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A Pharmacokinetic and Analgesic Efficacy Study of Carprofen in Female CD1 Mice

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The minimization of pain in research animals is a scientific and ethical necessity. Carprofen is commonly used for pain management in mice; however, some data suggest that the standard dosage of 5 mg/kg may not provide adequate analgesia after surgery. We hypothesized that a higher dose of carprofen in mice would reduce pain-associated behaviors and improve analgesia without toxic effects. A pharmacokinetic study was performed in mice given carprofen subcutaneously at 10 or 20 mg/kg. Plasma concentrations were measured at 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h after dosing (n = 3 per time point and treatment). At these doses, plasma levels were above the purported therapeutic level for at least 12 and 24 h, respectively, with respective half-lives of 14.9 and 10.2 h. For the efficacy study, 10 mice per group received anesthesia with or without an ovariectomy. Mice were then given 5 or 10 mg/kg of carprofen, or saline subcutaneously every 12 h. Orbital tightening, arched posture, wound licking, rearing, grooming, nesting behavior, and activity were assessed before surgery and at 4, 8, 12, 24, and 48 h after surgery. The von Frey responses were assessed before and at 4, 12, 24, and 48 h after surgery. The efficacy study showed that all surgery groups had significantly higher scores for orbital tightening, arched posture, and wound licking than did the anesthesia-only groups at 4, 8, 12, and 24-h time points. At the 8 h time point, the surgery groups treated with carprofen had significantly lower arched posture scores than did the surgery group treated with saline only. No significant differences were found between carprofen treatment groups for rearing, grooming, von Frey, activity, or nesting behavior at any time point. These results indicate that subcutaneous carprofen administered at these doses does not provide sufficient analgesia to alleviate postoperative pain in female CD1 mice.

Abbreviations: A, anesthesia only group; 10C, high-dose carprofen treatment; 5C, low-dose carprofen treatment; NC, no carprofen, saline treatment; NSAIDs, non-steroidal anti-inflammatory drugs; RFID, radio-frequency identification; S, surgical group

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Introduction

Relieving pain and distress in research animals is an ethical obligation. Untreated pain can increase surgical recovery time and affect the immune system and secretion of hormones, enzymes and neurotransmitters.[10] In rodents, some of the behavioral changes seen after surgery include a decrease in grooming and in the frequency of hanging on the cage bars and an increased time to restart burrowing.[12,25,32] When housed as dyads, mice will tend to spend more time together in the same area if one or both are in pain; social hierarchy seems to matter less.[29] Due to these physiologic and behavioral effects, uncontrolled pain could influence data collected from experimental animals.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to provide analgesia for research mice after surgery. These agents are preferred over opioids for behavioral studies, as they are less likely to influence mouse behavior.[7,23] One such NSAID is carprofen, which is a selective cyclooxygenase-2 (COX-2) inhibitor. COX-2 is one of 2 enzymes that mediates the formation of prostaglandins from arachidonic acid.[2,3,26,31] Prostaglandins, particularly PGE2, have proinflammatory effects and increase pain.[31] Inhibition of COX-1 and COX-2 reduces the formation of prostaglandins and consequently reduces inflammation and pain. Inhibition of COX-1 has been associated with undesirable side effects, particularly with regard to gastrointestinal and renal function.[31] Selective inhibition of COX-2 is preferred because it has fewer side effects and is more specific for decreasing proinflammatory prostaglandins.[4,26,31]

The purported therapeutic plasma concentration of carprofen is 20 to 24 µg/mL in dogs and horses,[22] and the recommended dose of carprofen for mice is 5 mg/kg.[6] Pharmacokinetic data suggest the 5 mg/kg subcutaneous dose has an effective duration of up to 12 h;[15] however, efficacy studies in female C57BL/6J suggest that this dose produces little or no amelioration of postoperative pain.[13] In rodent species other than mice, its efficacy is minimal beyond 6 h.[15,27] Increasing the dose may improve the analgesic effect by increasing the plasma levels and/or the duration in which plasma levels exceed therapeutic levels.[5,6,13,23]

The objectives of this study were to evaluate the pharmacokinetics of subcutaneous high-dose carprofen and to determine if using a higher dose would improve analgesic efficacy after surgery. The hypotheses were that a higher dose of carprofen would result increase plasma carprofen concentration and analgesic efficacy as compared with the current standard dose. Although plasma levels reached the purported therapeutic level, analgesic efficacy was not detected at either dose.

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Materials and Methods

Mice. Female, Crl:CD-1(ICR) mice, 6- to 8-wk-old, with a mean weight of 27.4 g, were purchased from Charles River Laboratories (Wilmington, MA). Based on vendor report, mice were free of Sendai virus, mouse hepatitis virus, minute mouse virus, mouse parvovirus, mouse norovirus, Theiler murine encephalitis virus, rotavirus, *Mycoplasma pulmonis*, pinworms, and ectoparasites. The mice were fed Teklad Irradiated Diet 2918 (Inotiv, West Lafayette, IN) and filter-sterilized water ad libitum. The room was on a 12:12-h light:dark cycle (lights on 0600 to 1800 h) at a temperature between 21 °C and 24 °C. For both the pharmacokinetic and efficacy studies, mice were housed 3 per cage in individually ventilated cages (No 1 cages, 19.56 × 30.91 × 13.34 cm, Thoren Caging Systems, Hazleton, PA). For the efficacy study, mice were separated into pairs and housed on a dedicated, static rack in a separate room, and randomly assigned a treatment group. All mice were housed on hardwood chip bedding (Sani-Chips, Teklad, Inotiv, West Lafayette, IN). All animal use was approved by the Institutional Animal Care and Use Committee and conducted at an AAALAC International accredited facility.

