Title: Evaluation of the Transmammary Delivery of Firocoxib in Sows to Alleviate Pain Associated with Piglet Castration, Teeth Clipping, & Tail Docking - NPB #16-118

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Industry Summary

Each year approximately 133 million piglets in the United States undergo processing procedures such as tail docking, castration and teeth clipping. Pain associated with piglet processing is an emerging animal welfare concern. We proposed that delivery of a non-steroidal anti-inflammatory drug, firocoxib, from the sow, through the milk, to nursing piglets would reduce pain associated with processing in piglets. The first study compared the drug concentrations, effectiveness, safety and tissue drug concentrations of four doses of firocoxib (0.5, 1.0, 1.5, or 2.0 mg/kg) when administered to sows and delivered to nursing piglets prior to processing. Sixteen sows, 5±2 d postpartum, were randomly assigned to one of four treatment groups. On d 0 sows received a single intramuscular dose of firocoxib at 7±1 h before piglet surgical castration, tail docking, and teeth clipping (males) or sham handling (females). Firocoxib, cortisol and prostaglandin E2 (PGE2) concentrations were determined from selected samples collected from sows and three piglets/litter (two barrows and one gilt) at 0, 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 h after drug administration. On d 21, piglets were weighed and all animals were euthanized and necropsied. Tissues were collected from 3 piglets/litter for histological examination and drug residue analysis. Firocoxib concentration were maintained in sows and piglets for a long time with the time taken for plasma drug concentrations to decrease by half ranging from 26 to 31 h in sows and 30 to 48 h in piglets. Barrows nursing sows that received 2.0 mg/kg firocoxib had a lower circulating concentrations of the stress hormone, cortisol, at 1±1 h after processing compared to barrows nursing sows that received 1.0 mg/kg (P=0.0416) and 0.5 mg/kg of firocoxib (P=0.0397). From processing to weaning, litters of sows receiving 2.0 mg/kg firocoxib gained more weight than litters of sows that received 0.5 mg/kg (P=0.008) or 1.0 mg/kg (P=0.005). No signs of toxicity were observed on
examination of the kidney, liver, stomach and small intestine and concentrations of firocoxib were below the limit of detection (0.01 µg/g) in all tissues examined from sows and piglets. These findings indicate that maternal delivery of firocoxib to suckling piglets before tail docking and castration may safely reduce processing-induced stress and enhance production by increasing weaning weights. In a second study, we evaluated changes in the temperature of the skin and the eye, using infrared thermography (IRT), and changes in gait, assessed using a pressure mat, as potential biomarkers of pain in piglets after delivery of firocoxib in the milk before processing. Eight sows (n=2 sows per replicate for 4 replicates), nursing approximately eight piglets per litter (male and female; 5 days old; minimum BW = 1.8 kg) were enrolled in the study. Replicates were conducted in January, February, July and August 2019. Sows were randomly assigned to 1 of 2 treatment groups (n = 4 sows (32 piglets)/ group). **Group 1** received 1.5 mg/kg Firocoxib intramuscularly (IM) in the right side of the neck at the time of study commence ment. **Group 2** served as a control and received a placebo injection consisting of physiological saline at a similar injection volume as sows in the firocoxib group. Treatments were administered at 6±1 h before piglet surgical castration, tail docking, and ear notching (males) or tail docking and ear nothing (females). IRT images were captured at 1 h, 2 h, 4 h, 7 h, 24 h, 30 h, 36 h and 48 h after processing. The effect of castration on piglet gait was assessed by briefly removing piglets from their pen and allowing them to walk across a pressure mat at 0.5, 7 h, 24 h, 36 h and 48 h post-processing. Female piglets had significantly greater skin temperatures compared to male piglets (P=0.0473). Skin temperature of the head was also significantly lower in piglets that were nursing sows that received firocoxib compared to control piglets at 2 h (P=0.0108) and 4 h (P=0.0316). However, skin temperatures of the head of piglets that were nursing sows that received firocoxib were higher than piglets nursing placebo-treated sows at 36h (P=0.0086) and 48h (P=0.0375) after processing. It is noteworthy that skin temperatures of piglets nursing the firocoxib-treated sows were higher than the control piglets in January and February but this effect was less evident in July and August. Eye temperatures in piglets that were nursing sows that received firocoxib were higher than piglets nursing placebo-treated sows at 1h (P=0.0207), 30h (P=0.0011) and 36 h (P=0.0024) after processing. Eye temperatures of piglets nursing the firocoxib-treated sows were also higher than the control piglets in August (P<0.0018). These observations suggest that season should be considered when infrared thermography is used to assess changes in eye and skin temperature after processing. Furthermore, compared to the skin temperature of the head, eye temperature assessment may be more robust for assessing pain in summer compared to winter. Piglets nursing sows in the control group demonstrated a significantly greater increase in the force applied to the front limbs at 7 h after processing compared to piglets nursing sows medicated with firocoxib (P=0.0127). We hypothesize that pain associated with castration results in a shift in force from the hind limbs to the front limbs of piglets to distribute weight away from the surgical site. In conclusion, these studies demonstrated the feasibility and safety of administering firocoxib in the milk to piglets before processing. Furthermore, the results of these experiments showed that at the higher doses tested, firocoxib delivered in the milk to piglets reduced markers of pain after processing. This was illustrated by a reduction in stress hormone concentrations, an increase in head and eye temperatures at later time points after processing and a reduction in processing-induced changes in weight distribution of the limbs in treated
compared to control piglets. These findings address current animal welfare concerns related to pain associated with processing in piglets and will have an immediate and significant impact on the sustainability of U.S. swine production systems. The results of this study support the use of transmammary firocoxib to mitigate pain associated with processing and thus help to maintain consumer confidence in pork production practices and keep American agriculture competitive as pain management expectations evolve.

