Evaluation of firocoxib inhibition of cyclooxygenase activity in alpacas

by
Abigail Chadwick

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Animal Sciences
(Honors Associate)

Presented May 26, 2017
Commencement June 2017

AN ABSTRACT OF THE THESIS OF

Abigail Chadwick for the degree of Honors Baccalaureate of Science in Animal Sciences presented on May 26, 2017. Title: Evaluation of firocoxib inhibition of cyclooxygenase activity in alpacas.

Abstract approved:_____

Katja F. Duesterdieck-Zellmer

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to control pain and inflammation, but can have serious adverse effects, including gastrointestinal ulceration. Non-selective NSAIDs inhibit both cyclooxygenase isoforms: COX-1 and COX-2. COX-2 is primarily responsible for the inflammatory response, while COX-1 is constitutively expressed, representing the dominant source of prostaglandins for gastric epithelial cytoprotection. The coxib class of drugs selectively reduces the activity of COX-2 while sparing protective effects of COX-1 activity. Our goal is to determine a safe and therapeutic dose of firocoxib in alpacas.

There is no established method for measuring inhibition of COX-1 and COX-2 activity in alpacas. It was our aim to validate quantification of clot-induced production of thromboxane B_2 (TXB_2) as a measure for COX-1 activity, and lipopolysaccharide-induced prostaglandin E_2 (PGE_2) to assess COX-2 activity and to determine how firocoxib impacts COX activity.

Concentrations of TXB_2 or its metabolite, 11-dehydrothromboxane B_2 were not different between alpaca plasma and serum with or without acetone or methanol purification, indicating inability of the utilized ELISA to quantitate TXB_2 or its metabolite in alpacas. PGE_2 increased with lipopolysaccharide exposure and 100 pg/ml firocoxib inhibited PGE_2 production by at least 80% in all animals tested, demonstrating firocoxib's therapeutic potential in alpacas.

Key Words: alpaca, firocoxib, NSAID, cyclooxygenase, camelid, prostaglandin, thromboxane

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Abigail Chadwick, Author

Acknowledgements

I would like to thank my mentor, Dr. Zellmer, for encouraging me to pursue research in an area of the veterinary field that I was unfamiliar with at the time we met. She has taught me so much about academic writing, jugular venipuncture, and everything in between. I simply would not have and could not have done this research without her.

I would also like to thank Maureen Larson, who not only showed me the ropes of all the bench-work and helped me trouble-shoot all the issues that came up with the ELISAs, but she became an important friend and moral support for me in hard times.

Thank you to Dr. Semevolos for her guidance and support as a valuable committee member.

Thank you to Dr. Klopfenstein and Dr. Vanegas in Rural Veterinary practice for helping me with exams on our alpacas and with sample collection. Thank you to Kasey Pedder, Peter McPartlin, and the rest of the Large Animal Hospital Husbandry Team for their assistance caring for and restraining animals used for sample collection.

Thank you to the OSU Department of Clinical Sciences for funding for this research.

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used in human and veterinary medicine to control pain and inflammation. Many commonly used NSAIDs, however, have serious adverse effects, including gastrointestinal erosion, ulcers, and perforation.[1] Non-selective NSAIDs inhibit the activity of both cyclooxygenase (COX) isoforms: COX-1 and COX-2. COX-2 is primarily responsible for the inflammatory response, while COX-1 is constitutively expressed in most cells and is the dominant source of prostaglandins for gastric epithelial cytoprotection.[2] Thus, an NSAID which selectively reduces the activity of COX-2 while sparing the protective effects of COX-1 activity would be preferred as therapeutic for pain and inflammation. The coxib class of drugs selectively inhibit COX-2 activity over COX-1 activity. One such medication, firocoxib, has been approved for use in dogs as a chewable tablet and in horses as an injectable and oral paste or tablet. However, firocoxib has not been studied in alpacas. Our goal is to determine a safe and therapeutic dose of firocoxib for use in this species. Our hope is that by selectively inhibiting COX-2, we can lower the instance of third compartment ulceration in alpacas treated with anti-inflammatory drugs.

Literature Review

NSAIDs are a class of medications that have analgesic, antipyretic, and anti-inflammatory effects.[3] They are commonly used for pain relief for anything from headaches and minor injuries to chronic inflammatory joint diseases. In veterinary medicine, NSAIDs are commonly used to treat osteoarthritis and postoperative pain.

These drugs work by inhibiting the synthesis of prostaglandins and thromboxanes from arachidonic acid by acting on prostaglandin G/H synthases, colloquially referred to as cyclooxygenases.[4] Cyclooxygenases are responsible for prostanoid production.[5] Prostanoids, a subclass of eicosanoids, include prostaglandins, thromboxanes, and prostacyclins. Generally speaking, prostaglandins are responsible for inflammatory reactions, thromboxanes are responsible for vasoconstriction, and prostacyclins are involved in the resolution phase of inflammation. Prostaglandin G/H synthases have two distinct enzymatic activities: cyclooxygenase and peroxidase.[6] The cyclooxygenase activity is characterized by the addition of two molecules of O_2 to the middle of free fatty acid chains of arachidonic acid. This process forms prostaglandin G, a five-member carbon ring with two peroxide linkages. The cyclooxygenase activity is what is inhibited by NSAIDs, which act by blocking fatty acids from binding to the COX site.[7] The peroxidase activity of prostaglandin G/H synthase is characterized by the formation of prostaglandin H, the precursor for all other prostanoids, via the removal of an oxygen from of one of the two peroxide linkages in prostaglandin G.[8] The peroxidase activity of prostaglandin G/H synthases is not altered by NSAIDs.[7]

There are two cyclooxygenase isoforms: COX-1 and COX-2. COX-1 and COX-2 have different roles within the body. COX- 1 is constitutively expressed in most cells throughout the body and is the dominant source of prostaglandins for "house-keeping" functions such as gastric epithelial cytoprotection[2] and regulation of glomerular filtration rate.[9] COX-1 expression in platelets leads to production of thromboxane A_2 (TXA_2), which is essential for the formation of clots.[10] TXB_2, a metabolite of TXA_2, is relatively stable and can be detected in urine and blood.[11] Low levels of prostaglandin are produced ubiquitously and are involved in the maintenance of local homeostasis through autocrine and paracrine signaling. In inflamed tissues, prostaglandin production increases dramatically in leukocytes and other immune cells when COX-2 is induced by cytokines and tumor promoters.[12] It is important to note, that while the general distinction is that COX-1 is involved in homoeostatic processes and COX-2 is involved in inflammatory processes, both isoforms are involved in homoeostatic and inflammatory processes.[11] Their relative expression is what determines the prostanoid profile of a cell or tissue.[8] For example, macrophages produce TXA_2 at much higher levels than PGE_2 at rest, but after exposure to lipopolysaccharide (an inflammatory stimulus), PGE_2 is produced in excess of TXA_2.[13]

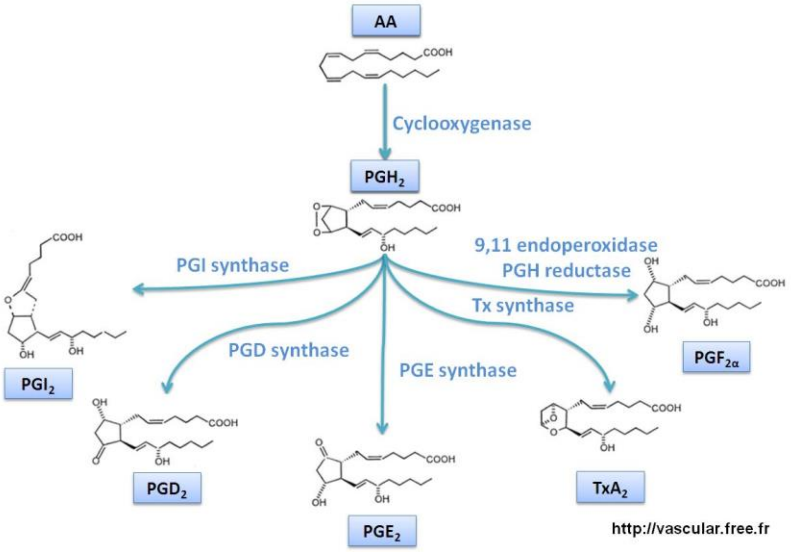

Figure 1 *– Biosynthesis of prostanoids via cyclooxygenase*

Traditional NSAIDs inhibit both COX isoforms and are efficacious in reducing pain and inflammation, but are also associated with irritation of gastric mucosa.[14] Since COX-1 is constitutively expressed in the gastrointestinal tract and produces prostaglandins that protect gastrointestinal mucosa by reducing the secretion of acid from parietal cells and increasing the secretion of mucus, nonselective NSAIDs have the adverse effects of gastrointestinal ulceration and erosion. There are also adverse effects of NSAID use on renal function, including renal papillary necrosis, interstitial nephritis, fluid retention, and electrolyte imbalances such as hyperkalemia.[15] This is not surprising given that prostaglandins are intimately involved in renal function, such as maintenance of blood flow through regulation of vascular tone and sodium re-absorption.[16]

The association of COX-1 inhibition with the gastrointestinal complications of NSAID use led to interest in the development of COX-2 selective NSAIDs. Structural differences between the two isoforms were exploited to accomplish this. The smaller valine residue allows for the formation of the hydrophobic side-pocket seen in COX-2 (Figure 2). Coxib drugs bind to this site, thus selectively inhibiting COX-2.[17]

