Abstract and Keywords

Objective: Analgesic regimes were compared in pregnant ewes after laparotomy by measuring thermal (TT) and mechanical (MT) nociceptive thresholds.

Study Design: Prospective randomised experimental study.

Animals: Pregnant ewes at 121 days gestation underwent laparotomy as part of another research project.

Methods: Thermal and mechanical thresholds were measured before, and 2, 6, 24 and 48 h after surgery. Thermal stimuli were delivered to the lateral aspect of the metatarsus via a skin-mounted probe, and mechanical stimuli to the contralateral site via a pneumatically driven 1.5 mm diameter pin. Each test was performed 5 times, alternating thermal and mechanical stimuli, with 10 minutes between thermal stimuli. At the end of surgery ewes received either: 75 µg h^{-1} transdermal fentanyl patch (medial thigh) (group FP) (n=8), or 3 µg $kg^{-1}h^{-1}$ intra-peritoneal medetomidine osmotic pump (group IPM) (n=8) inserted immediately prior to closure. Data were analysed using the Kruskal-Wallis RS Test ($p < 0.05$). Once a significant effect was identified, pairwise comparisons were performed using paired Wilcoxin RS tests. To compensate for multiple hypotheses testing, $p < 0.005$ was considered significant.

Results: Prior to surgery mean (SD) TT was 56.1(5.0)°C (FP) and 55.6(5.5)°C (IPM); MT was 5.3(2.6) N (FP) and 8.0(5.0) N (IPM). In FP there was no significant change in either TT or MT over time. In IPM there was no significant change in MT over time but TT increased at 2 hours to 59.2(3.0)°C (p=0.003). Skin temperature (ST) ranged from 33.0-34.7°C and did not change over time. There were no significant differences between groups in TT, MT or ST.

Conclusions: Administration of intra-peritoneal medetomidine (3 µg kg^{-1} h^{-1}) by an osmotic pump increases the thermal nociceptive threshold in the immediate post operative period in pregnant sheep.

Clinical Relevance: Medetomidine may have a role in providing post-operative analgesia in pregnant sheep.

Keywords: sheep, analgesia, nociceptive threshold testing

Introduction

The Australian code of practice for the care and use of animals for scientific purposes states that pain management appropriate to the species, the procedure and the circumstances must be provided (AustralianGovernment, 2004). This responsibility is difficult to fulfil in some species as assessment of analgesic drug efficacy requires robust pain assessment tools and sheep, despite being used extensively in biomedical research, have not been the target of comprehensive pain management studies. As a prey species sheep may not display overt signs of pain and suffering as they have evolved to be relatively stoical. This behavioural trait makes subjective assessment of pain especially difficult and the need for objective methods of pain assessment essential. If hyperalgesia (an exaggerated response to a noxious stimulus) is indicative of pain it may be useful to assess hyperalgesia as an indicator of pain in sheep (Fitzpatrick et al., 2006).

Nociceptive threshold testing involves the application of a potentially painful stimulus to an animal to elicit a specific response. Utilising this approach enables an objective assessment of hyperalgesia, hypoalgesia and analgesia as the threshold at which a response occurs can be measured and expressed as a number. To perform nociceptive threshold testing within an ethical framework requires the stimulus to provide quantitative information and to be applied to a body part where there are minimal variations in neurohistology, the stimulus delivered to be the minimum necessary to elicit a response, the response to be a natural behaviour of the animal (e.g. a left lift or head turn), termination of the stimulus the moment the response is observed, and avoidance of tissue damage (Nolan et al., 1987a, Beecher, 1957). Nociceptive threshold testing has been performed in a range of species including chickens (Hothersall et al., 2011), dairy cows (Rasmussen et al., 2011), horses (Love et al., 2011), pigs (Sandercock et al., 2009), cats (Dixon et al., 2007, Taylor et al., 2007), dogs (Bergadano et al., 2009, Bergadano et al., 2006) and sheep (Nolan et al., 1987a).