Key words: firocoxib, transmammary, swine, animal welfare, pain, castration

Abstract

Painful processing procedures in piglets such as tail docking, castration and teeth clipping are an emerging animal welfare concern. We hypothesized that transmammary delivery of a non-steroidal anti-inflammatory drug, firocoxib, would reduce pain associated with processing in piglets. The first study compared the pharmacokinetics, efficacy, safety and tissue residue concentrations of four doses of firocoxib (0.5, 1.0, 1.5, or 2.0 mg/kg) administered to sows and delivered to nursing piglets prior to processing. Sixteen sows, 5±2 d postpartum, were randomly assigned to one of four treatment groups. On d 0 sows received a single intramuscular dose of firocoxib at 7±1 h before piglet surgical castration, tail docking, and teeth clipping (males) or sham handling (females). Firocoxib, cortisol and prostaglandin E2 (PGE2) concentrations were determined from selected samples collected from sows and three piglets/litter (two barrows and one gilt) at 0, 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 h after drug administration. On d 21, piglets were weighed and all animals were euthanized and necropsied. Tissues were collected from 3 piglets/litter for histological examination and drug residue analysis. Mean (±SEM) peak plasma firocoxib concentrations (C\text{max}) were 107.90±15.18, 157.50±24.91, 343.68±78.89, and 452.83±90.27 ng/mL in sows receiving 0.5, 1.0, 1.5, and 2.0 mg/kg firocoxib, respectively, and 9.53±1.21, 31.04±6.79, 53.30±11.1, and 44.03±7.47 ng/mL in their respective piglets. Mean plasma terminal half-life values ranged from 26 to 31 h in sows and 30 to 48 h in piglets. Barrows nursing sows that received 2.0 mg/kg firocoxib had a lower mean plasma cortisol concentration at 1±1 h after processing compared to barrows nursing sows that received 1.0 mg/kg (P=0.0416) and 0.5 mg/kg of firocoxib (P=0.0397). Piglets nursing sows that received 1.5 mg/kg firocoxib had consistently the lowest concentrations of circulating PGE2 suggesting inhibition of the cyclooxygenase enzyme by firocoxib. From processing to weaning, litters of sows receiving 2.0 mg/kg firocoxib gained more weight than litters of sows that received 0.5 mg/kg (P=0.008) or 1.0 mg/kg (P=0.005). No signs of NSAID toxicity were observed on examination of the kidney, liver, stomach and small intestine and concentrations of firocoxib and the descyclopropylmethyl metabolite were below the limit of detection (0.01 µg/g) in all tissues examined from sows and piglets. These findings indicate that maternal delivery of firocoxib to suckling piglets before tail docking and castration may safely reduce processing-induced stress and enhance production by increasing weaning weights. The second study evaluated changes in cranial skin and ocular temperature, assessed using infrared thermography (IRT), and gait, assessed using a pressure mat, as biomarkers of pain in piglets after transmammary
delivery of firocoxib prior to processing. Eight postpartum sows (n=2 sows per replicate for 4 replicates), nursing approximately eight piglets per litter (male and female; 5 days old; minimum BW = 1.8 kg) were enrolled in the study. Replicates were conducted in January, February, July and August 2019. Sows were randomly assigned to 1 of 2 treatment groups (n = 4 sows (32 piglets)/ group). **Group 1** received 1.5 mg/kg Firocoxib intramuscularly (IM) in the right side of the neck at the time of study commencement. **Group 2** served as a control and received a placebo injection consisting of physiological saline at a similar injection volume as sows in the firocoxib group. Treatments were administered at 6±1 h before piglet surgical castration, tail docking, and ear notching (males) or tail docking and ear nothing (females). IRT images were captured at 1 h, 2 h, 4 h, 7 h, 24 h, 30 h, 36 h and 48 h after processing. The effect of castration on piglet gait was assessed by briefly removing piglets from their pen and allowing them to walk across a pressure mat at 0.5, 7 h, 24 h, 36 h and 48 h post-processing. IRT and pressure mat data were analyzed using commercial software. Statistical analysis was conducted with the piglet as the experimental unit. Female piglets had significantly greater cranial skin temperatures compared to male piglets (P=0.0473). Cranial skin temperature was also significantly lower in piglets that were nursing sows that received firocoxib compared to control piglets at 2 h (P=0.0108) and 4 h (P=0.0316). However, cranial skin temperatures in piglets that were nursing sows that received firocoxib were higher than piglets nursing placebo-treated sows at 36h (P=0.0086) and 48h (P=0.0375) after processing. It is noteworthy that skin temperatures of piglets nursing the firocoxib-treated sows were higher than the control piglets in January and February but this effect was less evident in July and August. Ocular temperatures in piglets that were nursing sows that received firocoxib were higher than piglets nursing placebo-treated sows at 1h (P=0.0207), 30h (P=0.0011) and 36 h (P=0.0024) after processing. Ocular temperatures of piglets nursing the firocoxib-treated sows were also higher than the control piglets in August (P<0.0018). These observations suggest that season should be considered when infrared thermography is used to assess changes in ocular temperature after processing. Furthermore, compared to cranial skin temperatures, ocular temperature assessment may be more robust for assessing pain during warm seasons compared to cool seasons. Piglets nursing sows that received the placebo demonstrated a significantly greater increase in force applied to the front limbs at 7 h after processing compared to piglets nursing sows medicated with firocoxib (P=0.0127). We hypothesize that pain associated with castration results in a shift in force from the hind limbs to the front limbs of piglets to distribute weight away from the surgical site. In conclusion, these studies identified a candidate dose of firocoxib for transmammary delivery by dose-titration and demonstrated the feasibility and target animal safety of administering firocoxib by the transmammary route to piglets prior to processing. Furthermore, the results of these experiments demonstrated that at higher doses, transmammary firocoxib mitigated pain associated with processing as illustrated by a dose-dependent reduction in plasma cortisol concentrations, an increase in cranial and ocular temperatures at later time points after processing and a reduction in processing-induced changes in gait in treated compared to control piglets. These findings address current animal welfare concerns related to pain associated with processing in piglets and will have an immediate and significant impact on the sustainability of U.S. swine production systems. The results of this study support the use of transmammary firocoxib to mitigate pain associated with
processing and thus help to maintain consumer confidence in pork production practices and keep American agriculture competitive as pain management expectations evolve.

**Introduction**

Each year, approximately 133 million piglets in the United States undergo painful processing procedures (USDA-NASS, 2018). Pain-related behaviors have been found to persist for up to 4 d after castration in piglets (Hay et al., 2003). Consumer concern about the animal welfare implications has resulted in investigation of methods to provide analgesia and alleviate distress associated with these practices (Sutherland, 2015). In the European Union (EU Council Directive 2008/120/EC, 2008) and Canada (NFACC, 2014), mandatory use of analgesia and anesthesia is required for castration in piglets older than 7 d. However, in the US, no drugs are currently labeled by the Food and Drug Administration (FDA) to mitigate pain in swine. Therefore, development of new pain mitigation strategies would be beneficial.

Firocoxib is an NSAID with FDA-approval in the US for control of pain and inflammation associated with osteoarthritis in horses and dogs. Firocoxib is 384 times more selective for canine COX-2, the inducible isoform of the enzyme that synthesizes prostaglandins that mediate pain and inflammation, than COX-1, the constitutively induced isoform that produces prostaglandins that maintain normal renal function and gastrointestinal integrity (Vane and Botting, 1995; McCann et al., 2002, 2004).

In the present studies we tested the hypothesis that transmammary delivery of firocoxib, from the lactating sow to the nursing piglets would achieve safe and effective analgesic drug concentrations in suckling offspring that underwent processing procedures. The specific aims of these studies were to (1) describe the pharmacokinetics and transmammary delivery of firocoxib to piglets after intramuscular (IM) administration to sows, (2) investigate the effects of transmammary-delivered firocoxib on the stress response, skin and eye temperature, and performance of piglets after castration, tail docking, and teeth clipping, and (3) to assess the safety and firocoxib tissue residue concentrations in both sows and piglets at weaning.

**Objectives**

1. To investigate the pharmacokinetics and transmammary delivery of firocoxib to piglets after intramuscular administration to sows
2. To investigate the impact of transmammary delivered firocoxib on the pain response and performance of piglets after castration, tail docking and teeth clipping
3. To determine the drug residue depletion profile of firocoxib in sows and piglets to ensure that extra-label drug use complies with the requirements of the Animal Medicinal Drug Use Clarification Act (1994) (AMDUCA)
Materials and Methods

a. Study 1.

The first study was conducted to (a) investigate the pharmacokinetics and transmammary delivery of Firocoxib after intramuscular administration to lactating sows at either 0.5, 1.0, 1.5 or 2 mg/kg and (b) to describe the residue depletion of Firocoxib in edible tissues of sows and select piglets at 21 days post treatment was initiated in February 2017. Sixteen sows, nursing approximately 9 piglets, aged 5 (+/- 2) days, per sow were clinically examined and weighed on Study Day 0 prior to dosing. Cross fostering of the litters was permitted in order to provide 6 male and 3 female piglets to be sampled at time point 0.

Bodyweights were used to randomly assign sows to 1 of 4 treatment groups (n=4 sows/group) as follows; Group 01 received 0.5 mg/kg Firocoxib IM once in the neck at the time of study commencement; Group 02 received 1.0 mg/kg Firocoxib IM once in the neck at the time of study commencement; Group 03 received 1.5 mg/kg Firocoxib IM once in the neck at the time of study commencement; Group 04 received 2.0 mg/kg Firocoxib IM once in the neck at the time of study commencement.

Firocoxib (Equioxx Injection, Merial) (Lot number 4VP07, Expiration Date 11/2017) was administered to each sow at Time 0 by intramuscular injection into the right neck using an 18G, 1.5 inch needle and a 20 mL syringe according to Table 1. In cases where the dose volume exceeded 20mL, the remaining dose was administered in the left neck.

Table 1. Dosing Information

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sow ID</th>
<th>Bodyweight (kg)</th>
<th>Required Dose (mg)</th>
<th>Dose volume administered IM (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T01 (0.5 mg/kg)</td>
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<tr>
<td>1427</td>
<td>266</td>
<td>133</td>
<td>6.5</td>
<td></td>
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<tr>
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<td>127.5</td>
<td>6.5</td>
<td></td>
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<td></td>
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<td>16175</td>
<td>179</td>
<td>89.5</td>
<td>4.5</td>
<td></td>
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<tr>
<td>T02 (1.0 mg/kg)</td>
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<tr>
<td>15177</td>
<td>241</td>
<td>241</td>
<td>12.0</td>
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<td>T03 (1.5 mg/kg)</td>
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<tr>
<td>16149</td>
<td>224</td>
<td>336</td>
<td>17.0</td>
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</tbody>
</table>
On Day -1 or Day 0, approximately 3 mL of blood was collected by jugular venipuncture from all sows and piglets for determination of baseline PGE2 and Firocoxib concentrations. Prior to sow dosing, the piglets had body weight data collected.

On Day 0, the pharmacokinetic portion of the study commenced. The test article was administered intramuscularly in the right neck to sows. Blood samples for Firocoxib determination were collected by jugular venipuncture from sows and 3 piglets/litter (2 male and one female at each time point) at 0, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 hours after drug administration to the sow.

Between the 6 hour and the 8 hour blood collection time points, all male pigs had the following procedures completed: castration, teeth clipping and tail docking. Female piglet did not have any of these procedures conducted and thus served as procedural controls.

At 21 days after drug administration, sows and piglets were weighed prior to weaning to calculate average daily gain.

