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DOTTORATO DI RICERCA IN : SCIENZE VETERINARIE

CICLO XXVII

Advances in sheep analgesia

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LIST OF PUBLICATIONS INCLUDED IN THIS THESIS

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RIASSUNTO

La pecora domestica (*Ovis aries*) è una delle specie animali più comunemente allevate ed utilizzate nella ricerca biomedica; nonostante questo l'analgesia in questo animale è stata a lungo trascurata.

I primi due studi presentati in questa tesi sono stati elaborati allo scopo di valutare la somministrazione di analgesici nell'ambito clinico e sperimentale. Il terzo studio valuta la farmacocinetica e gli effetti antinocicettivi del tramadolo e del suo metabolita O desmethyltramadol (M1).

Il primo studio consiste in una meta-analisi sull' uso di farmaci analgesici riportato nelle pecore utilizzate a fini sperimentali. Studi riguardanti procedure sperimentali in pecore effettuati in anni selezionati (2008-2011-2014) sono stati identificati utilizzando un motore di ricerca. In totale, sono stati selezionati 75 articoli scientifici. Lo studio evidenzia mostra che la terapia antalgica spesso non viene accuratamente riportata.

Il secondo studio consiste in un questionario on line redatto allo scopo di valutare l'attuale approccio dei veterinari italiani, che si occupano della specie ovina, alla valutazione ed al trattamento del dolore in questa specie. Il questionario era diviso in cinque sezioni riguardanti i dati demografici, l'uso di farmaci analgesici a tecniche utilizzate per apportare analgesia, e l'approccio utilizzato dai veterinari nella valutazione e trattamento del dolore nella specie ovina, ed, infine, la loro conoscenza riguardo tale argomento. Un numero limitato di veterinari ha completato il questionario. I farmaci più comunemente utilizzati dai veterinari che hanno risposto al questionario sono i farmaci antiinfiammatori non steroidei e gli anestetici locali. Secondo l'opinione dei veterinari, le ragioni principali per cui la terapia analgesica non viene effettuata nella specie ovina erano la mancanza di farmaci registrati, il loro costo, i tempi di sospensione e la regolamentazione riguardante il loro utilizzo. La maggior parte dei veterinari si dimostrava interessata a migliorare le proprie conoscenze riguardo l'analgesia nella specie ovina.

Il terzo studio investiga la farmacocinetica e gli effetti antinocicettivi del tramadolo ed M1 dopo somministrazione endovenosa nelle pecore. Due dosi di tramadolo, 4 mg/kg (T4) e 6 mg/kg (T6), e soluzione salina (SAL) sono state somministrate in due minuti a sei pecore adulte e sane in uno studio randomizzato “in cieco” con un periodo di sospensione di due settimane. A tempi predeterminati, sono stati effettuati i prelievi di sangue per l’analisi farmacocinetica, e sono stati registrati i parametri fisiologici e i valori dopo stimolazione nocicettiva meccanica (MNT). Tramadolo ed M1 presentano rispettivamente una cinetica bi-compartimentale e non-compartimentale. I parametri farmacocinetici sono simili per le due dosi T4 e T6. Le concentrazioni plasmatiche di tramadolo ed M1 sono rapidamente diminuite. I parametri fisiologici non sono risultati statisticamente diversi tra i gruppi. Non sono stati evidenziati effetti antinociettivi del tramadolo; infatti i valori di MNT non sono risultati statisticamente diversi tra i gruppi.

Concludendo, questi studi hanno dimostrato che ci sono ampi margini di miglioramento nella valutazione e trattamento del dolore nella specie ovina sia in ambito sperimentale sia clinico. Inoltre, sono necessari studi sperimentali e clinici riguardanti la farmacocinetica e farmacodinamica di farmaci analgesici nella specie ovina al fine di migliorarne il benessere.

ABSTRACT

Sheep (*Ovis aries*) are widely used in experimental settings and breeding system, nevertheless pain treatment in this species seems to be overlooked.

The first two studies described in this thesis were designed to evaluate administration of analgesics both in the experimental and clinical setting. The third study evaluated the pharmacokinetics and antinociceptive effects of tramadol and its metabolite O-desmethyltramadol (M1) in sheep in a preclinical model of pain.

The first study consisted of a meta-analysis of the reported use of analgesics in sheep for experimental purposes. Studies involving experimental procedures in sheep carried out in selected years (2008-2011-2014) were identified using a search engine. A total of 75 papers were selected. The study showed that analgesic treatment was often not accurately reported.

The second study consisted of an on-line questionnaire evaluating the current attitudes of Italian practitioners to assessment and treatment of pain in sheep. The questionnaire consisted of five sections regarding the demographic data, analgesic drugs and techniques used to treat pain, attitudes to pain relief and assessment of pain and the knowledge on the topic of sheep analgesia. Only a modest number of questionnaires were returned. The most commonly used drugs by sheep practitioners who replied to the questionnaire were non steroidal anti-inflammatory drugs and local anaesthetics. In the practitioners' opinion the main reasons for analgesic drugs not to be administered to sheep was the lack of licensed drugs followed by costs, withholding times and regulations. The vast majority of practitioners were interested in improving their knowlwdge on sheep analgesia.

The third study investigated the pharmacokinetic profile and antinociceptive effect of tramadol and M1 following intravenous administration in sheep. Six healthy adult sheep were administered 4 (T4) and 6 (T6) mg/kg of tramadol (T) and saline (SAL) over 2 minutes in a

cross over design with a two weeks wash out period. At predetermined time points blood samples were collected, physiological parameters and mechanical nociceptive threshold (NMT) values were recorded. Tramadol and M1 fitted a two compartmental model and a non compartmental model respectively. Pharmacokinetic parameters were similar for T4 and T6. Tramadol and M1 plasma concentrations decreased rapidly. Physiological parameters were not statistically different between groups. No mechanical antinociceptive effects of tramadol were detected, as MNT values did not statistically differ between groups.

In conclusion, these studies showed that there is great scope for improvement in pain assessment and treatment in sheep both in the research than clinical settings. Moreover more experimental and clinical studies regarding the pharmacokinetic and pharmacokinamic effects of analgesic drugs in sheep are advocated in order to improve their welfare.

Chapter 1

INTRODUCTION

1.1 Pain pathophysiology

1.1.1 Definition of pain

The International Association for the Study of Pain (IASP) define pain as a "*sensory and emotional experience associated with real or potential injuries, or described in terms of such injuries*" (Loeser & Treede 2008). This definition was then modified to include non-verbal humans who cannot self-report their feelings and the comment that "*the inability to communicate verbally does not negate the possibility than an individual is experiencing pain and is in need of appropriate pain-relieving treatment*". It was also clarified that "*pain is always subjective*".

An exhaustive definition of animal pain was provided by Molony and Kent: animal pain was defined as "*an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; unnecessary pain occurs when the intensity or duration of the experience is inappropriate for the damage sustained or when the physiological and behavioural responses to it are unsuccessful at alleviating it*" (Molony & Kent 1997).

1.1.2 Classification of pain

Pain can be classified according to different temporal phases. Acute pain occurs at the moment of the injury and can be followed by sub-acute pain. If pain is not properly treated or if the cause of pain is not eliminated chronic pain can arise (Millan 1999).

Another way to classify pain is by the source within the body as visceral, somatic or neuropathic pain; these types of pain differ by neurophysiological characteristics (McMahon 1997).

Pain can also be categorized as “adaptive” whose function is to protect the body from injury or injury progression or “maladaptive”, where pain itself is the disease (Woolf 2004).

Adaptive pain includes nociceptive pain and inflammatory pain.

The term nociception is used to define the processing of stimuli that damage (or may potentially damage) normal tissue into a conscious pain experience. Nociception is the physiological side of pain, without the aversive emotional component, as commented by Rutherford (Rutherford 2002).

1.1.3 Sensory processing

Sensory processing consists of different steps: transduction, transmission, modulation, projection and perception (Shilo & Pascoe 2014).

Transduction

A noxious stimulus (mechanical, thermal or chemical) is converted into electrical activity by nociceptors, which constitute the free endings of afferent primary peripheral neurons. The cell bodies of these neurons are located in the dorsal root ganglia and the trigeminal ganglion and extend central axonal endings into the dorsal horn of spinal cord or in the trigeminal nucleus caudalis in the caudal, where they synapse with second order neurons. Inflammatory mediators, such as bradykinin, serotonin,

prostaglandins, cytokines, are released from damaged tissue and can stimulate nociceptors directly medulla (Dubin & Patapoutian 2010).

Transmission

The neural impulse is propagated from the site of transduction, skin or viscera, to the dorsal horn of the spinal cord. Different nerve fibres are involved in this phase. The speed of transmission depends on the diameter of axons of sensory neurons and whether or not they are myelinated (Dubin & Patapoutian 2010). Aδ small myelinated fibres account for the initial “stabbing” of well localised pain, and respond to mechanical and thermal stimuli; while small non myelinated C fibres account for the “burning” more diffuse pain (McMahon 1997). C fibres are polymodal and respond to chemical, mechanical and thermal stimuli. In addition to Aδ and C fibres, there are also Aβ fibres carrying non noxious stimuli, such as touch. Aβ fibres are of large diameter and highly myelinated and are characterised by rapid signal conduction (Almeida et al 2004).

Modulation

Aδ and C fibres synapse with secondary afferent neurones in the dorsal horn of the spinal cord, where modulation takes place (Shilo & Pascoe 2014). At this level, there are sensory nuclei that receive and process incoming somatosensory information and can augment, inhibit or modify this information.

The dorsal horn is histologically organised into ten layers, called Rexed laminae. These second order neurons include projection cells, interneurons and propriospinal neurons. Propriospinal neurons belong to a polysynaptic pathway and control locomotion, reflex responses and transfer information to the brain. Interneurons can have either inhibitory or excitatory properties and release γ- aminobutyric acid (GABA) and/or glycine or glutamate and/or substance P respectively. Interneurons convey information from primary afferents to projection neurons. Projection neurons and interneurons responding to noxious stimuli only are located primarily in lamina I and lamina II, while the so called “wide dynamic range

neurons” WDR are located in lamina V and respond to both innocuous and noxious input (Basbaum & Jessell 2000). WDR neurons are involved in descending control and in induction of long term inflammatory or neuropathic pain states.

Projection

Nociceptive information are conveyed to the brain through the spinal cord, via two most important pathways: the spinothalamic tract and the spinoreticular tract (Shilo & Pascoe 2014).

In the spinothalamic tract, secondary afferent neurones decussate and ascend in the contralateral spinothalamic tract to nuclei in the thalamus. Third order neurones then ascend from this area to the somatosensory cortex, the periaqueductal grey matter (PAG) and convey information regarding pain localization.

In the spinoreticular tract fibres ascend the contralateral cord and reach the brainstem reticular formation; from here fibres project to the thalamus, hypothalamus and cortex. These fibres are involved in the emotional dimension of pain (Shilo & Pascoe 2014).

Perception

Nociceptive information is integrated by the brain and the overall conscious and emotional experience of pain is perceived. Indeed, nociception activation per se does not necessarily result in pain (Muir & Woolf 2001), but is a basic sensory ability (Sneddon et al. 2014). The somatosensory cortex, insular, anterior cingulate cortex and prefrontal cortex and the thalamus are involved in pain perception.

1.1.4 Pain modulation

Primary/peripheral sensitization

Local release of chemical mediators, the so called “inflammatory soup”, from primary afferent fibres and other cells increases pain sensitivity by decreasing the threshold for activation of nociceptors or by direct

activation of nociceptors (Farquhar-Smith 2007). These substances include hydrogen ions, adenosine triphosphate (ATP), bradykinin, prostaglandins, leukotrienes, etc. Nociceptors express ion channels for stimulus transduction and action potential generation. Peripheral sensitization consist in the modification of expression of these membrane proteins, such as Transient receptor potential cation channel subfamily V member 1 (TRPV1), tetrodotoxin-resistant voltage-gated sodium channel (TTX-r Na^+ channel). The clinical consequence of peripheral sensitization is primary hyperalgesia consisting of an increase in the painfulness of a noxious stimulus and reduced threshold for pain at the site of tissue injury (Shilo & Pascoe 2014).

Central sensitization

Peripheral sensitization increases inputs from primary afferent neurons to the spinal cord. In the dorsal horn of the spinal cord amplification mechanisms enable the peripheral neurons that are not involved in nociception, to carry painful sensations (Shilo & Pascoe 2014). Secondary hyperalgesia is caused by central sensitization and consists in the production of pain by mechanical stimulation around the site of injury. Allodynia, that is to say pain caused by a stimulus that does not normally provoke pain is another manifestation of central sensitization. N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors are involved in central sensitization and are activated by neuropeptides like substance P and calcitonin gene-related peptide (CGRP) (Farquhar-Smith 2007).

Descending modulatory pathways

Brainstem descending pathways can be either inhibitory (antinociceptive) or facilitatory (pronociceptive) and their activity contributes to the control of the output of second order neurons.

Descending inhibitory fibres from the periaqueductal grey (PAG) in the midbrain and the rostral ventromedial medulla (RVM), project to the dorsal horn and inhibit pain transmission by utilizing monoaminergic

neurotransmitters such as noradrenaline and serotonin. Other neurotransmitters involved in the descending modulation of spinal nociceptive processing include opioids, GABA, cannabinoids and adenosine (Shilo & Pascoe 2014).

"Gate control" theory of pain

This theory proposed by Melzack and Wall describes a process of inhibitory pain modulation at the spinal cord level. Activation of Aβ fibres by tactile non noxious stimulation, activates inhibitory interneurons in the spinal cord, which inhibit pain signals transmitted via the C fibres (Melzack & Wall 1965).

1.2 Sheep: experimental model and farm animal

The sheep (*Ovis aries*) is one of the most widely spread domestic animals and its domestication occurred about 9,000 years ago, in the Neolithic period, in the Fertile Crescent (Rocha et al. 2011). Due to their adaptability to extreme climatic conditions, nutrient poor diets and manageable size, sheep spread worldwide and they are raised for meat, milk, and wool.
Nowadays sheep are also widely used in experimental research.

1.2.1 Sheep: experimental model

The origins of animal testing dates back to the Ancient Greek when physician such as Herophilus, Galen, Aristotle, Hippocrates had been using animals as models of their anatomy and physiology (Franco 2013). Advances in medicine, surgery, pharmacology and biomedical sciences, including the discovery of blood circulation, organ transplantation, achievements in immunology and genetics, formation of modelling methods and drug testing, were obtained through experimentation on animals.
The selection of the animal model should be based on several factors including (Davidson et al. 1987):

- appropriateness as an analog
- transferability of information
- genetic uniformity of organisms, where applicable
- background knowledge of biological properties
- cost and availability
- generalisability of the results
- ease of and adaptability to experimental manipulation
- ecological consequences
- ethical implications.

The key factor in using animals in research is in the possibility of extrapolation of results to humans. Indeed translation of findings from the laboratory to clinical trials is not always straightforward and the type of

animal model is of fundamental importance (Perel et al. 2007). Sheep are considered as adequate models for several disciplines including orthopaedics, neurology, cardiology and internal medicine, because they closely correlate with human anatomy and physiology (Pearce et al. 2007; Potes et al. 2008; Guillamon & Clau 2010; Katz et al. 2011; Mageed et al. 2013; DiVincenti et al. 2014).

Sheep are widely used in experimental research due to their availability, temperament and body size, which allows easy handling, manipulation and access for sampling, monitoring and insertion or application of various devices. Moreover their anatomy and physiology has been widely studied so baseline data are available to scientists (Adams & McKinley 2009). Disadvantages of the use of sheep as experimental animals include the necessity of large animal facilities and equipment.

The benefits to science and medicine, does not relieve the scientists from responsibility to guarantee animal welfare during the experiments. Indeed, current opinion is favourable to the use of animals in experimental research, when the advantage of using them surpass the inconvenience and provided that no other alternative exists and no unnecessary suffering occurs (Flecknell 2002).

The basis for a more ethical use of animals in experimental settings were set by Russell and Burch in the late 1950s (Russell W.M.S. 1959). The 3Rs theory consists of: replacement, reduction and refinement. According to the “replacement” principle “non-sentient” alternative methods, such as in vitro techniques or bioinformatic model, should be used instead of experimental animals whenever possible. “Reduction” to a minimum of the number of animals can be achieved by improving the experimental study design by setting standardized conditions and by performing an appropriate power analysis before commencing it (Festing & Altman 2002). “Refinement” consists of alleviating pain and distress in animals as much as possible. As pain and distress may affect several physiological functions, safeguarding experimental animal’s welfare will be beneficial not only for the animal but for the experimental outcome as well (Baumans 2005).

These principles have influenced new legislation aimed at controlling the use of experimental animals worldwide (Flecknell 2002).

1.2.2 Sheep: farm animal

Sheep were one of the first farmed animals, reared for meat, milk and wool.

There are many concerns about sheep welfare in farms, related to management procedures, transport and illness caused by disease.

Castration, dehorning, tail docking and mulesing (removal of skin from perineal area to prevent flystrike) are common husbandry procedures performed in farms, while lameness caused by infectious pododermatitis (footrot), mastitis, external myiasis and urolithiasis are common diseases affecting sheep (Scott 2007). It has been shown that these procedures and conditions cause pain and distress in sheep, which may be long lasting (Stafford 2014).

Effective ways to alleviate pain and discomfort in farms animals are fundamental to ensure animal welfare. Indeed, according to the “Five freedoms” theory, animal welfare is based on “Freedom from pain, injury and disease”, together with “Freedom from hunger and thirst”, “Freedom from discomfort”, “ Freedom to express normal behaviour” and “Freedom from Fear and Distress” (FAWC 1993).

By analogy with the “3Rs” approach of “Replacement, Reduction and Refinement”, in order to minimise pain in farm animals, other authors have developed the “3S” theory accounting for “Suppress, Substitute and Soothe”. According to this theory, any source of pain for which negative effects outweigh potential benefits for the animal should be suppressed. Methods that have been proven to be more painful should be substituted with less painful ones. Finally, whenever the animal is experiencing pain treatments should be used to soothe it (Guatteo et al. 2012).

Provision of analgesia in animals is fundamental not only for ethical reasons but also because untreated pain may lead to significant economic losses, in terms of decreased productivity (Paul-Murphy et al. 2004).

1.3 Pain recognition and assessment in sheep

1.3.1 Pain recognition in animals

One of the pillars on which animal welfare is based is the recognition and proper treatment of pain (Rutherford 2002). Accurate pain assessment in the individual animal allows adequate analgesic treatment and avoids the changes in peripheral and central nervous system which may lead to primary and secondary hyperalgesia allodynia and spontaneous pain. Once peripheral and central sensitization has occurred, control of pain becomes more difficult to achieve (Woolf & Salter 2000). This is not only a theoretical concept, but something that animals experience in their lifetime; indeed it has been shown that some of the procedures normally carried out on farms in very young animals cause a lasting state of somatosensory sensitization (Vinuela-Fernandez et al. 2007).

In human pain assessment, self-reporting plays a fundamental role, but self-report is not possible in small children, handicapped or elderly people, who are unable to speak or have cognitive dysfunction (von Baeyer 2009; Hadjistavropoulos et al. 2014). The same problem applies to animals which cannot verbalise their suffering and report the sensations they are experiencing (Anand & Craig 1996).

There is a clear need for valid and reliable tools to assess pain in animals in order to allow effective pain recognition in experimental and clinical settings (Crook 2014).

Several methods are available to recognise and measure pain in animals, but no gold standard exists. The ideal method should be specific, sensitive, reliable and valid and the assessment should be comprehensive and practical (Kent & Molony).

Pain assessment can be performed using objective or subjective methods, as described by Kent & Molony (Kent & Molony) and by Crook (Crook 2014).

Pain can be recognised using the following different measures:

Physiological parameters:

- Measurement of heart rate, blood pressure, respiratory rate and temperature; assessment of pupillary dilation, sweating, defecation, urination; measurement of general bodily functions (food and water intake, etc.) or productivity (weight gain, milk production, etc.)

- Neurohumoral responses: plasma or salivary cortisol, catecholamines, glucose, endorphins
- Biochemical responses: plasma glucose, free fatty acids, lactic acid, acute phase proteins (APP)

Neurophysiological parameters:

- Changes in the electroencephalogram (EEG) activity: changes in the frequency of EEG can be considered as marked of nociception

Behavioural observations:

- Behavioural responses: introduction of new abnormal behaviours, decreased frequency of normal behaviours. These changes may comprise changes in gait, posture, vocalization, facial expression, activity, mental status, evoked behaviours and behavioural patterns.

Quantification of pain is based on:

Nociceptive threshold testing:

- the threshold at which a subject responds to a noxious stimuli applied to the body is measured

Pain scales:

- several pain scales have been developed, such as the visual analogue rating scale (VAS), simple descriptive scale (SDS), numerical rating system scale (NRS) or multi-dimensional/composite scales.

Methods developed to recognise and quantify pain in animals have been shown to have advantages and disadvantages.

Physiological parameters are easily obtained and provide objective measurements but are not sensitive and specific methods for pain detection and have been shown to be inconsistent (Auer et al. 2007). Many stressors other than pain may activate the sympatho-adrenal and hypothalamic-pituitary-adrenal system, including eating, exercise, noise and drug administration (Kent & Molony; Rutherford 2002). The same considerations apply to biochemical and neurohumoral parameters, which have not been proven to provide a practical application yet, as other stressors may alter their concentration in the body, including diseases and drug administration (Kent & Molony; Rutherford 2002).

Both physiological and biochemical/neurohumoral parameters are subjected to individual variabilities and to changes due to circadian rhythms; moreover they are invasive methods, as a blood sample is usually required, and handling itself can alter them (Kent & Molony). For these reasons their clinical use is of little importance (Holton et al. 1998).

Weight gain and speed of wound healing are only crude parameters for assessing pain and they lack of sensitivity and specificity (Stafford 2014).

Some difficulties may arise when assessing behavioural signs of pain; for example, some changes in posture can be involuntary and caused by spinal and brainstem reflexes.

When using pain scales, the training of the assessor plays a fundamental role. The VAS, SDS and NRS are considered to be unidimensional scales and measure principally the intensity of pain. In multidimensional scales more parameters likely to be indicative of the emotional effects of pain are considered.

The VAS scale consists of a 100mm long line; where 0mm is considered to be no pain and 100mm the worst possible pain imaginable or the worst possible pain for the procedure performed. The assessor marks the line at the point which they think represents the degree of pain that the animal is experiencing. The score is the distance, in mm, along the line from the zero anchor to the line marked by the assessor.

SDSs are very easy to use and simple and the assessor selects one of a small number of descriptors. NRSs are discontinuous scales, where

descriptors of pain are assigned numbers. Multidimensional pain scales usually integrate objective and subjective behavioural observations.
As pain scoring using scales is subjective, problems concerning intra and inter-observer variability may arise (Holton et al. 1998); some scales have been shown to be more sensitive than others, an example is the use of VAS over NRS in sheep (Welsh et al. 1993). Unidimensional pain scales do not consider pain in its multidimensional expressions, involving the sensory and affective components. Composite pain scales provide a more complete view of the pain the animal is experiencing but may be more time consuming to complete. Nociceptive threshold tests are based on stimuli that are different from clinical pain and they do not take into account the emotional dimension of pain.

Nowadays pain assessment in animals is based on measurement of physiological and behavioural indices (Rutherford 2002). Knowledge of the behavioural repertoire of the individual species is mandatory to identify signs of pain. Behavioural changes can be considered as the animal's way to self-report pain.

1.3.2 Pain assessment in sheep

Pain assessment in sheep lags behind progress made in the field in other species including small animals, horses and rodents. In other species including dogs (Reid et al. 2007), cats (Brondani et al. 2013), rodents (Langford et al. 2010; Sotocinal et al. 2011), horses (Dalla Costa et al. 2014), cattle (de Oliveira et al. 2014) pain scales have been designed and validated and they constitute a reliable and practical method to assess pain in clinical environments.
Pain recognition in ruminants is difficult; they are prey species and as such there was a strong evolutionary pressure to mask signs of pain as predators could likely detect these as indicators of weakness (Stafford 2014). Another reason for masking pain could be that adult ruminants have no advantage to show pain as other adult conspecifics would not

assist or help them (Stafford 2014). This is particularly true for sheep, which tend to remain silent during painful procedures, unlike goats and cattle, vocalisation is shown only in case of very severe pain (Stafford 2014). This does not mean that sheep do not feel pain; indeed, in sheep many medical conditions and standard husbandry procedures that are carried out on farms are a source of pain, such as lameness, caused by footrot or abscess, myiasis, tail docking, castration, dehorning, tail docking and mulesing (Scott 2007).

