Journal of the American Association for Laboratory Animal Science
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Vol 64, No 2
March 2025
Pages 204–211

Comparative Medicine Section

Efficacy and Effects of High-Dose Carprofen after Plantar Incision in C57BL/6J Mice

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Carprofen is a nonsteroidal antiinflammatory drug (NSAID) commonly used to control postoperative pain at 5 to 10 mg/kg once or twice a day in mice. Recent literature has suggested that higher doses of carprofen may be necessary to prevent postoperative pain in mice. We studied the efficacy and clinical safety of 3 doses of carprofen at 5 (Carp5), 25 (Carp25), and 50 (Carp50) mg/kg once a day through a plantar incision pain model, plasma carprofen levels, histopathologic evaluation, serum chemistry, CBC, and fecal occult blood testing. For the plantar incisional pain procedure, male and female C57BL/6J mice (n = 38) were randomly assigned to 1 of 4 groups; that is, saline, Carp5, Carp25, and Carp50, and all received subcutaneous injections of the treatment drug once a day for 3 d. Mechanical and thermal hypersensitivity assessments were performed 24 h before surgery and at 2, 6, 24, 48, and 72 h afterward. Thermal analgesia of saline and Carp5 were indistinguishable from each other at all timepoints, while Carp25 and Carp50 provided thermal analgesia that were indistinguishable from each other but differed from saline and Carp5. Mechanical analgesia showed similar but more graded distinctions. One Carp50 mouse displayed acute morbidity on day 4 and was euthanized. In a separate group of mice, plasma carprofen concentration for each dose was assessed following drug administration at 2, 6, 12, and 24 h (n = 3 per timepoint). Histopathological abnormalities were seen in 1 of 8 of Carp50 mice (neutrophilic gastritis) and in 1 of 8 of Carp25 mice (ulcerative gastritis). Mice in all carprofen treatment groups had elevated AST on chemistry and positive fecal occult blood. Our results indicate that Carp25 and Carp50 more effectively provided postoperative mechanical and thermal analgesia than did Carp5 and saline using an incisional pain model in C57BL/6 mice, although Carp50 carries more risk of gastrointestinal side effects.

Abbreviations and Acronyms: Carp5, carprofen 5 mg/kg once a day for 3 d; Carp25, carprofen 25 mg/kg once a day for 3 d; Carp50, carprofen 50 mg/kg once a day for 3 d; COX, cyclooxygenase; FOBT, fecal occult blood test; NSAID, nonsteroidal anti-inflammatory drug.

DOI: 10.30802/AALAS-JAALAS-24-108

Introduction

Effective clinical pain management is paramount to laboratory animal research not only from ethical and legal perspectives, but also for scientific reproducibility. Untreated nociception has the potential to significantly affect scientific study results.[18] Providing analgesia can be challenging due to interspecies differences and limited published evidence-based data on efficacious analgesia regimens for laboratory species. In mice, the response to analgesia is variable not only by procedure, but also by sex, strain, drug formulation, environment, and dose.[35,41,46]

NSAIDs, either as a sole agent or combined with buprenorphine, are frequently used to control postprocedural pain in mice. NSAIDs prevent pain by inhibiting cyclooxygenase (COX) production of prostaglandin. The ideal target is isoform COX-2, to disrupt its downstream inflammatory mediators that sensitize nociceptive nerves, causing pain.[51] However, the other isoform, COX-1, has homeostatic functions maintaining gastric mucosa and normal clotting. When COX-1 function is disrupted, common toxic side effects of NSAIDs are observed (melena, bleeding, gastritis). Carprofen provides greater inhibition of COX-2 than COX-1, minimizing the toxic effects of COX-1 inhibition.[11,23]

There is limited literature fully evaluating dosing regimens and efficacy of NSAIDs in rodents, including carprofen for mice.[40,42,45] Studies that have evaluated mouse NSAID dosing suggest that effective doses and dosing frequency for controlling postprocedural pain are much higher than what is frequently recommended in the research community (carprofen 5 to 10 mg/kg every 12 to 24 h).[13,14,16,21,24–26,39] For example, in a sham embryo transfer surgery in mice of different strains (C57BL/6J, DBA/2J, and B6D2-Tg(Pr-mSMalphaActin)V5rCLR-25), only the high dose of carprofen (50 mg/kg) improved nest complexity scoring, a proxy for pain assessment.[17] Another study in CD-1 mice indicated that carprofen can reduce pain following laparotomy only at higher doses of carprofen, that is, 20 and 25 mg/kg, as measured by mouse grimace score.[24] Toxicity is of particular concern when higher dosages of carprofen are used. A study in female CD-1 mice showed that 20 mg/kg carprofen once a day produces minimal renal or gastrointestinal toxic effects, though this study did not evaluate behavioral effects of analgesia.[25] The therapeutic plasma concentration of carprofen for mice is also unknown, but in other species the range is

Submitted: 04 Oct 2024. Revision requested: 29 Oct 2024. Accepted: 06 Jan 2025.
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This article contains supplemental materials online.

reported as 20 to 24 µg/mL.[21,22] A pharmacokinetic study of carprofen in female CD-1 mice found that by 4 h after administration of 5 mg/kg, the carprofen plasma concentration was below 40 µg/mL, and by 12 h it was well below 20 µg/mL.[21] Thus, these studies suggest that the therapeutic window for carprofen in mice may be very narrow, such that higher doses may improve analgesia but at the risk of increased toxicity and that doses at or below 20 mg/kg carprofen have not produced reliable analgesia.[16,20,24,25]

The aim of this study was to evaluate the efficacy of high-dose carprofen dosed at 5- or 10-fold higher than 5 mg/kg for providing thermal and mechanical analgesia (that is, decreased hypersensitivity) after plantar incision in C57BL/6J mice. We also evaluated the toxicity of these 2 dosing regimens by histopathologic analysis, CBC and serum chemistry, fecal occult blood test (FOBT), and measured plasma concentrations of these carprofen doses over a 24-h period. We hypothesized that higher doses of carprofen would improve analgesia, but that the highest dose of carprofen (50 mg/kg) would result in gastrointestinal toxicity.