Nociceptive threshold testing may involve the delivery of thermal, mechanical, chemical or electrical stimuli to the skin, teeth, muscles or viscera (Beecher, 1957). Contemporary literature, however, refers most commonly to the use of mechanical or thermal stimuli in pain and analgesic efficacy studies (Love et al., 2011, Robertson et al., 2003, Hoffmann et al., 2012). Nociceptive neurons associated with the transmission of pain are either small myelinated A fibres associated with sharp mechanical type stimuli or unmyelinated C fibres associated with dull, burning or longer lasting pain. While nociceptive neurons respond to more than one type of stimulus their sub-types may be more specific. A and C fibres can respond to both mechanical and thermal noxious stimuli but A fibre nociceptors can be sub-divided into those that respond to mechanical stimuli only, mechano-heat units that are activated by noxious and mechanical stimuli and mechano-cold units that are activated by noxious mechanical and noxious cold stimuli (Djouhri and Lawson, 2004). Ideally the type of

stimulus delivered in nociceptive threshold studies would be appropriate to the nociceptors where specific analgesic drug receptors are located.

Pregnant sheep are commonly utilised in biomedical research projects investigating the causes and consequences of preterm birth (Kemp et al., 2010) but given the paucity of data on safe and efficacious analgesia it is difficult to make evidence based recommendations for peri operative pain management. We aimed, therefore, to compare the analgesic efficacy of 2 different post-operative analgesic strategies that had previously been developed in this laboratory on empirical grounds for pregnant sheep undergoing a laparotomy, hysterotomy and instrumentation of the fetus. This comparison was made with both mechanical and thermal nociceptive threshold testing.

Materials and Methods

This study was approved by the Animal Ethics Committees of the University of Western Australia and Murdoch University. Merino singleton ewes at 118-121 days of gestation underwent anaesthesia and surgery as part of another study. The sheep were held in the Large Animal Facility at the University of Western Australia in raised group pens for at least 1 week prior to introduction to a raised single pen 2 days before surgery. Rooms were controlled for temperature (20.5 – 21.5 °C) and relative humidity (40-60%). On the morning of surgery ewes were weighed (weight range 53-68 kg). There was no difference in the weight of animals between groups.

Threshold testing

Mechanical nociceptive threshold (MT) testing was performed by positioning a 1.5 mm hemispherical blunt pin fixed in a rolling diaphragm actuator over the cranial aspect of the metatarsals of one hindlimb (Dixon et al., 2010). A preload force of 1 Newton was applied at the beginning of each test and after 1 minute a ramped force was applied to the actuator at 0.5 N/s, driving the pin into the skin until the leg was lifted from the ground and replaced in a stamping action. The force was delivered manually by a syringe connected to non-distensible tubing via a digital meter which displayed the force exerted (Figures 1 and 3). A consistent rate of force increase was ensured by traffic lights on the control module (ProdPlus, Topcat Metrology Ltd). The stimulus was removed as soon as the sheep responded and applied force at this point was held on the display and recorded as the MT.

Thermal nociceptive threshold (TT) testing was performed by positioning a 5 g thermal probe containing both heater element (contact area 24 mm^2) and temperature sensor over an area of clipped skin on the lateral aspect of the metatarsals of the opposite hindlimb. The probe was held in place with a Velcro strap and connected to the control module which was seated over the dorsal thorax and held in place by a thoracic strap. Heating was controlled from an infra red handheld remote control device (Figures 2 and 3) (WTT1, Topcat Metrology Ltd). The initial skin temperature was recorded after the

probe had been in place for at least 5 minutes. The probe was heated at 0.8 °C/s until the same leg lifting motion was observed and heating was stopped immediately. The temperature at this point was held on the display and recorded as the TT. The device cut out at 60 °C if no response occurred. The cut out temperature was decreased to 55 °C half way through the study.

Nociceptive threshold tests were performed the day before surgery (baseline) and 2, 6, 24 and 48 hours after surgery. At each time point each test was repeated 5 times and the mean of these 5 tests was used for analyses. Ten minutes was allowed between each thermal stimulus to allow for cooling of the probe and the skin. The probe remained in place during this cooling period. MT and TT were alternated. Observations were made by personnel at least 2 metres away from the sheep. Two operators performed all the testing.

