RESEARCH REPORT
ANIMAL WELFARE

Title: The Plasma And Tissue Pharmacokinetics And Pharmacodynamics Of Nonsteroidal Anti-Inflammatory Drugs In Neonatal Piglets – NPB #16-091

Investigator: Kristen M. Messenger.

Institution: North Carolina State University College of Veterinary Medicine, Department of Molecular Biomedical Sciences, Raleigh, NC, USA.

Co-investigators: Glen Almond, Ron Baynes, Emma Nixon, Alexandra Carlson, Liliana Hernandez, and Patricia Routh

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INDUSTRY SUMMARY

The provision of pain alleviation for painful procedures in piglets, including castration and tail-docking, is required by law in the European Union and will likely become required in the USA. Nonsteroidal anti-inflammatory drugs (NSAIDs) are currently being used in the EU and Canada for this purpose. However, there is conflicting research on which NSAID is the most effective and appropriate for this application, and currently there are no pain medications approved for use in swine in the US. This research examined and compared the effects of 3 different NSAIDs on pain and inflammation following piglet processing (castration and tail-docking).

At 4 days-of-age, male Yorkshire x Landrace piglets were removed from the sow and IV catheters and interstitial probes were placed. At 6 days-of-age, piglets were assigned to one of five treatment groups (saline sham [SAL SHAM], 0.1 mL saline and no processing; saline castration [SAL CAST], 0.1 mL saline and processed; Meloxicam treatment [MLX], 0.4 mg/kg meloxicam and processed; Flunixin treatment [FLU], 2.2 mg/kg flunixin meglumine and processed; or Ketoprofen treatment [KETO], 3 mg/kg ketoprofen and processed). All NSAIDs and saline were administered intramuscularly, two hours before processing. Pigs were sampled and observed up to 48 hours post-dose. Pain assessments included a behavior score, piglet grimace score, thermal imaging, and activity monitoring.

Flunixin was present in both the blood and interstitial fluid for longer than either meloxicam or ketoprofen in our study, and also had the longest-lasting effects of decreasing inflammation based on the measurement of prostaglandin E2. The dose was given 2 hours prior to processing, which was ideal as the interstitial fluid concentrations peaked between 2-4 hours; these tissue concentrations are more likely to be indicative of the effective concentrations since it reflects biologically active drug located at the site of action.

When comparing the SAL CAST and SAL SHAM groups, castration notably increased cortisol levels immediately at the time of processing in all groups. Treatment with flunixin lessened the increase in cortisol, when compared with the SAL CAST group. All the NSAIDs

decreased prostaglandin E2 levels in interstitial fluid (i.e., tissues) when compared to the SAL CAST group, however only flunixin was able to maintain that inhibition beyond 24 h post-dose.

Overall, meloxicam was the least effective NSAID when examining the behavior and grimace scores beyond 6 h post-dose. At the time of processing, all the NSAID treated groups had increased pain behaviors and increased pain scores. This may have been noted because the piglets were more active, making the active pain-related behaviors more obvious to the observers (e.g. scratching at castration site), compared to the SAL CAST group which showed more inactive behaviors (e.g. laying still and protecting the castrated site). Activity levels, obtained via Actical® monitoring, were also decreased in the SAL CAST and MLX piglets following processing. This was likely related to the reduced number of active pain-related behaviors.

In conclusion, this study found that the administration of NSAIDs 2 hours prior to castration and tail docking had a positive impact on pain alleviation in piglets, with flunixin demonstrating superiority compared with ketoprofen and meloxicam, and meloxicam being the least efficacious of the three treatments at the doses studied. Management strategies that include the administration of flunixin to reduce pain associated with processing will improve piglet health and welfare in the United States.

KEY WORDS: Analgesia, welfare, piglet, castration, nonsteroidal anti-inflammatory drugs (NSAIDs)

SCIENTIFIC ABSTRACT

Pain medications, predominately nonsteroidal anti-inflammatory drugs (NSAIDs), are used in the EU and Canada to decrease pain associated with castration and tail docking and to improve piglet welfare. However, in piglets, the efficacy and required dose of these NSAIDs is unknown.

Forty 4-day old male Yorkshire x Landrace piglets were removed from the sow and IV catheters, interstitial probes and activity monitors were placed. At 6 days-of-age the piglets were randomly assigned to one of five treatment groups (saline sham [SAL SHAM], 0.1 mL saline and no processing; saline castration [SAL CAST], 0.1 mL saline and processed; Meloxicam treatment [MLX], 0.4 mg/kg meloxicam and processed; Flunixin treatment [FLU], 2.2 mg/kg flunixin meglumine and processed; or Ketoprofen treatment [KETO], 3 mg/kg ketoprofen and processed. Two hours post-dose, all all piglets underwent routine castration and tail docking (with the exception of the SAL SHAM piglets, which were not castrated or tail docked, but handled for a similar length of time and in a similar manner).

Behavior (pain) scores, grimace scores, and activity counts were obtained at multiple time points before, and after processing. Blood samples and interstitial fluid samples were obtained at similar time points as the pain data. Plasma concentrations of meloxicam and ketoprofen were obtained by HPLC with fluorescence (KETO) or UV detection (MLX); plasma and interstitial fluid flunixin concentrations, and interstitial fluid meloxicam and ketoprofen were obtained with UPLC-MS/MS detection. Prostaglandin E2 concentrations in interstitial fluid were obtained with ELISA. Plasma cortisol concentrations were determined via RIA. All data was analyzed using ANOVA.

The time to maximum concentrations (Tmax) of meloxicam, flunixin and (S-)-ketoprofen in plasma were 1.21, 0.85 and 0.59 h, respectively. Plasma half lives ($T_{1/2}$) were 4.39, 7.69 and 3.50 h, respectively. The Tmax in ISF were 2.81 and 3.64 h for meloxicam and flunixin, and tissue half lives were 11.3 and 16.3 hours, respectively.

SAL CAST cortisol levels were significantly ($p=0.0031$) higher than that of SAL SHAM at the time of processing (2 h post-dose). This suggests that it is the procedure, rather than the handling of the animals that greatly increases cortisol and is indicative of stress. Cortisol concentrations were also significantly ($p=0.0488$) lower in the FLU group compared to the SAL CAST group at the time of processing.

All the NSAIDs decreased prostaglandin E2 levels in interstitial fluid when compared to the SAL CAST group, however only flunixin was able to maintain that inhibition beyond 24 h post-dose.

Overall, meloxicam was the least effective NSAID when examining the behavior and grimace scores beyond 6 h post-dose. At the time of processing, all the NSAID treated groups had increased pain behaviors and increased pain scores. This may have been noted because the piglets were more active, making the active pain-related behaviors more obvious to the observers (e.g. scratching at castration site), compared to the SAL CAST group which showed more inactive behaviors (e.g. laying still and protecting the castrated site). Activity levels, obtained via Actical® monitoring, were also decreased in the SAL CAST and MLX piglets following processing. This was likely related to the reduced number of active pain-related behaviors.

In conclusion, this study found that the administration of NSAIDs 2 hours prior to castration and tail docking had a positive impact on pain alleviation in piglets, with flunixin demonstrating superiority compared with ketoprofen and meloxicam, and meloxicam being the least efficacious at the doses and routes used. Management strategies that include the administration of flunixin to reduce pain associated with processing will improve piglet health and welfare in the United States.

INTRODUCTION

Piglets in the United States routinely undergo painful procedures, collectively known as processing. Processing includes tail-docking and castration of male piglets, which is considered painful and can negatively impact the welfare of these animals (Molony & Kent, 1997; Hay *et al.*, 2003; Prunier *et al.*, 2006; Von Borell *et al.*, 2009; Dzikamunhenga *et al.*, 2014). Currently, there is no requirement for the provision of analgesics for piglets in the US, however in the EU and Canada, legislation requires that piglets receive anesthetic and/or analgesic drugs during processing procedures (European Commission, 2010; NFACC, 2014). Additionally, routine tail-docking is forbidden in the EU and must only be performed when there is evidence that tail biting has occurred (European Union, 2009). Legislation in the United States will undoubtedly follow suit, and currently there are no FDA-approved analgesic drugs for swine in the US. The United States pork industry is a multi-billion-dollar business, and changes in legislation such as the provision of analgesia to piglets would have significant economic impact on pork producers.