Pharmacokinetic Study. Sixty mice were used to assess the pharmacokinetics of both 10 mg/kg (n = 30) and 20 mg/kg (n = 30) of subcutaneous carprofen (3 mice per dose per time point). Mice were randomized for dosing and then placed together in a cage corresponding to their time point. Baseline plasma was obtained from 3 mice that had not been treated with carprofen. The treated mice received a single subcutaneous injection of carprofen in the interscapular region. The stock carprofen (Zoetis, Kalamazoo, MI) was diluted to 2.5 mg/mL using sterile saline (Hospira, Lake Forest, IL). Three mice were euthanized by carbon dioxide at a 50% displacement rate, and blood was collected via cardiocentesis at each time point (0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h after injection). Samples were transferred to heparinized microcentrifuge tubes and then centrifuged at 3,000 ×g for 10 min. Plasma was stored at –80 °C until analyzed.

Determination of carprofen plasma concentrations. Liquid chromatography and tandem mass spectrometry (LC-MS were used to analyze the concentration of carprofen in plasma. Plasma calibrators were prepared by diluting carprofen solutions (Sigma Aldrich, St. Louis, MO) with drug-free mouse plasma to obtain concentrations ranging from 0.1 to 400 mg/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (mouse plasma with carprofen added at 3 concentrations within the standard curve) were included as an additional check of accuracy.

Prior to analysis, proteins were precipitated by diluting 50 µL of plasma with 200 µL of acetonitrile (ACN):1M ascorbic (9:1, v:v) that contained 0.1 ng/µL of an internal standard (D5-furosemide; Toronto Research Chemicals, Toronto, ON). The samples were then vortexed for 2 min to mix, refrigerated for 20 min, vortexed for an additional 1.5 min, centrifuged in a Sorvall ST 40R centrifuge (Thermo Scientific, San Jose, CA) at 4300 rpm/3830 g for 10 min at 4 °C. Aliquots of 30 µL were injected into the LC-MS system.

Quantitative analysis of plasma was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific) with an 1100 series liquid chromatography system (Agilent Technologies, Palo Alto, CA). The system was operated using negative electrospray ionization. The spray voltage was set at 3500 V, sheath gas and auxiliary gas pressure were 45 and 25 torrs, respectively, and the vaporizer temperature was 350 °C. Product masses and collision energies were optimized by infusing the standards into the mass spectrometer. Chromatography was performed using an ACE 3 C18 10 µm × 2.1 mm × 30 mm column (Mac-Mod Analytical, Chadds Ford, PA) and a linear gradient of ACN in water, both with 0.2% formic acid, at a flow rate of 0.4 mL/min. The initial ACN concentration was held at 5% for 0.33 min, ramped to 99% over 4.0 min and held at that concentration for 0.33 min, before re-equilibrating for 3.41 min at initial conditions.

Detection and quantification were conducted using selective reaction monitoring of the initial precursor ion for carprofen [mass to charge ratio (m/z) 271.9] and the internal standard [(m/z) 333.9]. The response ratio for the product ions for carprofen (m/z 225.9) and the internal standard (m/z 206.0, 290.1) were plotted, and peaks at the proper retention time integrated, using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantify analytes in all samples by linear regression analysis. A weighting factor of 1/concentration was used for all calibration curves.

Pharmacokinetic analysis. Noncompartmental analysis for sparse data was performed on plasma carprofen concentrations using commercially available software (Phoenix Winnonlin v8.2, Certara, Princeton, NJ). Plasma drug concentrations from each time point were analyzed simultaneously, enabling estimation of the standard errors for C_{max} and AUC_{last}. The standard error of the mean AUC_{last} and C_{max} values were calculated using a previously described modification.[9,24] Analysis was performed separately for the 2 carprofen doses.

The standards for carprofen were linear with concentration and had correlation coefficients of 0.99 or better. Accuracy was reported as percent nominal concentration, and precision was reported as percent relative standard deviation. For carprofen, accuracy was 89% for 0.25 mg/mL, 85% for 5 mg/mL and 113% for 100 mg/mL. Validation recommendations for chromatographic assays, according to the FDA's Guidance for Industry for Bioanalytical Method Validation, state that accuracy and precision of the quality control samples should be ± 15% of the nominal concentration; 113% falls within that range. Precision was 2% for 0.25 mg/mL, 4% for 5 mg/mL and 4% for 100 mg/mL. The technique was optimized to provide a quantification limit of 0.1 mg/mL and a detection limit of approximately 0.025 mg/mL for carprofen.

Efficacy study. Sixty mice were used to study the analgesic effects of carprofen in mice that had received a laparotomy. In this study, mice were dose once every 12 h with either 5 or 10 mg/kg or with saline. Mice were randomly assigned to 1 of 6 groups (10 per group): (1) surgery and 10 mg/kg (S10C), (2) surgery and 5 mg/kg (S5C), (3) surgery and saline (SNC), (4) anesthesia only and 10 mg/kg (A10C), (5) anesthesia only and 5 mg/kg (A5C), or (6) anesthesia only with saline (ANC). Surgery was performed between 0800 to 1000 h by 2 individuals (BM, HW) who received training prior to the study. Mice that did not have surgery were maintained under anesthesia for an amount of time equivalent to that of a mouse undergoing surgery (about 15 to 20 min). Carprofen was diluted to 2.5 mg/mL in sterile saline; carprofen was administered immediately after induction of anesthesia and then every 12 h for 36 h. Saline injections were the same volume as the high-dose carprofen volume based on mouse weight. Surgeries were performed in 5 cohorts of 12 mice each (2 mice per group per cohort, for a total of 10 mice per group), with each cohort undergoing surgery 72 or 96 h after the prior cohort.