Materials and Methods

Mice. Equal numbers of 2-month-old male and female C57BL/6J mice ($n = 98$, weight, 23.526 ± 3.957 g; The Jackson Laboratory, Bar Harbor, ME) were used ($n = 38$ for hypersensitivity testing; $n = 36$ to measure carprofen plasma concentration; $n = 24$ for clinical pathology). Mice were same-sex group housed (4 per cage) in static caging (Innovive, San Diego, CA) on prefilled ALPHA-dri bedding (Shepherd Specialty Papers, Watertown, TN) and kept in their original social group throughout the study. Throughout all experiments, sentinel animals at Stanford were free of minute virus of mice, mouse hepatitis virus, mouse rotavirus, Theiler murine encephalomyelitis virus, Sendai virus, murine adenovirus 1 and 2, ectromelia virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, respiratory enteric virus 3 (reovirus 3), *Mycoplasma pulmonis*, endoparasites, and ectoparasites. Mice were fed a commercial diet (Teklad Global 18% protein rodent diet 2018, Harlan Laboratories, Madison, WI), provided with acidified bottled water (Innovive, San Diego, CA) and Enviro-dri (Lab Supply, Fort Worth, TX) for bedding and enrichment. Rooms were maintained on a 12-h dark/12-h light cycle at 68 to 70 °F (20 to 21 °C) and 40% to 64% relative humidity. Experiments were approved by Stanford University's Administrative Panel for Laboratory Animal Care in an AAALAC-accredited facility.

Analgesic testing: study design. Mice ($n = 38$) were acclimated to the facility for a minimum of 5 d prior to baseline testing. For each assessment session, mice were tested in their housing room and acclimated to the testing enclosure for 15 min. All mice underwent baseline testing at 48 and 24 h prior to surgery as described below. Data collected at 24 h prior to surgery (day –1) was used as the baseline for subsequent comparisons. On the morning of surgery (day 0), each mouse in a same-sex cage was randomly assigned to 1 of 4 treatment groups by E.D.A. or M.K.H.: saline (0.9% NaCl, Hospira, Lake Forest, IL; $n = 9$; 1 mL/kg SC), Carp5 (5 mg/kg SC; $n = 10$), Carp25 (25 mg/kg SC; $n = 10$), Carp50 (50 mg/kg SC; $n = 9$). Estrous was not staged in female mice; however, randomization should ensure that estrous stages are represented.

Surgery. Mice were induced via 1% to 4% isoflurane with 100% O_2 in an induction chamber, and anesthesia was maintained using 0.8% to 2.5% isoflurane delivered via a nose cone. Surgery was performed by one investigator (R.M.C.). Sterile ophthalmic ointment was administered prior to surgery, and animals were placed on a circulating warm water blanket. Cefazolin (30 mg/kg SC; GlaxoSmithKline, Research Triangle Park, NC) was administered 5 min prior to skin incision once subcutaneously between the shoulders, and the animals were then placed in ventral recumbency. The plantar surface of the left (ipsilateral) hindpaw was aseptically prepped. The plantar incisional surgery was adapted from a previously described incisional pain model for mice.[10,37] Three millimeters from the tibiotarsal joint, an approximately 5-mm longitudinal incision through skin and fascia was made on the midline on the plantar surface of the foot, extending toward the digits. The underlying muscle bundle was elevated using curved forceps, and a stab incision was made into the muscle with the point of a #15 blade without disrupting muscle attachments or underlying structures. Fine-tipped forceps were then inserted into the incision and used to distract the muscle horizontally for 10 s. Saline was applied to the tissues and blotted with a sterile cotton-tip applicator. The skin was closed with a single horizontal mattress using 4-0 silk suture. Mice were left to recover in a clean cage placed over a warm-water blanket and monitored continuously during recovery. They were returned to the home cage once fully ambulatory and rehoused with their original social group.

All carprofen treatment groups were made by diluting carprofen (Rimadyl, Zoetis, Kalamazoo, MI) with 0.9% NaCl (Hospira, Lake Forest, IL) in an opaque 5-mL glass vial to 3 different concentrations: 0.5, 2.5, and 5 mg/mL. Dilutions were prepared each study week for surgery or pharmacokinetics evaluation for every group of animals tested. All drugs were administered subcutaneously with a 22-gauge needle over the left or right shoulder 1 to 2 min prior to incision. Drug treatments were administered every 24 h after surgery for 3 d (E.D.A. or M.K.H.). Mechanical and thermal analgesic testing was performed at 2, 6, 24, 48, and 72 h after surgery by the same experimenter (R.M.C.), who was blind to the treatment of the mice. All daily analgesic testing was performed between 0700 and 1000 except for the 2- and 6- h timepoints, which occurred between 1000 and 1300 and between 1400 and 1700 h, respectively, on day 0.