Analgesics are approved for use in pigs in multiple other countries, specifically, compounds belonging to the nonsteroidal anti-inflammatory drug (NSAID) class. However, there is conflicting data on the efficacy of some of these drugs (Keita *et al.*, 2010; Sutherland *et al.*, 2012; Kluivers-Poodt, 2013; Bates *et al.*, 2014; Dzikamunhenga *et al.*, 2014; Tenbergen et al., 2014), which may be due to the doses and/or methodology used to assess efficacy. Few studies extensively investigate both the pharmacokinetic and pharmacodynamic relationships of these drugs in neonatal piglets (those less than 10 days of age) the age at which routine processing occurs in the US (Fosse *et al.*, 2008; Fosse *et al*., 2010; Fosse *et al.*, 2011), and even fewer studies directly compare the pharmacokinetics and pharmacodynamics of multiple NSAIDs against one another (Viscardi & Turner, 2018)

Studies have suggested that plasma drug concentrations do not always reflect tissue drug concentrations, particularly for NSAIDs, which may become “trapped” at sites of inflammation (Lees et al., 2004; Brune & Furst, 2007; Messenger et al., 2016). A minimally invasive technique (in vivo ultrafiltration) has been previously utilized to collect tissue interstitial fluid (ISF) over time (Messenger *et al.*, 2012; Messenger *et al.*, 2016). This fluid can be analyzed for only the pharmacologically active, protein-unbound drug concentrations, critical to assess drug concentrations directly at the tissue level. In addition, unbound drug concentrations can be correlated with objective biomarkers of inflammation, such as prostaglandin E2 (PGE2) thereby establishing therapeutic drug concentrations directly at the effect site. No similar studies have been performed for NSAIDs in piglets.

The objectives of this study were to describe the pharmacokinetics and pharmacodynamics of 3 different NSAIDs in neonatal piglets, and to correlate plasma and tissue drug concentrations with anti-inflammatory and analgesic effects in animals undergoing routine tail docking and castration. *The importance of this work to producers was to identify the most effective NSAID to use to provide analgesia to piglets undergoing routine processing, in order to improve piglet welfare in the United States.*

Specific Objectives from the Research Proposal:

Objective 1: Describe the plasma and tissue pharmacokinetics of the following analgesic drugs in neonatal pigs: meloxicam, flunixin, and ketoprofen. We hypothesized that the plasma PK of NSAIDs in neonatal piglets will differ from those estimates reported in older pigs, and we hypothesize that tissue drug concentrations and kinetics will not reflect plasma data. Results from these studies can be used to make dosing recommendations that will maximize the therapeutic efficacy of these analgesics in piglets. Quantifying the tissue concentrations of these drugs will determine whether plasma concentrations reflect drug levels at the sites of inflammation. This determination could alter dosing recommendations and may significantly impact producers, particularly if drug concentrations remain elevated in the tissues far longer than that predicted by the plasma.

Objective 2: Determine the anti-inflammatory and analgesic effects of the previously listed analgesic drugs in neonatal piglets. We hypothesized that the NSAIDs studied will have significant long term anti-inflammatory and analgesic effects in neonatal piglets. The use of biomarkers to study inflammation at sites of action will be used in conjunction with pain scoring, and this information will be correlated with both plasma and tissue NSAID concentrations in piglets undergoing tail docking and castration. We expect that these results will demonstrate conclusively that NSAIDs reduce inflammation and provide long lasting analgesia to piglets.

MATERIALS AND METHODS

Animals

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC). A total of 48 Yorkshire/Landrace cross male piglets (weighing 3.80-3.21 kg) were housed in individual cages and were able to see each other. Lighting consisted of 12h/12h light/dark, and heat lamps were positioned above the piglets on one end of the individual pens. Ambient room temperature was maintained at 80-85 degrees Celsius. Once removed from the sow, piglets were fed non-medicated swine milk replacer (Milk Specialties Global, Eden Prairie, MN, USA) and offered fresh water every 4 hours from 7 am to 12 am. Piglets were weighed daily on a calibrated scale.

Piglets were randomized and allocated to one of five treatment groups. Treatments included: 1) Saline with sham castration (SAL SHAM; n=9), saline with processing (SAL CAST; n=8), meloxicam and processing (MLX; n=8), flunixin and processing (FLU; n=7) and ketoprofen and processing (KETO; n=8).

Catheter and Interstitial Probe Placement

At 4 days of age (±1 day), piglets were removed from the sow and moved to individual housing to prevent damage to sampling apparatus (instrumentation on piglet shown in Appendix 1). Piglets were anesthetized using sevoflurane (SevoFlo®, Zoetis, Parsippany, NJ) administered in 100% oxygen via face mask. An indwelling jugular catheter (22 Ga, 10 cm small animal long term venous catheterization kit, MILA International, Inc., Florence, KY, USA) was used for collection of blood samples. At the time of IV catheter placement, an ultrafiltration probe (Canine UF Probe, BASi systems, W. LaFayette, IN, USA) was placed subcutaneously along the epaxial muscles using a previously described technique (Messenger *et al.*, 2012). The interstitial probe allowed for continuous collection of interstitial fluid (ISF). Piglets were able to recover for 36-48 hours following the instrumentation procedure. During this recovery period, patency of the catheter was maintained by removing the heparin lock, flushing the catheter with saline and replacing the heparin lock every 12 hours.

Drug Administration and Processing

At 6 days of age (±1 day) piglets were injected intramuscularly with one of four treatments. One hundred microliters of saline (both SAL SHAM and SAL CAST), 0.4 mg/kg meloxicam (Meloxicam solution for injection 5 mg/mL, Putney, Inc., Portland, ME, USA), 2.2 mg/kg flunixin (Banamine-S®, Merck Animal Health, Summit, NJ, USA) or 3 mg/kg ketoprofen (Ketofen®, Zoetis, Inc., Kalamazoo, MI, USA). Two hours after drug administration, piglets were processed. Piglets were restrained to expose the anogenital region of the piglet, while a second person performed the procedure. An incision was made on each side of the scrotum using a scalpel, the testicles were pulled from the surrounding tissue and the scalpel was used to cut the testicles free. The tail was then docked using standard tail clippers. Both the castration site and tail were sprayed with betadine to disinfect the wounds. SHAM piglets were handled for approximately the same length of time as other pigs, and handled in a similar manner, but not castrated or tail-docked.

Sample Collection

Blood samples (1 mL) were collected and transferred into lithium heparin tubes via the jugular catheter at 0 (baseline), 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 hours post-dose. Blood samples were centrifuged at 3500 x g and the plasma collected for analysis of total drug concentrations and plasma cortisol levels. Interstitial fluid samples were collected at 0 (baseline), 2, 4, 6, 8, 12, 24, 36 and 48 hours post-dose and weighed to determine the volume collected. At the end of the experiment, the ISF probe was removed and the tubing length measured. A lag time for the ISF collection was calculated to account for the time taken for the sample to travel along the ISF probe tubing.

Interstitial fluid was collected via the preplaced collection probes, which was used to quantify the free (protein unbound/pharmacologically active portion) drug concentrations in

the tissues, as well as prostaglandin E2, a biomarker of inflammation. Both plasma and ISF were frozen at -80 ºC until analysis. Piglets were weighed daily (i.e. 48 and 24 hours pre-dose and 0, 24 and 48 hours post-dose).

Behavioral Data Collection/Pain Assessments

Photographs were taken of the left, right and front of each piglet's face at the same time points as ISF collection for grimace scoring (as previously described by Viscardi *et al.*, 2017). These images were then blinded (by naming the image files based on a random number generated in Microsoft Excel), and scored by 2 blinded observers (possible scores 0-5). If an image was scored with a difference of >1 between observers, a third observer scored that image. The scores were averaged between observers and across the 3 images to provide a single score at each time for each piglet.

A composite pain score (possible scores 5-22) was used to collect numerical data to quantify pain, made up of 6 categories; restlessness, vocalization, pain behavior, aggression, posture and feeding (Appendix 1). Pain behaviors included observations such as tail wagging, scratching at castration site or trembling.

Activity monitors (Actical®, Philips Respironics, Bend, OR, USA) were secured to the back of the piglets' neck with Ioban™ (3M, St. Paul, MN, USA). These were placed at the time of catheter and ISF probe placement and recorded continuously until 48 hours post-dose. Actical® monitors are omni-directional activity monitors previously described by Gruen *et al.* (2017). Activity (accelerometry) data was recorded at one-minute intervals throughout, and the 24 hours immediately prior to dose were used as baseline.

