Analgesia in the Horse

Various approaches for assessment and treatment of pain and nociception in equines

Thijs van Loon

2012

J.P.A.M. van Loon, 2012
Analgesia in the Horse, various approaches for assessment and treatment of pain and nociception in equines

Dissertation Utrecht University, Faculty of Veterinary Medicine
(with summaries in English and Dutch)

ISBN 978-90-8891-389-1

Cover: Philips Wouwerman (1619-1668): de Schimmel (ca. 1646). Rijksmuseum Amsterdam. Oil on panel.

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Omslag en lay-out: Proefschriftmaken.nl
Druk: Proefschriftmaken.nl
Uitgeverij: BOXPress, Oisterwijk

Analgesia in the Horse

Various approaches for assessment and treatment of pain and nociception in equines

Pijnstilling bij het Paard

Diverse benaderingen voor het meetbaar maken en behandelen van pijn en nociceptie bij paarden

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 maart 2012 des middags te 12.45 uur.

Door

Johannes Petrus Antonius Maria van Loon

geboren op 8 oktober 1972 te Roosendaal en Nispen

Promotoren: Prof. dr. L.J. Hellebrekers
Prof. dr. P.R. van Weeren

Co-promotor: Dr. W. Back

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van:

Dechra Veterinary Products
Pfizer Animal Health
AUV Dierenartsencoöperatie
MSD Animal Health
Stichting Nationaal Platform Pijn bij Dieren

Houdt de vlam brandende (Kees van Kooten)

Voor Fiene

The Veterinary Record

FOUNDED BY WILLIAM HUNTING, F.R.C.V.S., 1888.

No. 22. JUNE 1, 1935. Vol. XV.

General Article

SPINAL (EPIDURAL) ANÆSTHESIA IN THE DOMESTIC ANIMALS

A Review of Our Knowledge at the Present Time

GEOFFREY B. BROOK,
D.Sc. (EDIN.), M.R.C.V.S.

(*Continued from page* 608.)

Epidural Anæsthesia in the Horse

In the horse, as in the ox, one distinguishes between Posterior and Anterior Anæsthesia; in the ox the difference may be one not only of extent of anæsthesia but also of site of injection. In the horse, because the first coccygeal vertebra is often fused with the sacrum, only the intercoccygeal route of injection is available and both types of anæsthesia must be induced by puncture at this site. By Posterior Anæsthesia is understood the varying degrees of desensitisation that may be induced without abolishing control of the hind limbs; Anterior Anæsthesia is a term covering the degrees of epidural block which involve the motor nerves to the hind limbs. In the horse this difference is important. If the power of the hind limbs is to be interfered with, appropriate preparation must be made for control of an excitable patient, especially during the periods of development and recovery. Observations on one of the cases of subarachnoid anæsthesia in the mare reported by Hodgkins[64] amply verify this.

Special Anatomy: The sacrum of the horse is shorter than that of the ox. The five spines sloping upwards and backwards decrease in length from before to behind (fig. 20). With the exception of the first they are, therefore, difficult to palpate, the more so because of the regional development of ligaments and muscles. The first coccygeal vertebra is not infrequently fused with the sacrum with occlusion of the sacro-coccygeal interarcuate space. The lumbo-sacral dural cul-de-sac extends to the level of the second-third sacral segments. The spinal canal slopes backwards and downwards more steeply than in the ox (fig. 20). The first intercoccygeal interarcual space is smaller than that of the ox.

A.—POSTERIOR ANÆSTHESIA

This is discussed as follows:—

1. Estimation of site.
2. Needles to be used.
3. Mode of injection.
4. Failure to enter the epidural space.
5. Unsatisfactory development of anæsthesia.
6. Amounts of solution.
7. Progress of anæsthesia and Duration.
8. Indications for employment.
9. Accidents and complications.

1. Estimation of Site: The injection is made through the first intercoccygeal interarcual space, to locate which the method of Cuillé and Chelle[60] has been found most useful. An imaginary line is drawn between the hip joints (figs. 17, 18, 19). This intersects the mid-dorsal line at the level of the sacro-coccygeal junction, immediately behind which the spinous process of the first coccygeal vertebra may be felt as a bony eminence. Posterior to this is a depression (x, figs. 17 and 18) representing the space between the first and second coccygeal spines. It is into this depression that the needle is passed. McLeod and Frank[13] recommend the

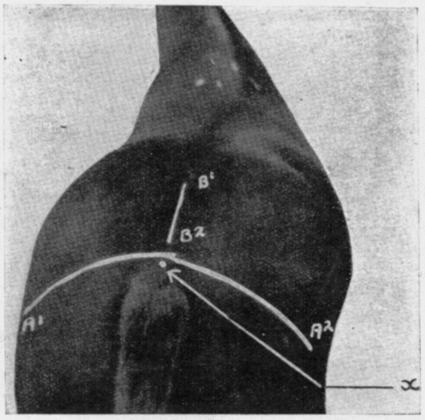

Fig. 17.—The Surface Markings for Location of Site of Injection.
A^1—A^2=line connecting hip joints.
B^1—B^2=position of summits of sacral spines.
x=site of injection, *i.e.*, depression between 1st and 2nd coccygeal spines.
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From: Geoffrey B. Brook (1935) Spinal (epidural) anaesthesia in the domestic animals. Veterinary Record 15, 659-667

Contents

1

General Introduction

"Dying is nothing, but pain is a very serious matter"
(Henry Jacob Bigelow, 1871)

Pain recognition and management in animals has advanced considerably in the last decade (Flecknell, 2008; Lerche, 2009) and animal welfare is receiving increased public interest (Nolen, 2001; Stafford and Mellor, 2007). However, pain assessment in all animals often remains subjective, as objective and reliable assessment of pain is difficult in nonverbal individuals. This thesis aims to improve our knowledge and expertise in this difficult area by focusing on pain assessment and various analgesic strategies in the horse. This introductory chapter presents various ideas, concepts and definitions of nociception and pain together with an historical overview of local anaesthetic and analgesic techniques. This chapter ends with a short outline of the thesis.

Nociception

Nociception is the physiologic perception of noxious stimuli. The term has been introduced by Sherrington in 1910 and comes from the Latin *nocere* ("to harm"). Nociception is triggered by stimuli that activate the peripheral terminals of nociceptors, a highly specialised subset of primary sensory neurons that respond only to intense stimuli. Classical theories dictate that nociceptors have either unmyelinated (C-fibre) or thinly myelinated (Aδ-fibre) axons (Figure 1) (McCleskey and Gold, 1999) and that all Aα/β-fibre afferents are low threshold mechanoreceptors (Doubell et al., 1999). However, a substantial proportion of nociceptors are innervated by Aβ-type fibres (Djouhri and Lawson 2004). Furthermore, Latremoliere and Woolf (2009) described a role for large low-threshold mechanoreceptor myelinated fibres in Aβ fibre-mediated pain after central sensitisation has occurred.

In general, the transducer ion channels on nociceptor nerve endings are nonselective cation or sodium channels that are gated not by voltage but by temperature, chemical ligands or mechanical shearing forces. Once activated,

the channels open and sodium and calcium ions flow into the nociceptor peripheral terminal, producing an inward electrical current that depolarises the membrane. The presence, specificity and threshold of the nociceptor transducers is thus the first and most important filter in the activation of nociception and defines different classes of primary sensory neurons. Action potentials from the peripheral nociceptor terminal of a nociceptor are conducted centrally by the peripheral sensory neuron axon, which runs within peripheral nerves to the dorsal root ganglion and into the spinal cord dorsal horn, where the central terminals of the neurons make synaptic contact with dorsal horn neurons. Transfer of signals from nociceptors to the dorsal horn neurons in the spinal cord projecting to the brain is mediated by either direct monosynaptic contact or through multiple interneurons, which are either excitatory or inhibitory. Some of these neurons have axon projections to the thalamus, transferring the sensory information to the brain. The thalamus receives only a small fraction of the sensory input that enters the dorsal horn of the spinal cord and relays this information to the cortex. Most input fails to evoke an action potential output and may alternatively be involved in local processing, refining, modulating and controlling of the sensory transfer. The central terminals of nociceptors contain presynaptic receptors that can alter transmitter release. Postsynaptic inhibition is effectuated by a hyperpolarising inhibitory potential evoked in dorsal horn neurons by the opening of potassium or chloride channels.

Pain

Pain is defined as *an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage* (Anon., 1983). This definition of pain highlights the fact that pain is both a sensory and an emotional experience. Experiencing emotional states is not restricted to higher levels of consciousness (as found in man and some animal species), but is considered common to all vertebrates (Flecknell, 2008). There is compelling evidence that horses do experience pain in a similar fashion as humans do and show highly emotional responses, suggesting that these animals have a definite

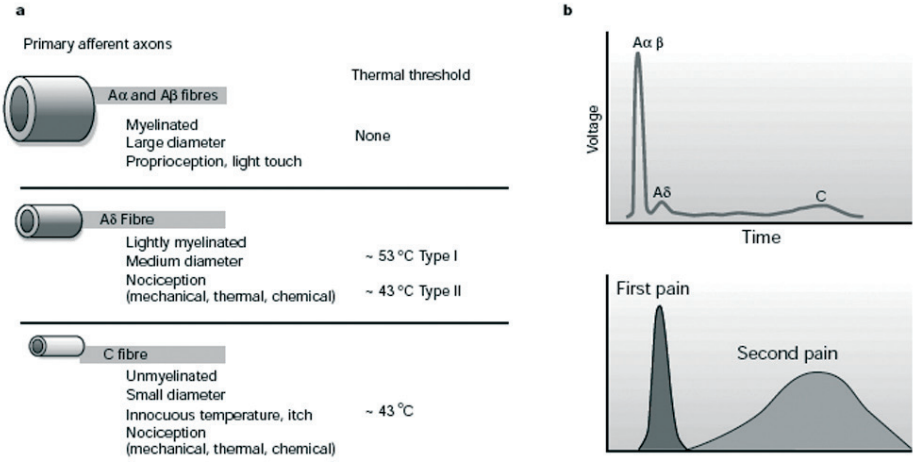

Figure 1 Different nociceptors detect different types of pain. A: Peripheral nerves include small-diameter (Aδ) and medium- to large-diameter (Aα,β) myelinated afferent fibres, as well as small-diameter unmyelinated afferent fibres (C). B: The fact that conduction velocity is directly related to fibre diameter is highlighted in the compound action potential recording from a peripheral nerve. Most nociceptors are either Aδ or C fibres, and their different conduction velocities (6-25 and 1.0 m/s, respectively) account for the first (fast) and second (slow) pain responses to injury (Modified from Julius and Basbaum, 2001).

affective component to their nociceptive experience (McMillan, 1999; McMillan and Rollin, 2001; Taylor et al., 2002). Verbal communication is used to describe emotions in humans, but it is not a prerequisite for emotion. Consequently, the lack of communication familiar to man does not imply an absence of emotions (Paul-Murphy et al., 2004). For these reasons, in 1994 the IASP (International Association for the Study of Pain) felt it necessary to add to its definition of pain the important *caveat* that "an inability to communicate does not negate the possibility that an individual is experiencing pain or is in need of appropriate pain-relieving treatment". This is not only important for children, mentally disabled and geriatric, neurologic or aphasic human patients, but holds true for animals as well. The general definition of pain has been modified for animals as follows by Molony and Kent (1997): *Animal pain is 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.*

In human literature, pain is nowadays considered as the fifth vital sign, next to temperature, heart rate, respiratory rate and blood pressure (Lynch, 2001). In 2000, the Joint Commission on Accreditation of Healthcare Organisations officially recognised that pain is a major health problem in humans and stated that pain assessment should be provided in all medical records. Pain in horses is to be considered a valuable clinical sign as it is often the first and only sign of a current or impending problem (Price et al., 2002). Since blood pressure measurements are not routinely executed in conscious horses, it is time to consider adding pain assessment as a "fourth vital sign" to good veterinary practice (Sellon, 2006).

Analogy postulate

The French mathematician and philosopher René Descartes (1596-1650) denied animals the ability to feel pain because they had no capacity for reasoning (with his famous quote "*The greatest of all prejudices we have retained from our infancy is that of believing that beasts think*") (Descartes, 1649). After him, many other philosophers considered this issue. Jeremy Bentham (English lawyer and philosopher, 1748-1832), one of the earliest protagonists of animal rights argued that the ability to suffer, not the ability to reason, must be the benchmark of how to treat other beings. He stated: *"It may one day come to be recognised that the number of the legs, the villosity of the skin, or the termination of the os sacrum are reasons equally insufficient for abandoning a sensitive being to the same fate. What else is it that should trace the insuperable line? Is it the faculty of reason or perhaps the faculty of discourse? But a full-grown horse or dog is beyond comparison a more rational, as well as more conversable animal, than an infant of a day or a week or even a month old. But suppose they were otherwise, what would it avail? The question is not, Can they reason? Nor can they talk? But, can they suffer?"* (Bentham, 1907).
The analogy postulate holds the view that in analogy to humans and based on the homologous anatomy of the perception and reception organs of humans and vertebrate animals, we should acknowledge that animals are able to experience pain. Verhoog and Wemelsfelder (1988) formulated it as: 'The analogy postulate implies that the presence or absence of consciousness in animals cannot directly

be scientifically proven. Based on similarities between humans and animals in anatomy, physiology and behaviour however, one may assume that there are also similarities in subjective perception.' Animals clearly have the anatomical and pharmacological components necessary for transduction, transmission and perception of pain (Livingston, 1994). The neurophysiologist Gentle (2001) studied pain in chickens and showed that pain reactions were modified by shifting the chicken's attention elsewhere. He therefore concluded that the pain response could not have been an unconscious adjustment of behaviour that happened automatically, but must have been mediated by conscious awareness of the pain. Despite the fact that the question whether animals are conscious or not has not been answered unequivocally, the premise holds that animals should be given the benefit of the doubt and the simple approach of "if in doubt about pain use an analgesic" may be the best we can do in most circumstances.

Various types of Pain

Different classifications of types of pain exist and are shown in Figure 2.

Physiological or adaptive pain operates to protect the body against further damage by warning for contact with tissue damaging stimuli (Woolf and Chong, 1993). This type of pain is produced by stimulation of nociceptors linked to high-threshold Aδ- and unmyelinated C-fibres. This nociceptive system extends from the periphery through the spinal cord, brainstem and thalamus to the cerebral cortex, where the sensation is perceived (Woolf, 2004). The nociceptive pain system is a key early warning device, an alarm system that announces the presence of a (potentially) damaging stimulus. Nociceptive pain needs to be controlled in specific clinical situations for both animal welfare and animal health reasons (i.e. during surgical or medical procedures that damage tissue, after trauma). It is important that this system is not chronically disabled (for instance during chronic pain syndromes), because loss of its protective function inevitably leads to tissue damage, including possible self-induced mutilation. Nociceptive pain therefore has a vital physiologic function. Lack of this function in patients with

congenital insensitivity to pain, as in cases of a mutation of the nerve growth factor tyrosine kinase A receptor, resulting in loss of high-threshold sensory neurons, reduces life expectancy (Miranda et al., 2002). Although physiological pain acts as a protective warning system, it is otherwise rarely beneficial. The notion for example, that pain-induced limitation of physical activity is beneficial is an inappropriate rationalisation, and as such more indicative of the severity of pain and inflammation than a real benefit (Muir 1998).

Clinical or maladaptive pain originates from peripheral tissue injury or damage to the nervous system. It can be categorised into inflammatory and neuropathic pain. Inflammatory pain is described as either visceral (thoracic and abdominal viscera) or somatic (skin, joints, muscles or periosteum) in origin. Visceral pain

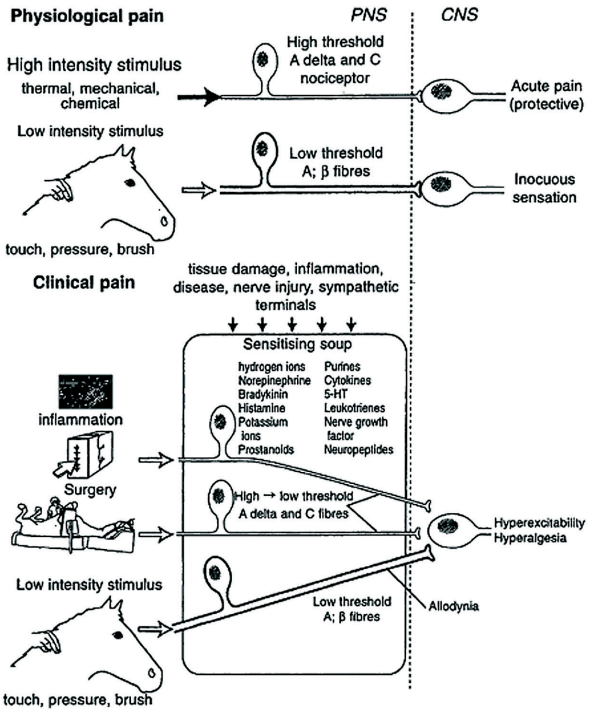

Figure 2 Differences between physiologic pain and clinical pain. Tissue damage and inflammation can lead to hypersensitivity to mechanical, chemical and thermal stimuli resulting in increased sensation or 'hyperalgesia'. PNS = peripheral nervous system, CNS = central nervous system (Courtesy of Muir, 1998).

is poorly localised and may be referred to cutaneous sites far from the site of injury (i.e. referred pain). Somatic pain on the other hand, is generally distinctly localised and occurs as cutaneous or incisional pain after surgery. Somatic pain is frequently referred to as superficial (skin) or deep (joints, muscle and periosteum). Neuropathic pain occurs as a direct result of more chronic damage to peripheral nerves or the spinal cord and is often poorly to unresponsive to treatment. Finally, idiopathic pain is pain that persists in the absence of an identifiable organic substrate. Idiopathic pain is often excessive and can be accentuated by activation of the sympathetic nervous system due to emotional stress, fear or excitement.

