Veterinary
Anaesthesia and Analgesia
Formerly the Journal of Veterinary Anaesthesia

Veterinary Anaesthesia and Analgesia, 2012, **39,** 206–217 doi:10.1111/j.1467-2995.2011.00684.x

RESEARCH PAPER

Effects of carprofen, meloxicam and deracoxib on platelet function in dogs

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Abstract

Objective To determine effects of anti-inflammatory doses of COX-2 selective NSAIDs carprofen, meloxicam, and deracoxib on platelet function in dogs and urine 11-dehydro-thromboxane B2.

Study design Randomized, blocked, crossover design with a 14-day washout period.

Animals Healthy intact female Walker Hounds aged 1–6 years and weighing 20.5–24.2 kg.

Methods Dogs were given NSAIDs for 7 days at recommended doses: carprofen (2.2 mg kg^{-1}, PO, every 12 hours), carprofen (4.4 mg kg^{-1}, PO, every 24 hours), meloxicam (0.2 mg kg^{-1}, PO, on the 1st day then 0.1 mg kg^{-1}, PO, every 24 hours), and deracoxib (2 mg kg^{-1}, PO, every 24 hours). Collagen/epinephrine and collagen/ADP PFA-100 cartridges were used to evaluate platelet function before and during and every other day after administration of each drug. Urine 11-dehydro-thromboxane B_2 was also measured before and during administration of each drug.

Results All NSAIDs significantly prolonged PFA-100 closure times when measured with collagen/epinephrine cartridges, but not with collagen/ADP cartridges. The average duration from drug cessation until return of closure times (collagen/epinephrine cartridges) to baseline values was 11.6, 10.6, 11 and 10.6 days for carprofen (2.2 mg kg^{-1} every 12 hours), carprofen (4.4 mg kg^{-1} every 24 hours), meloxicam and deracoxib, respectively.

Conclusions and clinical relevance Oral administration of some COX-2 selective NSAIDs causes detectable alterations in platelet function in dogs. As in humans, PFA-100 collagen/ADP cartridges do not reliably detect COX-mediated platelet dysfunction in dogs. Individual assessment of platelet function is advised when administering these drugs prior to surgery, particularly in the presence of other risk factors for bleeding.

Keywords carprofen, deracoxib, meloxicam, platelet function.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to reduce pain and inflammation and minimize post-operative opioid requirements in an attempt to speed recovery (Pibarot et al. 1997; Grisneaux et al. 1999; Lafuente et al. 2005). The anti-inflammatory and analgesic effects of NSAIDs result from the inhibition of cyclooxygenase (COX), an enzyme that converts arachidonic acid to a number of different prostanoids including the

prostaglandins, the prostacyclins, and thromboxane. There are two well described isoforms of this enzyme, COX-1 and COX-2 (Davies et al. 2004). COX-1 is thought to be constitutively expressed and is responsible for prostaglandin regulation in basal physiologic functions, including normal platelet activity, gastric cytoprotection, and renal blood flow. COX-2 is traditionally considered to be inducible and responsible for producing the prostaglandins that are involved in the inflammatory process (Jones & Budsberg 2000; Smith & Langenbach 2001). However, both COX isoenzymes have been found to have constitutive and inducible roles. COX-1 expression has been detected on mouse bone marrow-derived mast cells (Smith et al. 1997), and its induction has been found to play a role in differentiation of a human leukemia cell line (Smith et al. 1993; Kaufmann et al. 1996), Furthermore, COX-2 has been detected in the brain and kidney (Harris et al. 1994; Kaufmann et al. 1996), while its constitutive expression in normal endothelial cells has been controversial (Hla & Neilson 1992; Jones et al. 1993; Topper et al. 1996).

Non-selective NSAIDs such as aspirin (acetylsalicylic acid) bind to and affect the function of both COX-1 and COX-2, and impair platelet function by inhibiting thromboxane A_2 formation by platelets (FitzGerald 1991; Funk et al. 1991; Bergh & Budsberg 2005). Thromboxane A_2 is synthesized and released by activated platelets resulting in vasoconstriction, further platelet activation, and enhanced platelet aggregation. Inhibition of COX-1 mediated thromboxane A_2 synthesis leads to decreased platelet function (Moncada et al. 1976). In the past decade, much research has been devoted to the development of COX-2 selective NSAIDs that maintain analgesic and anti-inflammatory effects while minimizing the unwanted side-effects thought to result primarily from COX-1 inhibition, such as gastrointestinal ulceration, and susceptibility to bleeding (Jones et al. 2002; Streppa et al. 2002; Sessions et al. 2005).

Platelets, both in humans and in dogs, have historically been described as cells that exclusively express COX-1 and not COX-2 (Patrignani et al. 1999; Kay-Mugford et al. 2000a). Based on this assumption, it would be expected that COX-2 selective NSAIDs would have no direct effect on platelet function (Kay-Mugford et al. 2000a,b). Many 'COX-2 selective' NSAIDs, however, are not purely selective for COX-2, and selectivity appears to decrease as drug dose increases (Jones & Budsberg 2000; Streppa et al. 2002). Many NSAIDs, even some that are marketed as COX-2 selective, may therefore have the potential to inhibit COX-1 and thereby decrease platelet function.