Thereafter, sows and piglets in all groups were humanely euthanized and examined for macroscopic signs of NSAID toxicity. Approximately 200 g of muscle, liver, kidney, fat and injection site were harvested from sows for tissue residue determination. In addition, liver, 2 kidneys and 50 g of muscle and fat were harvested from at least 3 piglets/litter (2 male and 1 female) for tissue residue determination.

**Firocoxib in plasma**

Plasma concentrations of firocoxib were determined using high-pressure liquid chromatography (Agilent 1100 Pump, Column Compartment and Autosampler, Agilent Technologies, Santa Clara, CA, USA) with mass spectrometry detection (LTQ Ion Trap, Thermo Scientific, San Jose, CA, USA). Plasma samples, plasma spikes, plasma QC’s, and blanks, 100 µL, were protein precipitated in 1.5 mL microcentrifuge tubes with 400 µL of acetonitrile/0.1% formic acid. An internal standard, d6-firocoxib, was incorporated into the acetonitrile precipitating agent at a concentration of 200ng/mL. The samples were vortexed for 5 seconds after the addition of the acetonitrile and centrifuged for 20 minutes at 7500 rpm to sediment the protein pellet. Following centrifugation, the supernatant was poured off into cell culture tubes and evaporated to dryness in a
Turbovap at 48° C. The tube contents were reconstituted with 150 µL of 25% acetonitrile and transferred to autosampler vials equipped with 300 µL glass inserts. The samples were centrifuged at 2,500 rpm prior to LC-MS analysis.

For LC-MS analysis the injection volume was set to 25 µL. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a flow rate of 0.275 mL/min. The mobile phase began at 25% B with a linear gradient to 95% B in 5 minutes, which was maintained for 1.25 minutes at 0.325 mL/min, followed by re-equilibration to 25% B. Separation was achieved with a HypersilGoldC18 column, 100 mm x 2.1 mm, 3 µm particles, Thermo Scientific, San Jose, CA, USA) maintained at 50°C. Firocoxib and d6-firocoxib each eluted at 4.9 minutes. Full scan MS was used for analyte detection and three fragment ions were used for quantitation of each analyte species. The fragment ions for firocoxib were at 283, 265, and 237 m/z, while ions at 289, 270, and 243 m/z were characteristic of d6-firocoxib fragmentation. The descyclopropylmethyl metabolite produced a single fragment ion at 209 m/z. Firocoxib and d6-firocoxib were analysed in positive ion mode. The mass spectrometer was optimized for detection of firocoxib by infusion of a 10 µg/mL solution of firocoxib into the mobile phase of 80% B. Detection of firocoxib was greatly enhanced with a transfer capillary temperature of 350° C. Sequences consisting of plasma blanks (porcine plasma), calibration spikes, QC’s, and porcine plasma samples were batch processed with a processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA). The processing method automatically identified and integrated each peak in each sample and calculated the calibration curve based on a weighted (1/X) quadratic or linear fit. Twelve calibration spikes were prepared in blank porcine plasma covering the concentration range of 1 to 5000 ng/mL for porcine sow samples. Piglet samples were analysed with a narrower range of calibration spikes covering 1 to 500 ng/mL. A linear (1/X) fit was used for piglet plasma samples and the narrower 1-500 ng/mL concentration range. A quadratic (1/X) fit was used for sow plasma samples and the 1-5000 ng/mL concentration range. Plasma concentrations of firocoxib in unknown samples were calculated by the Xcalibur software based on the calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. Calibration curves exhibited a correlation coefficient (r^2) exceeding 0.995 across the concentration range. QC samples at 7.5, 15, 35, 75, 150, and 1500 ng/mL were within a tolerance of ± 15% of the nominal value. The limit of quantitation (LOQ) of the analysis was 1.0 ng/mL with a limit of detection (LOD) of 0.2 ng/mL.

**Firocoxib in tissues**

Tissue concentrations of firocoxib and its descyclopropylmethyl metabolite were determined using high-pressure liquid chromatography (Agilent 1100 Pump, Column Compartment and Autosampler, Agilent Technologies, Santa Clara, CA, USA) with mass spectrometry detection (LTQ Ion Trap, Thermo Scientific, San Jose, CA, USA).
The tissue samples analysed were muscle, injection site, kidney, liver, and fat. Tissue samples were thawed and homogenized in a Waring blender prior to extraction and analysis. Tissue samples, tissue spikes, and blanks, 1 gram of tissue homogenate, were extracted with 10 mL of a 4:1 mixture of acetonitrile:water in a 50 mL centrifuge tube. An internal standard, d6-firocoxib, was added to the tissue homogenate, prior to extraction with 25 µL of a 100 ng/µL solution. The solvent extraction was performed on a multi-tube vortex mixer at 2500 rpm for 15 minutes after the addition of the acetonitrile mixture. Subsequently, the extracted samples were centrifuged for 5 minutes at 3000 rpm and filtered through glass fiber filters into 15 mL centrifuge tubes. Finally, 1 mL of each extract was pipetted into cell culture tubes and evaporated to dryness in a Turbovap at 48°C. The tube contents were reconstituted with 150 µL of 25% acetonitrile and transferred to autosampler vials equipped with 300 µL glass inserts. The samples were centrifuged at 2,500 rpm prior to LC-MS analysis.

For LC-MS analysis the injection volume was set to 25 µL. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a flow rate of 0.275 mL/min. The mobile phase began at 25% B with a linear gradient to 95% B in 5 minutes, which was maintained for 1.25 minutes at 0.325 mL/min, followed by re-equilibration to 25% B. Separation was achieved with a HypersilGoldC18 column, 100 mm x 2.1 mm, 3 µm particles, Thermo Scientific, San Jose, CA, USA) maintained at 50°C. Firocoxib and d6-firocoxib each eluted at 4.9 minutes while descyclopropylmethyl–firocoxib eluted at 3.0 minutes. Full scan MS was used for analyte detection and three fragment ions were used for quantitation of each analyte species. The fragment ions for firocoxib were at 283, 265, and 237 m/z, while ions at 289, 270, and 243 m/z were characteristic of d6-firocoxib fragmentation. The descyclopropylmethyl metabolite produced a single fragment ion at 209 m/z. Firocoxib and d6-firocoxib were analysed in positive ion mode while detection of descyclopropylmethyl-firoxib was only possible in negative ion mode. The mass spectrometer was optimized for detection of firocoxib by infusion of a 10 ug/mL solution of firocoxib teed into the mobile phase of 80% B. Detection of firocoxib was greatly enhanced with a transfer capillary temperature of 350°C. Sequences consisting of tissue blanks, calibration spikes, and porcine tissue samples were batch processed with a processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA). The processing method automatically identified and integrated each peak in each sample and calculated the calibration curve based on a weighted (1/X) linear fit. Eight calibration spikes were prepared in blank porcine tissue covering the concentration range of 0.05 to 10 ug/g for porcine sow samples. Tissue concentrations of firocoxib and descyclopropylmethyl-firoxib in unknown samples were calculated by the Xcalibur software based on the calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. Calibration curves exhibited a correlation coefficient (r²) exceeding 0.99 across the concentration range. The limit of
Quantitation (LOQ) of the analysis was 0.05 ug/g with a limit of detection (LOD) of 0.01 ug/g.

Pharmacokinetic analysis of data

The firocoxib plasma concentration versus time profile from each sow of four treatment groups treated intramuscularly with firocoxib at dose of 2mg/kg, 1.5mg/kg, 1mg/kg and 0.5mg/kg, were subjected to PK analysis using commercially available software (Phoenix® Win-Nonlin® 7.0, Certara, Inc. Princeton, NJ, USA). The data were analyzed using non-compartmental methods implemented in the software with Model Type Plasma (200-202) with uniform weighting. The PK parameters determined were; slope of the terminal phase ($\lambda_z$), terminal half-life ($T_{1/2 \lambda_z}$), maximum plasma concentration ($C_{\text{max}}$), time to achieve peak concentration ($T_{\text{max}}$), area under the concentration time curve (AUC), area under the first moment of the concentration-time curve (AUMC), apparent volume of distribution during the elimination phase ($V_z/F$) and apparent systemic clearance ($CL/F$) and mean residence time (MRT). The rate constant ($\lambda_z$) associated with the terminal phase was calculated by means of linear regression of the terminal part of the log plasma concentration versus time curve, and the $T_{1/2 \lambda_z}$ was calculated using standard pharmacokinetic equations. A The Linear Trapezoidal Linear Interpolation method was used to determine AUC and AUMC. For the calculation of $AUC_{\text{last}}$ and $AUMC_{\text{last}}$, time range from the first measurement to the last measurement of drug concentration, as well as the extrapolation to infinity ($AUC_{\infty}$, $AUMC_{\infty}$) was used. An AUC and AUMC were extrapolated to infinity to account for the total exposure of firocoxib to sows.