Anaesthesia

Food was withheld for 18 hours before anaesthesia and free access to water was allowed until the premedication drugs were administered. Ewes were premedicated with a combination of acepromazine (0.03 mg/kg, A.C.P. 2 Injection, 2 mg/mL, Ceva Delvet Pty Ltd, NSW, Australia) and buprenorphine (0.01 mg/kg, Temgesic, 0.3 mg/mL, Reckitt Benckiser, NSW, Australia) administered by intramuscular injection 30-40 minutes prior to induction of anaesthesia. Anaesthesia was induced with a combination of diazepam (0.25 mg/kg, Ilium Diazepam injection, 5 mg/mL, Troy Laboratories, NSW, Australia) and ketamine (5 mg/kg, Ketamil, 100 mg/mL, Troy Laboratories, NSW, Australia) by intravenous injection and the trachea was subsequently intubated (7.5 mm internal diameter, cuffed, Portex Ltd, England). Anaesthesia was maintained with isoflurane (1-2.5%, Attane Isoflurane 1mL/mL, Bayer Australia Ltd, NSW, Australia) in 100% oxygen delivered through a circle breathing system. A line block of ropivacaine (100 mg, Naropin 1%, Astra Zeneca, NSW, Australia) was performed along the laparotomy incision site prior to surgery. Intermittent positive pressure ventilation was used to maintain normocapnia (ET CO_2 35-45 mmHg). Physiological monitoring included electrocardiogram, pulse oximetry, capnography, temperature and invasive blood pressure. At the end of surgery the ewes were randomly allocated to one of two treatment groups: intraperitoneal medetomidine (IPM) and fentanyl patch (FP).

Analgesia

Intraperitoneal medetomidine (IPM) was administered via a 2 mL osmotic pump (Alzet osmotic pumps, 10 µL/h, Durect, America). The pump was secured in a pocket of omentum just prior to closure of the linea alba, at the end of surgery. Pumps were loaded aseptically with 2 mL of medetomidine diluted with sterile saline to deliver 3 µg/kg/h. Medetomidine (Zalopine 30 mg/mL, Orion Corporation, Espoo, Finland) loaded pumps were primed overnight at 37^{o}C according to the manufacturer's instructions. Priming the pump ensures immediate and accurate pumping when placed

in vivo. The fentanyl patch (FP) (Durogesic 75 µg/h, Jannsen, NSW, Australia) was placed on clean skin of the medial thigh, adjacent to the udder, at the end of the surgery. The dose of fentanyl was 1.1-1.4 µg/kg/h.

Euthanasia

The ewes were euthanased 7 days after surgery (according to the original project's protocol) and skin from the TT test site and from the equivalent site on the opposing hindlimb were collected for histopathological examination post mortem. Tissues were stored in formalin until preparation for histopathology. Three sections from each tissue sample were examined.

Statistics

Data were analysed by Sigmaplot 12.0™ using the Kruskal-Wallis rank sum test ($p < 0.05$ was considered significant). Once a significant effect was identified, pairwise comparisons were performed using paired Wilcoxon rank sum tests ($p < 0.005$ was considered significant).

Results

The initial skin temperature was comparable between and within each group. The TT of IPM sheep was significantly higher 2 hours postoperatively compared to the baseline TT in that group (p=0.003). There were no other significant differences between or within the groups for the TT. The MT was not different between or within groups (Table 1).

The dose range of fentanyl was equivalent to 1.1-1.4 µg/kg/h for the sheep in this study. The anaesthesia and surgery time was 90-120 minutes and recovery from anaesthesia was uneventful in both groups. The ewes were standing within 30 minutes of extubation and eating within an hour.

The lesions created by the TT test were the shape and size of the thermal probe and were characterised by pale skin with a hyperaemic rim. Occasional pustules were apparent a few days after testing. The histopathological examination of the TT test site in 3 of the sheep revealed moderate to sub-acute focal epidermal and mid-dermal coagulative necrosis with mild to moderate suppurative dermatitis, pustule formation and epidermal erosion. Deep dermal early fibroplasia was also present. These findings are consistent with a second degree burn. The remaining sites were normal.