Administration of NSAIDs before surgery has the beneficial effects of reducing inflammation at the surgical site, minimizing peripheral and central amplification of nociceptive input, and decreasing pain after surgery (Lemke et al. 2002). Any NSAID-associated decrease in platelet function, however, has the potential to contribute to excessive peri-operative hemorrhage (Kitchen et al. 1982), leading to longer surgical and anesthetic times and an increased risk of intra- and peri-operative complications (Palmer et al. 1994). Human studies (Leese et al. 2003; Blaicher et al. 2004) have shown that many of the COX-2 selective drugs have less effect on platelet function than many non-selective NSAIDs, and therefore are expected to carry less risk for intra and peri-operative bleeding in surgical patients. The effects of these COX-2 selective NSAIDs on canine primary hemostasis, however, have not as yet been comprehensively evaluated using appropriate methodologies.

The goal of our study was to characterize changes in platelet function in dogs associated with the administration of standard anti-inflammatory doses of the veterinary-approved NSAIDs of varying COX-2 selectivity.

Materials and methods

Eight female intact purpose-bred adult Walker Hound dogs were used for the study. The dogs were intact females ranging in age from 1 to 6 years (median, 5.21 years) and weights ranged from 20.5 to 24.2 kg (median, 22.3 kg). Dogs were considered healthy if there were no abnormalities on physical examination and baseline testing including a complete blood count, serum chemistry, urinalysis, buccal mucosal bleeding time, prothrombin time, partial thromboplastin time, von Willebrand factor concentrations, heartworm testing and titers for rickettsial diseases and *Babesia* species. No vaccines or medications were administered to the dogs for at least 2 weeks prior to initiation of the project.

Baseline samples were obtained for platelet function analysis using a point-of-care platelet function analyzer (PFA-100;, Siemens Healthcare Diagnostics, IL, USA) and measurement of urine 11-dehydro-thromboxane B_2. Venous blood was collected from the jugular veins via a 20-gauge vacutainer

needle directly into glass tubes containing either 3.8% sodium citrate or ethylenediaminetetraacetic acid. Venipuncture was performed by alternating between both jugular veins. An automated hematologic analyzer (Abbott Cell-Dyn 3700; Abbott Laboratories, IL, USA) was used to determine an accurate platelet count and packed cell volume on each sample. Urine was collected via cystocentesis using a 22-gauge needle. All urine samples were batched and stored at −80 °C until analysis.

Platelet function analysis

The PFA-100 device was used according to the manufacturer's instructions, and has previously been described and evaluated and validated in dogs (Kundu et al. 1995; Callan & Giger 2001; Mischke & Keidel 2003). The instrument's alignment and calibration was performed according to the manufacturer's instructions. Briefly, vacuum test cartridges were inserted into each position within the carousel, and a cleaning procedure was initiated. Following this cleaning procedure, a trigger solution was utilized to maintain proper alignment of the instrument. Prior to sample analysis, the instrument ensured calibration of the vacuum chuck, carousel drive, trigger system, and incubation system. Sodium citrate anti-coagulated blood at a 9:1 blood-to-citrate ratio was used. Samples were analyzed within 30 minutes of collection, and kept at room temperature until analysis. For each sample, 800 μL of citrated blood was deposited in a test cartridge, which contained a biologically active membrane coated with either collagen and adenosine diphosphate (PFA Collagen/ADP Test Cartridge; Siemens Healthcare Diagnostics, GA, USA) or collagen and epinephrine (PFA Collagen/EPI Test Cartridge, Siemens Healthcare Diagnostics, Duluth, GA, USA). After a 3-minute incubation period at 37 °C, the blood sample was aspirated through an aperture in contact with the coated membrane, and the time until occlusion of the aperture (closure time) was recorded. The upper limit for the assay is 300 seconds.

Urine 11-Dehydro-Thromboxane B_2 Analysis

A commercial competitive enzyme immunoassay kit validated for analysis of canine urine for 11-dehydro-thromboxane B_2 (Luminex 11-dehydro Thromboxane B2 Kit; Cayman Chemical Co, MI, USA) was used to measure thromboxane production. Samples were analyzed according to the manufacturer's instructions. Based on manufacturer's recommendations and previously published studies (Baltzer et al. 2006), samples were diluted to different concentrations that fit within the working range of the instrument. Specific gravities of all samples were within 1.006 to 1.012. To minimize assay variability, all samples were batched and analyzed in duplicate and averaged. Results were reported in pg mL^{-1} of urine (a correction factor was applied to allow for the effects of sample dilution). According to the manufacturer, there was a 100% specificity with 11-dehydro-thromboxane B_2 and 11-dehydro-2,3-dinor thromboxane B_2. There was an inter-assay and intra-assay coefficient of variation of 67% and 10%, respectively. The analytic validation performed by the manufacturer had minimal cross-reactivity with the thromboxane metabolites, 2,3-dinor TxB_2 (3.8%), 2.3-dinor TxB_1 (0.2%), and TxB_2 (0.01%). Other related metabolites had minimal to no cross-reactivity, prostaglandin D_2 (0.1%), leukotriene B_4 (0.03%), and prostaglandin E_2 (<0.01).

Briefly, a 96-well Luminex plate was prepared by adding 100 μL of the diluted sample to the appropriate well, followed by the addition of 11-dehydro-thromboxane B_2 Phycoerythrin Tracer and 11-dehydro-thromboxane B_2 XMAPR Beads. The plate was placed on an orbital shaker and incubated in the dark at room temperature for 4 hours prior to analysis. Urine creatinine was measured using an automated chemistry analyzer (ACE Alera Clinical Chemistry System; Alfa Wasserman, Inc., NJ, USA). Urine 11-dehydro-thromboxane B_2 concentration was normalized to the individual's urine creatinine by determining the 11-dehydro-thromboxane B_2 to creatinine ratio using the diluted urine.