Laparotomy model. Isoflurane (Fluriso, VetOne, MWI Veterinary Supply, Boise, ID) was used to induce and maintain anesthesia during the procedure. All mice had their abdomens

shaved to create similar von Frey test conditions. The surgical site was aseptically prepared using alternating chlorohexidine surgical scrub and 70% isopropyl alcohol. A midline incision of approximately 2 cm was made through the abdominal skin. This was followed by a 1.5 cm incision in the muscular abdominal wall. A bilateral ovariectomy was performed by cauterizing the ovarian pedicle and distal uterine horn. The abdominal wall was closed using 5-0 Biosyn suture (Covidien, Mansfield, MA), and 9-mm wound clips (MikRon Precision, Gardena, CA) were used for skin closure. All mice were monitored during recovery from anesthesia and were returned to their monitoring cages once they could ambulate normally. The home cage was placed on the Mouse Matrix plate for activity monitoring and behavioral assessments.

Activity Monitoring. Five days before surgery, mice were anesthetized briefly with isoflurane to insert the RFID microchip (Unified Information Devices, Lake Villa, IL). The chip was placed subcutaneously in the lateral flank and was oriented perpendicular to the RFID reading plate, which laid parallel to the floor. Activity was monitored by RFID microchips and plates located under home cages (Unified Information Devices' Mouse Matrix, Lake Villa, IL). Each Mouse Matrix plate had 8 zones that check for the presence of an RFID chip. The settings were adjusted such that the plate would scan a maximum of 960 times a minute. Due to the cage being smaller than the plate size, only zones 2 through 7 were used, excluding zones 1 and 8 which are the two zones in the front-most portion of the plate which were not in contact with the cage.

The Mouse Matrix software calculates an activity index based on the movements of an individual mouse between zones. The activity index is the distance the mouse traveled during the observation period, calculated under the assumption that the mouse started in the center of the first zone and then traveled to the center of the next zone. Mice were housed in pairs and tested together in the home cage. If both mice were in the same zone, only the mouse closest to the antenna located in the center of the zone would be counted. Activity data were collected from 24 h before surgery to 48 h after surgery. The data were exported to a spread sheet in one-hour blocks, with total activity measured for each mouse over that hour. Data were assessed by averaging over 3 h periods (hours 3 to 5, 7 to 9, 11 to 13, 23 to 25 and 46 to 48 after surgery or anesthesia and are presented as hours 4, 8, 12, 24, and 48 after surgery or anesthesia.

Mice received nesting material (Bed-r'Nests, The Andersons, Maumee, OH) at the time of microchip placement, and mice had built nests prior to the baseline assessment. At each time point (4, 8, 12, 24 and 48 h after surgery or anesthesia), the zone containing the nest was documented by photograph. The Mouse Matrix system was then used to quantify nesting behavior. The system reported the hourly number of entries into each zone. The known zone location of the nest allowed a general determination of when the mouse was in the nest (that is, when the mouse was scanned in the nest zone). This value was used to quantify nesting behavior. The percentage of scans in the nest as compared with outside of the nest was calculated for each time point.

Behavior assessment. Two observers who were blind to the treatment groups (one male, one female) assessed pain markers over 5-min observation periods. This assessment was performed 24 h before surgery or anesthesia and at 4, 8, 12, 24, and 48 h thereafter. The target behaviors were orbital tightening, arching, rearing (both forelimbs off the floor), wound licking, and grooming. The frequency of rearing, wound licking, and grooming were counted as the behavior occurred during the observation period. Wound licking was considered to occur in the anesthesia-only groups when the mice licked their ventral abdomen. Orbital tightening was described using mouse grimace ratings of 0 to 2;[20] arched posture was scored as not present (0) or present (1). Both orbital tightening and arched posture were measured every 30 seconds during the 5-min observation period, and a cumulative score was determined. Room lights were turned on for observations made during the dark phase.

von Frey test. The von Frey test was used to assess the response to a mechanical stimulus at both 24 h before and at 4, 12, 24, and 48 h after surgery or anesthesia. The two mice from each treatment cage were placed individually into plastic cages with wire mesh bottoms (IITC Life Science, Woodland Hills, CA); cage space for each mouse was 9.5 × 9.5 cm. A timer was set to allow a 30 min acclimation period for the mice in the presence of the von Frey operator (female), who was blinded to the treatment groups. After the acclimation period, the von Frey test was performed using the IITC size 10 filament with a maximum of 13 grams of force in 3 trials, with roughly 5 min between the trials for each mouse. An electronic Almemo 2450 von Frey device (Ahlborn Mess, Holzkirchen, Germany) was used to read the maximum force applied before the mice pulled away. The filament was applied when the mice were not moving (that is, sitting in one position) and was placed at least a millimeter from the incision site for mice that had surgery or within a millimeter of midline for mice that had anesthesia only. After the data were collected, mice were returned to their home cages and the von Frey cages, mesh and probe were cleaned with soap and water.