Several methods have been used to recognise and evaluate pain in sheep including, physiological, biochemical, neurohumoral parameters, behavioural and EEG changes.

As commented before, physiological and neurohumoral parameters may be altered by handling itself, so researchers have also been looking for non-invasive methods of pain assessment via the use of telemetry (Stubsjoen et al. 2009).

Table 1.1 shows the studies evaluation pain in sheep.

Table 1.1 Studies evaluating pain in sheep classified by parameters used to identify it.

	Physiological / Neurohumoral / Biochemical parameters
(Shutt et al. 1988)	Lambs undergoing castration (rubber ring or surgery) plus docking showed a significant increase in beta-endorphins and cortisol. Surgery caused less distress than the application of rubber ring.
(Ley et al. 1991)	Sheep suffering from severe lameness showed increased plasma prolactin and decreased plasma cortisol concentrations, while plasma vasopressin did not consistently change with lameness.
(Kent et al. 1993)	Lambs underwent castration by different methods and tail docking, changes in plasma cortisol between pre and post procedure corresponded to behavioural changes.
(Ley et al. 1994)	Plasma cortisol concentrations did not show any correlation with the severity of lameness in sheep.
(Thornton & Waterman-Pearson 1999)	Several castration methods were assessed in lambs and they all caused an increase in cortisol
(Price & Nolan 2001)	In new-born lambs undergoing castration and tail docking haptoglobin levels were similar to control lambs.
(Mellor et al. 2002)	In lambs undergoing castration and tail docking a rapid increase in noradrenaline concentration and a marked increase in cortisol concentration were reported. No significant changes in adrenaline concentrations were shown.
(Peers et al. 2002)	In lambs undergoing castration and tail docking, BP and HR increased up to 4 hours after the procedure, while ACTH and cortisol increased markedly during the first hour, but returned to basal values by 2.5-3 hours. No significant changes in in mean plasma concentrations of renin, electrolytes, minerals, glucose, lactate, urea, creatinine, total carbon dioxide and total proteins were reported.
(Stubsjoen et al. 2009)	In sheep exposed to a noxious ischaemic stimulus heart rate variability was shown to be a sensitive non-invasive method to assess mild to moderate pain while changes in eye temperature measured using infrared thermography was a less sensitive method.

(Paull et al. 2007)	Mulesed lambs showed increased plasma cortisol, reduced lying and increased standing with a hunched back compared with sham mulesed animals. A combination of local anaesthetic and long acting non-steroidal anti-inflammatory drug decreased the pain response of lambs to mulesing.
(Paull et al. 2008)	The effect of NSAIDs on mulesing in lambs was examined. In comparison to control lambs, mulesed lambs showed an increase in plasma cortisol, beta-endorphin and haptoglobin, decreased body weight and changes in behaviour including spending less time lying ventrally and walking but more time standing with a hunched posture. NSAIDs administered before mulesing did not reduce the acute response of lambs to mulesing.
(Paull et al. 2009)	The physiological (plasma cortisol and haptoglobin) and behavioural responses suggest that ring castration has less impact on the lamb than surgical castration. NSAIDS and topic local anaesthetic formulation provided modest improvement in pain and discomfort.
(Paull et al. 2007)	Mulesed lambs showed increased plasma cortisol, reduced lying and increased standing with a hunched back compared with sham mulesed animals. A combination of local anaesthetic and long acting non-steroidal anti-inflammatory drug decreased the pain response of lambs to mulesing.
(Colditz et al. 2009)	Non-surgical mulesing by injection of cetrimide in lambs caused increased rectal temperatures, cortisol, haptoglobin, decreased daily gain, abnormal behaviours including hunched standing, stiff walking, pawing, lateral lying and lying intention. Carprofen ameliorated the behavioural responses, but was unable to provide relief from the intense and sustained physiological responses to non-surgical mulesing by intradermal injection of cetrimide.
	Behavioural observations
(Molony et al. 1993)	Several methods of castration in lambs of different ages were examined and it was shown that all the methods caused quantitative and qualitative changes in behaviour.
(Kent et al. 1993)	Lambs underwent castration by different methods and tail docking, changes in plasma cortisol between pre and post procedure corresponded to behavioural changes.
(Welsh et al. 1993)	NRS and VAS were used for subjective assessment of lameness in sheep and they showed intra-observer reproducibility, but more variation with sheep suffering from what was considered moderate

	lameness.
(Ley et al. 1994)	Plasma cortisol concentrations did not show any correlation with the severity of lameness in sheep.
(Kent et al. 1995)	In lambs undergoing tail docking different methods of castration were assessed. Foot stamping, restlessness, tail flicking, abnormal postures, rolling and kicking were observed.
(Lester et al. 1996)	Abnormal standing and lying were noticed in lambs undergoing castration and tail docking by different methods.
(Stafford et al. 1996)	Aversive behaviours were shown in rams undergoing electroejaculation.
(Thornton & Waterman-Pearson 1999)	In lambs undergoing castration by different methods, local anaesthetics abolished responses to rubber ring (RR) and combined rubber ring and Burdizzo clamp (CM), but did not affect the response to surgical castration (SX). General anaesthesia did not reduce responses to RR and SX but avoided the rise in MNT.
(Thornton & Waterman-Pearson 1999)	VAS scale combined with active behaviours, response to an observer and response to scrotal palpation was used to compare the pain caused by a number of castration techniques with or without analgesia.
(Dinniss et al. 1999)	Restlessness was shown in lambs up to 4 hours after ring castration. Clamp castration did not causes restlessness.
(Molony et al. 2002)	Behaviours recorded in sheep during and after castration included restlessness, rolling, jumping, foot stamping/kicking, easing quarters, tail wagging, head turning, vocalization, lip curl, teat seeking, trembling, normal lying, abnormal lying (ventral or lateral), normal standing, statue standing (hind limbs apart, further back than normal), abnormal standing (unsteady, backward, on knees, hops, circling, leaning, falling).
(Thornton & Waterman-Pearson 2002)	Several methods of castration were examined in lambs of different ages. Castration caused a reduction in time spent performing play behaviour in one week old lambs. In four to six week old lambs castration caused a decrease in lying behaviour and an increase in abnormal postures. Behavioural changes were present for 3 days post castration.
(Paull et al. 2007)	Mulesed lambs showed increased plasma cortisol, reduced lying and increased standing with a

	hunched back compared with sham mulesed animals. A combination of local anaesthetic and long acting NSAID decreased the pain response of lambs to mulesing.
(Paull et al. 2008)	The effect of NSAIDs on mulesing in lambs was examined. In comparison to control lambs, mulesed lambs showed an increase in plasma cortisol, beta-endorphin and haptoglobin, decreased body weight and changes in behaviour including spending less time in lying ventrally and walking but more time standing with a hunched posture. NSAID administered before the procedure did not reduce acute response of lambs to mulesing.
(Paull et al. 2009)	The physiological (plasma cortisol and haptoglobin) and behavioural responses suggested that ring castration has less impact on the lamb than surgical castration. NSAIDS and topic local anaesthetic formulation provided modest improvement in pain and discomfort.
(Lomax et al. 2008)	In lambs undergoing mulesing, topical anaesthesia decreased pain-related behaviour and improved wound healing.
(Lomax et al. 2010)	Topical anaesthesia alleviated the short-term pain of castration and tail docking in lambs according to behavioural observations and mechanical nociceptive threshold testing.
(Lomax et al. 2013)	In lambs undergoing mulesing, local anaesthetics decreased primary and secondary hyperalgesia and pain-related behaviours and provided analgesia for up to 24 hrs.
	Electrophysiological parameters
(Morris et al. 1997)	Painful electrical stimulation in sheep caused changes in EEG, which can be considered a useful tool to measure acute pain in these species.
(Ong et al. 1997)	Electrical stimulation was applied to sheep being implanted EEG electrodes. According to electroencephalogram changes recorded, this method could provide a measure of acute pain in this species.

The first studies performed evaluated husbandry procedures commonly carried out on farms without the use of analgesics, such as castration, mulesing and dehorning. The behaviour, physiological, biochemical and neurohumoral parameters of sheep undergoing painful procedures have been compared to sham controls or to themselves before the procedure (Paull et al 2007 & 2008). The findings of these early studies constitute the bases of pain assessment in sheep. Moreover, research focused also on the impact of different surgical methods and the choice of some procedural techniques over others was suggested and was then introduced into practice (Shitt et al 1988, Kent et al. 1993 & 1995, Molony et al. 1993). Finally, newly identified indicators of pain could be used to compare the efficacy of different drugs (Colditz et al 2009). Several studies included more than one type of assessment, such as evaluation of physiological parameters, neurohumoral/biochemical essays, observation of behaviours, response to observer, response to wound palpation, and measurement of pain by the use of subjective pain scales and nociceptive tests (Thornton & Waterman-Pearson 1999).

These studies showed that changes in plasma cortisol values are useful in assessing pain of moderate intensity but not severe pain, as a ceiling effect was reported. (Kent et al. 1993; Molony et al. 2002). Moreover changes in plasma cortisol values during chronic pain may not be consistent (Ley et al. 1991; Ley et al. 1994). Acute phase proteins were not reliable indicators of pain in lambs (Price & Nolan 2001).

Normal postures and gaits have been described in lambs (Molony et al. 1993; Molony et al. 2002) and this helped with the recognition of abnormal behaviours in sheep after painful procedures (Kent et al. 1995; Molony et al. 2002). Pain scoring scales have been used to evaluate lameness in sheep and the VAS was more sensitive than the NRS (Welsh et al. 1993). Nociceptive threshold tests were used to assess hyperalgesia in sheep undergoing painful procedures (Lomax et al. 2010; Lomax et al. 2013). Measurement of electrophysiological parameters can be potentially useful in pain recognition, but more research is necessary in this field and its practical use would be difficult.

Behavioural assessment of pain is recognised to be one of the most specific and sensitive ways of assessing pain in farm animal species (Molony & Kent 1997). Many studies have evaluated behavioural changes associated with painful procedures. Behavioural indicators of pain in sheep are shown in table 1.2.

The expression of pain through behaviour may include suppression or change in frequency of common behaviours as well as introduction of new behaviours indicative of pain. In summary, a high variability in behaviours has been shown which depends on the kind of procedure performed and the age of the animal. The most obvious behaviours such as vocalization and aggression after palpation of the affected area were reported only after very painful procedures. Moderate pain caused changes in postures, gait, eating and sleeping patterns. Sedation, illness or stress may affect short term behavioural observations and twenty four hours, or longer, monitoring period is required to detect changes in patterns of behaviour.

Table 1.2. Behavioural indicators of pain in sheep.

Behavioural indicators of pain in sheep		
Inappetence	Restlessness	Abnormal lying
Isolation	Rolling	Abnormal standing
Inactivity	Jumping	Abnormal postures
Decreased play	Head turning	Foot stamping
Aggression	Trebling	Easing quarters
Vocalization (severe pain only)	Lip licking	Itching quarters
Colic signs	Teeth grinding	Kicking
Dull expression	Tail flicking	Wound licking

Sources: (Kent & Molony; Kent et al. 1995; Molony & Kent 1997; Hardie 2000; Thornton & Waterman-Pearson 2002; Stafford 2014)

In conclusion, a combination of physiological and behavioural indices are useful for recognising and assessing pain in sheep, but they have shown a high variability according to the type of procedure performed and age of the animals.

1.4 Nociceptive threshold testing

1.4.1 Quantification of pain: nociceptive threshold testing

Animals cannot self-report the level of pain they are experiencing, and as explained before, neurohumoral/biochemical and electrophysiological parameters are not specific indicators of pain (Le Bars et al. 2001). Pain assessment in animals relies mainly on behavioural observations, and thus the importance of knowing each species behavioural repertoire in order to detect abnormal behaviours, which may be indicative of pain. Animal's pain scoring is subjective and affected by the assessor's observational skills, attitudes towards pain and knowledge of the species' ethogram (Hardie 2000). In order to overcome the subjectivity of the scoring which is implied in the observational method, a more objective way to quantify the degree of pain experienced by the animal consists in the measurement of nociceptive thresholds. The term " nociception" originates from the Latin " *nocere*" which means " to harm" and was first introduced in the early 1990s (Sherrington 1910). Nociception consists in the detection of potentially harmful stimuli, and in conscious animals gives rise to pain, which has both a sensory and emotional component (Sneddon 2004). Nociceptive systems have developed and become more complex during evolution; they have been extensively studied in vertebrates and recent studies have shown the presence of nociceptors in lower vertebrates too (Sneddon 2004).

Nociceptors are specialised peripheral sensory neurons which are activated by potentially damaging stimuli at the skin, mucosa, deep fascia, connective tissue of visceral organs, ligaments and articular capsules, periosteum, muscles, tendons and arterial vessels (Almeida et al. 2004). Nociceptors transduce these stimuli into electrical signals conveyed to higher brain centres (Dubin & Patapoutian 2010). Thermal, mechanical, chemical stimuli, with the potential to injure tissues, activate functionally distinct cutaneous nociceptors and the variety of receptors involved is

mirrored by the multiple characteristics of pain (Dubin & Patapoutian 2010).

Nociceptors are associated with free nerve endings and represent the more distal part of first-order neurons; the classification of first order afferent neuron fibres in terms of structure, diameter and conduction velocity is shown in table 1.3.

Nociceptors are generally electrically silent; once stimulated they transmit an action potential. Perception of pain does not directly come from their activation but peripheral inputs have to be transmitted to and modulated by higher centres (Dubin & Patapoutian 2010).

Nociceptive threshold testing (NTT), also referred to as Quantitative Sensory Testing (QST), are used in experimental and clinical settings in people (Rolke et al. 2006; Arendt-Nielsen & Yarnitsky 2009; Backonja et al. 2013; Grosen et al. 2013; Hubscher et al. 2013). NTT has also been widely used in the veterinary experimental research in studies evaluating the efficacy of analgesic drugs and in dose-finding studies (Love et al. 2011), and to assess the neural processing of noxious stimuli (Hothersall et al. 2011).

Many nociceptive stimuli have been used in research, including electrical, thermal, mechanical and chemical stimuli. As commented by Le Bars (Le Bars et al. 2001), studies carried out in conscious animals are referred to as " behavioural studies" and this terminology implies that "*all responses, including simple withdrawal reflexes, are part of an animal's behaviour repertoire*". Nociceptive tests comprise an input, the stimulus applied, and an output, the reaction of the animals to that stimulus (Le Bars et al. 2001). Nociceptive stimulation should be repeatable, reliable, quantifiable, with a clear end point non-invasive, and produce no harm to the animal (Beecher 1957).

The stimulus variables include intensity, duration and surface area of stimulation: "*these three parameters determine the global quantity of nociceptive information that will be carried to the central nervous system by the peripheral nervous system*" (Le Bars et al. 2001). Other variables

when performing nociceptive threshold test include the site on the body where the stimulus is applied and the status of the site, if healthy or inflamed (Le Bars et al. 2001). Tests based on the use of short duration stimuli and long duration stimuli as referred as “phasic pain” and “tonic pain” experiments respectively (Le Bars et al. 2001).

Limitations of NTT is that they provide a stimulation which is different from clinical pain, and in order to reproduce a stimulation as similar as possible to clinical pain more than one threshold testing modality should be used (Tyers 1980; Nielsen et al. 2009). That is the reason why these tests may not be sensitive enough to assess the analgesic properties of drugs in clinical settings (Love et al. 2011).

Table 1.3 Classification of first-order afferent fibres in mammals. [Adapted from (Almeida et al. 2004)]

Fibre type	Diameter	Conduction velocity	Structure	Stimuli	Comments
Aβ	>10 µm	30-100 m/sec	Myelinated	Respond to innocuous mechanical Stimulation.	Do not propagate noxious stimuli in normal situations. Involved in segmental suppression of pain.
Aδ	2-6 µm	12-30 m/ sec	Barely myelinated	Type 1: High threshold mechanoreceptors responding to high intensity mechanical stimuli and weakly to thermal and chemical stimuli and, after sensitization to harmful heat. Type 2: Mechano-termal receptors responding to very high and low temperatures. Later sensitization to vigorous mechanical stimuli at non-noxious thresholds.	Propagate information with marked intensity and short latency. Promote quick sensation and withdrawal actions.
C	0.4-1.2 µm	0.5-2 m/sec	Unmyelinated	Polymodal receptors responding to mechanical, thermal and chemical stimuli. Silent receptors activated by inflammation	Propagate information slower and their prolonged potentials undergo summation along time, and pain is felt as “dull”

1.4.2 Classification of nociceptive stimulation

Electrical stimulation

Application of an electrical stimuli has been used to test nociceptive withdrawals reflex in people and it has been proven to be a useful tool in pain assessment and research in central sensitization and chronic pain. It has also been used and validated in several species (Spadavecchia et al. 2004; Bergadano et al. 2007) including sheep (Rohrbach et al. 2014; Rohrbach et al. 2015). The input given by the electrical stimulation is repeatable, controllable and non-invasive; nevertheless, it stimulates directly all peripheral fibres included the ones not involved in nociception and it is not a stimulus naturally encountered by an animal, and as such does not reflect clinical pain (Le Bars et al. 2001). Indeed it activates large diameter fibres not implicated in nociception and Aδ and C fibres which not only mediate nociception but also sensation of cold and hot (Le Bars et al. 2001). It can be considered as a non-invasive method to evaluate central sensitization as a consequence of surgery in animals (Rohrbach et al. 2015).

Chemical stimulation

Chemical stimuli are characterised by being progressive, slow form of stimulation whose effects are long lasting and inescapable (Le Bars et al. 2001). This kind of stimulation differs greatly from the others not only because of the nature and duration but also because the output is not a threshold but a behavioural score (Le Bars et al. 2001).

Thermal stimulation

Thermal stimuli have been widely used in laboratory animals in a variety of test including the tail flick test, paw and tail withdrawal tests and hot plate tests (D'Amour & Smith 1941; Woolfe & Macdonald 1944; Luttinger 1985). These tests consists in either the application of a constant temperature and measurement of the time taken for the animal to respond (latency), or the application of ramped increasing temperatures and record of the

temperature at which the animal respond (threshold temperature) (Love et al. 2011). Heat stimulates thermosensitive and nociceptive fibres and so it stimulates cutaneous receptors in a more selective way (Le Bars et al. 2001). Thermal stimulation can be conveyed by radiant heat sources, thermode based systems or carbon dioxide laser thermal stimulators (Le Bars et al. 2001). Thermal nociceptive threshold has been use to evaluate the analgesic/antihyperalgesic effect of drugs and the nociceptive processing in horses (Dhanjal et al. 2009; Hoffmann et al. 2012; Love et al. 2012; McGowan et al. 2013; Poller et al. 2013), dogs (Hoffmann et al. 2012), cats (Slingsby et al. 2010; Slingsby et al. 2012; Ambros & Duke 2013; Farnworth et al. 2015), birds (Caplen et al. 2013; Hothersall et al. 2014) and sheep (Nolan et al. 1987a; Nolan et al. 1987b; Nolan et al. 1987c; Nolan et al. 1988). Thermal nociceptive tests performed using radiant heat have the advantage that the source is not directly in contact with the skin, but, on the other hand, measurements are affected by the radiation (reflectance, transmittance, absorbance), conduction properties and the initial temperature of the skin (Le Bars et al. 2001). Thermodes have the disadvantage of activating both nociceptors and low-threshold non –nociceptive nerves exerting inhibitory influences on pain mechanisms (Le Bars et al. 2001). In the past their use in animals was limited due to their fixed and rigid surface and the difficulty in standardization of skin contact pressure (Le Bars et al. 2001), but this disadvantage has been overcame as new probes have been designed specifically for animals, and the pressure at which the probe contacts the skin can be modified (Dixon et al. 2002; Love et al. 2011). Moreover a wireless thermal threshold testing system has been used in horses (Love et al. 2011). The carbon dioxide laser thermal stimulator overcomes the disadvantaged of the other thermal nociceptive threshold devices (Le Bars et al. 2001), and only recently its use has been investigated in veterinary medicine (Farnworth et al. 2013). Thermal threshold temperatures varies between and within the species and they are affected by skin pigmentation, hair density, skin thickness and composition, depth and density of Aδ and C fibre nociceptors within the tissue (Love et al. 2011;

Grint et al. 2015). Fluctuations in ambient temperature and rate of heating may also affects thermal threshold (Love et al. 2011). One disadvantage of assessing thermal nociceptive threshold in species which do not clearly show behavioural pain expression, is the potential for skin lesions; to prevent it a thermal cut off is usually set. Nevertheless there are reports of thermal injuries in horses (Robertson et al. 2005), sheep (Musk et al. 2014), and donkeys (Grint et al. 2015). In order to prevent burns, modifications to a thermal probe used in animals have been recently done (Dixon et al. 2015).

Mechanical stimulation

A rudimental form of mechanical stimulation used in practice is represented by hoof testers (Love et al. 2011). Von Frey filaments were the first type of mechanical stimulus used in research but they do not provide a specific noxious stimulation, as they activates both low threshold mechanoreceptors and nociceptors; difficulty in application to unrestrained animals also apply (Le Bars et al. 2001). Nowadays they are used in rodents to measure mechanical thresholds and in larger species to identify allodynia (Taylor & Dixon 2012b).

Several veterinary research and clinical experimental studies evaluated and validated the use of mechanical nociceptive threshold (MNT) devices in order to measure the level of pain after a procedure or to quantify the analgesic or antihyperagesic effect of a drug in several species including cats (Dixon et al. 2007; Bortolami et al. 2013), dogs (Dixon et al. 2010; Hunt et al. 2013), horses (Love et al. 2012), donkeys (Grint et al. 2014), cattle (Whay et al. 1997; Raundal et al. 2014), birds (Hothersall et al. 2011), pigs (Nalon et al. 2013), sheep (Nolan et al. 1987a; Nolan et al. 1987b; Lizarraga & Chambers 2006; Lizarraga et al. 2008) and rodents (Callahan et al. 2008). Measurement of mechanical nociceptive threshold is useful tool to assess pain in clinical conditions as well; this test can be considered a proper adjunct to clinical care, that is why in the United Kingdom this method can be used in practices without Home Office License (Jolliffe et al. 2009).

Mechanical nociceptive threshold tests can evaluate both somatic and visceral sensory systems.

Somatic MNT testing devices consists in a probe which is applied to the skin of the test area and an increasing force, usually generated by a pneumatic cylinder, is exerted to this probe until a clear response is evoked. The reflex withdrawal which is the first reaction seen can be considered as a spinal reflex, but then the animal can exhibit signs which involve supraspinal structures, such as escape behaviours and vocalization (Le Bars et al. 2001).

The dorsal aspect of the limb is the area mainly used in large animal species for MNT tests (Nolan et al. 1987a; Whay et al. 1997; Love et al. 2012), because of minimum amount of soft tissue present in that area and the minimal anatomical variations (Love et al. 2011). MNT values have been found to differ significantly between different anatomical locations in some species (Haussler & Erb 2006; Harris et al. 2015).

Both hand-held (Nolan et al. 1987a; Whay et al. 1997; Haussler & Erb 2006; Stubsjoen et al. 2010; Raundal et al. 2014) and limb-mounted mechanical algometers have been used (Love et al. 2012; Nalon et al. 2013; Musk et al. 2014) in large animal species. Hand held algometers allow measurement of thresholds on multiple body sites (Stubsjoen et al. 2010), and do not require restraint of animals and are useful when working with animals kept in loose-housing systems (Raundal et al. 2014) but the animals see the operator approaching it and so there is a higher predictability of the stimulus (Nalon et al. 2013). Limb-mounted mechanical algometers have the advantage of being operated remotely from the animal, thus preventing distraction or anxiety which may affect the result of the test (Taylor et al. 2015). Moreover limb actuators are usually securely strapped to the limb thus preventing slippage as observed when using a hand-held probe (Nalon et al. 2013). A dummy device can also be fitted to the contralateral limb so that the animal is not distracted by uneven balance (Nolan et al. 1988)

Another variable when using MNT devices is the probe design. In people probes with tips of different sizes are used to differentiate the origin of

pain; in animals it has been shown that MNT increases with tip diameter and that large tip diameters produced more variability in MNTs (Taylor & Dixon 2012b; Raundal et al. 2014; Taylor et al. 2015). It has also been shown that MNT values are not proportional to the area of the probe tip, but a relationship was shown with the square root of the probe tip diameter (Taylor & Dixon 2012a).