Mechanical analgesic testing. Responses to mechanical stimuli were evaluated using the von Frey monofilament nociceptive assay. Mice were placed in a clear acrylic enclosure (10.1 × 10.1 × 12.5 cm) on an elevated mesh stand (electronic von Frey mesh stand, IITC Life Science, Woodland Hills, CA) with 0.64-cm 'waffle' holes. Mice were acclimated to the testing enclosure for a minimum of 15 min before applying von Frey monofilaments with calibrated bending forces (0.4 g, Aesthesio, DanMic Global, San Jose, CA) for 10 trials per mouse. Each mechanical stimulus was applied for 1 s on locations adjacent to the surgical incision on the left hindpaw. Subsequent monofilament applications on a given mouse were performed at minimum 10 s later. Any clear nociceptive reflexive behavior (for example, withdrawal, shaking, or licking of the stimulated paw) was considered a positive response; absence of such behaviors was considered a negative response. Trials in which the behavioral response could not be clearly interpreted were omitted and repeated at least 1 min later. Trials were not performed or counted while mice were rearing, mesh chewing, grooming, or sleeping. Mechanical hypersensitivity or algesia was defined as a significant increase in the frequency of positive responses as compared with baseline (the values obtained at 24 h before the incision) and mechanical analgesia as nonsignificant compared with baseline values. Mechanical analgesic testing preceded thermal analgesic testing each day.

Thermal analgesic testing. Mice spent 10 min in the home cage within the testing room before undergoing thermal analgesic testing. Responses to thermal stimuli were evaluated with the hotplate assay. An aluminum hotplate (IITC Life Science, Woodland Hills, CA) with a transparent acrylic enclosure (275 × 263 × 15 mm) was set and calibrated at a constant 54 to 55 °C at least 30 min prior to testing. A mirror was placed behind the transparent enclosure, and a video of each session was recorded to be evaluated by one observer blinded to the treatment group. An unrestrained mouse was placed on the metal surface of the hotplate with a timer and video recording to detect thermal latency (for nociceptive reflexive behaviors). Nociceptive reflexive behaviors were defined as licking a hindpaw, jumping, stamping, hopping, or spinning. When any nociceptive reflexive behavior was observed, the timer was stopped and the mouse was removed from the hotplate. The testing cutoff time was 20 s to prevent tissue injury; no mice reached this threshold. Thermal latency was defined as the time taken to observe nociceptive reflexive behaviors. Thermal hypersensitivity or algesia was defined as a significant decrease in thermal latency.

Clinical observations. All study mice were monitored for abnormal behavior or clinical signs (decreased activity, rearing, and grooming, hunched posture, and limping) in cage daily for 3 min prior to any manipulation and during the acclimation period prior to analgesic testing (at least 15 min). Behaviors and clinical signs were also actively recorded during analgesic assessments. Mice were weighed preoperatively the day prior to surgery to ensure optimal accuracy for drug dosing, and daily after completion of analgesic testing. Observations were made by a single experimenter who was blind to the experimental group (R.M.C.).

CBC and serum chemistry. At the time of necropsy for mice used in analgesic testing, terminal blood collection was performed by cardiocentesis for CBC and serum chemistry. Due to limited sample volume of the initial submissions, an additional 24 mice were included across all treatment groups (saline, Carp5, Carp25, Carp50).

Plasma carprofen concentration analysis. Mice were given a single dose of Carp5, Carp25, or Carp50 and euthanized by isoflurane followed by cardiocentesis (as described) by R.M.C. for plasma collection at 2, 6, 12, and 24 h after administration ($n = 3$ per treatment drug at each timepoint and $n = 2$ for saline control; $n = 38$ mice total). Whole blood was placed into lithium-heparin microtainers (BD Microtainer tubes with lithium heparin, above) and centrifuged at 2500 rpm for 20 min. Plasma was separated, placed in cryogenic tubes, labeled, and stored at −80 °C until analyzed. Plasma carprofen concentrations were measured by the Pharmaceutical Sciences Research Institute at the McWhorter School of Pharmacy (Samford University, Birmingham, AL) via liquid chromatography–tandem mass spectrometry. Individual samples had a minimum volume of 50 µL and were shipped overnight on dry ice. Carprofen standard spiking solutions were prepared in 50:50 deionized water/acetonitrile to yield plasma concentrations ranging from 0.2 to 200 ng/mL. The carprofen plasma samples and standards (100 µL) were fortified with internal standard (50 ng/mL terfenadine). Acetonitrile (1 mL) was added to precipitate the plasma proteins, and the mixture was vortexed and centrifuged. The organic layer was transferred to a clean test tube and evaporated to dryness under nitrogen in a 50 °C water bath. The samples were reconstituted in dilution solvent and analyzed by liquid chromatography–tandem mass spectrometry. Matrix-matched standards and quality control samples were prepared by using blank control plasma.

Toxicity assessment: study design. Fecal occult blood testing and clinical, gross, and histopathologic evaluations were performed on mice.