Plasma and Interstitial Fluid Drug Concentration Analysis

Meloxicam Plasma Analysis

Plasma concentrations of meloxicam were determined using high-performance liquid chromatography (HPLC; 1260 Infinity HPLC system with a multiwavelength detector, Agilent Technologies, Wilmington, DE, USA). The UV detector was set at a wavelength of 365nm. The column was a 4.6 mm x 150 mm C18 column (Zorbax SB-C18; MAC-MODAnalytical, Inc., Chadds Ford, PA, USA) kept at a constant temperature of 40°C, and a flow rate of 1 mL/min. Mobile phase consisted of 60% 0.05M sodium acetate buffer (pH 3.75) and 40% acetonitrile (ACN).

Meloxicam plasma samples, calibration samples and blank (control) were prepared using solid phase extraction (1cc Waters Oasis Extraction Cartridges, Waters Corporation, Milford, MA, USA), conditioned with 1 mL methanol followed by 1 mL distilled water. A plasma sample was added to a conditioned cartridge, washed with 1 mL water:methanol (95:5 v/v), and then eluted with 1 mL 100% methanol. Samples were then evaporated at 40°C for 15-20 minutes. Each sample was then reconstituted with 200 µL of mobile phase and vortexed. Twenty-five microliters were then injected into the HPLC system.

All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification for both meloxicam in plasma was 0.01 µg/mL, which was determined from the lowest point on a linear calibration curve that produced an appropriate signal-to-noise ratio, using guidelines published by the United States Pharmacopeia (2007).

Ketoprofen Plasma Analysis

Plasma concentrations of ketoprofen were analyzed using the same HPLC system as the meloxicam plasma samples. For ketoprofen, the UV detector was set at a wavelength of 255nm. The column was a 4.6 x 150 mm, 5 um chiral column (Agilent Ultron ES-OVM; Agilent Technologies), kept at 25°C. Mobile phase consisted of 89% 0.02M potassium monobasic phosphate buffer and 11% ACN.

Ketoprofen plasma samples, calibration samples and blank (control) were prepared using solid phase extraction (3cc Waters Oasis Extraction Cartridges, Waters), conditioned with 1 mL methanol followed by 1 mL distilled water. A plasma sample was added to a conditioned cartridge, washed with 1mL water:ammonium hydroxide (95:5 v/v), and then eluted with 1 mL methanol:formic acid (98:2). Samples were then evaporated at 30°C for 20-

30 minutes. Each sample was then reconstituted with 200 µL of water and vortexed. Thirty microliters were then injected into the HPLC system. Standards spiked with S(-)-ketoprofen only, were also analyzed at the same time to determine the retention time, allowing separate identification of the S(-)- and R(+)-enantiomer.

All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification for both S(-)- and R(+)-ketoprofen in plasma was 0.05 µg/mL, which was determined from the lowest point on a linear calibration curve that produced an appropriate signal-to-noise ratio, using guidelines published by the United States Pharmacopeia (2007).

Flunixin Plasma Analysis

Flunixin plasma concentrations were quantified by ultra-high-pressure liquid chromatography (UPLC) with mass spectrometric (MS/MS) detection (Waters Corp., Milford, MA, USA). The UPLC-MS/MS system consisted of a Xevo TQD tandem quadrupole mass spectrometer (Waters Corp.) For all flunixin sample matrices, the UPLC-MS/MS analysis consisted of a 2.1 mm x 100 mm, 1.8 um HSS T3 column (Waters Corp.) A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in acetonitrile (70:30 v/v) with a flow rate of 0.4 ml/min for the first 2.5 minutes. The mobile phase then switched to (10:90 v/v) from 2.5 min - 3.5 min. For the last 1.5 min of the run, the mobile phase was (70:30 v/v). The MS/MS was run in ESI+ mode. The quantification trace used was 297 → 279. Column temperature was 35 °C and sample temperature was ambient.

Flunixin plasma samples were combined with 250 µL 0.5% citric acid in ACN, vortexed thoroughly and then centrifuged for 10 minutes at 3000 x g. The supernatant was collected and evaporated at 55 °C for 60 minutes under an 18-psi stream of air. Each sample was then reconstituted with 100 µL of water:ACN (50:50 v/v) and vortexed, filtered through a 0.2 µm filter and then injected. All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification for flunixin in plasma was 0.1 ng/mL.

Meloxicam ISF Analysis

Meloxicam ISF concentrations were quantified by UPLC-MS/MS (system information as mentioned previously). UPLC-MS/MS analysis consisted of a 2.1 mm x 50 mm, 1.7 um Waters Acquity BEH C18 column (Waters Corp.) A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in acetonitrile (65:35 v/v) with a flow rate of 0.4 ml/min for the first minute. The mobile phase then switched to (10:90 v/v) from 1.0 min – 1.1 min. For the last 1.9 min of the run, the mobile phase was (65:35 v/v). The MS/MS was run in ESI+ mode. The quantification trace used was 352.043 → 115. Column temperature was 35 °C and sample temperature was 10 °C. Fifteen microliters of ISF were combined with 50 µL MeOH, filtered through a 0.2 µm syringe filter and then injected. All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification for meloxicam in ISF was 1 ng/mL.

Flunixin ISF Analysis

Flunixin ISF samples were analyzed using the same UPLC system and conditions as described above for flunixin plasma samples. The sample preparation was the same as described for meloxicam ISF. All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification for flunixin in ISF was 0.1 ng/mL.

Plasma Cortisol Analysis

Plasma cortisol concentrations were determined using a commercial radioimmunoassay (RIA) kit ([ImmuChem™ Cortisol Coated Tube RIA Kit], MP Biomedicals, LLC., CA, USA). Samples were analyzed on a Packard Cobra gamma counter. Samples were assayed in triplicate with the reported concentration equaling the average cortisol concentration between replicates. R^2 for all calibration curves were >0.9990 and within the range of 1.0 – 25.0 µg/dL.

Interstitial Fluid Prostaglandin E2 Analysis

The concentration of plasma prostaglandin E2 (PGE2) was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Co., Ann Arbor, MI, USA). R^2 for all calibration curves were >0.98 and within the range of 7.81 – 1000 pg/mL. All samples were analyzed in duplicate.

Pharmacokinetic Analysis

A noncompartmental analysis of drug plasma concentration vs. time profiles was performed with Phoenix® WinNonLin® software (version 8.0; Certara, Princeton, NJ, USA). The area under the plasma concentration–time curve from time zero to infinity (AUC0→∞; h × µg/mL) was calculated by the linear trapezoidal rule. The AUC0→∞ was used to calculate clearance per fraction absorbed (Cl/F; L/h/kg) and half-life ($T_{1/2}$; h). The volume of distribution at steady state (per fraction absorbed) (Vdss/F; L/kg), peak concentration (Cmax; µg/mL) and time at which maximum concentration occurs (Tmax; h) were also calculated.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism (version 8.0.1, GraphPad Software, Inc., San Diego, CA, USA). All data were tested for normality by the Shapiro-Wilks test. Data that were not normally distributed were found to be lognormally distributed, and therefore were log transformed before statistical testing. All data except activity were analyzed using a mixed model procedure, including treatment, time and treatment x time interaction. Time was a repeated measure with piglet as the experimental unit. A post-hoc Tukey's test was conducted for significant outcomes.

Activity Count Statistical Analysis

For statistical analysis, activity was split into three 24-hour periods; baseline, 0-24 h post-dose and 24-48 h post-dose. Similarly to a previous study analyzing activity data (Lascelles et al., 2008), total activity counts for 6-hour periods were calculated and described as quarters (Q1, 08:00–13:59; Q2, 14:00–19:59; Q3, 20:00–01:59; and Q4, 02:00–07:59). The values for each quarter post-dose were compared to the baseline values within each treatment group. Data were analyzed using a mixed model procedure, with multiple comparisons and Tukey's test for significant outcomes. The treatment groups were also compared to the control (SAL SHAM) at each time point. This was also analyzed using a mixed model procedure but this time with Dunnett's post-hoc test.

RESULTS

Forty out of 48 piglets completed the study. Eight piglets were euthanized due to illnesses unrelated to the study (severe diarrhea, malaise, abdominal hernia, abdominal cyst), and their data were not included in the report.