A three pin configuration tip was designed, and used in some studies, in order to maintain better contact by preventing the probe from angling and sliding off the limb, but it was proven to provide more variable data (Taylor et al. 2015). In order to avoid the animal's reactions when the probe first makes contact with the skin, a spring loaded configuration, which maintains the probe in contact with the skin at 1-2 N, has been evaluated in sheep and horses, but the results are controversial (Musk et al. 2014; Taylor et al. 2015).

Several other factors influence the results of nociceptive threshold testing, such as skin thickness, coat density, gender, distractions, exposure to new ambient, disruption of social bounds. Marked variations in ambient temperature should be avoided because they may affect equipment performances and alter skin perfusion (Love et al. 2011). The assessor's experience, handling of the animal, rate of stimulus application may also affect the test results.

Finally, after prolong testing, animals may develop a sort of habituation to the procedure, with the consequent decrease in MNT values over time (Stubsjoen et al. 2010) or manifest a learned response, that is to say that the animal respond to the stimulus as soon as it is perceived rather than when or becomes aversive (Love et al. 2011)

In conclusion, nowadays there is no gold standard method or device for measuring MNT in any species (Taylor et al. 2015). The type of device, the probe configuration (dimensions, shape, profile), the area tested (thickness, coat, different innervation, soft tissue), the rate of stimulus application, but also ambient temperature, age of animals, companion status, distractions, exposure to new environment are factor which may account for the variability of data reported in the studies (Taylor et al.

2015). For these reasons these factors have to be specified when reporting an experiment (Taylor et al. 2015).

1.4.3 Nociceptive threshold testing in sheep

Quantitative sensory testing methods have been used in conscious painful and non-painful (naïve) sheep in order to assess the efficacy of analgesic drugs, including opioids (Nolan et al. 1988; Waterman et al. 1991a; Kyles et al. 1993b), non-steroidal anti-inflammatory drugs (NSAIDs) (Welsh & Nolan 1994; Welsh & Nolan 1995b) ,α_2-adrenergic agonists (Grant et al. 2001; Grant & Upton 2004), local anaesthetics (Lomax et al. 2008) and to assess hyperalgesia caused by husbandry procedures or pathological conditions (Ley et al. 1989; Welsh & Nolan 1995a). Both hand held algometers (Stubsjoen et al. 2010) and limb actuators have been used in this species (Nolan et al. 1987a; Chambers et al. 1994). Thanks to these studies the site of action of analgesic drugs was assessed (Brandt & Livingston 1990; Waterman et al. 1991b; Kyles et al. 1993a; Kyles et al. 1993b; Lizarraga & Chambers 2006).

Ambient temperature below 8˚C caused a marked decrease in thresholds (Chambers et al. 1994). In sheep kept for experimental use MNT values were less variable than the one measured from naïve sheep (Welsh & Nolan 1995b).

Species differences in the number and distribution of opioid receptors may account for the different activity of drugs, thus the different effect of opioid in different species (Nolan et al. 1987c) and different class of opioid may have more effective at suppressing thermally induced nociception than mechanical induced (Waterman et al. 1990). Finally breed can have an impact on the efficacy of analgesic drugs due to different metabolic pathways (Ley et al. 1990).

Table 1.4 shows the studies performed in sheep using a mechanical nociceptive threshold device.

In the study performed by the Author in chapter 4, a limb mounted mechanical nociceptive threshold device comprising a cuff with a 2 mm hemispheric blunt pin fixed on a rolling diaphragm actuator and applied perpendicular to the skin of the test area. The pin was pushed against the skin of the dorsal aspect of the right metacarpus, approximately 4 cm below the carpus, with a force which was applied manually by a syringe, connected to non-distensible tubing via a digital meter which displayed the force exerted, until a clear withdrawal response (leg lift, head turn, weight bearing on the contra-lateral limb) was evoked. The force at which the sheep responded with a clear withdrawal response was recorded as the MNT. As suggested by previous studies (Chambers et al. 1994), sheep were penned individually in specially designed pens, which allowed them to move without turning round, and the assessor to access their legs. Moreover sheep could be in visual and auditory contact with other usual flock mates, a measure recommended to limit stress in experimental sheep (Livingston et al. 1992). Sheep have been acclimatised to the environment, assessors and to the MNT test, as acclimatization and training have been shown to reduce the variability of the results (Welsh & Nolan 1995b). In order to avoid distractions, the same level of ambient noise was maintained constant during the experiments.

In conclusion, the measurement of nociceptive thresholds can be used to provide evidence that the animal is experiencing pain, to evaluate the analgesic/antihyperalgesic properties of drugs and to assess hypersensitization and correlate it with clinical conditions (Ashley et al. 2005).

Table 1.4. Studies performed in sheep using a mechanical nociceptive threshold device.

(Nolan et al. 1987a)	Reliable series of values for thermal and mechanical nociceptive thresholds tests were obtained.
(Nolan et al. 1987c)	Buprenorphine (3 and 6 µg/kg IV) showed no detectable mechanical antinociception. Prior treatment with naloxone (0.2 mg/kg IV) did not affect the response threshold. Subsequent injection of xylazine (50 µg/kg IV) increased the threshold to maximum. Buprenorphine showed thermal mechanical antinociception which was detectable up to 3 and ½ hours.
(Nolan et al. 1987b)	Xylazine (50µg/kg IV) caused an immediate increase of MNT to maximum value of 16 N, values returned to basal levels after 45 min. Clonidine (6µg/kg IV) increased threshold to maximum within 3 minutes at it remained like that for 45 min, reaching control values after 120 min. Prior administration of idazoxan (0.1 mg/kg IV) abolished the effects of both xylazine and clonidine, while naloxone (0.2 mg/kg IV) did not. MNT markedly increased with both xylazine and clonidine.
(Nolan et al. 1988)	Pethidine (5 mg/kg IV) produced a significant degree of antinociception to thermal pain for 30 min (on average) but gave only a few minutes of significant analgesia when tested with the mechanical pressure system.
(Waterman et al. 1988)	Intrathecal injections of small volumes of the α2-adrenoceptor agonists, xylazine (5-50 µg) and clonidine (100µ), into the cervical region of the spinal cord of conscious unrestrained sheep produced a dose-dependent analgesia of the forelimbs as measured using a mechanical pressure device. Intravenous injection of the α2-adrenoceptor antagonist, idazoxan completely abolished the analgesic effects of the intrathecally applied α2-adrenoceptor agonists.
(Ley et al. 1989)	Chronic pain from footrot in sheep caused a reduction in the threshold to mechanical pressure, with thresholds remaining lower for periods longer than 3 weeks in many cases, with some returning normal after 3 months. Footrot did not alter the threshold to the thermal test.
(Waterman et al. 1990)	After fentanyl (5 µg/kg IV) administration significant analgesia to thermal pain was reported for 30 minutes but mechanic antinociceptive activity was not detected. However fentanyl at a dose rate of 10 µg/kg produced both thermal (60 min) and mechanical (40 min) antinociceptive effects.

(Kyles et al. 1990)	Intrathecal administration of naloxone (5 mg) caused significant mechanical hyperalgesia.
(Ley et al. 1990)	Antinociceptive effects of xylazine administered intravenously varied with the breed of the sheep tested.
(Waterman et al. 1991a)	Butorphanol (0.05, 0.1, 0.2, 0.4 mg/kg IV) produced dose-dependent thermal antinociception, but no significant elevation in mechanical pressure threshold.
(Waterman et al. 1991b)	Buprenorphine (1.5, 12 µg/kg IV) caused significant increase in thermal threshold for 40 minutes, but no mechanical antinociception.
(Ley et al. 1991)	Intensity and duration of analgesia produced by xylazine (50 µg/kg IV) was significantly reduced in animals experiencing chronic pain (footrot). When the test was repeated after clinical resolution of the condition there was almost no change in the profile of the chronic pain animals with MNT values being significantly different from the controls.
(Kyles et al. 1993a)	Intrathecal xylazine (100 µg/kg) increased mechanical nociceptive thresholds in the sheep; this effects was abolished by prior intrathecal administration of a selective α_2-adrenoceptor antagonist.
(Kyles et al. 1993b)	Droperidol (5 µg/kg IV) combined with fentanyl (5 µg/kg IV) and zuclopenthixol (100µg/kg IV) combined with fentanyl (5 µg/kg IV) increased significantly MNT.
(Chambers et al. 1994)	Further development in the design of a MNT device was made. The device showed normal distribution of thresholds in both healthy and lame sheep; the mean threshold in lame sheep was slightly but significantly lower than that in healthy sheep.
(Welsh & Nolan 1994)	The tourniquet was placed on the sheep limb and it significantly decreased MNT values in the ipsilateral limb, but not in the contralateral. Pre-treatment flunixin (1 mg/kg IV) or carprofen (0.7 mg/kg IV) attenuated the development of mechanical hyperalgesia, and fentanyl (5 µg/kg IV) caused significant antinociceptive effects initially.
(Welsh & Nolan 1995a)	Abdominal surgery caused thermal hyperalgesia in the acute post-operative period, similar changes were not found with mechanical stimulation.
(Welsh & Nolan 1995b)	Lame sheep did show lower MNT values than healthy (non-experimental) sheep; but MNT values was significantly greater than that recorded from experimental animals. Flunixin (1or 2 mg/kg IV) had no effect on

	MNT in either lame or healthy sheep but its repeated administration to lame sheep reduced their thresholds to noxious mechanical stimulation.
(Ley et al. 1995)	Sheep suffering from severe lameness showed lower mechanical threshold values than their matched sound controls and their thresholds remained low when tested three months later, after the apparent resolution of the foot rot lesion. In flocks where lame sheep were less severely affected there was no difference in the threshold responses to a mechanical stimulus between the sound and lame sheep.
(Chambers et al. 1995)	Flunixin (2.2 mg/kg IV) and dipyrone (25 mg/kg IV) caused a small but statistically significant rise in mechanical pain thresholds. In the lame sheep a similar effect occurred but the response was smaller, much more variable and tended to be prolonged. Pre-treatment with naloxone or atipamezole prevented the rise in thresholds. Naloxone and atipamezole had no effect on thresholds when given alone to healthy sheep.
(Lizarraga & Chambers 2006)	Intrathecal cumulative concentrations (0.375–200 µM; 100 µL) of ketoprofen, phenylbutazone, salicylic acid and tolfenamic acid as well as a single IV dose (3, 8, 10 and 2 mg/kg, respectively) of each NSAID were administered to sheep. None of the NSAIDs administered by the intrathecal route increased MNT values, while only IV ketoprofen and tolfenamic acid raised the pain thresholds. The hypoalgesic effect of IV ketoprofen was prevented by intrathecal naloxone or atipamezole.
(Lizarraga et al. 2008)	In sheep, intrathecal administration of ketoprofen (200-3200 µM; 100 µL) and ketamine (25-400 µM; 100 µL), alone or in combination, produced no hypoalgesia; however, they prevented NMDA-induced mechanical hypersensitivity.
(Stubsjoen et al. 2010)	An electronic hand-held algometer, provided with a blunt plastic tip of 0.5 cm^2, was tested and proven useful to measure MNT in sheep. A decrease in MNT values over 3 consecutive test days was reported.

1.5 Pain treatment in sheep

Difficulty in pain treatment in animals arise from the difficulty in detecting pain-related behaviours, lack of cost-effective licensed analgesics, withdrawal times and fear of side effects (Valverde 2013).

1.5.1 Opioids

The use of opioids in sheep is not common due to issues related to licensing, schedule classification and fear of side effects.

Opioid receptors are protein G-protein coupled receptors and once activated they promote a cellular signalling cascade leading to closure of voltage-sensitive calcium channels, efflux of potassium and reduction of cyclic adenosine monophosphate production. The result is a decrease in neuronal excitability through cellular hyperpolarization, inhibition of neurotransmitters release (Duke-Novakovski 2014). Different opioid receptors have been classified as μ, ƙ, δ and nociception/orphanin and they are located at spinal (substantia gelatinosa) and supraspinal (periaqueductal grey area, amygdala, corpus striatum, hypothalamus) sites (Duke-Novakovski 2014).

The efficacy of opioids in sheep has been proven mainly in experimental settings. An increase in thermal nociceptive threshold values was observed with buprenorphine (Nolan et al. 1987c; Waterman et al. 1991b), pethidine (Nolan et al. 1988), fentanyl (Waterman et al. 1990) and butorphanol (Waterman et al. 1991a), while an increase in mechanical threshold values was detected only with fentanyl (Waterman et al. 1990) and pethidine (Nolan et al. 1988).

There are limited studies assessing the administration of opioids in anaesthetised sheep; it has been shown that opioids exert anaesthetic - sparing effects: oxymorphone and hydromorphone significantly decreased minimum alveolar concentration (MAC) of desflurane by more than 7% (Sayre et al. 2015), while, an infusion of fentanyl (10μg/kg/h) decreased

MAC of isoflurane by more than 22% (Funes et al. 2015). The analgesic effects of fentanyl administered transdermally in a clinical experimental setting seemed promising (Ahern et al. 2009; Christou et al. 2015). Nevertheless, an infusion of fentanyl has been shown to cause respiratory depression in sheep undergoing cardiac surgery (Kronen et al. 2005).
Other experimental studies tested the effects of opioids in sheep to evaluate their possible analgesic properties, physiological effects or adverse events in preclinical models (Booke et al. 1996; Upton et al. 1997; Swenson et al. 1998; Smith et al. 2004). The epidural administration of opioids was shown to be useful as part of the analgesic management of procedures on flank and hind limb. (DeRossi et al. 2015).

1.5.2 Tramadol

The main side effects of opioid therapy in people are nausea, vomiting, sedation, drowsiness, dizziness and cardiovascular and respiratory depression, urinary retention, constipation, miosis; other disadvantages are potential for abuse and dependency (Candiotti & Gitlin 2010). Research has focused on the synthesis of analgesic drugs with good analgesic efficacy but devoid of undesirable side effects (Giorgi 2012).
Tramadol seems a promising analgesic drug because although it is has been shown to have analgesic properties similar to other opioid agonist drugs, it was shown to minimally affect the respiratory, cardiovascular, and gastro-intestinal system and has minimal potential for misuse or dependency. Moreover tramadol is not a controlled drug or scheduled analgesic in most countries and this facilitates its access and use (Bonezzi 2008). For these reasons the pharmacokinetics and antinociceptive effect of this drug were evaluated in sheep by the Author.

Tramadol is a centrally acting synthetic analgesic drug structurally related to codeine and morphine; tramadol is 6000-times less potent than morphine and 10-times less potent than codeine (Vazzana et al. 2015).

Tramadol has shown a dual mechanism of action which results in the inhibition of both perception and transmission of pain (Mattia & Coluzzi 2005).

Noradrenaline and serotonin reuptake inhibition are predominantly caused by (-) and (+) enantiomers of the parent compound respectively, while the (+) enantiomer of O-desmethyltramadol and to a lesser extent (+) tramadol activates the μ opioid receptors (Grond & Sablotzki 2004).

In people, the active metabolite, O-desmethyltramadol (M1) has an affinity for the μ opioid receptor 300 times higher than that of the parental drug and its analgesic efficacy seems to be 6 times higher than that of the parent compound (Gillen et al. 2000).

In humans tramadol provides good analgesia with only mild effects on cardio-respiratory function and intestinal motility (Raffa et al. 1992) and is not a scheduled drug.

In people therapeutic serum concentrations of tramadol and M1 were 0.3 ± 0.2 to 590 ± 410 ng/ml and 0.08 ± 0.03 μg/ml respectively (Lehmann et al. 1990; Grond et al. 1999). The minimum effective concentration showed a wide variability between subjects due to genetic polymorphisms which affect tramadol metabolism (Pedersen et al. 2006).

Tramadol in veterinary medicine

The pharmacokinetics and biotransformation of tramadol have been studied in several animal species including the dog, cat, goat, llama, alpaca, horse and donkey (KuKanich & Papich 2004; Giorgi et al. 2007; de Sousa et al. 2008; Pypendop & Ilkiw 2008; Giorgi et al. 2009b; Cox et al. 2011; Stewart et al. 2011; Edmondson et al. 2012). Species-specific differences in the kinetic profiles of both the parent drug and its metabolites have been highlighted.

Tramadol was shown to have analgesic effects in dogs and cats undergoing surgical procedures (Kongara et al. 2013; Morgaz et al. 2013; Teixeira et al. 2013; Evangelista et al. 2014)

There are also studies confirming the analgesic efficacy of tramadol for the management of peri-operative pain in other ruminants (Bigham et al. 2010; Habibian et al. 2011; Dehkordi et al. 2012).

1.5.3 Alpha-2 adrenoceptor agonist drugs

Alpha$_2$-adrenoceptor agonist drugs are widely used in sheep because of their analgesic and sedative effects, nevertheless their effect is short lasting and they are not devoid of side effects, including cardiorespiratory depression, alteration of ventilator parameters leading to hypoxaemia, and increased myometrium tone (Kastner 2006; Kastner et al. 2007).

Adrenergic receptors are protein G-coupled receptors, are targeted by catecholamines and are present in many tissues including peripheral and central nervous system. Analgesic and antihyperalgesic effects are caused by activation of the descending noradrenergic-serotoninergic inhibitory pain pathway, while sedation is due to activation of receptors in the pontine locus coeruleus and which decrease activity of ascending neural projections to the cerebral cortex and limbic system (Seddighi 2014).

Analgesic activities of systemically administered α_2-adrenergic agonists has been proven by experimental pain models using nociceptive testing and by clinical experimental studies (Grant et al. 1996; Grant et al. 2001; Grant & Upton 2001b; Grant & Upton 2001a; Hughan et al. 2001; Grant & Upton 2004). Analgesic effect, onset and duration of action depend on dose and route of administration; analgesia was proven to be generally short lasting, approximately 60-90 minutes (Nolan et al. 1987b; Grant & Upton 2001a; Grant & Upton 2004).

The neuraxial administration of α_2-adrenergic agonists has been extensively investigated not only in the experimental but also in the practical setting (Waterman et al. 1988; Aminkov & Hubenov 1995; Scott et al. 1995; Scott & Gessert 1997a; Scott & Gessert 1997b; Vesal &

Oloumi 1998; Christina Haerdi-Landerer et al. 2005; Rostami & Vesal 2012).

1.5.4 Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory, anti-nociceptive, anti-pyretic, anti-endotoxemic and anti-neoplastic properties.

Briefly, NSAIDs inhibit the production of inflammatory mediators synthetized from arachidonic acid, by the activation of lipoxygenase and cyclooxygenase, such as leukotrienes, thromboxanes and prostaglandins (Clark-Price 2014).

Their mechanism of action, pharmacokinetics, and analgesics effects after surgical procedures have been widely studied in sheep (Cheng et al. 1998; Landoni et al. 1999; Arifah et al. 2001; Price & Nolan 2001; Ali 2003; Lizarraga & Chambers 2006; Paull et al. 2007; Paull et al. 2008; Colditz et al. 2009; Stock et al. 2013). NSAIDs have also been proven to be effective analgesics in cases of chronic pain (Welsh & Nolan 1995b).

Several NSAIDs are licensed for use in cattle, provide cost-effective analgesia of medium duration; for these reasons they are one of the most popular analgesics administered to sheep.

1.5.5 Local anaesthetics

Local and regional anaesthetic techniques are widely used in sheep; market authorization of different local anaesthetics varies from country; in general the most used local anaesthetics include lidocaine, mepivacaine and bupivacaine. The onset and offset of action, adverse effects and analgesic effects depend on the drug used.

Local anaesthetics cause reversible blockage of action potentials in sensory, motor and sympathetic fibres by blocking sodium channels and thus preventing depolarization of nociceptors (Lemke 2014).

Local anaesthetics are usually employed in neuraxial anaesthesia or are used for local infiltrations or peripheral nerve blocks (Vesal & Oloumi 1998; Ratajczak-Enselme et al. 2007; Habibian et al. 2011; DeRossi et al. 2012b; Rostami & Vesal 2012; DeRossi et al. 2015).

1.5.6 Dissociative anaesthetics

Ketamine is the most commonly used dissociative anaesthetic, and it is considered as a nontraditional analgesic agent, which has shown analgesic properties at subanaesthetic doses. It exerts antagonist action at the N-methyl-D-aspartate (NMDA) receptors, which are located in the brain and spinal cord and contribute to the development of hyperalgesia and central sensitization (Love & Thompson 2014).

Systemic administration of ketamine is used in sheep mainly for induction and maintenance of anaesthesia (Lin et al. 1993; Lin et al. 1994; Hughan et al. 2001; Ozkan et al. 2010; Raske et al. 2010; Walsh et al. 2012). The use of ketamine in neuraxial techniques has been also investigated (Guedes et al. 2006; Lizarraga et al. 2008).

1.6 AIMS OF THE THESIS

The studies described in this thesis were designed to evaluate administration of analgesics in sheep both in the experimental and in the clinical setting.

The first study consisted in a meta-analysis of the reported use of analgesics in sheep used for experimental purposes in 2008, 2011 and 2014.

The second study was a questionnaire evaluating the current attitudes of Italian practitioners on pain assessment and treatment in sheep.

Pain assessment and treatment in sheep lags behind progress made in the field in other species and there is paucity of data regarding pharmacokinetics and pharmacodynamics of analgesic drugs and limited number of drugs licensed for use in this species. Because of the favourable properties of tramadol reported in other species, the third study evaluated the pharmacokinetics and antinociceptive effect of tramadol and its metabolite O-desmethyltramadol (M1) in sheep in a preclinical model of pain.

Chapter 2

REPORTED ANALGESIC ADMINISTRATION TO SHEEP UNDERGOING EXPERIMENTAL SURGICAL PROCEDURES

2.1 Introduction

Sheep are widely used as experimental models because their anatomy and physiology is similar to humans, they are easy to handle, clinical procedures are easy to perform after an appropriate training and there are less ethical concerns than the use of other animals (Turner 2007; Potes et al. 2008; Guillamon & Clau 2010; DiVincenti et al. 2014).

In the last decades there has been an increasing interest in the welfare of animals used for scientific purposes and scientists have to comply with guidelines on their accommodation, care and use (Forbes et al. 2007; Anonymous 2011).

Some of the requirements include the use of appropriate sedation, anaesthesia and analgesia together with the provision of adequate veterinary care (Anonymous 2011). Provision of anaesthesia and analgesia is important not only for ethical reasons but also because pain can cause neurohumoral changes which might potentially interfere with the aims of the study, thus confounding and affecting the research study‘s outcomes (Flecknell 2008). Indeed, it has been shown that uncontrolled perioperative pain may cause hypertension, myocardial ischemia and poor wound healing, just to list some complications, and may lead to central and peripheral neural sensitization (Vadivelu et al. 2010).

Currently in both human and veterinary medicine, an analgesic "multimodal" approach is recommended (Corletto 2007; Gritsenko et al. 2014). Conventional analgesic drug classes include opioids, non-steroidal anti-inflammatories (NSAIDs) and local anaesthetics, but also α_2-adrenoceptors agonists and dissociative anaesthetics have analgesic properties. The use of different classes of drugs allow targeting pain along various pathways involving transduction, transmission, modulation and perception by the central nervous system (Gritsenko et al. 2014).

A recent survey evaluating analgesic administration in 2000-2001 and 2005-2006 has shown that "large" laboratory animals including sheep are more likely to receive pain relief in comparison to laboratory rodents, but provision of analgesia is still suboptimal (Coulter et al, 2009).

The current study can be considered as a continuation of the work previously done by researchers from Newcastle University. A literature review was carried out in order to assess the reported use of analgesics in experimental sheep in the years 2008, 2011 and 2014.

2.2 Materials and methods

Search strategy

Studies involving experimental procedures in sheep were identified using Scopus search engine (www.scopus.com), which was accessed from December 2014 to January 2015. The key words "sheep" and "surgery" were used. Original research papers published in English in 2008, 2011 and 2014 and available as full text in electronic format at the University of Padua were selected. Whenever the inclusion criteria were met (described below), a random number generator was used to select 25 papers from each time period.

Inclusion criteria

Inclusion criteria were similar to the ones described by Coulter and colleagues (Coulter et al, 2009). Papers describing experimental

procedures carried out in sheep under general anaesthetic including a minimum of 24 hours recovery period were selected.

Exclusion criteria

Exclusion criteria were similar to the ones described by Coulter and colleagues (Coulter et al, 2009). Papers describing/evaluating a) multiple studies on different species of animals, b) foetal surgery, c) procedures carried out in neonatal lambs or pregnant sheep, d) neuropathic models of pain, e) analgesic efficacy of drugs, , f) changes of anaesthetic protocol during the course of the experiment, g) only one animal, h) material and methods not in detail, i) similar studies from the same research groups, j) review articles, letters to the editor, case reports and meta-analysis were excluded.