FOBT. This test is designed to detect blood in feces that may not be macroscopically appreciable. Mice were selected by E.D.A. (not blinded) from hypersensitivity testing studies ($n = 9$ total) and put in acrylic boxes over paper towels to collect feces at behavioral study timepoints until defecation (less than 20 min) for Carp5, Carp25, and Carp50 at 0, 2, 6, 12, 24, 48, and 72 h. In addition, mice from the carprofen plasma analysis ($n = 27$ total) had feces collected by R.M.C. from clean cages at 0 h, then from colon at euthanasia timepoints 6, 12, and 24 h. This was accomplished by avoiding vessels or organs at abdominal opening, blotting any blood, and removing colon from the abdomen before incision to remove fecal pellets with clean forceps or expelled feces at euthanasia. Fecal pellets were collected into 1.5-mL Eppendorf tubes and immediately analyzed or frozen at 4 °C until analysis. Before use, pellets were rehydrated with 0.05 to 0.1 mL of sterile water (Hospira, Lake Forest, IL) and/or thawed. Hemoccult (Beckman Coulter, Brea, CA) testing kits were used following the manufacturer's label instructions.

Gross pathology and histology. Upon completion of the 72-h analgesic assessments, mice were euthanized either at 4 d (acute group) or 10 d (chronic group) postoperatively by carbon dioxide asphyxiation, followed by exsanguination by cardiac puncture, and they underwent postmortem pathologic examination for gross pathology and histopathologic analysis by a blinded board-certified veterinary pathologist (K.M.C.). Tissues from acute ($n = 16$ mice, $n = 4$ per group) and chronic ($n = 16$ mice, $n = 4$ per group) mice across all 4 treatment groups, including liver, spleen, kidney, heart, lung, stomach, duodenum, ileum, cecum, and colon, were immersion fixed in 10% neutral buffered formalin for 72 h. In addition, any grossly visible lesions in any organ were captured on histology. Formalin-fixed tissues were processed routinely, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (Histo-Tec, Hayward, CA).

Statistical analysis. A power analysis for group size determination was performed for analgesic testing mice. Analgesic data were analyzed using a restricted maximum likelihood repeated-measures mixed model in JMP 16 Pro for Windows, and data and analyses are supplied as human-readable equivalent SAS code in SI. A subject was nested within a treatment, and treatment, timepoint, and their interaction were included as experimental factors. Baseline performance was included as a covariate. A subject was treated as a random effect, making timepoint a within-subject repeated factor, treatment a between-subject factor, and their interaction a within-subject factor.

Because each subject acts as its own control, unspecified differences (for example, sex or weight) are inherently controlled for in this analysis. Appropriate error terms were applied (×1). Significant effects were further examined with post hoc Tukey tests. See Supplementary Materials for further discussion and detailed model descriptions.

Results

There were no significant differences in any of the evaluated parameters between sexes, except for body weight, and this linear model analysis controls for any potential differences; each animal is its own control.

Mechanical analgesia (von Frey). The interaction of timepoint-by-treatment was nonsignificant ($F_{12,136} = 1.319$; $P = 0.2148$). However, treatment ($F_{3,33} = 8.469$; $P = 0.0003$) and timepoint ($F_{4,136} = 11.83$; $P < 0.0001$) were independently and additively significant (that is, both drug and time contributed to analgesic response). Tukey tests revealed that the treatments differed progressively and that timepoints 2, 6, and 24 did not differ, but did differ

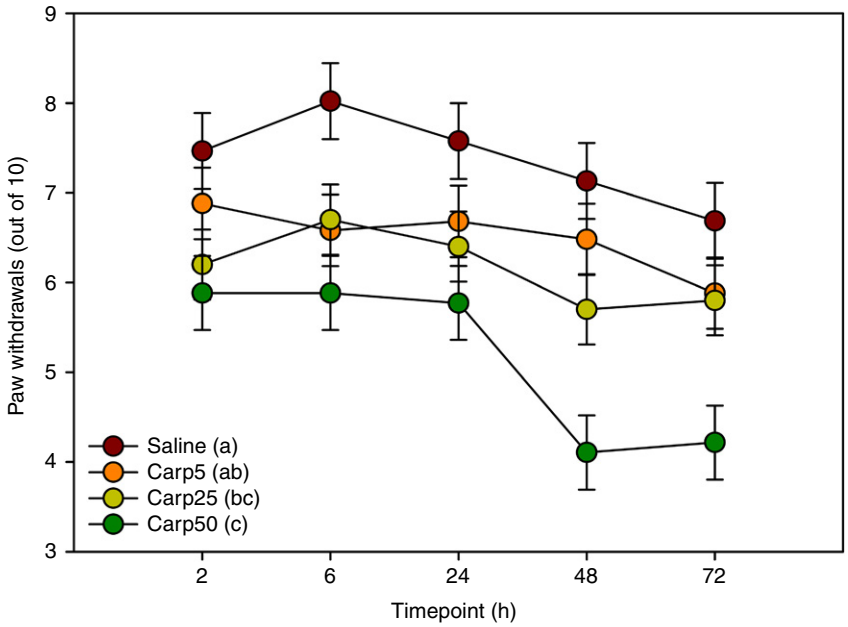

Figure 1. Mechanical analgesia. Measured as the number of paw withdrawals (mean ± SE) of ipsilateral (left) hindpaw. Each animal is its own control. Surgery was performed at 0 h. Groups with the same letter are not significantly different, and different letters between groups indicate statistically significant differences ($P < 0.05$). Saline, $n = 9$; Carp5, $n = 10$; Carp25, $n = 10$; Carp50, $n = 9$.

from timepoints 48 and 72. As shown in Figure 1, Carp50 provided significantly increased mechanical analgesia (decreased number of paw withdrawals) compared with saline and Carp5 throughout the study. At all timepoints, Carp25 provided significantly increased mechanical analgesia compared with saline but not compared with Carp5. Carp5 mechanical analgesia was indistinguishable from saline except at timepoints 6 and 24.