Recognition of pain and nociception in equines

Pain assessment

Although identification and treatment of animal pain is an increasingly important topic both from animal welfare and animal health perspective, and as such is receiving increasing public interest (Nolen, 2001; Stafford and Mellor, 2007), reliable pain assessment in different animal species remains difficult and thus highly subjective. Questionnaires by Dohoo and Dohoo (1996a, b), Capner et al. (1999), Lascelles et al. (2000) and Raekallio et al. (2003) investigated the attitude of veterinarians towards pain management in companion animals. The majority of the respondents appeared convinced that animals experience pain in a range of circumstances. However, the application of perioperative analgesics was inconsistent and tended to be limited for many surgeries (Hewson et al., 2006). The questionnaires of Dohoo and Dohoo (1996a, b) revealed that 84% of dogs and 70% of cats received postoperative analgesics after orthopaedic surgery. After castration, however, these percentages were 10% for dogs and 9% for cats. Difficulties in assessing the level of pain the animal experiences were thought to have contributed to this limited use of analgesics in veterinary practice. In the same study 77% of the veterinarians considered their knowledge of issues related to the recognition and control of postoperative pain inadequate. Raekallio et al. (2003) found that 40% of 411 Finnish veterinarians more or less agreed with

the statement 'it is difficult to recognize pain in animals'. Hugonnard et al. (2004) examined the attitudes of French veterinarians with regard to pain in animals and found 96% of the respondents to be "moderately or extremely concerned about recognition and alleviation of small animal pain". Equine analgesia has been relatively neglected until relatively recently, lagging clearly behind in attention and focus compared to small animals (Taylor, 2003). The problems in recognising pain cause doubt about the potential benefits of analgesic intervention in animals. There has been considerable professional debate about the necessity to provide analgesia following castration in horses. In a survey in the UK by Price et al. (2002) 70% of respondents estimated the severity of pain after castration in horses as 'low'. Another study by Price et al. (2005) found that 45.4% of respondents never administered postoperative analgesia following castration and only 36.9% of veterinarians in the UK did so routinely.

In a recent survey performed among equine practitioners in the Netherlands and Belgium, we assessed the attitude towards recognition of equine pain and the knowledge about analgesic therapy in the horse (Dujardin and van Loon, 2011). Results are shown in Figure 3A. Although a fair number of veterinarians consider their own level of knowledge to be sufficient, especially with respect to pain recognition, improvement is still possible and needed. Both academic

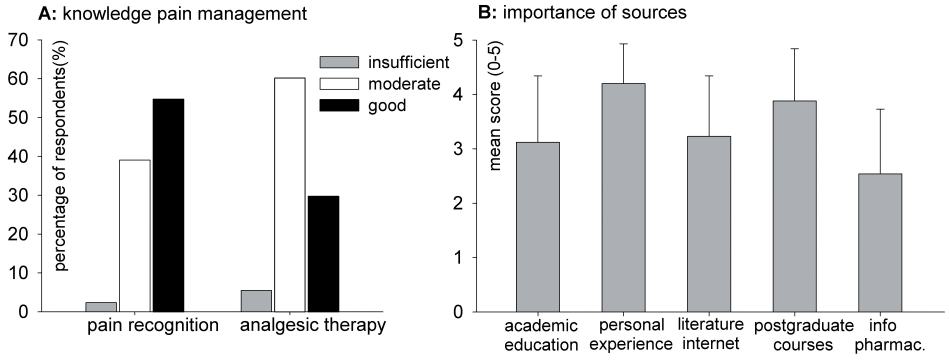

Figure 3 Pain management in horses.

A: Personal perception of knowledge about pain recognition and analgesic therapies in horses. B: mean (± SEM) scores for the estimated importance of several information sources regarding pain management in horses. N=128 respondents (Courtesy of Dujardin and van Loon, 2011).

and postgraduate education were regarded as important sources to increase knowledge in this area (Figure 3B).

The majority of the parameters most often used by equine practitioners to assess pain in horses are behavioural in character, while several physiological parameters such as heart rate are also mentioned (Table 1, Dujardin and van Loon, 2011). Heart rate was found to be the second most often mentioned (47%) pain-related parameter after weight-bearing (50%). This is in agreement with a recent survey in the UK (Price et al., 2002). However, objective studies demonstrating a positive correlation between heart rate and pain in horses are not published yet. This has distinct relevance as heart rate is also influenced by factors other than pain, such as endotoxaemia and hypovolemia as well as mental states like fear and stress (Sellon et al., 2004).

Table 1 Parameters used to assess pain in horses by equine practitioners in the Netherlands and Belgium (N=128 respondents) (Modified from Dujardin and van Loon, 2011).

Pain parameter	Percentage of respondents that cite this parameter (%)
Weight bearing	50
Heart rate	47
Sweating	40
Anorexia	40
Behaviour	37
Severity of colic/lameness	33
Breathing frequency	25
General impression of patient	14
Rolling	13
Body temperature	13
Response to therapy	9
Body posture	9
Response to palpation	8
Type of disorder	8
Outcome of clinical examination	8
Lateral recumbency	8
Sopor	8
Falling to the ground	7
Teeth grinding	6
Anxious look	6
Restlessness	5

Figure 4 shows the results of Visual Analogue Scale (VAS) pain scores attributed to 9 clinical conditions (Dujardin and van Loon, 2011). These results show that the variation in pain scores is substantial, with surgical castration for instance receiving pain scores ranging from 2 to 8 on a 0-10 scale. This wide range of pain scores implies that subjective evaluation by the individual attending veterinarian is of major impact.

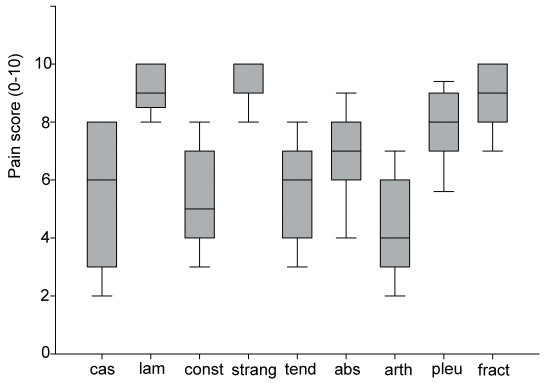

Figure 4 Simple numerical rating scale pain scores for 9 clinical conditions.
Median pain scores with 25th-75th percentiles (bars) and ranges (whiskers) are shown. Cas = surgical castration, Lam = severe acute laminitis, Const = colic due to constipation of the ascending colon, Stran = colic due to strangulation of small intestines or colon, Tend = acute tendinitis of superficial flexor tendon, Abs = solar abscess, Arth = fetlock joint arthrosis with chronic synovitis, Pleu = acute pleuritis, Fract = simple phalanx 3 fracture (N=128 respondents) (Courtesy of Dujardin and van Loon, 2011).

It is generally accepted that horses in severe or acute pain demonstrate recognisable changes in behaviour. These behavioural characteristics are not specific for the source and type of pain, nor do they correlate strictly with severity of pain or progression of a disorder. However, all individual observational aspects have the potential to be refined through further study, especially if a comprehensive knowledge of the equid's normal behaviour is used as a benchmark (Ashley et al., 2005). In order to use these various behavioural and non-behavioural pain-related parameters for assessment of pain in horses, they should be incorporated in objective pain scales.

In pain assessment, simple descriptive scales (SDS) classify pain in terms of absent, mild, moderate or severe. Visual analogue scales (VAS) and Numerical Rating Scales (NRS) provide continuous and discrete 0-10 pain scales. VAS scores are considered to be more sensitive than preset rating scales because they present the experience of pain as a continuum and do not force the observer to decide between limited numbers of restricted categories (Vettorato et al., 2010). In contrast to these simple one-dimensional and thus more or less subjective pain scales, combined interactive and observational multifactor pain behaviour rating scales, used in conjunction with physiological parameters, have been proposed as being more sensitive in identifying and documenting pain in animals (Dobromylskyj et al., 2000). Such composite pain scales have been described and validated for dogs (University of Melbourne Pain Scale, UMPS, by Firth and Haldane, 1999; Glasgow Composite Measures Pain Scale by Holton et al., 2001) and have been used in subsequent studies (Morton et al., 2005; Murrell et al., 2008). For horses, only a limited number of composite pain scales have been described for use in clinical patients (without validation under standardised experimental conditions) (Sellon et al., 2004; Sanz et al., 2009). These pain scales do not include physiological variables. Bussières et al. (2008) and Lindegaard et al. (2010) described and validated composite pain scales that also contain physiological parameters in equine experimental models of acute pain (amphotericin B induced and LPS-induced synovitis respectively). Dutton et al. (2009) described the use of a modified composite pain scale (based on the Glasgow composite scale) to assess the clinical condition of a horse with severe hoof pain and the influence of multimodal pain treatment.

History of local anaesthetic and analgesic techniques

In the last decade, an increased interest in local anaesthetic and analgesic procedures has evolved in equine veterinary medicine (Robinson and Natalini, 2002; Muir, 2005). These techniques offer possibilities for multimodal analgesic therapies, combining different classes of analgesics and different routes of administration and are used both in conscious animals and in combination with general anaesthesia. In the nineteenth century, when general anaesthesia was

not common practice, local anaesthetic techniques were also widely performed and this paragraph gives an historical overview of these techniques.

Epidural and intrathecal anaesthesia

In 1885 Corning, a New York neurologist, found that the injection of a cocaine solution into the spinal canal of the dog induced paralysis and loss of sensation in the hind limbs (Corning, 1885). He used a large dose of local anaesthetic (110 mg 3% cocaine) and fortunately the drug was delivered epidurally rather than in the subarachnoid space, since the latter route might have proven fatal to the patient and might have discouraged others from experimenting with neuraxial anaesthesia. Shortly thereafter, the German zoologist August Bier, the godfather of spinal anaesthesia, published his observations on the injection of cocaine solutions into the subarachnoid space in man (Bier, 1899). Bier performed the first true spinal block using only 15 mg of cocaine for his first patient and then reduced the dose to 5-10 mg for subsequent patients. August Bier and his associate, Dr. Hildebrandt, subjected themselves to spinal anaesthesia. What follows in Bier's paper is a delightful description of the spinal anaesthesia and the creative ways that they used to test the effectiveness of the block, which included the use of sharp instruments, burning cigars and strong blows with a hammer. Bier also described the classic symptoms of a post-dural puncture headache, together

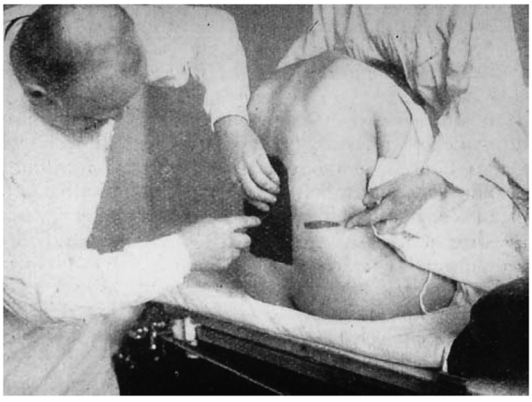

Figure 5 The versatile August Bier (1861-1949), godfather of spinal anaesthesia, performing a spinal anaesthesia around 1925 (Courtesy of Brill et al., 2003).

with nausea and vomiting. They used cocaine mixed with tap water without any apparent sterilisation of the drug or equipment. Possibly, nausea and vomiting were due to a mild form of aseptic meningitis (Mandabach, 2002).

In veterinary practice subarachnoid injections were first performed in France by Cuillé and Sendrail in 1901. They demonstrated the method in the horse, ox and dog, but due to perceived difficulties and dangers the technique was not widely adopted. In the Netherlands, Jacob Schotsman, an equine veterinarian in military service, published his thesis on epidural and subarachnoidal novocaine anaesthesia in the horse in 1927. He described over 40 clinical cases in detail in which epidural or subarachnoidal anaesthesia had been performed for various orthopaedic and soft tissue surgeries.
In the UK, epidural anaesthesia was introduced in veterinary practice in 1935 by Geoffrey Brook (Figure 6). He described epidural anaesthesia in several domestic animals (including the horse, dog and ox) with very detailed information on general anatomy of the central nervous system and the anatomic landmarks aiding spinal injections, modes of action of several anaesthetic solutions (including procaine, novocaine and tutocaine, as sole agents or combined with ephedrine, adrenalin or sodium bicarbonate), as well as the indications for the application of epidural anaesthesia.

The first published report on the use of opioids for intrathecal anaesthesia in humans is from a Romanian surgeon, Racoviceanu-Pitesti, who presented this in Paris in 1901. This was almost a century before the use of opioids for epidural analgesia was reported. Behar and his colleagues published the first report on the epidural use of morphine for the treatment of pain in *The Lancet* in 1979 and in the same year, Cousin and colleagues published their results on the use of epidural meperidine.

632 No. 22. Vol. xv. THE VETERINARY RECORD. June 1, 1935.

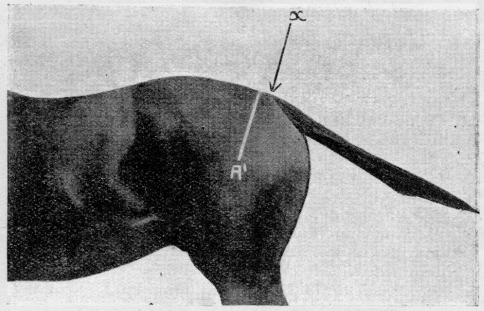

Fig. 18.—The Surface Markings for Location of Site of Injection.

A¹=line connecting hip joints. Note that this cuts the upper contour of animal at right angles.

x=site of injection; arrow depicts the direction in which needle is to be inserted.

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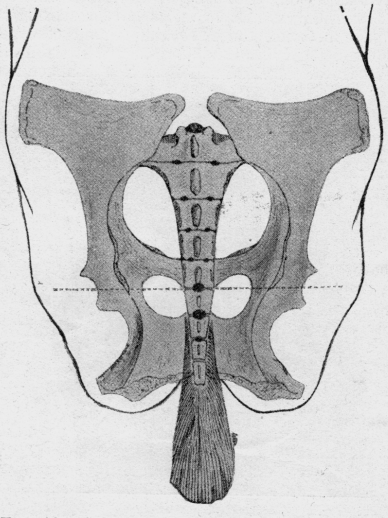

Fig. 19.—Schematic drawing of Pelvic Girdle in relation to Contour of the Animal.

Note.—A transverse line passing through the hip joints cuts the mid-line at the level of the sacro-coccygeal space. The injection is made through the first intercoccygeal space depicted in the figure a little further back.

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operator to palpate the contour of the "croup" backwards and downwards as far as the first obvious depression between two bony spines and state that "counting the spines from the first sacral backwards enables one to recognise the first intercoccygeal space with certainty."

The writer has found difficulty in estimating this site, particularly in heavy draught horses. A combination of both the above methods is recommended.

2. Needles to be Used: The Equine Coccygeal Epidural needle is similar in size and design to the corresponding needle for bovine work (see this journal, p. 601).

3. Mode of Injection.—An area of skin is clipped or shaved and treated with ether and spirit. The control of the patient depends on its temperament, a twitch and a sideline being applied if deemed necessary. In the young foal neither need be used, whereas in the intractable thoroughbred injection may be found impossible. A little local anæsthetic is injected under the skin. With an assistant holding the tail in the normal position the operator inserts the needle in the mid-line at right-angles to the upper contour of the tail. After this the procedure is quite similar to that in the ox (see this journal, p. 600), except that the canal is usually entered at the greater depth of 4 to 5 cm.

4. Failure to Enter the Epidural Space: The first three factors enumerated under the heading in the ox (see this journal, p. 601), apply to the horse. Failure is more common in the horse since the bony landmarks are not so easily palpated and the coccygeal canal is more deeply situated.

5. Unsatisfactory Development of Anæsthesia: This may depend upon the extraspinal escape of the solution. Cuillé and Chelle[60] believe that the intervertebral foramina are often incompletely shut off and permit leakage

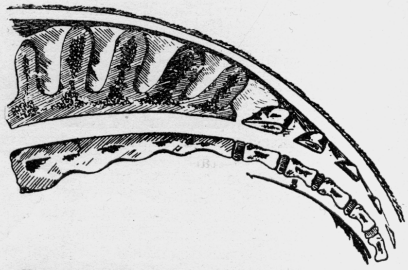

Fig. 20.—Sagittal Section of the Sacro-coccygeal Region of the Spine of the Horse.

Note the sharp backward and downward sweep of the spinal canal.

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Figure 6 Spinal (epidural) anaesthesia in the horse by Geoffrey B. Brook, The Veterinary Record (1935).

Locoregional anaesthetic and analgesic techniques as a component of multimodal pain management

Multimodal pain management refers to the practice of combining multiple analgesic drug classes or techniques to target different points along the pain pathway (Lamont, 2008). This technique takes advantage of additive or synergistic analgesic effects while allowing for lower doses of individual analgesic agents, thus limiting negative side-effects, and has therefore become widely accepted in veterinary (and human) medicine.

Systemically administered non steroidal anti-inflammatory drugs (NSAIDs) form the mainstay of many equine pain protocols. Using multimodal analgesic techniques, NSAIDs can be combined with locally applied analgesics, both in peroperative and postoperative analgesia. Especially for drugs like opioids, that may produce several side-effects when administered systemically (Bennet and Steffey, 2002; Boscan et al., 2006), locoregional administration has the advantage of obtaining relatively high target tissue concentrations while retaining low systemic plasma concentrations, thus reducing negative side-effects. By inhibiting the neural conduction from the surgical site to the spinal cord (transduction and transmission phases) as well as the processing of the afferent information in the spinal cord dorsal horn (modulation phase), locoregional analgesia can suppress subsequent neurohumoral activation and thus prevent spinal cord sensitisation and wind-up (Buvanendran and Kroin, 2009). Therefore, one of the aims in this thesis was to assess clinical efficacy of locally applied analgesic drugs.

Aim and outline of the thesis

The goal of this thesis and of the experiments that form the base of it is twofold. On the one hand the experiments aim to increase knowledge on pain recognition and quantification of nociception in horses. On the other hand, the aim was to determine clinical efficacy of locoregional analgesic techniques in horses using various approaches. Equine nociception has been assessed by neurophysiological techniques that quantify spinal cord dorsal horn activity and reflex tests that quantify nociceptive reflex patterns. Equine clinical pain has been assessed

by gait and behavioural analysis, systemic and synovial fluid inflammatory parameters in experimentally induced lameness and by composite pain scale analysis in clinical patients. In this way, the thesis aims to add both fundamental and clinically applicable knowledge to the equine pain and analgesia research field.