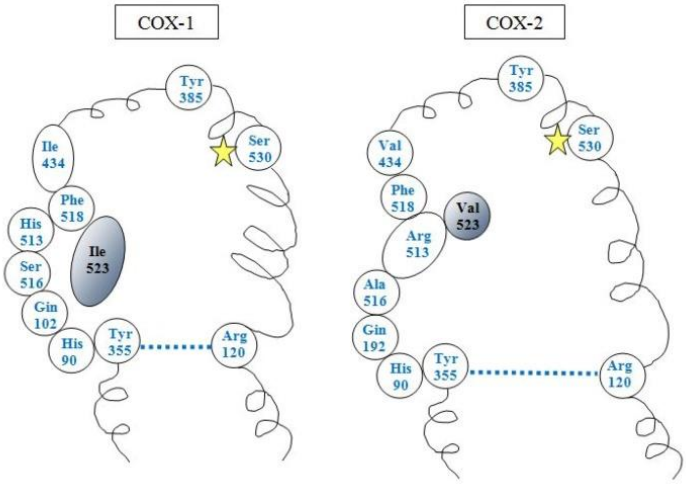

Figure 2– *Comparing structure of COX isoforms*[18]

Firocoxib is FDA approved for use in dogs and horses and is becoming widely used in veterinary medicine.[18–20] Firocoxib has been shown to be COX-2 selective,[21] and an effective treatment for equine osteoarthritis[22] and post-operative pain.[21] An *ex vivo* study in horses comparing the efficacies of canine chewable firocoxib tablets and equine firocoxib paste showed equal reductions of inflammatory-mediated (lipopolysaccharide-induced) PGE_2 synthesis in equine blood, thus suggesting that the two formulations of firocoxib inhibited COX-2 activity equally well.[23] The pharmacokinetics of firocoxib have been studied in horses.[24,25] Administering a loading dose of three times the 0.1mg/kg body weight recommended intravenous or oral maintenance dose decreased the time it took to reach average steady state drug concentrations as compared to a multi-dose regimen without this loading dose.[24] A pharmacodynamic study of firocoxib in horses established that a plasma concentration of 108 ng/ ml inhibited 80% of COX-2 activity *in vivo*.[25]

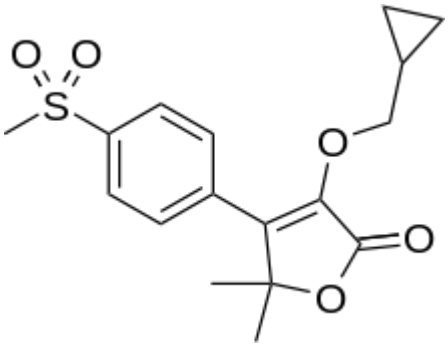

Figure 3– *Structure of firocoxib*

The COX-1 sparing effects of firocoxib have been studied in dogs. Firocoxib was shown to decrease PGE_2 concentrations in blood and synovial fluid, but did not decrease duodenal or gastric secretions of PGE_2 to the same extent as the non-specific NSAID tested, tepoxalin. Firocoxib has been shown to have a COX-1 sparing-effect in dogs *in vivo* and appears to reduce COX-2 activity associated with inflammation in the joints without inhibiting the PGE_2 production necessary in the stomach and duodenum for epithelial cytoprotection.[26]

In a study in horses, all gastric epithelium collected from stomachs of healthy horses expressed COX-1, but only two of the ten specimens showed expression of COX-2.[27] In ulcerated specimens, COX expression was varied, but overall, COX-2 expression was increased in ulcerated samples as compared to healthy tissue and COX-1 expression was decreased.[27]

Non-steroidal anti-inflammatory drugs studied in camelids include a non-specific cyclooxygenase inhibitor, phenylbutazone, and two COX-2 selective inhibitors, meloxicam and firocoxib. A 1997 study used High Performance Liquid Chromatography (HPLC) to investigate the pharmacokinetics and deposition of phenylbutazone administered intravenously and intramuscularly in Dromedary camels. It was shown that the elimination half-life for the intramuscular dosage was longer than that of the intravenous dosage.[28] More recently in 2012, a study in llamas used HPLC on serum samples to study pharmacokinetics and bioavailability of meloxicam following oral and intravenous administration. Gastrointestinal absorption was high with no noted adverse effects. Oral administration had higher peak plasma concentrations than the intravenous dose.[29] The pharmacokinetics of firocoxib have also been studied in Dromedary camels. Firocoxib could be detected with HPLC in camel serum 3-5 days after intravenous administration.[30] To date, the pharmacodynamics and pharmacokinetics of firocoxib have not been studied in New World camelids such as alpacas.

The effect of NSAIDs on the contractility of gastrointestinal tract has been studied in llamas. Samples from the third compartment of the stomach, in a tissue bath, responded to the addition of PGE_2 with reduced contractile strength of circular smooth muscle. Addition of indomethacin, a non-specific COX inhibitor, to the tissue bath

increased the contractile strength of circular smooth muscle. This effect was seen to be dose-dependent.[31] This study indicates that NSAID use could play a role in the incidence of ulceration of the third compartment of the stomach in camelids.

Objectives and Aims

To establish if firocoxib is COX-2 selective *ex vivo* in alpaca blood, we must first establish methodology to measure *in vitro* COX-1 activity and COX-2 activity in alpaca blood using a commercially available ELISAs. We must then determine if the concentration of firocoxib which inhibits *in vitro* COX-1 activity in alpaca blood by 50% is higher than that which inhibits *in vitro* COX-2 activity by 50%. We hypothesize that firocoxib will be COX-2 selective by this definition. We must also determine what concentration of firocoxib inhibits 80% of COX-2 activity *ex vivo* in alpaca blood, as an *ex vivo* therapeutic dose. We hypothesize that a firocoxib concentration of about 108 ng/ml will inhibit 80% of COX-2 activity *ex vivo* in alpacas, as this it concentration found to inhibit 80% of COX-2 activity *in vivo* in a pharmacodynamic study of firocoxib in horses.[25]

Materials and Methods

Sample Collection

The sample collection protocol was approved by Oregon State University's Institutional Animal Care and Use Committee. All animals belonged to the Oregon State University New World Camelid Research Herd. A total of ten healthy alpacas were used in this study and provided water and hay ad libitum while housed in pens at the Oregon State University Veterinary Teaching Hospital. Blood was collected while the animals were restrained by the husbandry crew of the Large Animal Veterinary Teaching Hospital. The fleece was clipped over the jugular vein in the upper third of the right side of the neck and isopropyl alcohol was applied to the venipuncture area. Jugular venipuncture was performed using a 20-gauge needle (Monoject™, Covidien Ltd., Dublin, Republic of Ireland) and blood was withdrawn into Monoject™ vacutainers (both EDTA and non-treated). After collection, the venipuncture site was held off for approximately 20 seconds before checking for hematoma formation. If a hematoma occurred, a neck bandage was applied for 1 minute.

Determination of COX-1 Activity

To determine COX-1 activity, we measured levels of the COX-1 product, TXB_2, using a competitive enzyme-linked immunoassay (Thromboxane B_2 ELISA Kit # 501020, Cayman Chemical, Ann Arbor, MI), following a procedure established in horses.[32] Measured TXB_2 concentrations in serum samples were compared to those in plasma as a negative control for evidence of COX-1 activity during clotting.

Experiment 1 – Initial Quantitation of Thromboxane B_2 in Alpaca Blood

Whole blood was collected from two alpacas as described above into non-treated vacutainers and allowed to clot at room temperature. Half of the samples were allowed to clot for 1 hour, and the other half for 2 hours before centrifugation at 2200 x g for 10 minutes to separate the serum from the clot. The serum was then pooled according to clotting-time. To generate TXB_2 negative controls, blood was also collected from the same two alpacas into EDTA-containing vacutainers, centrifuged at 2200 x g for 10 min and the plasma collected and pooled. For both, plasma and serum samples, one aliquot was assayed for TXB_2 without purification, a second aliquot underwent methanol purification as described below before TXB_2 assay and the remaining serum and plasma was stored at -80°C for future experiments. For methanol purification, serum and plasma samples were added to four volumes of methanol, vortexed for 5 seconds, centrifuged at 6,000 x g for 10 minutes, and the supernatant collected and stored at -80°C, as validated previously in horses.[21] Before the TXB_2 assay, samples were prepared in ELISA buffer to give dilutions of 1:50, 1:100, 1:300, 1:600, and 1:1200 in order to fit within the range of our TXB_2 standards (1.6 – 1000 pg/ml). The ELISA was run per manufacturer instructions with samples loaded in duplicate wells. Concentrations of TXB_2 were calculated from the standard curve, and concentrations in serum samples were compared to those in plasma as a negative control for evidence of COX-1 activity during clotting. All absorbances were read with a microplate spectrophotometer (Thermo Scientific Multiskan™ GO) at 420 nm. Readings were used when the adjusted B_0 (the maximum amount of tracer that the ELISA antibodies can bind in the absence of the sample) suggested by quality control analysis of the assay had been reached at 135 minutes and

only absorbances that fit within the range of our standards were used for calculations of concentrations of TXB_2.

Experiment 2 – Quantitation of TXB_2 in Alpaca Blood at Lower Dilutions and the Use of Equine Blood as a Positive Control

To ascertain that the processing of serum and plasma as well as assay procedures were appropriate to measure TXB_2, we measured concentrations of TXB_2 in more concentrated preparations of the banked alpaca samples from Experiment 1, along with the inclusion of equine blood samples as positive controls. We were provided blood in EDTA-containing and untreated vacutainers from one Oregon State University-owned healthy horse by veterinary students practicing jugular venipuncture. These samples were processed similarly to samples from alpacas, as described in Experiment 1 to give plasma, serum with a 1-hour clotting time, and serum with a 2-hour clotting time, all without purification. We used the same competitive enzyme-linked immunoassay as in Experiment 1. (Thromboxane B_2 ELISA Kit # 501020, Cayman Chemical, Ann Arbor, MI) to measure TXB_2. Alpaca serum and plasma was diluted in ELISA buffer to 1x (no dilution), 1:2.5, 1:10, and 1:50 and horse serum and plasma were diluted in ELISA buffer to 1:25, 1:50, 1:100, and 1:200. The ELISA was run, absorbance was read, and TXB_2 concentrations were calculated as described in Experiment 1.