Thermal analgesia (hotplate). The interaction of timepoint-by-treatment was nonsignificant ($F_{12,136} = 0.5514$; $P = 0.8771$). However, treatment ($F_{3,33} = 18.412$; $P < 0.0001$) and timepoint ($F_{4,136} = 5.961$; $P = 0.0002$) were independently and additively significant (that is, both drug and time contributed to analgesic response). Tukey tests revealed that at all timepoints, the saline and Carp5 treatments did not differ, but differed from the Carp25 and Carp50 treatments (see Figure 2) and that timepoints differed in a progressive manner. Both Carp25 and Carp50 provided significantly increased thermal analgesia (increased paw withdrawal latency) compared with both saline and Carp5 at all timepoints. Only at 6 h did Carp50 show significantly increased thermal analgesia compared with Carp25.

Weight and clinical signs. Baseline and interstudy body weights were not significantly different between treatment groups. There were no significant differences in weights across days or between group except by sex; males were heavier than females throughout the study ($P < 0.05$). All mice in this study were frequently evaluated for abnormal behavior, skin

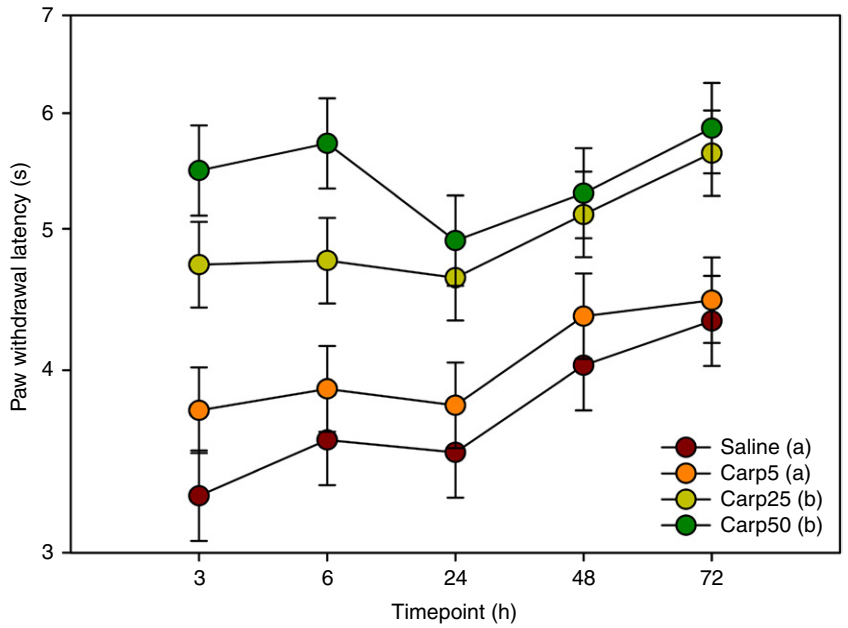

Figure 2. Thermal analgesia. Thermal latency was measured in seconds (mean ± SE) of ipsilateral (left) hindpaw to thermal stimulus. Each animal is its own control. Surgery was performed at 0 h. Groups with the same letter are not significantly different, and different letters between groups indicate statistically significant differences ($P < 0.05$). Saline, $n = 9$; Carp5, $n = 10$; Carp25, $n = 10$; Carp50, $n = 9$.

lesions, weight loss, or other clinical signs. No surgical site infections were observed at any timepoint. Across groups of analgesic tested mice, only lameness and licking of the incised hindpaw were observed postoperatively as expected in this model (100% of animals on day 1; days 2 to 3 intermittent and difficult to grade during testing). Animals receiving Carp25 or Carp50 were noted to be active and rear frequently during analgesic testing or in cage, with mild or no lameness after day 1. One animal euthanized due to acute illness on day 4 is described below.

CBC and serum chemistry. CBC and serum chemistry results on day 4 and day 10 are summarized in Table 1. AST was elevated on day 4 in mice receiving carprofen (Carp5, Carp25, and Carp50) compared with saline and not elevated at day 10, a week after cessation of administration. No other significant differences or trends of other analytes were found.

Plasma carprofen concentrations. Carprofen was not detected in the 2 negative control mice given saline (data not shown). The plasma concentration following a single dose of Carp5, Carp25, and Carp50 was determined at 2, 6, 12, and 24 h. The highest peak plasma concentration occurred at 2 h after administration for all groups. The peak plasma concentrations were 41.8 ± 2.65, 130.97 ± 10.02, and 191.57 ± 10.58 µg/mL for Carp5-, Carp25-, and Carp50-treated mice, respectively. Carp5-treated mice had a plasma concentration of 18.43 ± 0.59 µg/mL at 12 h and then 7.59 ± 1.37 µg/mL at 24 h, well below the purported therapeutic concentration. Both the mean carprofen plasma concentrations for the Carp25 and Carp50 groups remained above the purported therapeutic plasma concentration and Carp5 concentrations through 24 h (31.9 ± 1.7 and 57.13 ± 13.13 µg/mL, respectively). The serum concentration at 2 h of Carp5 was statistically equivalent to that of both Carp25 and Carp50 at 24 h (see Figure 3).