For each treatment, plasma firocoxib concentrations versus time data of piglets ($n=36$ per treatment) were subjected to non-compartment analysis using sparse data option available in the software (Phoenix® Win-Nonlin® 7.0, Certara, Inc. Princeton, NJ, USA). PK parameters; $\lambda_z$, $T_{1/2 \lambda_z}$, $C_{\text{max}}$, $T_{\text{max}}$, AUC, AUMC and MRT were generated. Firocoxib exposure to piglets through milk consumed from treated mothers were determined by comparing the firocoxib AUC determined in piglets with the drug AUC calculated for their corresponding sows (mothers) of each treatment. The % exposure was determined using the equation:

$$% \text{Exposure} = 100 \times \frac{AUC(\text{piglet})}{AUC(\text{sow})}$$

Plasma cortisol concentrations

Plasma cortisol concentrations were determined using a commercially available radioimmunoassay (CortiCote I-125, MP Biomedicals, Santa Ana, CA). Samples were run in duplicates and repeated if a large difference in cortisol levels between the samples were determined. The assay had a detection range of 0.64 to 150 ng/mL. The
coefficient of variation for the intra-assay variability was 9.33 % and the inter-assay variability was calculated to be 10.58%.

**Plasma prostaglandin E2 (PGE2) concentrations**

For PGE2 analysis, plasma proteins were precipitated using methanol in a 1:5 ratio of 1 part plasma and 5 parts methanol. Following protein precipitation, samples were centrifuged at 3,000 g for 10 minutes and the supernatant was used to determine PGE2. PGE2 concentrations were determined using a commercially available PGE2 ELISA kit (Cayman Chemicals, Ann Arbor, MI).

b. **Study 2. Assessment of piglet gait using a pressure mat and cranial skin temperatures using infrared thermography**

A study was conducted to assess the impact of processing, with or without transmammary delivery of firocoxib analgesia, on gait and cranial skin temperature in piglets. Eight sows (n=2 sows replicate for 4 replicates), nursing approximately 8 piglets per litter (male and female; 5 days old; minimum BW = 1.8 kg) were enrolled in the study. Replicates were conducted in January, February, July and August 2019. Sows were randomly assigned to 1 of 2 treatment groups (n = 4 sows/ group): Group 1 received 1.5 mg/kg Firocoxib intramuscularly (IM) in the right side of the neck at the time of study commencement (5 days after farrowing). Group 2 served as a control and received a placebo injection consisting of physiological saline at a similar injection volume as sows in the firocoxib group.

Approximately 6 hours after firocoxib was administered to sows, piglets within each litter were removed and weighed. All male piglets were then surgically castrated using a scalpel and two vertical incisions and testicles were removed by tearing the spermatic cord. Both male and female piglets were tail docked using side pliers. Ear notching for identification purposes was also performed on both male and female piglets at the time of processing.

Immediately after processing, piglets were placed individually in a holding cart and temperatures of their cranium were measured using an infrared thermography (IRT) camera (Fluke TiX580, Fluke Corporation, Everett, WA). Piglets were then placed back into their home pen. IRT images were captured at the following post-processing time points: 1 h, 2 h, 4 h, 7 h, 24 h, 30 h, 36 h and 48 h.

The impact of castration on piglet gait was assessed by briefly removing piglets from their pen and allowing them to walk across a pressure mat (MatScan, Tekscan, Inc., South Boston, MA). This occurred at 0.5, 7 h, 24 h, 36 h and 48 h post-processing.
Data were analyzed using a commercial software program. Results were summarized in Microsoft Excel. Data were analyzed using a commercial statistical software program (JMP® Pro 14.3.0, SAS, Cary, NC). The individual piglet was designated as the experimental unit. Data were analyzed using a Mixed Effects statistical model. Specifically, piglet nested in treatment was designated as a Random Effect in the statistical model with time, treatment and the time-by-treatment interaction was designated as Fixed Effects in the model. Statistical significance was designated a-priori as P<0.05. In addition, sex, replicate and their interaction with treatment were also included as covariates in the model. Interactions were retained in the statistical model where these were found to be statistically significance (P<0.05) and excluded where these interactions were not statistically significant (P>0.05).
Results

a. Study 1

Firocoxib pharmacokinetics in sows following IM administration

The pharmacokinetic parameters for firocoxib following IM administration in sows is presented in Figure 1 and Table 2. Mean (±SD) peak plasma firocoxib concentrations (Cmax) of 107.90 ± 30.37, 343.68 ± 157.50 and 452.83 ± 180.55 ng/mL was observed at 3.5, 5.5, 3 and 4.5 hours respectively. Firocoxib demonstrated a prolonged plasma elimination half-life of between 26.71 ± 5.77 hours and 31.09 ± 6.73 hours in sows.

Figure 1. Mean plasma firocoxib in sows

Table 2. Pharmacokinetic parameters in sows after IM administration

<table>
<thead>
<tr>
<th>Dose</th>
<th>0.5 mg/kg IM</th>
<th>1.0 mg/kg IM</th>
<th>1.5 mg/kg IM</th>
<th>2.0 mg/kg IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Units</td>
<td>Mean</td>
<td>Std Dev</td>
<td>Mean</td>
</tr>
<tr>
<td>λz</td>
<td>1/h</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>λz-HL</td>
<td>h</td>
<td>28.87</td>
<td>13.84</td>
<td>26.71</td>
</tr>
<tr>
<td>Tlag</td>
<td>h</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Tmax</td>
<td>h</td>
<td>3.50</td>
<td>1.91</td>
<td>5.50</td>
</tr>
<tr>
<td>Cmax</td>
<td>ng/mL</td>
<td>107.90</td>
<td>30.37</td>
<td>157.50</td>
</tr>
<tr>
<td>CL_F</td>
<td>mL/h/kg</td>
<td>0.33</td>
<td>0.07</td>
<td>0.21</td>
</tr>
<tr>
<td>AUC_0-24h</td>
<td>h*ng/mL</td>
<td>1030.88</td>
<td>123.15</td>
<td>2673.25</td>
</tr>
<tr>
<td>AUC_0-last</td>
<td>h*ng/mL</td>
<td>1534.25</td>
<td>363.44</td>
<td>5331.50</td>
</tr>
<tr>
<td>AUC_∞</td>
<td>h*ng/mL</td>
<td>1586.75</td>
<td>368.40</td>
<td>5624.75</td>
</tr>
<tr>
<td>AUC_%Exp</td>
<td>%</td>
<td>3.39</td>
<td>1.31</td>
<td>4.15</td>
</tr>
<tr>
<td>AUMC_0-24h</td>
<td>h²*ng/ml</td>
<td>31764.50</td>
<td>17060.71</td>
<td>164892.00</td>
</tr>
<tr>
<td>MRT_0-last</td>
<td>h</td>
<td>27.16</td>
<td>15.28</td>
<td>29.38</td>
</tr>
<tr>
<td>MRT_0-∞</td>
<td>h</td>
<td>29.36</td>
<td>13.85</td>
<td>34.80</td>
</tr>
<tr>
<td>Vz_F</td>
<td>mL/kg</td>
<td>13.82</td>
<td>8.50</td>
<td>7.82</td>
</tr>
</tbody>
</table>
Dose linearity was investigated by plotting the Cmax and AUC for firocoxib in sows against the administered dose (Figure 2 and 3).

Based on these data it is apparent that the Cmax and AUC increased in a linear manner across the 4 doses that were investigated.