Chapters 2, 3 and 4 focus on physiological nociception and the effects of nociceptive stimulation of the dorsal horn of the spinal cord. **Chapter 2** describes the first study that attempted to develop an equine model for quantification of caudal nociception using spinal cord somatosensory evoked potentials (SSEPs). In this study, caudal epidural placement of electrodes was used to measure spinal cord dorsal horn activity in conscious ponies. In **Chapter 3**, the SSEP model was optimised by using lumbosacrally placed spinal electrodes in anaesthetised ponies, furthermore quantification of physiological nociceptive input into the spinal cord dorsal horn is described. **Chapter 4** describes the effects of a low concentration of lumbosacral epidural ropivacaine in ponies, using the SSEP model in anaesthetised ponies and pressure algometry for assessing mechanical nociceptive thresholds (MNTs) and ataxia scores in conscious animals.

Chapters 5, 6 and 7 focus on clinical pain and treatment effects of various analgesics using an LPS induced acute synovitis model in healthy horses. In **Chapter 5**, the analgesic, anti-hyperalgesic and anti-inflammatory effects of epidural morphine are described using this LPS induced synovitis model. **Chapter 6** describes the analgesic and anti-inflammatory effects of intra-articular morphine. In both chapters 5 and 6 subjective and objective lameness scores are used to assess locomotion of horses walking and trotting on the treadmill. Additionally, behavioural analysis and synovial fluid inflammatory marker analysis is performed to assess the anti-inflammatory effects of morphine. Anti-hyperalgesic effects in chapter 5 are assessed by MNTs based on pressure algometry. In **Chapter 7** possible up-regulation of synovial membrane μ-opioid receptors is assessed using immuno-histochemistry during acute inflammatory conditions and the effects of non-steroidal anti-inflammatory drug (NSAID)

treatment are evaluated. **Chapter 8** focuses on the assessment of clinical pain in equine patients. It describes the use of a composite pain scale (CPS) in clinical equine patients that can be used to objectify and quantify equine pain. In this pilot study, usefulness and applicability of an existing composite pain scale, which had been validated for acute inflammatory joint pain are evaluated for wider use in equine patients with a variety of clinical painful problems. In **Chapter 9**, this CPS pain scoring system has been more extensively studied in a larger population of horses that received intensive care after emergency gastrointestinal (colic) surgery. Clinical "validation" has been performed by comparison with another visceral equine pain scale and the influence of individual components of the CPS on total pain scores has been assessed.

Chapter 10 contains the summary and the general discussion of this thesis.

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2

Use of epidurally derived evoked potentials for quantification of caudal nociception in ponies

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American Journal of Veterinary Research (2009) 70 (7) 813-819

Summary

Objective To determine whether epidurally derived evoked potentials (EPs) can be used to reliably assess nociception and antinociception in ponies.

Animals 7 ponies.

Procedures EPs and electromyograms (EMGs) from the quadriceps femoris muscles were recorded simultaneously, following electrical stimulation applied to the distal portion of the hind limb. The effect of increasing stimulus intensity, conduction velocities of the stimulated nerves, effect of epidurally applied methadone, and effect of systemically administered propofol were evaluated.

Results In the EP and EMG waveforms, 2 distinct complexes, the EP N25 and P50 and the EMG P27 and N62, respectively, were identified. On the basis of their latency and calculated conduction velocities, the EP P50 and EMG N62 were considered to be related to nociception (Aδmediated). All complexes increased significantly in amplitude with increasing stimulus intensity and decreased significantly following epidural administration of methadone or systemic administration of propofol.

Conclusions and Clinical Relevance Although the experimental setup allowed successful discrimination between tactile- and nociceptive-associated responses, the identified EPs, considered to reflect activity in the spinal cord, could not be definitively differentiated from activity in the lumbosacral epaxial musculature. Further research is required to refine measurement techniques to allow for discrimination between these 2 signals. Similar to other species, neurophysiologic variables such as EPs could potentially become a useful additional tool in quantifying nociception in equidae.

Introduction

The epidural technique for analgesia of the caudal portion of the body in horses is widely accepted (Olbrich and Mosing, 2003; Ganidagli et al., 2004; Doherty et al., 1997; Natalini and Linardi, 2006). Epidural opioid administration provides regional analgesia with minimal behavioural and gastrointestinal adverse effects (Natalini and Robinson, 2003; Mircica et al., 2003; DeRossi et al., 2004). However, options for valid quantitative assessment of antinociception are limited and primarily focused on NWRs (Price et al., 2003; Spadavecchia et al., 2004; Redua et al., 2002). Nociceptive withdrawal reflexes provide important but rather limited information on nociception because they rely primarily on afferent dorsal horn activation and subsequent ventral horn motor neuron activation. Suppression of the latter only, consequently resulting in the absence of the spinal reflex, could falsely be interpreted as antinociception, when nociceptive dorsal horn activity and subsequent nociceptive brain center activation remain partially intact (You et al., 2005; Kim et al., 2007). Valid quantitative assessment of caudal epidural analgesia by recording epidurally derived EPs, rather than NWRs, could potentially overcome this problem. Evoked potentials are epidurally recorded responses time-locked to stimulation of peripheral somatosensory fibres and are, as such, representative of spinal somatosensory processing. Similar to cortical somatosensory EPs (Stienen et al., 2004; Banoub et al., 2003), changes in EP waveform amplitude or latency may be considered to indicate altered spinal somatosensory processing. Therefore, following noxious stimulation, EPs may potentially be used to detect changes in spinal antinociception. In contrast to various other species, such as rabbits (De Haan et al., 1996), rats (Fehlings et al., 1988; Shanker et al., 1991; Winkler et al., 1988+1997), and humans (Hallstrom et al., 1989; Britt et al., 1986; Jeanmonod et al, 1991; Kumar et al., 2000), EPs have not been characterised in equidae or evaluated for their applicability to potentially improve the quantification of nociception. The purpose of the study reported here was to determine whether epidurally derived EPs can be used to reliably assess nociception and antinociception in equidae.

Our hypothesis was that by determining the conduction velocities of stimulated nerve afferents, the effect of increasing stimulus intensity, and the effect of epidural opioid and systemic hypnotic drug administration, we could validate our equine nociception technique on the basis of epidurally derived EPs.

Materials and Methods

Animals

The study design was approved by the institutional ethical committee for animal experiments. Selection and subsequent inclusion of the 7 pony geldings (mean ± SD age, 11 ± 1.1 years; weight, 172 ± 13.7 kg) was based on acceptance of handling. The ponies were acclimatised to the experimental room prior to the first experiment. During the experiment, a second pony accompanied the experimental pony to avoid social isolation stress responses.

Instrumentation

The site of insertion of the epidural catheter, between the first and second coccygeal vertebrae, was surgically prepared and locally anesthetised (2 mL of lidocaine HCl 2%,[a] SC). A 16-gauge Tuohy spinal needle[b] was inserted into the epidural space. For EP recording, an epidural electrode (custom made from an epidural catheter[b]) was advanced through the Tuohy needle for 22 to 30 cm up to the lumbosacral junction. A second Tuohy needle was placed epidurally to introduce a 19-gauge catheter for 10 cm for drug administration. While the catheter and the electrode were left in situ, the Tuohy needles were removed and the electrode and catheter were covered with sterile gauzes. Two needle electrodes were placed SC at the level of the lumbosacral junction on the back, one in the median plane (ground electrode) and one 4 cm out of the median plane to the right (reference electrode). Additionally, 3 needle electrodes were placed SC at the level of the right quadriceps femoris muscle with an interelectrode distance of 3 cm to measure the quadriceps femoris EMG. For generating EPs and EMGs, 2 stimulus electrodes (cathode proximal) were placed SC on the distal aspect of the right hindlimb, halfway between the coronary band and the metatarsophalangeal

(fetlock) joint. For proximal electrical stimulation, 2 stimulus electrodes (cathode proximal) were placed SC on the right hind limb just below the talocrural joint. The positions of the distal and proximal stimulating electrodes and the insertion site of the epidural electrode and catheter were recorded (Figure 1).

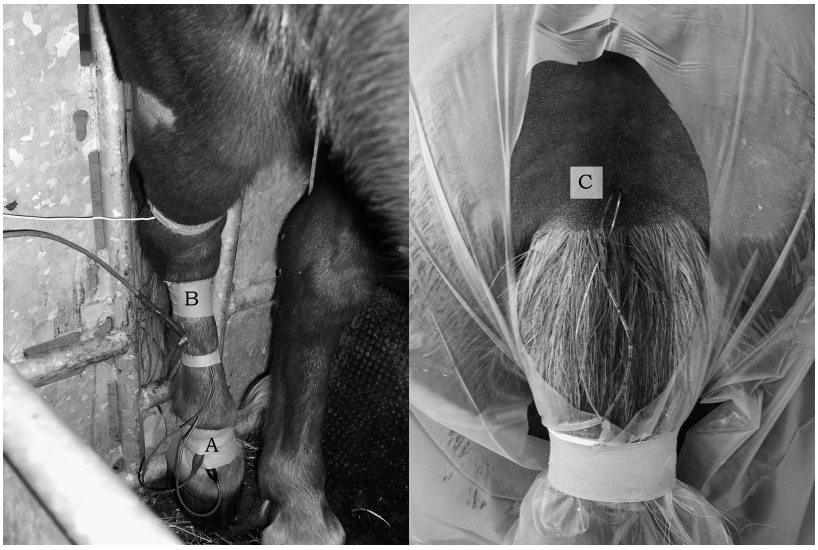

Figure 1 Photographs of the hindlimbs (left) and caudal lumbar area (right) of a pony in a study of epidurally derived evoked potentials for quantification of caudal nociception in ponies. Notice locations of distal stimulating electrodes (A) and site of insertion of epidural electrode and catheter (between coccygeal vertebrae 1 and 2 (C).

Generating and recording of EP and EMG

Following stimulation of the distal portion of the hindlimb, EPs and EMGs were recorded simultaneously. Stimuli for a single recording consisted of 32 square-wave electrical stimuli of 0.5 milliseconds' duration each, with an intensity of 0.2 to 4 mA in session 1, and 3 to 4 mA in sessions 2, 3, and 4 at a fixed stimulus frequency of 0.5 Hz. Stimuli were generated by a stimulator[c] triggered by software developed by one of the authors (AD). The stimuli were delivered to a stimulation isolation unit[d] and a constant current unit[e] that controlled the stimulus intensity. The EPs were recorded from the epidural electrode with the 2 needle electrodes close to the lumbosacral junction on the back serving as reference and ground,

respectively. Electromyogram was recorded from the 3 needle electrodes (active, reference, and ground electrodes) located at the right quadriceps femoris muscle. Signals were fed to separate but identical bioelectric amplifiers[f], amplified 5,000 times, and band-pass filtered between 1.59 and 1,000 Hz. A 50-Hz notch filter was used to eliminate electrical net interference. Subsequently, the analog signals were fed to the data acquisition hardware, digitised online at 10,000 Hz[g], and entered into the data acquisition software environment, also responsible for stimulus generation. Each EP-EMG recording consisted of 32 averaged, subsequent responses of 500 milliseconds (10 milliseconds before stimulus and 490 milliseconds after stimulus).

Experimental procedures

Four experimental sessions were performed. In session 1, data were recorded in response to distal stimulation with intensities ranging from 0.2 to 4 mA, administered in random order with the same order for all ponies. After collecting the electrophysiologic responses at the different intensities, data were collected in response to more proximal stimulation, just distal to the talocrural joint, by use of the maximum stimulus intensity (3 or 4 mA). In sessions 2, 3, and 4, electrical stimulation was performed by use of the optimal stimulus intensity for each pony (3 or 4 mA), as established in session 1. In all sessions, 3 baseline runs were recorded with 5-minute intervals.

Subsequently, in sessions 2 and 3, either methadone[h] (0.5 mg/kg in 10 mL of saline [0.9% NaCl] solution) or a corresponding volume of saline solution was administered epidurally and recordings were performed every 5 minutes for a 40-minute period. Next, naloxone[i] (0.04 to 0.06 mg/kg in 10 mL of saline solution) or a corresponding volume of saline solution was administered epidurally, and recordings were performed every 5 minutes for another 40 minutes. Four ponies received methadone-naloxone in session 2 and saline solution–saline solution in session 3. In the remaining 3 ponies, the order of treatment was reversed. A minimum of 10 days elapsed between each of the sessions. In session 4, 4 ponies were used. After 3 baseline runs, a slowly administered bolus of propofol[j] (0.5 mg/kg administered in 1 minute) was administered IV, followed by a constant

rate infusion of propofol (0.1 mg/kg/min). After 20 minutes of stabilization, 3 recordings were performed with 5-minute intervals. The ponies were physically supported with a sling during this part of the study. At the end of each session, the ponies were deinstrumented and administered antimicrobials (benzylpenicillin[k] [2 X 105 U/kg] and gentamicin[l] [6.6 mg/kg, IV]) and an NSAID (flunixin meglumine[m] [1 mg/kg, IV]).

Data and statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed by use of commercially available software[n]. Values of $P < 0.05$ were considered significant. The EPs and EMG recordings were evaluated by use of the RDF (van Oostrom et al., 2007). The RDF, an expression of the overall shape of the waveform (EP and EMG) in the latency range studied, is obtained by determining the mean of the absolute differences between all pairs of subsequent sampled data points y_k in a specified latency range from x to m, as follows:

$$\left(\text{Equation 1 : RDF} = \frac{1}{m - x}\sum_{k=x+1}^{m}\left|y_k - y_{k-1}\right|\right).$$

Decreases in amplitude and increases in latency of the waveform components will decrease the value of the RDF, whereas increases in amplitude and decreases in latency will increase the value of the RDF. When choosing the RDF latency range (x to m) in the group with the strongest signals (4-mA stimulation intensity) or in the baseline stimulations during the other sessions, individual signals can be analysed by use of this fixed latency range. The x and m boundaries that were used for calculation of RDFs were determined (Figure 2). Therefore, the RDF is a highly objective method to evaluate EP signals without the need for choosing peak amplitudes in individual signals, which is prone to confounding errors, especially when signals are weak (at low stimulus intensities or during methadone-propofol treatment in the present study). To establish the fibre types involved in the responses recorded in this study, we determined the CV by using the latency shifts for the EP N25/P50, calculated by subtracting the latency obtained after proximal stimulation from the latency obtained after distal stimulation. Subsequently, the CV was calculated per individual animal by

dividing the distance between the 2 stimulation sites (12 to 18 cm, measured on the distal portion of the hindlimb of every pony separately) by the latency shift of both peaks. Because we were interested in the Aδ-afferent transmitted EPs that were elicited in the spinal cord, we investigated the 30- to 80-millisecond latency range. The RDFs were calculated for the most consistent and prominent occurring complexes, that is, for the EPs in the latency range of 30 to 70 milliseconds (EP P50) and for the EMG in the latency range of 52 to 75 milliseconds (EMG N62 [Figure 2]). The EP N25 and EMG P27 were, on the basis of CV data, considered to be generated by stimulation of tactile Aβ-fibres and were not further analysed.
For statistical analysis, the RDF values of session 1 were normalised to the mean RDF following stimulation with 0.2 and 0.5 mA for each pony and response (EP-EMG) separately. For session 2, 3, and 4, the RDF values were normalised to their value of the 3 baseline stimulations (relative RDF) and subsequently expressed as the mean of 3 baseline stimulations 5 to 20 minutes after methadone administration (t1), 25 to 40 minutes after methadone administration (t2), 45 to 60 minutes after methadone administration (t3), and 65 to 80 minutes after methadone administration (t4). For session 1, a 1-way RM-ANOVA design was performed with factor intensity. For sessions 2 and 3, a 2-way RM-ANOVA design with factors treatment and time was performed. The RM-ANOVA designs were followed by post-hoc analysis when appropriate. Post-hoc tests that were used were 1-way RM-ANOVA design with factor time and, subsequently, paired *t*-tests. Results from the statistical analysis in sessions 2 and 3 were presented until 60 minutes after methadone administration (t3) because in 1 pony, recording was stopped after 60 minutes because of technical problems. For session 4, a paired *t*-test was performed.

Results

EP and EMG component definition

In the EP-waveform, a negative (EP N25) and a positive (EP P50) complex could be determined (Figure 2). The EMG-waveform consisted of 2 distinct complexes, EMG P27 and EMG N62. It was decided to evaluate the EP waveform with the

latency of 28 to 70 milliseconds and the EMG waveform with the latency of 52 to 75 milliseconds as 1 complex.

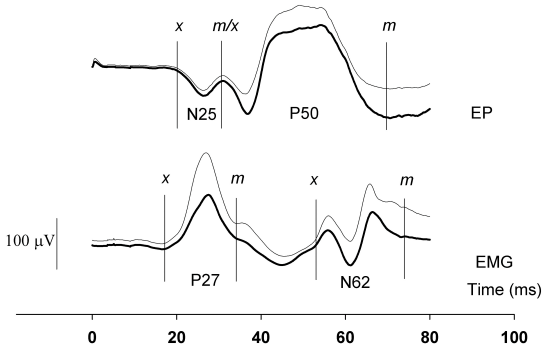

Figure 2 Mean (thick lines) + SEM (thin lines) EP-EMG waveforms in a pony resulting from 3 baseline recordings with stimulus intensity of 4 mA and stimulus frequency of 0.5 Hz. X-axis values indicate latency from distal stimulation 100 μV = Amplitude. Vertical lines (*x* to *m*) indicate boundaries for RDF calculation.

Determination of CV

Mean latency shifts allowed calculation of CV to be within the Aα/β domain (> 35 m/s) for EP N25 and EMG P27 (mean ± SEM, 47.89 ± 7.72 m/s and 70.71 ± 13.06 m/s, respectively) and within the Aδ domain (4 to 35 m/s) for EP P50 and EMG N62 (mean ± SEM, 25.57 ± 3.03 m/s and 31.10 ± 3.41 m/s, respectively). In 2 animals (pony 2 and 5), no EMG N62 and, thus, no CV based on the EMG could be determined after proximal stimulation. In 1 animal (pony 7), no distinct peaks and, thus, no CV could be determined in both the EP and EMG after proximal stimulation.

Stimulus intensity response characteristics

The RDF EP P50 ($P < 0.001$) and RDF EMG N62 ($P = 0.007$) significantly increased with increasing stimulus intensity (Figure 3). All ponies had aversive behaviour to some extent, including a withdrawal reflex and full-body movements, during the top range of electrical stimulation.