Experiment 3 – Quantitation of Thromboxane B_2 Using Different Purification Procedures in Alpaca and Equine Serum and Plasma

To determine if sample purification would increase our ability to detect TXB_2 in alpaca samples, we tried both methanol and acetone purification procedures before measurement with the same competitive enzyme-linked immunoassay described above.

Pooled plasma and serum (allowed to clot for 1 hour) from two alpacas, generated as described in Experiment 1 were separated into 2 aliquots. One aliquot was purified using a methanol procedure that was modified from the one which was used in Experiment 1. Plasma and serum samples were diluted to 1:5 in methanol, mixed by vortex for 5 seconds, incubated at room temperature for 15 minutes, and centrifuged at 6000 x g for 10 minutes. Equal amounts of supernatant (1 ml) were transferred to clean polypropylene test tubes and dried under nitrogen gas. The resulting precipitates were frozen at -80°C until reconstitution in ELISA buffer prior to immunoassay. Ultrapure (PURELAB Ultra, ELGA Labwater, Veolia Water Solutions & Technologies, France) water samples were spiked with TXB_2 standard and processed under the same conditions to verify recovery of TXB_2. A second aliquot was purified using an acetone purification procedure. Plasma and serum samples were diluted to 1:5 with cold acetone (-20°C), vortexed for 5 seconds, and incubated at 4°C for 15 minutes before 4°C centrifugation at 6000 x g for 10 minutes. 1 ml of supernatant was transferred to a clean polypropylene test tube and dried under nitrogen gas. The resulting solids were frozen at -80°C until being reconstituted in ELISA buffer prior to immunoassay. TXB_2 spiked water samples were processed similarly to verify TXB_2 recovery.

Blood samples from one Oregon State University-owned healthy horse provided by veterinary students practicing jugular venipuncture were processed and purified identically to the samples form alpacas described above. Before the assay, alpaca samples were reconstituted in ELISA buffer to give concentrations of 1x, 1:5, and 1:25, and horse samples were reconstituted in ELISA buffer to give concentrations of 1:50, and 1:100. A 1:25 alpaca plasma sample was spiked with TXB_2 standard and run alongside the other

samples to determine if elements of the plasma were interfering with binding to the ELISA antibodies. Concentrations of TXB_2 were determined in all samples as described in Experiment 1.

Experiment 4 – Quantification of 11-dehydrothromboxane B_2 in Alpaca Serum and Plasma, Using different Purification Procedures

We measured levels of 11-dehydrothromboxane B_2, a non-enzymatic metabolite of TXB_2, with a competitive enzyme-linked immunoassay (11-dehydro Thromboxane B_2 ELISA Kit – Monoclonal # 519510, Cayman Chemical, Ann Arbor, MI) to determine whether or not TXB_2 is hydrolyzed non-enzymatically *ex-vivo*[33] in alpaca serum. Serum and plasma were generated from one alpaca as described in Experiment 1 with slight modifications. The EDTA vacutainer contained 25.1 µl of 875 µg/ml indomethacin in dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, Waltham, MA) in phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA), resulting in a final concentration of about 10 µM once about 5 mL of blood filled the tube. The indomethacin was added to the blood for an approximate concentration of 10 µM per manufacturer instructions to prevent *ex vivo* formation of 11-dehydrothromboxane B_2. Blood collected in untreated vacutainers for generation of serum was allowed to clot for 1 and 3 hours. Plasma and serum samples were separated into 3 aliquots, the first of which was stored at -80°C until assay, representing a non-purified sample. The other two aliquots were processed using methanol or acetone precipitation procedures, as described in Experiment 3. Ultrapure water samples were spiked with TXB_2 standard and processed under the same conditions to verify recovery of TXB_2 from the acetone-precipitation process. Before the assay, samples were reconstituted in ELISA buffer to 1.75x, 1:2,

1:20, and 1:40. The ELISA was run per manufacturer instructions with samples loaded in duplicate wells. Concentrations of 11-dehydrothromboxane B_2 were calculated from the standard curve, and concentrations were compared between indomethacin-treated plasma as a negative control and non-treated serum samples for evidence of COX-1 activity during clotting. Absorbances were read with a microplate spectrophotometer (Thermo Scientific Multiskan™ GO) at 420 nm. Readings were used when the adjusted B_0 suggested by quality control analysis of the assay had been reached at 90 minutes and only absorbances that fit within the range of our standards were used for calculations of concentrations of 11-dehyrdoTXB_2.

Determination of COX-2 Activity

As a first step to eventually quantifying inhibition of COX-2 by firocoxib, we first had to ascertain the ability to detect PGE_2 in alpaca blood using a commercially available ELISA. Measured PGE_2 concentrations were compared between samples exposed to LPS and non-LPS exposed controls for evidence of COX-2 activity during inflammatory response.

Experiment 5 – Initial Quantitation of Prostaglandin E_2 in Alpaca Blood exposed to LPS

We measured levels of the COX-2 product, PGE_2, with a competitive enzyme-linked immunoassay (Prostaglandin E_2 ELISA Kit – Monoclonal # 514010, Cayman Chemical, Ann Arbor, MI), following a procedure established in horses.[21] Lipopolysaccharide (LPS) from E. coli 055:B5 (Sigma-Aldrich Co., St. Louis, MO) was dissolved in 0.1% bovine serum albumin (BSA) (HyClone™, Thermo Fisher Scientific,

Waltham, MA) in PBS. Whole blood was collected from two alpacas into EDTA vacutainers and then transferred into tubes representing five different conditions (PBS, 0.1% BSA in PBS, and 1, 100, and 500 µg/ml LPS in 0.1% BSA in PBS) and mixed thoroughly. The samples were then incubated for 24 hours at 37°C.[21] After incubation the blood was centrifuged at 1000 x g for 10 minutes per the established method to generate plasma in alpacas.[34] The plasma was collected and pooled for each condition. These pooled plasma samples were added to four volumes of methanol and centrifuged at 6,000 x g for 10 minutes, and the supernatant collected and stored at -80°C, per the established method in horses.[21] Before the assay, samples were diluted in ELISA buffer to give concentrations of 1:4, 1:20, and 1:60. The ELISA was run per manufacturer instructions with samples loaded in duplicate wells. Concentrations of PGE_2 were calculated from the standard curve, and concentrations were compared between samples treated with LPS and the controls (blood with PBS as a negative control and blood with 0.1% BSA in PBS as a vehicle control) for evidence of COX-2 activity during inflammatory response. Absorbance was read with a microplate spectrophotometer (Thermo Scientific Multiskan™ GO) at 420 nm. Readings were used when the adjusted B_0 suggested by quality control analysis of the assay had been reached at 95 minutes and only absorbances that fit within the range of our standards were used for calculations of concentrations of PGE_2.

COX-2 Inhibition by Firocoxib

In order to measure COX-2 inhibition by firocoxib, we compared lipopolysaccharide-induced production of PGE_2 in samples treated with varying concentrations of firocoxib to those in untreated samples.

Experiment 6 – Preliminary Experiment to Determine an Appropriate Range of Firocoxib Concentrations

Our preliminary experiment involved samples from two alpacas to determine an appropriate range of firocoxib concentrations for the inhibition of COX-2 activity. Blood was collected into EDTA vacutainers as described previously. For each animal, 493 µl of blood was transferred into each of eight tubes representing seven different treatments, containing 2 µl of either PBS (2 tubes, untreated controls), DMSO (vehicle control), 0.25, 2.5, 12.5, 25, or 125µg/ml firocoxib in DMSO, giving the final concentrations shown in Table 1. After each tube had been gently mixed, 5 µl of 100 µg/ml LPS in 0.1% BSA dissolved in PBS was added to all tubes other than the tube representing the LPS unconditioned, untreated control (0 ng/ml firocoxib, no DMSO; no LPS, no BSA in PBS). The unconditioned, untreated control sample had 5µl PBS added, instead. All samples were then gently mixed a second time before a 24-hour incubation at 37°C. Plasma from both animals was purified using an acetone purification procedure. Plasma and serum samples were diluted to 1:5 with cold acetone (-20°C), vortexed for 5 seconds, and incubated at 4°C for 15 minutes before 4°C centrifugation at 6000 x g for 10 minutes. 1 ml of supernatant was transferred to a clean polypropylene test tube and dried under nitrogen gas. The resulting solids were frozen at -80°C until being reconstituted in

ELISA buffer, and these reconstituted samples from both animals pooled prior to immunoassay. PGE_2 was quantified as described previously in Experiment 5.

***Table 1**: Identification of experimental groups with respect to firocoxib treatment and LPS conditioning, including control groups*

Group name	**Treatment**		**Condition**	
	Firocoxib [ng/ml]	**DMSO [%]**	**LPS [µg/ml]**	**BSA in PBS [%]**
Unconditioned, untreated control	0	0	0	0
LPS untreated control	0	0	1	0.001
LPS treatment vehicle control	0	0.4	1	0.001
LPS firocoxib treatment 1	1	0.4	1	0.001
LPS firocoxib treatment 10	10	0.4	1	0.001
LPS firocoxib treatment 50	50	0.4	1	0.001
LPS firocoxib treatment 100	100	0.4	1	0.001
LPS firocoxib treatment 500	500	0.4	1	0.001

The percent inhibition of COX-2 activity by each firocoxib concentration was calculated using the following formula:

$$\%\,COX-2\ inhibition = \left(\frac{PGE2control - PGE2firocoxib}{PGE2control}\right)\times 100$$

Here "PGE2control" is the measured PGE_2 concentration in the LPS conditioned non-firocoxib treated control and "PGE2firocoxib" is the measured PGE_2 concentration in the firocoxib-treated, LPS conditioned sample. Percent inhibition was calculated separately using LPS conditioned, untreated controls for one data set and using LPS conditioned, treatment vehicle (DMSO) controls to generate a second data set.

Experiment 7 –Determination of COX-2 Inhibition by Firocoxib in 3 Alpacas

The final experiment involved the same protocol as described in Experiment 6 with samples from 3 alpacas. In this experiment PGE_2 was measured in plasma from each individual animal, as opposed to in pooled plasma.