Histopathology. Mice were euthanized 48 h after surgery or anesthesia by using carbon dioxide. Tissue samples were collected (lungs, heart, liver, kidney, pyloric duodenal junction, and skin from the injection site) and placed in 10% buffered formalin. Samples were processed for hematoxylin and eosin staining and were evaluated for signs of drug toxicity.

Statistical analysis. Data analysis was performed by using Prism 8.1.2 for Mac (GraphPad Software, San Diego, CA). A mixed-effects model with Tukey multiple comparison tests was used to compare all treatment groups at each time point. Behavioral observation data were expressed as the average score or frequency of each specific behavior. For all tests, values are expressed as mean ± SD. A P value less than 0.10 was considered statistically significant. The 2 observers were assessed for agreement using the Cohen k statistic (idostatistics.com, Giacomo Scarpellini).

Results

Pharmacokinetics. At all time points of the pharmacokinetic study, mice that received the 20 mg/kg dose had a higher plasma concentration of carprofen than did the 10 mg/kg mice (Figure 1, Table 1). The maximum plasma concentration of the 10 and 20 mg/kg groups occurred at 2 h after administration (C_{max} = 103 µg/mL, 185 µg/mL, respectively). The terminal half-life in the plasma was 14.9 h and 10.2 h when dosed at 10 mg/kg and 20 mg/kg respectively. The plasma concentration remained above the purported therapeutic level of 20 to 24 µg/mL[22] 12 and 24 h after administration of the low and high doses, respectively.

Laparotomy and post-procedural behavioral assessments. Five of 60 mice did not survive to the study endpoint. All were mice that had surgery; all were euthanized or found dead between the 24 h and 48 h time points. One SNC mouse was euthanized prior to anesthetic recovery due to surgical complications. One S10C mouse was euthanized before the 48 h time point due to severe neurologic deficits. Two other mice, one SNC and one S10C, were euthanized before the 48-h time point due to lack of responsiveness to manipulation. Necropsy revealed that the

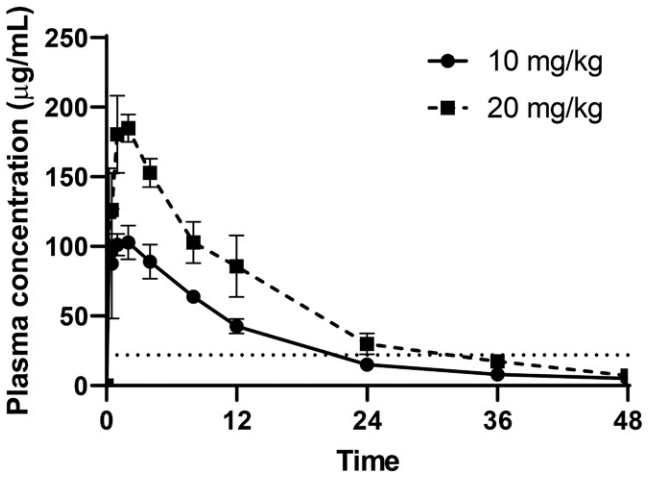

Figure 1. Mean plasma carprofen concentrations (μg/mL) after 10 mg/kg and 20 mg/kg subcutaneous dosing of female CD1 mice. The dotted line shows the purported therapeutic plasma level of carprofen.

Table 1. Noncompartmental analysis of carprofen pharmacokinetic variables after subcutaneous administration at 10 and 20 mg/kg in female CD1 mice

		Doses (mg/kg)	
Parameter	Units	10	20
λz	1/h	0.046	0.068
HL λz	h	14.9	10.2
T_{max}	h	2	2
C_{max}	μg/mL	103	185
C_{max} SE	μg/mL	6.97	5.62
AUC_{last}	h* μg/mL	1458	2656
AUC_{last} SE	h* μg/mL	45	126
AUC 0→∞	h* μg/mL	1565	2761
$AUC_{\%Extrap}$	%	6.85	3.80

λz, elimination rate constant; HL λz, terminal half-life; T_{max}, time of maximum concentration; C_{max}, maximum concentration; C_{max} SE, standard error of C_{max}; AUC_{last}, area under the concentration-time curve from the time of dosing to the last measurable concentration; AUC_{last} SE, standard error of AUC_{last}; AUC 0→∞, area under the concentration time curve from time 0 extrapolated to infinity; $AUC_{\%Extrap}$, percentage of AUC0→∞ due to extrapolation from the last measured time point to infinity.

SNC mouse had necrosis of the small intestine. An S10C mouse that was found dead at 36 h after surgery also had necrosis of the small intestine. Given the location in the small intestine, this was likely associated with surgery, rather than carprofen toxicity.

Behavioral results are summarized in Table 2, and individual values are shown as a scatter plot of Figure 2. All surgery groups scored significantly higher in orbital tightening scores than did the anesthesia-only groups (F (5.3, 45.5) = 7.9; $P < 0.001$). All surgery groups had their maximum orbital tightening scores at 4 h after surgery. None of the surgery groups differed significantly from the others at any time point. Arched posture scores of mice that had surgery were significantly higher than those of the anesthesia-only groups, and all of the surgery groups had peak scores at the 4 h time point (F (4.5, 39) = 9.5; $P < 0.001$). At 8 h after surgery, the S10C ($P = 0.08$) and S5C ($P = 0.07$) arched posture scores were significantly lower than the SNC score and anesthesia only groups. All scores continued to decrease with time. Orbital tightening and arched posture scores did not vary significantly from baseline scores.