Classification

Experimental procedures were classified using similar criteria used by Coulter (Coulter et al., 2009) and Richardson (Richardson & Flecknell 2005), but were modified as some procedures that are commonly performed in other species are not carried out in sheep. Classification included: thoracotomies, orthopaedic surgeries, neurological surgeries, maxillofacial surgeries, soft tissue surgeries, vascular surgeries, and other procedures.

The procedure described in the paper was classified based on the most invasive procedure when either multiple procedures were described in one paper or when the study was carried out in two different time periods (e.g. experimental induction of infarction followed by its treatment days later).

Analgesic and anaesthetic drugs used in the papers were classified as previously done by Coulter (Coulter et al., 2009). Opioids and non-steroidal anti-inflammatory drugs were considered as systemic analgesic agents (SAA). Dissociative anaesthetic agents and α_2-adrenoceptors agonists were considered as anaesthetic agents with analgesic properties (AAAP). Preanaesthetic medications (i.e. acepromazine) and anaesthetic agents without analgesic properties (i.e. propofol, inhalant agents and

benzodiazepines) were classified as anaesthetics. The use of local anaesthetic (LA) was reported.

Whenever specified in the paper the dose, duration, frequency of analgesic administration were noted.

The number and gender of animals used in each study and the duration of the study was noted as well as the specification of institutional ethical committee authorization and country where approval was given.

Statistical analysis

Data analyses were performed using SAS statistical software (version 9.3, SAS Institute). The Chi-square test was used for all comparisons among years, except study size which was analysed between time periods using a non-parametric Kruskal-Wallis test. *P* values < 0.05 were deemed significant.

2.3 Results

A total of 75 papers were selected, 25 from each time period (2008-2011-2014).

The papers were published in the following 47 journals:

American Journal of Roentgenology, Annals of Biomedical Engineering, ANZ Journal of Surgery, Archives of Cardiovascular Diseases, Artificial Organs, ASAIO Journal, Biomaterials, BioMed Research International, BMC Musculoskeletal Disorders, BMC Veterinary Research, Bone, Clinical Oral Implants Research, Cytokine, European Journal of Cardio-thoracic Surgery, European Spine Journal, Gene Therapy, Injury, International Journal of Cardiology, International Journal of Developmental Neuroscience, International Urogynecology Journal and Pelvic Floor Dysfunction, Journal of Biomechanics, Journal of Biomedical Materials Research - Part A, Journal of Bone and Joint Surgery - American Volume, Journal of Bone and Joint Surgery - Series A, Journal of Bone and Joint Surgery - Series B, Journal of Cardiac Failure, Journal of Clinical Neuroscience, Journal of Cranio-Maxillofacial Surgery, Journal of Heart

and Lung Transplantation, Journal of Interventional Cardiology, Journal of Materials Science: Materials in Medicine, Journal of Minimally Invasive Gynecology, Journal of Orthopaedic Research, Journal of Rehabilitation Research and Development, Journal of Surgical Research, Journal of the American College of Cardiology, Journal of Thoracic and Cardiovascular Surgery, Journal of Vascular Surgery, Knee Surgery, Sports Traumatology, Arthroscopy, Medical science monitor basic research, Oral and Maxillofacial Surgery, PLoS ONE, Small Ruminant Research, Spine, Surgical Endoscopy and Other Interventional Techniques, Translational Stroke Research, World Journal of Surgery.

Study size

The median, minimum and maximum number of sheep enrolled in each year can be visualised in Table 2.1. There was a statistically significant decrease in the median number of animals involved in surgical experimental procedures in 2014 compared to 2011 and 2008 (P = 0.01).

Table 2.1. Median number of animals enrolled in each study (minimum, maximum "study size") each year and in all years together.

	2008	**2011**	**2014**	**ALL YEARS**
NUMBER OF SHEEP				
Median	12	15	8	12
Minimum	3	5	2	2
Maximum	58	58	40	58

Classification of experimental procedures

The most commonly performed procedures in sheep were thoracotomies, orthopaedic procedures and neurosurgeries. Table 2.2 and Figure 2.1 and 2.2 indicate the number of procedures in the time period selected. Thoracotomies were performed mainly in studies evaluating myocardial infarction, valvular bioprosthesis, ventricular assistance devices and

cardio-pulmonary bypass. Orthopaedic and neurosurgery studies' aims were the assessment of new surgical techniques, biomaterials and grafts.

Table 2.2. Number of papers included in the review classified by type of procedure performed and by year. All years includes years 2008, 2011 and 2014.

PROCEDURES /YEAR	2008	2011	2014	ALL YEARS
Maxillofacial surgery	3	0	3	6
Neurosurgery	4	5	1	10
Orthopaedic surgery	9	8	7	24
Other procedures	1	0	1	2
Soft tissue surgery	1	2	3	6
Thoracotomy	7	10	5	22
Vascular surgery	0	0	5	5

Figure 2.1. Number of papers included in the review classified by type of procedure performed and by year.

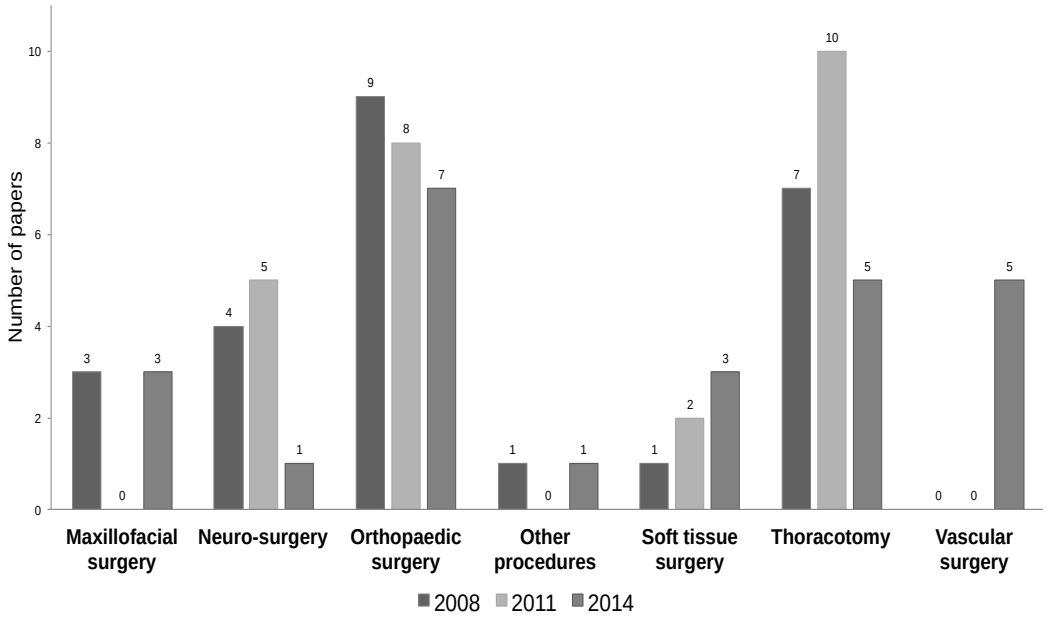
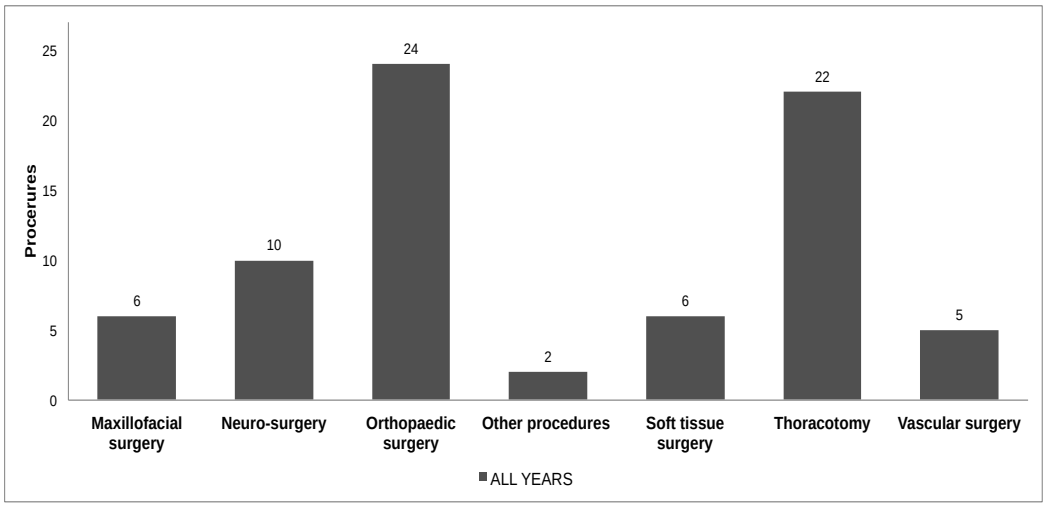


Figure 2.2. Number of papers included in the review classified by type of procedure performed in all years (2008+2011+2014).



Specification of Institutional Ethical Approval and Countries where the study was conducted

Specification in the papers of Institutional Ethical Committee approval or compliance with Guidelines for the Use and Care of Laboratory Animals approval was noted.

As shown in Table 2.3 there was an increase in the report of ethical approval in 2014 but this was not statistically significant ($P = 0.15$).

Table 2.3. Specification in the paper of ethical approval or compliance with guidelines for use and care of laboratory animals. “N” refers to number of papers and % to percentage of papers.

	2008		**2011**		**2014**		**ALL YEARS**	
	N papers	%	N papers	%	N papers	%	N papers	%
Ethical approval	21	84	19	76	24	96	64	85
Guidelines	1	4	2	8	1	4	4	5
No ethical approval	3	12	4	16	0	0	7	9

The country where the study was conducted according to the institution where the approval was granted was noted. The studies were more commonly performed in the United States followed by Germany and United Kingdom, as shown in Table 2.4.

Table 2.4. Country where the research study was conducted and total number of paper published in all years (2008-2011-2014).

COUNTRY	**Number of papers**	**Country**	**Number of papers**
United States of America	19	Austria	2
Germany	10	Belgium	2
United Kingdom	7	Canada	2
Australia	5	Iran	1
Brazil	4	Ireland	1
Italy	4	Korea	1
Switzerland	4	New Zealand	1
China	3	Spain	1
France	3	The Netherlands	1
Israel	3	Turkey	1

Animal's gender

Sheep gender was noted when specified in the papers. Female animals were most commonly chosen as described in Table 2.5.

Table 2.5. Number of papers specifying animal gender classified by year.

	2008	2011	2014
NUMBER OF PAPERS SPECIFYING GENDER	16/25	16/25	14/25
Number of papers where female sheep were enrolled in the study	14/16	9/16	13/14
Number of papers where male sheep were enrolled in the study	2/16	6/16	1/14
Number of papers where female and male sheep were enrolled in the study	0/16	1/16	0/14

Duration of experiments

Duration of experiments was noted when specified in the papers. When there were different end points, for example a group of animals was euthanized at 2 weeks and another at 4 weeks, the longest time point was considered as the end of the experiment. Table 2.6 shows the duration of experiment by procedure in all years considered (2008-2011-2014).

Table 2.6. Experiment duration indicated in weeks classified by kind of procedures in all years considered (2008-2011-2014)

	EXPERIMENT DURATION (weeks)	
	Median	Range
PROCEDURES		
Maxillofacial surgery	14	4-26
Neuro-surgery	12	7-40
Orthopaedic surgery	16	2-96
Other procedures	24.5	<1-48
Soft tissue surgery	9	<1-56
Thoracotomy	9	<1-72
Vascular surgery	16	2-36

Use of analgesics

The perioperative use of analgesic drugs was noted when specified. This study shows that when all years are considered together (2008-2011-2014) opioids, NSAIDs, local anaesthetics, dissociative agents and α2-receptor agonists were administered in 37.3%, 38.6%, 14.6%, 42.6% and 18.6% of the papers respectively.

Table 2.7 shows the number of papers specifying the use of α_2-adrenoceptor agonists, dissociative agents, NSAIDs, local anaesthetics and opioids. There were no statistically significant differences in the use of each analgesic drug between the years, as indicated by the *P* value >0.05. When considering all 75 papers, the most commonly reported analgesics drugs were xylazine (18.6%), ketamine (40%) carprofen (13.3%), flunixin (12%), buprenorphine (14.6%), bupivacaine (8%) and lidocaine (5.3%) with respect to α_2-adrenoceptors agonists, dissociatives, NSAIDs, opioids, and local anaesthetic drugs (data not shown).

Table 2.7. Percentage of papers specifying the use of analgesic drugs and the relative percentage is reported classified by year. P value refers to the statistical difference between years.

DRUGS / YEAR	**2008**	**2011**	**2014**	**ALL YEARS**	***P value***
α_2- adrenergic agonists	20%	24%	12%	18.6%	*0.54*
Dissociative anaesthetics	36%	52%	40%	42.6%	*0.49*
Local Anaesthetics	20%	8%	16%	14.6%	*0.74*
Opioids	28%	40%	44%	37.3%	*0.47*
NSAIDs	32%	40%	44%	38.6%	*0.60*

Range of doses reported

When specified in individual papers the range of doses reported for drugs with analgesic properties is shown in table 2.8.

Table 2.8. Range of reported doses of analgesic drugs.

DRUG	DOSE RANGE (mg/kg)	DRUG	DOSE RANGE (mg/kg)
Xylazine	0.02-1.5	Aspirin	3
Tiletamine-Zolazepam	4-4.4	Carprofen	1-4
Ketamine	10-25	Dipyrone	20-25
Buprenorphine	0.005-10	Flunixin	1-2.2
Butorphanol	0.06-0.4	Ketoprofen	50
Fentanyl	0.002-0.0025	Ketorolac	0.5
Meperidine	2	Meloxicam	0.4
Morphine	0.2	Tolfenamic acid	2
Tramadol	2		

Duration of post-operative analgesia

The duration of post-operative analgesia was noted when specified. When more than one class of analgesic was used in the post-operative period the longest duration of analgesia was noted. Table 2.9 shows the number of papers specifying the duration of post-operative analgesia classified by procedure in all years (2008-2011-2014). Table 2.10 shows the percentage of papers classified by year specifying the use of post-operative analgesia. There were no differences in the duration of post-operative analgesia between years (P= 0.49)

Table 2.9. Number of papers specifying the duration of post-operative analgesia classified by procedure in all years considered (2008-2011-2014)

PROCEDURES	1 day	2 days	3 days	4-5 days	1 week	>1 week	Not specified
Maxillofacial surgery		1	2				6
Neuro-surgery			2	1	1		6
Orthopaedic surgery	1	1	4	5		2	11
Other procedures		1					1
Soft tissue surgery				1			5
Thoracotomy		2	1	1		1	17
Vascular surgery							5

Table 2.10. Number and percentage of papers classified by year specifying the use of post-operative analgesia.

POST-OPERATIVE ANALGESIA	2008	2011	2014	ALL YEARS
Number of papers indicating post-operative analgesia	11	9	7	27
Percentage of papers indicating post-operative analgesia	44%	36%	28%	36%
Minimum days of post-operative analgesia	1	2	2	1
Maximum days of post-operative analgesia	5	30	10	30
Median days of post-operative analgesia	3	5	3	3

Multimodal analgesia

The reported use of analgesic drugs was noted. Analgesic drugs were classified as systemic analgesics agents (opioids and NDSAIDs), anaesthetic agents with analgesic properties (dissociatives and α_2-adrenergic agonists) and local anaesthetics. The use of these 3 classes of analgesics was investigated. The use of analgesics drugs was not statistically significant between year (P>0.05). Systemic analgesic agents were used in 53% of all 75 papers, a combination of systemic analgesic agents and anaesthetic agents with analgesic properties was reported in 33.3% of the papers and only 8% of the paper reported the use of 3 different classes of analgesics (Table 2.11).

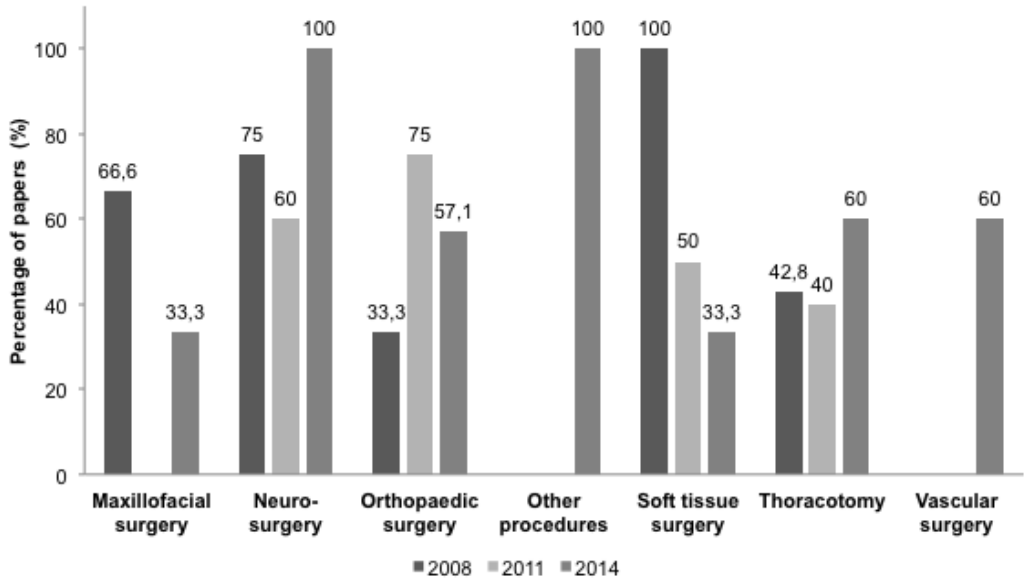
Table 2.11. Percentage of papers by years reporting the class of analgesic agents used.

	2008	**2011**	**2014**	**ALL YEARS**	**P value**
SAA	48%	56%	56%	53.3%	0.8
AAAP	44%	52%	44%	46.6%	0.8
SAA + AAAP	28%	36%	36%	33.3%	0.78
SAA + AAAP + LA	12%	4%	8%	8%	0.58

Legend: SAA: systemic analgesic agents (opioids and NSAIDs), AAAP: anaesthetic agents with analgesic properties (α_2- adrenoceptors agonists and dissociatives); LA: local anaesthetics.

The analgesic multimodal approach was investigated by type of procedure (Table 2.12). When all years were considered together, systemic analgesic agents (opioids and NSAIDs) were given more often during neurosurgeries (78.3%), orthopaedic surgeries (59.3%) and thoracotomies (47.6%) than during other types of surgery.

Table 2.12. Reported use of systemic analgesic agents by year and by procedures.



2.4 Discussion

In this meta-analysis the reported use of analgesic agents in experimental sheep in 2008, 2011 and 2014 was assessed. Prior to clinical use in people, new biomaterials, drugs or surgical procedures often have to be tested in an animal model in order to test the biological responses and cellular interactions (Potes et al. 2008). Sheep are widely used as experimental models in many biomedical research fields, including orthopaedics, neurology, cardiology and internal medicine, due to physiological and anatomical similarities with the human body (Pearce et al. 2007; Potes et al. 2008; Guillamon & Clau 2010; Mageed et al. 2013; DiVincenti et al. 2014; Katz et al. 2015).

Nowadays it is widely accepted that experimental animals should be provided with adequate analgesia not only for welfare and ethical considerations, but also because pain alters homeostasis and so could affect the end points of the research and thus become a source of experimental error (Anonymous 2011). Indeed pain causes changes in cardiovascular indices, ventilatory, renal and immunological functions,

coagulation parameters, behaviour and many other changes as described by Otto (Otto K.A. 1998).

Guidelines regarding husbandry and anaesthetic/analgesic management have been designed for scientists (Forbes et al. 2007; Anonymous 2011) and the drugs used, the surgical procedure and the methods of euthanasia should be reported in the scientific paper (Kilkenny et al. 2010).

One of the requirements for publication in some journals is declaration of approval by an ethical committee. In this study, specification of institutional ethical approval was noted and the number of papers reporting it increased in 2014 with 96% of papers indicating ethical approval, nevertheless the increase was not statistically significant. When all years were considered together the institutional ethical approval was specified in 85% of the papers.

This study has shown a statistically significant decrease in the number of sheep used in experimental research in 2014 in comparison to the previous years. Indeed the median number of animals decreased from 12 and 15 in 2008 and 2011 to 8 in 2014. Data relative to years 2008 and 2011 can be considered comparable to data obtained by Coulter and colleagues (Coulter et al. 2009), where the median number of sheep used in 2000-2001 and 2005-2006 were 15 and 12 respectively. Although these are not precise numbers relative to the number of sheep used for experimental purposes in the European Community (EU), a recent report showed a reduction of over half a million of all animals used in the EU from just above 12,0 million in 2008 to just under 11,5 million in 2011. Moreover ungulates represented the 1.4% and 1.2% of the total number of animals used in the Member States in 2008 and 2011 respectively (Anonymous 2013). So the trend found in the current study is in accordance with this EU report.

A decreased number of animals used in research is one of the goals required by the Three R's theory by Russell and Burch (Russell W.M.S. 1959), which is considered to be a guiding principle for a more ethical use of experimental animals. The 3Rs theory asks for the replacement of the use of animals with alternative methods, the reduction of the number of

animals use in experimental studies and refinement of methods in order to alleviate pain and distress as much as possible. In particular, a reduction in the number of animals used in experimental research should be achieved by finding alternatives to animal use or by using other methods in order to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals. Reduction of the number of animals focuses indeed on optimal experimental design with robust and appropriate statistical analysis including estimating the size of the experiment using power and sample size calculations (Festing & Altman 2002).

This study showed that orthopaedic and neurological surgical procedures were the most commonly performed; original research papers were indeed classified by type of procedure and, when considering all years together (2008-2011-2016), 32% and 29% of papers describe orthopaedic and neurological surgeries respectively. These results are consistent with the ones reported in the previous study performed at Nottingham University (Coulter et al. 2009) where considering the studies published in two different time periods, 2000-2001 and 2005-2006, 26% and 43% of papers describe orthopaedic and neurological surgeries respectively. When considering the duration of the experiment, the longer lasting experiments involved vascular surgery, followed by orthopaedic and maxillofacial surgery. The expected level of pain after neurosurgery procedures, orthopaedic surgeries and thoracotomies can be considered very high and proper analgesia should be provided in the post-operative period. In this study, provision of post-operative analgesia was reported in 27 out of 75 papers, and the duration was of 3 to 5 days in orthopaedic and neurological procedures and 2-4 days after thoracotomies. If we translate these procedures to small animals practice, in the author's clinical experience analgesia is usually provided for at least one week: in the first 48 hours postoperatively the use of full μ opioid agonists is suggested followed by partial μ agonist or other drugs such as tramadol or NSAIDs. In veterinary as well as in human medicine, a multimodal approach to analgesia is recommended: it implies the concurrent use of different

classes of analgesics, such as opioids, NSAIDs, local anaesthetics and α_2-adrenoceptors agonists and its aim is to decrease the doses and as a consequence the side effects of each single drug, while improving pain relief (Gritsenko et al. 2014; Mathews et al. 2014).

When considered as all years together and all types of procedures, the reported use of multimodal analgesia was generally low, with only 8% of the papers reporting the use of systemic analgesics, anaesthetic agents with analgesic properties and local anaesthetics was reported. The fact that the analgesic regimen was not reported in the studies does not imply per se that pain relief was not administered to sheep; the goal of the vast majority of the papers was to describe new surgical techniques or therapies and so the anaesthetic regimen might have been overlooked.

The analgesic plan should be not only multimodal but also “preventive”, which means that the analgesic drug should be administered before noxious stimulation thus preventing changes in the central nervous system, leading to central sensitization which manifests as allodynia and hyperalgesia (Katz et al. 2011). In the current study xylazine was administered preoperatively in 18.6% of papers. In sheep, xylazine induces dose dependent sedation and central nervous depression (Kastner 2006). Moreover α2-adrenoceptors agonists have analgesic properties (Yaksh 1979) and interact synergistically with opioid agonists (Drasner & Fields 1988). Xylazine is usually administered in conjunction with ketamine for induction of anaesthesia (Carroll & Hartsfield 1996); in this study ketamine was used in 40% of the papers.