Data Analysis

All data is presented as individual data points. No statistical analysis was performed, due to the low sample sizes used in the experiments. In Experiment 7, a software program was used to fit a model to the data points (Excel, Microsoft Office 2010).

Results

Sample Collection

No hematoma formation occurred during sample collection.

Determination of COX-1 Activity

Experiment 1 – Initial Quantitation of Thromboxane B_2 in Alpaca Blood

Determined concentrations of TXB_2 in alpaca serum subjectively were not higher than those in the plasma control. This was the case for unpurified plasma and serum (Figure 4) as well as methanol-purified samples (Figure 5). Calculated TXB_2 concentrations (based on sample dilutions) subjectively appeared to increase with increasing sample dilution (Figures 4-5). However, when subjectively assessing the absorbance readings (Table 2), there did not seem to be differences in absorbance quantity between different dilutions prior to calculation of the TXB_2 concentration based on sample dilution. Allowing the blood to clot for 2 hours compared to 1 hour did not appear to make a difference in determined TXB_2 concentrations (Figures 4-5).

Table 2 *– Measured Absorbances in alpaca plasma and serum (allowed to clot for 1 or 2 hours) at different dilutions in ELISA buffer, with and without methanol purification*

	Conc.(pg/ml)	**Absorbances (run in duplicate)**
Plasma 1:100	1393	0.5414, 0.5414
Plasma 1:300	3648	0.5393, 0.5648
Plasma 1:600	8019	0.5556, 0.5341
Plasma 1:1200	9366	0.5757, 0.5808
Plasma 1:100 Methanol Purified	955	0.5624, 0.573
Plasma 1:300 Methanol Purified	2702	0.5663, 0.5757
Plasma 1:600 Methanol Purified	4191	0.5817, 0.5846
Plasma 1:1200 Methanol Purified	14527	0.5259, 0.5794
Serum 1 hour 1:100	1274	0.5391, 0.558
Serum 1 hour 1:300	4009	0.526, 0.5641
Serum 1 hour 1:600	8106	0.5416, 0.5462
Serum 1 hour 1:1200	22171	0.4974, 0.5343
Serum 1 hour 1:100 Methanol Purified	1938	0.5591, 0.468
Serum 1 hour 1:300 Methanol Purified	3925	0.5563, 0.5367
Serum 1 hour 1:600 Methanol Purified	13177	0.5158, 0.4787
Serum 1 hour 1:1200 Methanol Purified	6939	0.6033, 0.5763
Serum 2 hour 1:100	1040	0.5651, 0.5601
Serum 2 hour 1:300	2756	0.5929, 0.5466
Serum 2 hour 1:600	12375	0.5364, 0.4742
Serum 2 hour 1:1200	17064	0.5563, 0.5236
Serum 2 hour 1:100 Methanol Purified	1418	0.5579, 0.5226
Serum 2 hour 1:300 Methanol Purified	4137	0.538, 0.5465
Serum 2 hour 1:600 Methanol Purified	7392	0.5533, 0.5487
Serum 2 hour 1:1200 Methanol Purified	14976	0.573, 0.5277

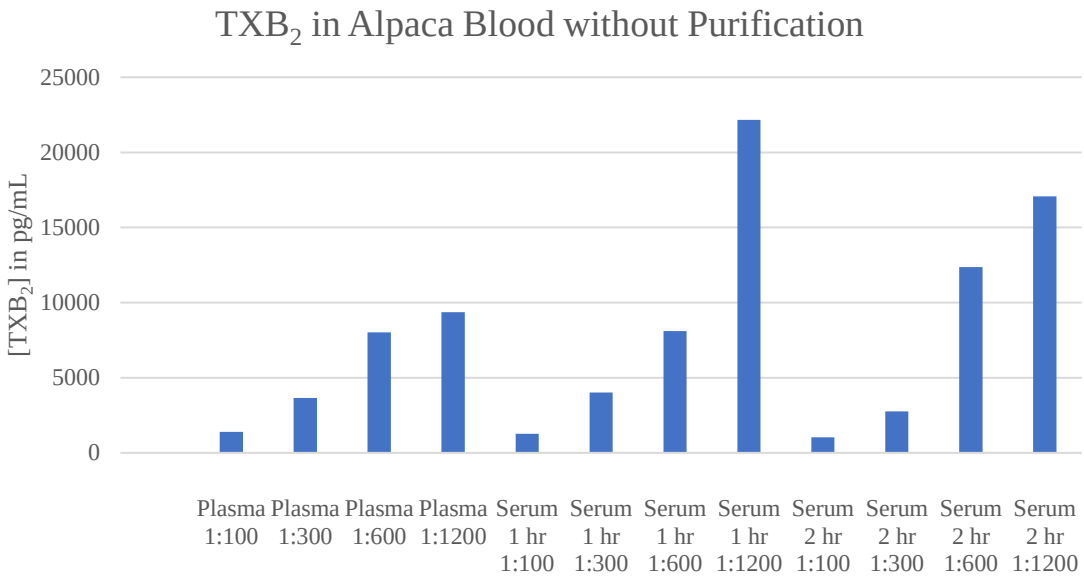

Figure 4 *– Clot-induced TXB_2 concentrations measured using a commercially available ELISA (Thromboxane B_2 ELISA Kit, Cayman Chemical, # 501020) in alpaca serum (1 and 2-hour clotting times) and plasma (as negative control) at higher dilutions, without purification. Data represents pooled plasma or serum from two animals.*

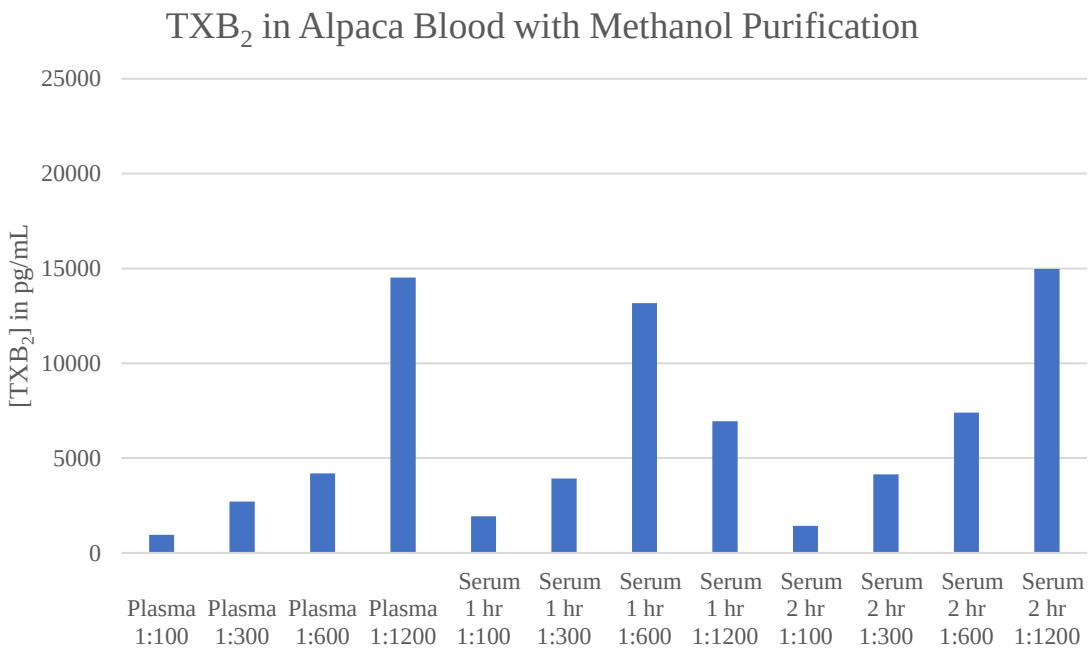

Figure 5 *– Clot-induced TXB_2 concentrations measured using a commercially available ELISA (Thromboxane B_2 ELISA Kit, Cayman Chemical, # 501020) in alpaca serum (1 and 2-hour clotting times) and plasma (as negative control) at higher dilution, with methanol purification. Data represents pooled plasma or serum from two animals.*

Experiment 2 – Quantitation of TXB_2 in Alpaca Blood at Lower Dilutions and the Use of Equine Blood as a Positive Control

To determine if the concentration of TXB_2 in alpaca serum was lower than our initial estimate in Experiment 1, we ran more concentrated versions of the same samples. Similar to results from Experiment 1, the determined of TXB_2 in alpaca serum were subjectively not higher than those in the plasma control (Figure 6). Determined TXB_2 concentrations in these samples with lower dilutions appeared lower or within the same range of concentrations as those determined in Experiment 1 in samples with higher dilutions. In contrast to this, TXB_2 concentration in equine serum was at least 56 times higher than in equine plasma (Figure 7). It appeared that allowing the equine serum to clot for 2 hours, compared to a clotting time of 1 hour, slightly decreased the concentration of TXB_2 in the serum.