Wound licking peaked for all groups at the 4 h time point and was significantly higher than that of the anesthesia groups (F (4.6, 39.7) = 3.0; $P = 0.02$). Instances of wound licking decreased over time, with all groups having fewer than 3 counts per 5 min by the 24 h time point. The wound licking frequency among the surgery groups were not significantly different at any time points. Mice that had surgery also had fewer instances of grooming compared with anesthesia-only mice at 4 and 8 h after the procedure (F (5.8, 498) = 1.6; $P = 0.2$) . The grooming frequency was the lowest for all groups at the 8 h timepoint. Rearing was the least variable parameter among the groups (F (1.4, 12.4) = 1.6; $P = 0.2$), with the frequencies of SNC and S10C groups being similar to those of the anesthesia-only groups for the duration of the experiment. The range of rearing frequency of the S5C mice at 24 h before and 48 h after surgery were wider than those of the other groups due to stereotypical back-flipping that was displayed by 2 mice in the S5C group. If these individuals are excluded from analysis, the values for the S5C group was similar that of the other 5 groups at all time points.

No significant differences were detected for von Frey testing between any of the groups at any of the time points (F (5.1, 40.7) = 1.5; $P = 0.2$). Overall, the von Frey values had a negative trend over time, so the amount of force the mice tolerated was higher before surgery and less after surgery. At 4 h after surgery, all 3 surgery groups had a lower force threshold than the anesthesia mice.

Activity Monitoring. The Mouse Matrix was used to perform 2 assessments: activity, measured by distance traveled by each mouse and time spent in the nesting area (Table 3). The table shows the data in 3-h interval averages for both parameters. Movements of individual mice were recorded and summed over each hour. No significant differences were detected in total activity level of any of the treatment groups at any of the time points (F (240, 1974) = 1.1; $P = 0.3$). Furthermore, no significant differences were seen among groups in the amount of time spent in the nesting area (F (25, 316) = $P = 0.8$).

Histopathology. No gross abnormalities were noted at the surgical or injection sites in mice euthanized at 48 h after the procedures. Histologic evidence of carprofen toxicity was not seen, and only incidental lesions were identified. One A10C mouse had a large area of focal hepatic necrosis. Three mice (of groups S10C, S5C, and A5C) had focal submucosal lymphoid aggregation at the pyloric-duodenal junction, and one S10C mouse had mild suppurative gastritis. One S10C mice had focal renal tubular regeneration. No histologic lesions were identified in the 2 mice that died with a grossly necrotic bowel.

Discussion

Mice given either 10 or 20 mg/kg of carprofen showed similar pharmacokinetic profiles. The mice given 20 mg/kg had higher plasma levels at all time points measured. The peak plasma concentration occurred approximately 2 h after subcutaneous injection regardless of dose. This supports the hypothesis that a higher dose would increase plasma drug concentration, and would presumably be expected to provide greater analgesic efficacy. In a previous study, 20 mg/kg of carprofen had minimal toxic effects in mice.[14] In our efficacy study, the 10 mg/kg dose was administered every 12 h to avoid toxicity while maintaining purported therapeutic levels.

Mice that had a midline laparotomy for ovariectomy and had received either dose of carprofen (5 or 10 mg/kg q12 h) had similar behavioral results, indicating no difference between saline

Table 2. Post-procedural summary statistics of behavior scores and frequencies (mean ± SD) in female CD1 mice