Plasma and ISF Drug Concentrations (OBJECTIVE #1)

All concentration versus time data for each NSAID in plasma and ISF are presented in Figures 1-3. The plasma and ISF pharmacokinetic data for each NSAID are presented in Tables 1 and 2. Overall, our hypothesis was correct in that plasma NSAID concentrations did not predict or reflect the tissue concentration data; for example, the time to maximum concentrations and half-life were longer in the tissues and although the maximum concentrations were lower in the tissues, these data represent only plasma unbound drug concentrations. These plasma unbound concentrations are more pharmacodynamically relevant, and are expected to be lower because all NSAIDs are highly protein bound (generally > 98%).

Plasma cortisol (OBJECTIVE #2)

The effect of time was significant ($p=0.0012$) and the effect of treatment was not significant ($p=0.3145$). The interaction between treatment and time was also not significant ($p=0.2092$). However, both the SAL SHAM and FLU groups exhibited significantly lower plasma cortisol levels at 2 h than the SAL CAST group ($p=0.0031$ and $p=0.0488$, respectively). FLU piglets also had significantly lower cortisol than KETO piglets at 24 h ($p=0.0221$). The plasma cortisol results for each group are presented in Figure 4.

Interstitial Fluid Prostaglandin E2 (OBJECTIVE #2)

While no significant differences were shown between treatment groups, the SAL CAST group exhibited higher PGE2 concentrations in the ISF. All the NSAID groups lowered PGE2, however the MLX and KETO groups' PGE2 concentrations increased again after 24h.

Pain Scores (OBJECTIVE #2)

Following mixed effect model, time, treatment and treatment x time effects were all significant ($p<0.0001$, $p=0.0143$ and $p=0.0059$, respectively). Many pain behaviors that were observed in the NSAID treated groups consisted of active behaviors, such as scratching at castration site (which was designated a score of 4). It is possible that the pain scores appear higher because these pigs are more active, rather than because they experience more apparent pain, as other pain behaviors scored were recumbency and protecting of the affected limb (designated a score of 3 or 2, respectively), which did not occur concurrently with more active behaviors. The pain score data for each group over the 48 hour study period are presented in Figure 6.

Piglet Grimace Score (OBJECTIVE #2)

There were no significant differences in grimace scores between groups or over time in this study, although the SHAM group had the lowest overall scores compared to the other groups. The SAL CAST and MLX groups had the highest scores. Overall the grimace scores were low, and similar across groups (average scores between 2-3). Figure 7 presents the grimace score results over the 48 hour study period, and Appendix 3 shows an example of the 3 view photographs that were taken at each time point for each piglet.

Activity Monitoring (OBJECTIVE #2)

Time and treatment were both shown to have statistically significant effects on results ($p<0.0001$ and $p=0.0347$, respectively). The SAL CAST and MLX groups at 0-24h Q3 and Q4 were shown to be significantly lower activity levels compared to baseline at Q3 (SAL CAST, $p=0.0323$; MLX, $p=0.0480$) and Q4 (SAL CAST, $p=0.0234$; MLX, $p=0.0234$), respectively.

Both FLU and KETO groups were significantly more active when compared to SAL CAST at 24-48 h Q3 (p=0.0121 and p=0.0239, respectively). FLU piglets were also significantly more active from SAL CAST piglets at 24-48 h Q1 (p=0.0265). Finally, the FLU group were significantly more active at 24-48 h compared to 0-24 h at Q3 (p=0.0143).

Weight gain

The change in daily body weight was not significant between treatment groups (Figure 9). All piglets gained weight over the course of the study.

DISCUSSION

Meloxicam, flunixin and ketoprofen are commonly studied analgesic candidates for the provision of pain relief to piglets at processing. Each of these NSAIDs have previously demonstrated anti-inflammatory properties, reduction of plasma cortisol levels or a measurable decrease in observed pain behaviors in a variety of food animal species (Keita et al., 2010; Kluivers-Poodt et al., 2013, Bates et al., 2014; Gottardo *et al.*, 2016; Levionnois, Fosse & Ranheim, 2018; Melendez *et al.*, 2018). However, there is conflicting evidence describing the beneficial effects of meloxicam and ketoprofen when administered to piglets at castration (Keita et al., 2010; Tenbergen et al., 2014; Viscardi & Turner, 2018) and limited data available regarding the effect of flunixin or ketoprofen administration at these painful procedures (Viscardi & Turner, 2018), thus making it difficult to determine optimal recommendations for piglet pain management. This study achieved a direct comparison of meloxicam, flunixin and ketoprofen efficacy and pharmacokinetics, measuring both physiological and observational measures of pain and inflammation, and comparing to non-treated castrated and non-castrated controls. A study this detailed has not been previously described in piglets undergoing routine processing.

This study found that flunixin was the most efficacious NSAID analgesic based on the pain assessments in our study, followed by ketoprofen. Results of the present study also found that meloxicam is largely ineffective in its provision of analgesia to piglets following castration and tail-docking. Similar findings have been reported by other investigators (Viscardi & Turner, 2018). Our study was the first to employ novel methods to study behavioral indices of pain in piglets; specifically activity monitors have never been used to assess pain in piglets. The activity count data (presented in Figures 8 a and 8 b) show that both flunixin and ketoprofen-treated piglets had greater activity counts (subsets c and d) when compared to the saline castration group, and were similar to the activity counts of the saline sham group (the group that did not undergo any painful processing). In addition, the activity counts reinforce our findings on meloxicam, which was the least effective of our 3 treatment groups (subset b).

Based on the tissue (ISF) pharmacokinetic data, administration of each NSAID 2 hours prior to processing was an ideal time to administer these drugs as maximum tissue concentrations were achieved within 2 –4 hours of administration. NSAIDs are highly protein bound in the plasma, so tissue concentrations of drugs are more likely to be representative of the effective concentration at the site of action. As can be seen in Figures 1 and 2, tissue concentrations of both meloxicam and flunixin were detected at the last time point assessed (48 hours; results still pending for S(-) ketoprofen). These results are different from those reported by Fosse et al., (2008), which reported meloxicam concentrations in the exudate only to 12 hours (although higher concentrations were reported, which is likely reflective of the sampling methodology, which was a tissue cage versus an ultrafiltration probe). More importantly, this study found that flunixin inhibited PGE2 production in the tissues when compared with meloxicam (Figure 5), which suggests that flunixin may be a more potent and longer lasting COX-2 inhibitor in piglets.

The untreated controls (administered saline and either castrated or sham-castrated) accounted for any physiological or behavioral effects due to piglet handling. The results of the SHAM group demonstrate that the saline injection and handling for the sham castration had a negligible effect on pain behaviors, grimace scores, activity, cortisol, or PGE2 concentrations.

This study was the first to describe both the plasma *and tissue* pharmacokinetics of each NSAID in the intended population of animals: 6-day old piglets undergoing surgical castration and tail-docking. Our plasma pharmacokinetic results are comparable to previous reports on pharmacokinetics of meloxicam and ketoprofen in piglets of similar age or older, which was contrary to our hypothesis in Objective 1, although across studies the routes of administration, doses, and methods of pharmacokinetic analysis differ slightly (Fosse et al., 2008; Fosse et al., 2011). This study is the first to report on the tissue pharmacokinetics of each of these drugs in piglets, using a novel, minimally invasive sampling technique of in vivo ultrafiltration, and demonstrated the feasibility of this technique in neonatal piglets for the first time. The tissue concentrations of NSAIDs are more likely to represent the pharmacodynamic activity (anti-inflammatory and analgesic effects) of these drugs, which we demonstrated using PGE2 as a biomarker of inflammation. Ultrafiltration is a novel technique in piglets that could be extended to other studies to investigate the effects of new drugs, or to identify objective biomarkers of pain in neonatal piglets.

This study does have some limitations that are worth consideration. The first is that piglets were removed from their litters/sows and had to adjust to individual housing. While this is not reflective of management on the farm, it was necessary in order to maintain patency of the IV catheters and ISF probes, as well as to prevent damage to the activity monitor device. All piglets were treated, handled and housed in an identical manner, thus we do not believe this limitation affected our primary outcomes. Another consideration is that we administered the NSAID analgesics 2 hours prior to processing. In a production setting, the additional time that was taken to administer a drug, then return 2 hours later may not be practical. A 2 hour period was chosen purposefully to ensure that NSAID concentrations would reach maximum levels in the tissues- one of the main sites of anti-inflammatory and analgesic actions of these drugs. This prediction was shown to be correct– while plasma levels of NSAIDs might be high after administration, tissue levels take longer to reach maximum concentrations due to limitations in drug absorption and distribution. A final consideration, while subjective, was that it was noticed that some of the piglets tended to bleed more from their castration sites. This is suspected primarily to be a side effect of the NSAIDs, particularly ketoprofen which is known to be a nonselective COX inhibitor in many species (Lees et al., 2004). The COX 1:2 selectivity of commonly used NSAIDs in piglets has not been reported and may be an area of future research for our laboratory and would have clinical relevance if significant bleeding occurred due to the use of these drugs.