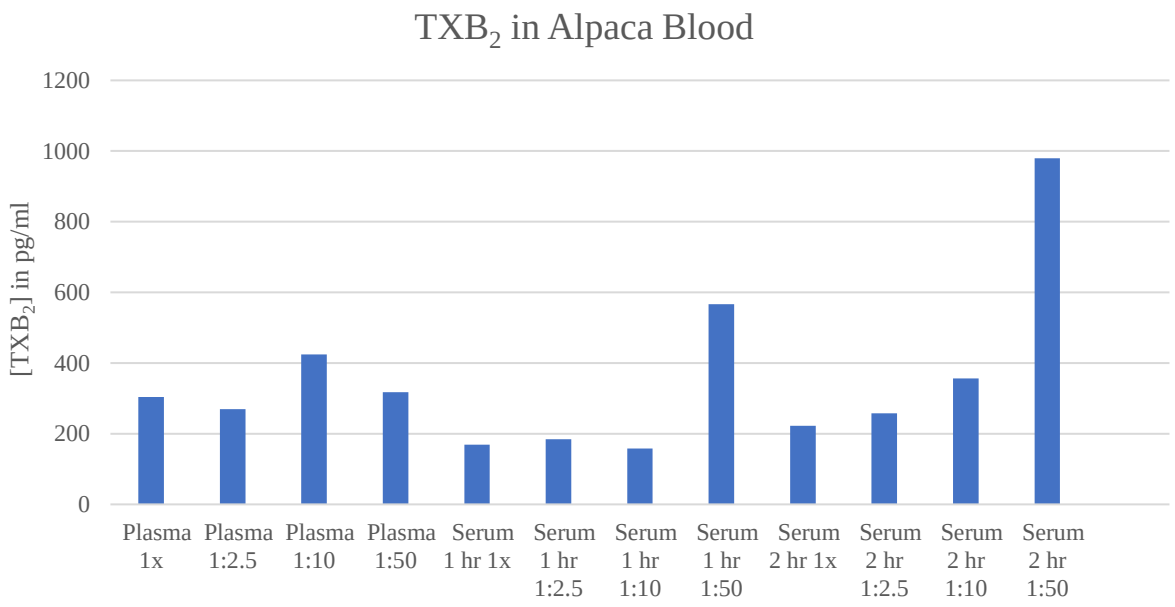

Figure 6*– Clot-induced TXB_2 concentrations measured using a commercially available ELISA (Thromboxane B_2 ELISA Kit, Cayman Chemical, # 501020) in alpaca serum (1 and 2-hour clotting times) and plasma (as negative control) at lower dilutions, without purification. Data represents pooled plasma or serum from two animals.*

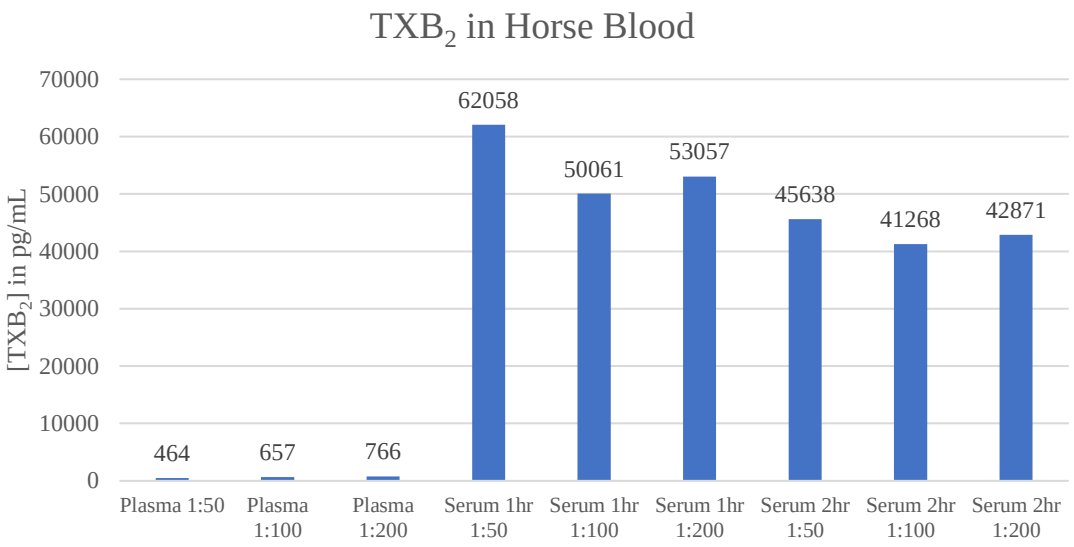

Figure 7 *– Clot-induced TXB_2 concentrations measured using a commercially available ELISA (Thromboxane B_2 ELISA Kit, Cayman Chemical, # 501020) in equine serum (1 and 2-hour clotting times) and plasma (as negative control) at various dilutions, without purification. These samples were used to ascertain adequate performance of the ELISA in our laboratory. Data represents measurements in blood from n=1 horse.*

Experiment 3 – Quantitation of Thromboxane B_2 Using Different Purification Procedures in Alpaca and Equine Serum and Plasma

Measured concentrations of TXB_2 in horse serum were at least 100-fold higher than those in the plasma control. Measured concentrations were similar among methanol-precipitated and acetone-precipitated serum (Figure 8). Absorbances for methanol purified equine plasma were below the values of the standard curve. In TXB_2 spiked water samples, recovery of TXB_2 was 251% for acetone-precipitation and 103% for methanol-precipitation. For TXB_2 spiked 1:25 alpaca plasma, recovery of TXB_2 was 4100% acetone-precipitated plasma and 4080% for methanol-precipitated plasma.

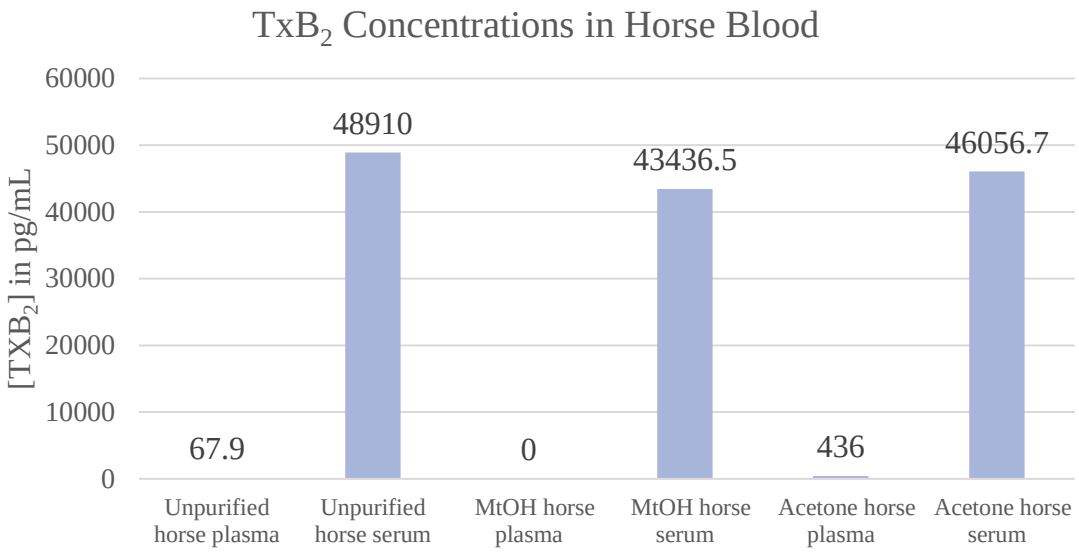

Figure 8 *- Clot-induced TXB_2 concentration in horse serum (allowed to clot for 1 hour) and plasma (as negative control), measured with a commercially available ELISA (Thromboxane B_2 ELISA Kit, Cayman Chemical, # 501020) from 1 animal, using different purification methods.*

In contrast to what was found for equine blood, measured concentrations of TXB_2 in alpaca serum were not subjectively higher than those in the plasma controls and this was the case no matter what purification procedure was used (Figure 9).

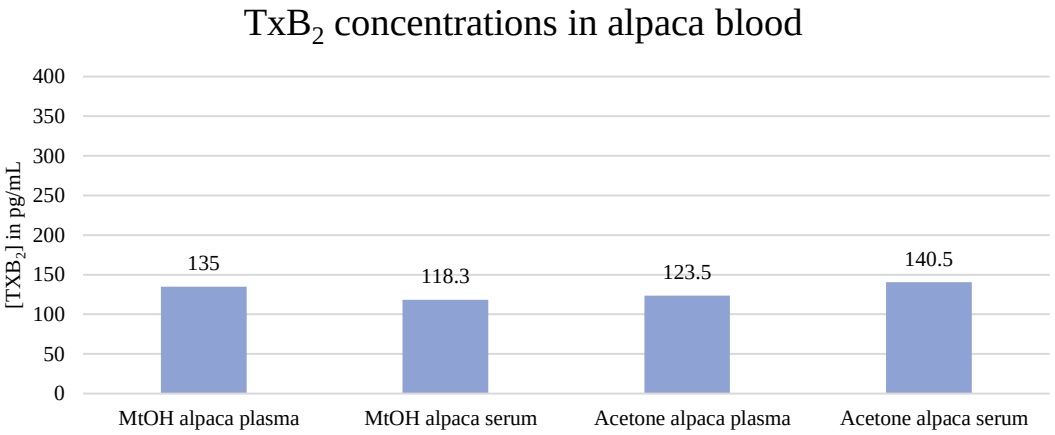

Figure 9 *- Clot-induced thromboxane B_2 concentration in alpaca serum (allowed to clot for 1 hour) and plasma (as negative control), determined with a commercially available ELISA (Thromboxane B_2 ELISA Kit, Cayman Chemical, # 501020) from pooled blood of 2 animals, using different purification procedures.*

Experiment 4 – Quantification of 11-dehydrothromboxane B_2 in Alpaca Serum and Plasma, Using different Purification Procedures

Measured concentrations of 11 dehydro-TXB_2, a non-enzymatic metabolite of TXB_2, in alpaca serum were not subjectively higher than those in the plasma control. This was the case for unpurified, methanol-precipitated, and acetone-precipitated plasma and serum (Figure 10). For 11 dehydro-TXB_2 spiked water the recovery was 67% after acetone-precipitation.