**Firocoxib pharmacokinetics in piglets after transmammary delivery**

The pharmacokinetic parameters for firocoxib following transmammary delivery to piglets is presented in Figure 4 and Table 3. Mean peak plasma firocoxib concentrations (Cmax) of 9.53 ng/mL, 31.04 ng/mL, 53.30 ng/mL and 44.03 ng/mL, was observed at 24h after firocoxib was administered to the sows. Firocoxib demonstrated a prolonged plasma elimination half-life of between 30.86 hours and 48.71 hours in piglets after transmammary delivery.
Table 3. Pharmacokinetic parameters in piglets after transmammary delivery

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>0.5 mg/kg IM</th>
<th>1.0 mg/kg IM</th>
<th>1.5 mg/kg IM</th>
<th>2.0 mg/kg IM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax</td>
<td>ng/mL</td>
<td>9.53</td>
<td>31.04</td>
<td>53.30</td>
<td>44.03</td>
</tr>
<tr>
<td>Tmax</td>
<td>h</td>
<td>24.00</td>
<td>24.00</td>
<td>24.00</td>
<td>24.00</td>
</tr>
<tr>
<td>Lamda_Z</td>
<td>1/h</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Lamda_HL</td>
<td>h</td>
<td>30.86</td>
<td>48.71</td>
<td>37.91</td>
<td>32.42</td>
</tr>
<tr>
<td>AUC_{last}</td>
<td>h*ng/mL</td>
<td>635.36</td>
<td>2,468.00</td>
<td>3,897.55</td>
<td>3,220.90</td>
</tr>
<tr>
<td>AUC_{∞}</td>
<td>h*ng/mL</td>
<td>690.47</td>
<td>3,178.00</td>
<td>4,615.17</td>
<td>3,652.32</td>
</tr>
<tr>
<td>AUC_{extrpl}</td>
<td>%</td>
<td>0.08</td>
<td>0.22</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>AUMC_{last}</td>
<td>h<em>h</em>ng/mL</td>
<td>28,443.00</td>
<td>136,805.00</td>
<td>204,246.00</td>
<td>166,016.00</td>
</tr>
<tr>
<td>MRT_{last}</td>
<td>h</td>
<td>44.76</td>
<td>55.42</td>
<td>52.41</td>
<td>51.54</td>
</tr>
<tr>
<td>AUC 0-24h</td>
<td>h*ng/mL</td>
<td>175.98</td>
<td>406.67</td>
<td>662.37</td>
<td>608.69</td>
</tr>
</tbody>
</table>

Translactational delivery of firocoxib

The concentration of firocoxib in the plasma of the piglets as a percentage of the plasma firocoxib concentrations in the medicated sows at the same time point are illustrated in Figure 5. The concentration of firocoxib in the piglets as a percentage of the firocoxib in the sow at 48 hours was significantly greater in the sows that received 0.5 mg/kg and 1.5 mg/kg firocoxib compared to the other 2 groups (P<0.05). After 48h the concentration of firocoxib in the piglets compared to the sows plateaued in the piglets nursing on the sows that received 2.0 mg/kg firocoxib. At 120 h after firocoxib administration, the concentration of firocoxib in the piglets as a percentage of the concentration in the plasma of sows was significantly greater in the 1.0 mg/kg and 1.5 mg/kg groups compared to the 0.5 mg/kg and 2.0 mg/kg groups. Upon closer examination, there was an absence of dose linearity as assessed using Cmax (Figure 6) and AUC (Figure 7). These data suggest that the passage of firocoxib from the sow plasma into the milk is likely a saturable process. This
implies that an increase in firocoxib dose to the sow above 1.5 mg/kg is unlikely to result in higher concentrations of firocoxib in the milk. Therefore 1.5 mg/kg is likely the optimal dose for transmammary delivery of firocoxib from the sow to the piglets.

Given that Area Under the plasma drug concentration Curve (AUC) represents total firocoxib exposure over time, an alternative approach to investigating the transmammary delivery of firocoxib from the sow to the piglets is to express the AUC for firocoxib in piglets as a percentage of the AUC for firocoxib in sows (Table 4).
Table 4. Total firocoxib exposure in piglets after transmammary delivery

<table>
<thead>
<tr>
<th>Sow Dose</th>
<th>Parameter</th>
<th>Sow (h*ng/mL)</th>
<th>Piglet (h*ng/mL)</th>
<th>% Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/kg</td>
<td>AUC (_{\text{last}})</td>
<td>1,534.00</td>
<td>635.36</td>
<td>41.42</td>
</tr>
<tr>
<td></td>
<td>AUC (_{\infty})</td>
<td>1,587.00</td>
<td>690.47</td>
<td>43.51</td>
</tr>
<tr>
<td>1.0 mg/kg</td>
<td>AUC (_{\text{last}})</td>
<td>5,332.00</td>
<td>2,468.00</td>
<td>46.29</td>
</tr>
<tr>
<td></td>
<td>AUC (_{\infty})</td>
<td>5,625.00</td>
<td>3,178.00</td>
<td>56.50</td>
</tr>
<tr>
<td>1.5 mg/kg</td>
<td>AUC (_{\text{last}})</td>
<td>8,323.00</td>
<td>3,897.50</td>
<td>46.83</td>
</tr>
<tr>
<td></td>
<td>AUC (_{\infty})</td>
<td>8,657.00</td>
<td>4,615.20</td>
<td>53.31</td>
</tr>
<tr>
<td>2.0 mg/kg</td>
<td>AUC (_{\text{last}})</td>
<td>12,722.00</td>
<td>3,220.90</td>
<td>25.32</td>
</tr>
<tr>
<td></td>
<td>AUC (_{\infty})</td>
<td>13,652.00</td>
<td>3,652.32</td>
<td>26.75</td>
</tr>
</tbody>
</table>

Piglets nursing on sows administered 0.5 mg/kg to 1.5 mg/kg firocoxib as a single intramuscular injection received between 41% and 46% of the total sow firocoxib exposure based on the AUC calculated using the last time point. When AUC was extrapolated to infinity, piglets nursing on sows that received 0.5 mg/kg to 1.5 mg/kg were exposed to 43% to 56% of the firocoxib that sows were exposure to. In contrast, piglets nursing on sows that received 2 mg/kg of firocoxib IM were exposed to 25% of the firocoxib that sows were exposed to. These data confirm that 1.5 mg/kg of firocoxib is likely the optimal dose to be administered to sows for transmammary delivery to piglets.

**Serum cortisol concentrations**

Plasma cortisol concentrations in piglets were determined at 6 hours after firocoxib was administered to the sows (Figure 8a). This represented the time point immediately prior to castration, tail docking and teeth clipping.

There was evidence of an effect of treatment group (P=0.0009), time (P<0.0001) and a time X treatment interaction.
interaction (P<0.0001) on plasma cortisol concentrations after castration, tail docking and teeth clipping (Figure 8a). Differences in LSMeans were compared using Student’s t-test. There was a significant difference in plasma cortisol concentrations between female piglets and processed male piglets at 2 and 6 hours after processing. Furthermore, piglets nursing on sows that received 0.5 mg/kg of firocoxib (T1) had a higher mean plasma cortisol concentration at 2 hours after processing (T8) compared to piglets nursing of sows that received 1.5 mg/kg of firocoxib (T3) (P=0.003) and 2.0 mg/kg of firocoxib (P=0.0001). Furthermore, piglets nursing on sows that received 1.0 mg/kg of firocoxib (T2) had a higher mean plasma cortisol concentration at 2 hours after processing (8 hours after sows were treated) compared to piglets nursing of sows that received 1.5 mg/kg of firocoxib (T3) (P=0.004) and 2.0 mg/kg of firocoxib (T4) (P<0.0001).

Prostaglandin E2 (PGE2) determination

PGE2 data were analyzed with treatment, time and their interaction included in the model. Baseline PGE2 concentrations were included as a covariate. There was evidence of a time X treatment interaction (P=0.0022) on plasma PGE2 concentrations after castration, tail docking and teeth clipping (Figure 8b). PGE2 concentrations were significantly higher at 2 h after the sows were injected in piglets nursing sows that received 1.0 mg/kg firocoxib compared to the other groups (P=0.0132). Similarly, PGE2 concentrations were significantly higher in piglets nursing sows that received 2.0 mg/kg firocoxib compared to the other treatment groups at 8h (P=0.0246) and 12 h (P=0.0403) after the sows were injected. Piglets nursing sows that received 1.5 mg/kg firocoxib had consistently the lowest concentrations of circulating PGE2 suggesting inhibition of the cyclooxygenase enzyme by firocoxib. However at 96 h after the sows...
were injected, piglets nursing animals that received 1.5 mg/kg firocoxib had significantly higher concentration of PGE2 compared to the other groups (P=0.0017).

**Piglet Average Daily Gain (ADG)**

Average daily gain in bodyweight over the 21 days from processing to weaning was calculated by subtracting the bodyweight at processing from the bodyweight at weaning and dividing this by 21 days (Figure 9).

![Figure 9. Mean average daily gain in piglets after processing](image)

There was evidence of an effect of treatment on average daily gain in bodyweight over the 21 days between processing and weaning (P=0.0257). Bodyweight change in the piglets appeared to increase with increasing doses administered to the sows. Specifically, piglets nursing on sows that received 2 mg/kg of firocoxib at 6 hours prior to processing gained more weight than piglets nursing on sows that received 0.5 mg/kg firocoxib (P=0.0073) and sows that received 1.0 mg/kg (P=0.0038).