In summary, flunixin administered at 2.2 mg/kg IM 2 hours prior to processing, was shown to be the most efficacious and longest-lasting treatment in this study. Additionally, meloxicam may not be the most appropriate analgesic to prevent pain associated with piglet processing (at a dose of 0.4 mg/kg IM), despite its current use in the EU and Canada. Flunixin is already FDA approved in the US for use in pigs (although not for treatment of pain) and is a good candidate for provision of analgesia in piglets for pain and inflammation associated with processing.

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TABLES

Plasma Pharmacokinetic Parameters

Parameter	Units	Meloxicam (MLX) n= 8 piglets	Flunixin (FLU) n= 7 piglets	S(-)-Ketoprofen (KETO) n= 8 piglets
Dose	mg/kg	0.40	2.20	3.00
$T_{1/2}$	h	4.39 (1.66)	7.69 (2.67)	3.50 (0.80)
λ_z	1/h	0.19 (0.10)	0.10 (0.04)	0.21 (0.05)
T_{max}	h	1.21 (0.68)	0.85 (0.70)	0.59 (0.27)
C_{max}	µg/mL	1.61 (0.34)	4.66 (0.56)	9.13 (1.75)
AUC_{last}	h*µg/mL	10.65 (3.87)	31.99 (10.09)	52.26 (14.61)
AUC_{inf}	h*µg/mL	11.05 (4.02)	32.81 (10.83)	53.74 (14.79)
AUC_{extrap}	%	3.59 (4.51)	2.06 (2.39)	2.82 (2.30)
Vd/F	L/kg	0.23 (0.03)	0.76 (0.17)	0.29 (0.04)
Cl/F	L/h/kg	0.04 (0.02)	0.08 (0.03)	0.06 (0.02)

Table 1: Noncompartmental plasma pharmacokinetic parameters following intramuscular administration of NSAIDs (meloxicam 0.4 mg/kg, flunixin 2.2 mg/kg and ketoprofen 3 mg/kg) to 6-day-old piglets. Data are shown as mean (SD).

ISF Pharmacokinetic Parameters

Parameter	Units	Meloxicam (MLX)	Flunixin (FLU)
Dose	mg/kg	0.40	2.20
$T_{1/2}$	h	11.26 (4.15)	16.34 (7.09)
T_{max}	h	2.81 (1.00)	3.64 (1.63)
C_{max}	ng/mL	31.88 (4.13)	24.02 (8.96)
AUC_{last}	h*ng/mL	536.95 (240.33)	476.21 (210.55)
AUC_{inf}	h*ng/mL	612.78 (215.84)	577.39 (295.30)
AUC_{extrap}	%	15.86 (16.41)	14.18 (10.07)

Table 2: Noncompartmental ISF pharmacokinetic parameters following intramuscular administration of NSAIDs (meloxicam 0.4 mg/kg and flunixin 2.2 mg/kg) to 6-day-old piglets. Data are shown as mean (SD). Ketoprofen data not presented, as samples are still undergoing analysis.

FIGURES

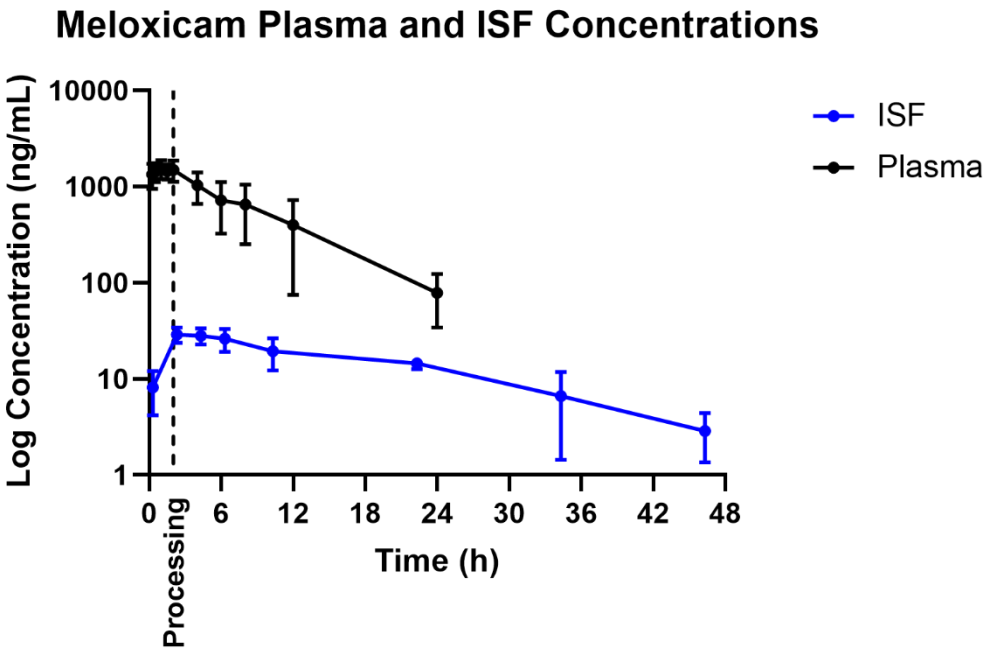


Figure 1: Mean =/- SD for total plasma concentrations and free/unbound ISF concentration over time following intramuscular injection of 0.4 mg/kg meloxicam in 6-day-old piglets (n = 8). ISF plotted with lag time (*1.68 h). Dose was administered at 0 h and processing was performed at 2 h, as indicated by the vertical dotted line.*

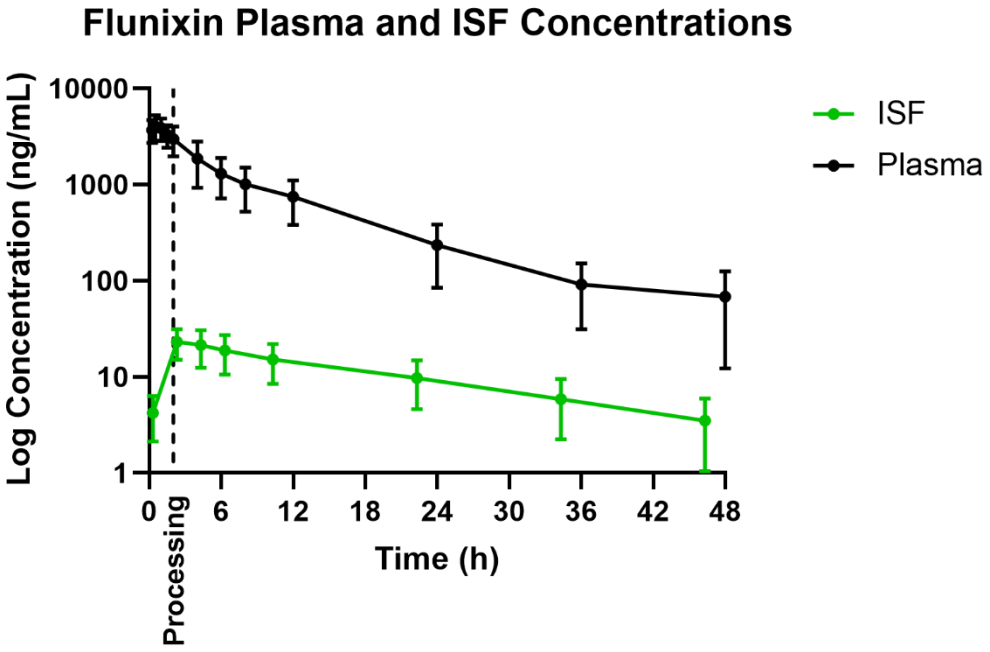


Figure 2: Mean +/- SD of total plasma concentrations and free/unbound ISF concentration over time following intramuscular injection of 2.2 mg/kg flunixin in 6-day-old piglets (n=7). ISF plotted with lag time (*1.68 h). Dose was administered at 0 h and processing was performed at 2 h, as indicated by the vertical dotted line*