A drug can only manifest its analgesic effects when administered at the proper dose. Doses in sheep cannot be extrapolated from other species, as pharmacokinetic parameters, such as absorption, distribution, metabolism and excretion are species-specific, but there is paucity of data regarding pharmacokinetics and pharmacodynamics of analgesics in this species. Xylazine was used at doses between 0.02-1.5 mg/kg; the recommend dose of xylazine in sheep is 0.05-0.2 mg/kg (Valverde 2013), as higher doses could provoke marked side effects such as cardiovascular depression, alteration of ventilatory mechanics, hypoxia and pulmonary

oedema (Kastner 2006). Ketamine was used at doses ranging from 10 to 25 mg/kg: suggested doses are 3-5 and 10-15 mg/kg for intravenous and intramuscular administration respectively (Riebold 2007). NSAIDs usually used in small ruminants are ketoprofen, flunixin, meloxicam, carprofen and tolfenamic acid (Hodgkinson 2007; Riebold 2007). Doses used in the selected papers were pretty similar to the ones suggested in textbooks, with some exceptions, such as ketoprofen. Doses reported for opioids in this study are similar to those reported in textbooks (Valverde 2013). Posology of analgesic administration was reported only in a few cases. Moreover in many cases the doses were not reported in the appropriate way (mg/kg) but as ml/animal or ml/kg without specification of the concentration of the drug administered. Moreover often the brand name was indicated and not the active principle; spelling mistakes have also been noted. Finally, none of papers described how pain was assessed post-operatively, and this is in agreement with previous reports (Coulter et al. 2009).

The provision of adequate analgesia in experimental animals is one of the concepts included in the 3Rs' theory; in particular refinement of methods consist also in avoiding or minimizing discomfort and pain (Russell W.M.S. 1959). Pain treatment can be provided only if pain is recognised and assessed. If pain is not recognised it is unlikely to be treated. Recognition of pain may be difficult in sheep as they are prey animals and behavioural signs of pain may be subtle (Fitzpatrick 2006). Moreover a pain scale for sheep has not yet been designed; pain scales for rodents have been developed (Langford et al. 2010; Sotocinal et al. 2011) and have been used by scientists to assess the efficacy of post-operative analgesics (Matsumiya et al. 2012).

2.5 Conclusions

This study showed that the number of sheep involved in experimental research has decreased in 2014 compared with 2011 and 2008 and that anaesthetic and analgesic treatment is not properly reported in research

papers. The veterinary surgeon, or other trained professionals, should play an important role in the experimental research setting in recognising, assessing and treating pain adequately in animals used for scientific purposes.

Chapter 3

ATTITUDES OF ITALIAN VETERINARIANS TO PAIN AND THE USE OF ANALGESICS IN SHEEP

3.1 Introduction

"Freedom from pain, injury and disease", one of the principles included in the "Five freedoms" theory (FAWC 1993) is one of the conditions required to ensure an animal's welfare. Provision of analgesia in animals is fundamental not only for ethical reasons but also because untreated pain may lead to significant economic losses (Paul-Murphy et al. 2004). Pain recognition is mandatory to provide effective pain management, but recognising pain in sheep may be difficult as they are prey species and they tend to mask pain to their potential predators (Stafford 2014). The inability to appreciate signs of pain in cattle has led some practitioners to think that "*farm animals are not as sensitive to pain as small animals*" (Raekallio et al. 2003). In recent years research into pain assessment and recognition has increased and nowadays pain scales for detection of pain in dogs (Holton et al. 2001), cats (Brondani et al. 2013), horses (Dalla Costa et al. 2014) and cattle (de Oliveira et al. 2014) have been designed and are available to guide practitioners in pain treatment. Unfortunately a pain scale for assessment of pain in sheep has not yet been validated. In general, pain management in farm animals has not progressed as much as for small animals. Analgesic treatment in food producing animals is

particularly challenging due to the legal implications concerning prescription, record keeping, withdrawal times and lack of registered drugs. Nevertheless with the market authorization of α_2-adrenoceptor agonist drugs and NSAIDs for use in cattle, more efficacious and longer lasting analgesia can be provided to this species (Stafford 2014). The risks of adverse effects, such as sedation by α_2-adrenoceptor agonist or decreased ruminal motility by opioids may be another factor limiting the use of analgesics in ruminants. For the same reasons, analgesics were not administered to cats as often as in dogs (Capner et al. 1999), but thanks to research, market authorization of licensed drugs and teaching, feline pain management has improved massively in the last few years (Bortolami & Love 2015). Questionnaires regarding the practitioners' attitudes towards pain and analgesics are important to assess changes in pain management over time, to address the research towards specific topics and to potentiate continuing education programmes.

The aim of this study was to assess the attitudes of practicing sheep veterinarians working in Italy towards this topic.

3.2 Materials and methods

An on line questionnaire was designed based on other surveys previously carried out worldwide to investigate the use of analgesia in cattle (Huxley & Whay 2006; Laven et al. 2009; Thomsen et al. 2010; Lorena et al. 2013). It was distributed to Italian veterinarians belonging to the Italian Society of Pathology and Breeding of small ovine and caprine species (SIPAOC) and SEMENTUSA project from April to June 2015. The questionnaire was sent on the behalf of the Department of Animal Medicine, Production and Health of the University of Padua and an e-mail accompanied the questionnaire explaining its aims, stating its anonymity and the estimated time (10 minutes) required to complete it.

The questionnaire was divided into 5 sections:

- Section I concerned veterinarian's demographic data and working activity, including gender, year of graduation, post-graduate degree obtained, working area, species treated, proportion of working time spent dealing with sheep. Information regarding flock characteristics, such as number of sheep and purpose (milk, meat, wool, mix, pet animals), were also asked.
- Section II solicited information on provision of analgesia: drugs administered and anaesthetic techniques performed in the perioperative period (considered as 24 hours from the beginning of the procedure), or for specific medical conditions, including lameness, mastitis, and abscesses. Factors affecting drug choice, anaesthetic techniques used, use of non-pharmacological analgesic techniques were also investigated.
- Section III investigated the drugs commonly used for specific procedures, such as castration, dehorning and caesarean section.
- Section IV asked the veterinarian to rate the level of pain associated with specific surgical procedures or medical conditions, including castration, dehorning, caesarean section, fracture, lameness, abscess: a 10-point numerical rating scale was used, on which 1 represented no pain, and 10 the worst possible pain. Veterinarians were asked to state which physiological and behavioural changes could be considered as indicators of pain in sheep and the reasons why provision of analgesia is not common in sheep.
- Section V investigated the veterinarian's opinion on their knowledge on the topic of sheep analgesia and which form of continuing education they would prefer to improve it. A copy of the questionnaire is included in the Annex A.

Statistical analysis

Data analyses were performed using SAS statistical software (version 9.3, SAS Institute). Chi-square test was used to compare the percentages between the different levels of the variables of interest, whereas non parametric Kruskal-Wallis test was used to compare pain scores. *P* values < 0.05 were deemed significant.

In order to be used in the data analysis, a questionnaire had to include all demographic data and 80% of the questions had to be answered.

3.3 Results

Section I. Demographic data

A total of 31 questionnaires were returned, two of which were discarded as the veterinarians did not work with sheep, but only goats and cattle. The percentage of respondents cannot be calculated as the precise number of veterinarians who received the questionnaire was unknown, but it can be roughly estimated as 100-150 veterinarians.

Of the respondents, 69% were male and 31% were female (Figure 3.1); 44.8% and 55.2% of veterinarians graduated less and more than 10 years ago respectively (Figure 3.2). The vast majority of the veterinarians achieved a post graduate degree, with 3 people having more than one (Figure 3.3). Geographical distribution of veterinarians responding to the questionnaire is shown in figure 3.4.

Figure 3.1. Gender of Italian veterinarians responding to the questionnaire.

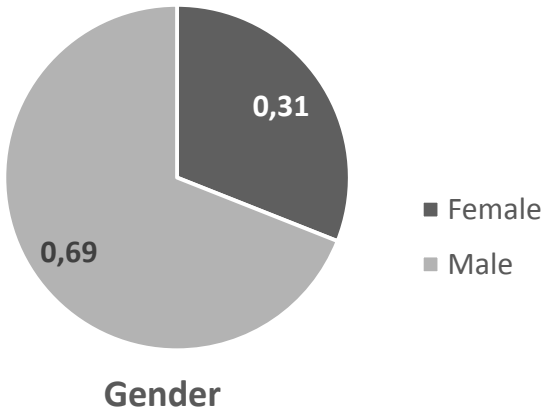


Figure 3.2. Year of graduation of Italian veterinarians responding to the questionnaire.

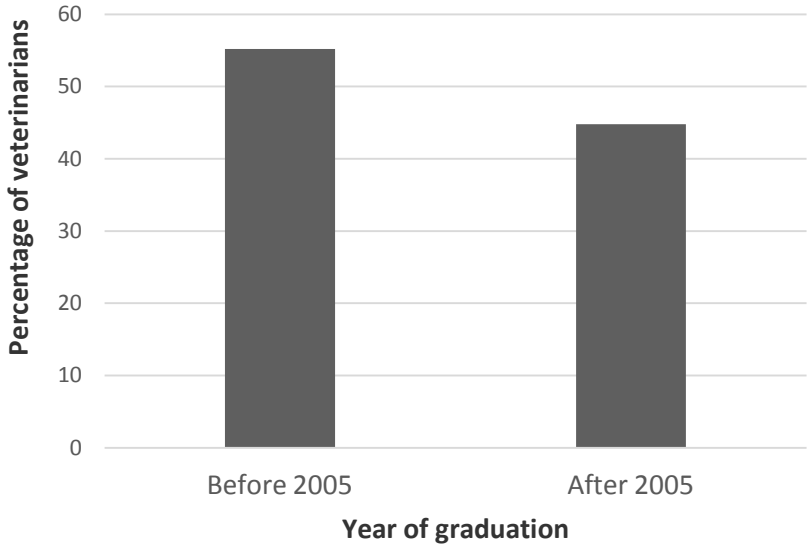


Figure 3.3. Post-graduation studies done by Italian veterinarians responding to the questionnaire.

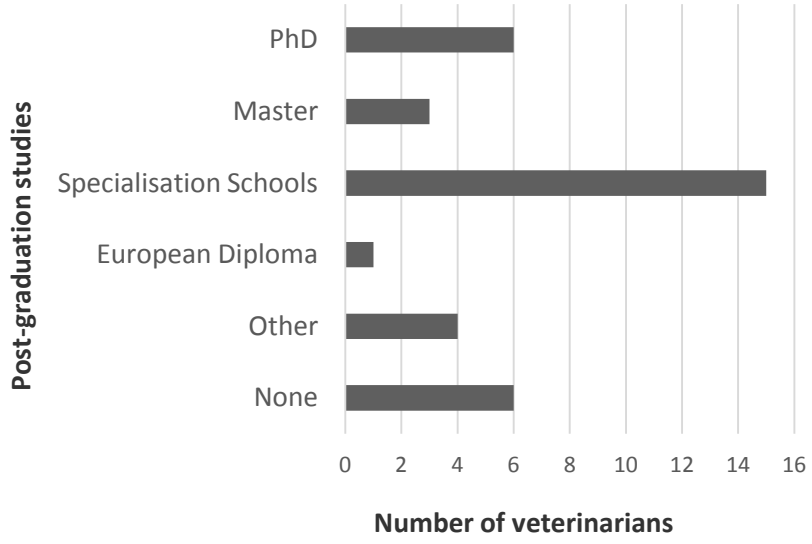
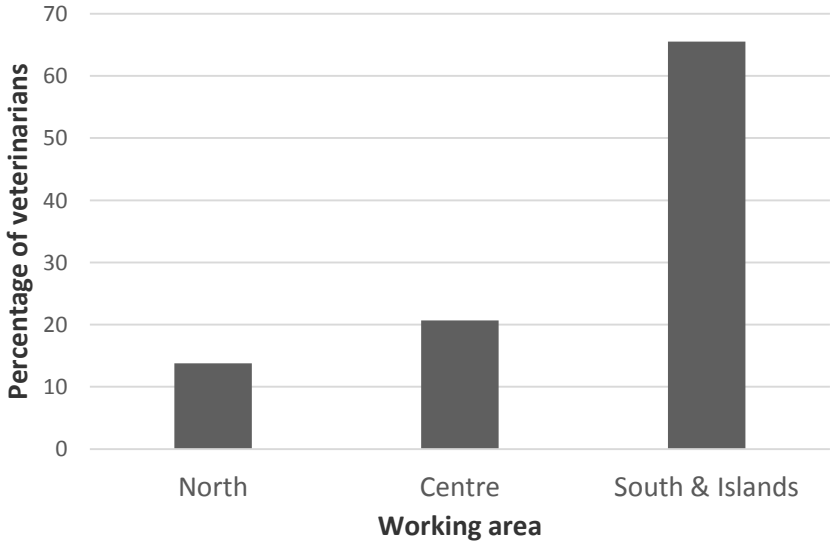
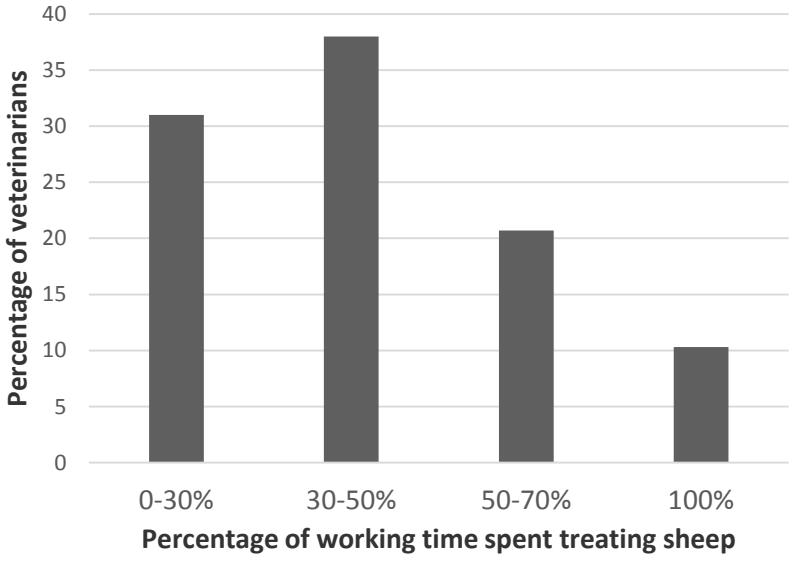


Figure 3.4. Geographical distribution of working areas in Italy of veterinarians responding to the questionnaire.



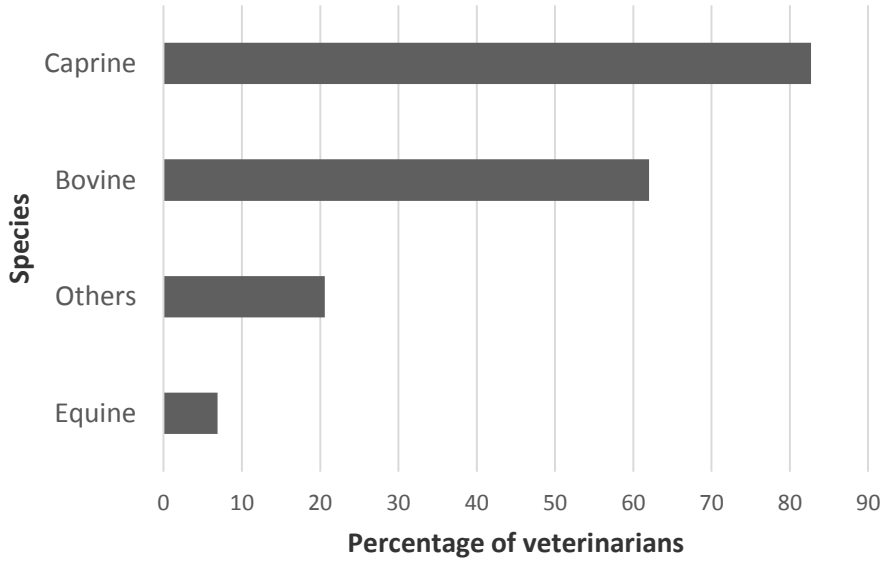
Only 10.35% of veterinarians worked only with sheep, while the greatest percentage, 38%, spent from 30 to 50% of their time treating sheep (Figure 3.5).

Figure 3.5. Percentage of working time spent treating sheep by Italian veterinarians responding to the questionnaire.



Indeed, most of the respondents treated not only sheep but also other species, goats and cows in particular (Figure 3.6).

Figure 3.6. Other species treated by Italian veterinarians responding to the questionnaire.



Flock size and purpose are summarised in Table 3.1.

Table 3.1. Size and purpose of flocks treated by Italian veterinarians responding to the questionnaire.

	FLOCK SIZE	>100	50-100	10-50		
Percentage of veterinarians		82.7%	6.9%	10.4%		
	FLOCK PURPOSE	Milk	Meat	Mix	Pet	Wool
Percentage of veterinarians		79.3%	24.1%	17.2%	10.3%	3.4%

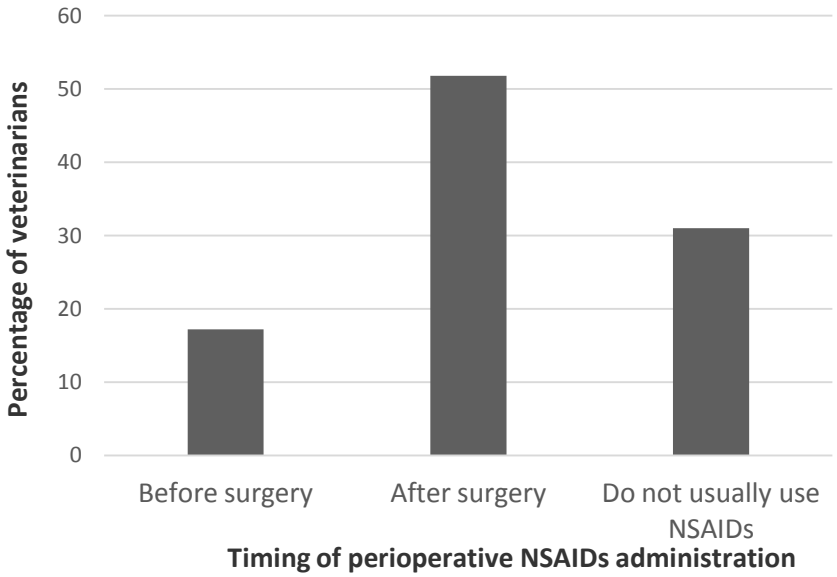
The level of association between the percentage of working time spent treating sheep and the flock size was found to be statistically significant (P=0.04): veterinarians who spent more time treating sheep dealt with bigger flocks (Data not shown). The level of association between the flock size and geographical area was investigated: it was found that bigger flocks are distributed in Southern Italy and Islands (P=0.02).

Section II. Drugs used

None of the veterinarians used opioids in sheep, not even in any particular circumstance.

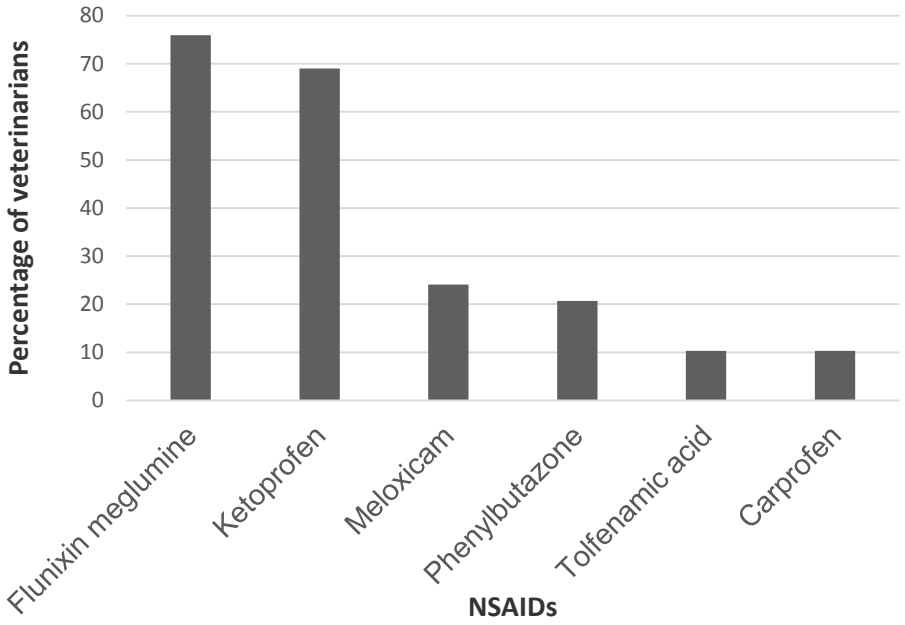
More than one half of veterinarians (51.8%) administer NSAIDs after the surgical procedure, while 31% do not usually administer them in the perioperative period (Figure 3.7). In case of lameness, mastitis, abscesses, 83%, 76% and 10% of veterinarians administer NSAIDs respectively (data not shown).

Figure 3.7. Timing of perioperative NSAIDs administration.



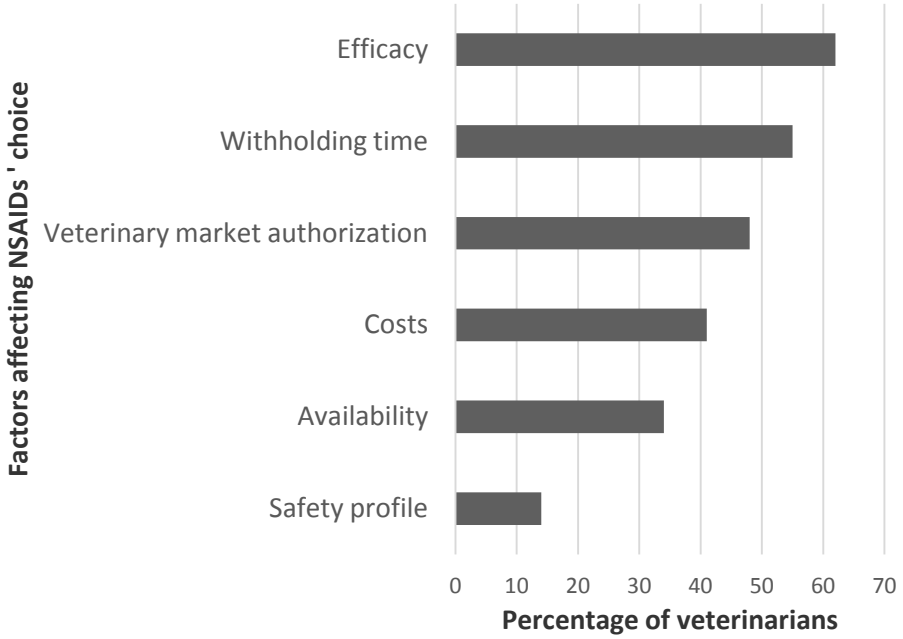
The two most commonly use NSAIDs were flunixin meglumine and ketoprofen (Figure 3.8).

Figure 3.8. Most commonly used NSAIDs by Italian veterinarians responding to the questionnaire.



The most important factors affecting the choice of NSAIDs were efficacy, withholding time, veterinary market authorization, followed by costs, availability and safety profile (P=0.003) (Figure 3.9).

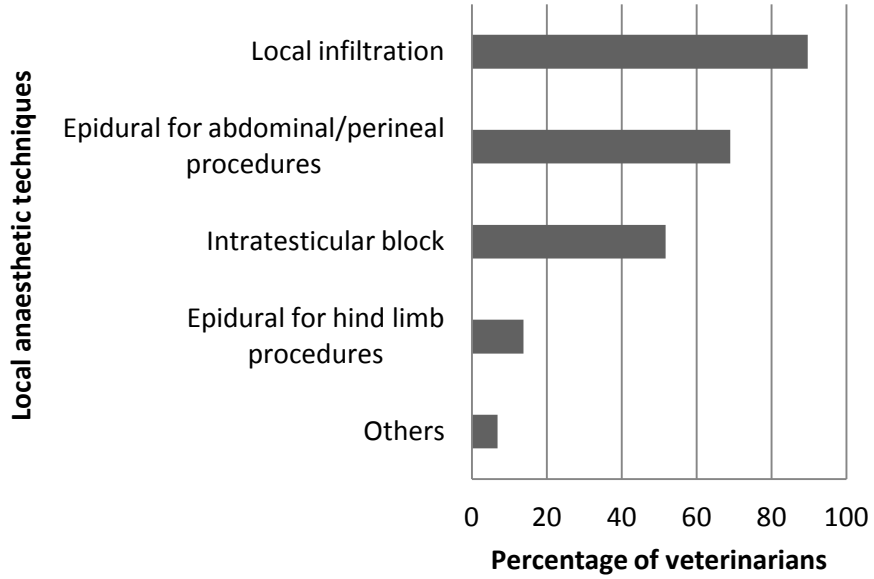
Figure 3.9. Factors influencing NSAIDs choice by Italian veterinarians responding to the questionnaire.