Study design

This study was designed in accordance with the Policy on the Humane Care and Use of Laboratory Animals and was approved by the Mississippi State University Institutional Animal Care and Use Committee (protocol no. 08-057). The eight dogs were randomly assigned at the initiation of the study to one of four groups (two dogs/group) by drawing their information from a hat. The dogs were treated for 7 days with one of the following treatments, with each animal rotating through all drug treatments in the same order of a crossover design: carprofen (Rimadyl; Pfizer Animal Health, NY,

USA) 2.2 mg kg^{-1} orally every 12 hours (Car2), carprofen 4.4 mg kg^{-1} orally every 24 hours (Car4), meloxicam (Metacam suspension (0.5%); Boehringer Ingelheim Vetmedica, MO, USA) 0.2 mg kg^{-1} orally the first day then 0.1 mg kg^{-1} orally every 24 hours (Met), or deracoxib (Deramaxx; Novartis Animal Health, NC, USA) 2 mg kg^{-1} orally every 24 hours (Der). Drug doses were based on manufacturer's recommendations. A 14 day washout period was established between each rotation.

Prior to drug administration, blood was collected and baseline values were established for the PFA-100 collagen/epinephrine and collagen/adenosine diphosphate (ADP) cartridges, platelet count, and packed cell volume. Additional baseline values for the PFA-100 collagen/epinephrine and collagen/ADP cartridges, platelet count, and packed cell volume were obtained prior to administration of each NSAID, at the end of each preceding washout period. During the period of administration of each NSAID, blood was obtained for PFA-100 analysis using collagen/epinephrine cartridges on days 1, 2, 3, 5, and 7 of drug administration. According to previous studies comparing the utility of collagen/ADP and collagen/epinephrine cartridges (Nielsen et al. 2007; Blois et al. 2010; Thomason et al. 2010), collagen/epinephrine is a more sensitive measure of COX mediated platelet effects; therefore, we chose collagen/epinephrine cartridges to evaluate drug effects over time. On day 7 for each NSAID, blood was also collected for packed cell volume, platelet count, and PFA-100 analysis using collagen/ADP cartridges. During the washout periods, PFA-100 analysis using collagen/epinephrine cartridges was performed every other day until measurements within 10% of each individual dog's normal baseline were obtained on two consecutive occasions. Urine was collected for 11-dehydro-thromboxane B_2 analysis at baseline prior to the administration of any drug, and again on day 7 of administration of each NSAID. Samples collected during drug administration were obtained midway through the dosing interval for each NSAID and all drugs were expected to have reached steady state plasma concentrations by day 3. (Lipscomb et al. 2002; Montoya et al. 2004; Deramaxx; Novartis Animal Health US, Inc).

Statistical analysis

Following visual assessment of qqplots of the data (PROC UNIVARIATE, SAS for Windows 9.2; SAS Institute, Inc., Cary, NC, USA), platelet count, packed cell volume, and urine 11-dehydro-thromboxane B_2 results were found to be approximately normally distributed while data from both PFA-100 cartridges (ADP and EPI) were judged to not be normally distributed. The effects of carprofen (both dosing regimens), deracoxib, and meloxicam on platelet count, packed cell volume, and urine 11-dehydro-thromboxane B_2 results were individually assessed by analysis of variance using the GLIMMIX procedure in SAS for Windows version 9.2. Drug, rotation, sample time, and the drug × sample time interaction were included as fixed effects for the models for platelet count and packed cell volume. In addition, fixed effects variables were included to account for the drug used to treat a dog in the prior rotation, thereby accounting for any effects due to the sequence of treatments. The repeated measures of the dogs were accounted for in the mixed models, assuming an autoregressive covariance structure. Due to the different sampling protocol for the 11-dehydro-thromboxane B_2 outcome, a similar model was used but without sample time or the drug × sample time interaction. To accommodate the lack of normality of the PFA-100 results, nonparametric methods of analysis that accounted for the repeated measures were utilized (Brunner & Puri 2001; Shah & Madden 2004). For each outcome, the data were ranked and then analysis of variance type statistics were obtained through the MIXED procedure in SAS for Windows 9.2 by using the ANOVAF option and the MIVQUE0 estimation method for the covariance parameters and a REPEATED statement specifying dog identity within rotation as the subject and an autoregressive covariance structure. The fixed effects included in the models were the same as for platelet count and packed cell volume. Differences in least square means with the SIMULATE adjustment of *p*-values were used for multiple comparisons where appropriate. Kruskal–Wallis Tests using the NPAR1WAY procedure in SAS were used to determine if there were significant differences in the PFA-100 cartridge results among the groups of dogs at the initial time point. A simple analysis of variance model using the GLIMMIX procedure in SAS was used to determine if there were significant differences in 11-dehydro-thromboxane B_2 results among the groups of dogs at the initial time point. A *p*-value of <0.05 was considered to be significant for all analyses.

Results

All dogs completed the study, with one dog receiving oral sucralfate (Sucralfate; Teva Pharmaceuticals, PA, USA) 1 g orally every 8 hours for 5 days due to an episode of mild hematochezia observed during the washout period after receiving meloxicam. No other adverse effects were observed.

The dogs were weighed at the beginning of the study and these weights were used to calculate all subsequent dosing. The mean (±SD) administered doses of drugs were Car2, 2.18 ± 0.12 mg kg^{-1} orally every 12 hours, Car4 4.36 ± 0.25 mg kg^{-1} orally every 12 hours, Met 0.19 ± 0.013 mg kg^{-1} orally for the first dose then 0.097 ± 0.006mg kg^{-1} orally every 24 hours, and Der 2.18 ± 0.12 mg kg^{-1} orally every 24 hours.

