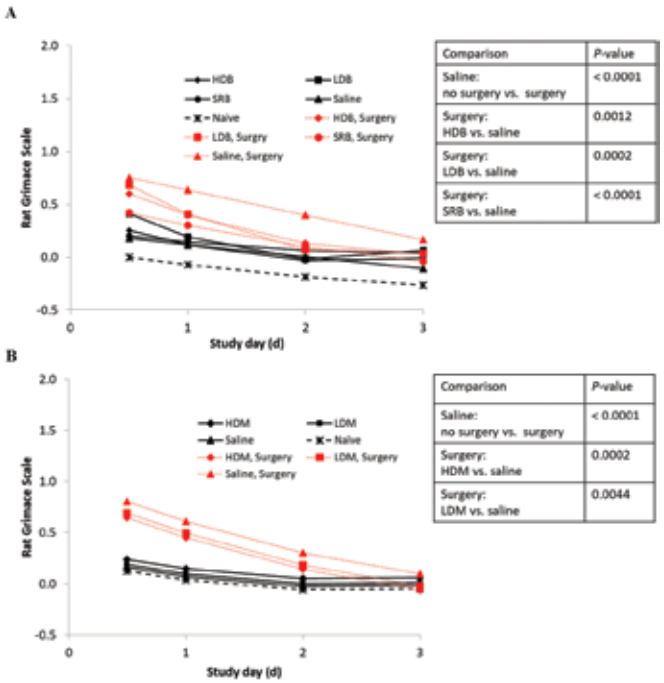

Figure 6. Model-fitted curves of the mean real-time RGS score (n = 10 per group) over days 0.5 through 3. P values are reported for each pairwise curve comparison. (A) Curves for rats in buprenorphine groups and saline controls (groups 1, 4 through 7, and 10 through 13). (B) Curves for rats in meloxicam groups and saline controls (groups 1 through 3, 7 through 9, and 13).

abstract idea that is difficult to appreciate or describe in animals. For the purposes of this discussion, we define 'complete pain relief' to mean surgery and control groups did not differ significantly for all parameters measured, a goal that none of the analgesics tested achieved.

Body weight frequently is used to monitor and assess postoperative pain in rats.[4,5,7,19,24,32] However, the results presented herein call in to question the utility of body weight loss to describe pain during the postoperative period in this surgical model. All surgical groups exhibited average body weight loss ranging from 2.4% to 6.2%, depending on the experimental group, similar to findings from previously published studies.[4,5,7,37] We also found that animals that were anesthetized, received an analgesic, but did not undergo surgery lost weight (Figure 4)—as high as 9.1%, in the case of one rat that received LDB with anesthesia. Given the variability in weight loss across all groups, we do not recommend using body weight as the sole postoperative monitoring tool to identify pain in rats that may (or may not) be receiving adequate analgesia.

Observational scoring systems have been used to assess rodent pain following surgical procedures.[31,32,35] Cageside observations specifically assessing body posture, activity, and coat appearance were sensitive indicators of postoperative pain, with significant differences during the first 48 h between rats given saline alone compared with saline plus surgery (Figure 5). Cageside scoring was simple to perform, involved a minimal time commitment, and required no handling of the animals, making this technique ideal for assessing postoperative pain in rodents. In addition, we saw reasonably good agreement between different observers using the same cageside scoring system. Although the κ statistic indicated 'moderate' agreement between observers, this level (which is 50% above chance agreement) is regarded as a favorable outcome for most observation-based systems.[20,21] In addition, all disparate observation scores were within 1 point of each other. Rats that received either dose of meloxicam, LDB, or SRB all had lower cageside scores than those that received saline after surgery. These data suggest that these analgesics, at the doses administered, provide some pain relief after laparotomy. In contrast, HDB did not appear to provide pain relief according to this parameter, perhaps due to a ceiling effect of buprenorphine between doses of 0.03 and 0.1 mg/kg in female rats.[8,29] This finding for HDB is consistent with previous findings demonstrating decreased analgesic efficacy for orofacial pain 1 h after administration for buprenorphine doses greater than 0.03 mg/kg in female Sprague–Dawley rats.[29]

Facial grimace scoring has been described as a very accurate way to detect pain in rodents.[22,23,38] Previous studies using facial scoring in rats videorecorded the rats and then processed the images for observers to score at a later time point.[38] Unfortunately, the process of videotaping the rats and processing the images is time-consuming and impractical for routine postoperative assessment. However, real-time RGS scoring is relatively quick and easy to perform and may be a practical option for postoperative evaluation of pain.[22,23] Similar to what we saw with the cageside scoring method, both doses of meloxicam induced significant decreases in real-time RGS scoring after surgery. In addition, LDB, HDB, and SRB all resulted in significantly lower scores compared with saline when given after surgery. However,