Almost all veterinarians usually administer a local anaesthetic in the perioperative period (96.6%) and in particular after the surgery (96.6%); lidocaine was commonly used by all veterinarians (data not shown).

Data regarding the use of local anaesthetic techniques can be visualised in figure 3.10: local infiltration was performed by most of veterinarians (89.7%), followed by epidural for abdominal/perineal procedures (69%), and intratesticular block (51.7%) ($P<0.0001$). When an epidural was performed, 86% of the veterinarians used a local anaesthetic only.

Figure 3.10. Local anaesthetic techniques performed in sheep by Italian veterinarians responding to the questionnaire.



The association between the use of NSAIDs and local anaesthetic was investigated and although not statistically significant (P= 0.67), it was found that 68% veterinarians who administer NSAIDS administer also local anaesthetics, and 88.9% veterinarians who do not use NSAIDs administer a local anaesthetic peri-operatively.

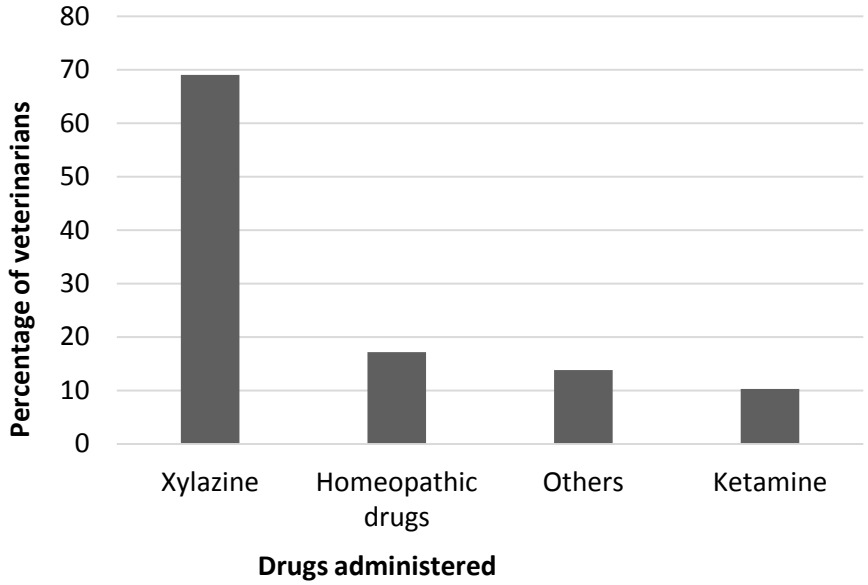
The association between the use of advanced local anaesthetic techniques, such as epidural anaesthesia, and the time of graduation and post-graduate education was investigated.

When considering the year of graduation, before or after 2005, 56.2% and 84.6% of veterinarians performed neuraxial anaesthesia respectively, but this was not found to be statistically significant (P=0.21).

Of the veterinarians with a post graduate degree 80% performed epidurals, while 44.4% of veterinarians without a post graduate degree did not: this was not considered statistically significant (P=0.14).

The most commonly used drugs in practice can be visualised in figure 3.11. None of the veterinarians ever used tramadol in sheep.

Figure 3.11. Other drugs commonly used in the perioperative period by Italian veterinarians responding to the questionnaire.



Section III. Drugs used for specific conditions

- Castration and dehorning

Local anaesthetics were commonly used for castration in sheep with 41.6% of the veterinarians using them; a multimodal analgesic approach consisting of α_2-adrenoceptor agonists, NSAIDs and local anaesthetics was used by 20.7% (Figure 3.12). The same multimodal approach was used only by 13.9% of veterinarians performing dehorning (Figure 3.13).

Figure 3.12. Most commonly used drugs for castration by Italian veterinarians responding to the questionnaire.

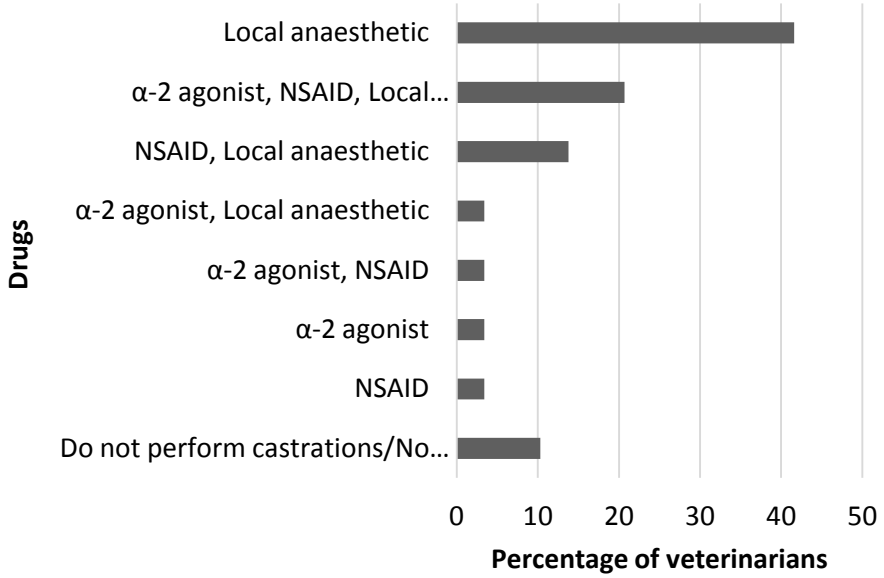
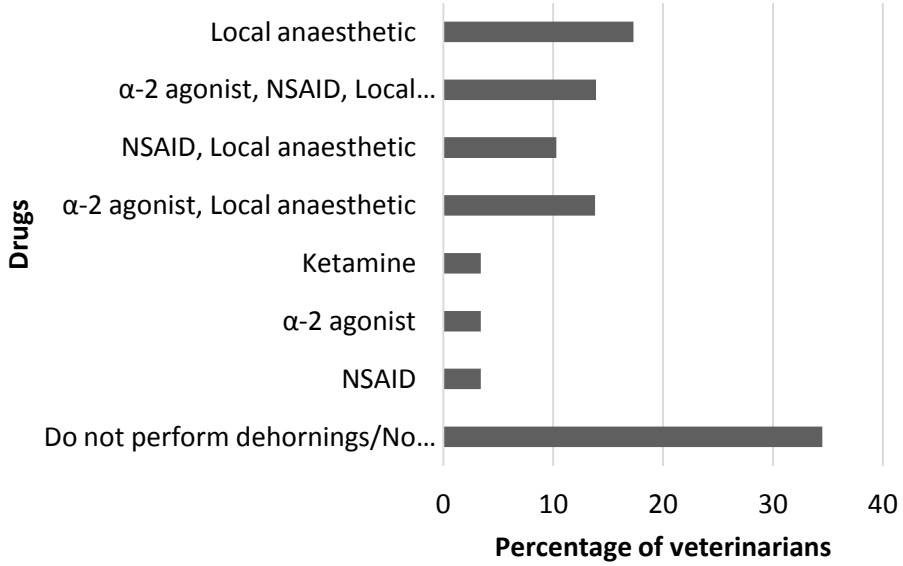


Figure 3.13. Most commonly used drugs for dehorning by Italian veterinarians responding to the questionnaire.



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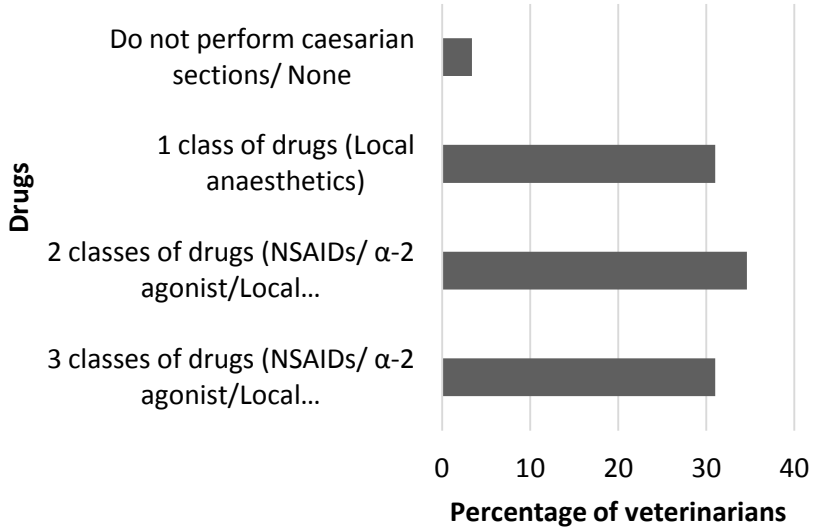
- Caesarean section

There was a great variability among practitioners in drugs and techniques used for caesarean section; the number of classes of analgesic drugs used, classified as NSAIDs, dissociative anaesthetic agents and local anaesthetics, is shown in figure 3.14. Epidural anaesthesia alone or in combination with other drugs was performed by 55% of veterinarians. Multimodal analgesia with NSAIDs, α_2-agonists and local anaesthetics was provided by 24% of veterinarians, while 34.5% of them used 2 classes of analgesic drugs (from α_2-agonists, NSAIDs, local anaesthetics, ketamine).

Figure 3.14. Most commonly used drugs for caesarean section by Italian veterinarians responding to the questionnaire.



Section IV. Estimated pain score

Italian veterinarians responding to the questions regarding the pain experienced by sheep undergoing different surgical procedures or suffering from different conditions considered fracture to be the most

painful condition, followed by mastitis and lameness (P<0.0001) (Table 3.2).

Table 3.2. Estimated pain scores of Italian veterinarians responding to the questionnaire. Each cell shows the percentage of respondents who recorded that pain score; shaded cells show the mode.

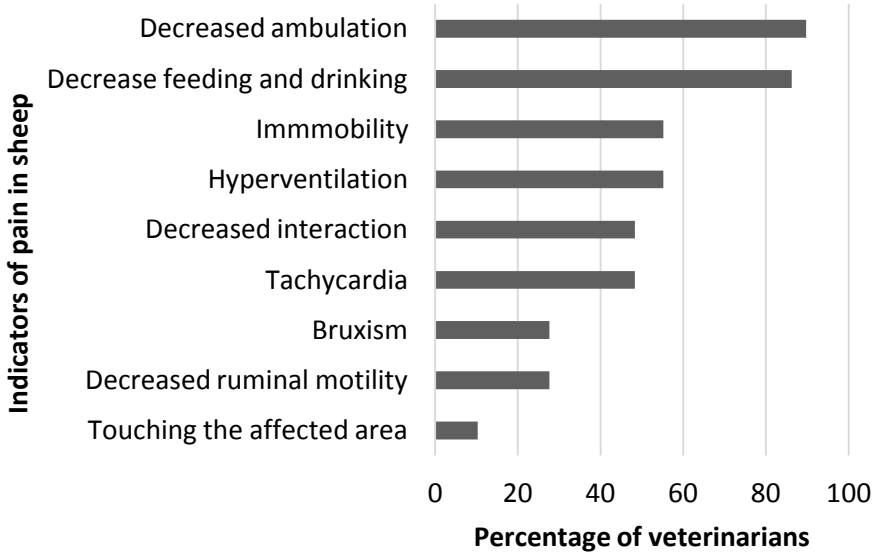
		ESTIMATED PAIN SCORE										
PROCEDURE / CONDITION	*n*	1	2	3	4	5	6	7	8	9	10	Median
Castration	*29*	3.4	3.4	20.7	10.3	24.1	10.3	14	10.4	3.4	0	5
Dehorning	*29*	0	6.9	14	20.7	17.2	6.9	10.3	17.2	3.4	3.4	5
Caesarean Section	*29*	0	10.3	13.8	10.3	10.3	10.3	24.1	14	6.9	0	6
Fracture	*29*	0	0	0	6.9	10.4	10.4	17.2	17.2	17.2	20.7	8
Mastitis	*29*	0	3.4	10.4	6.9	20.7	17.2	10.4	27.6	0	3.4	6
Lameness	*29*	0	3.4	3.4	10.4	14	6.9	24.1	27.5	6.9	3.4	7
Abscess	*29*	6.9	10.4	27.7	17.2	17.2	0	17.2	0	3.4	0	4

The effect on pain scoring of year of graduation, gender and percentage of working time spent treating sheep was evaluated. Veterinarians who graduated before 2005 attributed higher median pain scores than the ones who graduated after 2005, with the median being 6 and 5 respectively (P= 0.04). When all the conditions/procedures were considered together, the median pain scores attributed by female veterinarians was higher than males, with the median pain scores being 7 and 5 respectively (P<0.001). Nevertheless when the procedures/conditions were considered separately, a statistical significant difference was found only for lameness and caesarean section. No statistically significant difference was reported between veterinarians spending less or more than 50% of their working time treating sheep, with the median pain scores being 5 and 6 respectively (P=0.1).

The association between the numbers of analgesic classes (NSAIDs, alpha 2 agonists, local anaesthetics, dissociative anaesthetic agents) used for the different surgical procedures on the pain scoring was evaluated, but no statistically significant results were found (P>0.05).

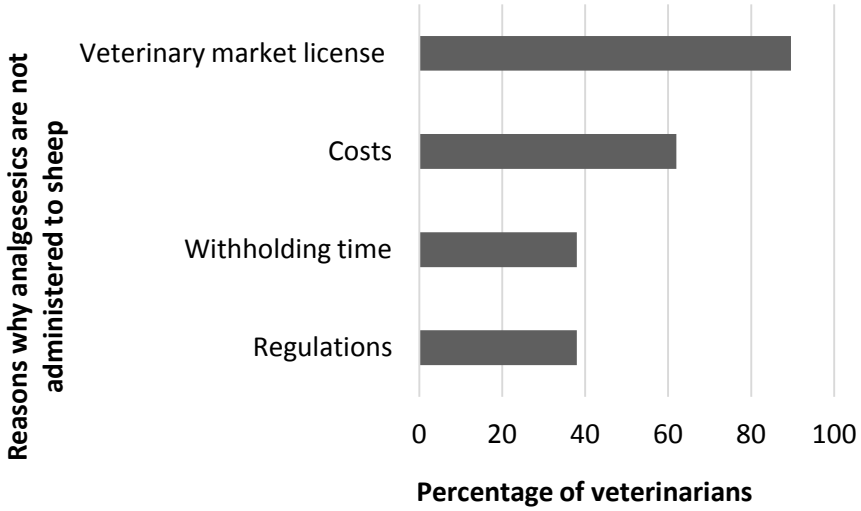
The vast majority of veterinarians considered decreased ambulation, feeding and drinking as signs of pain in sheep, followed by immobility, hyperventilation, decreases interaction with other sheep and tachycardia (Figure 3.15).

Figure 3.15. Indicators of pain in sheep according to Italian veterinarian responding to the questionnaire.



In the respondents' opinion, the main reason why analgesic drugs were not administered to sheep was the lack of licensed drugs, followed by costs, withholding times and regulations (Figure 3.16).

Figure 3.16. Reasons why analgesics drugs were not administered to sheep according to Italian veterinarians responding to the questionnaire.



Section IV. Knowledge and continuing education on sheep analgesia

The great majority of veterinarians (89.6%) considered their knowledge on sheep analgesia to be limited and were keen to improve it (96.5%) by attending congresses and seminars (79%), or with distance learning continuing professional education programmes (51.7%), or by reading scientific journals (51.7%) (Data not shown).

3.4 Discussion

To the author's knowledge, this is the first questionnaire assessing the attitude of veterinarians towards recognition, assessment and treatment of pain in sheep. Similar published questionnaires have evaluated the same topic for other large animal species, in particular cattle, horses and pigs (Raekallio et al. 2003; Huxley & Whay 2006; Hewson et al. 2007a; Thomsen et al. 2010; Lorena et al. 2013).

Only 31 veterinarians took part in the survey; the response rate is not known as the total number of veterinarians who received the e-mail was not known, but the number can be approximated to be 100-150. Moreover, the exact number of veterinarians dealing with sheep in Italy is unknown.

The current questionnaire is shorter than used in previous studies, this was done purposefully to encourage more responses, but this strategy was not successful and the number of responses obtained was very low. Publicity in specialised journals or direct mailing to practitioners could have increased the response rate, but it was not done for reasons of cost. It is difficult to tell if the respondents were representative of the sheep profession as a whole because of the voluntary nature of the questionnaire and the mailing database was not “audited” to reduce the risks of biased distribution, as previously commented by Huxley (Huxley & Whay 2006). Nevertheless considering the heterogeneity of gender and time since graduation, the dataset could be considered representative of sheep profession in Italy. This survey has considerably fewer respondents in comparison to other surveys but this was expected, as the questionnaire focused only on one species and sheep are not as common in Italy in comparison to other countries such as the United Kingdom or Australia. Similar questionnaires investigating analgesia in a single species, specifically in cattle, included only 137 (Becker et al. 2013) and 166 (Laven et al. 2009) answers in contrast to 641 and 666 replies obtained in the United Kingdom (Huxley & Whay 2006) and in the States (Fajt et al. 2011) respectively. Of the 31 replies, two had to be discarded as these two practitioners dealt only with goats and cattle but not with sheep; all the remaining 29 questionnaires were included in the study as all background data were present and more than 80% of questions were answered. More robust results might have been obtained with an increased number of replies; the sample size of this study is limited and this could have affected the outcome.

Small ruminants breeding is performed mainly in the Southern Italy and Islands and this is mirrored by the results of this study which indicate that 65.5% of veterinarians who responded to the survey work in this area where bigger flocks are located. Sheep practitioners who replied to the questionnaire were mainly males (69%), who graduated before 2005 (55.2%) and achieved a post-graduation degree (86.3%). According to Italian statistics, the number of female graduating from veterinary school in

Italy has increased in the last few years (Mazzanti 2013), but according to this study it seems that sheep veterinarians are mainly male.

None of the veterinarians usually administer opioids to sheep in the perioperative period. Experimental pain models using nociceptive stimulation have provided evidence for efficacy of intravenously administered opioids in sheep (Nolan et al. 1987c; Nolan et al. 1988; Waterman et al. 1990; Waterman et al. 1991a; Waterman et al. 1991b). Systemic and neuraxial administration of opioids has been reported in sheep undergoing experimental procedures and they have been proven to provide perioperative analgesia (Ahern et al. 2009; DeRossi et al. 2015), but they are not commonly used by practitioners (Hodgkinson 2007). This is because although opioids could be used in an extra-label manner according to the cascade principles, practitioners are put off by the long withdrawal times, labelling and records requirement, costs and limited knowledge of the pharmacokinetics and pharmacodynamics of these drugs.

None of the respondents had ever used tramadol in sheep. Tramadol is a centrally acting opioid analgesic drug (Raffa et al. 1992) and is exempted from the safe custody requirements; it could potentially be a valuable option for use in sheep as it has been proven to be an useful adjunct in the management of perioperative pain in ruminants (Bigham et al. 2010; Habibian et al. 2011; Dehkordi et al. 2012). Moreover the author has investigated the pharmacokinetic and pharmacodynamics of tramadol in conscious sheep (Bortolami et al. 2015), but further studies are warranted before tramadol can be safely used in practice.

The two most commonly used NSAIDs were flunixin meglumine and ketoprofen, followed by meloxicam and phenylbutazone. Similar results have been reported in other studies where flunixin meglumine, meloxicam and ketoprofen were the most frequently cited NSAIDs by British cattle practitioners (Huxley & Whay 2006), and flunixin meglumine, ketoprofen and phenylbutazone were the most frequently used NSAIDs in large animals in Brazil (Lorena et al. 2013). The great majority of practitioners (69%) administer NSAIDs in the perioperative period, especially after the

surgical procedure and this result is similar to that reported by Huxley in cattle (Huxley & Whay 2006). The use of NSAIDs has been proven to improve pain control following several commonly performed surgical procedures (Ting et al. 2003; Milligan et al. 2004) and so they should be routinely used as standard practice for these procedures (Huxley & Whay 2006). Indeed, not only do NSAIDs have potent anti-inflammatory and analgesic properties, but they are also characterised by ease of parenteral administration and long duration of action (Valverde 2013).

In the perioperative period provision of analgesia was provided by almost all practitioners by the use of local anaesthetics. Local anaesthetic techniques are indeed very common in small ruminants and cattle as the vast majority of the procedures can be carried out with standing sedation and local anaesthetics (Hodgkinson 2007; Valverde 2013). The importance of the use of neuraxial anaesthesia in sheep can be also deducted by the number of experimental studies evaluating it (Habibian et al. 2011; Moll et al. 2011; Dehkordi et al. 2012; DeRossi et al. 2012a; DeRossi et al. 2012b; Rostami & Vesal 2012; DeRossi et al. 2015). Advanced local anaesthetic techniques, such as epidural anaesthesia, were performed most commonly by practitioners who had been graduated for less than ten years, a result that was consistent with other studies (Lorena et al. 2013).

Almost all practitioners, believed that procedures and medical conditions listed in the survey cause pain in sheep, with the condition perceived as causing the greatest pain being a fracture, followed by lameness and caesarean section and mastitis.

In this study the overall median pain score assessed by women was higher than the one attributed by men and this was consistent with other studies' findings (Huxley & Whay 2006; Laven et al. 2009; Lorena et al. 2013). In the same studies higher pain scores were assigned by more recent graduates (Huxley & Whay 2006; Laven et al. 2009; Lorena et al. 2013), but this was not found in this study.

As in other studies, pain scores attributed by practitioners varied widely between them, and this confirms the subjective nature of pain assessment

in animals (Huxley & Whay 2006). Unlike people, animals are unable to verbalise their feelings, so pain assessment is based primarily on behavioural and physiological evaluations (Hardie 2000; Rutherford 2002). According to the practitioners' opinion collated in this survey, sheep manifest their pain by decreasing movement, food and water intake and physiological changes such as tachycardia and hyperventilation can be observed. These opinions are consistent with indicators of pain previously reported in cattle (Stafford 2014).

Not only pain recognition and assessment is difficult in sheep but also its treatment.

Indeed, to the practitioners' opinion the main reason why analgesics drugs were not administered to sheep was the lack of licensed drugs, followed by costs, withholding times and regulations. Registration for use in food producing animals is the most important factor to be considered, and this explains why practitioners do not administer opioids. In the author's opinion the approval of drugs for treatment of painful states in food producing animal may increase the use of analgesics by veterinarians. Nevertheless costs were also considered an important aspect to take into account; which is consistent with other studies (Huxley & Whay 2006; Hewson et al. 2007b). The practitioners' use of analgesics is influenced by the farmers' concerns about the cost effectiveness of drugs compared to the value of the livestock (Huxley & Whay 2006; Gottardo et al. 2011), and the industry's narrow profit margins. Spending money on analgesia can be cost effective, as the provision of analgesia for dehorning and castration has been proven to improve growth rates in cattle (Faulkner & Weary 2000; Stafford & Mellor 2005b; Stafford & Mellor 2005a; Vickers et al. 2005). Finally, analgesia improves not only animals' wellbeing and productivity but also handling calm and pain-free animals will increase practitioner's safety. In order to improve farmers and veterinarians' knowledge regarding the benefits of analgesia, continuing education should be provided. The vast majority of the practitioners were interested in improving their knowledge regarding pain assessment and treatment in

sheep; this willingness is therefore the first step towards a better management of sheep analgesia.

3.5 Conclusions

Welfare of animals has its basis in pain treatment. Surveys investigating practitioners' attitudes on analgesia are important to provide understanding of their practices and help to target professional development programs in order to improve animal care.

Chapter 4

PHARMACOKINETICS AND ANTINOCICEPTIVE EFFECTS OF TRAMADOL AND ITS METABOLITE M1 FOLLOWING INTRAVENOUS ADMINISTRATION IN SHEEP

4.1 Introduction

Sheep are widely used as an experimental model for various surgical procedures (Coulter et al. 2009). In spite of this, there is a paucity of data regarding the pharmacokinetics and efficacy of analgesic drugs in this species. There is a clear need to identify analgesic drugs, dose and dose interval for use in sheep during invasive experimental procedures.

Tramadol is an analgesic drug widely used in people and in small animals; it possesses a weak agonist action against the Mu (μ) opioid receptor and inhibits the reuptake of norepinephrine and serotonin (Raffa et al. 1992). The active metabolite, O-desmethyltramadol (M1) has an affinity for the μ opioid receptor that is 300 times greater than that of tramadol (Grond & Sablotzki 2004). No studies investigating the analgesic efficacy of tramadol in sheep have been performed so far. However, the pharmacokinetics and biotransformation of tramadol have been studied in several animal species including the dog, cat, goat, llama, alpaca, horse and donkey (KuKanich & Papich 2004; Giorgi et al. 2007; de Sousa et al. 2008; Pypendop & Ilkiw 2008; Giorgi et al. 2009b; Cox et al. 2011; Stewart et al. 2011; Edmondson et al. 2012), highlighting species-specific differences in the kinetic profiles of both the parent drug and its metabolites.