Platelet function analysis (PFA-100)

Pretreatment (baseline) closure times using collagen/epinephrine cartridges ranged from 92 to 212 seconds and were not significantly different ($p = 0.5724$) among the four starting groups of dogs. Collagen/epinephrine cartridge closure times were significantly longer than baseline values ($p \leq 0.0098$) at all time points during treatment with carprofen (both dosing regimens), meloxicam, and deracoxib (Fig. 1), except for Day 7 ($p = 0.0781$). However, there was no significant difference among any time points after baseline ($p = 0.1538$), and there were no significant differences between the four treatments ($p = 0.4921$) or for the interaction between drug and time ($p = 0.0829$). When individual dog responses to various NSAIDs were evaluated, six of eight dogs returned to baseline closure times (defined as within 10% of their baseline value) at least once (range 1–3 times) with Car2, 5 of 8 returned to baseline closure times twice with Car4, 3 of 8 returned to baseline closure times at least once (range 1–3 times) with Met, and six of eight returned to baseline closure times at least once (range 1–4 times) with Der (Fig. 2). During the washout period, the average number of days from the time of drug discontinuance until return of closure times to

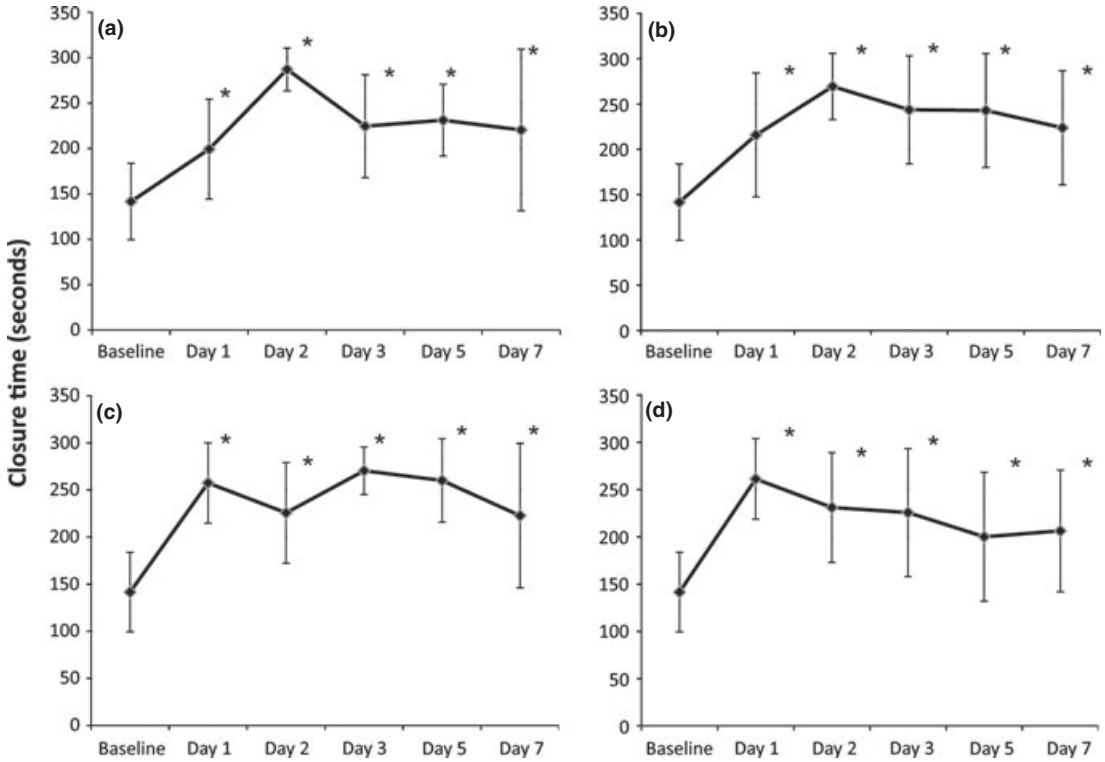

Figure 1 Mean PFA-100 closure time with the collagen/epinephrine cartridge (±SD) over time of eight healthy dogs after treatment with drug (*significantly prolonged over baseline value; $p \leq 0.05$). (a) Carprofen (2.2 mg kg^{-1} orally every 12 hours for 7 days). (b) Carprofen (4.4 mg kg^{-1} orally every 24 hours for 7 days). (c) Meloxicam (0.2 mg kg^{-1} first day then 0.1 mg kg^{-1} orally every 24 hours for 6 days). (d). Deracoxib (2 mg kg^{-1} orally every 24 hours for 7 days).

