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Development of a transdermal technology to deliver analgesia to cattle undergoing surgical husbandry procedures

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Executive summary

Cattle undergo a range of necessary surgical husbandry procedures, including dehorning, castration and ovariectomy. Analgesia may be insufficient during these procedures, especially in extensively managed properties where access to veterinary assistance is limited. However, public interest groups and welfare advocates are increasingly concerned about the welfare of animals undergoing painful procedures. Therefore development of practical and effective methods of delivering pain relief to cattle undergoing painful husbandry procedures is a high priority.

The transdermal route of drug administration offers many advantages, including avoiding first pass effects and the high costs of producing parenteral formulations (particularly sterility), plus can be easily administered by lay personnel. The problem arises that formulations cannot be extrapolated between species, and movement through skin is strongly dependent on the specific active drug and the vehicle the drug is dissolved in. Therefore, the current project sought to develop a new vehicle formulation to drive a registered non-steroidal anti-inflammatory (NSAID) through bovine skin to provide analgesia to cattle for at least 24 hr after undergoing surgical husbandry procedures.

An initial in vitro study, using Franz-type diffusion cells, screened over 150 vehicle combinations with the four NSAIDs currently registered for cattle in Australia: ketoprofen, meloxicam, flunixin meglumine and tolfenamic acid. The NSAIDs differed in their ability to penetrate through bovine skin, but one experimental formulation yielded promising results.

Pilot studies were undertaken *in vivo* to confirm that therapeutic concentrations of active drug would reach the systemic circulation. A bioavailability study was then conducted using a cross-over design on 12 calves, which had IV, IM or transdermal NSAID administered alternately, with a week wash-out period. The transdermal formulation had a slightly slower absorption than IM, although the peaks were equivalent at 30 minutes. A peak (C_{MAX}) occurred ~ 2 hr after administration. A bioavailability of 50% and an area under the curve exceeding that following IM administration suggested that the transdermal formulation had at least similar if not greater systemic absorption than IM administration.

A clinical efficacy study was then performed, with groups of Holstein calves (n=10) to be dehorned allocated to a placebo, IM treatment or transdermal treatment group; a further six untreated calves underwent sham dehorning. NSAID treatment, whether by IM or transdermal administration, resulted in significantly lower total plasma cortisol concentration and higher post-operative live weight gain than placebo-treated calves. Further, analysis of calf behaviour within 24 hr of dehorning demonstrated a significant treatment effect for the positively associated pain variables (head rubbing+ head shaking+ear flicking+ tail wagging+ lying down+neck extended.

A proteomic analysis of ~ 300 proteins in cattle plasma was incomplete at the time of writing this report, although preliminary results suggested that the NSAID, particularly by the transdermal route, induced changes in the type and ratio of plasma proteins that were significantly different from placebo.

In summary, a transdermal NSAID has been developed that produces rapid and therapeutically effective concentrations of active drug in cattle. The analgesia provided would appear to be similar to or better than IM administration of the same drug, which in turn would suggest analgesia for 24 hr following a surgical husbandry procedure. This is the first analgesic product that has been developed for topical administration to cattle that has been shown to have clinical efficacy and has the potential to revolutionize provision of analgesia to cattle undergoing painful husbandry procedures.

Table of contents

1 Background

1.1 Analgesia for surgical husbandry procedures in cattle

Industry and welfare groups are advocating for the provision of adequate analgesia for cattle undergoing a range of surgical husbandry procedures. Topical application of analgesics provides a potentially highly effective approach to pain control, in terms of practical application, efficacy, and maximising analgesic drug concentrations at the site of surgery. However, research is required to confirm efficacy and safety of potential transdermal analgesic formulations in cattle.

Cattle are routinely subjected to painful husbandry procedures such as dehorning, spaying, and surgical and non-surgical castration of males (Hemsworth, 2006; Phillips, 2006). From a welfare point of view, it would be highly desirable to control the pain associated with these procedures so animals experience a level of anaesthesia and analgesia equivalent to that offered to humans and companion animals for similar levels of pain (Viñuela-Fernández et al., 2007). Furthermore, pain leads to transient production losses, such as a decreased weight gain in cattle dehorned older than 4-6 months of age (Loxton, 1982, Winks 1977, Goonewardene, 1991). Acute pain and the associated cortisol response following surgery last for 6 – 9 hours because the nerve fibres supplying the wound site remain activated for that period (Mellor and Stafford, 1999). Infusion of a short acting local anaesthetic has a temporary effect on the cortisol response associated with, for example, surgical dehorning in calves, but the long acting local anaesthetic agent, bupivacaine, provides more sustained analgesia. However, there is still a pain response when the bupivacaine wears off. Non-steroidal anti-inflammatory drugs (NSAIDs) can significantly reduce the pain response following surgery by limiting the inflammatory changes induced by surgery (Stafford and Mellor, 2005). Importantly, it is well recognised that aggressive management of acute pain is preferable and reduces the likelihood of the development of maladaptive (neuropathic) pain which is difficult to control with conventional analgesics.

In the context of beef cattle, particularly those on extensively managed properties, a pain control regime must fulfil the following criteria:

- The treatment would be applied once only when the animal is presented for the procedure so animal handling (stress and cost) is minimised. The agent must have a rapid onset of action, yet have a long duration of effect to ensure prolonged analgesia.
- The treatment must be easily applied so as not to increase animal stress, and it must not affect the level of consciousness of the animal.
- The analgesic treatment must significantly reduce the immediate and longer term (~ 24 hr) pain and inflammation associated with surgery.
- The treatment must be cost-effective without unacceptable occupational health safety risks.
- It would be desirable if all constituents are currently registered for use in cattle in Australia to reduce costs of manufacturing, registration, and importantly provision of withholding periods for slaughter.

There are no current or effective alternatives. Commonly used approaches to pain management used in other species, such as infiltration of a local analgesic, systemic administration of analgesics, including opioids, and general anaesthesia, are impractical and too expensive for use with routine surgical husbandry procedures conducted on beef cattle. There are no topical analgesic formulations registered for use in cattle in Australia. A formulation containing flunixin meglumine (FINADYNE® Transdermal) has been registered overseas, but only claims to reduce pyrexia and not pain. Part of the reason for this lack of alternatives is that there is little known about drug movement through bovine skin and it is well established that substantial species variation in transdermal drug

delivery exists, so any potential formulation must be confirmed in the target species (Mills & Cross, 2006a).

1.2 Aims for this project

There were three primary aims for this project:

- To identify suitable NSAIDs and vehicle combinations effectively move through cattle skin using *in vitro* techniques.
- To optimise a transdermal formulation to control pain in cattle for up to 24 hr.
- To determine the efficacy of the candidate formulation to control pain associated with surgical husbandry techniques.

2 Project objectives

2.1 To confirm transdermal penetration of candidate formulations using *in vitro* techniques

2.1.1 Regional differences in transdermal drug penetration

There is often significant variation in transdermal drug penetration when applied at different sites on an animal (Mills et al 2004 a,b; 2007 b,c). Pour-on treatments in cattle are normally applied along the backline, but some may spill down to the thoracic region. It would also be advantageous if some skin regions were more permeable to specific drug formulations, since this could be used to enhance the penetration of a drug if insufficient penetration is achieved along the backline. The purpose of this study is to assess the relative permeability of several regions of cattle skin.

2.1.2 Screening of NSAID and vehicle combinations to maximise active drug penetration through cattle skin

It is currently unknown which, if any, NSAIDs will penetrate skin. Since there are four NSAIDs currently registered to use in cattle in Australia (flunixin meglumine, ketoprofen, meloxicam and Tolfenamic acid), these will be tested to determine the relative amount of penetration through cattle skin. Since the vehicle (the matrix the active drug is dissolved in) has a major impact on drug permeability (Mills et al 2005; 20076 a,c), this study will use *in vitro* techniques to screen a comprehensive array of vehicles, selected based on then literature, on the permeability of the four NSAIDs through cattle skin.

2.1.3 Optimising the candidate formulations

Once the promising vehicle candidates are identified, the final formulation will be selected after optimisation studies to determine the most effective vehicle. These studies will consist of varying the relative proportions of the various vehicle components (excipients) and also the concentration of active, again using *in vitro* techniques.

2.1.4 Stability studies of the final formulation

It is essential that any formulation is stable in terms of both the active drug and the excipients under a range of environmental conditions. This study will store the final formulation under a range of conditions to ensure stability under expected conditions of use.

2.1.5 Analysis of NSAIDs in biological samples

Analytical methods will be developed to measure the concentrations of the various NSAIDs in receptor fluids (for *in vitro* studies) and in plasma (for *in vivo* studies). Ultra-High Performance Liquid Chromatography with tandem mass spectrometry (UPLC-MS) methods will be validated for this project.

2.2 To determine the efficacy of candidate transdermal formulations to control pain associated with surgical husbandry formulations

Once a final formulation is produced, it will need to be applied to cattle *in vivo* to confirm that it can pass through skin of age-appropriate cattle (i.e. calves ~ 6 months or less that may undergo surgical husbandry procedures, such as dehorning and castration). A clinically-effective dose of a transdermal formulation will be determined if active drug reaches similar systemic concentrations (i.e. in cattle plasma) as commercially-available injectable formulations and/or plasma drug concentrations identified in the literature as being effective for analgesia and to control inflammation.

2.2.1 Pilot study 1

A pilot study where the final formulation will be applied at different volumes to cattle to determine:

(i) If active drug reaches the systemic circulation at detectable concentrations.

(ii) If there is a dose (volume) relationship, so final dose rate can be regulated by volume of formulation applied.

The working formulation will be studied, although it was uncertain how the in vitro findings would translate to the in vivo applications, since a number of factors may affect final systemic drug concentrations once a formulation is applied topically. An appropriate working volume of administration would be ~ 10 mL. However, translation of in vitro to in vivo dosing can involve up to a 10 fold increase in dose rate (i.e. a transdermal dose rate is often 10X or more than the parenteral dose rate, depending on the drug and formulation). It was decided to apply a 10 mL and 40 mL volume to ~ 6 months old calves (n=2 for each dose) since these would be an expected age for dehorning.

2.2.2 Pilot study 2

Once the initial pilot study has been performed, the lag time (delay in appearance in then plasma after application) and total amount of active drug (based on pharmacokinetic parameters) can be estimated. A second pilot study will then permit sampling (blood collection) more closely aligned to drug appearance in the plasma and to better characterise the pharmacokinetics of the active drug following topical administration. The pharmacokinetic parameters will include: (i) Maximum plasma concentration (C_{MAX}); Time to C_{MAX} (T_{MAX}); (iii) area under the plasma-concentration-time curve (AUC).

2.2.3 Bioavailability study

Bioavailability compares the amount of active drug reaching the plasma from any particular route of administration, compared to intravenous (IV, considered 100%). Relative bioavailability compares the amount of active drug reaching the plasma from non-IV routes of administration. Since parenteral (injectable) formulations of all four test NSAIDs are currently registered for cattle in Australia, a bioavailability study comparing IV, intramuscular (IM) and transdermal routes of administration will provide absolute and relative bioavailability of the active drug in the final formulation which can then be used to determine dose rate and act as a surrogate measure of safety

and efficacy (i.e. if a similar amount of systemic drug concentration can be attained following topical administration compared to IM, a similar efficacy and safety can be assumed).

2.3 To develop a formulation containing an NSAID to control pain associated with routine surgical interventions for up to 24 hr

2.3.1 Clinical study

The field testing of the efficacy of the final formulation will involve application during the performance of a surgical husbandry procedure. Dehorning was selected since this procedure will induce pain and inflammation when performed in the field. Importantly, NSAIDs have been shown to be effective in providing analgesia for this procedure (Taulkner & Waery, 2000; Stafford et al. 2003; Stafford & Mellor, 2005; Allen et al., 2013). This study will involve treated and non-treated (placebo) animals.

2.3.2 Conventional parameters to assessment pain and inflammation

The currently accepted parameters to measure pain and inflammation in cattle including total plasma cortisol concentration, behavioural changes and some acute phase proteins, such as haptoglobin and serum amyloid A, as well as Prostaglandin E2 (PGE2) (Allen et al 2013).

2.3.3 Proteomic assessment of pain and inflammation

Collection and analysis of blood has long been used to diagnose and monitor animal disease. The non-cellular component contains a wide range of proteins and peptide molecules, many of which are used as biomarkers for physiological and pathological conditions. While routine biochemical analysis can quantify large and abundant proteins, specialised immunoassays, such as Enzyme-Linked Immunosorbent Assays (ELISA) and radioimmunoassays (RIA), are commonly used techniques to identify and quantify proteins that are less common or in low concentration. However, there are several limitations associated with these assays, particularly since they rely upon species-specific antibodies, which must be validated for each species, while separate kits are required for each protein, increasing costs and time required. In addition, Apart from this, they also suffer from lack of concordance, hook's effect and production of autoantibodies and anti-reagent antibodies (Hoofnagle and Wener, 2009).

An increasingly popular approach to protein measurement in human medicine is mass spectrometry-based proteomics. Proteomics is the study of the protein component of a cell, tissue, or an organism at a given time under given conditions (Wilkins et al., 1996) and complements the study of genomes and transcriptome through interpreting a true biochemical consequences of the genomic data. Proteomics provides highly sensitive and specific identification of proteins and has therefore been used to understand and diagnose important human diseases, such as cancer, neurological disorders and several cardiovascular conditions.