FOBT. No saline mice had positive fecal occult blood at any timepoint and no mice in any group or study (analgesic or pharmacokinetic) had positive fecal occult blood prior to carprofen administration. No mice from any group had positive FOBT ($n = 9$, 3 per group) at 2 h. A positive FOBT was noted in Carp5 (1/6 mice), Carp25 (1/6 mice), and Carp50 (2/6 mice) at 6 h. At 12 h, positives were Carp5 (3/6), Carp25 (3/6), and Carp50 (6/6). At 24 h, positive FOBTs were Carp5 (4/6 mice), Carp25 (6/6 mice), and Carp50 (6/6 mice). Once animals tested positive, they remained positive at 48 and 72 h across all groups ($n = 3$ per group). There was no correlation between sex of mouse and positive fecal occult blood.

Gross pathology and histopathology. Histopathology lesions were restricted to the gastrointestinal tract. Mild gastric ulceration was seen in 1 out of 4 mice (Carp25; 4 d after operation) and moderate neutrophilic gastritis was seen in 1 out of 4 mice (Carp50; 4 d after operation). A single mouse receiving Carp50 was acutely clinically ill and euthanized at day 4 after analgesic study due to dehydration, a 0.5-cm skin ulceration on the flank, tachypnea, and hunched posture. Histologically, this mouse (1/9 Carp50 analgesic mice) exhibited ulcerative enterotyphlocolitis with secondary septic peritonitis. No other treatment-related histologic findings were noted within any of the examined organs.

Discussion

Recent work evaluating carprofen dosing regimens in mice indicates that the commonly used dose of 5 to 10 mg/kg may

Table 1. Serum chemistry and CBC following carprofen dosing.

	Day 4				Day 10			
Treatment group:	Saline	Carp5	Carp25	Carp50	Saline	Carp5	Carp25	Carp50
Glucose (mg/dL)	255.1(50.2)	272.1(35.7)	237(41.4)	246.3(58)	245(45.3)	228.9(43.5)	224.1(63.7)	234.4(26.8)
Chol (mg/dL)	91.6(19.6)	104.2(24.4)	75.2(39.3)	106.8(21.9)	91.2(17.5)	79.7(33.1)	91.4(18.2)	105.8(17.8)
BUN (mg/dL)	23.9(4.4)	28.4(6.7)	24(6)	22.7(1.8)	22.4(2.4)	23.6(2.9)	22.7(2.1)	24.3(2.2)
Creatinine (mg/dL)	0.2(0.1)	0.2(0.05)	0.3(0.1)	0.3(0.1)	0.2(0.1)	0.2(0.03)	0.3(0.1)	0.3(0.1)
Phos (mg/dL)	10.3(2.7)	9.4(3)	11.6(2.3)	10.3(3.6)	9.5(2.7)	8.1(2)	8.2(2.2)	9.4(2.7)
Calcium (mg/dL)	10(0.8)	9.2(0.3)	9.8(1.1)	9.9(1.4)	9.9(0.8)	9.5(0.3)	9.9(0.9)	9.9(0.8)
TP (g/dL)	5(0.3)	5.3(0.6)	5.4(0.5)	5.2(0.7)	4.9(0.4)	4.8(0.3)	5(0.5)	5.8(1.2)
Albumin (g/dL)	3(0.2)	3.2(0.4)	3.3(0.2)	2.9(0.6)	2.9(0.3)	2.9(0.2)	3(0.3)	3.4(0.8)
Globulin (g/dL)	2(0.3)	2.2(0.3)	2.1(0.3)	2.3(0.3)	1.9(0.2)	2(0.2)	2(0.2)	2.2(0.3)
T.bili (mg/dL)	0.3(0.2)	0.2(0.1)	0.3(0.1)	0.3(0.2)	0.3(0.1)	0.2(0.04)	0.2(0.1)	0.3(0.1)
ALP (IU/L)	113(48)	117(22.6)	111.2(25.6.)	89.7(21.6)	101.6(22.9)	102.2(28.6)	107.4(32.5)	92.5(22.5)
ALT (IU/L)	37.3(18.5)	69.1(37.8)	86.6(51.5)	79.7(42.1)	46.2(12.4)	39.2(20.6)	57.9(63.2)	39.3(20.8)
AST (IU/L)	87.8(26.8)	200.1(110.8)+	230.4(166.8)+	240.7(194.3)+	110.7(36.4)	110.9(41.1)	139.3(116.7)	120.7(160)
GGT (IU/L)	4.2(1.1)	6(5.4)	9.5(3.9)	11.7(8.6)	4(1.1)	4.1(2.5)	4.8(2.2)	4.8(1.8)
Na (mEq/L)	150.8(6)	146.8(4.3)	151.6(6.6)	151.8(4.6)	148.7(3.4)	147.3(0.8)	151.9(6.3)	147.7(3.4)
K (mEq/L)	7.6(3.2)	7.4(2.2)	8.9(2.2)	8(2.3)	7.7(3)	4.9(1.5)	6.7(2.7)	7.2(2.2)
Cl (mEq/L)	109.3(0.8)	109.2(1.3)	109.5(3.5)	109.6(3.5)	109.3(0.8)	109.3(0.8)	109.1(1.6)	107.3(1)
Anion gap (mEq/L)	28.8(6.5)	28.8(4.7)	35.3(11.5)	29.6(6.4)	26(3.2)	24.5(1)	29.3(11.3)	27.2(4.2)
HCO_3 (mEq/L)	18.8(3.2)	15.9(5.3)	15.8(10.4)	20.5(4.7)	19.5(2.6)	18.4(1.4)	20.1(3.6)	20.3(3.9)
WBC (K/µL)	5.8(1.3)	7(2.2)	6.1(0.8)	6.8(2.1)	4.3(2)	4.8(1.4)	7.2(1.5)	6.7(1.8)
RBC (M/µL)	10.7(1.6)	10.3(1.4)	8.9(1.2)	9.4(1.7)	9.7(2.7)	10(0.9)	9.9(1.1)	9.8(1)
Retic (% cells)	3.9(0.8)	4.1 (0.5)	5.4(2.7)	7.7(5.2)	3.8(0.8)	3.8(0.6)	5.6(3.1)	4.3(0.8)
Lympho (% cells)	87.8(5.5)	85.8(5.5)	76.6(11.1)	78(10.8)	86.2(4.4)	86(3.1)	85.6(3.8)	81.8(6.4)
Neut (% cells)	8.4(5)	7.8(4.9)	15.6(10.4)	13.7(10.9)	10.2(3.9)	9.8(3.6)	9.6(4.7)	12.8(4.7)