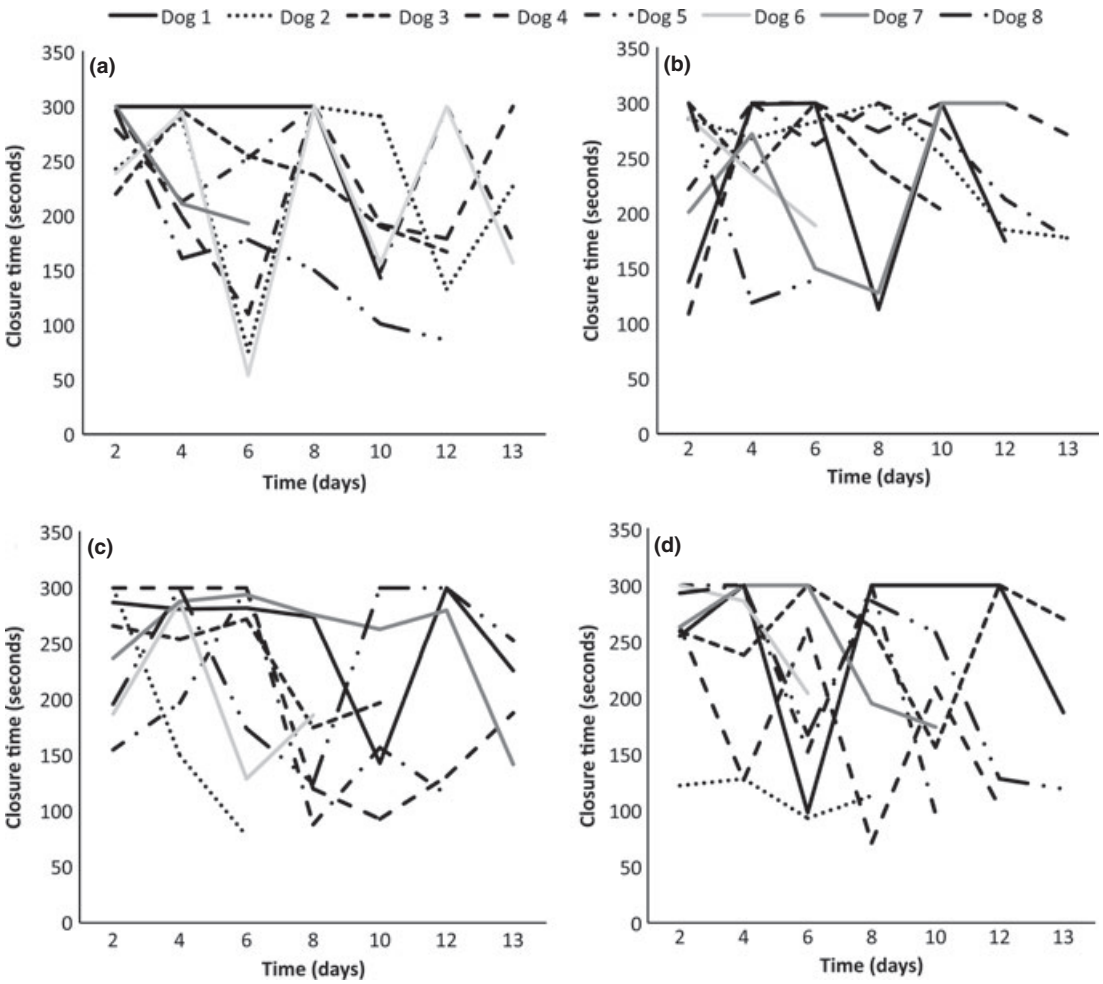

Figure 2 PFA-100 closure times with the collagen/epinephrine cartridge over time from eight healthy dogs during the washout period after treatment with drug. (a) Carprofen (2.2 mg kg^{-1} orally every 12 hours for 7 days). (b) Carprofen (4.4 mg kg^{-1} orally every 24 hours for 7 days). (c) Meloxicam (0.2 mg kg^{-1} first day then 0.1 mg kg^{-1} orally every 24 hours for 6 days). (d) Deracoxib (2 mg kg^{-1} orally every 24 hours for 7 days).

near baseline values as measured by the collagen/epinephrine cartridges was 11.6 days for Car2 (range 3–13 days) with two dogs not returning to 10% of baseline by 18 seconds, 10.6 days for Car4 (range 2–10 days) with three dogs not returning to 10% of baseline by 37 seconds, 11 days for Met (range of 2–13 days) with one dog not returning to 10% of baseline by 1 second), and 10.6 days (range 2–12 days) for Der. Because most of the dogs in the rotation had returned to within 10% of baseline, all dogs continued to the next drug rotation. Significant differences ($p = 0.0112$) were found in the PFA-100 collagen/epinephrine cartridges responses among the rotations.

The PFA-100 collagen/ADP cartridges did not detect differences in platelet function between the four groups of dogs at the initial baseline time point ($p = 0.1038$). Pretreatment (baseline) values ranged from 67 to 148 seconds. There were no significant differences in PFA-100 collagen/ADP closure times seen among drugs ($p = 0.6895$), between baseline and day 7 ($p = 0.7123$) (Fig. 3), among rotations ($p = 0.8161$) or for the interaction between time and drug ($p = 0.2603$).

Urine 11-dehydro-thromboxane B_2 analysis

There was no significant difference in urine 11-dehydro-thromboxane B_2 concentrations among the four groups of dogs at the baseline time point ($p = 0.9230$). The 11-dehydro-thromboxane B_2 concentrations did not decrease significantly

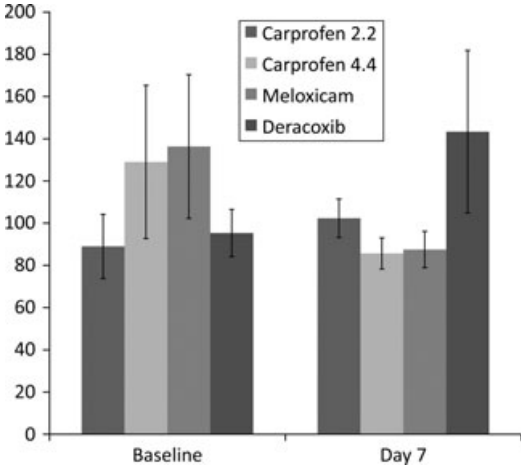

Figure 3 Mean PFA-100 closure time with collagen/ADP cartridge (±SD) over time of eight healthy dogs after treatment with carprofen (two doses), meloxicam, and deracoxib (See Fig. 1. for doses). No significant difference found.