The major advantage in using proteomic techniques to identify biomarkers is that it is a highly specific and sensitive approach to measuring proteins that are crucial to the regulation of physiology and energy metabolism, reflecting changes in homeostasis. For example, acute phase proteins (APP) are already recognised as biomarkers of pathological changes and perturbations in the innate immune response, and thus can be used as sensitive and reliable indicators for diagnosis of many inflammatory diseases (Horadagoda et al., 1999).

3 Methodology

Approval for these studies was obtained for the University of Queensland Animal Ethics Committee (approval #: SVS/455/14/MLA).

3.1 To confirm transdermal penetration of candidate formulations using *in vitro* techniques

3.1.1 Regional differences in transdermal penetration of lignocaine hydrochloride

Cattle skin was harvested from Black Angus steers (*n* = 5, age 1.5 - 2 years, 5/5 body condition score and weighing 508-570 kg). Hair was removed using an electric clipper and the subcutaneous fat and musculature carefully trimmed away before the skin was cut into circular sections (approximately 2 cm diameter) using a metal wad punch, and placed in airtight ziplock bags, stored at -20 °C within 5 hr of slaughter.

The skin samples were defrosted immediately prior to use and mounted on Franz diffusion cells. A donor solution of radiolabeled lignocaine (Lidocaine [carbonyl-14C] HCl in ethanol, 0.1 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO, USA) solution was added, while the receptor solution was phosphate-buffered saline containing 4% Bovine serum albumin (Mills et al., 2003, 2005). The concentration of lignocaine (C_v) was 100 mg/mL, resulting in a dose per area of 88.42 mg/cm^2. A glass cover slip was applied to each donor chamber to prevent evaporation of the donor solution. A sample (200 µL) were collected at regular intervals from the receptor chambers via the sampling port and immediately replenished with equal volumes of fresh receptor solution. At the completion of each study, the diffusions cells were dismantled and the skin discs were rinsed and stored. Both receptor fluid samples and skin samples were analysed for lignocaine concentration using a Tri-carb® 3110TR Liquid Scintillation Analyser.

The pseudo-steady state flux (J_{ss} or driving force) of lignocaine was derived from the gradient of the regression line through the data points of the linear part of the cumulative amount of lignocaine penetration per skin area against time profile (permeation profile) (Cross et al., 2003b; Mills et al., 2004a). The time lag or delay before drug first appearing in the receptor fluid (t_{lag}, which correlates to the initial onset of drug activity) was designated as the x-intercept of the regression line (Ahlstrom et al., 2009) and the permeability of active drug in the specific vehicle (k_p or permeability coefficient) was calculated using the known values of J_{ss} and the concentration of lignocaine hydrochloride in the vehicle (C_V) (Williams, 2003; Ahlstrom et al., 2007) as:

$$k_p = \frac{J_{ss}}{C_v}$$

The amount of lignocaine in the receptor cell was analysed with respect to length of freezing and sampling time, using factorial ANOVA on STATISTIC™ (version 12; StatSoft Inc., Tulsa, OK, USA). With respect to the length of freezing, the derived k_p and t_{lag} were analysed with repeated measures ANOVA. The amount of lignocaine retained within the skin each at the end of each experimental was analysed using Kruskal Wallis ANOVA since it had a non-parametric distribution.

3.1.2 Screening of NSAID's and vehicle combinations to maximise active drug penetration through cattle skin

Four NSAIDs are currently registered for use in Australia: Flunixin meglumine, Ketoprofen, Meloxicam and Tolfenamic acid. *In vitro* techniques, as decided in 3.1.1, were used to measure the penetration of each of these four NSAIDs in over 150 vehicle combinations.

3.1.3 Optimising the candidate formulations

Once the relative permeability of each NSAID was determined, the most suitable drug-vehicle formulation was selected and optimised, in terms of additional excipients and active drug concentration, to enhance transdermal drug penetration.

3.1.4 Stability studies of the final formulation

At the request of Elanco, preliminary stability studies were performed on the optimal formulation to ensure no obvious or major concerns with storing the final formulation. This consisted of storing the final formulation in glass and plastic (polypropylene) containers at varying temperatures (0, 20 and 40^{o}C) for 6 months. The samples were examined for visual appearance, precipitation and active drug concentration at 0 (when first prepared), 2, 4 and 6 months.

3.1.5 Analysis of NSAIDs in biological samples

Plasma Protein Precipitation:
Cold Acetonitrile (0.2% formic acid) precipitation was added to samples at 1:1 ratio and vortexed for 30 s. Protein crashing carried out by 2 rounds of centrifugation for 15min at 20,000 *g*. Interval between "crashing" was 30 min at 4 ˚C. Supernatant of each sample collected and analysed by LCMS/MS.

NSAID concentration determination by LC-MS/MS (16 March – 28 May 2016)
Instrument: Shimadzu Nexera uHPLC in tandem with LCMS 8030.
CID gas: Argon
Column: Phenomenex Kinetix C18, 1.7 µm pore size.
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in Acetonitrile
LOD: 0.01; QC samples instrument stability (0.1, 2 and 5 µg/mL); Calibration range 0.019 – 5 µg/mL (similar range to previous experiments in this lab).

Test recoveries, using spiked plasma from protein crashing ranged from 91.2 – 102.6%.
Detection m/z 255.10>105.20 and confirming ion 255.10>194.25

Samples: must be prepared with less than 90% solvent prior to analysis.

Instrument performance and stability: All samples were analysed on the instrument continuously over 4 days. QCs were analysed every 20 samples. Three sets of calibration curves were assessed. Instrument stability was reasonable.

Standard		**CV (%)**
QCH	(5 µg/mL)	9.287
QCM	(2 µg/mL)	11.274
QCL	(0.1 µg/mL)	10.385
CC	0.0195 µg/mL	14.681
CC	0.0781 µg/mL	10.581
CC	0.3125 µg/mL	14.148
CC	1.25 µg/mL	12.132
CC	5 µg/mL	1.84

3.2 To determine the efficacy of candidate transdermal formulations to control pain associated with surgical husbandry formulations

3.2.1 Pilot study 1

The study design consisted of a pre-treatment blood sample being collected, then the formulation applied (10 or 40 mL) along the backline between the withers and lumbar region of four calves. Further blood samples were then collected at 2, 4, 8, 12 and 24 hr later, then analysed for NSAID concentrations using UPLC-MS. The calves were all Droughtmasters (approximately 50% Bos indicus, 50% Bos taurus), as follows:

Table 1. Cattle details for Pilot study 1

Calf ID	Gender	Weight (kg)	Dose volume (mL)
(mg/kg)			
3405	Male	241	40
3383	Female	191	40
3389	Male	248	10
3385	Female	216	10

3.2.2 Pilot study 2

The study design for the second pilot study was the same as the first pilot study, except that all animals received a transdermal dose volume of 10 mL. All calves were estimated to be of similar weight and were from the same cohort of calves as the first experiment. Body weight was estimated as per industry standard. The animals were closely observed for any grooming, particularly during the first 2 hr following application. Blood samples were collected at 0, 1, 2, 3, 4 and 6 hr following application.

Table 2. Cattle details for Pilot study 2

Calf ID	Gender	Weight (kg)	Dose volume (mL)	Dose rate (mg/kg)
3401	Male	~ 220	10	~ 8
3415	Female	~ 200	10	~ 10
3417	female	~ 200	10	~ 10
3421	Female	~ 200	10	~ 10

3.2.3 Bioavailability study

This study will be performed at the Yarrandoo site of Elanco in NSW.

This was a randomized, non-blinded, crossover pharmacokinetic study. Cattle (n=14, including two spares) of approximately 250–350 kg body weight were selected from the Yarrandoo herd. The cattle were randomized to three treatment groups (A, B and C), each of four individuals. Each group received each treatment once, as described in Table 1. The treatments were: (1) NSAID IV; (2) NSAID IM; (3) NSAID topical (transdermal formulation). A minimum one week 'washout' period was allowed between each treatment. Cattle receiving the topical treatment were washed, with warm water and shampoo (Fido's Everyday shampoo), applied with a soft brush, and then thoroughly

rinsed, 12 hr after application and after all samples had been collected to remove any residual product. The cattle facilities used for handling of topically-treated animals was appropriately washed after all samples had been collected. Cattle receiving the IM and IV treatments were handled using facilities separate to any cattle treated with the topical treatment on the same day.

Blood samples (9 mL) were collected at regular intervals (Table 2). Blood samples were centrifuged and plasma separated for NSAID concentration analysis *via* UPLC-MS to determine pharmacokinetic parameters. The pharmacokinetic analysis was performed by Dr Jane Owens from Elanco, using Phoenix WinNonLin and a non-compartmental model.

Table 3. Bioavailability study treatment allocation

	Day	Treatment		
		IV	**IM**	**topical**
1st treatment	0	A1*	B1	C1, C2
	1	A2	B2	C3, C4
	2	A3, A4	B3, B4	–
2nd treatment	7	C1	A1	–
	8	C2	A2	B1, B2
	9	C3	A3	B3, B4
	10	C4	A4	–
3rd treatment	15	B1	C1	A1, A2
	16	B2	C2	A3, A4
	17	B3, B4	C3, C4	–

*Indicates treatment group and animal (*e.g.* A1 is animal 1 in Group A)

Table 4. Bioavailability study blood sampling times

Treatment	**Blood sampling times (± 1 minute)**
IV	0, 2, 5, 10, 15, 25, 40 min and 1, 2, 3, 4, 6 and 8 hr
IM	0, 10, 20, 30 and 45 min, then 1, 2, 4, 6, 8 and 12 hr
topical	0, 10, 20, 30 and 45 min, then 1, 2, 4, 6, 8 and 12 hr

For application of the transdermal formulation, the test item was administered topically once to each individual as a single dose. Each dose was individually calculated based on the animal's weight, rounded up to the most accurate graduation on the syringe(s) The test item was equilibrated to ambient temperature prior to administration.
Cattle were dry at the time of treatment and remained dry until the completion of blood collection. The dose was administered as a pour-on, using a suitably-sized syringe, applied as a single band along the spine between the shoulders and approximately halfway along the back. Cattle were restrained within a suitable crush at the time of treatment. Elbow-length gloves, fully enclosed boots and overalls were worn as a minimum during administration.

The animals were observed regularly during the bioavailability study. Following treatment on day 0 and during the remainder of the working day, the animals were examined regularly for adverse reactions at the time of blood collections. For animals receiving the topical formulation, the treatment site was specifically examined at approximately 1, 4 and 8 hours post-treatment to ensure no local reactions were evident. Animals receiving the topical formulation were housed individually until the completion of blood collection on the day of administration and specifically observed for grooming behavior. During the study, the cattle were inspected at least daily for general health and well-being.

3.3 To develop a formulation containing an NSAID to control pain associated with routine surgical interventions for up to 24 hr

3.3.1 Clinical study

This study was performed at the Queensland Animal Science Precinct (QASP) at the University of Queensland Gatton Campus.

A total of 36 weaners (male and female ~ 200 kg, Holstein-Fresian calves) were randomly assigned according to bodyweight to one of four groups:

(i) Disbudded and placebo transdermal treatment (n=10).
(ii) Disbudded and NSAID transdermal (n=10).
(iii) Disbudded and NSAID intramuscular (n=10).
(iv) Sham (handling only) (n=5).

To ensure accurate timing of blood sampling and behaviour observations the calves were managed as a series of replicates each containing two calves from each treatment group. All treatments were administered 30 min before dehorning whilst the animals were restrained in the crush. The transdermal NSAID or placebo (vehicle only) was applied along the backline, approximately from the shoulder to the mid-point of the backline. Each replicate of calves were subsequently brought back into the race and individual calves were restrained in the crush, a blood sample collected (0 hr) and then amputation dehorning was performed. Wherever possible severed arteries were grasped with a pair of haemostats and then pulled and twisted to provide effective haemostasis. Subsequent blood samples were collected at 1, 2, 4, 8, 24, 48 and 96 hr. Blood samples were analysed for cortisol and protein biomarkers.

Calf behaviour was monitored before and after dehorning in suitable time periods. The day before dehorning, the behaviour of all 36 calves were monitored for two hours to record the baseline variation, which was taken as 0 hour observation. Next day after dehorning, behaviour was recorded at 2-4 hour, 4-8 hour, 8-12 hour, 24 hour and 48 hours. There were 12 behaviours monitored as follows: head shaking, ear flicking, tail wagging, head rubbing, lying, ruminating, neck extending, grooming, walking, vocalising, feeding and drinking. Each animal was observed for 3 min at each observation period and the frequency of each behaviour parameter during this period was recorded. This protocol was adapted from a spay study conducted by Petherick et al (2013), with the subsequent Ethogram used to analyse the frequency of expression of behaviours during this study.

3.3.2 Conventional parameters to assess pain and inflammation

The conventional parameters used to asses pain and inflammation were measurement of plasma total cortisol concentration (ELISA using a commercial kit), live weight change at 2 and 5 weeks after dehorning and animal behaviour as described above.