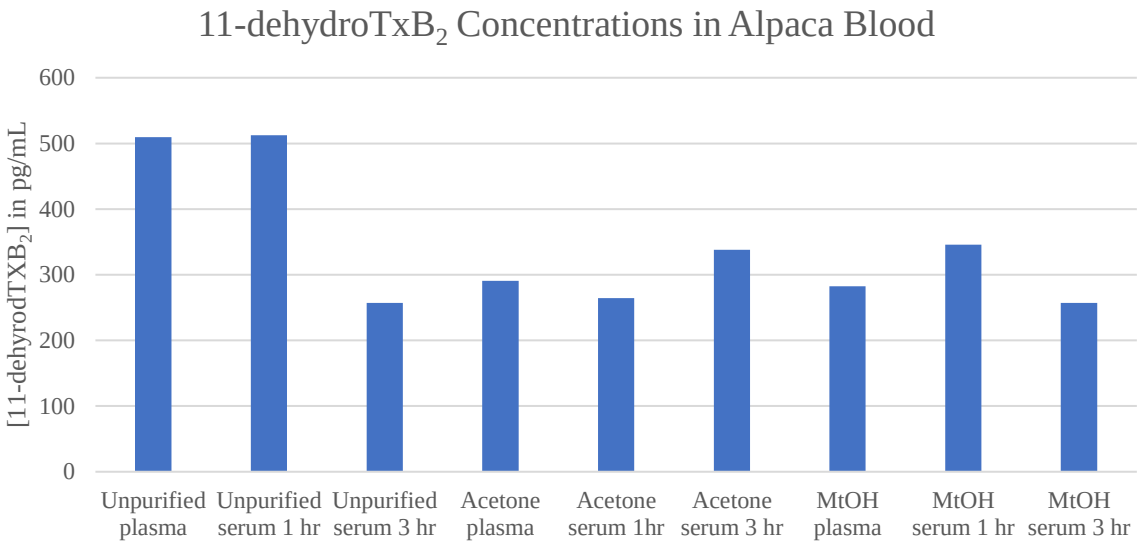

Figure 10 *- Clot-induced 11-dehydroTXB_2 concentration in alpaca serum and plasma (as negative control), determined with a commercially available ELISA (11-dehydro Thromboxane B_2 ELISA Kit - Monoclonal, Cayman Chemical, # 519510) from 1 animal using different purification procedures.*

Determination of COX-2 Activity

Experiment 5 – Initial Quantitation of Prostaglandin E_2 in Alpaca Blood exposed to LPS

Measured concentrations of PGE_2 in alpaca plasma were subjectively higher in LPS-conditioned samples than the unconditioned controls (Figure 11). PGE_2 concentration in LPS unconditioned control plasma was relatively low. Addition of the vehicle for LPS (0.1% BSA in PBS) increased the PGE_2 concentration slightly. Addition of LPS to the blood was associated with a more substantial increase in PGE_2 concentration in plasma. Highest PGE_2 concentration was measured after addition of 50µg/ml LPS.

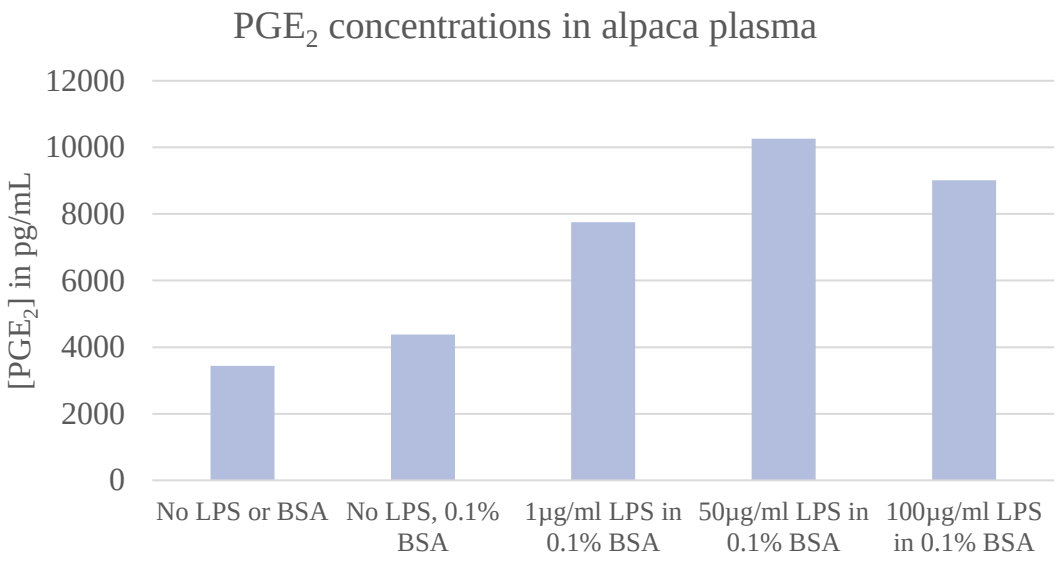

Figure 11 *- PGE_2 concentrations in alpaca plasma as measured with a commercially available ELISA (Prostaglandin E_2 ELISA Kit - Monoclonal, Cayman Chemical, # 514010) from pooled blood of 2 animals.*

COX-2 Inhibition by Firocoxib

Experiment 6 – Preliminary Experiment to Determine an Appropriate Range of Firocoxib Concentrations

Measured concentrations of PGE_2 in alpaca plasma were subjectively higher in LPS-conditioned samples than the unconditioned controls. Addition of the firocoxib vehicle (DMSO) appeared to increase PGE_2 production in the alpaca blood and addition of 10 ng/ml firocoxib then appeared to decrease PGE_2 production with 500 ng/ml of firocoxib decreasing PGE_2 production close to the levels in unconditioned, untreated controls (Table 3).

Table 3 *– Measured PGE_2 concentrations in alpaca plasma as measured with a commercially available ELISA (Prostaglandin E_2 ELISA Kit - Monoclonal, Cayman Chemical, # 514010) from pooled plasma of 2 animals.*

Group name	Firocoxib [ng/ml]	Measured PGE_2 [pg/ml]
Unconditioned, untreated control	0	1396
LPS untreated control	0	4158
LPS treatment vehicle control	0	10438
LPS firocoxib treatment 1	1	9619
LPS firocoxib treatment 10	10	4186
LPS firocoxib treatment 50	50	2525
LPS firocoxib treatment 100	100	1859
LPS firocoxib treatment 500	500	885

In the pooled plasma from two alpacas, percent COX-2 inhibition depended on which sample was used as control sample for the calculation of % COX-2 inhibition (Figure 12).

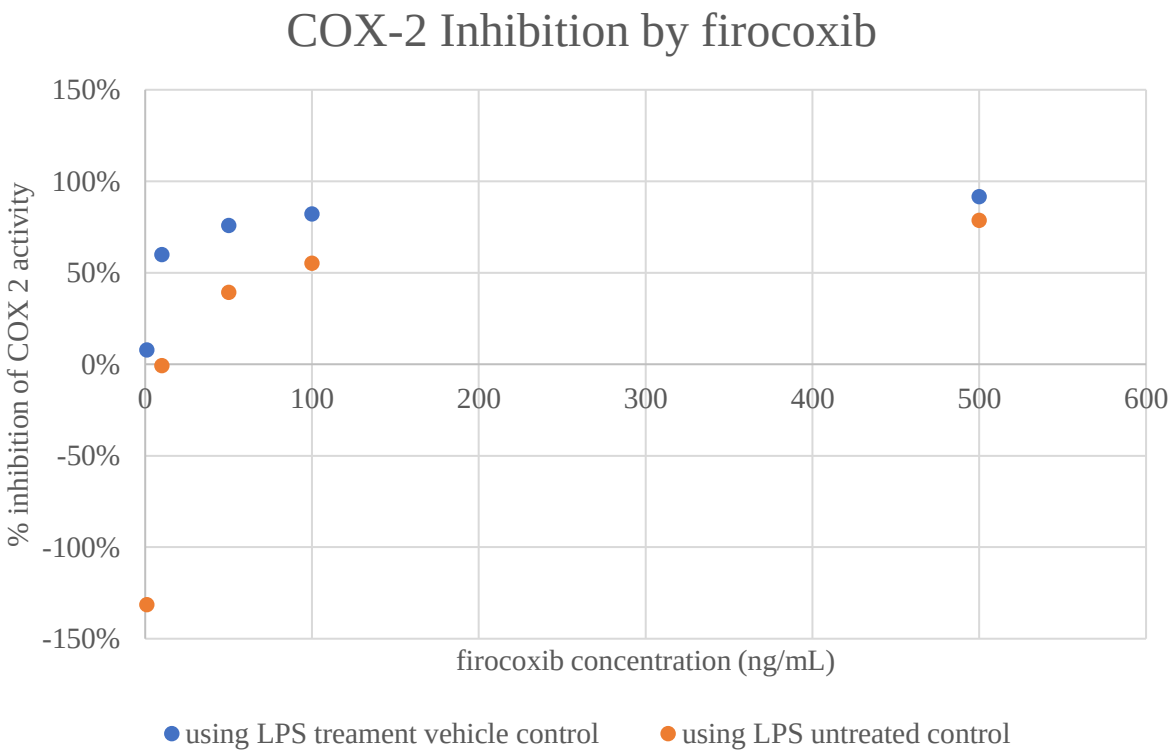

Figure 12 *– Percent Inhibition of COX-2 activity in alpaca plasma as measured with a commercially available ELISA (Prostaglandin E_2 ELISA Kit - Monoclonal, Cayman Chemical, # 514010) from pooled plasma of 2 animals. Percent inhibition of COX-2 activity was calculated by the following formula:* $\% COX-2\ inhibition = \left(\frac{PGE2control - PGE2firocoxib}{PGE2control}\right) \times 100$ *with the blue dots representing a dataset calculated using the LPS conditioned samples that had the treatment vehicle added (DMSO) as control sample, and with the orange dots representing a dataset calculated using the LPS conditioned samples that did not have the treatment vehicle added as control sample.*

Experiment 7 – Determination of COX-2 Inhibition by Firocoxib in 3 Alpacas

In contrast to results from Experiment 6, addition of the treatment vehicle to LPS–conditioned alpaca blood did not increase PGE_2 production in any of the samples (n=3; Table 3). Firocoxib appeared to decrease PGE_2 concentrations of LPS conditioned samples at concentrations of equal to or greater than 10 ng/ml. In general, the higher the firocoxib concentration was, the lower the PGE_2 concentration was in the samples tested (Table 4).