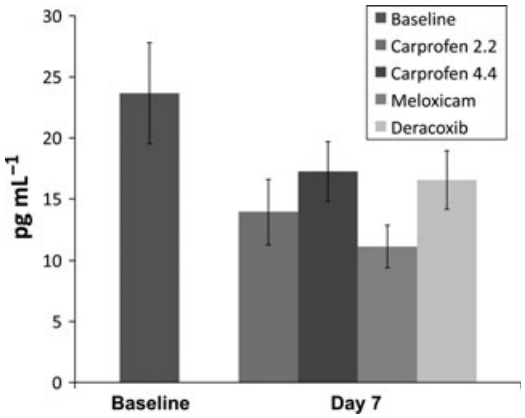

Figure 4 Mean urine 11-dehydro-thromboxane B_2 concentrations (±SD) of eight healthy dogs after treatment with carprofen (two doses), meloxicam, and deracoxib (See Fig. 1. for doses). No significant difference found.

($p = 0.9658$) below baseline concentrations during the period of drug administration for any of the NSAIDs studied (Fig. 4). Although significant differences ($p = 0.0140$) were found in 11-dehydro-thromboxane B_2 concentrations among rotations, specific reasons for the differences were not pursued.

Other testing

No significant differences were seen in platelet count among drugs ($p = 0.7152$), time points ($p = 0.3393$), rotation ($p = 0.9777$), or the interaction between time and drug ($p = 0.1207$). Similarly, no significant differences were seen in packed cell volume among drugs ($p = 0.0973$), time points ($p = 0.1839$), rotation ($p = 0.1551$), or the interaction between time and drug ($p = 0.8470$). Due to a laboratory error, the baseline measurements for packed cell volume and platelet count for the second rotation in the crossover trial (rotation B) were unavailable therefore these variables for rotation B were excluded from the analysis.

Discussion

Non-selective NSAIDs such as aspirin have long been considered to be contributing risk factors for perioperative hemorrhage in both humans and dogs due to their effects on platelet function (Luna et al. 2007; Li et al. 2009). One of the perceived benefits of the more COX-2 selective NSAIDs is that they would be expected to have less effect on platelet function than drugs like aspirin. The primary purpose of our study was therefore to determine the effects of the more COX-2 selective NSAIDs on platelet function in the dog.

When the mean response in all dogs was evaluated, all of the NSAIDs in our study caused a rapid and sustained decrease in platelet function that persisted for the majority of drug administration and for several days after drug discontinuation. As measured by PFA-100 (collagen/epinephrine cartridges), closure time was significantly prolonged from baseline to all time points, except for Day 7, during the period of drug administration for all drugs. Closer evaluation of individual dog responses to the COX-2 selective NSAIDs, however, suggests that there is a high level of dog-to-dog variability in both the degree and duration of drug-induced platelet dysfunction. Previous work has shown that anti-inflammatory doses of aspirin, in contrast, cause a marked and consistent prolongation of PFA-100 (collagen/epinephrine) closure times in all dogs receiving the drug, with minimal dog-to-dog variability (Thomason et al. 2010).

Yet to be determined is whether the altered platelet function associated with the COX-2 selective NSAIDs correlates with intra-operative hemorrhage. In human medicine, the finding that NSAIDs affect laboratory measures of platelet function has led to the recommendation that, whenever possible, NSAIDs should be avoided prior to surgeries where significant hemorrhage is expected (Møiniche et al. 2003; Li et al. 2009; Ng et al. 2009). Based on our

results, while we cannot conclude that the COX-2 selective NSAIDs will predispose to clinical bleeding, we have demonstrated that they do have an effect on some laboratory measures of platelet function, and thus perhaps should be used with caution in surgical patients that are at risk for bleeding similar to the recommendation in humans. However, further work is needed to clarify the best means for platelet function testing in dogs for the evaluation of COX inhibition, and additional prospective studies in clinical patients with larger subject numbers would be needed to further evaluate whether the use of NSAIDS in surgical patients increases the risk of clinical bleeding.

While our study is very similar in overall design to two recently published studies by Blois et al. (2010) and Brainard et al. (2007), important aspects of our results and our general conclusions are very different. Blois et al. (2010) evaluated the effects of 1 week of anti-inflammatory doses of oral aspirin, carprofen, deracoxib and meloxicam on platelet function in 10 healthy dogs using a comprehensive panel of tests including platelet function analysis using the PFA-100 collagen/ADP cartridge and plasma thromboxane B_2, and concluded that these NSAIDs had minimal effect on platelet function. In another study, Brainard et al. (2007) evaluated the effects of 10 days of anti-inflammatory doses of oral aspirin, carprofen, deracoxib and meloxicam on platelet function in eight dogs with osteoarthritis, and concluded that the more COX-2 selective NSAIDs had minimal and variable effects on most measures of hemostasis, though platelet aggregometry was significantly impaired in dogs receiving aspirin and carprofen. We believe that these differing results are due to significant differences in the techniques used for measuring platelet function in the two previous studies compared to our study.

Firstly, Blois et al. (2010) measured platelet function using only the collagen/ADP cartridge, whereas our study predominantly utilized the collagen/epinephrine cartridge. In humans, Kundu et al. (1995) described the use of the PFA-100 collagen/epinephrine measurements to detect aspirin-induced platelet dysfunction and collagen/ADP measurements to evaluate for other congenital and acquired platelet function defects. Similarly, in dogs, Nielsen et al. (2007) and Thomason et al. (2010) both found that administration of aspirin prolonged PFA-100 closure times when using collagen/epinephrine cartridges, but not when collagen/ADP cartridges were utilized. Our current study results are very comparable to these two previous studies, and strongly suggest that, in dogs as in people, prolongation of PFA-100 closure time utilizing the collagen/epinephrine cartridge is a sensitive indicator of NSAID-induced platelet dysfunction, whereas in contrast the collagen/ADP cartridge appears to be a very insensitive indicator.