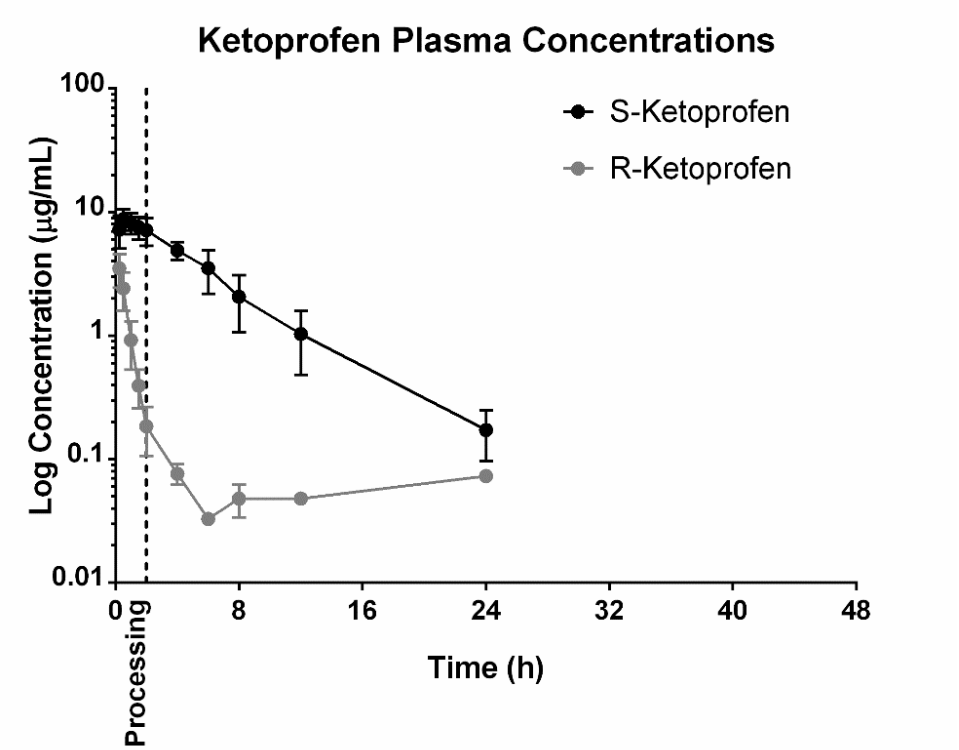


Figure 3: Mean +/-SD of total plasma concentration over time following intramuscular injection of 3 mg/kg ketoprofen in 6-day-old piglets (n=8). Both the S(-)- and R(+)-enantiomers of ketoprofen are shown, but only the S(-) enantiomer is considered pharmacologically active. Dose was administered at 0 h and processing was performed at 2 h, as indicated on the plot by a vertical dotted line.

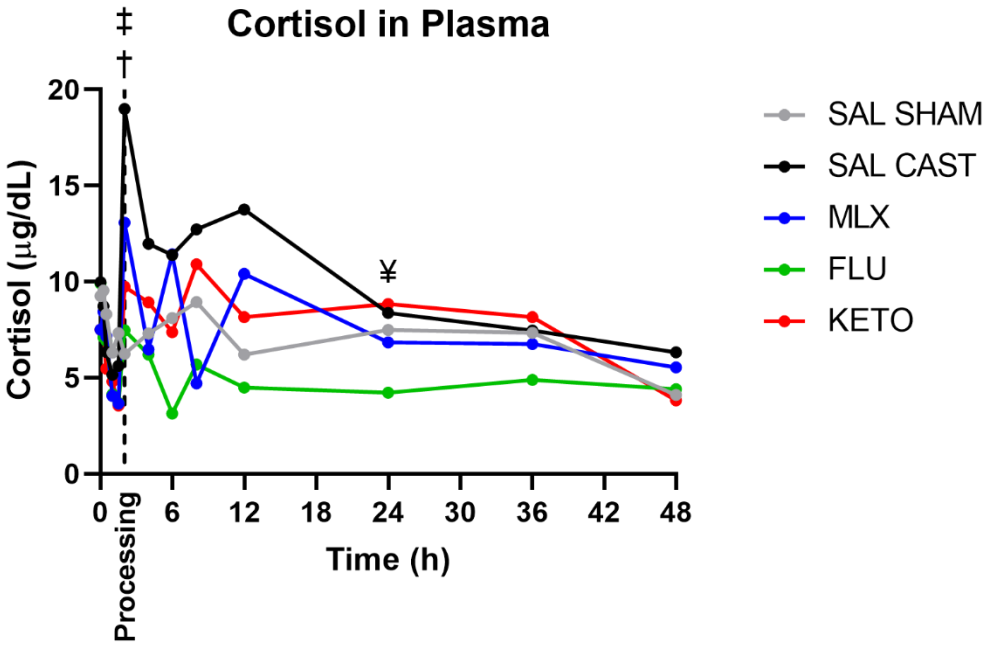


Figure 4: Total plasma cortisol concentration over time following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3 mg/kg ketoprofen in 6-day-old piglets. All piglets were processed (castrated and tail-docked) at 2 h as shown by the vertical dotted line, except for the SAL SHAM group. † indicates significant difference between SAL CAST and SAL SHAM; ‡ indicates significant difference between SAL CAST and FLU; ¥ indicates significant difference between FLU and KETO.

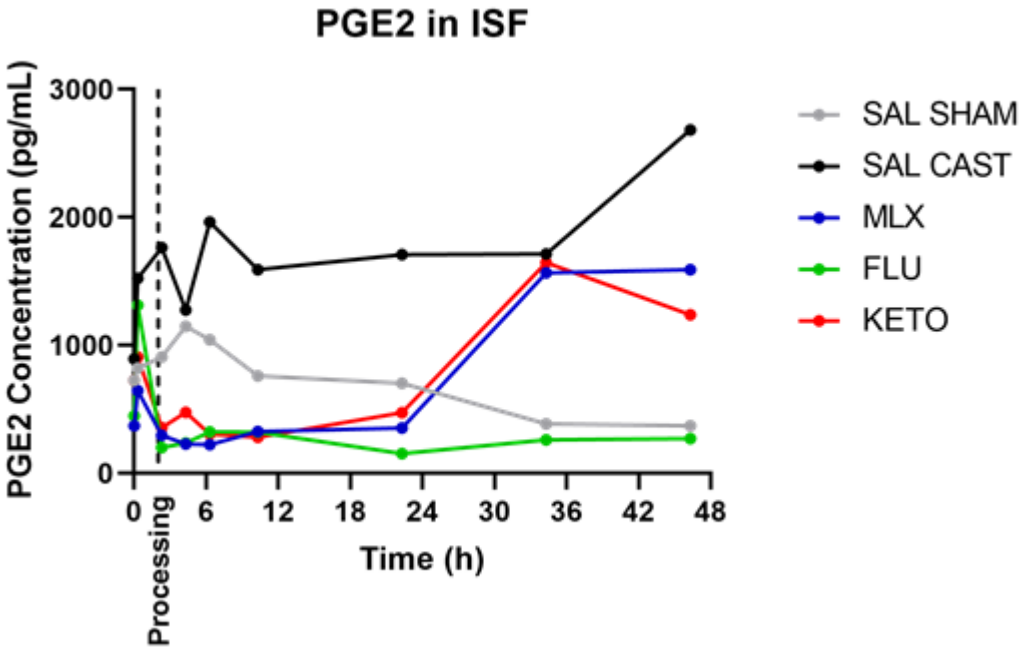


Figure 5: Total ISF PGE2 concentration over time following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3 mg/kg ketoprofen in 6-day-old piglets. All piglets were processed (castrated and tail-docked) at 2 h as shown by the vertical dotted line, except for the SAL SHAM group.