Table 2. Real-time RGS score (mean ± 1 SD; n = 10 per group)

Group	Day 0 Morning	Day 0 Afternoon	Day 1 Morning	Day 2 Morning	Day 3 Morning
1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
3	0.0 ± 0.0	0.4 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
4	0.0 ± 0.0	0.5 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
5	0.0 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
6	0.0 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
7	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
8	0.0 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
9	0.0 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	–0.1 ± 0.0
10	0.0 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.0 ± 0.0	0.1 ± 0.0
11	0.0 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
12	0.0 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.0 ± 0.0
13	0.0 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.1 ± 0.1

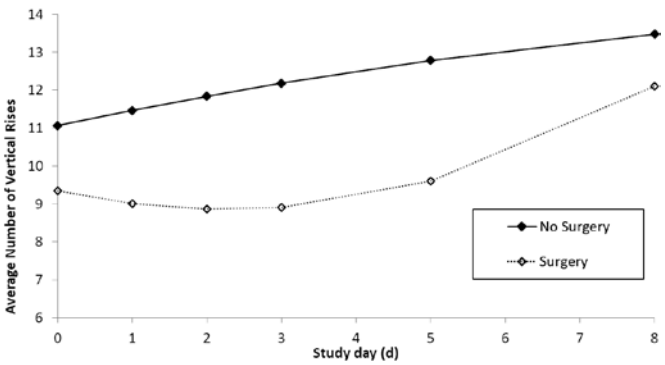

Figure 7. Model-fitted curves of the mean vertical rises in control rats (n = 10 per group). The curves differ significantly ($P < 0.0001$) between rats that received saline and anesthesia only (group 7) and those given saline after anesthesia and ovariohysterectomy (group 13) for 3 d.

the real-time RGS scores were not assessed by blinded observers, thus calling into question the validity of these data, particularly given that the blinded facial scoring failed to detect pain. The most likely reason for the failure of the photograph-based blinded RGS was the use of ketamine and xylazine anesthesia. Authors who used isoflurane anesthesia found significant scores only during the first 12 h postoperatively in their laparotomy test.[22] Because our 12-h time point was only a few hours after the injection of ketamine and xylazine, the effects of these drugs were likely still present in the control groups. It also is possible that the limited data set evaluated by the blinded observers contributed to the inability to detect differences between groups, but the data sets were similar to those of other published studies in rodents.[22,23,38] Future studies using blinded observers are necessary to determine the usefulness of the RGS in real time and the ideal timeframe for its use (12 h, 24 h, or 3 d).

Assessing the sufficiency of analgesia by using practical behavioral tests that can be widely applied to rodents postoperatively in a research setting remains challenging, and several of the tests we used in the current study were unsuccessful. The ineffectiveness of the vertical rise and activity score tools may have been due to differences in the type of pain or surgical procedure performed compared with those used in references. Vertical rises were likely unsuccessful in the current study because these animals had abdominal surgery rather than the tibial defect model,[10] for which vertical rises was a successful indicator of pain. In addition, sex and stock may have played a role in the expression of pain and thus contributed to a lack of detectable difference in activity level, which has been seen in other abdominal surgery studies in male Wistar and Fisher rats.[32,34] Furthermore, we noted additional indicators of pain during the postoperative period. Rats that only received saline after surgery exhibited writhing, tensing of the abdomen, and increased vocalization with handling. Although these signs were inconsistently displayed among animals, it is important to look for and educate investigators regarding these signs of unrelieved pain. Furthermore, the single rat that had surgery, received LDB, but exhibited writhing on the morning of day 1 illustrates the importance of dosing interval. In the current study, rather than treating every 12 h as is recommended, we used morning (0800 to 1000) and evening (1600 to 1800) dosing, according to common practice among investigators. This dosing regimen increases the potential for breakthrough or inadequate pain control, as demonstrated by the rat that received LDB and exhibited breakthrough pain. It is important to limit the interdose interval to a maximum of 12 h when using buprenorphine, and 8-h intervals may be more appropriate for some animals. Animals should be assessed regularly for breakthrough pain so that additional analgesic can be administered as needed, particularly when using a 12-h dosing schedule.[33] Alternatively, multimodal analgesic dosing strategies can be used to protect against inadequate pain control.