**Firocoxib and the descyclopropylmethyl metabolite concentrations in tissues**

There were no samples of muscle, liver, kidney, fat or the injection site that yielded detectable concentrations of firocoxib and its descyclopropylmethyl metabolite above the limit of quantitation of 0.05 µg/g at 21 days after IM administration.
Assessment of the safety of Firocoxib in sows and piglets

Histopathology Examination of Tissues

Formalin-fixed sections of kidney, liver, small intestine and stomach from sows and 3 piglets/ litter were trimmed and positioned in cassettes loaded into an automated tissue processor (Sakura VIP 5, Sakura Finetek, Torrance, CA) for overnight paraffin infiltration. Processed tissues in cassettes were then placed in a paraffin bath (Sakura Tissue-Tek TEC 5, Sakura Finetek, Torrance, CA) after which they were removed from the cassette and oriented in molds. The paraffin-embedded tissues were then fully exposed through sectioning on a microtome (HM 355S Automatic Microtome, Thermo Fisher, Waltham, MA). Tissue sections were cut at 4 microns from the cooled blocks. Paraffin ribbons with tissue were then laid out on a water bath and the floating tissue sections were collected onto microscope slides. The unstained tissue sections were then mounted on the slide and dried at 60°C for 20 minutes. Finally, the tissue was deparaffinized and rehydrated for staining by transfer through xylene and a series of decreasing concentrations of alcohol to hematoxylin on an automated stainer (Sakura Tissue-Tek Prisma, Sakura Finetek, Torrance, CA). After a tap water rinse, the tissue on the slide was counterstained with eosin, dehydrated in an alcohol series, cleared in xylene and cover slipped (Sakura Tissue-Tek Glas g2) prior to histological examination by a veterinary diagnostician with experience in the histological examination of swine tissues.

No macroscopic lesions were evident on post-mortem examination of the kidney, liver, stomach and small intestines. Upon histological examination, all sections of liver and small intestine from lactating sows and nursing piglets across all 4 treatment groups were within normal limits (Tables 5 and 6). Ecstatic tubules, which are considered a congenital anomaly in swine, were observed in 5 sow and 10 piglet kidneys. These findings were not associated with increasing dose of firocoxib and were therefore considered an incidental finding. Mild gastritis was observed in 19 sections of the stomach lining of the piglets but this finding is considered to be not specific for a singular etiology. Interstitial nephritis lesions in sow kidneys are also considered an incidental finding. No macroscopic or histological evidence of NSAID intoxication were observed in any planes of the sections of sow and piglet tissues examined.

NSAID toxicity causes renal papillary necrosis which is considered a pathognomonic lesion for this condition. No evidence of NSAID toxicity was found on post-mortem examination of the kidney, liver, stomach and small intestines of the sows and piglets enrolled in the present study. Ecstatic tubules are considered a congenital anomaly in pigs. Gastric changes observed in the present study were considered mild and not specific for a singular etiology. Interstitial nephritis lesions in sow kidneys are considered an incidental finding.
Table 5. Histopathological findings in lactating sows at 21 days after intramuscular administration of firocoxib at 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, or 2.0 mg/kg.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Histopathology findings</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg/kg</td>
</tr>
<tr>
<td>Liver</td>
<td>Within normal limits</td>
<td>4</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>Within normal limits</td>
<td>4</td>
</tr>
<tr>
<td>Kidney</td>
<td>Within normal limits</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mild tubular ectasia</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Focal interstitial nephritis</td>
<td>2</td>
</tr>
<tr>
<td>Stomach</td>
<td>Within normal limits</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 6. Histopathological findings in nursing piglets (n=48) at 21 days after intramuscular administration of firocoxib to lactating sows at 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, or 2.0 mg/kg.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Histopathology findings</th>
<th>Treatment Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 mg/kg</td>
</tr>
<tr>
<td>Liver</td>
<td>Within normal limits</td>
<td>12</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>Within normal limits</td>
<td>12</td>
</tr>
<tr>
<td>Kidney</td>
<td>Within normal limits</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rare tubular ectasia</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mild tubular ectasia</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Moderate ectasia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Prominent ectasia</td>
<td>5</td>
</tr>
<tr>
<td>Stomach</td>
<td>Within normal limits</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mild vasculitis, muscle layer</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Mild gastritis</td>
<td>5</td>
</tr>
</tbody>
</table>
None of the histological changes that were reported were over-represented in any of the four treatment groups suggesting that these observations were not dose-dependent. Therefore, it is reasonable to conclude that firocoxib was safe for transmammary delivery from medicated sows to piglets at the doses that were tested.

b. Study 2.

Cranial Skin Temperature

There was evidence of an effect of time (P<0.0001), sex (P=0.0473) and replicate (P<0.0001) on the cranial skin temperature of the piglets after processing. Furthermore, there was evidence of a significant treatment-by-time (P<0.0001) and replicate-by-time (P<0.0001) interaction. Specifically, female piglets had significantly greater cranial skin temperatures compared to male piglets (P=0.0473) (Figure 10). We hypothesize that this may be due to the fact that male piglets were castrated and experienced a tail docking procedure at processing while the female piglets only experienced a tail docking procedure at processing.

Cranial skin temperature was significantly lower in piglets that were nursing sows that received firocoxib at 2 h (P=0.0108) and 4 h (P=0.0316). However, cranial skin temperatures in piglets that were nursing sows that received firocoxib were higher than piglets nursing
placebo-treated sows at 36h (P=0.0086) and 48h (P=0.0375) after processing. This was consistent with our hypothesis that pain associated with processing causes peripheral vasoconstriction presenting as lower skin temperatures and that this effect could be mitigated by the transmammary administration of the analgesic, firocoxib.

It is noteworthy that our results found a significant effect of replicate and a replicate-by-time interaction on cranial skin temperatures. Specifically, cranial skin temperatures taken in January and February were significantly lower compared to those assessed in July and August (Figure 12). There was a tendency towards a significant 3-way interaction of replicate, treatment and time (P=0.073). Specifically, skin temperatures of piglets nursing the firocoxib-treated sows were higher than the control piglets in January and February but this effect was less evident in July and August. These observations suggest that season should be considered when infrared thermography is used to assess changes in cranial skin temperature after processing.

**Ocular Temperature**

There was evidence of an effect of time (P<0.0001), treatment (P=0.0047) and replicate (P<0.0001) on the ocular temperature of the piglets after processing. Furthermore, there was evidence of a significant treatment-by-time (P=0.023), replicate-by-treatment (P=0.0005) and replicate-by-time (P<0.0001) interaction. There was also a tendency towards a 3-way interaction between replicate, treatment and time (P=0.0695).
Ocular temperatures in piglets that were nursing sows that received firocoxib were higher than piglets nursing placebo-treated sows at 1h (P=0.0207), 30h (P=0.0011) and 36 h (P=0.0024) after processing (Figure 13). This was consistent with our hypothesis that pain associated with processing causes peripheral vasoconstriction presenting as lower ocular temperatures and that this effect could be mitigated by the transmammary administration of the analgesic, firocoxib.

It is noteworthy that our results found a significant replicate-by-treatment interaction on ocular temperatures. Specifically, ocular temperatures taken in January and February were significantly lower compared to those assessed in July and August (Figure 14). There was a tendency towards a significant 3-way interaction of replicate, treatment and time (P=0.0695). Specifically, ocular temperatures of piglets nursing the firocoxib-treated sows were higher than the control piglets in August (P<0.0018). These observations suggest that season should be considered when infrared thermography is used to assess changes in ocular temperature after processing. However, compared to cranial skin temperatures, ocular temperature assessment may be more robust for assessing pain during warm seasons compared to cool seasons.

**Gait analysis**

There was evidence of an effect of time (P<0.0001), and a time-by-treatment interaction (P=0.0033) on the percent change in force applied to the front limbs of the piglets after processing compared to baseline measurements (Figure 15). Specifically, piglets nursing sows that received the placebo
demonstrated a significantly greater increase in force applied to the front limbs at 7 h after processing compared to piglets nursing sows medicated with firocoxib (P=0.0127). We hypothesize that pain associated with castration results in a shift in force from the hind limbs to the front limbs of piglets to distribute weight away from the surgical site. It is noteworthy that this effect was not evident at 24 h after processing.

V. Discussion

To our knowledge, this is the first report examining the pharmacokinetics and effectiveness of firocoxib in swine. NSAIDs are the most commonly administered class of analgesic drugs in swine production systems in the US due to their effectiveness, availability and relatively low cost. However, there are currently no analgesic drugs that have FDA-approved label indications for pain relief in pigs. Consumer concern about the welfare of farm animals experiencing pain during routine management procedures has increased efforts to develop effective, safe and practical analgesic protocols for use in piglets (Sutherland, 2015). Specifically, the “European Declaration on alternatives to surgical castration of pigs” required that from 1 January 2012, surgical castration of pigs would only be performed with prolonged analgesia and/or anesthesia in piglets over 7 days of age in all EU countries with the intent of phasing out the procedure by 2018. However, a 2015 survey of swine producers in 24 European countries found that only 5% of piglets received both anesthesia and analgesia and 41% of piglets received only analgesia at the time of surgical castration (De Briyne et al., 2016). In over 50% of the countries surveyed, (1) increased production costs; (2) the need for additional labor and (3) the lack of practical and effective analgesic/anesthetic protocols were identified as the primary factors that reduced compliance with the EU Declaration. The results of the present study suggest that a single injection of firocoxib administered to sows resulted in successful transmammary delivery of analgesia to nursing piglets prior to processing. This finding could potentially address many of the current impediments to routine analgesic drug use in piglets at the time of processing by reducing labor costs and improving piglet welfare through reduced stress. Furthermore, the cost of analgesia may be offset by enhanced production through increases in piglet weaning weights.