	Time (h)	S10C[f]	S5C	SNC[g]	A10C	A5C	ANC
No. per group		10	10	9	10	10	10
Orbital tightening score	−24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	4	17.7 ± 2.9	14.9 ± 3.1	18.2 ± 2.6	3.5 ± 4.0[a,b,c]	3.8 ± 4.3[a,b,c]	3.1 ± 3.9[a,b,c]
	8	11.1 ± 4.9	13.3 ± 4.7	15.3 ± 5.4	2.0 ± 2.9[a,b,c]	1.3 ± 2.8[a,b,c]	1.4 ± 2.2[a,b,c]
	12	10.2 ± 7.4	10.0 ± 4.7	11.9 ± 7.2	0.3 ± 0.8[a,b,c]	0.2 ± 0.3[a,b,c]	0.6 ± 1.3[a,b,c]
	24	7.4 ± 7.5	6.9 ± 7.7	6.2 ± 6.5	0 ± 0	0.1 ± 0.3[a]	0.1 ± 0.2
	48	3.6 ± 4.9	2.7 ± 4.7	2.4 ± 2.7	0 ± 0	0 ± 0	0.1 ± 0.2
Arching score	−24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	4	7.6 ± 3.5	7.6 ± 2.8	9.4 ± 1.1	1.0 ± 1.6[a,b,c]	0.4 ± 0.7[a,b,c]	1.4 ± 2.1[a,b,c]
	8	4.3 ± 3.2	6.6 ± 2.8	8.9 ± 1.9[a,b]	0.8 ± 1.1[a,b,c]	0.1 ± 0.3[a,b,c]	0.3 ± 0.8[a,b,c]
	12	3.9 ± 4.5	5.8 ± 3.6	7.9 ± 2.4	0 ± 0	0 ± 0	0.6 ± 1.3[b,c]
	24	4.0 ± 5.0	3.0 ± 4.1	1.8 ± 3.5	0.1 ± 0.2	0 ± 0	0.1 ± 0.3
	48	2.1 ± 3.8	1.5 ± 3.4	0.8 ± 1.5	0.1 ± 0.2	0 ± 0	0 ± 0
Rearing frequency (events/5 min)	−24	0.6 ± 0.7	10.3 ± 29.8	0.4 ± 1.1	1.9 ± 5.5	0.3 ± 0.6	1.0 ± 1.7
	4	0.4 ± 0.6	0.5 ± 1.0	0.1 ± 0.2	0.4 ± 0.5[e]	2.0 ± 1.4[c]	0.2 ± 0.3[e]
	8	0.2 ± 0.6	0.4 ± 0.8	0.5 ± 1.0	0.1 ± 0.3	1.0 ± 2.1	1.0 ± 2.3
	12	2.1 ± 3.0	2.0 ± 2.1	0.9 ± 2.2	2.1 ± 3.5	2.2 ± 2.7	1.5 ± 2.7
	24	0.8 ± 2.4	7.4 ± 23.1	1.3 ± 1.9	4.2 ± 3.6	3.5 ± 3.1	1.8 ± 2.5
	48	1.5 ± 2.1	35.8 ± 75.4	3.6 ± 5.6	2.5 ± 4.1	2.5 ± 3.8	0.2 ± 0.4
Wound licking frequency (events/5 min)	−24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	4	5.4 ± 4.7	5.2 ± 2.0	4.7 ± 4.9	0.6 ± 0.8[a,b,c]	0.8 ± 0.9[a,b,c]	0.4 ± 0.4[a,b,c]
	8	3.7 ± 5.2	1.8 ± 3.3	4.7 ± 3.5	0.2 ± 0.5[c]	0.4 ± 0.7[c]	0.3 ± 0.7[c]
	12	3.4 ± 3.4	2.5 ± 1.7	2.4 ± 3.0	0.4 ± 0.9[a,b]	0.8 ± 0.9	0.2 ± 0.3[b]
	24	0.5 ± 0.7	1.1 ± 1.8	2.1 ± 2.2	0.6 ± 0.9	0.3 ± 0.5	0.1 ± 0.2
	48	1.4 ± 2.2	0.8 ± 1.9	1.1 ± 1.7	0.2 ± 0.5	0.1 ± 0.2	0.3 ± 0.5
Grooming frequency (events/5 min)	−24	0.8 ± 1.5	3.3 ± 3.5	1.1 ± 1.8	2.5 ± 3.7	1.5 ± 3.2	2.8 ± 2.9
	4	1.7 ± 2.0	2.2 ± 2.2	1.7 ± 2.4	2.9 ± 2.9	5.6 ± 4.2	2.8 ± 3.5
	8	1.5 ± 1.8	1.0 ± 2.1	1.3 ± 2.1	1.4 ± 3.2	1.3 ± 2.0	1.8 ± 1.9
	12	2.1 ± 2.1	3.7 ± 2.9	1. 9 ± 3.1	2.7 ± 2.9	2.7 ± 2.9	1.3 ± 1.8
	24	1.0 ± 1.7	2.4 ± 2.6	2.1 ± 2.6	4.2 ± 2.7[a]	1.0 ± 0.9[d]	1.3 ± 1.9
	48	1.7 ± 1.6	1.2 ± 2.0	1.8 ± 1.4	2.2 ± 1.8	1.0 ± 0.9	2.2 ± 1.7
Von Frey response (g)	−24	9.4 ± 3.7	7.2 ± 6.8	9.8 ± 4.8	9.0 ± 3.6	10.9 ± 6.9	9.5 ± 6.4
	4	3.5 ± 2.6	3.8 ± 2.9	3.7 ± 3.8	6.7 ± 7.0	7.5 ± 5.4	7.6 ± 6.7
	12	4.3 ± 3.5	3.0 ± 2.1	2.6 ± 2.0	4.4 ± 4.6	3.4 ± 3.7	5.0 ± 5.1
	24	2.6 ± 2.4	2.9 ± 4.4	2.6 ± 1.3	2.9 ± 2.5	2.7 ± 2.9	3.3 ± 3.4
	48	2.8 ± 1.9	3.1 ± 3.6	2.8 ± 3.2	3.6 ± 4.6	1.2 ± 0.6	1.8 ± 1.8

Mice were treated with a high dose carprofen (10C, 10 mg/kg bid), low dose carprofen (5C, 5 mg/kg bid), or saline (NC). Groups that had surgery are designated as 'S'; those that had anesthesia only are designated as 'A'.
[a]Value significantly ($P < 0.10$) different from the S10C group.
[b]Value significantly ($P < 0.10$) different from the S5C group.
[c]Value significantly ($P < 0.10$) different from the SNC group.
[d]Value significantly ($P < 0.10$) different from the A10C group.
[e]Value significantly ($P < 0.10$) different from the A5C group.
[f]One mouse was euthanized at 48 h, one was euthanized at 48 h, and one was found dead at 36 h after surgery.
[g]One mouse was euthanized prior to recovery from anesthesia; one was euthanized at 48 h after surgery.

and carprofen treatment of mice with surgery except in arched posture at 8 h after surgery. Similarly, no differences in activity or nesting behavior were detected between any groups at any time point. Mice with adequate analgesia would be expected to have less pain and more movement as compared with mice that had inadequate analgesia. Given that only a single behavioral value differentiated the groups, with no differences in activity, our results indicate that neither dose of carprofen provided sufficient analgesia for surgical pain.