Secondly, Blois et al. (2010) measured the effects of NSAIDs on platelet thromboxane production by measuring plasma thromboxane B_2 concentrations whereas, in our study, we utilized urinary 11-dehydro-thromboxane B_2 as an indicator of platelet thromboxane production. Whereas Blois et al. (2010) found that aspirin had little effect on plasma thromboxane B_2 concentrations in dogs, Thomason et al. (2010) showed that anti-inflammatory doses of aspirin induced a significant decrease in urine 11-dehydro-thromboxane B_2 concentrations in all dogs. These results are similar to human studies (Catella & FitzGerald 1987; Perneby et al. 1999) that suggest that when compared to the measurement of plasma thromboxane B_2 concentrations, the measurement of urinary thromboxane B_2 metabolites is a much more sensitive and reliable indicator of NSAID-induced inhibition of platelet thromboxane production. Thromboxane B_2 is metabolized by the tissues into 11-dehydro-thromboxane B_2, which is then excreted in urine. Urine 11-dehydro-thromboxane B_2 assays are therefore not subject to sampling artifact related to *in vitro* platelet activation (Roberts et al. 1981; Catella et al. 1986). While our study did not show a statistically significant decrease in urine 11-dehydro-thromboxane B_2 concentrations with the more COX-2 selective NSAIDs, urine 11-dehydro-thromboxane B_2 concentrations were lower than baseline concentrations for all drugs evaluated at the same time that platelet function was demonstrably impaired via PFA-100 analysis, suggesting that platelet dysfunction may be mediated by inhibition of thromboxane A_2 synthesis.

Finally, the previous study by Brainard et al. (2007) that concluded that aspirin and the more COX-2 selective NSAIDs had relatively minimal effects on hemostasis, utilized thromboelastography, platelet aggregometry using collagen and ADP as agonists, platelet thromboxane B_2, and plasma thromboxane B_2 and 6-keto-prostaglandin F-1α as measures of hemostasis, and therefore lacked several of the more sensitive indicators of NSAID-induced platelet dysfunction such as urinary thromboxane B_2 metabolites and utilization of epinephrine as a platelet agonist.

In dogs, the so-called 'COX-2 selective' NSAIDS actually vary in their degree of COX-2 selectivity, with many also having at least some small degree of documented COX-1 inhibition (Kay-Mugford et al. 2000a,b; Brideau et al. 2001; Streppa et al. 2002; Wilson et al. 2004). Many of the studies performed to determine this, however, are carried out *in vitro* and are highly dependent on the cells or tissue being evaluated. Meloxicam, for example, has some measurable effect on COX-1 function, although the drug reportedly inhibits canine COX-2 activity 12 times more effectively than COX-1 activity (Kay-Mugford et al. 2000b). The reported COX-2 selectivity of NSAIDS can also vary widely from study to study. For example, while one study reported that the potency of carprofen for COX-2 was more than 100-fold greater than for COX-1 in dogs (Ricketts et al. 1998), another study reported carprofen to be only 1.75 times more selective for COX-2 than COX-1 (Kay-Mugford et al. 2000b). Since even very low doses of aspirin are known to impair platelet function in many people via inhibition of platelet COX-1, it is possible that even a small amount of relative COX-1 inhibition imparted by a COX-2 'selective' NSAID may similarly have an impact on platelet function, particularly at higher drug doses. Our study certainly suggests that each of the COX-2 selective NSAIDs evaluated has the potential to impair platelet function in individual dogs.

Even when similar methodologies for testing canine platelet responses to NSAIDs are employed using the same drug at comparable doses, conflicting results are occasionally reported. Gaal et al. (2007), for example, evaluated the effects of carprofen on platelet function and found no difference in PFA-100 closure times between control and carprofen-treated dogs with either collagen/epinephrine or collagen/ADP cartridges. This contrasts with the observation in our study that carprofen significantly prolonged collagen/epinephrine PFA-100 closure times. When administering meloxicam, Luna et al. (2007) reported prolonged bleeding times in dogs within 7 days of administration of meloxicam compared to control dogs, while Mathews et al. (2001) reported that the administration of meloxicam to dogs did not result in a prolonged buccal mucosal bleeding time. Variability in the platelet responses of the individual dogs in our study to the COX-2 selective NSAIDs, and perhaps even apparent discrepancies between the results of previously published studies that used comparable drugs, doses and methodologies, may potentially be explained by innate dog-to-dog variability in NSAID responsiveness. In our study, it was interesting that individual dog responses to the COX-2 selective NSAIDs were much more variable as compared to responses to anti-inflammatory doses of aspirin (Thomason et al. 2010). In humans, while high doses of aspirin have predictable effects on platelet function, the lower levels of COX-1 inhibition provided by low dose aspirin do not impair platelet function in all individuals. The precise mechanism or mechanisms by which some patients are 'resistant' to low dose aspirin remain undetermined (Brideau et al. 2001; Wilson et al. 2004; Meen et al. 2008), but the mechanism may be similar to that resulting in variable platelet responsiveness in COX-2 selective NSAIDS. Recent studies have shown that, contrary to previously held assumptions, human platelets actually contain COX-2 as well as COX-1 (Weber et al. 2002), and it has been confirmed that canine platelets also contain measurable quantities of COX-2 (Thomason et al. 2010). Since COX-2 provides a potential alternative pathway to COX-1 for platelet thromboxane production, it is conceivable that varying individual-to-individual concentrations of platelet COX-2 may explain variable responsiveness to low dose aspirin and COX-2 selective NSAIDs. Further work comparing the platelet responses in individual dogs to low dose aspirin with responses to more COX-2 selective NSAIDs and looking at relative levels of platelet COX-1 and COX-2 in responders compared to non-responders is warranted.