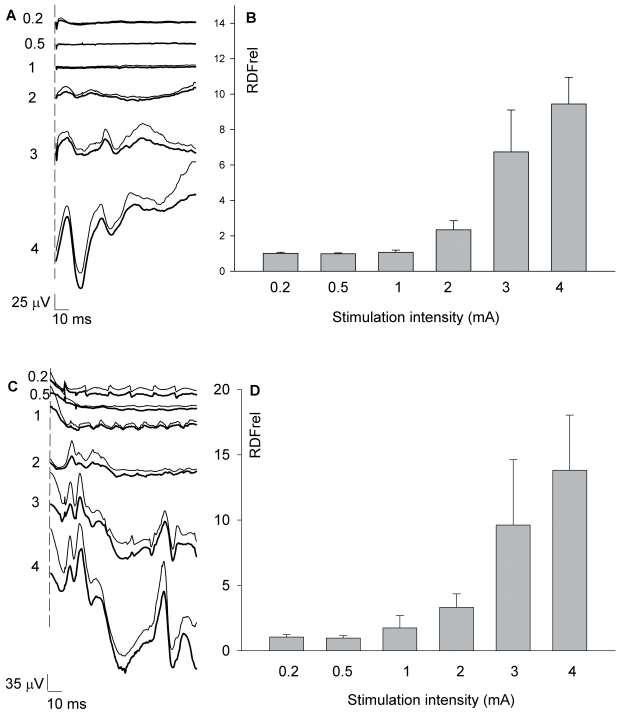

Figure 3 Mean (thick lines in A and C) + SEM (thin lines in A and C) stimulus intensity response characteristics in 7 ponies. A: EP readings. B: Relative RDF of EP P50. C: EMG readings. D: Relative RDF of EMG N62. RDFrel = relative RDF, normalised to mean of 0.2 and 0.5 mA stimulations. Y axis in A and C indicates stimulus onset.

Effect of epidural opioid administration

In 6 of 7 ponies, the 4-mA stimulation intensity was used. In 1 pony, this was not tolerated; therefore, 3-mA stimulation was used. Administration of either methadone or saline solution led to slight and transient (seconds) behavioural responses during the injection phase, characterised by looking backward and slight restlessness. No excitation or sedation occurred. The overall mean EP and EMG Aδ-related waveforms with the influence of epidurally administered methadone and the partial reversal effect of epidurally administered naloxone were determined (Figure 4). Overall statistical analysis revealed that the mean relative EP P50 RDF was affected differently by saline solution and methadone administration over time ($P = 0.03$). Post hoc analysis revealed that the mean relative EP P50 RDF decreased significantly following both saline solution

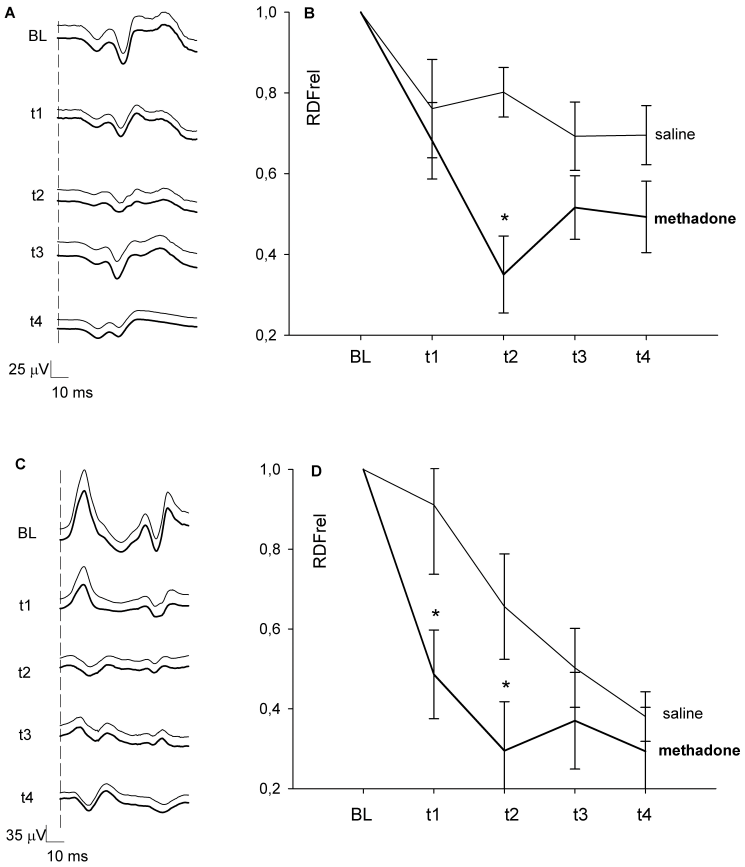

Figure 4 Mean (thick lines in A and C) + SEM (thin lines in A and C) methadone response characteristics in 7 ponies. A: EP readings. B: Relative RDF of EP P50. C: EMG readings. D: Relative RDF of N62 RDF after epidural administration of methadone (0.5 mg/kg) and naloxone (0.04-0.06 mg/kg) or saline solution. BL = baseline. t1 = Time points 5 to 20 minutes after administration of methadone or saline solution. t2 = Time points 25 to 40 minutes after administration of methadone or saline solution. t3 = Time points 45 to 60 minutes after administration of methadone or saline solution. t4 = Time points 65 to 80 minutes after administration of methadone or saline solution. At t3, naloxone (methadone group) or saline solution (saline group) was administered. *Significant ($P < 0.05$) difference between groups. *See* Figure 3 for remainder of key.

($P = 0.008$) and methadone ($P < 0.001$) injection. However, the decrease of the mean relative EP P50 RDF was significantly ($P = 0.011$) greater following methadone administration than following saline solution administration at t2. Overall statistical analysis revealed that the mean relative EMG N62 RDF was affected differently by saline solution and methadone administration over time

($P = 0.035$). Post-hoc analysis revealed that the mean relative EMG N62 RDF significantly decreased following both saline solution ($P = 0.006$) and methadone ($P < 0.001$) injection. However, the decrease of the mean relative EMG N62 RDF was significantly greater following methadone than following saline solution at t1 ($P = 0.032$) and t2 ($P = 0.018$).

Effect of systemically administered hypnotic agents

Administration of a loading dose plus constant rate infusion of propofol led to standing sedation in all ponies. While remaining in a standing position, they were physically supported by a sling. The NWR disappeared, and statistical analysis revealed that the mean relative RDF of the EP P50 ($P = 0.001$) and the EMG N62 ($P < 0.001$) decreased significantly (Figure 5).

Discussion

In the present study, EPs were characterised in ponies and subsequently evaluated for their applicability to quantifiably determine epidural antinociception in equidae. On the basis of the CV determined in this model, the EP P50 component was determined to be primarily related to nociceptive Aδ-fibre activity. Furthermore, the EP P50 increased with a stepwise increase in stimulus intensity and decreased following epidural methadone administration. These combined results strongly suggest the EP P50 to be quantitatively related to caudal nociception in equidae, thereby consequently proposing the EP P50 to be used as a valid quantitative method for determining the level of analgesia, compared with the more traditionally applied reflex. However, although the EP waveform characteristics (peak latency and general waveform) differed from the EMG waveform, the presence of a considerable propofol effect on the EPs prohibits the definitive exclusion of EMG or ventral horn interference in the EPs. The combined results suggest that the stimulation and recording setup described in this report allows discrimination between nociceptive and other somatosensory responses. However, it ultimately remains undetermined whether EPs are of specific neural rather than muscular origin. To use EPs for quantifying epidural antinociception,

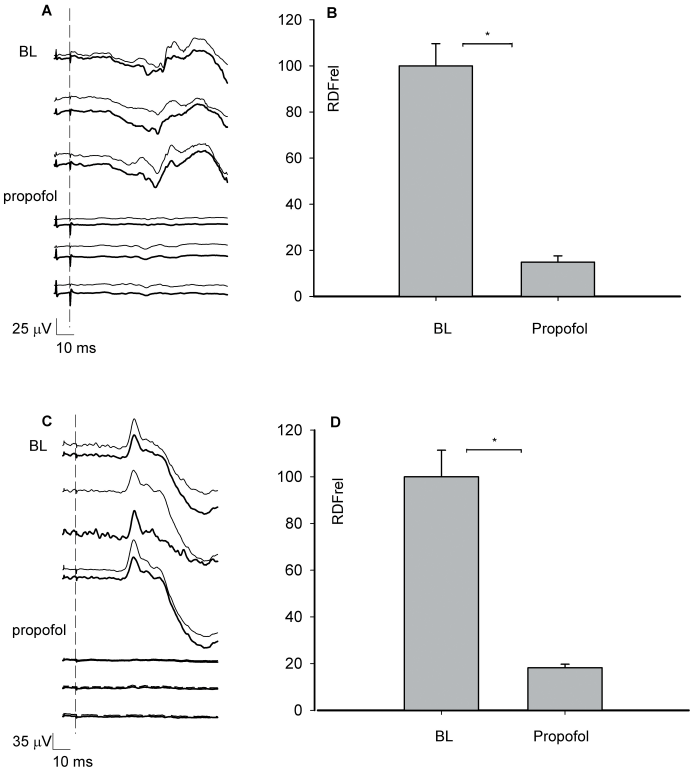

Figure 5 Mean (thick lines in A and C) + SEM (thin lines in A and C) propofol-response characteristics in 4 ponies. A: EP readings. Y axis indicates stimulus intensity (mA). B: Relative RDF of EP P50. C: EMG readings. Y axis indicates stimulus intensity (mA). D: Relative RDF of EMG N62, during administration of propofol (0.5 mg/kg loading dose and 0.1 mg/kg/min constant rate infusion). *See* Figures 3 and 4 for remainder of key.

by definition the EP must be nociception-related, that is, mediated by peripheral afferent fibres such as Aδ- and C-fibres (Bromm and Lorenz, 1998). The fibre type mediating a specific response can be determined by calculating its CV. With a mean distance of 1 m between the stimulation and recording site in the present study and considering a peripheral CV of 15 to 35 m/s for Aδ-fibres, < 15 m/s for C-fibres, and 35 to 75 m/s for Aα/β-fibres (Stienen et al., 2004; Shaw et al., 1999); EP components from 28 to 67 milliseconds, such as the present EP P50, can be considered Aδ-fibre–related. In the present study, the results obtained with this approach indicated that the EP P50 was mediated by Aδ-fibres and the EP N25 was mediated by Aα/β-fibres. This was further supported by the latency shifts and

calculated CVs of the N25 and P50 complexes. The same is true for the early and late complex of the EMG. On the basis of the distance between the stimulation and EMG recording site, conduction velocities of Aβ- and Aδ-fibres, and a 5- to 10-millisecond delay of ventral horn efferent fibres to muscles (Spadavecchia et al., 2004), the EMG P27 and N62 was ascribed to Aβ-and Aδ-mediated stimulation, respectively. This was further supported by the latency shifts of the P27 and N62 complexes, documented by a fixed recording site after both proximal and distal stimulation. These findings are consistent with findings of Spadavecchia et al. (2004), who described a short-latency (0 to 80 milliseconds) Aβ-mediated EMG response and a longer-latency (80 to 250 milliseconds) Aδ-mediated EMG response in Warmbloods. Taken together, although the stimulus modality used in the present study (electrical stimulation) appeared to be non-nociceptive specific, the relatively long afferent pathways (approx. 1 m) allowed for a clear distinction between the different types of afferent fibres and, consequently, the evaluation of specific nociceptive (ie, primarily Aδ-fibre–mediated) responses after electrical stimulation. To further validate the techniques used here, we compared the effects of a μ-opioid analgesic and a nonanalgesic hypnotic between the EPs and EMGs. With respect to the effect of μ-opioid analgesics, μ-opioid receptors are primarily presynaptically located on the primary afferent fibres and in the neurites of dorsal root ganglion cells (Yaksh, 1987) and, to a lesser degree, postsynaptically in the different layers of the dorsal horn of the spinal cord (Dickenson, 1995; Yaksh, 1981; Kelly et al., 2001). Although μ-opioids are considered to primarily affect nociceptive Aδ-/C rather than tactile Aβ-afferent fibres (Yeomans et al., 1995), methadone in the present study decreased both the Aδ-related EP P50/EMG N62 and the Aβ-related EP N25/EMG P27. Similar findings were described by Carpenter et al. (2000) and Clarke et al. (1988), who determined that opioids inhibit Aβ-, Aδ-, and C-fibre evoked responses. Differences in selectivity for Aδ-/C- and Aβ-afferent fibres as reported in the literature may be explained by differences in doses or route of administration as well as by species differences.

In the present study, the high dose of methadone (0.5 mg/kg) may have led to high opioid concentrations in the dorsal horn and, thus, may have accounted for the effect on both nociceptive- (Aδ) and tactile- (Aβ) mediated sensory transmission.

The fact that following epidural saline solution injection, both the RDF EP and RDF EMG changed can be explained by habituation to the repeated electrical stimulation. Besides validation with the application of opioids, we also used the nonanalgesic hypnotic propofol for additional validation studies. Propofol primarily suppresses ventral horn spinal cord neurons rather than dorsal horn spinal cord neurons (Kim et al., 2007; Barter et al., 2008). Therefore, during application of relatively low doses of propofol, absence of the NWR relying on afferent dorsal horn activation and subsequent ventral horn motor activation could mistakenly be interpreted as antinociception, although nociceptive dorsal horn activity and subsequent nociceptive brain centre activation can remain partially intact (You et al., 2005; Kim et al., 2007). The same has been reported for inhalation anaesthetics, like isoflurane and halothane (You et al., 2005; Jinks et al., 2003). In this line of reasoning, in the present study, we expected EMG to be decreased or even absent, whereas EPs were expected to be intact following propofol administration. However, both EMG and EP decreased to similar values following propofol administration. From these combined results, we concluded that the EP did not definitively reflect solely afferent dorsal horn activity. A possible explanation could be found in the location of the epidural electrode. During advancement of the epidural electrode, the measuring tip could have been translocated from a dorsal position to a more ventral position. Therefore, the disadvantage of the caudal epidural placement of the electrode is found in the uncertainty regarding its ultimate location, both in relation to the lumbosacral junction and in relation to the dorsal horn section of the spinal cord. In addition, both the active (epidural electrode) and the reference electrode were located close to the lumbosacral epaxial muscles (longissimus dorsi) of which activity might have interfered with the EP signals from the epidural space as well.

Although the stimulation setup reported here allows the successful discrimination between nociceptive and other somatosensory responses, additional research is needed for further optimisation of the recording configuration to obtain clean (ie, EMG-free) dorsal horn neural activity. This would contribute to the development

of a valid and quantitative measure of spinal nociceptive processing for the assessment of new analgesics or improvement of analgesia protocols in equidae.

Manufacturers' addresses

[a] Lidocaine HCl 2%, B. Braun, Melsungen, Germany.

[b] Perifix 402 filter set, B. Braun, Melsungen, Germany.

[c] Stimulator Model S88, Grass Medical Instruments, Quincy, Mass.

[d] Model SUI5, Grass Medical Instruments, Quincy, Mass.

[e] Model CCU 1A, Grass Medical Instruments, Quincy, Mass.

[f] Bio electric amplifier AB 601 G, Nihon Kohden, Tokyo, Japan.

[g] PCI 6251, Labview, National Instruments, Woerden, The Netherlands.

[h] Methadon HCl, Eurovet Animal Health, Bladel, The Netherlands.

[i] Naloxon HCl, University Pharmacy, Utrecht, The Netherlands.

[j] Propovet, Abbott Animal Health, Zwolle, The Netherlands.

[k] Benzylpenicilline Natrium, Eurovet Animal Health, Bladel, The Netherlands.

[l] Gentamycine 5%, Eurovet Animal Health, Bladel, The Netherlands.

[m] Bedozane, Eurovet Animal Health, Bladel, The Netherlands.

[n] SPSS, version 12.0, SPSS Inc, Chicago, Ill.

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3

Lumbosacral spinal cord somatosensory evoked potentials for quantification of nociception in horses

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Equine Veterinary Journal 2010 42 (3) 255-260

Summary

Reasons for performing study There is a need for objective evaluation and quantification of the efficacy of analgesic drugs and analgesic techniques in horses.

Objectives To determine whether lumbosacral spinal cord somatosensory evoked potentials (SSEP) can be a useful and reliable tool to assess nociception in equines.

Methods SSEPs and electromyograms (EMG) from the epaxial muscles were recorded simultaneously, following electrical stimulation applied to the distal hindlimb in lightly anaesthetised Shetland ponies (n = 7). In order to validate the model, the effect of increasing stimulus intensity was documented and the conduction velocities (CV) of the stimulated nerves were calculated. The effect of epidurally applied methadone (0.4 mg/kg bwt) in a randomised, crossover design was investigated.

Results Two distinct complexes (N1P1 and N2P2) were identified in the SSEP waveform. Based on their latency and conduction velocity and the depressant effect of epidurally applied methadone, the SSEP N2P2 was ascribed to nociceptive Aδ-afferent stimulation. The SSEP N1P1 originated from non-nociceptive Aβ-afferent stimulation and was not influenced by epidurally applied methadone.

Conclusions and potential relevance The nociceptive Aδ-component of the SSEP, the N2P2 complex, is presented as a valid and quantitative parameter of spinal nociceptive processing in the horse. Validation of the equine SSEP model enables the analgesic effects of new analgesics/analgesic techniques to be quantified and analgesia protocols for caudal epidural analgesia in *equidae* to be improved.

Introduction

Somatosensory-evoked potentials (SEPs) evoked by stimulation of peripheral somatosensory fibres appear as a waveform consisting of positive and negative peaks with different amplitudes and times of onset (latency) after stimulation (Stienen et al., 2006). Spinally derived SEPs (SSEPs) are responses, recorded at the level of the spinal cord, that are time-locked to stimulation of peripheral somatosensory fibres, and as such are considered to be representative of spinal somatosensory processing. Similar to cortical SEPs (Banoub et al., 2003; Stienen et al., 2004), changes in SSEP waveform amplitude and/or latency may be considered to indicate altered somatosensory processing. Therefore, SSEPs recorded following noxious stimulation may potentially be used to detect changes in spinal (anti) nociception. Spinally derived evoked potentials (SSEPs) have been described in various species, including humans (Hallstrom et al., 1989; Jeanmonod et al., 1991; Kumar et al., 2000), rats (Shanker Sharma et al., 1991; Winkler et al., 1998), monkeys (Yates et al., 1982) and dogs (Uzuka et al., 1997; Cuddon et al., 1999) for various purposes. The potential of SSEPs for measuring nociception and analgesia in humans has been suggested by Athayde and Franklin (2005).