Some published data suggest that carprofen may not provide adequate analgesia after surgery in mice. In one study, C57BL/6J mice underwent a sham embryo transfer and the effects of a single subcutaneous dose of carprofen on nest complexity (a proxy pain behavior) were assessed 9 h after surgery.[13]

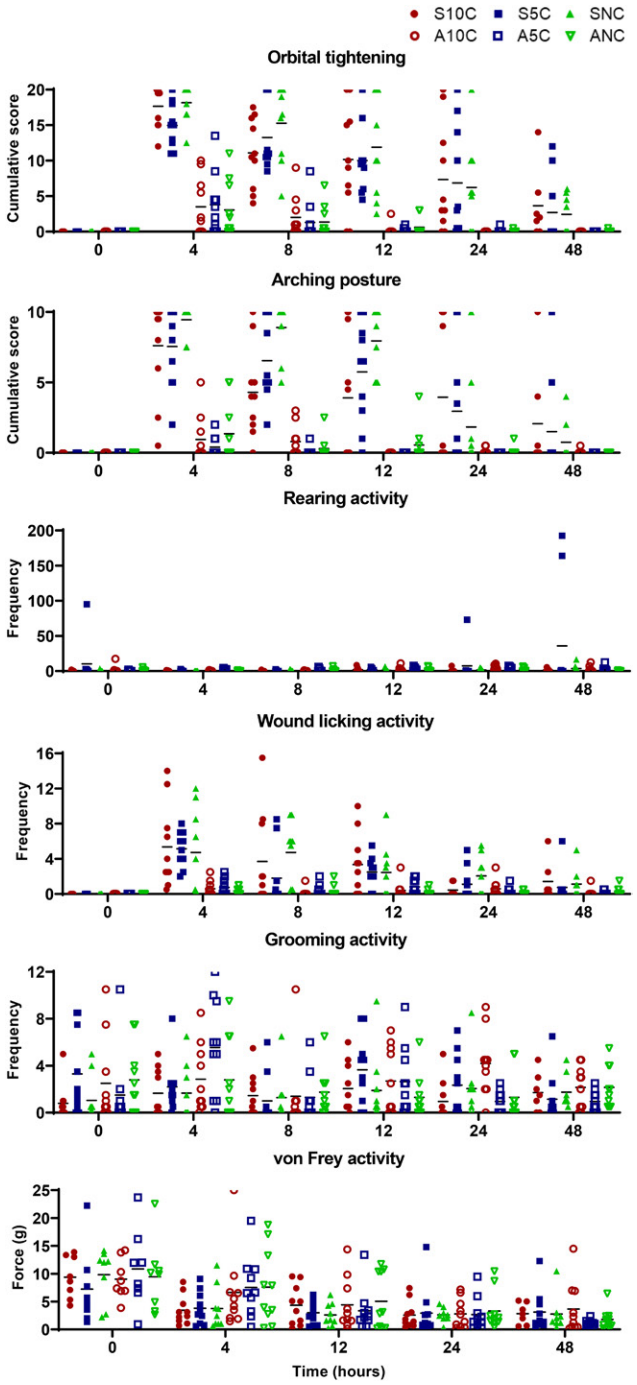

Figure 2. Individual behavioral observations pre- and postoperatively in mice treated with carprofen following surgical laparotomy or anesthesia only. Group means are identified for each as a dash (-). Summary statistics are provided in Table 2.

Mice that received 5 mg/kg of carprofen or no treatment after surgery did not make nests; this failure was interpreted to indicate postoperative pain. In contrast, mice that received 50 mg/kg of carprofen after surgery made nests that were comparable to those of mice that received anesthesia but not surgery. However, in that study, fewer than 10% of the mice treated with either 5 or 50 mg/kg of carprofen made discernible nests after surgery, as compared with 20% to 60% of the anesthesia-only mice and 75% prior to surgery. If nesting behavior is a valid proxy for postoperative pain, then these doses of carprofen appear to be inadequate.

The mouse grimace scale has also been used to evaluate the efficacy of subcutaneous carprofen after ventral ovariectomy in CD-1 mice.[23] Mice received various doses of carprofen (5, 10, 15, 20, and 25 mg/kg) after surgery and before recovery from anesthesia. The mouse grimace scale was used as an indicator of pain. A significant difference in the scores was not detected until the doses had reached 20 or 25 mg/kg, with a calculated half-maximal analgesic dose suggestion of 29 mg/kg.

The use of continuous oral delivery of carprofen at 10 mg/kg in water was assessed in a CD-1 mouse after laparotomy.[25] Mice were given carprofen-treated water from 24 h before until 48 h after surgery. The nest consolidation scores of mice that received the carprofen after surgery were lower than their baseline scores and similar to the scores of the saline-treated mice. The mechanical threshold forces and grooming scores of the carprofen-treated mice were similar to those of the anesthesia-only mice; grooming scores were also similar to those of the saline-treated mice. Like our study, these findings also indicated the inadequacy of carprofen for treating postoperative pain in mice.

To understand the analgesic effectiveness of high-dose carprofen (10 mg/kg bid) on post-surgical pain, its behavioral effects must be assessed in conjunction with plasma concentrations. The mouse grimace scale is a common criteria used to assess mouse pain.[20,23] It was simplified to focus only on orbital tightening because that sign is readily visualized and personnel can easily be trained in its use, as described previously.[16-18] The grimace score is a commonly used marker of mouse pain, with higher scores indicating more pain.[16,17,28] Although orbital tightening is easy to evaluate, its drawbacks are that mice are prey species and frequently mask their pain, and sleep or posture can obstruct a view of their eyes, leading to difficulty in determining an accurate orbital tightening score. In this study, the orbital tightening scores of the mice that received carprofen following surgery were high compared to the anesthesia only groups, and similar to the mice that received saline following surgery. Multiple other behavioral assessments used to evaluate analgesic efficacy in mice, including arched posture, grooming, and wound licking, which were used in this study.[1,15-17,21,30] Greater pain is expected to cause less rearing, less grooming, and longer durations of wound licking. These signs could also be difficult to evaluate if mice were in a curled position in the nest. Although we found no differences in the grooming activity between any of the groups, the mice that received surgery may have a falsely elevated incidence of grooming as they groomed the residual disinfectant from the surgical skin preparation, as the surgical skin preparation was not performed on anesthesia-only mice. Nonetheless, the carprofen treated mice did not have more grooming activity as compared with saline-treated mice after surgery.