Discussion

Without sound methods for identifying and describing pain it is difficult to determine the efficacy of analgesic drugs. A range of qualitative and quantitative methods have been investigated for pain assessment in sheep (Lizarraga and Chambers, 2011), but this species continues to be a challenge. We investigated thermal and mechanical nociceptive threshold testing to determine whether there was

a difference between the analgesic efficacy of intraperitoneal medetomidine and a fentanyl patch in pregnant sheep during the post operative period. These methods of pain assessment were employed to measure the development of hyperalgesia, and from this, to infer the degree of pain. The techniques worked well and a clear end point was easily determined for both thermal and mechanical stimuli.

Pain is a complex experience and in sheep it is dependent on not only the severity of the insult to nociceptive pathways and the degree of tissue or nerve damage but on previous pain experiences and social position within the flock (Fitzpatrick et al., 2006). Surgical trauma may damage nerve fibres but it also stimulates an inflammatory response which in turn causes pain by activation and sensitisation of unmyelinated and myelinated sensory nerves fibres by chemical mediators (Fitzpatrick et al., 2006). Hyperalgesia is a common sequelae to inflammatory pain (Nolan, 2000) and is described as primary and secondary. Primary hyperalgesia refers to exaggerated responses to painful stimuli at the site of trauma while secondary hyperalgesia occurs in the surrounding uninjured tissues (Nolan, 2000). When hyperalgesia develops animals (and humans) experience more pain than they would do otherwise. It follows therefore, that if hyperalgesia has developed, both the TT and MT will decrease from the preoperative baseline. Conversely, hypoalgesia is associated with an increase in TT and MT from the preoperative baseline. Analgesic drug efficacy is often inferred if thresholds increase but there are few studies investigating analgesic drug efficacy following a painful procedure. It is possible that the magnitude of increase in a nociceptive threshold may diminish following a painful procedure. Our results suggest that none of the sheep in this study developed secondary hyperalgesia.

Alpha 2 adrenoreceptor agonists are consistently reported to provide analgesia for sheep (Kästner, 2006, Grant and Upton, 2004, Grant et al., 2001) and have recently been demonstrated to achieve therapeutic plasma concentrations when delivered by the intra-peritoneal route (Murdoch et al., 2013). Transdermal fentanyl has also been reported to provide analgesia in sheep. When applied 12 h prior to orthopaedic surgery, analgesia was superior to intramuscular administration of buprenorphine (Ahern et al., 2009). It does, however, require 12 hours to achieve maximum plasma concentrations (Ahern et al., 2010). We expected a window in the immediate post operative period in the FP sheep where the TT and MT would be decreased, coinciding with the decline in analgesic effects of drugs included in the anaesthetic protocol (buprenorphine, ketamine, ropivacaine) before the fentanyl took effect. Both the TT and MT, however, were stable and did not differ from the pre-operative baseline. The increase in TT in the IPM group suggests antinociceptive effects from the medetomidine at the 2 hour time point but this effect was not sustained.

Fentanyl has previously been reported to increase both TT and MT in sheep (Waterman et al., 1990). While the response to TT testing was immediate and reached the cut-out of 70 °C within minutes of administration of the drug, the response to MT testing was delayed and did not reach the cut-out of 16 N (Waterman et al., 1990). It is, however, difficult to compare between studies as the dose of fentanyl

196 is not equivalent. The sheep receiving fentanyl in our study did not demonstrate any measurable
197 difference in TT or MT over the 48 hours. It is possible that the dose of fentanyl in this study was too
198 low to alter TT or MT during the 48 hour study period. The potential for interaction between fentanyl
199 and buprenorphine is also important but is unlikely given that the nociceptive effects of
200 buprenorphine last 3.5 hours in sheep (Nolan et al., 1987b) and transdermal fentanyl is unlikely to
201 reach therapeutic plasma concentrations within this time frame. In mice α_2 adrenoreceptor agonist
202 drugs increase TT (Hunter et al., 1997) so it is possible that the plasma concentration of
203 medetomidine was sufficient to provide some analgesic effect in pregnant sheep.