Values are presented as mean (SD). +, $P < 0.05$ compared with saline. Chol, cholesterol; Phos, phosphorus; TP, total protein; T.bili, total bilirubin; Retic, reticulocytes; Lympho, lymphocytes; Neut, neutrophils.

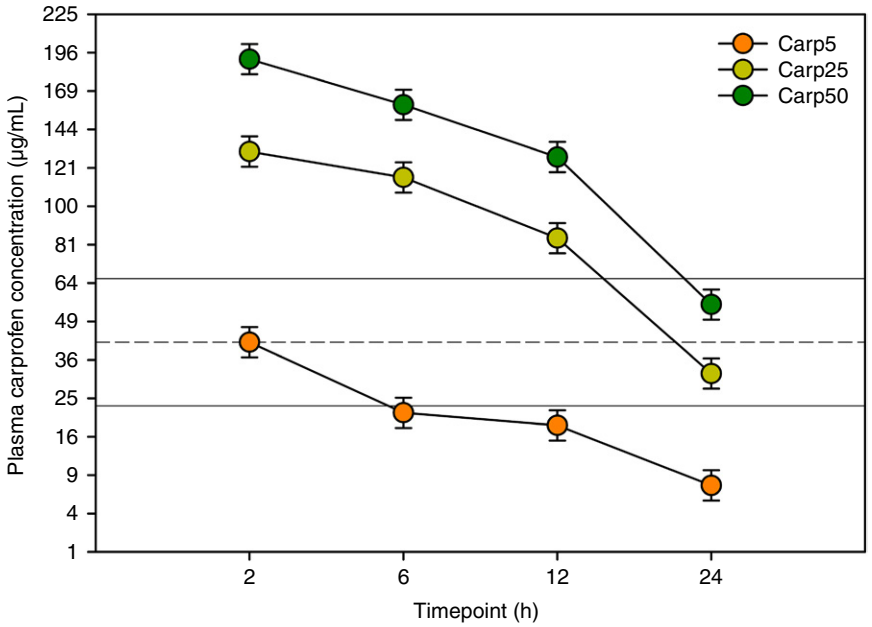

Figure 3. Plasma carprofen concentrations (µg/mL, mean ± SE) in mice (n = 3/group/timepoint) treated with either Carp5, Carp25, or Carp50. Plasma drug concentrations were determined at timepoints 2, 6, 12, and 24 h after dosing. Solid lines indicate confidence intervals, and the dashed line indicates the mean.

not be sufficiently high enough to provide postoperative analgesia.[1,6,7,24,25] Additional evaluation of the currently used dosing regimens of carprofen in mice with specific consideration given to the species,[6,10] strain,[4,27,46] sex,[46] and surgery performed[1,7,25] are needed. This study evaluated whether carprofen dosed 5- or 10-fold higher than 5 mg/kg effectively provides analgesia for an incisional pain model in C57BL/6 mice and whether there are any clinical or pathologic sequelae with these higher doses. Our results indicated that the analgesia provided by Carp5 was primarily indistinguishable from saline and that both Carp25 and Carp50 provided more consistent analgesia for postoperative mechanical and thermal pain.

The paw incisional pain model produces testable mechanical and thermal hypersensitivity for evaluation of analgesic efficacy in mice,[2,4,33,37] where complete analgesia would be indicated by continued baseline responses. Saline-treated C57BL/6 mice in this study exhibited mechanical and thermal hypersensitivity lasting 3 d, consistent with other studies using the incisional pain model in C57BL/6 or NSG mice.[4,33] There are a variety of ways to test mechanical and thermal analgesia. For this study, the von Frey and hotplate tests facilitated efficient timepoint testing given that mice were appropriately active and ambulatory, which can make performing and interpreting specific test modalities for minor pain challenging.[12]

As an NSAID, carprofen provides analgesia through inhibition of cyclooxygenase enzymes that produce prostaglandins, mediating inflammatory pain at the injury.[8] To test the effectiveness of analgesics, there needs to be enough inflammatory pain present to evaluate whether carprofen can ameliorate it.[48,49] Acute postprocedural pain begins with tissue damage (here, incision) eliciting a local inflammatory response that sensitizes peripheral sensory neurons.[31] In cats, an intradermal injection of kaolin was used as a model of inflammatory pain for which carprofen administration prevented mechanical hypersensitivity after intradermal kaolin injection for mimicking inflammatory pain.[50] Our previous work with this model of acute incisional pain shows that control saline mice experience both thermal and mechanical pain.[2–4,33] Studies evaluating buprenorphine formulations for this model in rodents indicated that buprenorphine more consistently provided mechanical analgesia but not thermal analgesia.[3,7,33] In our study, Carp50 reduced mechanical hypersensitivity compared with Carp5 and saline, and both Carp25 and Carp50 reduced thermal hypersensitivity compared with Carp5 and saline. The differences in the thermal and mechanical analgesic response provided by buprenorphine (an opioid) and carprofen further support the need for the provision of multimodal analgesia.