Overall activity was assessed using Mouse Matrix system, which provides objective indirect measures of pain, with higher activity after surgery correlating with better analgesia.[8,17,19] The Mouse Matrix tracks individual mouse activity using an RFID chip and plate reader that assess distance traveled, active time and stationary time. In our study, mice were pair-housed. The Mouse Matrix system can only scan a single RFID in each zone during each recording. The system records only one mouse if 2 mice are in the same zone, so some data, especially the nesting evaluation, may be skewed. We occasionally noted that when a surgery and anesthesia-only mouse were housed together, they would build a barrier between themselves (4 of 19), which would further confound the nesting percentages. The pair housing of mice may have confounded our interpretation of efficacy because social housing has been shown to improve analgesic

Table 3. Post-procedural summary statistics of activity and nesting (mean ± SD) of female CD1 mice

	Time (h)	S10C	S5C	SNC	A10C	A5C	ANC
Activity Index	−24	716 ± 342	1,438 ± 1,426	462 ± 172[a]	478 ± 259	559 ± 337[a]	814 ± 417
	4	540 ± 558	865 ± 1,013	327 ± 480	718 ± 701	368 ± 341	1,049 ± 1,415
	8	483 ± 713	1,054 ± 1,376	815 ± 572	997 ± 1215	336 ± 633	622 ± 778
	12	810 ± 992	388 ± 320	386 ± 369	624 ± 481	290 ± 171	608 ± 468
	24	301 ± 289	835 ± 951	334 ± 271	385 ± 298	351 ± 226	577 ± 699
	48	580 ± 550	1,018 ± 812	368 ± 329	524 ± 425	618 ± 1,015	695 ± 1,011
Time spent in nest (%)	−24	68 ± 38	62 ± 36	60 ± 37	80 ± 27	62 ± 34	67 ± 40
	4	30 ± 32	45 ± 45	28 ± 28	46 ± 44	41 ± 13	57 ± 43
	8	57 ± 49	58 ± 42	33 ± 40	50 ± 42	32 ± 38	47 ± 45
	12	48 ± 45	65 ± 39	44 ± 42	50 ± 39	65 ± 36	63 ± 40
	24	77 ± 29	75 ± 20	67 ± 39.1	54 ± 34	54 ± 33	56 ± 31
	48	65 ± 33	55 ± 29	75 ± 39	61 ± 36	58 ± 35	64 ± 38

Mice were treated with a high dose carprofen (HC, 20 mg/kg), low dose carprofen (LC, 10 mg/kg), or saline (NC). Mice that received surgery are denotes by 'S'; mice that received anesthesia only are denoted by 'A'.
[a]Value significantly ($P < 0.10$) different from the S10C group.

efficacy.[11,29] Housing mice individually after surgery may have generated different outcomes and made differences between treatment groups more apparent.

The time points assessed in this study for both activity and behavior occurred primarily during the light phase of the circadian cycle; mice in a research setting are most likely to be evaluated during this period, the exception of the 12 h time point. Mice are typically more active during the dark phase of the circadian cycle, and an evaluation of their activity and behavior during the dark phase may alter the interpretation of the data. However, the anesthesia-only mice did not have significantly more activity than did the surgical mice, suggesting that our observations at the 12 h time point were not affected by the dark phase of the light cycle.

The ANC group was used as a control to assess behavioral effects of carprofen by comparison to A10C and A5C mice. We found no effect of carprofen treatment compared with saline across all time points and parameters examined. Thus, in our study, carprofen did not appear to affect mouse behavior, as has been reported by others.[13]

After surgery, the mice were also observed by veterinary staff who were aware of the treatment groups; rescue analgesia was planned if the saline-treated surgical group displayed behaviors indicative of postoperative pain. Based on the assessments by both the blind observers and the veterinary staff, the mice treated with saline after surgery did not display behaviors indicative of pain that would warrant rescue analgesia.

Despite our numerous behavioral assessments, mechanical stimuli testing, and activity monitoring, we found no analgesic effect after surgery in mice that received either the 5 or 10 mg/kg of carprofen every 12 h. While the current and previous studies suggest carprofen may not be an adequate analgesic in mice, this conclusion may be limited to the strain, sex and procedure evaluated in this study. If the purported carprofen therapeutic level of 20 to 24 µg/mL is applied to female CD1 mice, our pharmacokinetic data indicate the persistence of appropriate plasma concentrations for 12 h. However, efficacy did not correlate with the pharmacokinetics, suggesting that the true minimum therapeutic plasma concentration of carprofen is likely above 20 to 24 µg/mL. Overall, this study did not find postoperative analgesic activity in mice treated with carprofen administered subcutaneously at 5 and 10 mg/kg q 12 h. Thus, we recommend using alternative pain management strategies in postsurgical mice, such as a more effective NSAID, opioids or multimodal therapy.

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