Rotation was found to be a significant factor with the PFA-100 collagen/EPI results and 11-dehydrothromboxane B_2 concentrations, but it is unclear from where this significance stems.

The dogs were randomly assigned to one of the four COX-2 selective NSAIDS at the beginning of the study with the order of administration in subsequent rotations following the same pattern within each pair of dogs. Consequently, each drug was equally represented within a rotation. Furthermore, our statistical methodology accounted for the sequence in which the drugs were administered. While it is possible that failure to return to baseline in some dogs may have had an impact on rotation effects, the mechanism for this is difficult to understand. In light of the life span of the canine platelet, there should have been no exposed platelets in circulation after 14 days. When drug pharmacokinetics with reversible enzyme inhibition and drug clearance by day 14 is taken into consideration, there should have been

no residual drug effects unless these drugs act irreversibly on megakaryocytes in the bone marrow. Further work would be needed to clarify potential mechanisms for residual drug effects. The rotation effect may also be related to changes in laboratory substrates or machine calibrations over time. Regardless of the factors contributing to the differences in rotation, statistical models were adopted to account for this source of variation.

Our study had several limitations. The PFA-100 was our single measure of ex vivo platelet function. While optical, or light transmission, aggregometry is considered the 'gold standard' for the evaluation of platelet function, it is difficult to use in the clinical setting and methods for measuring platelet function are not standardized in the dog. Additionally there is relatively poor correlation among different laboratory assays of platelet function and poor correlation between ex vivo functional assays and clinical outcome. Our study demonstrated that NSAID administration can alter platelet function; but there was variability among dogs, requiring individual patient assessment of platelet function, ideally using multiple methodologies to evaluate platelet function. Further work is needed to fully phenotype platelets and their responses to cyclooxygenase inhibition with several methods employed for evaluation of functional alterations. In addition, variation noted in PFA-100 results may have been improved by performing duplicate collagen/epinephrine cartridges. While a baseline TxB_2 level was obtained prior to study initiation, no samples were obtained at the end of the washout periods to ensure the concentrations returned to pre-study level. These post washout measures would have been of considerable value for identifying potential causes for the rotational effects seen in this study, although the statistical model applied accounted for the potential impact of drug rotation. Lastly, our study population was small. The power of the study was insufficient to detect differences among the drugs. A larger study population would likely allow us to distinguish differences in effect among the COX-2 selective drugs. Future studies in this area should involve larger study populations, allowing better characterization of individual drug effects. Future studies should employ additional measures of platelet function and cyclooxygenase expression including ex vivo platelet function testing with both impedance whole blood aggregometry and light transmission aggregometry. Work in this area needs to include a comparison of methodologies applied for platelet aggregometry in dogs to identify the optimal anticoagulants and agonists for evaluation of cyclooxygenase active drugs.

Pharmacodynamic measures of NSAID effects on platelets should also be expanded to include a comparison of the assays for serum and urinary thromboxane metabolites as well as measures of prostacyclin production and thromboxane/prostacyclin ratios. Another interesting direction for future research would be evaluation of platelet function in clinical patients receiving COX-2 selective NSAIDs for an existing medical condition (i.e. osteoarthritis or acute musculoskeletal trauma) that could potentially activate platelets and alter platelet function. Research is currently being performed by our group investigating the possibility that some dogs, like humans, may be non-responders or partial-responders to aspirin. Larger studies evaluating platelet responses to COX-2 selective inhibitors may detect similar inter-individual variability in drug effects. If this is the case, future research involving platelet phenotyping may help to identify the mechanism or mechanisms for variability in platelet responses to cyclooxygenase inhibiting drugs.

In conclusion, all of the COX-2 selective NSAIDs evaluated in our study caused significantly prolonged PFA-100 closure times as measured using collagen/EPI cartridges, suggesting NSAID-induced alterations in platelet function. When evaluated with collagen/ADP cartridges, however, there was no change in PFA-100 closure time, indicating that, as is the case with aspirin, NSAID-induced platelet dysfunction cannot be accurately assessed using collagen/ADP cartridges (Brainard et al. 2007; Blois et al. 2010; Thomason et al. 2010; Dudley et al. 2011). Detectable impairment of platelet function persisted after drug discontinuation, but there was marked variability among dogs. While we did not undertake to show a correlation between the results of laboratory findings and clinical bleeding, our study does suggest that hemostatic evaluation of surgical patients known to be exposed to NSAIDs may be warranted, particularly in the presence of other risk factors for bleeding.

Acknowledgements

Funded by the Mississippi State University College of Veterinary Medicine Office of Research and Graduate Studies Competitive Research Grant Program. The authors thank Ryan Butler, Cory Fisher, Leonardo Baez, Ryan Taggart, Jamie Allison, Ruby

Lynn Carter, Chase Atwood, Frank Fasanella, Mandy Wallace, John Stokes, and Sumalee Givaruangsawat for their technical assistance.

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Received 18 February 2011; accepted 7 July 2011.