3.3.3 Proteomic assessment of pain and inflammation

Proteomics is the branch of science that involves identification, quantification and characterization of all proteins in the living individual. It also plays an indispensable role in assessing the changes in protein expression, occurring in response to various physiological state as well as disease conditions. In last few years, proteomics has been increasingly used in development of novel biomarkers for early diagnosis of diseases and monitoring of treatment strategies of different diseases in human medicine as well as in veterinary medicine

Mass spectrometry (MS) -based proteomics

Over the last two decades technological advancement in the field of proteomics has created a lot of opportunities in exploring the complex biological system of living organism. Especially, MS -based techniques have emerged as the most powerful tool to interpret available genomic information and to create quantitative protein profile from plasma, tissues or cell lines of various species. These techniques also proved useful for analysis of protein-protein interaction, post-translational modifications and gene annotation (Mann and Aebersold, 2003). Mass spectrometry -based proteomics analysis can be broadly divided into two main domains that include discovery proteomics and targeted proteomics. The former enables the unbiased identification and quantification of hundreds to thousands of proteins from complex mixtures using shotgun proteomics techniques (explained below), however, in somewhat stochastic manner whereas targeted proteomics focuses only on the subset of selected protein targets important for the biological process under study. Quantification may be either relative to control sample or on an absolute scale. Targeted proteomics is more and more commonly employed to validate shotgun proteomics findings and started to replace Western blotting approach.

Shotgun (or discovery) proteomics

Traditionally, proteomics and especially proteomics in veterinary field, has been associated with 2D gels-based approaches. 2D gels, however suffer from several issues such as lack of reproducibility, being laborious, underrepresentation of certain classes of proteins and most importantly each protein spot in fact consists of multiple proteins. The idea of shotgun proteomics was introduced in order to avoid having to do 2D gels. Shotgun proteomics is one of the most widely used approaches for identification and/or quantification of maximal number of proteins in a given samples. It has achieved high throughput analysis as compared to other conventional MS-based techniques because of advantages associated with it. First is proteolytic digestion of proteins into short peptides followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) based sequencing, simplifies the sample handling and increase its efficiency of protein identification (Nesvizhskii and Aebersold, 2005). In shotgun proteomics, proteins are denatured, cysteine residues alkylated and the proteins digested into peptides. Various fractionation strategies can be introduced at the protein or peptide level. These peptides are then desalted and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in data dependent acquisition mode (explained below). The most frequently used instruments for shotgun proteomics include ion-trap, hybrid ion-trap/orbitrap, hybrid quadrupole/orbitrap and hybrid quadrupole/TOF mass spectrometer. However, spectra obtained in all these methods vary with regard to their quality (resolution and mass accuracy). There are several other DDA-based quantitative proteomics strategies (label free and label-based, the most popular being spectral counting, SILAC, TMT tags, iTRAQ tags).

Application in veterinary science

Shotgun proteomics techniques support formulation of new hypotheses and are ideally suited for discovery studies due to theirs high sensitivity and ability to screen hundreds to thousands of proteins at once. Its use has been increased in veterinary medicine in the last few years especially in farm animals. Either plasma or other body fluids or tissues have been utilized with a various degree of success. Shotgun analysis has been conducted on plasma samples from dairy cattle suffering with foot rot, to understand plasma protein profile and to discover potential disease-associated proteins. Plasma proteins were separated by SDS-PAGE and finally LC-MS/MS was performed. A total of 648 proteins were identified in healthy plasma samples, of which 234 were non-redundant proteins and 123 were high-confidence proteins; and 712 proteins were identified from infected animal plasma, of which 272 were non-redundant proteins and 138 were high-confidence proteins (Sun et al., 2013). Another study has conducted comparison of the bovine uterine flushing (UF) and plasma proteome using iTRAQ labelling and SCX separation of the iTRAQ labelled peptides and finally LC-MS/MS technique have enabled identification of 53 proteins, out of that 35 proteins were higher in UF compared to plasma (Faulkner et al., 2012). Serum proteome has analysed from normal and FMD virus infected piglets using LC-MS/MS after removing high abundance proteins with depletion kit. In control group, 8 proteins were identified while in virus infected piglets total 9 proteins were identified with good reproducibility and 3 proteins appeared after FMD infection which were absent in healthy (control) animal (Liu et al., 2011). In one study of swine, serum proteomic analysis was done using shotgun (LC-MS/MS) approach after depleting high abundance proteins using SepproTip columns. A total of 1,096 proteins were identified and quantified, of which 182 were identified with multiple unique amino acid sequences and high peptide ID confidence (Bell et al., 2010). Finally, data dependent acquisition is critical for spectral library collection used for deconvolution of SWATH data.

3.3.4 Skin irritation study

During the bioavailability study (at Yarrandoo) skin lesions were observed in some calves observed following application of the transdermal formulation. However, distinct skin changes were not observed during the clinical study (at Gatton campus). Several factors may account for these differences, including:

- The Yarrandoo study washed the site of transdermal application with 'Fido's everyday shampoo' 12hrs after application to remove the remaining formulation.
- Breed differences – mixed breeds were used at Yarrandoo, while Holstein-Friesians were used at Gatton.
- Age/weight differences – average volume at Gatton was 10 mL, while at Yarrandoo it was 15-19 mL, reflecting the larger size of the cattle.
- Ambient conditions, relating to sun exposure, temperature, etc.

To investigate this apparent difference, a further study was conducted at the Gatton campus using the same study design and protocol as used in the Yarrandoo study.

A total of 30 Holstein-Friesian calves were enrolled into the study. The calves (males only) were ~ 150 kg bodyweight and between 2-4 months old. The calves were weighed and then randomly assigned according to weight to one of three groups:

Group 1 (n=10): The transdermal formulation was applied as a single application along the spine from shoulders to lumbar region.

Group 2 (n=10): The transdermal formulation was applied as a single application as in Group 1. The site of application was washed off 12 hr later using Fido's everyday shampoo and use of a scrubbing brush, as per the Yarrandoo protocol.

Group 3 (n=10): The transdermal vehicle only, in an equivalent volume to the formulation, was applied as a single application as in Group 1.

Each calf was photographed shortly after formulation administration and then proceeded to a holding yard (with no cover against sun or rain). The calves were then casually monitored while in the holding yard for grooming and rubbing and then were observed as they came through the race at 12 hrs after application. The calves were managed in a small pasture paddock for the remainder of the study. During the first week the site of application was observed and palpated daily by a large animal internal medicine specialist who was blinded to the treatments applied. The calves were monitored for a further week on alternate days. Any calf showing visible or palpable skin lesions was photographed, and at 2 weeks post application a representative number of calves (n=4) with skin lesions underwent a skin biopsy.

4 Results

4.1 To confirm transdermal penetration of candidate formulations using *in vitro* techniques

4.1.1 Regional differences in transdermal penetration of lignocaine hydrochloride

In all animals, the cumulative amount of lignocaine hydrochloride penetrating through skin significantly increased over time (P < 0.001) (see Figure 1). There was a significantly higher cumulative amount of lignocaine penetrating through the skin from animals 2 and 4, compared to animals 1 and 3 (*P* < 0.001). From a pair-wise comparison, the amount of lignocaine penetrating through the skin in animal 5 did not significantly differ from other animal (*P* > 0.1), except animal 1 (*P* < 0.05)

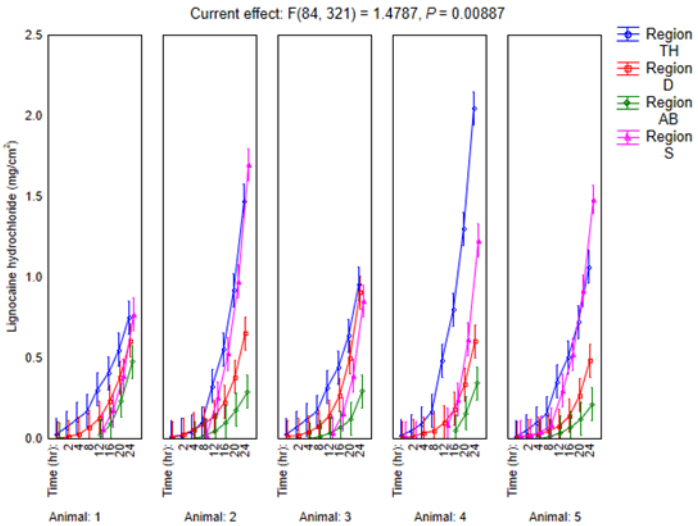

Figure 1. Permeation profiles of lignocaine hydrochloride through skin obtained from the thorax (TH), dorsal midline (D), ventral abdomen (AB) and scrotum (S). The cumulative amount is marked on the y-axis. Each data point represents a mean value and standard error of mean from a number of replicates (n = 3).

There were significant differences in the cumulative amount of lignocaine that penetrated through all the skin regions (*P* < 0.001) (See Figure 2), with the thorax significantly higher than the other three regions (*P* < 0.001). The amount of lignocaine penetrated through the scrotal skin was also

significantly higher than the skin from the dorsal midline ($P < 0.001$) and ventral abdomen ($P < 0.001$). After 24 hr exposure, the concentration of lignocaine hydrochloride retained within the scrotal skin was significantly higher than the other regions ($P < 0.001$) (See Figure 3). The concentrations of lignocaine in the skin from the ventral abdomen, dorsal midline and thorax did not significantly differ ($P > 0.5$).

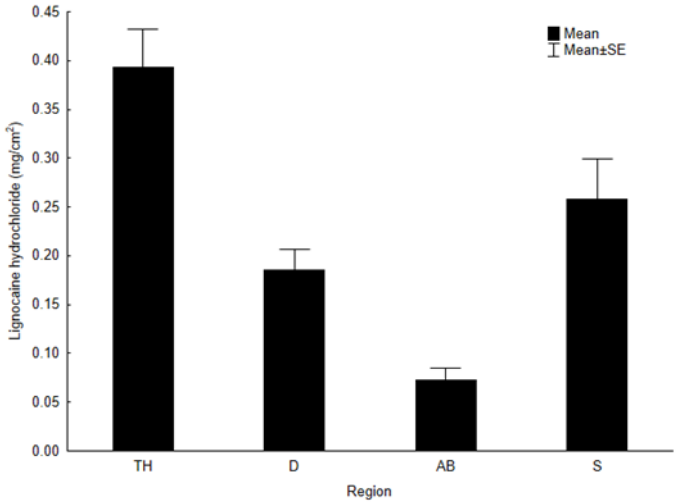

Figure 2. The mean cumulative of lignocaine hydrochloride in the receptor phase across four regions over 24 hr.

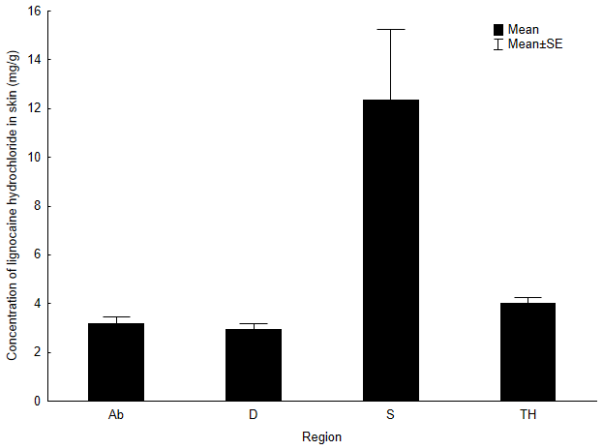

Figure 3. The mean of the amount of lignocaine hydrochloride retained in the skin at the complete of each experiment.

4.1.2 Screening of NSAID's and vehicle combinations to maximise active drug penetration through cattle skin

Over 150 vehicle combinations were screened in this study. The four NSAIDS registered for use in cattle in Australia were compared for their solubility in the various vehicles and their efficacy in moving through cattle skin *in vitro*. One NSAID was significantly more effective in penetrating through cattle skin in all of the vehicles used in this study. A full list of the vehicles and raw data can be found as an Excel file ('NSAID screening data April-May2016 final') in the confidential background information to this report.

4.1.3 Optimising the candidate formulations

Once it was decided to move forward with one of the NSAIDs, the vehicles were refined to maximise transdermal drug penetration. Several solubilising agents were tested, and a range of active concentrations were considered. The costs of the most promising formulation would be ~ $3.20 per 10 mL dose, which compares highly favourably with parenteral formulations (which would need to also include needle and syringe), particularly when considering the convenience and safety factors.

4.1.4 Stability studies of the final formulation

There was no change in the final formulation at 2 or 4 months storage, in terms of precipitation, crystallisations, colour and evaporation. There was some evidence of evaporation, particularly from the glass vessels, when stored at 40^{o}C.

At 6 months, evaporation was evident in all samples stored in glass at 40 ^{o}C and some at ambient temperature. A small colour change was observed in samples stored at 40 ^{o}C.

4.1.5 Analysis of NSAID in plasma

Cold Acetonitrile (0.2% formic acid) precipitation was added to samples at 1:1 ratio and vortexed for 30 s. Protein crashing carried out by 2 rounds of centrifugation for 15min at 20,000 *g*. Interval between "crashing" was 30 min at 4 ˚C. Supernatant of each sample collected and analysed by LCMS/MS.

It is noted that further cleaning and processing of samples will enable better sensitivity to the assessment. For the purpose of continuity method of sample preparation was not changed throughout the project.