Both NSAID and opioids have been associated with side effects. NSAID can cause gastrointestinal ulcers and renal ischemia. In rats, blocking of both COX1 and COX2 is required for the formation of gastrointestinal ulcers.[41,47] Although it was unlikely that a COX2-selective NSAID would cause problems, we performed fecal occult blood testing of all rats that received meloxicam injections as well as of any rat that had dark-colored stool. Four positive tests were obtained over the course of the entire study, and 2 were from the same rat at subsequent sampling time points. The false-positive rate reported for this test in humans is 1% to 2% (Hemoccult package insert). These 4 positive results total less than 2% of the tests that were run, making it likely that meloxicam does not cause any significant gastrointestinal bleeding in rats, even after multiple doses.

Injection-site reactions were an unexpected side effect of meloxicam. Many rats had ulcers (diameter, 0.3 to 1 cm) near or in the area where meloxicam was injected, with more ulcers forming in the HDM group than LDM group. The rats did not appear to be bothered by the lesions, given that we noticed no scratching, and the ulcers were dry. Injection-site reactions associated with meloxicam have not been reported in rats previously and might be due to the specific brand we used here

Table 3. Vertical rises (mean ± 1 SD; n = 10 per group) in control rats

	Day 0		Day 1	Day 2	Day 3	Day 5	Day 8
Group	Morning	Afternoon	Morning	Morning	Morning	Morning	Morning
7	12.3 ± 3.2	2.8 ± 3.4	12.2 ± 5.5	13.4 ± 6.5	14.1 ± 5.5	12.7 ± 3.4	15.3 ± 5.7
13	10.3 ± 3.6	0.2 ± 0.2	6.0 ± 3.6	7.7 ± 3.8	8.9 ± 4.0	9.4 ± 3.4	12.1 ± 6.1

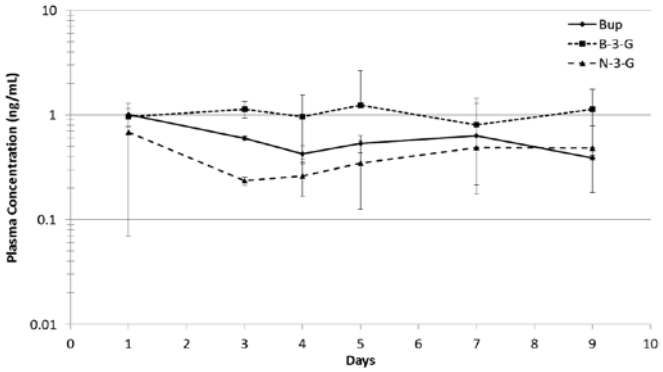

Figure 8. Plasma concentrations of buprenorphine and metabolites after a single injection of 1.2 mg/kg SC SRB in female Sprague–Dawley rats over 9 d. The average plasma concentrations (ng/mL; n = 3 per time point) of buprenorphine (BUP), buprenorphine-3-glucuronide (B-3-G), and norbuprenorphine-3-glucuronide (N-3-G) are reported.

or perhaps to sensitization due to the repeated injections over 3 d. This finding should be remembered when giving multiple injections of meloxicam and may be a reason to avoid the drug or choose a different brand if an injection-site reaction would interfere with the needs of the study. Additional studies to evaluate and identify the underlying cause of the meloxicam injection-site reaction are needed.

At the dose given, SRB (1.2 mg/kg SC) remains in the plasma for at least 9 d in Sprague–Dawley rats. Although the plasma concentration we saw is similar to that when buprenorphine is given to rats over 3 d,[10] our finding suggests a potential for prolonged side effects and aberrant influences on experimental parameters depending on the intended purpose of the rats on study. Our rats demonstrated pica behavior during only the first 24 h after injection. Further evaluation is necessary to understand the full extent of the potential complications of having opioids in the body for this extended period. Evaluation of other doses is warranted also.

The collective results of the current study suggest that midline laparotomy with ovariohysterectomy in female Sprague–Dawley rats is associated with pain on the day of surgery and for 2 d afterward. Our current results suggest that both buprenorphine and meloxicam provide some degree of pain relief after laparotomy in female Sprague–Dawley rats. Given the analgesic dosing regimens evaluated in this study, we recommend buprenorphine at 0.05 mg/kg SC at least twice daily or a single dose of sustained-release buprenorphine at 1.2 mg/kg SC. Alternatively, meloxicam at 1 to 2 mg/kg SC once daily might be used, but rats should be monitored closely for the development of injection site reactions when repeated-dosing regimens are used. Other doses of buprenorphine or meloxicam that were not evaluated in this study may be appropriate also. Furthermore, although we did not evaluate multimodal analgesia, it may be superior to any of the tested single-drug options in rats and should be considered for future studies. We further recommend cageside observation of body posture, activity level, and coat appearance and real-time scoring of facial grimace as practical methods for daily observation of Sprague–Dawley rats after surgery. Rats may need to be observed more often than once daily depending on the analgesic regimen used and the potential for breakthrough pain before the next day.

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