Table 4 - PGE_2 concentrations in samples from three individual alpacas, at different firocoxib concentrations

Group name	[firocoxib] in ng/ml	Alpaca #1 [PGE_2] in pg/ml	Alpaca #2 [PGE_2] in pg/ml	Alpaca #3 [PGE_2] in pg/ml
LPS untreated control	0	1536	6131	3034
LPS treatment vehicle control	0	699	1131	1974
LPS firocoxib treatment 1	1	862	3350	3040
LPS firocoxib treatment 10	10	1412	1195	2105
LPS firocoxib treatment 50	50	360	3009	1734
LPS firocoxib treatment 100	100	249	998	549
LPS firocoxib treatment 500	500	73	325	637

A concentration dependent inhibition of COX-2 activity by firocoxib, based on measurement of the COX-2 product PGE_2, was apparent, with higher firocoxib concentrations decreasing COX-2 activity to a greater extent than lower concentrations did (Table 5, Figure 12). Variability between animals was apparent (Figures 12-13), but it appeared that a firocoxib concentration of 100 ng/ml decreased COX-2 activity by at least 82% in all 3 animals using the LPS untreated control. However, when using the vehicle control (Table 6), the calculated percent inhibitions were lower than with the LPS untreated control (Table 5).

Table 5 - Calculated % inhibition of COX-2 activity by firocoxib for samples from three individual alpacas, at different firocoxib concentrations, using the LPS untreated control

[Firocoxib] in pg/ml	Alpaca #1	Alpaca #2	Alpaca #3	Average
1	44%	45%	0%	30%
10	8%	81%	31%	40%
50	77%	51%	43%	57%
100	84%	84%	82%	83%
500	95%	95%	79%	90%

Table 6 *- Calculated % inhibition of COX-2 activity by firocoxib for samples from three individual alpacas, at different firocoxib concentrations, using the LPS treatment vehicle control*

[Firocoxib] in pg/ml	Alpaca #1	Alpaca #2	Alpaca #3	Average
1	-23%	-196%	-54%	-91%
10	-102%	-6%	-7%	-38%
50	48%	-166%	12%	-35%
100	64%	12%	72%	49%
500	90%	71%	68%	76%

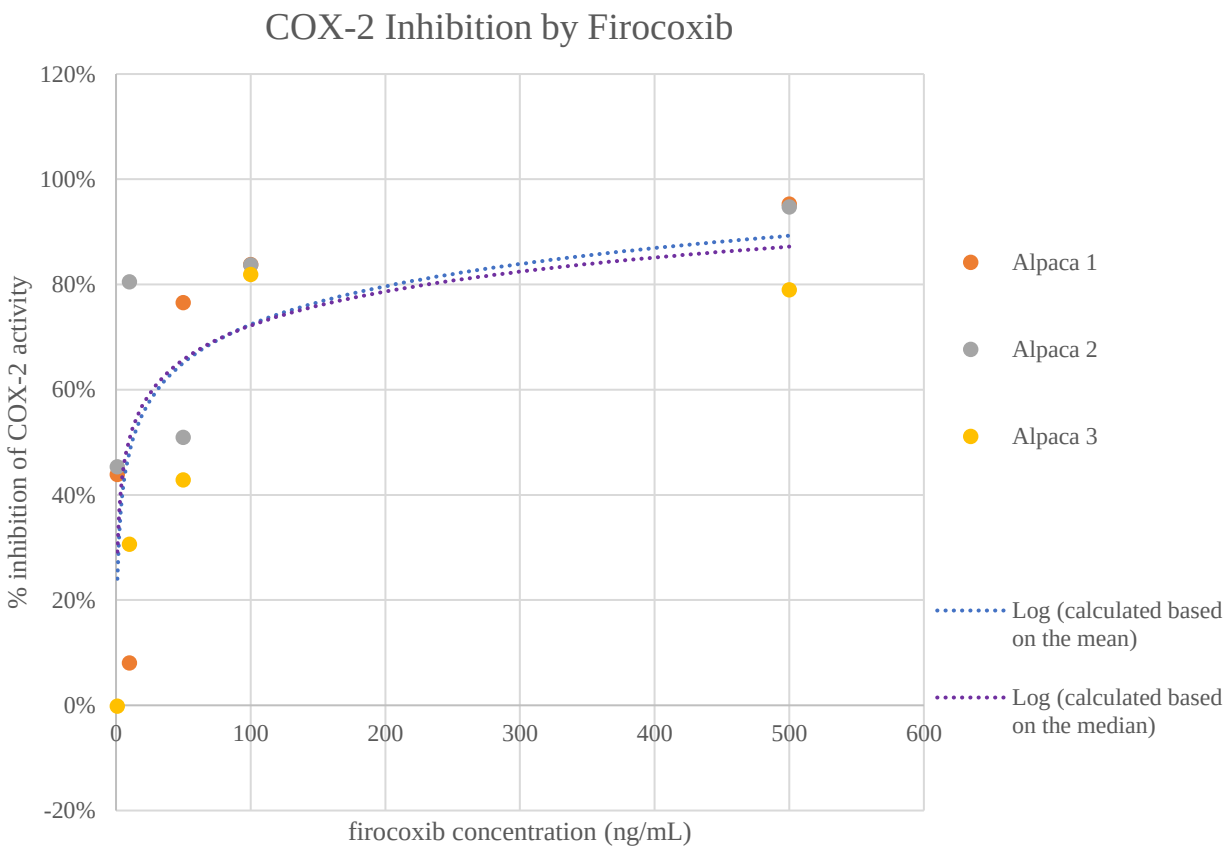

Figure 13 *- COX-2 Inhibition by Firocoxib calculated using LPS untreated control. Data represents individual measurements in blood from 3 alpacas. The blue dotted curve represents the best fit curve based on data averages (y = 0.1049ln(x) + 0.2405) and the purple dotted curve represents the best fit curve based on median values (y = 0.0933ln(x) + 0.2921)*

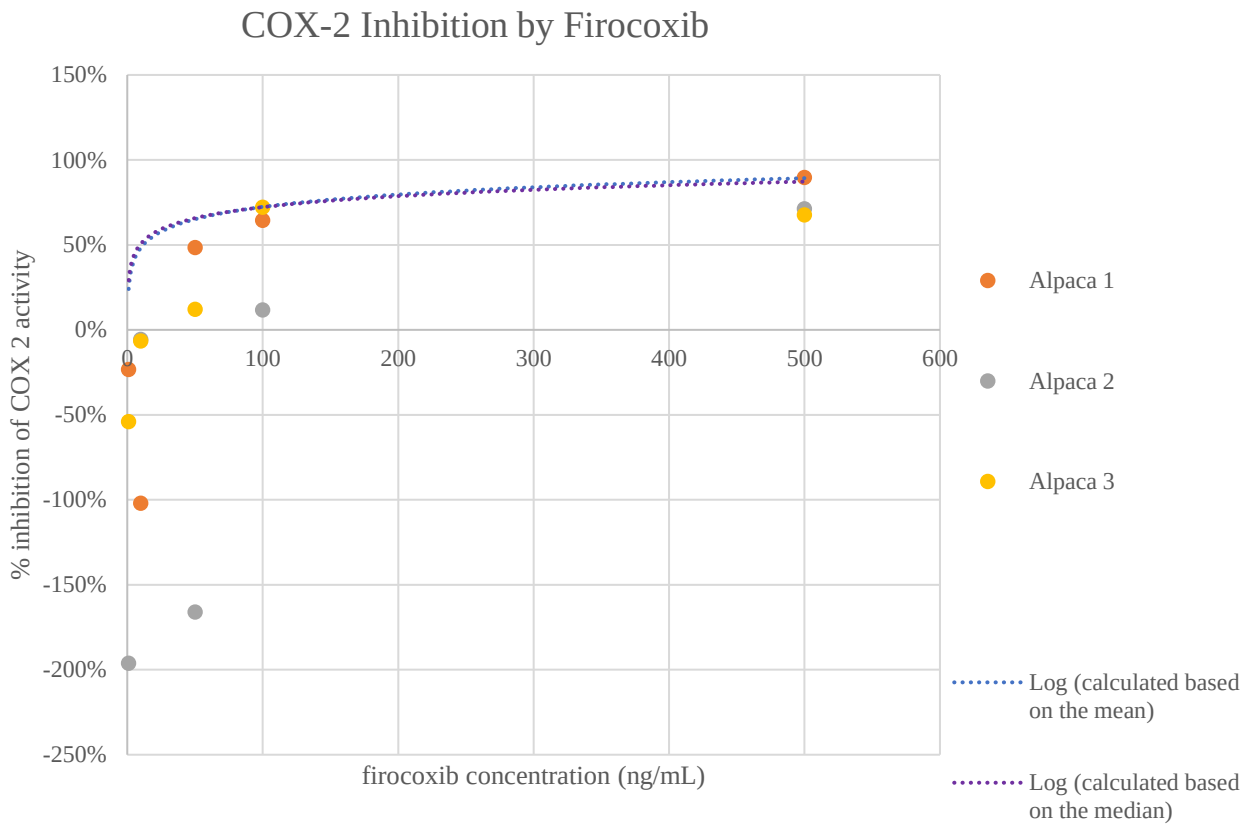

Figure 14 – COX-2 Inhibition by Firocoxib calculated using LPS treatment vehicle control. Data represents individual measurements in blood from 3 alpacas. The blue dotted curve represents the best fit curve based on data averages and the purple dotted curve represents the best fit curve based on median values.