NSAID concentration determination by LC-MS/MS (9-12th Jan 2017)
Instrument: Shimadzu Nexera uHPLC in tandem with LCMS 8030.
CID gas: Argon
Column: Phenomenex Kinetix Evo C18, 1.7µm pore size.
Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in Acetonitrile
LOD: 2 ppb; QC samples instrument stability at 0.02 µg/mL; Calibration range 0.0049 - 5µg/mL.

NSAID: Detection m/z 255.10>105.20 and confirming ion 255.10>194.25, 255.10>209.30
Collision energies for each transition was optimised Co-elution of all peaks are checked and verified in all analysis files. The right chart verifies the retention time %CV of each transition across all files.

ISTD Ibuprofen: Unsuccessful – instrument negative ion mode was out of order and the column length was likely to be too short, and a longer chromatography programme may have been required. Modifications to be considered, or another surrogate NSAID to be used as an ISTD.

Samples: must be prepared with less than 90% solvent prior to analysis.

Instrument performance and stability: All samples were analysed on the instrument continuously. QC was analysed every 20 samples, CV <5%. Five sets of calibration curves were assessed.

4.2 To determine the efficacy of candidate transdermal formulations to control pain associated with dehorning

4.2.1 Pilot study 1

The plasma NSAID concentrations (µg/mL) in each calf were as follows:

Time (hr)	Calf 3405	Calf 3383	Calf 3389	Calf 3385
0	0.057	0.021	0.027	0.024
2	12.21	42.768	1.905	3.498
4	1.092	2.103	3.132	0.963
8	0.603	0.252	1.503	0.048
12	0.375	0.102	0.303	0.108
24	0.075	0.045	0.111	0.285

Plasma NSAID concentrations were detectable and approached what may have been expected following IM administration. The lag time (between application and appearance of active in the plasma) was unexpectedly very short (most transdermal formulations exhibit a lag time of several hours). It was uncertain if the peaks for the applications had been missed. It was also unsure if any grooming had contributed to the short lag time but casual observation indicated this was unlikely to have been significant.

4.2.2 Pilot study 2

The plasma NSAID concentrations (µg/mL) were as follows:

Time (hr)	Calf 3401	Calf 3415	Calf 3417	Calf 3421
0	0.021	0.039	0.258	0.321
1	2.301	0.405	0.192	0.048
2	2.904	5.199	6.624	9.912
3	1.965	2.616	4.182	8.103
4	1.251	0.711	1.314	2.616
6	0.951	0.426	0.744	0.933

There was no grooming observed at any stage during the second pilot study. Again, a short lag phase was observed, with peak plasma concentrations ~ 2 hr following application. Although we did not weigh the calves, there did seem to be a weight-related effect, since the lowest plasma concentrations were in calf 3401 and the highest in calf 3421, which were estimated to be the heaviest and lightest calves, respectively, although calf 3401 was male and there may be some gender differences.

4.2.3 Bioavailability study

This study was performed at the Yarrandoo site of Elanco in NSW.

The transdermal formulation had a slower absorption than IM, although the peaks were equivalent at 30 minutes. A peak (C_{MAX}) occurred ~ 2 hr. A bioavailability of 50% and an area under the curve (AUC; 3940 µg.mL/min) exceeding that following IM administration (2376 µg.mL/min) suggested that the transdermal formulation had a greater systemic effect than IM administration.

Unfortunately, evidence of skin irritation was observed several days after administration of the transdermal formulation, continuing to marked skin inflammation and excoriation in the 3-4 weeks following application. It was uncertain if this irritation was due to photosensitisation by the NSAID, the fact that the transdermal formulation had been administered three times to each animal, or other environmental factors.

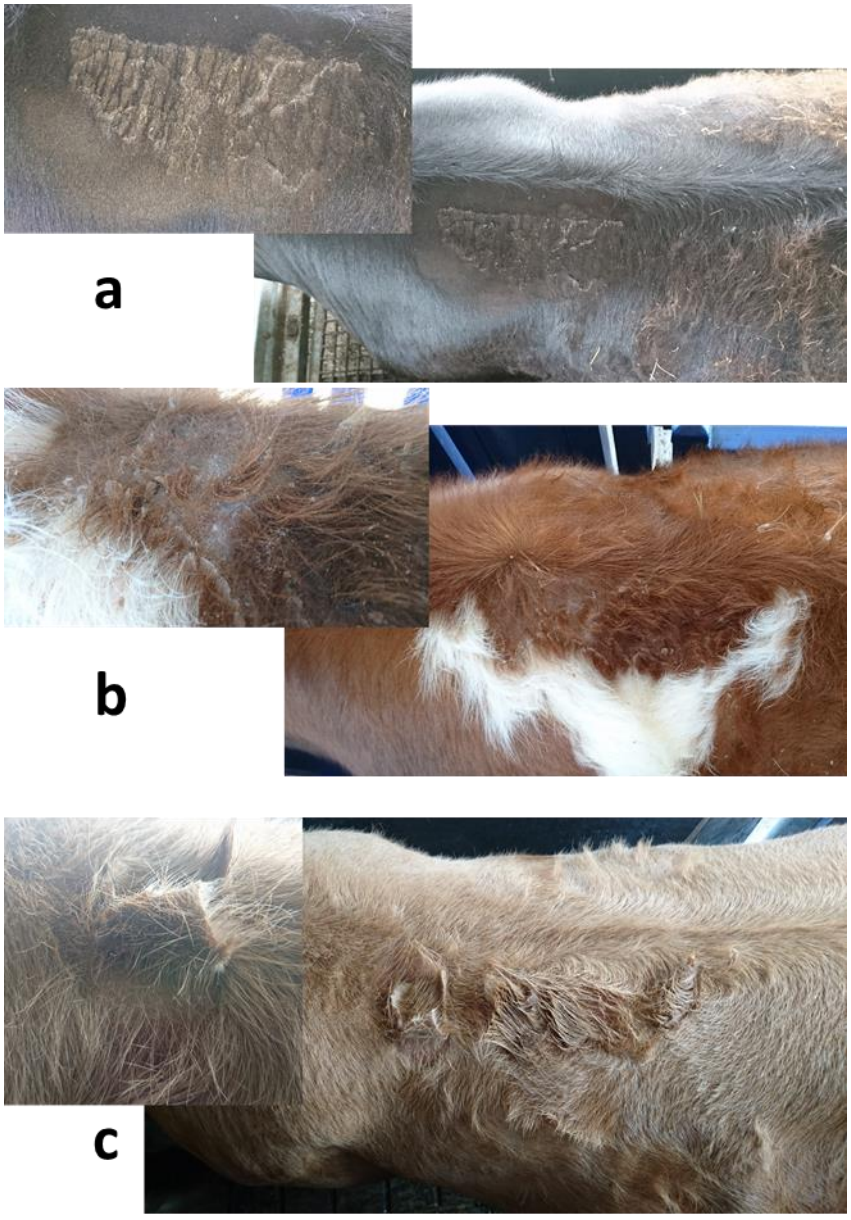

Figure 4. Skin lesion observed following administration of transdermal NSAID formulation in the Yarrandoo study. The figures illustrate the irritation at various time points, with inserts showing a closer view of the actual site of irritation; (a) calf #10, 8 days post application (area shaved for biopsy); (b) calf #169: 14 days post application; (c) calf #181: 27 days post application

4.3 To develop a formulation containing an NSAID to control pain associated with surgical husbandry procedures for up to 24 hr

4.3.1 Clinical study

This study was successfully performed and all 36 animals completed the study. Some calves required attention to continued bleeding from the dehorning site, which was undertaken manually. All animals remained in good health throughout the study.

4.3.2 Conventional parameters to assessment pain and inflammation

Total cortisol

The total plasma cortisol concentration (ng/mL) in each animal at each time point can be observed in table 6.

Table 5. Total plasma cortisol concentration (ng/mL)

		Time						
Group	Animal	0	1	2	4	8	24	96
Placebo	50	3.5	18.2	8.2	27.7	4.0	5.6	16.1
Placebo	54	10.0	19.2	36.9	32.6	24.1	19.5	22.5
Placebo	60	6.4	36.6	21.4	30.0	19.8	12.9	25.7
Placebo	61	2.0	17.2	21.8	24.9	21.1	24.6	7.5
Placebo	68	13.7	13.1	19.2	21.8	18.8	11.8	9.3
Placebo	76	8.4	28.4	35.7	31.8	22.9	37.8	25.6
Placebo	74	16.8	29.7	24.6	34.9	37.1	30.8	19.3
Placebo	47	15.9	30.9	9.6	15.5	15.8	20.1	8.7
Placebo	44	19.0	24.2	17.6	39.9	41.9	14.6	4.8
Placebo	52	6.8	8.1	46.6	8.8	5.6	7.0	6.0
	mean	**10.2**	**22.5**	**24.2**	**26.8**	**21.1**	**18.5**	**14.5**
	SEM	**1.8**	**2.8**	**3.9**	**3.0**	**3.7**	**3.3**	**2.6**
TransD	72	6.5	4.6	3.9	14.5	10.4	1.9	1.9
TransD	78	4.0	6.6	1.9	4.2	Missing	1.9	10.9
TransD	75	8.8	1.9	1.9	14.7	16.6	21.9	2.7
TransD	64	1.9	28.0	2.6	6.0	19.9	2.7	19.1
TransD	48	3.2	27.5	13.4	3.1	17.0	1.9	16.7
TransD	67	1.9	Missing	4.8	22.1	14.2	16.5	9.9
TransD	39	8.0	16.5	4.8	9.0	11.4	12.7	9.9
TransD	51	8.4	16.3	6.1	1.9	13.3	9.1	9.4
TransD	46	Missing	4.0	1.9	1.9	22.8	1.9	2.3
TransD	43	2.4	29.1	10.7	19.0	28.7	6.8	16.8
	mean	**5.0**	**15.0**	**5.2**	**9.7**	**17.1**	**7.7**	**9.9**
	SEM	**1.0**	**3.7**	**1.2**	**2.4**	**2.0**	**2.3**	**2.0**
Sham	69	1.9	1.9	8.5	5.4	10.1	14.0	8.2
Sham	58	2.7	8.9	5.6	2.4	7.7	1.9	17.1
Sham	66	1.9	2.9	1.9	7.0	12.8	4.5	6.7
Sham	49	5.1	12.1	2.4	1.9	19.5	3.8	2.6

Sham	53	1.9	2.3	1.9	11.3	16.4	1.9	1.9
Sham	38	1.9	1.9	2.2	1.9	1.9	1.9	Missing
	mean	**2.6**	**5.0**	**3.7**	**5.0**	**11.4**	**4.7**	**7.3**
	SEM	**0.5**	**1.8**	**1.1**	**1.5**	**2.6**	**1.9**	**2.7**
IM	82	2.9	3.3	4.1	29.9	23.3	21.9	30.0
IM	77	3.2	7.9	4.4	24.6	20.9	10.6	4.5
IM	65	7.8	5.9	9.0	15.8	18.9	6.7	26.9
IM	70	6.1	3.4	3.7	25.2	8.6	1.9	8.3
IM	56	11.8	13.5	4.6	16.2	12.9	6.9	30.7
IM	63	3.1	7.1	8.1	8.3	10.3	16.7	11.7
IM	35	13.0	30.8	6.4	19.7	28.8	6.5	2.3
IM	42	2.8	42.6	39.1	19.2	10.7	9.6	16.6
IM	40	3.7	7.6	10.1	4.4	7.9	2.8	4.1
IM	55	4.0	6.1	15.5	6.3	18.8	22.5	16.7
	mean	**5.8**	**12.8**	**10.5**	**17.0**	**16.1**	**10.6**	**15.2**
	SEM	**1.2**	**4.2**	**3.4**	**2.7**	**2.2**	**2.3**	**3.4**

When looked at graphically, it can be seen that there are differences between the groups when looked at over the entire 96 hr (Fig 15) and, particularly, during the first 4 hr (Fig 16).