204 The initial skin temperature was recorded to ensure a comparable starting point for the TT tests. Data
205 from these tests may be analysed as either the difference between the initial temperature and the
206 threshold temperature or the absolute value for the threshold temperature. We analysed the latter
207 given the initial temperatures were comparable. Increasing plasma concentration of medetomidine is
208 directly proportional to systemic vascular resistance (Talke et al., 2000) due to α_2 adrenoreceptor
209 stimulation (Kästner, 2006). Since dermal vasoconstriction is likely to influence skin temperature it
210 was anticipated that the initial skin temperature of the IPM sheep would be lower than the FP sheep.
211 However, no difference was observed, probably because these effects are dose dependent and the
212 relatively low dose of medetomidine used in this study correlates with maximum plasma
213 concentrations of 2.9 ng/mL (Murdoch et al., 2013). This concentration is associated with minimal
214 cardiovascular side effects (Kästner, 2006).

215 A preload force of 1 Newton was applied during MT testing to avoid a premature leg lift in response
216 to the touch-on of the pin. During pilot testing of the equipment it became apparent that a number of
217 sheep would lift their leg at the beginning of the MT test. They would then stand at ease and lift the
218 leg again at threshold. The 1 Newton preload force brought the pin into contact with the limb at very
219 low pressure. The sheep's responses then became considerably more consistent.

220 The lesions created by the TT test in 3 of the sheep were of concern. The cut-out for the TT test was
221 initially set at 60 °C in an effort to avoid damage to the skin. The cut-out was subsequently decreased
222 to 55 °C towards the end of the study in an effort to avoid thermal injury. We also allowed at least 10
223 min between each TT test to allow the skin to cool. Moreover, the thermal probe was repositioned for
224 each set of tests. Any impact of these lesions on the TT data is not obvious. The sheep did not react to
225 palpation of the lesions and if they had contributed to hyperalgesia a decrease in TT and MT over
226 time could be expected. Creating lesions that may take days to resolve and are prone to secondary
227 infection is not ideal. However, it was clear that that the lesions did not impact upon the welfare of
228 the sheep in this study, which may in part be due to the analgesic drugs.

There are a number of limitations to this study. After extensive pilot work we settled on the hind limb site for application of both the thermal and mechanical stimuli. A site more proximal to the ventral midline surgical site may have resulted in a greater difference between pre and postoperative thresholds but we could not elicit consistent responses to the stimuli at that site. Consistent responses have been achieved in other studies using thermal stimuli applied to the pinnae of sheep (Nolan et al., 1987a), and mechanical stimuli applied to the forelimb of sheep over the metacarpal area (Lizarraga and Chambers, 2006, Lizarraga et al., 2008). During the pilot studies we tested the ventral abdomen, lateral thorax and pinnae. Since secondary hyperalgesia is evidence of central nervous system sensitisation we expected that, although the stimulus site was distal to the surgical incision, changes in the response threshold would be evident if hyperalgesia had developed. Another limitation of this study was the absence of a negative control group which did not receive postoperative analgesia. For ethical reasons this was not possible. Analgesic drugs were also incorporated into the anaesthetic protocol and this may confound the results as such agents are likely to interfere with the development of hyperalgesia. However, since every sheep received buprenorphine, ketamine and ropivacaine as part of the anaesthetic protocol the lack of persistent differences between each group could be interpreted as demonstration of the analgesic efficacy of the anaesthetic protocol itself. It is unlikely that these drugs would be present in any appreciable concentration 12 hours after surgery.

Conclusion

Both TT and MT testing were viable options for nociceptive threshold testing in sheep in an animal house environment. Both methods reliably elicited a consistent leg lift response when applied to the distal hind limb of the sheep. Furthermore, IPM provided temporary analgesia for pregnant sheep in the immediate post-operative period.

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