Recently, epidurally derived evoked potentials were reported in conscious ponies (van Loon et al., 2009) and placement of the epidural electrode using the caudal approach led to interference of the recording of dorsal horn spinal cord activity by lumbar epaxial muscle activity. Therefore, a model was developed in order to record spinal cord dorsal horn activity and quantify caudal nociception in the horse, by means of a lumbosacral approach for placing spinal bipolar electrodes in anaesthetised ponies. To the authors' knowledge, no studies have reported the use of lumbosacrally placed bipolar spinal electrodes in horses before.

The hypothesis was that by using a lumbosacral approach for placing spinal electrodes, we would be able to measure nociceptive spinal cord dorsal horn activity without electromyogram (EMG) interference.

Materials and methods

Animals

The study design was approved by the institutional Ethical Committee for Animal Experiments. The experiment was performed with Shetland ponies (n = 7, age mean ± s.d. age 9.8 ± 2.9 years, weight 203 ± 32.3 kg, all geldings).

Instrumentation and anaesthesia

After fasting overnight with *ad libitum* availability of water, the ponies were prepared for anaesthesia. The jugular vein was prepared aseptically and a 14 gauge catheter placed and sutured to the skin. The ponies were sedated with 0.25 mg/kg bwt propofol (Propovet)[1] i.v. The site of insertion of the epidural catheter, between the first and second coccygeal vertebrae, was surgically prepared and locally anaesthetised (2 ml lidocaine HCl 2% s.c.)[2]. A 16 gauge Tuohy spinal needle (Perifix)[2] was inserted into the epidural space and a 19 gauge catheter was advanced for 10 cm through the Tuohy needle for drug administration. While leaving the catheter *in situ*, the Tuohy needle was removed and the catheter was covered with sterile gauzes.

The mouth was rinsed with water, anaesthesia was induced with propofol (4–5 mg/kg bwt i.v. to effect) and the ponies placed in left lateral recumbency on soft mattresses. The ponies were intubated with a 16 mm silicone cuffed orotracheal tube and subsequently secured to a circle system. Anaesthesia was maintained with 1.7–1.9% end tidal isoflurane (Isoflo)[3] in O_2. The ponies were ventilated mechanically with intermittent positive pressure ventilation, using a tidal volume of 8–10 ml/kg bwt and a breathing frequency of 8–10 breaths/min, in order to maintain the end tidal CO_2 between 4 and 5 kPa. Heart rate and rhythm were monitored by means of ECG. Arterial oxygenation and peripheral pulse were monitored by means of pulse oximetry. Lactated Ringer's solution[4] was infused at 5 ml/kg bwt/h. Rectal temperature was monitored during the experiment. When the ponies were positioned properly, the lumbosacral junction was clipped and prepared aseptically and a bipolar SSEP recording electrode

(custom-made from a Perifix epidural catheter, containing a flexible tip of 1.5 cm and a distance between the 2 poles of 3 cm) was advanced through a Tuohy needle, which was placed into the subarachnoidal space and was confirmed by obtaining liquor during placement of the needle, with the tip placed spinally at the lumbosacral junction facing cranially. After removal of the needle, the electrode was recovered until 4–5 cm was left in the subarachnoidal space. In this way we were able to standardise placement of the measuring electrode in all ponies. A needle electrode, serving as ground electrode for SSEP recording was placed subcutaneously close to the lumbosacral junction in the median plane.
Additionally, 3 needle electrodes were placed subcutaneously at the level of the epaxial muscles, 5 cm from the lumbosacral junction with an inter-electrode distance of 3 cm in order to measure the epaxial EMG.

Generating and recording of spinally derived somatosensory-evoked potentials and electromyograms

For generating SSEPs and EMGs, 2 stimulus electrodes were placed intradermally on the right distal hindlimb, just proximally of the fetlock joint for intradermal electrical stimulation (Inui et al., 2002; van Oostrom et al., 2009). For proximal electrical stimulation (see experimental set-up), 2 stimulus electrodes were placed intradermally on the right hindlimb just below the talocrural joint. Figure 1 shows the distal stimulating electrodes, the insertion site of the epidural catheter and spinal electrode and the needle electrodes for epaxial muscle EMG. Following distal hindlimb stimulation, SSEPs and EMGs were recorded simultaneously. For each recording, 32 square-wave electrical stimuli of 0.5 ms duration with the same stimulation intensity were applied at a stimulus frequency of 0.5 Hz. Stimuli were generated by a stimulator (Stimulator Model S88)[5], triggered by software developed in-house (AD). The stimuli were delivered to a stimulation isolation unit (Model SUI5)[5] and a constant current unit (Model CCU 1A)[5] that controlled the stimulus intensity. SSEPs were recorded from the spinal bipolar electrode with the needle electrode close to the lumbosacral junction on the back serving as ground (Figure 1). EMGs were recorded from the 3 needle electrodes (active electrode, reference and ground) at the epaxial muscles. Signals were

fed to separate, but identical bioelectric amplifiers, amplified 5000 times and band-pass filtered between 1.59 and 1000 Hz. A 50 Hz notch-filter was used to eliminate electrical net interference. Subsequently, the analogue signals were fed to the data acquisition hardware, digitised online at 10,000 Hz and entered into the data acquisition software built in the same Labview environment, which was responsible for stimulus generation. Each SSEP/EMG recording consisted of 32 averaged, subsequent responses of 500 ms (10 ms prestimulus and 490 ms post stimulus).

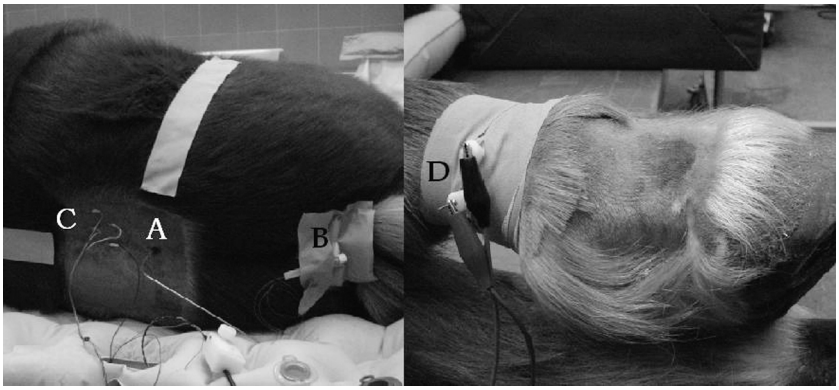

Figure 1 Location of spinal electrode, epidural catheter and stimulating electrodes The left panel shows an anaesthetised pony in left lateral recumbency with A) the spinally placed measuring electrode (between L6 and S1), B) the covered epidural catheter (between C1 and C2) for administration of analgesics and C) the needle electrodes for epaxial muscle EMG recording and the ground electrode for the spinal bipolar electrode. The right panel shows the distal stimulating electrodes just above the fetlock joint of the hind limb (D).

Experimental set-up

In Session 1 of the crossover design, data were recorded in response to distal stimulation with intensities ranging from 0 to 5 mA, administered in random order with the same order for all animals. Based on pilot study results, which were obtained under identical anaesthetic conditions, distal stimulation was chosen with a stimulus intensity of 1.5 mA for the methadone and naloxone sessions. Subsequently, either methadone (Methadon HCl)[6] (0.4 mg/kg bwt in 10 ml saline) or a corresponding volume of saline was administered epidurally and recordings

were repeated at 15 and 30 min. Next, naloxone (Naloxon HCl)[7] (10 mg in 10 ml saline) or a corresponding volume of saline was administered epidurally and recordings were again performed at 5 and 20 min after administration of naloxone. In Session 2, data were recorded in response to proximal stimulation (just below the tarsus) with a stimulus intensity of 1.5 mA. Thereafter, distal stimulation with 1.5 mA and subsequent epidural administration was performed. Similar recordings like in Session 1 were performed. Four ponies received methadone/naloxone in Session 1 and saline/saline in Session 2. In the remaining 3 ponies, the order of treatment was reversed. A minimum of 14 days elapsed between the 2 sessions. At the end of each session, the ponies were de-instrumented and administered antibiotics (benzylpenicillin; benzylpenicilline Natrium[6], 2 x 10^5 iu/kg bwt and gentamicin; Gentamycine 5%[6], 6.6 mg/kg bwt i.v.) and a nonsteroidal anti-inflammatory drug (flunixine; Bedozane[6], 1 mg/kg bwt i.v.), all administered once.

Data and statistical analysis

All data are expressed as mean ± s.e. Analysis was performed with the aid of Microsoft Excel 2000. Statistical analysis was performed using SPSS version 12.0[11]. Statistical significance was accepted at $P < 0.05$.

SSEPs and EMG recordings were evaluated by measuring amplitudes and latencies of the various peaks in the waveforms. Because the main interest was in the Aδ- afferent transmitted SSEPs elicited in the spinal cord, the 30–150 ms latency range was specifically investigated, in which the Aδ-activity can be expected based on the distance between stimulation and recording site and a proposed conduction velocity of Aδ-afferents of 4–35 m/s (Whalen et al., 1994; Spadavecchia et al., 2002). In order to establish the fibre types involved in generation of the various peaks recorded in this latency, we determined the conduction velocity (CV) by using the latency shifts of the various peaks, calculated by subtracting the latency obtained after proximal stimulation from the latency obtained after distal stimulation. Subsequently, the CV was calculated per individual animal by dividing the distance between the 2 stimulation sites (10–12 cm, measured on the distal hindlimb of every pony separately) by the latency shift in ms of all

peaks. A one-way ANOVA with Bonferroni correction for *post-hoc* tests was used to evaluate the differences in CVs between different SSEP peaks.
For the stimulus intensity data, a one-way repeated measures analysis of variance (RM-ANOVA) design was performed with factor intensity. For the methadone and naloxone results, a 2-way RM-ANOVA design with factors treatment and time was performed. RM-ANOVA designs were followed by *post hoc* analysis when appropriate, by means of paired *t*-tests.

Results

Spinally derived somatosensory-evoked potential component definition
Figure 2 shows the mean baseline SSEP waveform that was generated after 1.5 mA distal stimulation. The different peaks used for further analysis of our SSEP waveforms (N1, P1, N2 and P2) as well as the amplitudes of N1P1 (A) and N2P2 (B) are shown. The corresponding mean ± s.e. latencies are for N1: 12.5 ± 0.3 ms, for P1: 29 ± 1.6 ms, for N2: 72 ± 3.1 ms and for P2: 122 ± 3.8 ms.

Stimulus intensity response characteristics
Figure 3 shows both the SSEP and EMG responses after increasing stimulus intensity. Stimulus intensity significantly affected the N2P2 amplitude ($F(3,21) = 5.30$, $P=0.045$), whereas the effect of stimulus intensity on N1P1 amplitude was not significant ($F(3,21)=4.61$, $P=0.063$). Figure 3D demonstrates that no EMG signals could be recorded following any of the stimulus intensities.

Determination of conduction velocity
Mean latency shifts allowed calculation of conduction velocities (CV) to be within 1) the Aα/β domain (>35 m/s) for N1 (mean ± s.e. CV = 76.3 ± 12.8 m/s) and P1 (mean ± s.e. CV = 51.8 ± 8.5 m/s), and 2) within the Aδ domain (4–35 m/s) for N2 (mean ± s.e. CV = 10.5±2.4 m/s) and P2 (mean±s.e. CV = 5.4±1.2 m/s) (Figure 4). Statistical analysis revealed an overall significant difference between CVs ($F(3)=15.66$, $P<0.001$) and significant differences between P1 and N2 ($P=0.004$) in *post-hoc* tests.

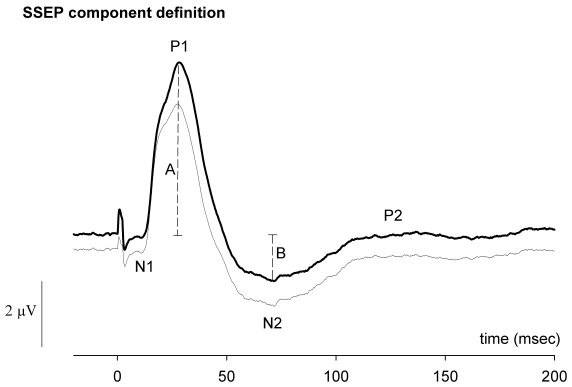

Figure 2 Mean overall BL SSEP waveform (bold line) - SEM (plain line), resulting from two averaged baseline recordings of each pony with stimulus intensity 1.5 mA and stimulus frequency 0.5 Hz. X-axis: latency from distal stimulation (ms). N1 (mean±SEM latency 12.5±0.3 msec) and P1 (29±1.6 msec) represent Aβ-mediated dorsal horn activity, N2 (72±3.1 msec) and P2 (122±3.8 msec) represent Aδ-mediated dorsal horn activity. A is the amplitude of N1P1; B is the amplitude of N2P2.

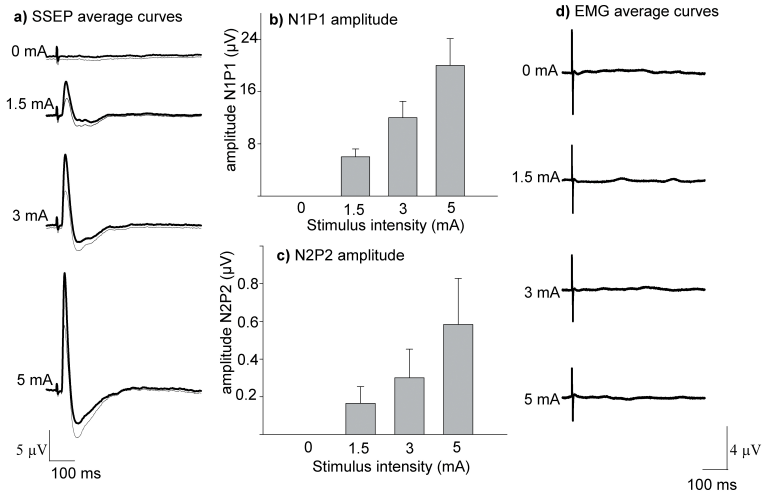

Figure 3 A) shows the mean total SSEP waveforms (bold lines) – SEM (plain lines). B) shows the SSEP N1P1 amplitude and C) shows the SSEP N2P2 amplitude, in response to increasing stimulus intensities. All data are shown mean ± SEM. D) shows the mean total EMG waveforms (bold lines).

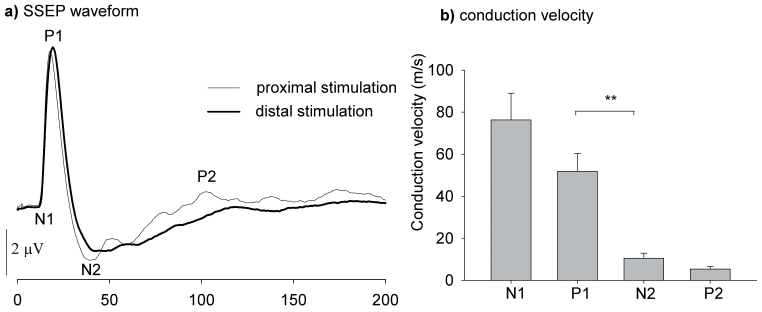

Figure 4 A) shows a typical example of SSEP waveforms, resulting from distal and proximal stimulation in one pony (stimulus intensity 1.5 mA and stimulus frequency 0.5 Hz, distance between proximal and distal stimulation site: 14.5 cm). X-axis: latency from distal stimulation (ms). B) shows CVs, calculated for N1, P1, N2 and P2. ** = $p < 0.01$.

Effect of epidural opioid administration

Figure 5 shows the results of epidural administration of methadone, naloxone and saline. Methadone depressed N2P2 amplitude and after naloxone administration, N2P2 amplitude increased towards pre methadone levels again. Overall statistical analysis revealed that methadone and naloxone had a significant effect on N2P2 amplitude ($F_{time}(2.5, 29.4) = 3.64$, $P=0.031$, $F_{time \times treatment}(2.5, 29.4) = 3.55$, $P=0.033$). *Post hoc* analysis showed a significant difference between methadone and saline at time points t15, t30 and t35 ($t15_{(6)}=3.29$, $P=0.017$, $t30_{(6)}=4.03$, $P=0.007$, $t35_{(6)}=3.28$, $P=0.017$). Methadone and naloxone did not significantly influence N1P1 amplitude ($F_{time}(4, 48)=2.30$, $P=0.073$, $F_{time \times treatment}(4, 48)=0.091$, $P=0.99$).

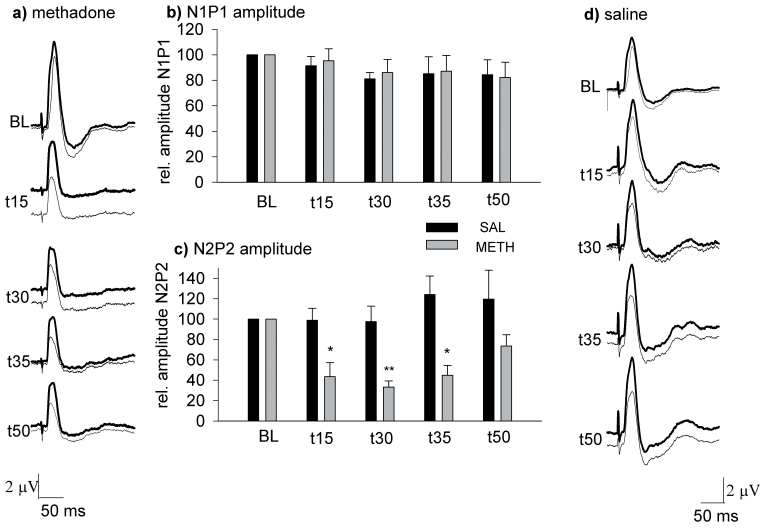

Figure 5 A) shows the mean SSEP waveforms (bold lines) – SEM (plain lines) in relation to methadone and naloxone administration. After BL, methadone (0.4 mg/kg) was epidurally administered. After t30, naloxone (10 mg) was epidurally administered. B) and C) show relative amplitudes of N1P1 and N2P2. METH = methadone, SAL = saline. * = $p < 0.05$, ** = $p < 0.01$. D) shows the mean SSEP waveforms (bold lines) – SEM (plain lines) in relation to saline administration.