Although the effectiveness of tramadol is still unclear in veterinary medicine (Giorgi 2012), there are studies confirming the analgesic efficacy of tramadol for the management of peri-operative pain in other ruminants (Habibian et al. 2011; Dehkordi et al. 2012).

To evaluate the analgesic or antihyperalgesic efficacy of opioid drugs, nociceptive threshold testing, or analgesiometry, can be used. It consists of the application of a measurable stimulus, usually mechanical, thermal or electrical, in order to obtain a clear behavioural response and record the threshold at which the animal responded. If the tested drug exerts analgesic or antihyperalgesic effect, the threshold will either increase or remain unchanged (for example, when thresholds are measured following induction of inflammation). Mechanical nociceptive threshold (MNT) testing devices have already been tested and validated in sheep (Nolan et al. 1987a; Musk et al. 2014).

The aim of this study was to investigate the pharmacokinetic profile and antinociceptive efficacy of two different doses of tramadol administered intravenously to sheep.

4.2 Materials and methods

Animals and treatments

Six female adult Brogna sheep, body mass between 38 and 55 kg, were enrolled in this study, which was performed with approval from the ethical committee for animal experimentation of the University of Padua (CEASA 80/2012, 30 April 2013) and according to EC Council Directive 86/609EEC (Council of the European Communities, 1986). All animals were considered healthy based on clinical examination and haematological analyses. Sheep were kept indoors in a group pen (400 cm x 400 cm) in the Large Animal Facility at the University of Padua and fed a commercial pellet and hay diet. On the day of the experiment, a group of three sheep were moved into individual stalls where animals remained in visual contact with each other. The dimensions of each pen were: length 160 cm, width 66 cm and height 110 cm. Pens were bedded with straw. Sheep were

acclimatized to the stalls, handlers, mechanical nociceptive threshold (MNT) probe and MNT testing procedure prior to commencing the study. Sheep were deprived of food for 8 h prior to the start of the experiment while water was available ad libitum. Hay and water were available ad libitum 2 h after treatment administration. Two 14 G catheters (Delta Ven, DeltaMed) were placed in the right and left jugular veins, to allow both treatment administration and collection of blood for the pharmacokinetic analysis. All six sheep received the following three treatments intravenously over 2 min via the left jugular catheter: Tramadol 4 mg/kg (Group T4) (Tramadolo Hexal Ag, Hexal), Tramadol 6 mg/kg (Group T6), and 5 mL of Sodium Chloride 0.9% (Group SAL). Drugs were administered in a randomly allocated, crossover design with a 2-week wash out period between treatments. Investigators were blinded to treatment allocation.

Blood sampling and clinical evaluation

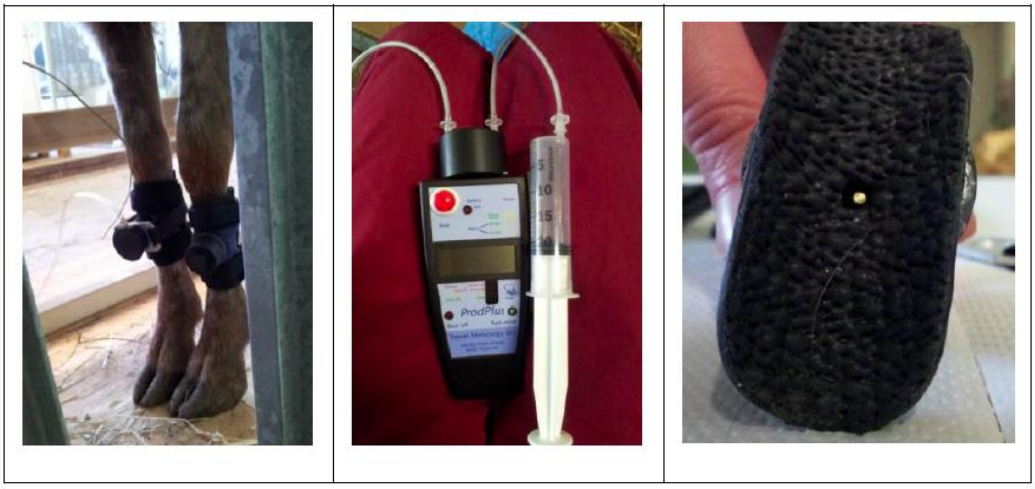
Five mL of blood was collected from the right jugular vein before drug (or saline) administration, 5, 10, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 12 and 24 h after administration. Whole blood was placed in lithium-heparinized tubes and centrifuged at 2000 *g* for 5 min. The harvested plasma was frozen at -80 °C until pharmacokinetic analysis was performed. Immediately before and 15, 30 min and 1, 1.5, 2, 4, 6, 8, 10 and 12 h after drug administration, heart and respiratory rates were determined by thoracic auscultation and observation of thoracic excursions respectively. Rectal temperature and reticulo-ruminal motility, assessed by auscultation of the rumen (number of cycles in 5 min), were monitored starting from 30 min after drug administration. Sedation was quantified using a 0-100 mm visual analogue scale (VAS) scale where 0 mm was considered no sedation and 100 was considered very deep sedation / unconsciousness. Any adverse events attributed to the drug treatment were noted throughout the course of the study.

MNT Testing

MNT was measured by a single investigator using the ProdPro (Topcat Metrology Ltd), as described elsewhere (Dixon et al. 2010). Briefly, this is a mechanical testing device comprising a cuff with a 2 mm hemispheric blunt pin fixed on a rolling diaphragm actuator and applied perpendicular to the skin of the test area, in this case the dorsal aspect of the right metacarpus approximately 4 cm below the carpus. The pin was pushed against the skin with a force which was applied manually by a syringe, connected to non-distensible tubing via a digital meter which displayed the force exerted, until a clear withdrawal response (leg lift, head turn, weight bearing on the contra-lateral limb) was evoked. The force at which the sheep responded with a clear withdrawal response was recorded as the MNT. A dummy actuator, identical to the test actuator apart from the fact that it did not contain the pin was secured to the contra-lateral limb. A cut off point was set at 25 N in order to prevent tissue trauma should a clear withdrawal response not be elicited. Figure 4.1 shows the MNT device used in this study.

The MNT was measured prior to blood collection at time point 0, immediately before drug administration (baseline), 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10 and 12 h after drug administration. In order to calculate the MNT, 3 measurements were performed at each time point with an interval of at least 2 min between each measurement and the mean was used for statistical analysis; five tests were performed and averaged to obtain the baseline MNT.

Figure 4.4. MNT device uses in this study.



Tramadol and M1 determination in blood

Based on a previously published High Performance Liquid Chromatography (HPLC) technique (Giorgi et al. 2009a), the analytical method was briefly re-validated in sheep plasma. The HPLC was a Liquid Chromatographic system (Jasco) consisting of high-pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus) and a loop of 20 µL. Data were processed by Borwin software (Jasco). Chromatographic separation assay was performed by a Luna C18 ODS2 analytical column (150 x 4.6 mm inner diameter, 3 µm particle size, Phenomenex) maintained at 25 °C. The mobile phase consisted of acetonitrile:buffer (20 mM sodium dihydrogen phosphate, 30 mM Sodium Dodecyl Sulfate, and 15 mM triethylamine, adjusted to pH 3.9 with phosphoric acid) (40:60 V/V) at a flow rate of 0.8 mL/min. Excitation and emission wavelengths were 275 and 300 nm, respectively. The analytical method used in this study was able to differentiate the three main metabolites (M1, M2 and M5). However, the M2 and M5 plasma concentrations are not presented here as they are inactive metabolites and hence of negligible importance for the study.

Pharmacokinetic analysis

The pharmacokinetic parameters were calculated for each subject from tramadol and M1 plasma concentrations vs. time curves using WinNonLin v 5.3 (Pharsight Corp). The comparison between competing models (one- vs. two-compartment) was made using the Akaike test. The best fit was described by a two-compartment open and a non-compartmental model, for tramadol and M1, respectively. The area under the concentration vs. time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule (Gibaldi and Perrier, 1982).

Statistical analysis

Sample size calculations were performed before commencing the study. For a two way repeated measures ANOVA with a difference between Δ MNT means (Δ MNT= MNT value at a specific time point minus baseline MNT value) of 3.5 N, standard deviation (SD) = 2, β = 0.8 and α = 0.05, a minimum of 6 animals per group were required. Residuals of repeated measures for Δ MNT, heart rate, respiratory rate, body temperature were analyzed for normality using the Shapiro-Wilk test.

Normally distributed data were analysed by a repeated mixed linear model with the fixed effects of treatment, time and their interaction and animal as a random effect (Littell et al., 1998). Reticulo-ruminal motility was analysed by a non-parametric approach (Kruskal-Wallis) to test the effect of treatment at the different time points. Data analyses were performed using SAS statistical software (version 9.3, SAS Institute). *P* values < 0.05 were deemed significant.

4.3 Results

Pharmacokinetics

The tramadol and M1 concentrations vs. time after IV administration of 4 and 6 mg/kg of tramadol are reported in Fig. 4.2 and Fig 4.3 respectively. The limits of detection (LOD) were 1 ng/mL and 3 ng/mL and the limits of

quantification (LOQ) were 5 ng/mL and 10 ng/mL for T and M1, respectively. The values of precision for both analytes were always lower or equal to 9.8 (CV%), while accuracy was less than 7.3%.

Serum concentrations after IV administration of 4 mg/kg and 6 mg/kg of tramadol in sheep are shown in Table 4.1, while serum concentrations of M1 after IV administration of 4 mg/kg and 6 mg/kg of tramadol in sheep can be visualised in Table 4.2. At the first time point (5 min) the plasma concentrations of tramadol were 1.29 ± 0.17 µg/mL and 1.56 ± 0.10 µg/mL following treatment with 4 mg/kg and 6 mg/kg tramadol respectively. At the subsequent time points, tramadol plasma concentrations decreased rapidly for both treatments and were detectable in all animals only up to 4 h post-administration. At 6 h, tramadol was detectable in 5 out of 6 sheep after treatment with 6 mg/kg and following administration of 4 mg/kg, was detectable at this time point in 4 out of 6 animals. The active metabolite (M1) was detectable in the plasma 5 min after tramadol administration, with a concentration equal to 0.13 ± 0.02 and 0.14 ± 0.03 µg/mL after administration of 4 and 6 mg/kg of tramadol, respectively. Similar plasma concentrations were maintained up to 45 min and then plasma concentrations decreased over the next 4 h. At time points later than 4 h, plasma concentrations of M1 were < LOQ.

The most important pharmacokinetic parameters of tramadol and M1 are reported in Tables 3 and 4, respectively.

Figure 4.2. Average tramadol (solid line, tringle) (-▲-) and M1 (dotted line, square) (-■-) concentrations vs. time after IV administration of tramadol 4 mg/kg (Group T4) (n=6). Bars represent the standard deviation.

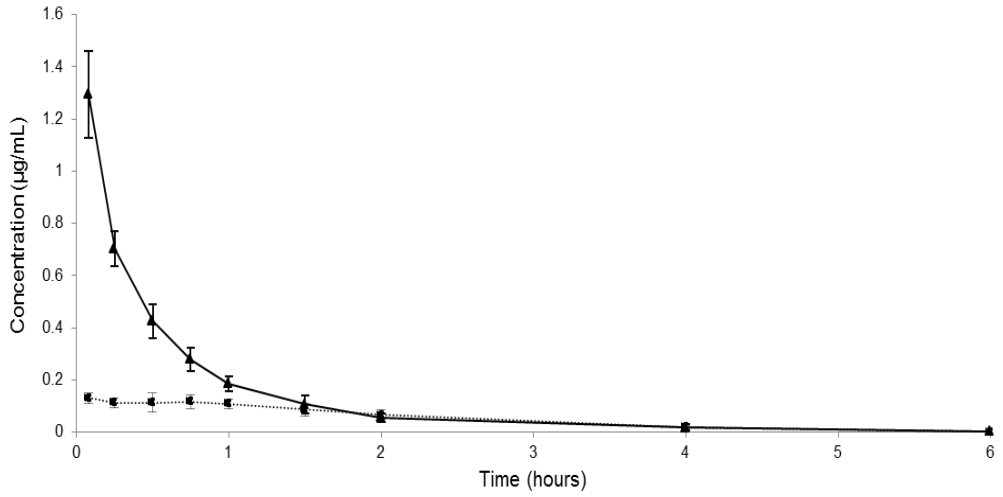


Figure 4.3. Average tramadol (solid line, tringle) (-▲-) and M1 (dotted line, square) (-■-) concentrations vs. time after IV administration of tramadol 6 mg/kg (Group T6) (n=6). Bars represent the standard deviation.

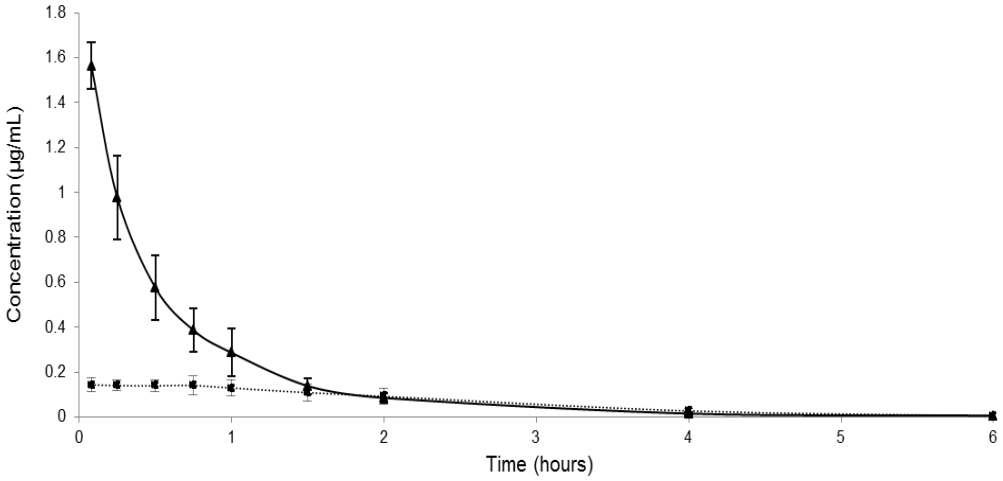


Table 4.1 - Serum concentrations (mean ± standard deviation) of Tramadol after IV administration of 4 mg/kg (Group T4) and 6 mg/kg (Group T6) of tramadol in sheep (n=6).

TIME POST-ADMINISTRATION (min)	GROUP T4: Tramadol Concentration (µg/mL) mean (± S.D.)	GROUP T6: Tramadol Concentration (µg/mL) mean (± S.D.)
5	1.295 ± 0.167	1.564 ± 0.103
15	0.702 ± 0.067	0.978 ± 0.186
30	0.426 ± 0.065	0.576 ± 0.144
45	0.280 ± 0.045	0.386 ± 0.096
60	0.187 ± 0.028	0.286 ± 0.107
90	0.106 ± 0.034	0.138 ± 0.036
120	0.057 ± 0.016	0.085 ± 0.021
240	0.018 ± 0.012	0.015 ± 0.007

Table 4.2 - Serum concentrations (mean ± standard deviation) of M1 after IV administration of 4 mg/kg (Group T4) and 6 mg/kg (Group T6) in sheep (n=6).

TIME POST-ADMINISTRATION (min)	GROUP T4: Tramadol Concentration (µg/mL) mean (± S.D.)	GROUP T6: Tramadol Concentration (µg/mL) mean (± S.D.)
5	0.130 ± 0.022	0.143 ± 0.028
15	0.113 ± 0.017	0.140 ± 0.024
30	0.113 ± 0.036	0.139 ± 0.028
45	0.116 ± 0.028	0.140 ± 0.042
60	0.108 ± 0.020	0.129 ± 0.035
90	0.086 ± 0.025	0.108 ± 0.039
120	0.067 ± 0.020	0.092 ± 0.035
240	0.019 ± 0.007	0.027 ± 0.012

Table 4.3. Main average pharmacokinetic parameters of tramadol following tramadol IV administration at 4 mg/kg (Group T4) and 6 mg/kg in sheep (Group T6) (n = 6).

		GROUP T4		**GROUP T6**	
PARAMETER	Unit	Mean	SD	Mean	SD
k10	1/h	6.895	7.350	2.210	0.381
k12	1/h	7.652	10.137	1.658	1.188
k21	1/h	3.102	1.243	3.062	1.269
t $1/2_{\alpha}$	h	0.091	0.078	0.161	0.118
t $1/2_{\beta}$	h	0.671	0.419	0.573	0.116
V1	L/kg	1.572	1.151	2.870	0.120
CL1	L/kg/h	4.862	1.191	6.315	0.949
V2	L/kg	1.694	0.890	1.415	0.796
CL2	L/kg/h	4.466	1.473	4.732	3.509
AUC 0-∞	µg/mL*h	0.870	0.236	0.968	0.145
AUMC	µg/mL*h2	0.539	0.245	0.671	0.215
MRT	h	0.651	0.337	0.686	0.137
Vss	L/kg	3.266	1.919	4.285	0.745

AUC 0-∞, area under serum concentration-time curve from time zero to infinity;
AUMC, area under moment curve;
CL1, clearance of central compartment;
CL2, clearance of peripheral compartment;
k10, the rate at which the drug leaves the system from the central compartment (the elimination rate);
k12, the rate at which the drug passes from central to peripheral compartment;
k21, the rate at which the drug passes from peripheral to central compartment;
MRT, mean residence time;
$t½_{\alpha}$, distribution half-time;
$t½_{\beta}$, elimination half-time;
V1, volume of distribution in central compartment;
V2, volume of distribution in peripheral compartment;
Vss, volume of distribution at steady state;
SD, standard deviation.

Table 4.4. Average pharmacokinetic parameters of M1 following tramadol IV administration at 4 mg/kg (Group T4) and 6 mg/kg (Group T6)in sheep (n = 6).

		GROUP T4		**GROUP T6**	
PARAMETER	Unit	Mean	SD	Mean	SD
λz	1/h	0.606	0.084	0.580	0.142
$t_{½\,\lambda z}$	h	1.163	0.163	1.266	0.350
$T_{max\ obs}$	h	0.373	0.334	0.402	0.267
$C_{max\ obs}$	µg/mL	0.141	0.020	0.159	0.037
$AUC_{0-\infty\ obs}$	µg/mL*h	0.317	0.077	0.414	0.128
$MRT_{0-\infty\ obs}$	h	1.810	0.244	1.974	0.388

$AUC_{0-\infty\ obs}$, area under serum concentration-time curve from time zero to infinity;

$C_{max\ obs}$, Maximum concentration observed;

$MRT_{0-\infty\ obs}$, mean residence time from time zero to infinity;

$T_{max\ obs}$, Time of maximum concentration observed;

$t_{½\lambda z}$, terminal half-time.

SD, standard deviation.

Clinical evaluations

Mild self-limiting adverse events were noticed in all animals in Group T6 and in 4 animals in Group T4. These included tremors, muscle fasciculation, ataxia, agitation, urination and defecation that started 15-30 s after the beginning of drug administration and lasted for a maximum of 10 min. The severity of adverse events was greater in Group T6 but in all cases they spontaneously resolved. No adverse events were recorded in Group SAL.

Heart rate, respiratory rate, temperature and reticulo-ruminal motility were not statistically different within each group in comparison to baseline values nor between groups at any time points ($P > 0.05$).

Tables 4.5, 4.6, 4.7, 4.8 show the results relative to the physiological parameters.

No sedation was observed during the experiment in any group (VAS = 0 mm) (data not shown).

Table 4.5. Mean (± SD) heart rate after administration of Tramadol 4 mg/kg (Group T4), Tramadol 6 mg/kg (Group T6) and Sodium Chloride 0.9% (SAL) at different time points in sheep (n=6). Values are expressed in beats per minute.

TIME POINTS	SAL	GROUP T4	GROUP T6
Basal values	78 (±8,29)	74 (±2,19)	78,33 (±12,027)
0,25 h	73 (±8,74)	74 (±8,67)	89,33 (±25,63)
0,5 h	72,66 (±6,40)	72,66 (±9,26)	82,33 (±20,87)
0,75 h	70 (±7,04)	77,66 (±7,20)	73,33 (±11,77)
1 h	72,33 (±6,74)	72,66 (±11,14)	75,33 (±12,75)
1,5 h	70 (±6,57)	77,66 (±8,16)	77,33 (±13,30)
2 h	72 (±9,38)	74,66 (±8,64)	77,33 (±13,06)
4 h	76 (±6,69)	76 (±8,76)	78 (±11,52)
6 h	72 (±10,43)	76 (±9,46)	77,33 (±10,93)
8 h	73,66 (±8,80)	73,33 (±11,77)	70 (±12,83)
10 h	70,66 (±8,26)	71,66 (±8,98)	66,66 (±18,53)
12 h	69 (±8,17)	69,33 (±10,32)	74,66 (±6,53)

Table 4.6. Mean (± SD) respiratory rate (beat per minutes) after administration of Tramadol 4 mg/kg (T4), Tramadol 6 mg/kg (T6) and Sodium Chloride 0.9% (SAL) at different time points in sheep (n=6).

TIME POINTS	SAL	GROUP T4	GROUP T6
Basal values	71 (±15,78)	67,33 (±29,97)	75,33 (±32,04)
0,25 h	86,66 (±32,75)	78 (±14,47)	73,33 (±21,11)
0,5 h	70,33 (±37,53)	79,33 (±22,96)	71,33 (±28,33)
0,75 h	66 (±20,35)	76,66 (±31,12)	64,33 (±15,92)
1 h	60,66 (±13,24)	85,33 (±17,82)	73,33 (±25,12)
1,5 h	76,66 (±26,94)	76,66 (±16,13)	86,66 (±32,26)
2 h	72,66 (±22,96)	66,66 (±22,54)	80 (±32,32)
4 h	74 (±16,92)	68 (±22,05)	60,66 (±13,24)
6 h	61,66 (±13,17)	64,33 (±16,89)	65,33 (±15,31)
8 h	58,66 (±17,09)	62,33 (±23,40)	63,33 (±35,63)
10 h	60,33 (±14,66)	57,33 (±15,31)	57,66 (±15,92)
12 h	56 (±19,59)	58,33 (±14,01)	52,66 (±17,42)

Table 4.7. Mean (± SD) rectal temperature, in degree Celsius (°C), after administration of Tramadol 4 mg/kg (T4), Tramadol 6 mg/kg (T6) and Sodium Chloride 0.9% (SAL) at different time points in sheep (n=6).

TIME POINTS	SAL	GROUP T4	GROUP T6
Basal values	39,98 (±0,49)	40,03 (±0,55)	39,91 (±0,48)
0,25 h	39,85 (±0,52)	40,1 (±0,56)	39,8 (±0,61)
0,5 h	39,7 (±0,44)	40,11 (±0,52)	39,96 (±0,67)
0,75 h	39,68 (±0,48)	40,08 (±0,49)	39,9 (±0,55)
1 h	39,53 (±0,45)	39,98 (±0,59)	39,73 (±0,56)
1,5 h	39,51 (±0,47)	39,7 (±0,38)	39,58 (±0,58)
2 h	39,6 (±0,33)	39,71 (±0,45)	39,53 (±0,58)
4 h	39,48 (±0,51)	39,56 (±0,39)	39,4 (±0,55)
6 h	39,56 (±0,51)	39,5 (±0,44)	39,36 (±0,50)
8 h	39,31 (±0,49)	39,45 (±0,40)	39,33 (±0,50)
10 h	39,33 (±0,33)	39,41 (±0,34)	39,26 (±0,34)
12 h	39,51 (±0,29)	39,11 (±0,38)	39,38 (±0,37)

Table 4.8. Mean (± SD) ruminal cycles (assessed by auscultation of the rumen for 5 minutes) after administration of Tramadol 4 mg/kg (Group T4), Tramadol 6 mg/kg (Group T6) and Sodium Chloride 0.9% (SAL) at different time points in sheep (n=6). Values are expressed number of cycles in 5 min.