The clinically effective plasma level of carprofen in mice has not been definitively established, so we sought to correlate the analgesic results with carprofen plasma levels. For all 3 carprofen treatment groups, the carprofen plasma level rose rapidly 2 h following administration and declined quickly throughout a 24-h period, consistent with previous studies.[2,15,16,21,25] At 2 h, Carp25 and Carp50 mice had carprofen plasma levels near 131 ± 17.4 and 191 ± 18.3 µg/mL, respectively, and the results follow closely with another study.[15] These results complement research suggesting that the therapeutic threshold may be above 75 to 100 µg/mL for CD1 and NSG mice following a minor pain procedure.[2,25]

In cases of suspected gastrointestinal bleeding where blood cannot be seen grossly, FOBTs may be used antemortem to detect trace amounts of blood in the feces.[34,38,47] Positive FOBT results were present in all 3 carprofen groups in at least one mouse after 6 h, despite limited correlative histopathologic evidence—specifically, mild ulceration in 1 of 8 Carp25 mice and moderate gastritis in 1 of 8 Carp50 mice. These positive test results may be attributed to the transient gastrointestinal irritation or ulceration that occurs with the use of carprofen[22,30] or to false positives. There are interstudy discrepancies between FOBT results in mouse strains (this study in C57BL/6J mice; others in CD1[25] and NSG[2] mice) receiving the same doses of carprofen. Such discrepancies could be due to the FOBT not being optimized for a low enough detectable threshold,[32,36] strain-specific nuances, quick healing of gastrointestinal mucosa, or untimed elimination of positive fecal samples given mouse gut transit time of 2 to 4 hours.[19] In addition, in multiple species, diets containing peroxidases from reducing agents, heme, or myoglobin are known to increase the rate of false-positive FOBT,[9,32,36,47]

and the effect of commercial rodent diets on FOBT is not published. Conversely, acidic or reducing agents in the diet such as vitamin C[32] may increase the rate of false-negative FOBT results; acidified water's effects are unknown. In both cats and humans, sequential samples are advised to increase specificity,[43,47] which lends credibility to findings but no quantitative indication of FOBT severity, only absence or presence. Taken together, these results suggest that mice receiving carprofen with a positive FOBT may have minor transient gastrointestinal irritation or ulceration from a combination of carprofen, surgery, and fasting during behavior testing that was not found in the selected histopathologic sections.

Measurements of incisional thermal and mechanically evoked hypersensitivity can themselves be impacted by treatment-related comorbidities, such as NSAID-induced gastroenteritis. In the present study, one Carp50 C57BL6/J mouse exhibited acute clinical morbidity necessitating euthanasia. The histologically observed ulcerative typhlocolitis in this mouse may have been a direct result of NSAID administration; however, the severity and distribution of lesions was atypical.[2,5,20,28,29,44] Given the severity of the histopathologic findings for the acutely ill Carp50 mouse, we expected other Carp50 mice to show signs of clinical morbidity or to have found other study mice with histopathologic or clinical pathology abnormalities if the findings were indeed related to NSAID toxicity. However, only one additional Carp50 (1/8) mouse exhibited neutrophilic gastritis with no notable gastrointestinal ulceration or concurrent kidney pathology. In contrast, a study using the same model of incisional pain evaluating the efficacy and safety of high doses of carprofen for NSG mice found that only Carp25 attenuated mechanical hypersensitivity and that Carp50 was associated with gastric ulceration (2/4) and kidney lesions (3/4).[2] Thus, the possibility exists that C57BL/6J mice exhibit a unique distribution of pathology following NSAID administration as compared with NSG mice.[4]

Limitations in the present study include those associated with the specific timepoints evaluated and methodologies. First, due to the time required for hypersensitivity testing, limited timepoints were evaluated. Second, only 2 higher doses of carprofen were evaluated, and the therapeutic window may lie within the doses chosen (25 to 50 mg/kg). Third, distinct groups of mice were used for analgesia testing and plasma analysis, and therefore the carprofen plasma level may not be precisely the same in both groups. Fourth, the model chosen was an incisional pain model, which will not be equivalent to the analgesia provided by carprofen for other surgical models. Finally, in any study using histopathology, only specific areas are captured on slides for evaluation.

Our study is consistent with recent studies in mice,[2,21,25] where results indicate that 5 mg/kg carprofen alone does not provide sufficient analgesia for incisional surgical pain. Higher doses of carprofen at 25 or 50 mg/kg daily were more efficacious than saline or 5 mg/kg carprofen. Postmortem histopathology indicated that carprofen at 50 mg/kg carried an increased risk of toxicity, including acute adverse gastrointestinal effects. Ultimately, these results indicate that Carp25 provides improved analgesia for C57BL/6J mice with minor incisional pain as compared with Carp5 mice and without the histopathologic findings seen in Carp50 mice.

Acknowledgments

We thank Elias Godoy, Alyssa Gavidia, John Gregor Duque, Roberta Moorhead (Stanford University) and Dr. Gregory Gorman (plasma analysis, Samford University).

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by the ACLAM Foundation and Stanford University School of Medicine, Department of Comparative Medicine.

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