Discussion

In the present study, SSEPs generated in anaesthetised ponies were characterised and have shown that the neurophysiological approach, using SSEPs, can lead to objective quantification of spinal nociception in equines. The SSEP N2P2 complex was found: 1) to increase in response to increasing stimulus intensity; 2) it was related to stimulation of afferent fibres with a CV that falls within the nociceptive Aδ-range; 3) it was affected by epidurally administered methadone and 4) it was devoid of EMG interference. Based on these characteristics, it is proposed that SSEPs can be used reliably to determine equine spinal nociception and analgesia.

Compared to previous work (van Loon et al., 2009), our model has been further implemented by several modifications. First, to ascertain the correct placement of the electrode, the lumbosacral approach was employed to enter the subarachnoidal space. In this way, the electrode could be reliably and accurately

placed dorsal to the spinal cord at a fixed distance from the lumbosacral junction. By performing this experiment in anaesthetised animals, optimal placement of the electrodes was guaranteed and decreased the possible EMG interference in the SSEP waves while obtaining highly reproducible SSEP recordings. In the stimulus response studies, the obtained stimulus-intensity dependent SSEPs were shown to be devoid of EMG interference. This corresponds with other studies (Jinks et al., 2003; Kim et al., 2007; Barter et al., 2008; Spadavecchia et al., 2010) showing the effects of both hypnotics, such as propofol and inhalants such as isoflurane, to affect the ventral horn (containing motor neurons generating efferent activity) more profoundly, rather than the dorsal horn of the spinal cord (containing sensory neurons generating afferent activity). Consequently, the (reflex) muscular activity is abolished at a lower anaesthetic level than the (non-)nociceptive afferent stimulation and activation of the spinal dorsal horn. This concept was used to generate EMG-free dorsal horn activity under stable anaesthetic conditions. This means that SSEPs show nociceptive activity of the spinal cord at anaesthetic levels that abolish pedal withdrawal reflexes, creating a more sensitive parameter for nociception, compared to nociception models using withdrawal reflexes. The μ-opioid methadone was found primarily to affect the N2P2 amplitude in our SSEP waveforms, while the early N1P1 amplitude was not significantly influenced. By evaluating the differential effect of the μ-opioid methadone on the various parts of the SSEP complex and comparing it to data from the literature regarding localisation of opioid receptors on various types of somatosensory afferents and the spinal dorsal horn (Yaksh 1981, 1987; Dickenson, 1995; Kelly et al., 2001), we could further characterise our SSEP signals. The differential effects of opioids on Aβ- and Aδ-afferents we found using low stimulation intensities are in accordance with the results of Yeomans et al. (1995), who documented the influence of systemic morphine on various components of the flexion reflex in nonhuman primates.

Separate studies would be warranted to investigate the influence of higher stimulus intensities on the effects of antinociceptive drugs on SSEP characteristics. Taken together, the present study showed that 1) the N1P1 complex was generated by means of fast conducting tactile afferents with CVs falling in the Aβ-range

and was not significantly influenced by the depressing effects of methadone, and 2) the N2P2 complex was generated by means of slower conducting afferent fibres with CVs falling in the Aδ-range and was sensitive to the depressing effects of methadone.

It was striking that, while using the intradermal stimulation technique described to be selective for Aδ-fibre stimulation in dogs (van Oostrom et al., 2009) and humans (Inui et al., 2002), both Aβ- and Aδ-related SSEP components were detected. However, the former studies based their conclusions on cortically evoked potentials and therefore the results might not be comparable to those of the present study. Furthermore, lack of specificity in stimulating afferent fibres could also result from differences in intradermal stimulation, due to differences between canine, human and equine skin. The advantage of the described equine model to study SSEPs over the frequently used rodent models was that, due to the relatively long afferent fibre tracts, these Aδ- and Aβ-related components of the SSEP waveform, originating from nonspecific electrical stimulation, could be differentiated. Spinally derived evoked potentials have been described in various species for various purposes. Much research has been performed in rodents, mainly focusing on spinal cord trauma and regeneration (Shanker Sharma et al., 1991; Winkler et al., 1998; Wang et al., 2005). In humans, SSEPs are used under clinical circumstances for monitoring spinal cord integrity during neurosurgery in, for example, patients with scoliosis (Ryan et al., 1986; Bernard et al., 1996; Ku et al., 2008). Cuddon et al. (1999) and Uzuka et al. (1997) described SSEPs in dogs, in studies assessing dorsal nerve root and spinal cord dorsal horn function. Their waveforms show similarities with the present data and related the early positive waveform (our P1) to dorsal horn interneuron depolarisation and the subsequent negative waveform (our N2P2) to primary afferent depolarisation.

As SSEPs are not measured as single neuron activities, they reflect electrical activity of neurons, adjacent to the recording electrode. Therefore, it is questionable whether it is possible to relate certain parts of the SSEP complex to, for example, interneurons or primary afferents. By means of the characteristics

used to describe SSEP signals, however, it is possible to relate various parts of the SSEP complex to different types of stimulated afferents. With this information, SSEPs were used in order to quantify spinal nociception. The potential of spinal cord derived evoked potentials to be used in measuring analgesia in humans has also been suggested by Athayde and Franklin (2005).

In conclusion, our equine SSEP model reported here represents a valid and quantitative measure of spinal nociceptive processing that allows for an objective discrimination between nociceptive and non-nociceptive stimulation of the spinal cord dorsal horn. Furthermore, this model enables the future investigation of new analgesics and supports the improvement of analgesia protocols for caudal epidural analgesia in *equidae*.

Acknowledgement

The authors thank Peter Stienen for his assistance during analysis of the results.

Manufacturers' addresses

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4

Antinociceptive effects of low dose lumbosacral epidural ropivacaine in healthy ponies

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The Veterinary Journal (2011) in press doi:10.1016/j.tvjl.2011.11.004

Abstract

The objective of this study was to evaluate the safety and efficacy of low dose lumbosacral epidural ropivacaine in ponies. Antinociceptive effects of epidural ropivacaine were evaluated by means of mechanical nociceptive thresholds (MNTs) at several spinal levels in conscious ponies. The effects of ropivacaine on nociceptive afferent transmission to the spinal cord were also assessed by measuring spinal cord somatosensory evoked potentials (SSEPs) in anaesthetised ponies. Ataxia scores were determined in conscious ponies to assess the effects on motor function. A randomised, placebo controlled, double blind cross-over design was used. Low dose lumbosacral epidural ropivacaine led to increases in MNTs at various anatomical locations with a maximum effect at the lumbosacral and sacrococcygeal regions, both with respect to increase in threshold and duration of effect. Analysis of SSEPs showed that epidural ropivacaine influenced both Aδ and Aδmediated afferent transmission to the spinal cord at the level of the lumbosacral junction. Ponies showed mild ataxia after low dose lumbosacral epidural ropivacaine, but all ponies remained standing. Application of low dose lumbosacral epidural ropivacaine provided safe and efficacious antinociceptive effects in conscious and anaesthetised ponies, and could therefore be a valuable addition to multimodal analgesic protocols in Equidae.

Keywords

Equine; Evoked potential; Epidural analgesia; Ropivacaine; Pressure algometry

Introduction

Multimodal pain management refers to the practice of combining multiple analgesic drug classes or techniques to target different points along the pain pathway (Lamont, 2008). It takes advantage of additive or synergistic analgesic effects while allowing for the use of lower doses of individual analgesic agents and has therefore become widely accepted in human and veterinary analgesia.

In these multimodal analgesic techniques, epidurally applied local anaesthetics can play a very important role in both peri- and postoperative analgesia (Robinson and Natalini, 2002). By inhibiting neural conduction from the surgical site to the spinal cord, this form of regional anaesthesia is able to prevent spinal cord sensitisation. In human anaesthesia, local anaesthetics are very often used for this purpose (Skinner, 2004; Tang et al., 2009). In standing procedures in equines however, the possible detrimental effects on motor function, depending on technique, volume and concentration of local anaesthetics, may limit the use of these drugs for peri- and postoperative epidural analgesia of the hindlimb. Therefore, epidurally applied local anaesthetics are mostly used for caudal perineal anaesthesia in horses (Robinson and Natalini, 2002).

Ropivacaine is a long-acting, S-enantiomer, amide-type local anaesthetic with a high pKa and low lipid solubility that blocks the Aδ- and C-nerve fibres involved in pain transmission (McClure, 1996; McLellan and Faulds, 2000; Hansen, 2004). In the human literature, there is abundant information on low dose ropivacaine epidural analgesia (Zaric et al., 1991; Halpern and Walsh, 2003; Zink and Graf, 2004; Heid et al., 2007). Casati et al. (2001) concluded that ropivacaine produces a marked differential blockade between sensory and motor fibres, especially at the low concentrations used for postoperative analgesia. This was confirmed by Eledjam et al. (2001) who described that a differential sensory/motor block was only apparent at low concentrations (≤0.2% or less). Apart from these differential sensory/motor blocking capacities, ropivacaine has fewer cardiotoxic side-effects, than bupivacaine (Graf et al., 2002). Neuraxial use of ropivacaine

has been described in few equine studies. Ganidagli et al. (2004) used 0.1 mg/kg ropivacaine (8 mL of a 0.5% solution) for caudal epidural administration in adult thoroughbred horses. With this volume and concentration, the large motor nerves supplying the hindlimb were not influenced. Skarda and Muir (2003) applied 0.02 mg/kg (5 mL of a 0.2% solution) ropivacaine spinally at the midsacral (S2-S3) vertebrae in adult horses. This dosage produced perineal analgesia without motor effects. Bussières et al. (2008) applied 0.15 mg/kg of ropivacaine (11 mL of a 0.7% solution) by means of a caudally placed (between first and second coccygeal vertebra) epidural catheter that was advanced cranially for 10 cm up to the lumbosacral area, in an epidural multimodal procedure and combined it with opioids, α_2-agonists and ketamine. No motor effects of epidurally applied ropivacaine were mentioned. Because of the multimodal analgesic approach, the specific contribution of ropivacaine to the total clinical antinociceptive effect could not be quantified.

The aim of this study was to evaluate the antinociceptive efficacy and clinical safety (i.e. motor side effects) of low dose lumbosacral epidural ropivacaine in standing and anaesthetised ponies. Antinociceptive effects were evaluated by means of determination of mechanical nociceptive thresholds (MNTs) in conscious ponies. MNTs have been described in horses in several studies (Varcoe-Cocks et al., 2006; Haussler and Erb, 2006a, b; Haussler et al., 2007). In addition, the effects of ropivacaine on nociceptive afferent transmission to the spinal cord were assessed by measuring spinal cord somatosensory evoked potentials (SSEPs) in anaesthetised ponies using a model described by van Loon et al. (2010). To evaluate the clinical applicability of epidural ropivacaine for standing surgical procedures and pain management in the hindlimb, ataxia scores were measured in conscious ponies.
We hypothesised that low concentration epidural ropivacaine would influence both MNTs and SSEPs at the level of the lumbosacral junction, while generating no effects on motor function other than subtle signs of ataxia.

Materials and methods

Animals

The study design was approved by the institutional Ethical Committee for Animal Experiments. The experiment was performed with eight Shetland ponies (mean ± SD age 11 ± 3 years, weight 206 ± 39 kg, all geldings). All animals were physically examined prior to the experiments. They were found to be healthy and showed no locomotor abnormalities.

Experimental set-up

In part one of the experiment, the effect of 0.15 mg/kg epidurally applied ropivacaine (Naropin, AstraZeneca) (10 mL of a 0.3 % saline solution) was assessed in conscious ponies. This dosage and concentration of ropivacaine was based on the study by Bussières et al. (2008) and unpublished pilot studies. After sedation with 0.25 mg/kg propofol (Propovet, Abbott Animal Health) intravenously, the site of insertion of the epidural catheter, between the 1st and 2nd coccygeal vertebrae, was surgically prepared and locally anaesthetised (2 mL lidocaine HCl 2%, BBraun). A 16 gauge Tuohy spinal needle (Perifix) was inserted into the epidural space. Correct epidural placement was verified by means of the hanging drop and loss of resistance technique. A 19-gauge catheter (Perifix, BBraun) was advanced cranially for 15 cm through the Tuohy needle and ropivacaine or placebo (saline) was administered through the epidural catheter. Effects on MNTs, determined by means of a pressure algometer (FPK 60, Wagner Instruments), were assessed at baseline (BL) and at 1 (T1), 4 (T4), 6 (T6), 8 (T8) and 24 (T24) h later. At BL, T1, T4 and T24 videos were taken for neurological assessments. These recordings were scored afterwards by a board certified equine internal medicine specialist, who was blinded for treatment and time after the intervention. In part two of the study, the ponies were anaesthetised and effect on SSEPs of the same dose and volume of epidural ropivacaine, administered using the same route was evaluated. Effects on SSEPs were determined at BL and at 15 (T15), 30 (T30), 45 (T45) and 60 (T60) min after ropivacaine administration. For both MNT and SSEP

experiments, a randomised cross-over design was used with at least 10 days wash-out between the ropivacaine and placebo treatments.

MNT measurement

A non-electrical pressure algometer (FPK 60, Wagner Instruments) was used, consisting of a force gauge with a 1 cm^2 rubber tip and a capacity of 3 to 30 kgf. MNT measurements were made as described by Haussler et al. (2007). In order to avoid excitation caused by social isolation, a second pony always accompanied the pony that was being assessed (De Heus et al., 2010). Pressure was applied perpendicularly to the surface at a constant rate of 5 $kg/cm^2/s$, half the speed used by Haussler and Erb (2006a) to allow for better interpretation of the animal's reaction. The pressure stimulus was applied until an avoidance reaction was evoked (muscle fasciculations, cutaneous trunci reflexes, active vertebral movement or stepping away; Haussler and Erb, 2006a, De Heus et al., 2010). When an avoidance reaction was observed, pressure application was stopped immediately and the value recorded as the MNT value. In order to decrease the effect of inter-measurement variability, three repeated measurements were taken in succession at one site at 3 to 4 s intervals, and the median of these three measurements was taken (Haussler et al., 2007).

MNT Measurement Sites

Twenty-five locations were used in this study: five over the dorsal midline at the spinal processes of different vertebrae and 20 over the extensor muscles of the back (10 on the left and 10 on the right) (Figure 1). Measurements were made at the level of the sacrococcygeal region (SC), the lumbosacral region (LS), the lumbar region (L), the thoracolumbar region (TL) and mid-thoracic region (MT) at fixed distances. The anatomical measurement sites were visually marked with a white, fast-drying marking fluid. The MNTs were measured in a fixed order from cranial to caudal. The area of the sacrococcygeal region was clipped as part of preparation for caudal epidural catheterisation; the other areas where MNTs were determined were not clipped.

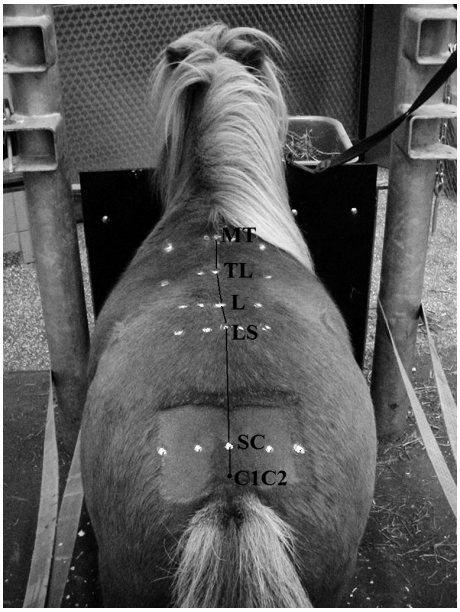

Figure 1 Specific locations at which mechanical nociceptive thresholds were determined for every individual pony. C1C2; first intercoccygeal space where epidural catheter was introduced into epidural space; SC; sacrococcygeal region (S5); LS , lumbosacral region (L6); L , lumbar region (5 cm cranial from LS); TL , thoraco- lumbar region (10 cm cranial from L); MT, mid- thoracic region (15 cm cranial from MT).

Ataxia scores

Videos from neurological examinations, consisting of walk and trot on a straight line, circling and backwards in walk and pulling laterally on the tail during walk, were evaluated using a standardized 0-5 Simple Descriptive Scale (modified from Mayhew, 1989) (Table 1).

Table 1 Simple Descriptive Scale (SDS) ataxia scores

SDS score	*Description*
0	*normal gait, no abnormalities*
1	*slightly swaying gait, only visible at circle and when pulled at tail*
2	*obviously swaying gait, mainly visible at circle and when pulled at tail*
3	*obviously swaying gait, visible at straight line*
4	*serious instability with stumbling and nearly falling at straight line*
5	*animal cannot stand on hindlegs*

SSEP measurement

After fasting overnight with ad lib availability of water, the ponies were prepared for anaesthesia. The jugular vein was prepared aseptically and a 14 gauge catheter was placed and sutured to the skin. The ponies were sedated with 0.25 mg/kg propofol (Propovet, Abbott Animal Health) intravenously. An epidural catheter was placed as described previously. Anaesthesia was induced with propofol (4-5 mg/kg intravenously to effect) and the ponies were placed in left lateral recumbency. The ponies were intubated and connected to a circle anaesthesia system.

Anaesthesia was maintained with 1.8-2.0% end tidal isoflurane (Isoflo, AST Pharma) in O_2. The ponies were ventilated mechanically with intermittent positive pressure ventilation using a tidal volume of 8-10 mL/kg and a breathing frequency of 8-10 breaths/min, in order to maintain the end tidal CO_2 between 4 and 5 kPa. Heart rate and rhythm were monitored by means of ECG. Arterial oxygenation and peripheral pulse were monitored by means of pulse oxymetry. Lactated Ringer's solution (Baxter) was infused intravenously at 5 mL/kg/hour during the whole procedure.

The lumbosacral junction was clipped and prepared aseptically and a bipolar SSEP recording electrode (custom made from a Perifix epidural catheter) was advanced through a Tuohy needle that was placed into the spinal space at the lumbosacral junction with the tip advanced for 4 cm in cranial position. A needle electrode, serving as ground electrode for SSEP recording was placed subcutaneously, close to the lumbosacral junction in the median plane. The generation and recording of SSEPs was undertaken as described by Van Loon et al., (2010). Two stimulus electrodes were placed intradermally on the left distal hindlimb, just proximally of the fetlock joint for intradermal electrical stimulation with 1.5 mA.