Plasma elimination half-life is the pharmacokinetic parameter that describes the time taken for the plasma drug concentrations to decrease by half. The long plasma elimination half-life of firocoxib reported in sows in the present study (26.7 - 31.1 h) was similar to the results obtained in horses (29.6 - 31.1h) and calves (31.8h) (Holland et al, 2015; Kvaternick et al, 2007; Stock et al, 2014). A long terminal half-life is desirable from a clinical perspective because this may result in a longer duration of analgesia following a single dose that could reduce dosing frequency. In contrast, a short half-life of 5.9 ± 1.1 h has been reported for firocoxib in dogs and 5.75 h in camels in previous studies (McCann et al, 2004; Wasfi et al., 2015). In comparison to other commonly used NSAIDs in pigs, the elimination half-life of firocoxib in sows was approximately 10-fold longer than ketoprofen (3 h) (Raekallio et al., 2008), 5-fold longer than meloxicam (6 h) (Pairis-Garcia et al., 2015) and 4-fold longer than flunixin (7.5 h) (Pairis-Garcia et al., 2013). These data support the hypothesis that firocoxib is a suitable analgesic for single
dose administration in swine resulting in reduced labor costs and stress associated with frequent injections.

Volume of distribution is the pharmacokinetic measurement that describes the tendency of a drug to move from the blood into the tissues. A large volume of distribution (7.75-13.8 L/kg) for firocoxib in sows in the present study was similar to calves (6.54 L/kg) (Stock et al., 2014) but greater than previously reported results in camels (2.34 L/kg) (Wasfi et al., 2015), horses (1.81 L/kg) (Holland et al., 2015) and dogs (2.9 L/kg) (McCann et al., 2004). A large volume of distribution is associated with high lipophilicity leading to greater distribution of a drug to tissues and body fluids. This property of firocoxib has been previously demonstrated in a radioresidue study that reported penetration of up to 30% of plasma concentrations of firocoxib into equine synovial fluid (Kvaternick et al., 2007). In comparison to other commonly used NSAIDs in pigs, the volume of distribution of firocoxib in sows was approximately 26-fold larger than flunixin (0.30 L/kg) (Paires-Garcia et al., 2013), 22-fold greater than ketoprofen (0.35 L/kg) (Raekallio et al., 2008) and 18-fold greater than meloxicam (0.42 L/kg) (Paires-Garcia et al., 2015). These data suggest that firocoxib could be expected to have a greater tendency to distribute into the mammary gland and milk compared to other NSAIDs that demonstrate a smaller volume of distribution.

Transmammary transfer of NSAIDS has been demonstrated in lactating females of other mammalian species (Jacqz-Aigrain et al., 2007; Malreddy et al., 2013). Previously, our group described the successful transmammary delivery of the NSAID, meloxicam, from medicated sows to 5-day old piglets before processing (Bates et al., 2014). Piglets nursing sows that received oral meloxicam at 30 mg/kg for 3 consecutive days demonstrated a significant reduction in plasma cortisol concentrations over 10 hours after processing compared to piglets nursing unmedicated sows. The results of the present study advance our understanding of transmammary delivery of analgesic compounds to manage pain in the offspring by demonstrating that this can be accomplished with a single injection using a dose volume that is attainable in a swine production environment.

The results of the pharmacokinetic analysis of the plasma firocoxib concentrations in the piglets indicate that the passage of firocoxib from the sow plasma into the milk was not linear. This suggests that transport across the blood-milk barrier may be a saturable process. Therefore, an increase in the sow dose above 1.5 mg/kg may not result in higher firocoxib concentrations in the milk. Furthermore, the AUC values represented total firocoxib exposure over time. Expressing the AUC values for firocoxib in piglets as a percentage of the AUC for firocoxib in sows is an alternative approach to investigation of the extent of the transmammary delivery of firocoxib from sows to piglets. Based on the AUC values calculated from 0 h to the last time point, piglets nursing on sows administered 0.5 mg/kg to 1.5 mg/kg firocoxib as a single IM injection received between 41% and 46% of the total sow firocoxib exposure. In contrast, the piglets nursing on sows that received 2.0 mg/kg firocoxib were exposed to 25% of the sow exposure. These results suggest that firocoxib doses above 2 mg/kg IM will likely not be associated with a proportional increase in drug transfer to nursing piglets.
Piglet behavior after castration, tail docking and teeth clipping was not assessed in the present study due to the likelihood that the frequency of animal handling for blood sample collection after processing would have had a negative impact on the expression of pain-related behaviors in the barrows thus confounding the experiment. The results of this study suggest that a dose of 2 mg firocoxib/kg reduced stress and improved the average daily gain in barrows. Therefore, this would be a candidate dose for a subsequent experiment focused on assessing behavior in medicated piglets after processing.

Increased plasma cortisol concentrations are associated with stressful events such as those performed during processing. Specifically, assessment of the stress response using cortisol has been used as a proxy for measuring pain in livestock (Carroll et al., 2006). However, an increase in plasma cortisol is not specific to any type of physical or mental stress. Routine animal handling procedures have been found to increase plasma cortisol concentrations in piglets (Moya et al., 2008). However, a study comparing plasma cortisol concentrations of surgically castrated animals to sham-castrated animals found that animals that did not experience castration pain had lower peak cortisol concentrations and returned to baseline concentrations faster than surgically castrated animals (Prunier et al., 2005). Persistent elevated plasma cortisol concentrations in the surgically castrated group could be a result of tissue damage or procedural pain (Tenbergen et al., 2014a). Until a pain-specific biomarker is identified and validated, the use of cortisol (with its limitations) as proxy measure for assessing pain in livestock will remain widespread in studies assessing the impact of production procedures and analgesic drugs on animal welfare.

In the present study, plasma cortisol concentrations reached a peak at approximately 30-60 min after the processing procedures in the piglets sampled at that time point. The time to peak plasma cortisol concentration and the magnitude of the response following processing procedures reported herein was similar to that reported in other studies (Prunier et al., 2005; Carroll et al., 2006; Marchant-Forde et al., 2009; Reiner et al., 2012; Tenbergen et al., 2014a). Furthermore, the results of the present study suggest that plasma cortisol concentrations in male piglets nursing sows that received the higher doses of firocoxib (1.5 mg/kg IM or 2.0 mg/kg IM), at 6 to 8 hours before processing, were lower compared with plasma cortisol concentrations in piglets nursing sows that received lower doses of firocoxib (0.5 mg/kg IM and 1.0 mg/kg IM). To account for the fact that not all piglets were sampled at this time point CortCmax concentrations were compared. These further support the conclusion that piglets from sows that received 2.0 mg/kg of firocoxib tended to have a lower observed peak cortisol concentration. However, the observation of a dose dependent reduction in peak cortisol concentrations was less conclusive in this analysis because the actual Cmax may have occurred before or after the sparse sampling time point. To our knowledge, this is the first published report demonstrating that NSAID administration reduces plasma cortisol concentrations after processing in a dose-dependent manner. This finding supports the use of plasma cortisol as a surrogate biomarker of pain in dose-titration studies in swine.
Several studies have reported that NSAIDs, including meloxicam, ketoprofen and flunixin, reduce plasma cortisol concentrations when administered prior to processing (Reiner et al., 2012; Schwab et al., 2012; Bates et al., 2014; Tenbergen et al., 2014a). Specifically, a recent meta-analysis of 14 studies involving 634 animals found that mean cortisol concentrations within 60 min of castration in piglets were 93.59 units lower (range: 48.74 – 138.44 units lower) in piglets receiving an NSAID compared to control animals (O’Connor et al., 2014). Although it is recognized that NSAIDs do not mitigate the acute, incisional pain associated with castration, these results suggest that transmammary delivery of firocoxib administered to sows at 1.5 mg/kg and 2.0 mg/kg reduces cortisol and therefore processing stress in piglets.