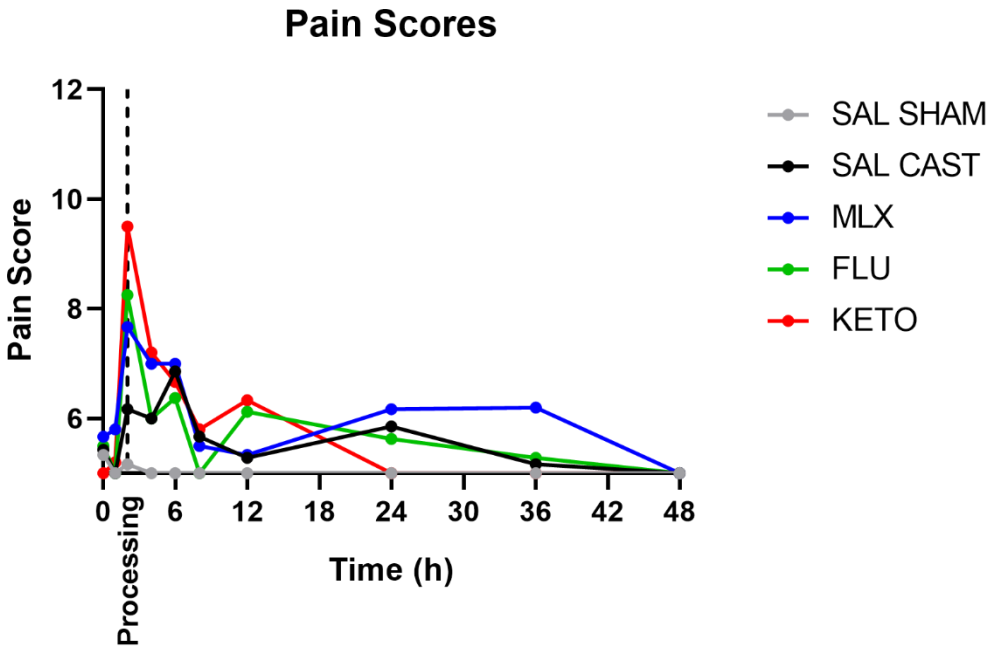


Figure 6: Pain scores over time following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3 mg/kg ketoprofen in 6-day-old piglets. All piglets were processed (castrated and tail-docked) at 2 h as shown by the vertical dotted line, except for the SAL SHAM group.

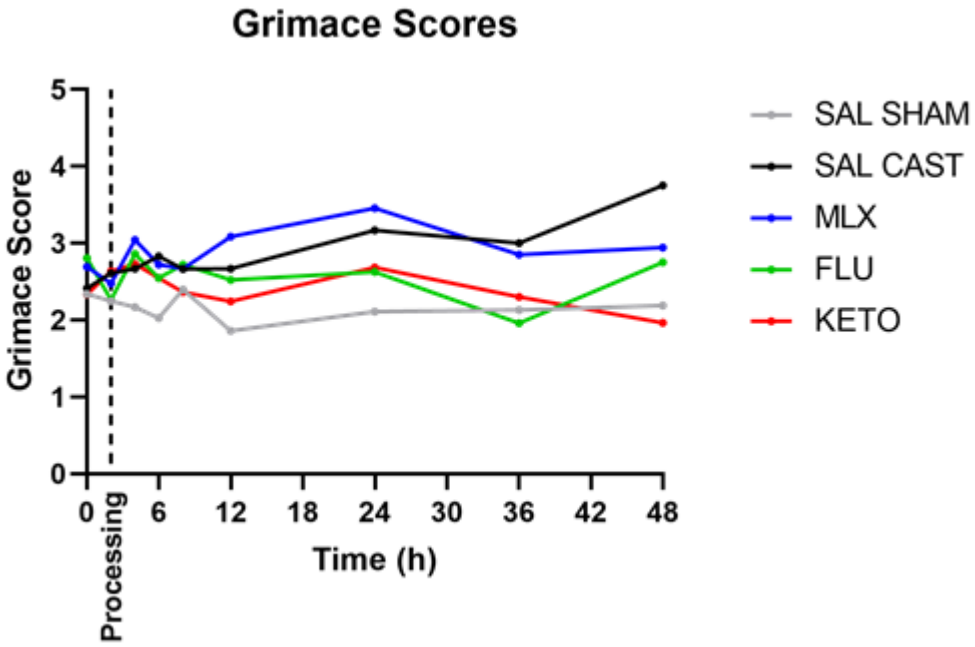


Figure 7: Average piglet grimace scores over time following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3 mg/kg ketoprofen in 6-day-old piglets. All piglets were processed (castrated and tail-docked) at 2 h as shown by the vertical dotted line, except for the SAL SHAM group. Images were scored by two blinded observers.

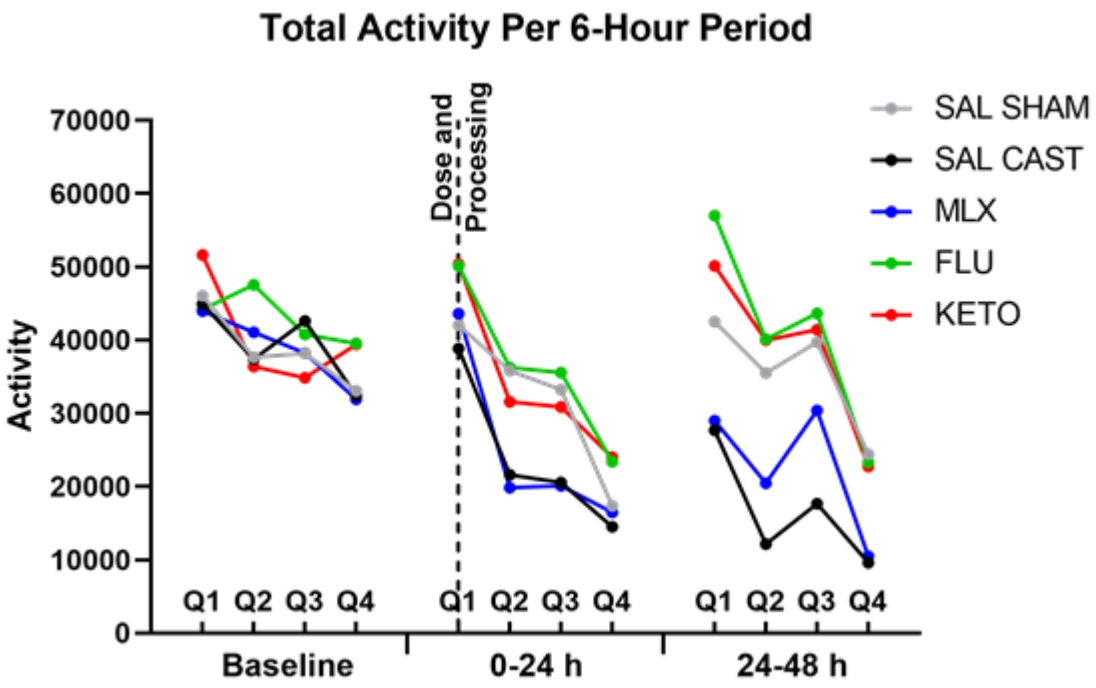


Figure 8a: Shows the total activity levels for each quarter (Q, 6-hour period) Q1, 8:00-13:59; Q2, 14:00-19:59; Q3, 20:00-01:59; Q4, 02:00-07:59.