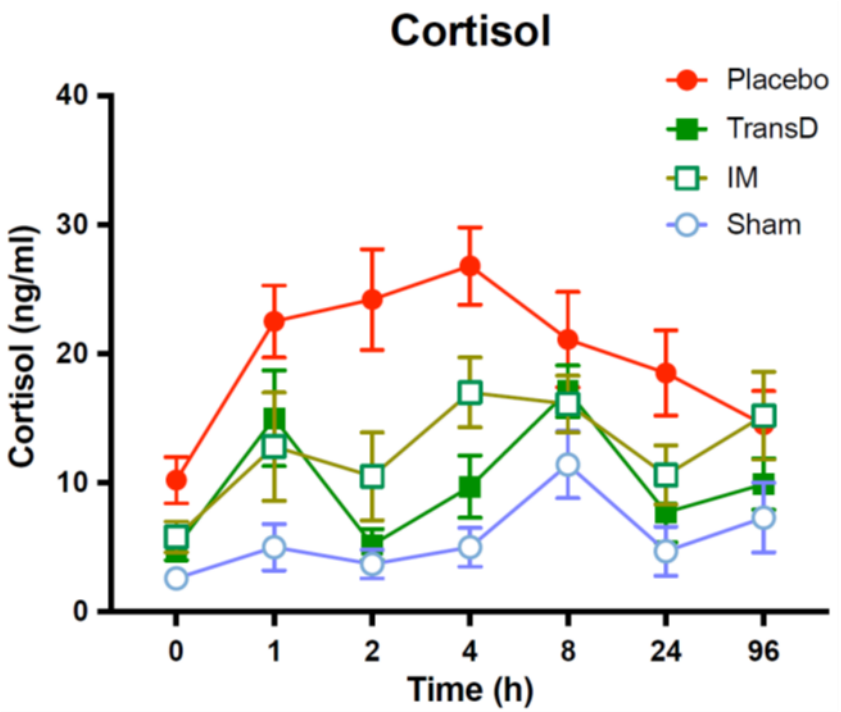

Figure 5. Total plasma cortisol (ng/mL) over 96 hr

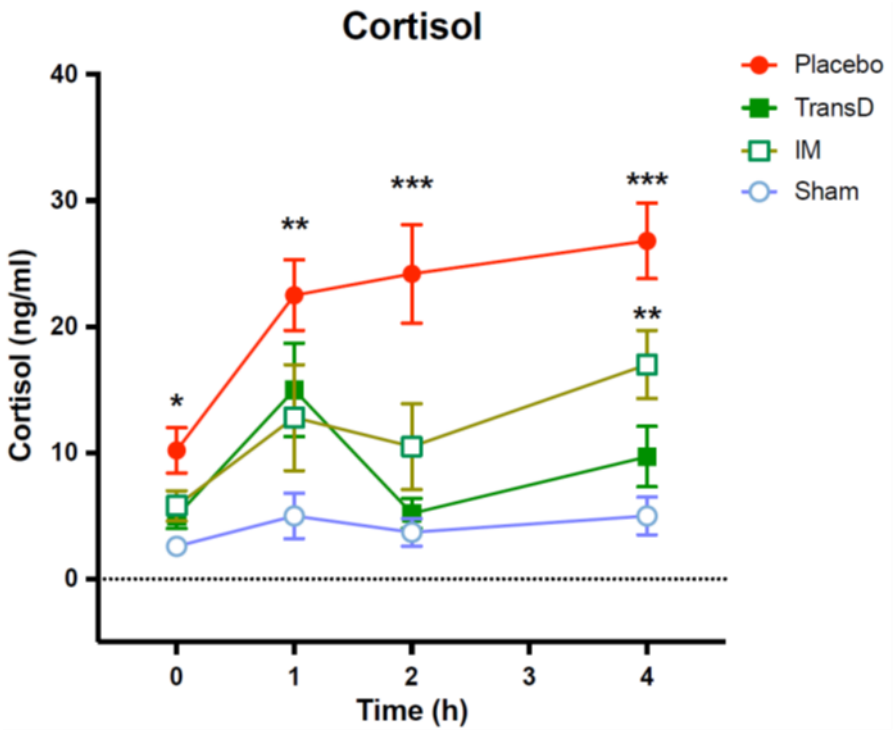

Figure 6. Total plasma cortisol (ng/mL) during the first 4 hr only

The most obvious differences are in the first 4 hr. At 8 hr all treatments had an increase and there was no treatment effect post 8 hr. An ANOVA on each time point and the statistical result is shown on the 4 hr (Fig 6). At the start (time 0) the placebo were slightly higher than others, while placebo increased markedly over time being significant from sham at all time points up to 4 hr.

The IM and Trans D increased from time 0 and were essentially the same (not different from each other at any time point). At 1 hr the Trans D and IM were not different from either sham or placebo (i.e. Intermediate). But at 2 hr both the Trans D and IM were not different from sham, but they were obviously both lower than placebo. At 4 hr the IM increases a bit to become significant from sham, but not Trans D.

These results mean that there was no difference in cortisol concentration between Trans D, sham OR IM at any time point. In Trans D there was a mild NS increase in cortisol at 1 hr compared to sham. There was a significant blunting of the stress response by transD and IM and 1, 2 and 4 hr post dehorning. The increase in cortisol at 8 hr for the sham group may reflect apprehension at repeated sampling.

Body weight

There were no significant differences between either treatment group and the sham group, while both IM and transdermal groups had significantly ($P<0.05$) higher body weight gain, compared to the placebo group, during the first 2 weeks following dehorning (Table 7).

Table 6. Body weight changes in dehorned calves compared to sham-treated calves, compared to the effects of NSAID, administered IM or transdermally

Treatment	Animal no.	Animal ID	Body weight		
			26/10/2016	10/11/2016	1/12/2016
Placebo (Y)	1	74	89	118	143
	2	76	99	125	153
	3	68	115	142	160
	4	61	135	165	201
	5	60	150	169	212
	6	54	161	186	190
	7	50	177	211	238
	8	47	188	205	246
	9	44	191	205	249
	10	52	212	233	266
	Mean		151.7	175.9	205.8
	SD		41.6	38.8	43.8
TD (R)	11	72	92	112	132
	12	78	98	118	145
	13	75	112	139	171
	14	64	127	155	184
	15	48	150	180	211
	16	67	165	195	225
	17	39	176	213	246
	18	51	180	206	241
	19	46	205	242	281
	20	43	215	252	292
	Mean		151.9	181.2	212.8
	SD		43.5	49.2	54.4
IM (B)	21	82	93	107	135
	22	77	94	120	152
	23	65	110	141	164
	24	70	116	134	169
	25	56	150	179	207
	26	63	156	187	224
	27	35	191	210	252
	28	42	191	216	257
	29	40	205	240	278
	30	55	212	250	287
	Mean		151.8	178.4	212.5
	SD		46.4	50.9	55.2

Sham control (G)	31	69	104	126	148
	32	58	107	126	162
	33	66	145	172	208
	34	49	149	183	215
	35	53	184	216	246
	36	38	236	264	311
	Mean		154.16	181.16	215
	SD		49.9	53.4	59.2

Behaviour Ethogram and analysis

The initial challenge was to determine which variables were indicators of pain and these could then be further investigated and compared between groups. The method used to identify those variables was Confirmatory Factor Analysis (CFA) within Structural Equation Modelling Framework (SEM). This method is far more superior to Principal Component Analysis (PCA), Multiple Component Analysis (MCA) or Factorial Analysis (FA) because it permits the use of information from all the variables in the dataset to describe which ones are better predictors of another latent variable, in this case, Pain. SEM is a conceptual map and hypothesis-driven process. Relationships between predictor variables (sems language 'observed variables') have to be plausible for SEM to produce reliable estimates that describe the relationship between the observed variable and the latent variable outcome.

The model output below describe the final, and most stable, models fitted using sems function in STATA 13. Only statistically significant variables remain ($P<0.05$) in the final model. The conceptual framework of the model is also plotted to make the interpretation of model outcome more accessible to the reader.

In the conceptual model map (Figure 7), the oval shape is the latent variable (Pain). Square shapes are the observed variables. The circles are the standard error terms of each of our variables. The arrows are pathways between the latent variable and observed variables and between the error terms of the observed variables (i.e. allows for those variables to correlate with each other). The values shown on those arrows are the values estimated from the sem model (variable loading). Those values are standardised across the entire model, and they are used quantify the association between the latent and observed variables. They are interpreted roughly like regression coefficients as a function of standard deviation change in the latent and observed variables.

Seven variables (ear flick, tail wag, ruminating, head shake, lying down, grooming and neck extending) had substantial loading and were statistically significant. The interpretation is as follow: if an animal is One Standard Deviation higher in Pain (or pain score), it will have/score 0.78 Standard Deviation (SD) higher (positive coefficient) Ear flick count, 0.66 SD higher in Tail wag, 0.36 SD lower (negative coefficient) in Rumination and 0.25 SD lower in Grooming. You can also not ignore the negative correlation between tail wagging and lying down, or that between earflick and headrub. This confirms that the model framework is sound and reliable.

These observed variables can then be compared over time and between groups using separate Poisson models. Ideally (i.e. to demonstrate efficacy), there would be lower counts of positively correlated variables with pain and higher counts of those variables negatively correlated with pain.

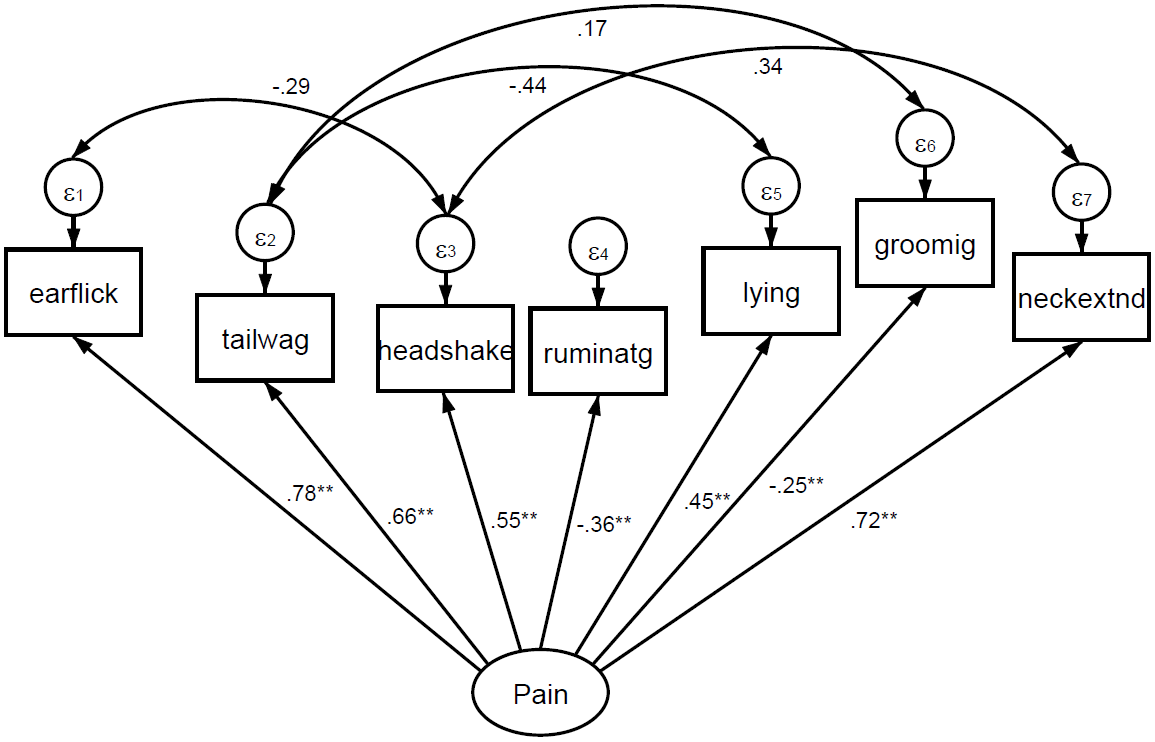

Figure 7. A conceptual map of the parameters indicating pain

Separate models were used for each of the observed animal behavioural traits (Figure 7). A mixed effect Poisson model with an animal fitted as random intercepts and time as random slope, and unstructured error term for the residuals. Behaviours trait at baseline (time 0) was centred and added to the fixed effect part of the model (essentially turning the model into a random slope model too). Overdispersion was assessed and it was ruled out as being problematic (the scale is ~ 1 and not significant. Overdispersion was not an issue). Overdispersed models (also those with excess zeros) were refitted using a negative binomial model. Poisson: ear flick, tail wag and negative binomial: headshake, head rub, lying, ruminate, neck extend, grooming.

Experimental time was fitted as a categorical variable to derive estimates at each time point; time 2 hr was used as the reference category. Treatment was also fitted as categorical variable, placebo as reference category.

The lack of statistical differences is related to the lack of statistical power in this study. To clarify, assuming a power of 80%, significance level percentage of 95%, balanced groups size, a probability of observing an outcome in the control group of 80%, the required sample size for this study to detect an odd ratio (or IRR) of 4 or greater is 88 animals in each group, with the current study having a power of 17%.