A potential criticism of the present study was to use of gilts as a procedural control for the castration and tail docking procedures. Data describing a sex difference in the stress response in young piglets are deficient in the published literature. However, Zupan and Zanella (2017) reported that cortisol response was similar between barrows and gilts exposed to various stressors including a human test, transportation, a novel object test and a novel arena test (P>0.10). Based on these data, it would be reasonable to assume that response to stress between 6 day old barrows and gilts would be similar, thus justifying their use as procedural controls. Furthermore, based on salivary cortisol concentrations, Gallagher and others (2002) observed that circadian rhythms only became established after Day 6 in piglets. This suggests that circadian fluctuations in plasma cortisol would be expected to have a minimal impact on the results of the present study.

A reduction in plasma PGE2 concentrations was expected since firocoxib exerts its anti-inflammatory effects by inhibition of the enzyme cyclooxygenase which is responsible for prostaglandin synthesis. It was surprising that PGE2 concentrations were not suppressed to a greater extent in piglets nursing sows that received 2 mg/kg. However, we observed that the increase in plasma drug concentrations was not linear which might explain why sows that received 1.5 mg/kg firocoxib had the lowest plasma PGE2 concentrations compared to the other groups. Further research is needed to establish the relationship between plasma firocoxib concentrations and suppression of PGE2 synthesis. This work may result in the characterization of an EC50 for firocoxib in swine which is the concentration where 50% of the cyclooxygenase activity is suppressed.

Previous studies examining the impact of meloxicam or ketoprofen administered immediately before castration on growth rates in piglets found no effects of NSAID administration on piglet average daily gain (Hansson et al., 2011; Kluivers-Poodt et al., 2012; Cassar et al., 2014; Tenbergen et al., 2014a; Bonastre et al., 2016). However, these studies focused on the administration of the NSAID individually to each piglet at the time of processing. Therefore, one explanation for the beneficial effect of transmammary delivered firocoxib on piglet performance reported herein was that the NSAID had a positive effect on material milk production or sow welfare. This hypothesis is supported by the observation that ADG increased in both barrows and gilts in the present study regardless of processing status.
Specifically, parturition is associated with weight loss, reduced feed intake and an increase in stress, acute phase proteins and pain-related behaviors in sows (Mainau and Manteca, 2011). The negative impacts of parturition on sows may be mitigated by postpartum administration of an NSAID resulting in reduced weight loss, reduced lying times and improved growth rates in piglets (Mainau et al., 2012; Viitasaari et al., 2014; Tenbergen et al., 2014b). Furthermore, oral meloxicam administration to sows at the start of farrowing has been found to increase piglet serum IgG concentrations, weaning weights and average daily gain (Mainau et al., 2016). Recently, several investigators have reported that NSAID therapy immediately after calving resulted in a significant increase in milk production and milk fat and protein composition over the course of a lactation (Farney et al., 2013; Carpenter et al., 2016; Shock et al., 2018; Swartz et al., 2018). Taken together, these findings support the hypothesis that the dose-dependent increase in ADG observed in the present study may have resulted from the beneficial effects of the NSAID, firocoxib, on postpartum physiology and behavior in the sows. Further large scale studies focusing on changes in feed intake, bodyweight and milk composition of sows medicated with firocoxib are needed to elucidate if the NSAID improves the welfare of the sows in addition to impacting the welfare of the nursing piglets.

NSAID toxicity causes renal papillary necrosis and gastric ulceration which is considered a pathognomonic lesion for this condition (Black, 1986). No evidence of NSAID toxicity was found on post-mortem examination of the kidney, liver, stomach and small intestines of the sows and piglets enrolled in the present study at 21d after treatment. Ecstatic tubules are considered a congenital anomaly in pigs (Wells et al., 1980; Jansen and Nordstoga, 1992). Gastric changes observed in the present study were considered mild and not specific for a singular etiology. Interstitial nephritis lesions in sow kidneys are considered an incidental finding (Kongsted and Sorensen, 2017). None of the histological changes that were reported were over-represented in any of the four treatment groups suggesting that these observations were not dose-dependent. Therefore, it is reasonable to conclude that firocoxib was safe for transmammary delivery from medicated sows to piglets at the doses that were tested.

Firocoxib administered to swine by any dose, route, for any duration or frequency constitutes extra-label drug use (ELDU) because currently there are no analgesic drugs specifically approved for pain management in pigs in the US (Smith et al, 2008). Under the Animal Medicinal Drug Use Clarification Act (AMDUCA), ELDU is permitted for relief of suffering in pigs provided specific conditions are met (AMDUCA, 1994). These conditions include that (1) ELDU is permitted only by or under the supervision of a veterinarian, (2) ELDU is allowed only for FDA-approved animal and human drugs, (3) ELDU is permitted only when the health of the animal is threatened and not for production purposes, (4) ELDU in feed is prohibited, and (5) ELDU is not permitted if this results in a violative food residue. In the present study there were no detectable concentrations of firocoxib or its descyclopropylmethyl metabolite detected above the limit of quantitation (0.05 µg/g) for the assay in both sow and piglet tissues at 21 d after IM injection. In the EU, a maximum residue limit (MRL) of 10 µg/kg has been established in muscle and kidney, 15 µg/kg in fat and 60 µg/kg in the liver of horses.
Based on these data, tissue concentrations in the present study were below the MRL for liver at 21 d after administration but the assay was not sensitive enough to quantify concentrations below the MRL for the other tissues, although none of these concentrations were above the limit of detection (0.01 µg/g) for the assay. Based on these data, additional studies conducted in accordance with FDA Guidance for Industry (GFI) #207 (Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs In Food Producing Animals: Marker Residue Depletion Studies to Establish Product Withdrawal Periods) and GFI #3 (General Principles for Evaluating the Human Food Safety of New Animal Drugs Used in Food Producing Animals) are needed to characterize the tissue depletion of firocoxib after IM administration in sows.

IRT evaluates changes in surface temperature. Processing can release epinephrine, which causes changes in sympathetic tone. The adrenergic effects on cutaneous blood flow results in a decrease in cranial skin temperature after castration as previously described by Bates et al. (2014). Ocular temperature has also been reported to decrease due to the stimulation of the autonomic nervous system resulting in peripheral vasoconstriction (Stewart et al., 2010). Both outcomes are consistent with the results of our study. However, our study described a significant effect of season on both cranial skin and ocular temperature. These data suggest that season should be considered when assessing pain by cutaneous thermography.

Recently gait analysis was used to assess pain associated with castration in cattle and parturition in adult dairy cows. Specifically, Kleinhenz et al (2018a) used a pressure mat to demonstrating that cows that received 1 mg/kg of meloxicam PO within 26 h of calving placed 48.9% (95% CI: 47.4% to 50.5%) of total force on the rear limbs compared to 46.3% (95% CI: 44.7% to 47.9%) in placebo-treated cows \((P = 0.02)\). Total impulse on their rear limbs in the meloxicam-treated cows was 50.5% (95% CI: 48.6% to 52.4%) compared to 46.7% (95% CI: 44.8% to 48.7%) for cows in the placebo group \((P = 0.01)\). No differences in contact pressure of the rear limbs were observed \((P = 0.27)\). However, cows in the placebo-treated group had a longer stride length (101.3 cm with a 95% CI: 95.9% to 106.6 cm) vs. 90.8 cm (95% CI: 85.4% to 96.1 cm) \((P = 0.03)\).

Kleinhenz et al. (2018b) also demonstrated that calves undergoing surgical castration placed more force onto their fore limbs \((P = 0.02)\) indicating a shift in their weight distribution to the front limbs. However, there were no measured differences in total step contact area and step contact pressure. Calves in the control group also had lower total impulses compared to surgically castrated calves \((P = 0.004)\). These data support the results of the present study that found that piglets placed more force on their front limbs after processing and that this effect was mitigated by transmammary delivery of firocoxib.

The results of this study suggest that IM administration of firocoxib to sows at 7 ± 1 h before performing piglet processing procedures resulted in successful transmammary drug delivery to the nursing piglets. Transmammary delivery of firocoxib resulted in a dose-dependent reduction of plasma cortisol concentrations after processing with barrows nursing sows that received 1.0 mg/kg and 2.0 mg/kg IM recording lower plasma cortisol concentrations than barrows nursing sows that received 1.5 mg/kg and 1.0
mg/kg IM. Furthermore, a dose-dependent increase in average daily gain was observed at 21 d after processing. Drug concentrations in tissue samples taken 21 d post-maternal administration were below the level of detection of the assay. When given via the transmammary route, firocoxib has potential as a therapeutic drug used for analgesia, to reduce processing-induced pain and stress thus improving piglet welfare, and enhance production through increases in weaning weights.

LITERATURE CITED


Kongsted., H and Sorensen, J.T. 2017. Lesions found at routine meat inspection on finishing pigs are associated with production system. Veterinary Journal 223; 21 - 26