Discussion

To determine COX-1 activity, we attempted to quantify clot-induced TXB_2 production in alpaca serum. This methodology has been used previously in other species, such as the cat, horse and dog.[35] Plasma is typically used as a negative control or as COX-1 baseline activity control and it is expected that serum from the same animal has a much higher TBX_2 concentration than plasma, due to clot-induced COX-1 activity by platelets. Surprisingly, our results for alpaca serum were not different from those in alpaca plasma. This could be explained if TXB_2 in alpaca serum is binding very poorly to the antibody in the ELISA utilized. This in turn could be due to either poor affinity of the antibody for alpaca TXB_2 or due to another protein interfering with the binding between TXB_2 and antibody. We were concerned that there may be proteins in alpaca blood that were interfering with the binding of TXB_2 to the ELISA antibody, so tested different purification methods to attempt to remedy this, with no success: measured serum concentrations of TXB_2 remained at levels that were as low as those in plasma. In TXB_2 spiked water samples, recovery of TXB_2 was 251% for acetone-precipitation and103% for methanol-precipitation. The abnormally high recovery in the acetone-purified samples could be due to pipetting error or perhaps residual acetone interfered with the ELISA, or acetone precipitation may not be the best purification for this ELISA. For TXB_2 spiked 1:25 alpaca plasma, recovery of TXB2 was 4100% for acetone-precipitation and 4080% for methanol-precipitation, indicating that there may be a protein in the alpaca plasma that is binding to the ELISA antibodies.

The fact that we had nearly identical absorbance readings for different dilutions in the initial TXB_2 experiments is interesting, because this means that there was not a

difference in the amount of TXB_2 tracer (Figure 15) binding to the ELISA antibodies. This may also indicate that something in the sample interfered with the ability of TXB_2 in the sample to bind to the ELISA antibody.

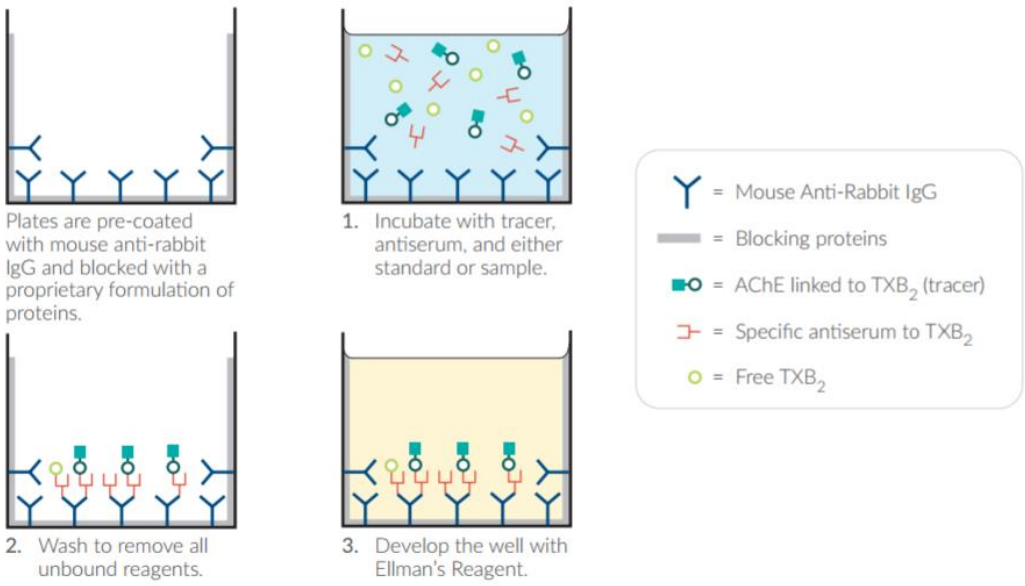

Figure 15 *– Schematic of the AChE ELISA, from the Cayman TXB_2 ELISA Kit Booklet – the tracer acts competitively with the TXB_2 in the sample such that the lower the concentration of the TXB_2 in the sample the darker the color of the sample (more tracer bound)*

In order to determine that these results were not due to a flaw in our sample processing, we ran the same ELISA with horse plasma and serum. The goal was to obtain results consistent with existing literature on horses, which states that equine clot-induced TXB_2 concentrations in serum are about ten times the baseline concentration in plasma[35]. Similarly, in our study, there was a notable increase in the TXB_2 concentration in horse serum as compared to plasma (Figure 2), suggesting that equine TXB_2 has a higher affinity for the ELISA antibody than alpaca TXB_2, or equine serum is missing the interfering proteins presumably present in alpaca serum. The acetone-precipitated samples and methanol-precipitated samples were subjectively the same, contradicting the

earlier concern that acetone-precipitation may not be a viable purification method for use with this ELISA.

Another study in camelids failed to detect TXB_2 production by platelets: in a pharmacokinetic study of phenylbutazone in camels, TXB_2 concentrations in serum were below the limit of detection with radioimmunoassay.[28]

An OSU study on platelet aggregation in llamas showed that while arachidonic acid has been shown to stimulate platelet aggregation in other species including guinea pigs, horses, humans, and rabbits,[36] it failed to stimulate platelet aggregation in llama blood.[37] This may indicate that camelids have a different clotting mechanism than that of species previously studied. If this is the case, our assumption that alpaca platelets produce thromboxane by COX-1 activity during the clotting process may be invalid.

It is interesting to note that the measured TXB_2 concentrations were lower in horse blood allowed to clot for two hours than horse blood allowed to clot for only one hour. It is possible that this was due to TXB_2 being hydrolyzed *ex-vivo* to 11-dehydroTXB_2. Thus, we thought it possible that the low measured thromboxane levels in alpaca serum may also be due to non-enzymatic conversion of TXB_2 to 11-dehydrothromboxane B_2. However, when we tried measuring 11-dehydrothromboxane B_2 in alpaca blood, we once again failed to see an appreciable difference between measured 11-dehydrothromboxane B_2 concentrations in serum and the plasma control, despite the use of both methanol and acetone-extraction steps prior to assay. Reasons for these results are possibly similar to those for results using the TXB_2 ELISA. Based on the results from one alpaca plasma sample that was spiked with TXB_2 standard, we conclude that something in the alpaca plasma is interfering with TXB_2 binding to the ELISA

antibodies. Further work is needed to establish a methodology to measure COX-1 activity in alpaca blood.

We were successful in utilizing a methodology previously used in equine blood[21] to measure COX-2 activity in alpaca blood. There was an appreciable difference between measured PGE_2 concentrations in LPS-conditioned and non-LPS conditioned samples, which is what we would expect as PGE_2 is synthesized in response to inflammatory stimuli such as the presence of LPS.[6] We saw an increase in PGE_2 production in whole blood with exposure to an LPS concentration of just 1 µg/ml, which is similar to what has been reported in canine blood.[32] However, for feline and equine blood, exposure to LPS at a final concentration of 100 µg/ml was required to see an increase in PGE_2 production.[35] We did see lower PGE_2 production in the experiments with samples from individual alpacas as compared to our pooled samples. This may be due to variability in PGE_2 production among alpacas. In the preliminary experiment with firocoxib, we saw that the addition of DMSO increased PGE_2 production quite a bit, but this was not seen in the final experiments with individual animals. This could be due to the hypertonic nature of DMSO causing hemolysis, but no hemolysis was noted qualitatively. It would make sense that the vehicle control would have lower LPS-induced PGE_2 production than the control without the DMSO vehicle, as DMSO has been shown to have *ex vivo* anti-inflammatory activity in human blood exposed to E. coli.[38] It is possible that in the preliminary COX-2 inhibition study, the two controls were labeled incorrectly. It is unclear why the LPS-induced PGE_2 production in samples with lower firocoxib concentrations was higher than in the samples with the DMSO vehicle control.

In LPS-conditioned alpaca blood *ex vivo*, firocoxib appeared to inhibit COX-2 activity at concentrations that were similar to those considered clinically effective *in vivo* in horses. A pharmacodynamic model based on an *in vivo* pharmacokinetic and pharmacodynamics study of firocoxib in horses suggested that an approximate plasma concentration of 108 ng/ml should inhibit 80% of COX-2 activity *in vivo*[25] and in our study, 100 ng/ml firocoxib inhibited LPS-conditioned PGE_2 production by at least 80% in all alpacas tested.

However, given our small sample size, we cannot make claims about what plasma concentration may inhibit COX-2 in alpacas *in vivo* or even *ex vivo*. Based on our data, we conclude that there is a positive association between firocoxib concentration and inhibition of COX-2 in alpaca blood. A logarithmic model has been used in other species and appears to describe the trend of our data (Figure 13), with a correlation coefficient of $R^2 = 0.895$ for the trendline using the average of the three alpacas and a correlation coefficient of $R^2 = 0.6558$ for the trendline using the median of the three alpacas. This means that these models can explain 90% and 66% of the variability of the data, respectively. Repeating the COX-2 inhibition experiment with more animals will improve our ability to predict a therapeutic concentration of firocoxib ex and *in vivo*.

Interestingly, it has been shown that Eltenac while a COX-2 selective inhibitor *ex vivo*, is not selective in the *in vivo* environment. This shows that *ex vivo* models are not always predictive of COX selectivity of NSAIDs *in vivo*.[39] Thus, *in vivo* pharmacodynamic and pharmacokinetic studies will be necessary to determine a safe and efficacious dose of firocoxib for alpacas, as well as to determine whether or not firocoxib is COX-2 selective in alpacas *in vivo*.

Conclusions

We cannot draw any conclusions as to the COX selectivity of firocoxib, given that we have only successfully measured the activity of the cyclooxygenase isoform COX-2. Possible areas of expansion include testing different purification and preparation methods of samples for the thromboxane immunoassays, as well as including the use of a more lipophilic solvent for precipitation of proteins. It is also a possibility that the alpaca thromboxane naturally has a lower affinity for the ELISA antibody than that of the horse or dog. Other possibilities for our results are that alpacas do not produce as much or any thromboxane during the clotting process as do other species or that the thromboxane is metabolized non-enzymatically to a different metabolite than we have been able to test for.

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Abbreviations

AA – arachidonic acid

BSA – bovine serum albumin

COX – cyclooxygenase

DMSO – dimethyl sulfoxide

HPLC – high-performance liquid chromatography

LPS – lipopolysaccharide

NSAID – nonsteroidal anti-inflammatory drug

PBS – phosphate-buffered saline

PGE_2 – prostaglandin E_2

TXB_2 – thromboxane B_2