Table 7. Marginal effects on frequency of head rub, head shake and ear flick

Variable	Animal Behaviour								
	Head rub			Head shake			Ear Flick		
	Coef (SE)	IRR* 95% CI	P	Coef(SE)	IRR 95% CI	P	Coef(SE)	IRR 95% CI	P
Constant	-3.75 (0.98)	0.02 (0.00 to 0.16)	<0.01	-3.12 (0.36)	0.04 (0.02 to 0.09)	<0.01	-3.37 (0.17)	0.03 (0.02 to 0.05)	<0.01
Behaviour's count at Baseline (time 0)	0.96 (0.93)	2.61 (0.42 to 16.00)	0.30	-0.32 (0.52)	0.72 (0.26 to 2.02)	0.53	-0.05 (0.04)	0.95 (0.87 to 1.03)	0.22
Time									
2h	Reference			Reference					
4h	-1.84 (0.44)	0.16 (0.07 to 0.38)	<0.01	-0.32 (0.52)	0.72 (0.26 to 2.02)	0.53	-0.04 (0.17)	0.96 (0.69 to 1.33)	0.80
8h	-3.64 (1.01)	0.03 (0.00 to 0.19)	<0.01	-1.17 (0.58)	0.31 (0.10 to 0.97)	0.04	-0.36 (0.18)	0.70 (0.49 to 1.00)	0.05
24hrs	-2.14 (2.95)	0.12 (0.00 to 38.00)	0.21	-2.27 (0.75)	0.10 (0.02 to 0.45)	<0.01	-1.73 (0.30)	0.18 (0.10 to 0.32)	<0.01
48hrs	-1.99 (0.62)	0.14 (0.04 to 0.46)	<0.01	-2.67 (0.85)	0.07 (0.01 to 0.37)	<0.01	-1.35 (0.26)	0.26 (0.16 to 0.43)	<0.01
Treatment									
Placebo	Reference			Reference					
Trans	-0.03 (0.49)	0.97 (0.37 to 2.55)	0.96	-1.22 (0.51)	0.30 (0.11 to 0.81)	0.02	-0.07 (0.18)	0.93 (0.65 to 1.32)	0.68
IM	-0.77 (0.70)	0.46 (0.12 to 1.83)	0.27	-0.12 (0.52)	0.89 (0.32 to 2.45)	0.82	-0.10 (0.19)	0.91 (0.62 to 1.32)	0.60

* IRR = Incidence Risk/count Ratio

Table 8. Marginal effects on frequency of tail wag, lying down and neck extending

Variable	Animal Behaviour								
	Tail wag			Lying			Neck extended		
	Coef(SE)	IRR* 95% CI	P	Coef(SE)	IRR 95% CI	P	Coef(SE)	IRR 95% CI	P
Constant	-3.13 (0.18)	0.04 (0.03 to 0.06)	<0.01	-2.62 (0.45)	0.07 (0.03 to 0.18)	<0.01	-2.42 (0.17)	0.09 (0.06 to 0.12)	<0.01
Behaviour's count at Baseline (time 0)	-0.12 (0.06)	0.88 (0.79 to 0.99)	0.03	-0.51 (0.34)	0.60 (0.31 to 1.16)	0.13	-0.58 (0.28)	0.56 (0.33 to 0.97)	0.03
Time									
2 hr	Reference			Reference			Reference		
4 h	-0.37 (0.23)	0.69 (0.44 to 1.07)	0.09	-0.62 (0.47)	0.54 (0.21 to 1.35)	0.18	-0.58 (0.28)	0.56 (0.33 to 0.97)	0.04
8hr	-0.88 (0.27)	0.42 (0.25 to 0.70)	<0.01	-0.77 (0.49)	0.46 (0.18 to 1.21)	0.11	-0.83 (0.31)	0.44 (0.24 to 0.81)	<0.01
24 hr	-1.39 (0.32)	0.25 (0.13 to 0.47)	<0.01	-2.56 (1.04)	0.08 (0.01 to 0.59)	0.01	-1.16 (0.34)	0.31 (0.16 to 0.61)	<0.01
48 hr	-1.16 (0.30)	0.31 (0.18 to 0.56)	<0.01	-2.56 (1.04)	0.08 (0.01 to 0.59)	0.01	-1.16 (0.37)	0.31 (0.15 to 0.64)	<0.01
Treatment									
Placebo	Reference			Reference			Reference		
Trans	-0.22 (0.22)	0.80 (0.52 to 1.24)	0.32	-0.74 (0.58)	0.48 (0.15 to 1.49)	0.20	-0.07 (0.26)	0.93 (0.55 to 1.56)	0.78
IM	-0.08 (0.22)	0.92 (0.60 to 1.41)	0.71	-0.61 (0.53)	0.54 (0.19 to 1.53)	0.25	0.05 (0.30)	1.05 (0.59 to 1.88)	0.85

* IRR = Incidence Risk/count Ratio

Table 9. Marginal effects on frequency of grooming and ruminating

Variable	Animal Behaviour					
	Grooming			Ruminating		
	Coef(SE)	IRR* 95% CI	P	Coef(SE)	IRR 95% CI	P
Constant	-7.15 (1.42)	0.00 (0.00 to 0.01)	<0.01	-11.17 (3.19)	0.00 (0.00 to 0.01)	<0.01
Behaviour count at Baseline (time 0)	0.09 (0.26)	1.10 (0.67 to 1.82)	0.71	-0.08 (0.43)	0.93 (0.40 to 2.16)	0.86
Time (continuous variable)	0.66 (0.32)	1.94 (1.04 to 3.62)	0.03	2.11 (1.06)	8.22 (1.03 to 65.45)	0.04
Treatment						
Placebo	Reference					
Trans	0.08 (2.12)	1.08 (0.02 to 68.22)	0.97	0.64 (0.77)	1.91 (0.42 to 8.65)	0.40
IM	0.90 (1.78)	2.46 (0.08 to 80.6)	0.61	-0.30 (1.10)	0.74 (0.09 to 6.37)	0.78

* IRR = Incidence Risk/count Ratio

Table 10. Predicted marginal means derived from the models above for an animal with behavioural count at baseline set to the average observed for that trait.

Behaviour	Time														
	2 hr			4 hr			8 hr			24 hr			48 hr		
	Placebo	KetoTrans	KetoIM	Placebo	KetoTrans	Ket IM	Placebo	KetoTrans	KetoIM	Placebo	KetoTrans	KetoIM	Placebo	KetoTrans	KetoIM
Head rub	6.17	6.00	2.85	0.98	0.95	0.45	0.16	0.16	0.08	0.00	0.00	0.00	0.84	0.82	0.39
Head shake	4.44	1.32	3.94	3.21	0.70	3.42	1.38	0.47	1.20	0.46	0.42	1.03	0.31	7.09	0.17
Ear flick	2.02	1.88	1.83	1.94	1.97	2.09	1.41	1.34	1.26	0.36	0.49	0.43	0.53	0.52	0.50
Tail wag	1.29	1.04	1.19	0.89	0.92	0.95	0.54	0.69	0.63	0.32	0.40	0.30	0.40	0.37	0.42
Lying	4.60	2.87	2.91	2.47	2.18	2.22	1.33	1.66	1.69	0.72	1.27	1.29	0.39	0.96	0.98
Neck extended	0.98	0.91	1.03	0.55	0.76	1.31	0.43	0.46	0.69	0.31	0.38	0.69	0.31	0.42	0.69
Grooming	0.19	0.21	0.34	0.36	0.42	0.50	0.70	0.84	0.73	1.36	1.68	1.07	2.63	3.39	1.56
Ruminating	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.18	0.15	0.15

* IRR = Incidence Risk/count Ratio

One issue that became apparent was that the statistical analyses reported above considered changes to 48 hr. However, NSAIDs are known to have a duration of effect of a maximum of 24 hr. The analysis was therefore re-done to consider only the initial 24 hr period. As described above, the model output below describes the final, and most stable models fitted using sems function in STATA 13. Only statistically significant variables remain ($P<0.05$) in the final model. The conceptual framework of the model is also plotted to make the interpretation of model outcome more accessible to the reader.

Seven variables (Ear flick, tail wag, …) had substantial loading and were statistically significant. The interpretation is as follow: if an animal is One Standard Deviation higher in Pain (or pain score), it will have/score 0.78 Standard Deviation (SD) higher (positive of) Ear flick count, 0.66 SD higher in Tail wag, 0.36 SD lower (negative coef) in Rumination and 0.25 SD lower in Grooming. You can also not ignore the negative correlation between tail wagging and laying down, or that between earflick and headrub, which confirms that the model framework is sound and reliable. These observed variables were then compared over time and between groups using separate Poisson models.

To fit the mixed effect Poisson and negative binomial models, the results from the initial (48 hr) outcomes were examined to determine the following approaches:

- Aggregate positively associated variables (i.e. Head rub+ Head shake+Ear Flick+ Tail wag+ Laying+Neck extended) with Pain.
- Aggregate negatively associated variables with Pain (i.e. Grooming + Ruminating).

Each of those combined variables became an outcome that is carried forward in to the final analysis, as described previously. Separate models were used for each of the observed animal behavioural traits i.e. all the variables showed above (minus the latent variable). A mixed effect Poisson model with an animal fitted as random intercepts and time as random slope, and unstructured error term for the residuals. Behaviours at baseline (time 0) were centred and added to the fixed effect part of the model (essentially turning the model into a random slope model too). Overdispersion was assessed and it was ruled out as being problematic (the scale is ~ 1 and not significant. Overdispersion was not an issue). Overdispersed models (also those with excess zeros) were refitted using a negative binomial model. Poisson: earflick, tailwag and Negative binomial: headshake, headrub, lying, ruminate, neck extend, grooming. Experimental time was fitted as a categorical variable to derive estimated at each time point, with time 2 hr used as the reference category. Treatment was also fitted as categorical variable, placebo as reference category. Two-way interaction with Treatment was forced and remained in the final model.

Tables 12 and 13 describe the outcomes of analysis of the positively associated and negatively associated coefficients. The interaction terms can be observed in Figures 8 and 9. The coefficient for NSAID Trans (compared with Placebo) is -0.42, which means that, on average, the expected log count of positively associated pain variables in NSAID Trans was reduced by 42%, compared with those for the Placebo calves. Alternatively, on average, the incident risk ratio of positively associated pain variables for NSAID Trans was 0.66 (IRR 0.66 95% CI 0.47 to 0.92; $P = 0.02$) times that of the Placebo group. The highest incidence risk ratio of positively associated pain variables was observed 2 hr and 4 hr after dehorning. Although the interaction term results were less impressive, the positively associated pain variables count followed a similar trend across all groups, which indicates a significant ($P<0.05$) treatment effect.

Table 11. Marginal effects on positively associated pain variables (Head rub+ Head shake+Ear Flick+ Tail wag+ Lying down+Neck extended)

Variable	Coef (SE)	IRR* 95% CI	P
Constant	-2.79 (0.29)	0.06 (0.04 to 0.11)	<0.01
Behaviour count at Baseline (time 0)	0.07 (0.03)	0.93 (0.87 to 0.99)	0.03
Time			
2 hr	Reference	1.00	
4 hr	-0.28 (0.17)	0.75 (0.54 to 1.01)	0.09
8 hr	-0.72 (0.18)	0.49 (0.34 to 0.70)	<0.01
24 hr	-1.85 (0.24)	0.16 (0.09 to 0.25)	<0.01
Treatment			
Placebo	Reference	1.00	
Trans	-0.42 (0.17)	0.66 (0.47 to 0.92)	0.02
IM	-0.17 (0.17)	0.84 (0.60 to 1.20)	0.32
Treatment and Time interaction			
Placebo at 2 hr	Reference	1.00	
Trans at 4 hr	0.16 (0.25)	1.18 (0.72 to 1.92)	0.49
Trans at 8 hr	0.19 (0.26)	1.21 (0.72 to 2.02)	0.46
Trans at 24 hr	0.59 (0.32)	1.81 (0.96 to 3.42)	0.07
IM at 4 hr	0.26 (0.25)	1.31 (0.80 to 2.14)	0.28
IM at 8 hr	0.11 (0.27)	1.12 (0.66 to 1.91)	0.67
IM at 24 hr	0.38 (0.35)	1.46 (0.75 to 2.85)	0.27

* IRR = Incidence Risk/count Ratio

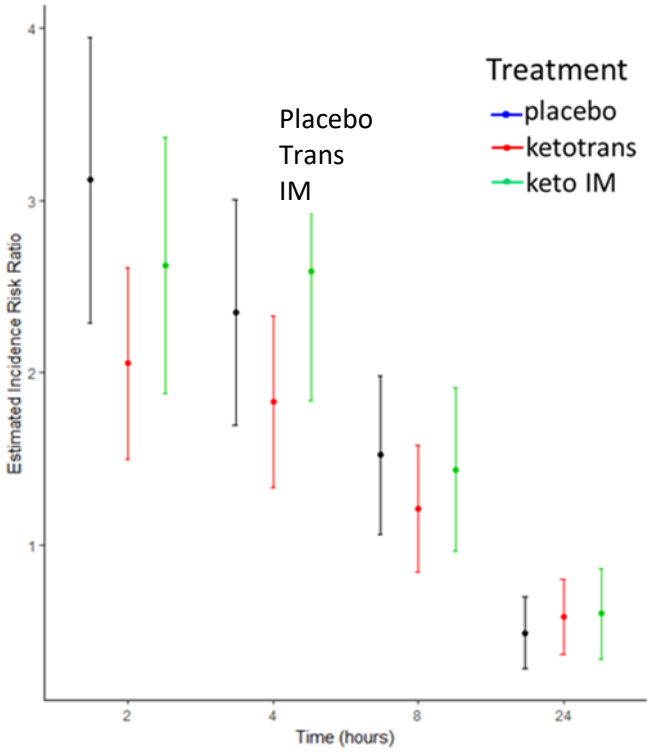

Figure 8. Treatment group by time interaction for the positively associated pain variables

Table 12. Negative pain indicators group (Ruminating and Grooming).