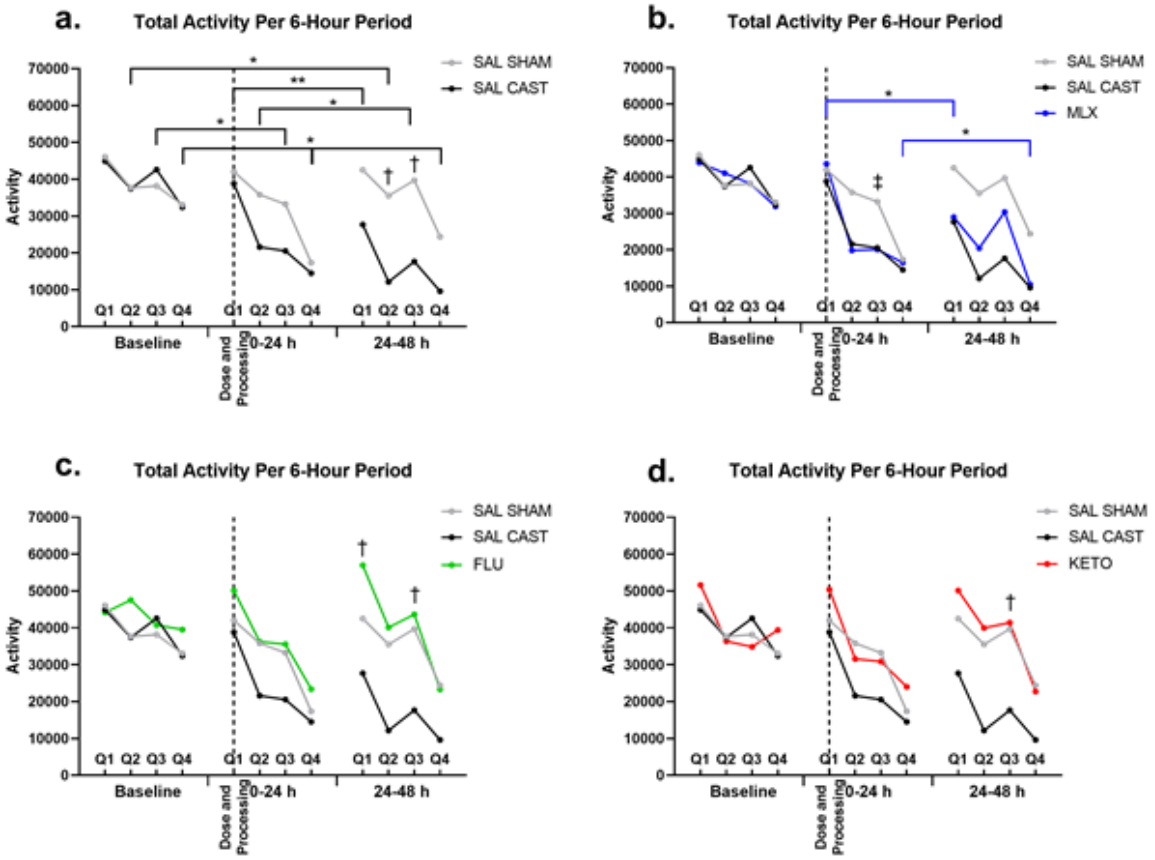


*Figure 8b: Same data as Figure 8a, but divided by treatment. Total activity levels for each quarter (Q, 6-hour period) Q1, 8:00-13:59; Q2, 14:00-19:59; Q3, 20:00-01:59; Q4, 02:00-07:59. * indicates significant difference ($p<0.05$) between the indicated time periods for CAST (graph a) and MLX (graph b). ‡ indicates significant difference between MLX and SHAM ($p<0.05$). † indicates significant difference between CAST and SHAM (graph a) or NSAID-treated group (graphs b-d).*

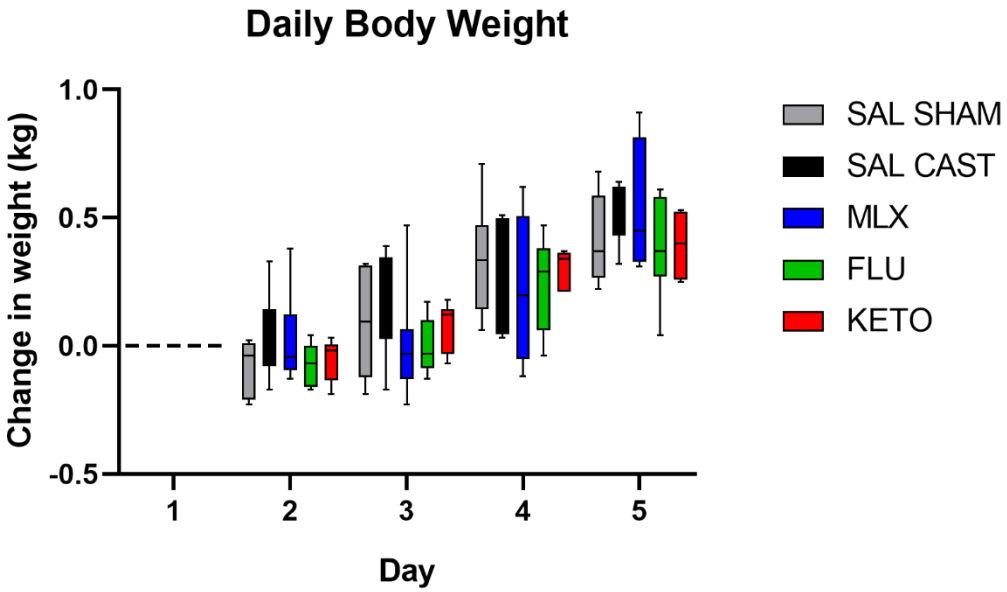
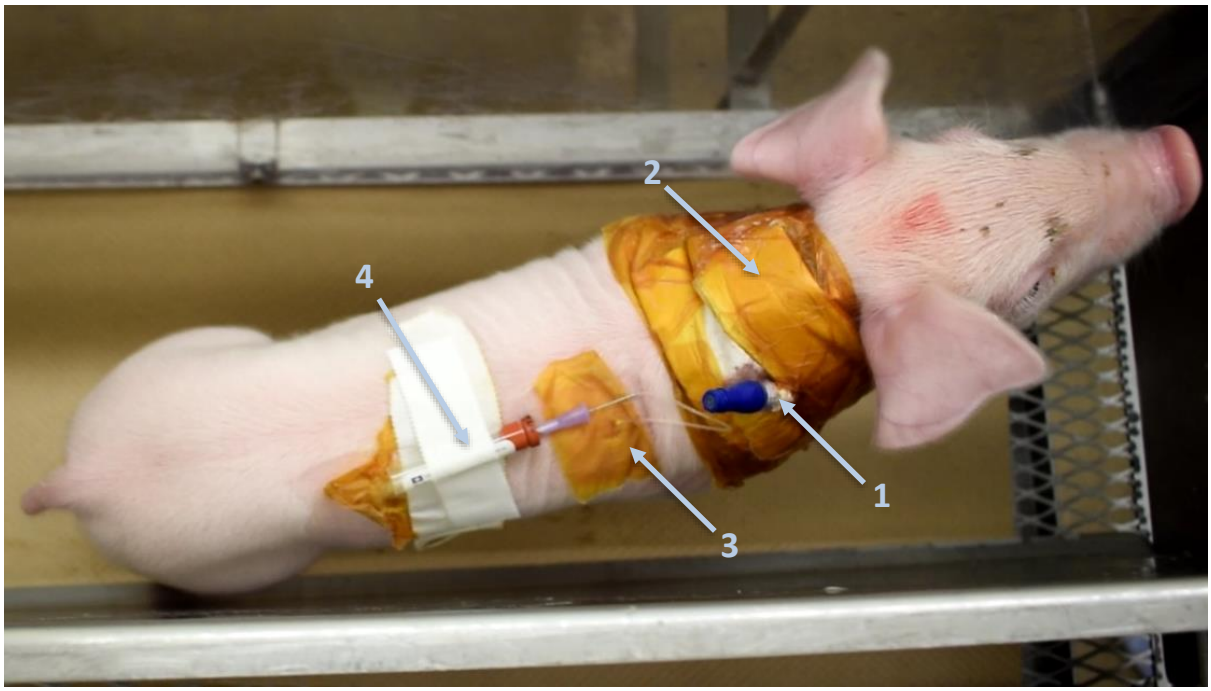


Figure 9: Change in piglet body weight over time compared to day 1. Day 1 is catheter/ISF probe placement. Day 2 is recovery. Day 3 is intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3 mg/kg ketoprofen in 6-day-old piglets. All piglets were processed (castrated and tail-docked) 2 hours after dose on day 3, except for the SAL SHAM group.

APPENDICES

Appendix 1: Image of piglet with instrumentation. 1; Catheter site is protected with Ioban, and the extension is stored in a hand-made pouch. 2; Activity monitor is hidden under Ioban to protect from damage or removal. 3; Indicates the site at which the interstitial probe comes out of the skin. 4; the vacutainer that collects the ISF.



Appendix 2: A composite pain score (possible scores 5-22) used to collect numerical data to quantify pain

Piglet Behavior Score

Reference	Score	Description
Restlessness	1	Normal sleep, rests comfortably
	2	Disrupted sleep/wake times
	3	Minimal sleeping
Vocalization	1	Low volume, occasional cries
	2	Low volume, frequent cries
	3	High volume, frequent cries
	4	Screaming when touched
Pain behavior	0	No pain behaviors
	2	Head is lower than shoulders
	3	Tail wagging: up/down or side to side
	4	Scratching at castration site/rear
	4	Trembling regularly
Aggression	1	Quite friendly
	2	Tendency to avoid interaction
	3	Biting or screaming when touched
	4	Biting or screaming without being approached
Posture	1	Slightly hunched back
	2	Protecting affected limb
	3	Recumbent or hardly moving
Feeding	1	Eating well, normal time spent at bowl
	2	Mild decrease in appetite
	3	Minimal interest in food, but does eat
	4	No appetite, ignores food

.

Appendix 3: Grimace photograph examples (score From Viscardi et al., 2017)

Pig 35

Before castration

After castration (2h)