At the end of each session, the ponies were given 2 x 10^5 IU/kg benzylpenicillin (Benzylpenicilline Natrium, Eurovet Animal Health); 6.6 mg/kg gentamycin (Gentamycine 5%, Eurovet Animal Health), and 1 mg/kg flunixin (Bedozane,

Eurovet Animal Health), all administered iv. Recovery from general anaesthesia was unassisted and was subjectively evaluated by one researcher. After recovery from anaesthesia, the animals were kept in a group stable to monitor them for several days before they returned to their herd.

Generating and recording of SSEP

For each SSEP recording, 32 square-wave electrical stimuli of 500 ms duration (10 ms pre-stimulus and 490 ms post-stimulus) with the same stimulation intensity were applied at a stimulus frequency of 0.5 Hz. Stimuli were generated by a stimulator (Model S88, Grass Medical Instruments) triggered by custom-built software (AD). The stimuli were delivered to a stimulation isolation unit (Model SUI5) and a constant current unit (Model CCU 1A) that controlled the stimulus intensity. Signals were fed to separate, but identical bioelectric amplifiers (Bio electric amplifier AB 601 G, Nihon Kohden), amplified 5 000 times and band-pass filtered between 1.59 and 1 000 Hz. A 50 Hz notch-filter was used to eliminate electrical net interference. Subsequently, the analogue signals were fed to the data acquisition hardware (PCI 6251, Labview, National Instruments), digitised online at 10 000 Hz and entered into the data acquisition software built in the same Labview environment that was responsible for stimulus generation. After three baseline stimulations with a 5 min interval, either ropivacaine or saline was administered epidurally and single SSEP measurements were performed at 15, 30, 45 and 60 minutes after epidural administration. In accordance with an earlier study (Van Loon et al., 2010) we evaluated the N1P1 complex (latency 12.5-30 ms) as a measure of Aβ-afferent mediated activity and the N2P2 complex (latency 70-120 ms) as a measure of Aδ-afferent mediated activity.

Data processing and statistical analysis

Three consecutive MNTs from each measurement site were evaluated for sensitisation (decrease in threshold) or habituation (increase in threshold). Within each of the five regions, differences between measurement locations were tested by means of one-way ANOVA with post-hoc Bonferroni correction. If no statistically significant differences between left and right were found, data

were pooled. In order to rule out systematic habituation or sensitisation between the first and second round of the cross-over trial, we compared first and second round baseline MNTs by means of paired *t*-tests. Statistical analysis of MNTs and SSEPs was performed using a linear mixed model for repeated measures, with horse as a random effect, and time and treatment as fixed effects and repeated factors. When significant treatment effects were found, univariate post-hoc tests were used for comparisons at each time point, using Bonferroni's correction for multiple comparisons. Categorical ataxia scores were statistically evaluated by means of Friedman tests to evaluate changes over time and Wilcoxon signed ranks tests for comparison between placebo and ropivacaine treatment. Statistical analysis was performed using SPSS version 12.0 (SPSS).

Results

MNTs

Across all measurements the three consecutive values sequentially increased in 8.5% (habituation), sequentially decreased in 19.9% (sensitisation) and showed no change or an inconsistent pattern in 71.7% of the measurements. The mean range (min-max) over three successive measurements across all measurements was 1.2 (0-9.2) kg/cm^2. Figure 2 shows all 25 measurement sites with their accompanying BL MNT values. No significant differences were noted between BL MNTs in the first and second round of the cross-over trial. As there were no statistically significant differences, left and right MNT values were pooled, which led to three values for each region. Table 2 shows relative MNTs (expressed as a percentage of BL) for each region at each time point. This table shows differences between placebo and ropivacaine administration at each time point and at each measurement location. Ropivacaine led to increased MNTs compared to placebo treatment, with the most prominent and long-lasting differences (up to 8 hours after ropivacaine administration) being recorded at the level of the sacrococcygeal junction (Table 2).

Table 2 Mechanical nociceptive thresholds in various regions following epidural administration of 0.15 mg/kg ropivacaine in 10 mL saline. Mean threshold (SEM), as a percentage of baseline measurement ($n = 8$ ponies). Statistical differences between placebo and ropivacaine administration for each time point and each segment are shown. * $P < 0.05$, ** $P < 0.010$, *** $P < 0.001$.

		Time after ropivacaine administration (h)				
Segment	**treatment**	**1**	**4**	**6**	**8**	**24**
Mid-thoracic	Placebo	91.5 (2.2) *	91.5 (4.6)	91.4 (5.1)	81.7 (6.3)	87.8 (6.0)
lateral	Ropivacaine	110.8 (2.0)	92.7 (7.3)	96.3 (4.5)	83.4 (2.9)	92.1 (2.9)
Mid-thoracic	Placebo	90.9 (4.6)	94.9 (5.1)	86.4 (3.1)	86.1 (5.5)	87.1 (5.3)
medial	Ropivacaine	103.5 (5.3)	95.8 (3.3)	95.5 (5.5)	88.2 (3.9)	92.6 (4.6)
Mid-thoracic	Placebo	90.7 (4.5) *	95.3 (5.6)	88.7 (3.7)	84.5 (5.5)	84.4 (7.2)
midline	Ropivacaine	107.2 (9.6)	93.2 (7.9)	94.4 (6.5)	95.4 (6.5)	92.5 (12.0)
Thoracolumbar	Placebo	97.7 (4.3) *	96.0 (4.1)	93.8 (3.2)	90.3 (4.2)	90.3 (5.8)
lateral	Ropivacaine	112.6 (4.5)	97.7 (4.9)	94.7 (6.7)	88.1 (7.0)	87.2 (3.7)
Thoracolumbar	Placebo	92.6 (6.2) **	87.0 (2.5) *	85.6 (3.0)	83.6 (5.5)	81.8 (4.8)
medial	Ropivacaine	108.5 (5.2)	98.7 (6.9)	89.7 (5.5)	89.1 (4.3)	87.6 (4.5)
Thoracolumbar	Placebo	93.4 (11.0)	91.2 (6.8)	81.8 (6.6)	84.4 (5.9)	88.2 (9.9)
midline	Ropivacaine	98.4 (9.0)	87.0 (8.2)	96.5 (8.9)	92.3 (9.7)	90.7 (9.3)
Lumbar	Placebo	110.7 (5.5) *	102.1 (5.9)	105.1 (6.3)	94.9 (5.6)	92.2 (3.9)
lateral	Ropivacaine	123.8 (4.6)	108.6 (5.8)	102.9 (5.4)	99.8 (6.5)	94.2 (3.7)
Lumbar	Placebo	102.6 (5.0)	96.6 (4.6)	96.5 (5.6)	92.3 (5.8)	83.3 (6.0)
medial	Ropivacaine	115.1 (6.5)	104.2 (5.6)	95.0 (5.5)	91.3 (3.0)	91.0 (3.9)
Lumbar	Placebo	109.5 (10.0)	106.9 (7.7)	102.8 (5.2)	101.7 (6.6)	100.7 (4.5)
midline	Ropivacaine	108.2 (7.5)	102.2 (6.4)	110.7 (6.8)	96.0 (4.5)	95.6 (7.8)
Lumbosacral	Placebo	108.1 (7.5) **	108.3 (6.3)	104.3 (7.2)	101.1 (7.6)	99.7 (4.1)
lateral	Ropivacaine	132.7 (10.1)	120.9 (5.1)	108.0 (4.9)	100.1 (6.4)	105.6 (4.4)
Lumbosacral	Placebo	102.5 (4.0) ***	106.5 (4.9)	101.4 (6.4)	97.4 (5.0)	86.3 (4.3)
medial	Ropivacaine	128.8 (7.9)	115.7 (3.7)	105.8 (4.4)	102.7 (4.3)	95.8 (3.6)
Lumbosacral	Placebo	104.5 (5.1) ***	107.2 (2.9)	109.5 (5.7)	99.1 (5.2)	100.2 (4.0)
midline	Ropivacaine	149.3 (15.7)	116.9 (9.5)	114.4 (7.7)	102.5 (6.8)	99.0 (4.6)
Sacrococcygeal	Placebo	97.2 (4.5) ***	101.2 (5.5)	88.4 (3.7)	85.0 (4.9)	91.2 (7.6)
lateral	Ropivacaine	129.9 (12.6)	111.8 (6.0)	91.9 (7.1)	90.4 (8.0)	93.3 (6.6)
Sacrococcygeal	Placebo	94.6 (3.2) ***	94.3 (4.2) **	88.9 (3.9)	84.2 (5.4)	84.0 (5.4)
medial	Ropivacaine	137.5 (9.0)	115.2 (5.0)	95.8 (5.5)	92.5 (5.7)	91.2 (4.2)
Sacrococcygeal	Placebo	91.6 (6.1) ***	94.0 (2.6) ***	87.9 (5.2) **	81.2 (5.4) **	85.3 (5.1)
midline	Ropivacaine	147.7 (13.2)	135.6 (10.1)	120.4 (12.7)	107.7 (11.9)	103.8 (6.4)

Ataxia scores

Ropivacaine led to mild increases in ataxia scores in conscious ponies. Significant differences between ropivacaine and placebo treatment were found at 1 and 4 h after ropivacaine administration ($P = 0.016$ and 0.023, respectively) (Figure 3).

Recovery from general anaesthesia after SSEP measurements was uneventful and all ponies had gentle and quiet recoveries with one attempt to stand without obvious signs of ataxia.

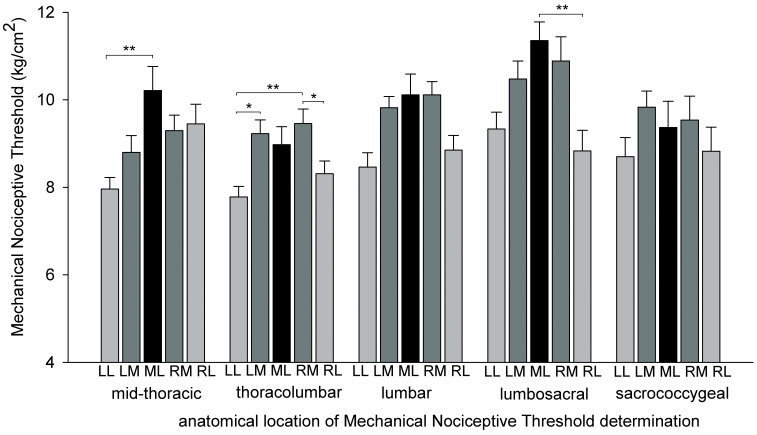

Figure 2 Mean (SEM) baseline mechanical nociceptive threshold values (n= 8; measured twice at baseline during each round of cross-over trial). X-axis represents one midline bony and four parasaggital muscular sites for each mid-thoracic to sacrococcygeal region. LL, left lateral; LM, left medial; ML, midline; RM, right medial; RL, right lateral. Statistical differences are shown between different MNT measurement sites within one region. * = $P < 0.05$; ** = $P < 0.01$

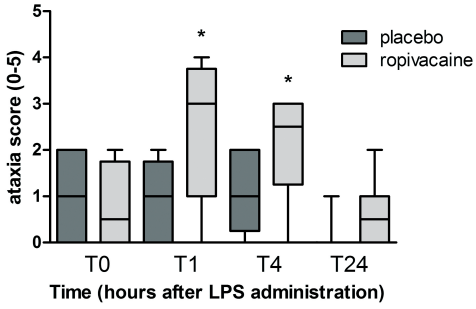

Figure 3 Median ataxia scores with 25th-75th percentiles (boxes) and ranges (whiskers) (SDS scale 0-5) before (T0) and at 1 (T1), 4 (T4) and 24 (T24) hours after epidural ropivacaine administration (0.15 mg/kg in 10 mL saline); (n = 8). Statistical differences between placebo and ropivacaine administration for each time point are shown. * = P <0.05.

SSEPs

SSEP measurements were performed in six of eight ponies. In two ponies, spinal application of the bipolar electrode technically failed and no measurements were made. Significant effects were found for both treatment ($P < 0.001$) and time ($P < 0.01$), but no significant time-treatment interactions ($P = 0.86$). Post-hoc tests showed significant differences between placebo and ropivacaine treatment at T15, T30, T45 and T60 for Aβ-afferent related SSEP complexes (N1P1) (T15: $P = 0.021$, T30, T45 and T60: $P = 0.001$) and at T30, T45 and T60 for Aδ-related SSEP complexes (N2P2) (T30: $P = 0.004$, T45: $P = 0.001$ and T60: $P < 0.001$ (Figure 4).

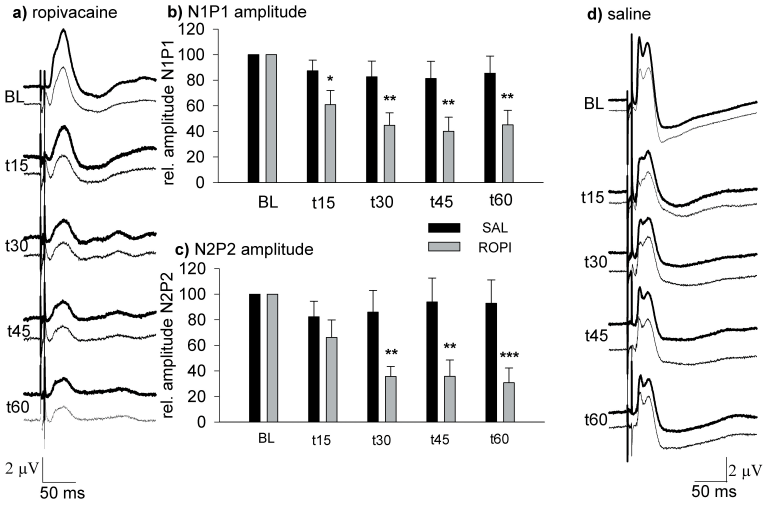

Figure 4 A) Mean somatosensory evoked potential (SSEP) waveforms (bold lines) – SEM (plain lines) in relation to ropivacaine administration. After baseline (BL); ropivacaine (0.15 mg/kg in 10 mL saline) was epidurally administered. B) and C) Relative amplitudes of N1P1 and N2P2 complexes. D) Mean SSEP waveforms (bold lines) – SEM (plain lines) in relation to saline administration ($n = 6$). SAL, saline; ROPI, ropivacaine; t15-t160, time 15-60 minutes after epidural administration. Statistical differences between placebo and ropivacaine administration for each time point are shown. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Discussion

This study supports the hypothesis that low dose ropivacaine provides effective antinociceptive activity after lumbosacral epidural administration in ponies, as demonstrated by changes in MNTs and SSEPs. At the same time, motor function remained (relatively) uncompromised, as demonstrated by the fact that only mild ataxia was measured in seven out of eight ponies and none of the ponies had problems retaining a standing posture. While seven ponies showed only mild ataxia, one pony had an ataxia score of four 1 h after ropivacaine administration. This was one of the smallest and thinnest ponies and body weight, size and amount of epidural fat might have influenced motor effects of ropivacaine. We feel that these ataxia scores allow for clinical use in animals that do not need to be walked during analgesic treatment.

MNTs result from mechanical stimulation of nociceptive afferents in the epidermis and deeper layers, consisting of free nerve endings of A- and C-fibre nociceptors (Treede et al., 2002). Due to the mechanical character of stimulation with pressure algometry, involved afferents are probably mostly Aβ-afferents (Linden and Millar, 1988). Besides C- and Aδ-afferents, local anaesthetics will also influence conduction of Aβ-afferents. However, the effects will be smaller on afferents with thicker myelin sheaths (Hall et al., 2001). We found the largest differences between ropivacaine and saline treatment at the sacrococcygeal and lumbosacral junctions with significant differences for up to 8 hours at the sacrococcygeal junction. Only in this region, the skin was clipped because of epidural catheterisation. The differences in coat thickness between the sacrococcygeal region and the MNT locations might partly account for the observed differences in both size and duration of treatment with ropivacaine, as reviewed by Love et al. (2011). The use of MNTs permitted us to evaluate the effects of ropivacaine at several anatomical locations simultaneously. In this way, spread of epidural ropivacaine and the duration of analgesic effect could be assessed over time. In a study of Haussler et al. (2007), MNTs were measured in horses with experimentally induced osteoarthritis in the carpal joints. The osteoarthritic limb showed significantly

reduced MNT values from 2 to 6 weeks after surgery. We have recently found (J. P. A. M. van Loon, in press) that MNTs are also reduced in horses with acute inflammatory synovitis and that these decreases are eliminated by treatment with epidural morphine. These data support the conclusion that decreases in MNTs do have clinical significance.

Since MNTs are dependent on both the afferent and efferent pathways of the spinal reflex arch, ropivacaine could theoretically lead to increased MNTs by an effect on either or both pathways. To discriminate between these pathways and to assess the specific effect of epidural ropivacaine on the afferent nociceptive transmission to the spinal cord at the lumbosacral junction, the use of the equine SSEP model (van Loon et al., 2010) was crucial. With this SSEP model, we showed that ropivacaine influenced both Aβ- and Aδ-related afferents (N1P1 and N2P2 complexes of the SSEP waveform). Explanatory normal SSEP waveforms with the various components are described by van Loon et al. (2010). In that study, we showed that epidural methadone significantly reduced the late Aδ-afferent mediated dorsal horn activity, while not influencing the early Aβ-afferent mediated component of the SSEP complex. While opioids have a differential effect on Aβ- and Aδ-afferents, local anaesthetics are sodium channel blockers and block both Aβ- and Aδ-afferents. However, the SSEP model has some limitations. It was not possible to assess antinociceptive effects over prolonged time periods because the animals were anaesthetised and it was not possible to assess analgesic effects on different anatomical locations simultaneously. The combination of MNTs and SSEPs provided complementary information and enabled us to assess analgesic efficacy of epidural ropivacaine in a comprehensive way.

Conclusions

We evaluated the antinociceptive efficacy of low dose lumbosacral epidural ropivacaine in both conscious and anaesthetised horses. We used the SSEP model to quantify the effects of ropivacaine on afferent transmission to the spinal cord. Furthermore, we evaluated the effects of epidural ropivacaine on MNTs. By this approach, we were able to assess both spread of epidural ropivacaine and the effect of the drug over time. Ataxia scores were obtained in conscious animals after ropivacaine or placebo administration. Epidural application of ropivacaine led to mild increases of ataxia, however all animals were able to remain in standing position unaided. It is concluded that epidural ropivacaine, as applied in this study, can be a useful part of multimodal pain management in standing horses and has potential for clinical use.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors thank Suzanne Berlinski for her help with MNT measurements and Inge Wijnberg for her help with the evaluation of the ataxia scores.