Variable	Coef (SE)	IRR** 95% CI	P
Constant	-3.26 (0.30)	0.04 (0.02 to 0.07)	<0.01
Behaviour's count at Baseline (time 0)	-0.03 (0.10)	0.97 (0.84 to 1.14)	0.74
Time			
2 hr	Reference	1.00	
4 hr	0.01* (0.38)	1.00 (0.47 to 2.13)	1.00
8 hr	0.10 (0.36)	1.10 (0.55 to 2.21)	0.79
24 hr	0.70 (0.32)	2.00 (1.07 to 3.75)	0.03
Treatment			
Placebo	Reference	1.00	
Trans	0.10 (0.36)	1.10 (0.53 to 2.23)	0.02
IM	0.20 (0.35)	1.23 (0.62 to 2.45)	0.32
Treatment and Time interaction			
Placebo at 2 hr	Reference	1.00	
Trans at 4 hr	-0.01* (0.55)	1.00 (0.34 to 2.94)	0.49
Trans at 8 hr	-0.01 (0.53)	0.99 (0.34 to 2.81)	0.46
Trans at 24 hr	-0.20 (0.47)	0.82 (0.32 to 2.07)	0.07
IM at 4 hr	-0.10 (0.55)	0.91 (0.31 to 2.68)	0.28
IM at 8 hr	-0.10 (0.50)	0.99 (0.37 to 2.65)	0.67
IM at 24 hr	-0.45 (0.49)	0.63 (0.24 to 1.65)	0.27

*Coefficient X 1000; ** IRR = Incidence Risk/count Ratio

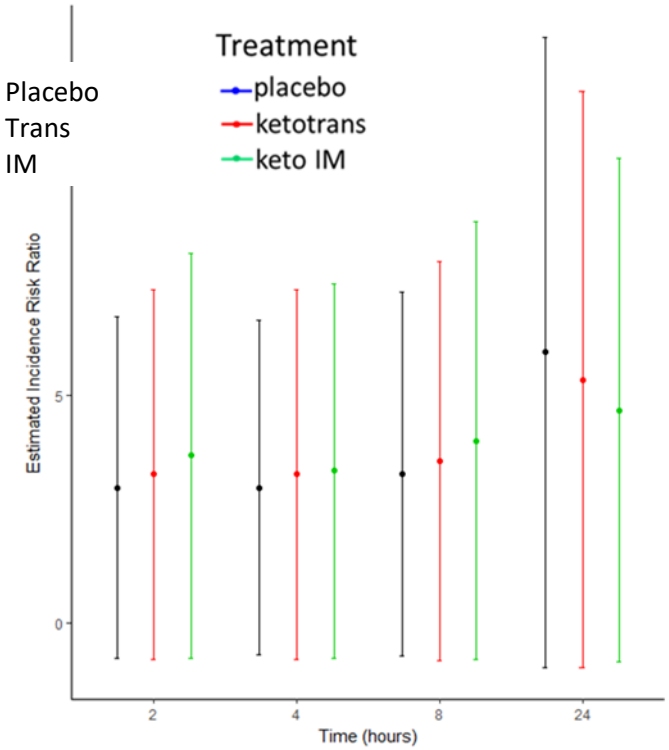

Figure 9. Treatment by time interactions for the negatively associated pain variables

4.3.3 Proteomic assessment of pain and inflammation

Currently, data collection to create an expanded cattle plasma spectral library is in progress. Plasma samples from a wide range of animals (age, breed/species and health conditions) are being interrogated in order to identify as many plasma proteins as possible. Spectral library construction is an inevitable component of SWATH-MS analysis. This analysis uses information contain in a library to analyse the acquired fragment ion maps to identify and quantify the targeted peptides because all the information about fragment ion signals, their intensities and chromatographic concurrence of targeted peptide can be access from this library. If the targeted peptide is not present in the spectral library then it cannot be identified and quantified in SWATH-MS analysis of samples.
We have processed eight plasma samples from adult cattle suffering with different pathological conditions. Sample preparation was done using in solution digestion method. Desalting was performed using stage tips. After sample preparation, mass spectrometer analysis was conducted using LC-MS/MS technique on ABSciex platform. Data generated through mass spectrometer were analysed using ProteinPilot search engine against bovine protein database. Data were analysed at 1% global FDR to eliminate falsely discovered proteins. We also removed proteins with very low unused score and very low peptide number to avoid false positive results.
Currently, approximately 165 proteins, including immunoglobulins, have been identified from all of these samples. Table 14 shows proteins of interest that include acute phase proteins, oxidative stress marker and few highly abundant cytokines. This list will be expanded further using additional protein separation techniques. The list will be benchmarked to plasma protein spectral library published from human proteins.

Table 13. Potential Pain Biomarkers found in first few plasma sample analysis

No	Name of Protein	Function of Protein
1	Alpha-1 acid glycoprotein	**APP**; anti-inflammatory properties, immune system modulation
2	Alpha-2-macroglobulin	**APP**; protease inhibition and clearance, inflammatory regulation
3	Alpha-2-macroglobulin variant 20	
4	Alpha-2-macroglobulin variant 4	
5	Alpha-2-macroglobulin variant 5	
6	Apolipoprotein A-I	**APP**; reverse cholesterol transport, anti-inflammatory and anti-coagulant activities
7	Apolipoprotein A-II	
8	Apolipoprotein C-III	
9	Ceruloplasmin (ferroxidase)	**APP**; iron oxidation, free radical scavenging
10	Chain D, The Three-Dimensional Structure Of Bovine Platelet Factor 4 At 3.0 Angstroms Resolution	**Cytokine**; Neutralizes the anticoagulant effect of heparin, chemotactic for neutrophils and monocytes. Inhibits endothelial cell proliferation.
11	C-reactive protein precursor	**APP**; It regulate the immune system during the early stage of an infection, plays a role in destroying infectious agents, minimizing tissue damage, and facilitating tissue repair and regeneration.
12	Fibrinogen beta chain	**APP**; involved in blood coagulation, cellular and matrix interactions, endothelial cell proliferation, inflammatory response, angiogenesis, neoplasia.
13	Fibrinogen, alpha polypeptide precursor	

14	Haptoglobin	**APP**; haemoglobin binding protein, iron elimination control, anti-oxidant/ anti-inflammatory role, immunomodulation.
15	Lactoferrin	**APP**; iron-binding glycoprotein, anti-microbial, anti-inflammatory, anti- cancer activities, immune system modulation.
16	Lipopolysaccharide binding protein	**APP**; innate immune response, Pro-inflammatory activity in high concentration and anti-inflammatory activity in low concentration.
17	Paraoxonase 1	Enzyme having antioxidant effect. It has a protective effect against oxidation of lipoproteins.
18	Plasminogen	**APP**; Anti-coagulant properties by fibrinolysis, cell migration enhancer, inflammatory response regulation.
19	Plasminogen precursor	
20	Regakine-1 precursor	**Cytokine**; Chemotactic activity for neutrophils and lymphocytes. Binds to heparin.
21	Serum amyloid A	**APP**; Immunomodulation, binding to cholesterol and opsonisation.
22	Transferrin	**APP**; Iron binding blood plasma glycoprotein.

Cytokines and neuropeptides are very low abundance proteins and are not likely to be in concentrations detectable in plasma by SWATH-MS analysis. However, multiple reaction monitoring (MRM) is the technique that allow to detect proteins with very low concentration, such as cytokines. With SWATH-MS, few highly abundant bovine cytokines like Regakine 1, Bovine platelet factor-4 (PF4/CXCL4) and Hemofiltrate CC Chemokine-1 (HCC-1/CCL14) can be detected and we have already detected Regakine 1 and platelet factor 4(PF4/CXCL4) in cattle sample analysis for the spectral library. Regakine 1 is a naturally abundant cytokine in bovine serum (100 ng/mL).

A preliminary analysis of the proteins identified was undertaken using Principal Component Analysis (PCA) within the MarkerView software package (Figure 10). This analysis looked at a comparison of groups for all time points (Figure 10 a), a comparison of dehorning vs. sham (Figure 10 b) and a comparison of treatment vs. placebo (Figure 10 c). Although further analysis and validation is required, there appears to be clear effects of treatment and, possibly, a better outcome (efficacy) with the transdermal formulation compared to IM.

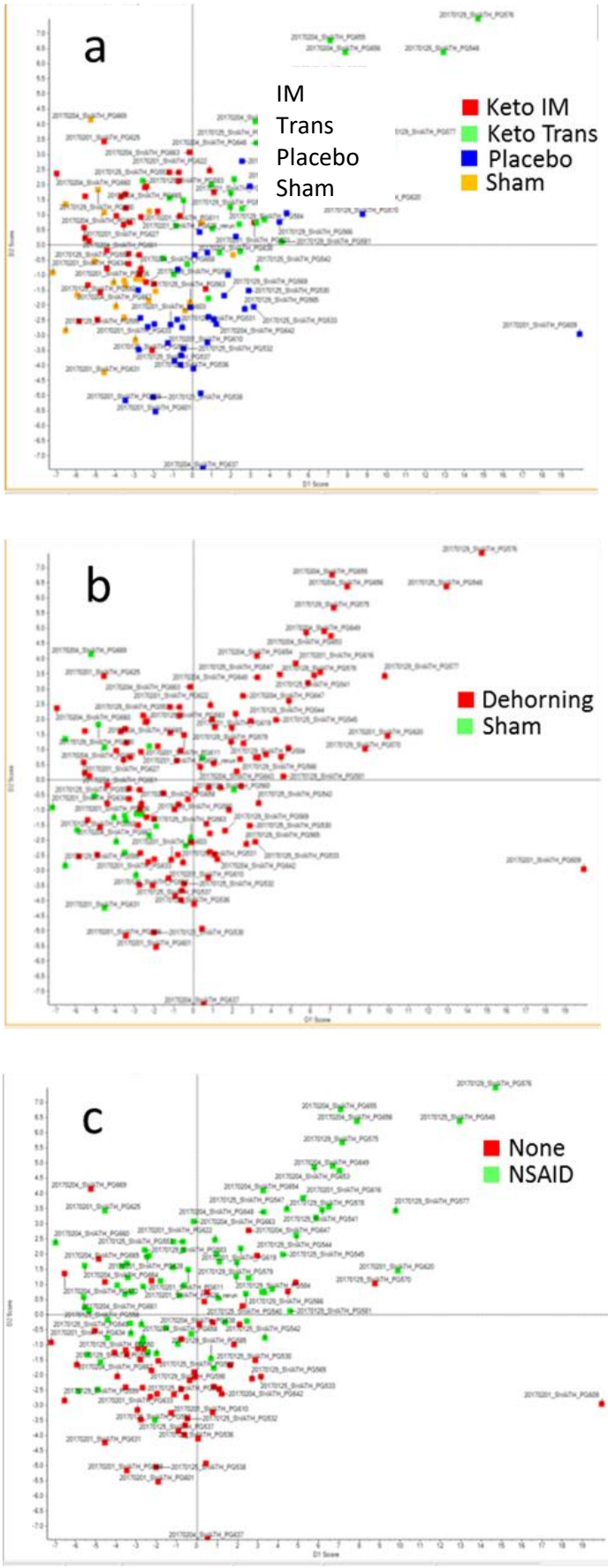

Figure 10. Analysis of proteins measured in plasma during the clinical study

4.3.4 Skin irritation study

The results of this study were possibly equivocal due to pre-existing Dermatophilosis (confirmed by biopsy) present in some of the calves (Figure 11). In the calves administered the transdermal formulation grossly mild skin lesions (palpably mild thickening and crusting of skin) developed in 5/10 calves between days 3-13 after application. In the group administered the transdermal formulation and had it washed off 12 hr later 3/10 calves developed similar mild lesions and for one calf with clinically diagnosed Dermatophilosis the severity of its skin lesions did not change. In the group that received the vehicle only similar mild skin lesions were detected in 5/10 animals between 6-13 days later, and for one calf with clinically diagnosed Dermatophilosis the severity of its skin lesions did not change. Histologic examination of biopsies taken from a representative affected calf from each group revealed mild to moderate hyperplastic superficial perivascular dermatitis with crusting. In summary, some mild skin lesions were detected during this study, although this did not appear to be specifically related to the formulation (or washing it off) or the vehicle.

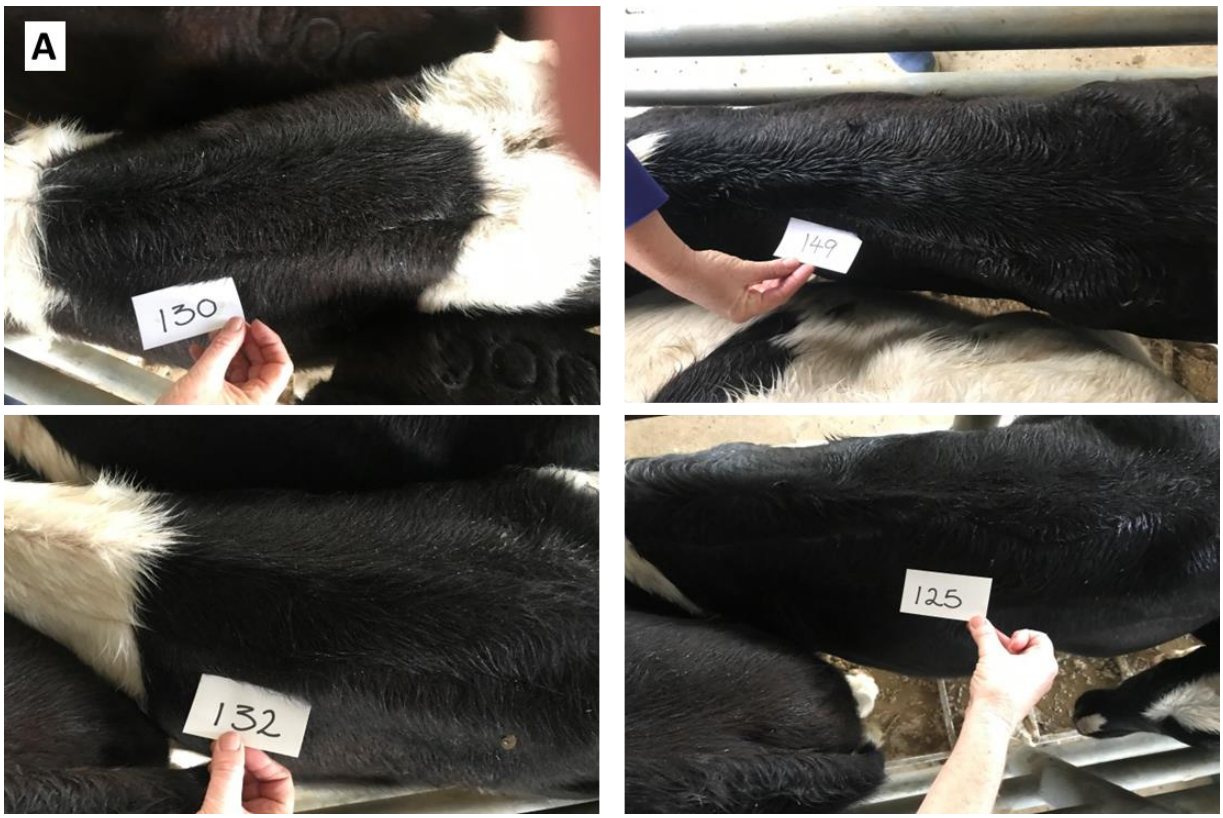

Figure 11. Examples of calves with normal skin (A) and Dermatophilosis associated skin (B) prior to the application of treatments.