TIME POINTS	SAL	GROUP T4	GROUP T6
Basal values	3,5 (±0,54)	3,33 (±1,21)	3,66 (±1,36)
0,5 h	3,66 (±0,81)	3,5 (±0,83)	3,16 (±0,98)
1 h	3,66 (±0,81)	4 (±0,63)	4 (±1,26)
1,5 h	3,83 (±1,47)	3,83 (±0,98)	3,66 (±1,36)
2 h	3,5 (±1,3)	4 (±0,63)	3,66 (±1,03)
4 h	4 (±0,89)	4,66 (±1,36)	4,5 (±0,83)
6 h	4,5 (±1,37)	3,83 (±0,98)	3,66 (±0,81)
8 h	4,5 (±0,83)	4,16 (±1,32)	3,83 (±0,98)
10 h	4,5 (±0,83)	4,66 (±0,81)	4 (±1,09)
12 h	4,33 (±1,03)	4 (±0,89)	4 (±1,26)

MNT Testing

Animals reacted to the MNT stimulation with a leg lift or head turn. The cut off value of 25 N was never reached during the study and no signs of tissue trauma or lameness were observed in sheep. There were no significant differences between groups in MNT baseline values; the overall baseline MNT was 8 ± 1.9 N. Table 4.9 shows results relative to MNT test.

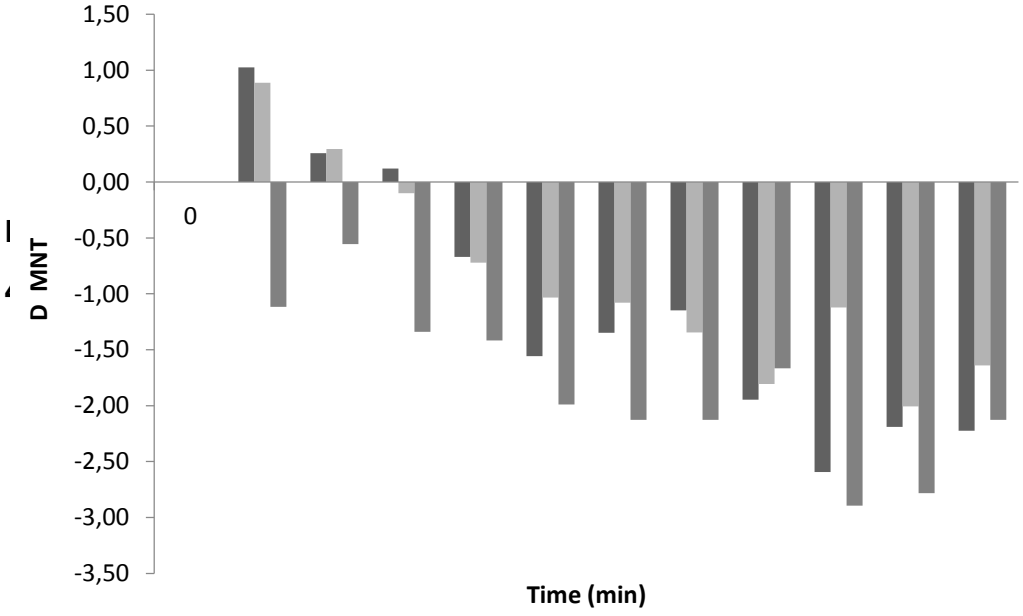
There were no differences in ∆ MNT between groups at any time point ($P > 0.05$). Independently from treatment, at 15 and 30 min post-administration the ∆ MNT values were significantly higher than those observed from the 360 min time point onwards ($P < 0.001$).

∆ MNT values are shown in Fig. 4.4. Within-group comparisons showed that there were no statistically significant differences between the basal MNT and the MNT at any different time point ($P > 0.05$).

Table 4.9. Median (± SD) MNT data after administration of Tramadol 4 mg/kg (Group T4), Tramadol 6 mg/kg (Group T6) and Sodium Chloride 0.9% (SAL) at different time points in sheep (n=6). Values are expressed in Newton (N).

TIME POINTS	SAL	GROUP T4	GROUP T6
Basal values	8,88 (± 2,45)	7,37 (±1,01)	7,94 (± 1,06)
0,25 h	7,96 (± 3,32)	8,63 (± 1,59)	8,92 (± 2,39)
0,5 h	8,22 (± 2,52)	7,76 (± 1,55)	8,36 (± 1,67)
0,75 h	7,64 (± 2,99)	7,57 (± 1,24)	7,43 (± 3,22)
1 h	7,57 (± 1,66)	6,85 (± 1,50)	7,14 (± 1,82)
1,5 h	6,85 (± 2,31)	6,52 (± 0,84)	6,26 (± 1,98)
2 h	6,94 (± 2,50)	6,56 (± 1,44)	6,41 (± 1,09)
4 h	7,31 (± 1,67)	6,22 (± 0,85)	6,56 (± 1,25)
6 h	6,93 (± 1,29)	5,90 (± 0,99)	5,95 (± 1,39)
8 h	5,80 (± 1,04)	6,44 (± 0,71)	5,56 (± 1,14)
10 h	6,08 (± 1,71)	5,75 (± 0,75)	5,64 (± 1,25)
12 h	6,73 (± 1,71)	5,72 (± 0,46)	5,59 (± 1,76)

Figure 4.4. ∆ MNT values at the different time points in the three groups of sheep (n = 6). Saline = grey; T4 = light grey; T6= dark grey. Bars represent the standard deviation



4.4 Discussion

Sheep are widely used for invasive biomedical research but there are limited data surrounding analgesic drug administration in this species. Few analgesic drugs have Market Authorisation for use in ruminants but those that are available include non-steroidal anti-inflammatory drugs (NSAIDs), α_2-agonists and local anaesthetic agents. In people tramadol provides good analgesia with only mild effects on cardio-respiratory function and intestinal motility (Raffa et al., 1992) and is not currently subject to Controlled Drug legislation in Europe. The tramadol doses chosen in the present study were extrapolated from previous studies in other ruminant species (de Sousa et al. 2008; Cox et al. 2011; Edmondson et al. 2012). A pharmacokinetic study in goats evaluated 2 mg/kg tramadol (de Sousa et al. 2008) and the resulting data suggested that 4 mg/kg would be an appropriate dose to achieve plasma concentrations that might be consistent with analgesia, although antinociceptive / analgesic efficacy was not measured concurrently in that study.

The plasma concentration vs. time profiles (Fig. 1) of tramadol and M1 were similar after the two doses. Blood concentrations of tramadol in sheep declined quickly as evidenced by the very short half-life and high clearance value after administration of 4 and 6 mg/kg. The elimination half-life values in this study were lower than those observed in other species such as goats (0.94 h) (de Sousa et al. 2008), alpacas (0.78-0.85 h) (Giorgi et al. 2010; Edmondson et al. 2012), and llamas (2.12 h) (Cox et al. 2011). In the present study, the formation of the active metabolite M1 was observed in all sheep. This is in agreement with an earlier study in goats (de Sousa et al. 2008), while in alpacas (Giorgi et al. 2010) M1 was detected in only 1 out of 8 treated animals. In this study, the ratio of AUCs for M1/T was equal to 0.36 and 0.43 after IV administration of 4 mg/kg and 6 mg/kg of tramadol, respectively. These similar values suggest that the metabolic system of the sheep is not saturated at doses up to 6 mg/kg. This ratio value is similar to that found in dogs (0.31) (KuKanich & Papich 2004), and in goats (0.28) (de Sousa et al. 2008), and lower than that

observed in llamas (0.94) (Cox et al. 2011) and in cats (AUCs ratio M1/T >1) (Pypendop & Ilkiw 2008). These comparisons indicate that M1 has a more prominent role in the pharmacokinetics of tramadol in cats and llamas compared to sheep. In people, the minimum effective concentrations reported for tramadol and M1 are 0.3 ± 0.2 µg/mL (Lehmann et al. 1990) and 0.08 ± 0.03 µg/mL (Grond et al. 1999) respectively. In this study, tramadol in plasma was above the human therapeutic concentration up to 45 min after drug administration while the M1 plasma concentrations considered effective in people were maintained in sheep plasma up to 2 h post treatment. Surprisingly, in the present study a mechanical antinociceptive effect of tramadol was not detected in the first h after drug administration, when plasma levels of tramadol and M1 were similar to analgesic concentrations reported in people.

Quantitative sensory testing methods have been used in conscious painful and non-painful/healthy sheep in order to assess the efficacy of analgesic drugs, including opioids (Nolan et al. 1988; Waterman et al. 1991a; Kyles et al. 1993b; Musk et al. 2014), NSAIDs (Welsh & Nolan 1994; Welsh & Nolan 1995b; Lizarraga & Chambers 2006) and α_2-agonists (Grant et al. 2001; Grant & Upton 2004; Musk et al. 2014). In this study no statistically significant difference in MNT was found between groups. These results are consistent with other studies performed in conscious healthy sheep. Buprenorphine (6 µg/kg IV) was found to exert antinociceptive activity in a thermal nociceptive threshold test but not in the mechanical one (Nolan et al. 1987c); butorphanol (0.1-0.4 mg/kg IV) did not cause any significant elevation in mechanical pressure threshold (Waterman et al. 1991a); pethidine (5 mg/kg IV) increased thermal threshold for 30 min but pressure threshold only for a few minutes (Nolan et al. 1988) and pethidine and fentanyl caused a brief increase in mechanical threshold values (Nolan et al. 1987a). Clearly a more complete evaluation of analgesic effects of a drug should be performed using more than one type of stimulus (Tyers 1980). Thermal nociceptive threshold testing was not performed in this study, not only because of the unavailability of the equipment and for economical reasons but also because it has been reported to cause skin

damage in sheep (Musk et al. 2014), most likely because of the stoic attitude of this pray species. Moreover, when tramadol was tested in conscious horses at the dose of 2 mg/kg, no changes were detected with a thermal nociceptive threshold model (Dhanjal et al. 2009).

The lack of efficacy of tramadol observed in the present study may be due to several reasons. It might be that the achieved plasma concentrations of tramadol were not sufficient to promote antinociception in sheep and that higher plasma concentrations would be required. Genetic variabilities were shown to affect tramadol metabolism in people (Pedersen et al. 2006) and this may apply to sheep as well. A variation in the analgesic effect of xylazine in different breeds of sheep has been reported (Ley et al. 1990). Another reason may be that sheep tend to mask signs of nociception, nevertheless in the current study very clear behavioral end points to the MNT test were produced and sheep did not reach the cut-out values. In this study xylazine, which has been shown to cause an increase in the mechanical nociceptive threshold in sheep (Nolan et al. 1987b), was not used as a positive control: it would have increased the mechanical nociceptive threshold but it would be difficult to differentiate between sedation and analgesia.

It should be pointed out that a major limitation of nociceptive threshold testing is that it does not provide the same stimulus as clinical pain, as commented by Love and colleagues (Love et al. 2011). It may be possible that the analgesic effects of tramadol would be detected in clinical pain states.

The MNT decreased with time in all groups, which might be explained by a sensitization to the MNT test. This finding is consistent with previous reports of MNT measurement in sheep (Stubsjoen et al. 2010) and could be another reason why no analgesic effect of tramadol was detected in this study. On the other hand, in another study the mechanical nociceptive threshold did not vary over 14 days in conscious healthy sheep (Abu-Serriah et al. 2007). In this study, in order to prevent bias, the same observer performed the MNT test and animals were acclimatized to research personnel, equipment, procedures and stables.

After tramadol administration, adverse events, including muscle fasciculation, tremors, agitation and ataxia, were noticed in the majority of animals, but these were short lasting and self-limiting and not deemed to be clinically problematic. This is consistent with findings described in alpacas (Giorgi et al. 2010; Edmondson et al. 2012), llamas (Cox et al. 2011), and horses (Giorgi et al. 2007; Stewart et al. 2011). Although drugs were injected over 2 min, adverse events were still observed. In people, dose and speed of infusion of tramadol affect the incidence of adverse events (Grond & Sablotzki 2004). Therefore in the clinical setting in sheep, a slow infusion rate, over 10 min, may produce less adverse effects. Compared to saline, tramadol administration did not affect physiological parameters including heart rate, respiratory rate and rectal temperature. Respiratory rates, including the basal ones, were high but this was due to the fact that respiratory rate was the last parameter to be evaluated, after blood collection, MNT test and other physiological parameters record. Other Authors have also observed an absence of change in these parameters after epidural administration of tramadol in goats and cows (Bigham et al. 2010; Dehkordi et al. 2012). In contrast, a study conducted in lambs has shown changes in rectal temperature and heart and respiratory rate (Habibian et al. 2011). These incongruities might be the result of having adult versus juvenile subjects and differences in route of administration. In this study tramadol was shown not to affect gut motility; this might be due to the low affinity of tramadol for the μ-opioid receptor and thus tramadol may be advantageous in this species. Tramadol administered to horses at the dose of 2 mg/kg IV was shown not to alter the faecal output although a short lived (40 min) decrease in borborygmus score was reported (Dhanjal et al. 2009). Further studies could be performed to assess the effect of tramadol on gastrointestinal motility by quantification of faecal output (Love et al. 2012) or using radiopaque spheres (Sano et al. 2011).

4.5 Conclusions

Intravenous administration of tramadol at 4 and 6 mg/kg in sheep was associated with rapid metabolism and a transient presence of M1 in plasma; antinociceptive effects were not detected with an MNT model. This study provided the pharmacokinetic data of tramadol in sheep; further studies are warranted to assess its clinical efficacy in animals experiencing pain.

Notes

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Chapter 5

GENERAL CONCLUSIONS

Sheep are widely used in experimental settings and in breeding systems, nevertheless pain treatment in this species seems to be overlooked.

In particular, in the vast majority of research papers, anaesthetic and analgesic treatment is not properly and accurately reported, while in the clinical setting administration of analgesic drugs is limited due to financial concerns and lack of licensed drugs.

Tramadol is a drug exerting opioid and non-opioid analgesic activity, which exhibited low incidence of adverse effects in people and is not a scheduled drug. For this reason it seemed to have potential for its use in sheep.

The study carried out showed that tramadol was rapidly metabolised in sheep and that O-desmethyltramadol's plasmatic concentrations decreased quickly. A mechanical antinociceptive effect of tramadol was not detected in sheep. A major limitation of nociceptive threshold testing is that it does not provide the same stimulus as clinical pain and more than one nociceptive stimulus should be used. It may be possible that the analgesic effect of tramadol would be detected in clinical pain states. Further studies would include the evaluation of tramadol in sheep undergoing a surgical procedure or suffering from a painful condition.

These studies showed that there is a lot of room for improvement in pain assessment and treatment in sheep and the veterinary surgeon has the important role of improving sheep welfare in the experimental and clinical setting.

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ANNEX A: On line questionnaire sent to Italian practitioners regarding analgesia in breeding sheep

QUESTIONARIO SULL' ANALGESIA NELLA SPECIE OVINA

*Campo obbligatorio

PARTE I

Per favore, rispondi alle seguenti domande. E' possibile indicare più di una risposta.

In che anno ti sei laureato/a? * _____

Hai effettuato studi post-lauream? *

- ☐ Master
- ☐ Dottorato di ricerca
- ☐ Scuola di specializzazione
- ☐ Diploma Europeo
- ☐ Non ho effettuato studi post-lauream
- ☐ Altro: _____

Sesso: *

- ○ Maschio
- ○ Femmina

In che regione lavori? * _____

Di quali specie animali ti occupi? *

- ☐ Specie ovina
- ☐ Specie caprina
- ☐ Specie bovina

- ☐ Specie equina
- ☐ Altro: ____

Che percentuale del tuo tempo dedichi alla cura della specie ovina? *

- ○ 0-30%
- ○ 30-50%
- ○ 50-70%
- ○ 100%

Quale è la grandezza media del gregge con cui hai a che fare? *

- ○ 1-10 capi
- ○ 10-50 capi
- ○ 50-100 capi
- ○ più di 100 capi

Qual è l'attitudine degli ovini a cui dedichi le tue cure? *

- ☐ Carne
- ☐ Latte
- ☐ Lana
- ☐ Mista
- ☐ Sperimentazione
- ☐ Compagnia
- ☐ Altro: ____

PARTE II

Le seguenti domande riguardano l'uso di analgesici/tecniche analgesiche nel periodo peri-operatorio nella specie ovina. Per periodo peri-operatorio si intende il periodo che intercorre tra l'inizio dell'anestesia fino a 24 ore dopo la procedura. Per favore, indica quali farmaci/tecniche utilizzi per fornire analgesia nella specie ovina, anche in deroga secondo normativa vigente. E' possibile indicare più di una risposta.

Oppioidi. Quale dei seguenti farmaci utilizzi? *

- ☐ Butorfanolo
- ☐ Buprenorfina
- ☐ Metadone
- ☐ Morfina
- ☐ Petidina
- ☐ Nessuno
- ☐ Altro: _____

Somministri abitualmente oppioidi nel periodo perioperatorio in pecore sottoposte a chirurgie (es. castrazione, decornuazione, cesareo, laparotomia, etc)? Se si, quando somministri gli oppioidi? *

- ☐ Non utilizzo oppioidi abitualmente
- ☐ Prima dell'anestesia
- ☐ All' inizio dell'anestesia o durante la chirurgia
- ☐ Dopo la chirurgia

Se non somministri abitualmente gli oppioidi nella specie ovina, li somministri in qualche circostanza particolare? *

- ○ Si
- ○ No

Se si, in che particolare circostanza somministri gli oppioidi?

_____

In base a quale criterio scegli l'oppioide da somministrare? *

- ☐ Non somministro oppioidi
- ☐ Efficacia analgesica
- ☐ Sicurezza riportata
- ☐ Costi
- ☐ Reperibilità
- ☐ Altro: _____

Farmaci anti-infiammatori non steroidei. Quale dei seguenti farmaci utilizzi? *

- ☐ Acido tolfenamico
- ☐ Carprofen
- ☐ Fenilbutazone
- ☐ Flunixin meglumine
- ☐ Ketoprofene
- ☐ Meloxicam
- ☐ Non dispongo di farmaci anti-infiammatori non steroidei
- ☐ Altro: _____

Somministri abitualmente farmaci anti-infiammatori non steroidei (FANS) nel periodo perioperatorio in pecore sottoposte a chirurgie (es. castrazione, decornuazione, cesareo, laparotomia, etc)? Se si, quando somministri i FANS? *

- ☐ Prima dell'anestesia
- ☐ All'inizio dell'anestesia o durante la chirurgia
- ☐ Dopo la chirurgia
- ☐ Non utilizzo abitualmente FANS

Per il trattamento di quali patologie somministri i FANS? *

- ☐ Zoppie
- ☐ Mastiti
- ☐ Ascessi
- ☐ Altro: _____

In base a quale criterio scegli i FANS da somministrare? *

- ☐ Registrazione per la specie ovina
- ☐ Efficacia analgesica
- ☐ Tempi di sospensione
- ☐ Sicurezza riportata
- ☐ Costi
- ☐ Reperibilità
- ☐ Altro: _____

Anestetici locali. Quale dei seguenti farmaci utilizzi?

- ☐ Procaina
- ☐ Bupivacaina
- ☐ Lidocaina
- ☐ Non dispongo di anestetici locali
- ☐ Altro: _____

Somministri abitualmente anestetici locali nel periodo perioperatorio in pecore sottoposte a chirurgie (es. castrazione, decorazione, cesareo, laparotomia, etc)? *

- ○ Si
- ○ No

Quale delle seguenti tecniche loco-regionali utilizzi? *

- ☐ Infiltrazione di anestetico locale attorno al punto di incisione della cute
- ☐ Blocco intratesticolare per castrazione
- ☐ Anestesia epidurale per procedure su addome e perineo
- ☐ Anestesia epidurale per procedure sugli arti posteriori
- ☐ Infiltrazione di anestetico locale attorno al dente per procedure dentali
- ☐ Blocco del nervo mandibolare/mascellare per procedure dentali
- ☐ Blocco del plesso brachiale
- ☐ Non utilizzo tecniche loco regionali
- ☐ Altro: _____

Che farmaci utilizzi quanto effettui un'epidurale? *

- ○ Anestetico locale
- ○ Anestetico locale e oppioide
- ○ Non effettuo epidurali
- ○ Altro: _____

Altri farmaci. Quale dei seguenti farmaci utilizzi? *

- ☐ Detomidina
- ☐ Dexmedetomidina

- ☐ Medetomidina
- ☐ Romifidina
- ☐ Xylazina
- ☐ Ketamina
- ☐ Farmaci anti-infiammatori steroidei
- ☐ Farmaci omeopatici
- ☐ Nessuno di questi
- ☐ Altro: ____

Hai mai somministrato tramadolo nella specie ovina? *

- ○ Si
- ○ No

Se si, in che circostanza/patologia hai utilizzato il tramadolo?

____

A che dose e per quanto tempo hai somministrato il tramadolo?

____

Hai notato effetti collaterali dopo la somministrazione di tramadolo?

- ○ Si
- ○ No

Se hai notato effetti collaterali, quale dei seguenti hai notato dopo la somministrazione di tramadolo?

- ☐ Agitazione
- ☐ Atassia
- ☐ Tremore
- ☐ Tachipnea
- ☐ Tachicardia
- ☐ Altro: ____

Per apportare analgesia, quali altre tecniche utilizzi? *

- ☐ Agopuntura
- ☐ Chiropratica
- ☐ Nessuna di queste
- ☐ Altro: ____

PARTE IV

Le seguenti domande riguardano l'uso di analgesici nel periodo peri-operatorio nella specie ovina. Per periodo peri-operatorio si intende il periodo che intercorre tra l'inizio dell'anestesia fino a 24 ore dopo la procedura. E' possibile indicare più di una risposta.

Che farmaci utilizzi per le castrazioni? *

- ☐ Alpha 2 agonista
- ☐ FANS
- ☐ Ketamina
- ☐ Anestetico locale
- ☐ Nessuno
- ☐ Altro: ____

Che farmaci utilizzi per le decornuazioni? *

- ☐ Alpha 2 agonista
- ☐ FANS
- ☐ Ketamina
- ☐ Anestetico locale
- ☐ Nessuno
- ☐ Altro: ____

Che farmaci/tecniche usi per un cesareo? *

- ☐ Alpha 2 agonista
- ☐ FANS
- ☐ Ketamina
- ☐ Epidurale
- ☐ Infiltrazione di anestetico locale sul fianco
- ☐ Oppioidi per via sistemica

- ☐ Nessuno
- ☐ Altro: _____

PARTE V

Per favore, rispondi alle seguenti domande. E' possibile indicare più di una risposta.

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora durante/dopo la castrazione? *

1 2 3 4 5 6 7 8 9 10

○ ○ ○ ○ ○ ○ ○ ○ ○ ○

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora durante/dopo la decornuazione? *

1 2 3 4 5 6 7 8 9 10

○ ○ ○ ○ ○ ○ ○ ○ ○ ○

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora durante/dopo un cesareo? *

1 2 3 4 5 6 7 8 9 10

○ ○ ○ ○ ○ ○ ○ ○ ○ ○

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora affetta da frattura ossea? *

1 2 3 4 5 6 7 8 9 10

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora affetta da mastite? *

1 2 3 4 5 6 7 8 9 10

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora affetta da zoppia? *

1 2 3 4 5 6 7 8 9 10

Indica in una scala da 1 a 10, dove 1 indica assenza di dolore e 10 il peggior dolore possibile, quale intensità di dolore prova una pecora con un ascesso? *

1 2 3 4 5 6 7 8 9 10

Quali atteggiamenti riconosci in una pecora che prova dolore? *

- Diminuzione della locomozione
- Immobilità
- Diminuzione dell'interazione con le altre pecore
- Diminuzione dell'alimentazione e abbeveraggio
- Diminuzione dell'attività reticolo-ruminale
- Calci / leccamento / sfregamento della zona dolorante
- Digrignamento dei denti

- ☐ Tachicardia
- ☐ Iperventilazione
- ☐ Nessuna di queste
- ☐ Altro: ____

Secondo il tuo parere, per quali motivi l'utilizzo dell'analgesia nella specie ovina è limitato? *

- ☐ Mancanza di farmaci registrati per l'uso negli ovini
- ☐ Motivi economici
- ☐ Tempi di attesa dopo la somministrazione del farmaco
- ☐ Incombenze burocratiche
- ☐ Nessuno di questi
- ☐ Altro: ____

PARTE VI

Per favore, rispondi alle seguenti domande. E' possibile indicare più di una risposta.

Consideri adeguata la tua conoscenza nell'ambito della terapia del dolore nella specie ovina? *

- ○ Si
- ○ No

Come preferiresti aggiornare le tue conoscenze riguardo la terapia del dolore nella specie ovina? *

- ☐ Riviste specialistiche
- ☐ Congressi / Seminari
- ☐ Aggiornamento a distanza
- ☐ Non mi interessa l'argomento
- ☐ Altro: ____

GRAZIE MILLE PER AVER COMPILATO QUESTO QUESTIONARIO!