5 Discussion

5.1 To confirm transdermal penetration of candidate formulations using *in vitro* techniques

5.1.1 Regional differences in transdermal drug penetration

This study has shown that there are significant differences in the penetration of lignocaine hydrochloride through different regions of cattle skin in vitro. Regional differences in transdermal drug penetration has also been reported in cats (Hill et al., 2015), horses (Mills and Cross, 2006b, 2007a, b) and dogs (Mills et al., 2004b, a, 2005). However, the specific differences between regions will depend on both the active drug and the vehicle used. In the current study, lignocaine hydrochloride was dissolved in an alcohol-based vehicle containing a surfactant, which was associated with a significantly higher penetration of active drug through skin harvested from the thorax and scrotal regions.

Specific reasons for the regional differences are uncertain, although the scrotal skin was observed to be grossly thinner with finer hair present on the skin, compared to the skin from dorsal midline, thorax and ventral abdomen. Regional differences may relate to the thickness of the stratum corneum (Monteiro-Riviere et al., 1990), regional cutaneous blood flow (Monteiro-Riviere et al., 1990), the size of corneocytes (Kashibuchi et al., 2002; McEwan et al., 2009) and density of hair follicles (Damodaram and Seshadri, 1984; Steelman et al., 1997). Since the current study used *in vitro* diffusion cells, cutaneous blood flow did not contribute to the outcomes, although it is generally higher in the ventral abdomen compared to the buttocks, ear and shoulder and dorsal

midline (Monteiro-Riviere et al., 1990). In contrast, the epidermis thickness in the skin from ventral abdomen is generally lower than other regions. However, the primary barrier to drug penetration is the stratum corneum and can be directly evaluated using diffusion cells in vitro (Mills and Cross, 2006).

The selection of the four regions used in the current study was based on potential sites where topical drug may be applied to cattle. The scrotal skin is incised during castration and an effective local anaesthetic would significantly reduce pain associated with this procedure, yet could be applied by lay operators. Topical drugs are usually applied to the thorax or dorsal midline for convenience, but this also facilitates larger volumes of formulation to be applied, if required, for adequate systemic effects. Moreover, many pour-on drugs applied along the dorsal midline will tend to run ventrally over the thorax. The abdomen was included in this study since studies in other species have demonstrated that some drugs will penetrate through the abdomen and/or groin region to a greater extent than other regions (Mills et al., 2004a, b; Mills and Cross, 2006b, 2007a, b). However, the current study showed that the thorax was more permeable to the specific formulation used, although lignocaine hydrochloride also penetrated through the other regions tested.

5.1.2 Screening of NSAID and vehicle combinations to maximise active drug penetration through cattle skin

Of the four NSAIDs currently registered for use in cattle in Australia, only two actually penetrated through skin in the vehicles tested. The other two were either poorly soluble in almost every vehicle tested, or had poor percutaneous penetration.

5.1.3 Optimising the candidate formulations

The final formulation was determined based of a number of factors, such as flammability and efficacy as a solubilising agent.

The amount of active drug required in the formulation was unknown. Other commercial formulations containing NSAIDs for human topical application contain 10+% of active drug. Up to 30% NSAID could be dissolved in the final vehicle formulation, although some precipitation occurred, while 20% dissolved easily without precipitation. A higher concentration would minimise the volume of formulation that needs to be applied.

5.1.4 Stability studies of the final formulation

The stability study was intended as a preliminary assessment to provide some support for the final formulation if advanced to production. The environmental conditions used (0, 20 and 40 ^{o}C) approximated potential storage or use conditions in the field. Despite a mild colour change after several months at 40 ^{o}C, the concentration of active drug remained stable (20 ± 1%). It was concluded that the formulation was quite stable under a range of conditions and storage.

5.1.5 Analysis of NSAID in biological samples

The analytical methodology was reliable and consistent, with a low and appropriate limit of detection. The use of mass spectrometry ensured that parent drug was detected and any metabolites identified.

5.1.6 Summary

All objectives were met for this section.

5.2 To determine the efficacy of candidate transdermal formulations to control pain associated with surgical husbandry formulations

5.2.1 Pilot study 1 and 2

Despite the *in vitro* data, it was uncertain how much active drug would reach the systemic circulation and how quickly. *In vitro* studies using Franz cells are widely acknowledged as useful for proof-of-concept studies and to screen large numbers of potential drug and vehicle combinations. However, other factors must be considered when applying formulations to the living animal, including metabolism in the skin (both phase 1 and phase 2 metabolism occurs), the effects of cutaneous blood flow and the potential of the active to move away for the dermis and enter the systemic circulation (Mills and Cross, 2006a). Significant concentrations of NSAID were detected systemically in plasma following transdermal application of the novel formulation. The 40 mL dose resulted in obvious precipitation of the skin of the calves and resulted in peak (C_{MAX}) concentrations of up to 43 µg/mL in the plasma, with drug appearing within 30 min (this was the first blood collection time) and peaking at ~2 hr. This was much faster than expected. The study was repeated with only the 10 mL dose, which resulted in a C_{MAX} of 3-10 µg/mL, again around 2 hr, within initial drug appearing in the plasma after ~ 30 min. These concentrations approached what had been reported following IM administration (3 mg/kg) and, as such, could be considered therapeutic (see Plessers et al 2015). No adverse effects were observed in the cattle.

5.2.2 Bioavailability study

This was a major study to determine the bioavailability of the NSAID from the transdermal formulation and to compare the pharmacokinetics with IM administration. The use of a cross-over study with animals in the target age group (i.e. when dehorning is normally undertaken) added power and relevance to the study design.

The bioavailability of the NSAID was ~ 50%, which is a good outcome for a topically-administered drug. More importantly, the lag phase (the time between application and first appearance in the plasma) was exceptionally short for the transdermal route, with drug appearing within 10-15 min. Furthermore, the C_{MAX} and AUC were almost twice that measured after IM administration, although the T_{MAX} for IM (74 min) was faster than transdermal delivery (115 min). These pharmacokinetic findings suggest that the transdermal route is a rapid and effective route to deliver ketoprofen to calves. Further modelling and dose administration should be undertaken, but a smaller dose rate (i.e. volume) of the transdermal formulation could be applied to achieve a similar efficacy (determined by C_{MAX} and AUC) as IM administration. Moreover, reducing the volume of drug applied could also reduce the T_{MAX}, meaning that the transdermal formulation could have a similar pharmacokinetic profile as IM administration.

The skin irritation and excoriation noted in the bioavailability study was a concern and had not been observed in the calves in the pilot studies or the clinical study. It should be noted, however, that the formulation had not been washed off in the other studies. The exact cause of this irritation is uncertain, although ketoprofen is a known photosensitising agent (Guy, et al., 2014; Seto et al., 2015). Since the active drug was absorbed so rapidly following topical administration, less of the active drug should have remained on the skin.

5.3 To develop a formulation containing an NSAID to control pain associated with routine surgical interventions for up to 24 hr

5.3.1 Clinical study

This study was performed on calves at an age when they would normally be dehorned. A cornual nerve block was not used (this was approved by the animal ethics committee) since this represents the industry standard currently. A sham-handled group was used as a negative control to allow for the stress induced by handling and multiple blood samples collected. The remaining calves were all dehorned and no adverse effects were observed.

An important consideration for the clinical study was that the bioavailability study had already demonstrated that the transdermal formulation resulted in a higher systemic concentration of NSAID than the IM administration and, while slower in onset, persisted in the plasma for longer. These findings suggested that the efficacy of the transdermal formulation would be similar, if not better than, IM injection, from a bioequivalence perspective. The literature has already shown that IM NSAID has an analgesic effect lasting at least 24 hr in cattle following surgical husbandry procedures.

5.3.2 Conventional parameters to assessment pain and inflammation

All the conventional parameters indicated that the NSAID had a significant analgesic effect in the cattle. Total plasma cortisol and body weight gain were significantly different between treated and untreated calves, but there were no significant difference between the routes of administration of the NSAID. Dehorned calves that received NSAID had a body weight gain that was similar to the sham-treated cattle and significantly higher than the placebo-treated cattle. These findings indicated that transdermal NSAID had a similar or higher efficacy than IM injetion and this significantly reduced pain and stress in cattle following dehorning.

5.3.3 Proteomic assessment of pain and inflammation

Unfortunately, the analysis of the proteomic data was continuing while this report was being combined. Over 300 proteins had been isolated and identified, consisting of two or more peptides each. The software packages to automatically identify the proteins proved inaccurate and much of the spectral library was confirmed manually. Irrespective, some important trends were emerging, backing up the conventional parameters. The response to dehorning (Figure 10b) was substantially different from sham. There was a difference between untreated and treated cattle, meaning that the NSAID significantly affected key biomarkers of pain and inflammation, while the transdermal NSAID appeared to have greater efficacy than IM delivery.

6 Conclusions/recommendations

6.1 Drug movement through cattle skin

The transdermal route appears to be an effective and useful approach to delivering analgesic drugs to cattle. It is more convenient and able to be applied by lay (untrained) operators. There are

regional differences in the permeability of cattle skin to topically applied drugs, with thoracic/backline skin being the highest.

It was possible to move NSAIDs through cattle skin, although, of the NSAIDs registered for cattle use in Australia, only two were suitable.

6.2 Optimising a transdermal NSAID formulation

The most effective transdermal formulation was stable under a range of conditions, such as may be expected under use in the field.

Pilot studies revealed that 10 mL of the transdermal formulation delivered systemic NSAID concentrations that were likely to be effective to control pain and inflammation.

6.3 Determining the efficacy of the transdermal NSAID formulation

A bioequivalence study revealed that the bioavailability of the transdermal formulation was ~ 50%. Importantly, the lag time was low, with active drug appearing in the systemic circulation after 15-20 min and a peak concentration ~ 2 hr. These outcomes suggest that the transdermal formulation should therefore have similar efficacy as IM delivery.

The clinical study confirmed that similar efficacy between IM and transdermal NSAID occurred when used during a routine surgical husbandry procedure, dehorning. Total plasma cortisol concentrations and body weight gain showed that NSAID, by either route, reduced stress and pain associated with dehorning. Behavioural parameters supported the analgesic effects of the NSAID and, while not demonstrating overall significance, the trends were all towards analgesia and may have been significant in the study had sufficient power. Some individual behaviour parameters, such as head shaking, were significantly different from the placebo group, demonstrating that NSAID significantly reduced this indicator of pain.

The outcome from this project was that a formulation has been developed to effectively move NSAID through cattle skin. When applied to the backline, a 10 mL provided similar if not greater efficacy to control stress and pain as IM administration. Pharmacokinetic modelling also demonstrated a similar extent and duration of active drug following topical administration as from IM, in itself providing strong support for efficacy.

The transdermal formulation is easy to administer, effective and relatively cheap, providing a highly encouraging means of providing analgesia for surgical husbandry procedures in Australia. This will be encouraging for producers, veterinarians and welfare advocates.

Proteomics has the potential to replace or at least support more traditional methods of assessing pain, such as plasma cortisol and behavioural parameters. Further analysis is required to finalise the protein spectral library for cattle, before a single blood sample could accurately and quantitatively assess pain and stress.

7 Key messages

- Cattle producers and veterinarians will have access to an inexpensive (particularly compared to parenteral) and effective form of analgesia that can be administered by lay users under veterinary supervision when performing surgical husbandry procedures.
- Effective analgesia will be able to be administered prior to any surgical husbandry procedure or if required for clinical analgesia.
- The outcomes from this project highlight a major role for topical (transdermal) delivery of pharmacological and possibly biological agents to cattle.
- The cattle production industry can provide strong evidence of a major advance in on-property improvement in welfare of cattle undergoing routine painful husbandry procedures.
- The outcomes from this project may have relevant and application to other species.
- Potentially new quantitative and objectives approaches to assessing pain, inflammation and stress have been identified.

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