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5

Analgesic and anti-hyperalgesic effects of epidural morphine in an equine LPS induced acute synovitis model

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The Veterinary Journal (2012) in press doi:10.1016/j.tvjl.2012.01.015

Abstract

Although epidural morphine is widely used in veterinary medicine, there is no information about the anti-hyperalgesic and anti-inflammatory effects in acute inflammatory joint disease in horses. Therefore the analgesic, anti-hyperalgesic and anti-inflammatory effects of epidural morphine (100 mg/animal, or 0.17±0.02 mg/kg) were investigated in horses with acute synovitis. In a cross-over study, synovitis was induced in the talocrural joint by intra-articular lipopolysaccharide (LPS). The effect of epidural morphine was evaluated using physiological, kinematical and behavioural variables. Ranges of motion (ROM) of metatarsophalangeal and talocrural joints were measured, clinical lameness scores and mechanical nociceptive thresholds (MNTs) were assessed and synovial fluid inflammatory markers were measured.

LPS injection caused transient synovitis, resulting in clinical lameness, decreased ranges of motion in talocrural and metatarsophalangeal joints, decreased limb loading at rest and increased composite pain scores. Epidural morphine resulted in a significant improvement in clinical lameness, increased ROM and improved loading of the LPS-injected limb at rest, with no effects on synovial fluid inflammatory markers. Morphine prevented a decrease in MNT and hence inhibited the development of hyperalgesia close to the dorsal aspect of inflamed talocrural joints. Our study shows that epidural morphine offers analgesic and anti-hyperalgesic effects in horses with acute synovitis, without exerting peripheral anti-inflammatory effects.

Keywords

Analgesia; epidural; morphine; kinematics; algometry

Introduction

The analgesic effects of epidurally administered morphine have been described extensively in horses (Natalini and Robinson, 2000; Goodrich et al., 2002; Fischer et al. 2009). However, in these reports, the analgesic effect of morphine was tested either in healthy horses or in combination with detomidine in horses with acute orthopaedic pain. Currently, there is no literature on the analgesic, anti-hyperalgesic and anti-inflammatory effects of epidural morphine in horses in the acute phase of synovitis. Recent studies have shown that local intra-articular application of morphine not only leads to potent analgesic effects, but has anti-inflammatory effects as well (Santos et al., 2009; Lindegaard et al., 2010; van Loon et al., 2010).

The peripheral anti-inflammatory effects of intrathecally applied morphine have been described in rodents (Zhang et al., 2005; Brock and Tonussi, 2008; Boettger et al., 2010). These peripheral anti-inflammatory effects can possibly be explained by pre-synaptic μ-opioid receptor binding on primary afferents, which cause morphine-induced decrease in the release of glutamate (Brock and Tonussi, 2008) and substance P (Mudge et al., 1979) from pre-synaptic terminals, leading to reduced dorsal root reflexes (Willis, 1999). Since all vertebrates have μ-opioid receptors in the dorsal horn of the spinal cord (Natalini, 2010), it was hypothesised that neuraxial morphine could produce peripheral anti-inflammatory effects in horses, in addition to providing analgesia and anti-hyperalgesia. However, no studies to date have described peripheral anti-inflammatory effects of neuraxial morphine in horses.

The aim of this study was to assess the analgesic, anti-hyperalgesic and anti-inflammatory effects of epidural morphine in horses, using a lipopolysaccharide (LPS)-induced acute synovitis model (van Loon et al., 2010). The effect of epidural morphine on the inhibition of hyperalgesia during the early phase of joint inflammation was also determined using pressure algometry. It was hypothesised that the analgesic effects of epidural morphine improved locomotion and

behavioural variables, while producing peripheral anti-inflammatory effects and inhibiting hyperalgesia during acute synovitis in horses.

Materials and methods

Animals

The study design was approved by the institutional Ethics Committee on the Care and Use of Experimental Animals in compliance with Dutch legislation on animal experimentation. The experiment was performed with eight Dutch Warmblood mares (age (mean ± SD) 6.3 ± 1.9 years, bodyweight (BW) 593.1 ± 60.6 kg), which were all clinically sound.

Experimental design

A randomised placebo-controlled observer-blinded cross-over design was used, in which left and right talocrural joints and order of treatment were randomly assigned for each animal. A 3 week wash-out period between the first and second experimental periods was used. Four horses were treated with morphine in the first round of the cross-over study, while the other four horses were treated with placebo. In the second round, the treatment was reversed. Treatment order was randomly assigned.

All horses were trained to walk and trot on a treadmill (Graber AG, Fahrwangen) for a minimum of eight training sessions (Buchner, 2001). Furthermore, horses were habituated to the test stables for 2 weeks. Baseline kinematics and video recordings for clinical lameness scoring were performed on the treadmill the day before synovitis was induced. At the same time, behavioural assessment videos were made, composite pain scores and mechanical nociceptive thresholds (MNTs) were assessed and joint circumference was measured. The following day, baseline synovial fluid (SF) was collected, after which synovitis was induced by injection of LPS (T0). One hour after induction of synovitis (T1), morphine or a similar volume of saline was administered epidurally. Blood (jugular venepuncture) and SF (routine arthrocentesis) was collected at 4, 8 and 28 h after treatment

with morphine or placebo (T4, T8 and T28), then hindlimb kinematics were measured and video recordings were made for clinical lameness evaluation on the treadmill and for behavioural variables. Horse behaviour was videotaped by remote operation of the camera. Before each arthrocentesis, joint circumference was measured. Fifty-six and 168 h after treatment with morphine or placebo (T56 and T168), further SF samples were collected and video recordings for clinical lameness evaluation were made at T56.

Synovitis induction

At T0, the left or right tarsus of each horse was clipped and surgically prepared for dorsomedial arthrocentesis of the talocrural joint. Sterile LPS from *Escherichia Coli* O55:B5 (catalogue number L5418, Sigma-Aldrich) was diluted to a final concentration of 0.21 ng/mL in sterile saline solution. A twitch was applied to each horse and arthrocentesis was performed using a 21 G x 40 mm needle. After withdrawal of 5 mL of the synovial fluid sample at T0, 0.8 mL LPS solution (containing 0.17 ng LPS) was delivered aseptically into the talocrural joint.

Epidural analgesia

The site of insertion of the epidural catheter, between the 1st and 2nd coccygeal vertebrae, was surgically prepared and locally anaesthetised using 2 mL lidocaine HCl 2% (B. Braun) subcutaneously (SC). A 16 G Tuohy spinal needle (Perifix, B. Braun) was inserted into the epidural space, the hanging drop technique and loss of resistance were used to ensure correct placement. A 19 G catheter was advanced through the Tuohy needle for 20 cm and reached the second sacral vertebral level. The Tuohy needle was removed and the catheter left in situ while either 100 mg (0.17 ± 0.020 mg/kg diluted to 15 mL) of preservative-free morphine (Centrafarm) or placebo (15 mL saline) was injected over 3 min. The catheter was then withdrawn from the epidural space.

Subjective and objective lameness evaluation

Subjective lameness evaluation was based on the retrospective scoring for lameness of the video recordings by a board certified equine orthopaedic

surgeon, using a standardised 0-5 scale (Ross and Dyson, 2003). Kinematic gait analysis was performed with the horse walking (1.7 m/s) and trotting (3.3 m/s) on a treadmill. Spherical reflective markers 1.5 cm in diameter were attached to the skin of both hindlegs over the following anatomical landmarks: lateral surface of the hoof wall (hoof); lateral surface of the distal portion of the metatarsal condyle (metatarsophalangeal joint); lateral malleolus of the tibia (talocrural joint), and the distal attachment of the lateral collateral ligament of the femorotibial joint on the lateral aspect of the proximal tibia (head of fibula). Six infrared video cameras linked with an automatic digitising system (Qualisys AB) were placed on both sides of the treadmill to record horses' movements at 240 Hz in a pre-calibrated measuring volume of approximately 3 x 2 x 1 m.

Behavioural variables

Horses were housed individually in box stalls where they received water and silage ad libitum. Thirty-minute behavioural video recordings were made at baseline (T0), between 5 and 6 h (T5-6), at 12 h (T12) and between 29 and 30 h (T29-30) after treatment with morphine or placebo. Ten predefined behaviours (foraging, pawing on the floor, box walking, lying down, standing still, weight shifting, rolling, head shaking, tail movement and flehmen) were evaluated for their frequency of occurrence and total duration. These videos were scored using the Observer, version 5.03 (Noldus Information Technologies). A composite pain scale (Bussières et al., 2008) was used to score pain status and the horses were videotaped for 15 min to determine the percentage of time the injected limb was loaded at T0, T4, T8 and T28.

Mechanical nociceptive thresholds (MNTs)

MNTs were determined with a non-electrical pressure algometer (FPK 60, Wagner Instruments), which has a force gauge with a 1 cm^2 rubber tip and a capacity from 3 - 30 kgf. Pressure algometer measurements were performed as described by Haussler and Erb (2006). Pressure was applied perpendicular to the surface with a constant rate of approximately 5 $kg/cm^2/s$. The median of three repeated measurements with intervals of 3-4 s was used as site-specific MNT (Nussbaum

and Downes, 1998). MNTs were measured at: the lateral malleolus and at the proximodorsal aspect of the third metatarsal bone of both left and right talocrural joints for detection of secondary hyperalgesia;, the midline at the spinal process of L6 and the centre of left and right gluteus medius muscles at the level of L3 for central sensitisation; and the ipsilateral scapula for generalised sensitisation unrelated to the segmental innervation of the hindlimb.

Synovial fluid and inflammatory mediators

After arthrocentesis, 5 mL of SF was collected at each time point. Part of each SF sample was placed in an EDTA tube for macroscopic evaluation, routine SF white blood cell count and total protein measurement (refractometer), while the remainder was centrifuged in plain tubes at 3400 *g* for 15 min, aliquoted and stored at -80 °C for further analysis. Following RP-18 extraction of SF samples, prostaglandin E_2 (PGE_2) concentration was measured by means of mass spectrometry (de Grauw et al., 2009).

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using a linear mixed model for repeated measures, with horse as a random effect, and time and treatment as fixed effects and repeated factors. When significant treatment effects were found, univariate post-hoc tests were used for comparisons at each time point, using Bonferroni's correction for multiple comparisons. Categorical clinical variables (lameness score, joint distension score, reaction to palpation of the injected joint, composite pain score) are presented as median values ± 25 and 75 percentiles and were analysed using Friedman tests to evaluate changes over time and Wilcoxon signed ranks tests for comparison between placebo and morphine treatment. Correlations between MNTs and inflammatory synovial fluid markers were determined with Pearson's correlation analysis. Statistical analyses were performed using SPSS version 16.0 (SPSS) and significance was set at $P<0.05$.

Results

LPS-injection in placebo-treated animals led to transient lameness with maximal lameness scores of 3 out of 5 in trot at T4 that resolved spontaneously within 12 hours. Horses did not sweat, had normal appetite and no long term effects were seen. Rescue analgesia (IV butorphanol), in case of lameness scores exceeding 4 out of 5 or if horses would get recumbent, was deemed unnecessary.

Evaluation of systemic effects

No changes in haematological variables (white blood cell count, percentage granulocytes and packed cell volume), heart rate or respiratory rate were found and rectal temperature also remained within physiological limits (37.5 – 37.9°C).

Lameness evaluation

Clinical lameness scores

Morphine significantly decreased the lameness scores at trot at T4 ($P = 0.039$). At T28, lameness scores had returned to BL levels in all horses (Figure 1).

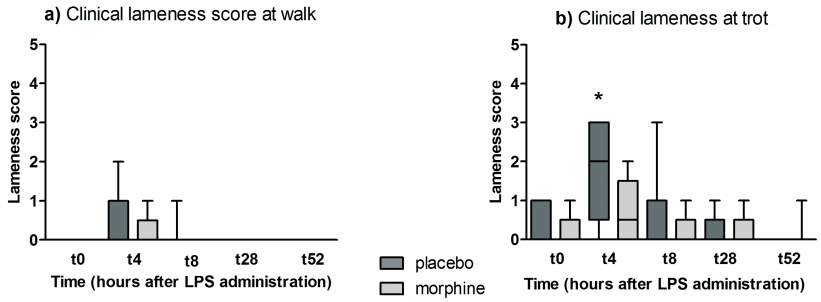

Figure 1 Median clinical lameness scores with 25th-75th percentiles (boxes) and ranges (whiskers) at walk (a) and at trot (b) at 0, 4, 8, 28 and 52 hours following intra-articular administration of 0.17 ng lipopolysaccharide (LPS). Horses (n = 8) were treated epidurally with morphine (100 mg) or placebo at one hour after administration of LPS. * = $p < 0.05$.

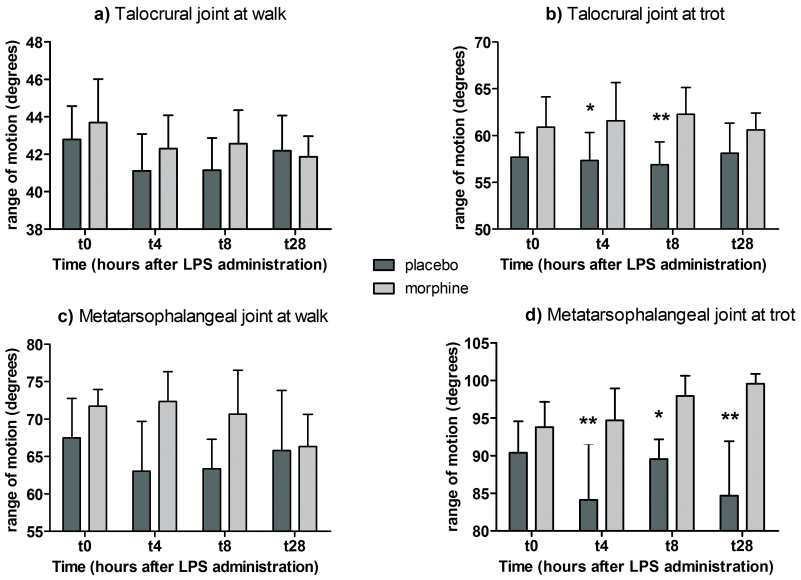

Figure 2 Mean ± SEM ranges of motion (ROM) of the tarsus at walk (a) and at trot (b) and of the metatarsophalangeal joint at walk (c) and at trot (d) at 0, 4, 8 and 28 hours following intra-articular administration of 0.17 ng lipopolysaccharide (LPS). Horses (n = 8) were treated epidurally with morphine (100 mg) or placebo at one hour after administration of LPS. * = $p < 0.05$, ** = $p < 0.01$.

Kinematic gait analysis

After LPS administration, ranges of motion of both talocrural and metatarsophalangeal joint decreased during trot. Epidural morphine significantly counteracted the decrease in ROM in both talocrural (T4: $P = 0.030$, T8: $P = 0.004$) and metatarsophalangeal joints (T4: $P = 0.004$, T8: $P = 0.015$, T28: $P = 0.007$) (Figure 2).

Weight-bearing at rest and joint swelling

LPS administration led to decreased weight-bearing of the injected limb during stance at 4 and 8 h after administration, which was significantly improved by morphine at T8 ($P = 0.001$) (Figure 3a). Joint circumference increased until T28 and then gradually declined. Morphine treatment did not influence joint circumference.

Behavioural variables

A significant main effect for time was found in composite pain scale (CPS) scores ($P < 0.01$). Post-hoc tests did not show significant differences in CPS scores at single time points (Figure 3b). Neither significant increases compared to baseline nor significant differences between placebo and morphine treatment were found for any of the 10 behavioural variables.

Mechanical nociceptive thresholds

Repeatability

Across all measurements, the three consecutive values sequentially increased in 16.8%, sequentially decreased in 7.6% and showed no change or a consistent pattern in 75.6% of the measurements. The mean range of three successive measurements across all measurements was 2.2 ± 1.41 kg/cm^2. The results of T0 MNTs at nine anatomical landmarks (Figure 4) indicated lower MNTs at extremities compared to the trunk. T0 MNTs at both left and right lateral malleolus and left and right proximodorsal metatarsal bone landmarks showed no statistical differences between the first and second round of the cross-over trial.

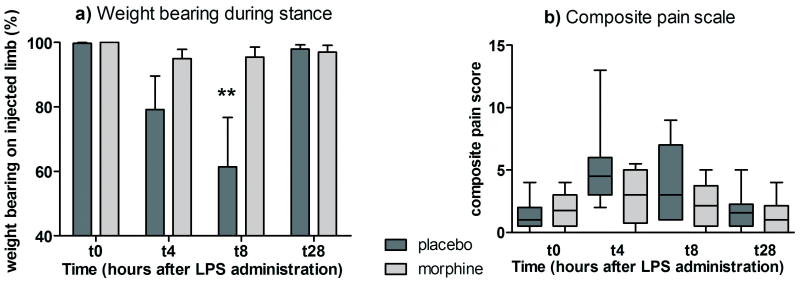

Figure 3 Mean ± SEM relative time spent weight bearing on injected limb during stance (a) during 15 minute video observations and Median ± 25-75th percentiles (boxes) and ranges (whiskers) Composite Pain Scale scores (b) at 0, 4, 8 and 28 hours following intra-articular administration of 0.17 ng lipopolysaccharide (LPS). Horses (n = 8) were treated with morphine (100 mg epidurally administered) or placebo at one hour after administration of LPS. ** = p< 0.01.

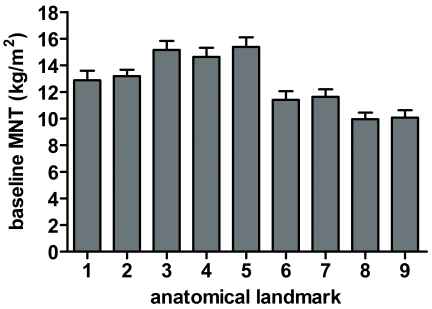

Figure 4 Mean ± SEM Baseline (BL) Mechanical Nociceptive Treshold (MNT) at various anatomical landmarks (n = 16, except for scapula: n = 8). 1 = left scapula, 2 = right scapula, 3 = processus spinosus L6, 4 =left gluteus medius L3, 5 = right gluteus medius L3, 6 =left lateral malleolus, 7 = right lateral malleolus, 8 = left proximodorsal aspect of third metatarsal bone, 9 = right proximodorsal aspect of third metatarsal bone.

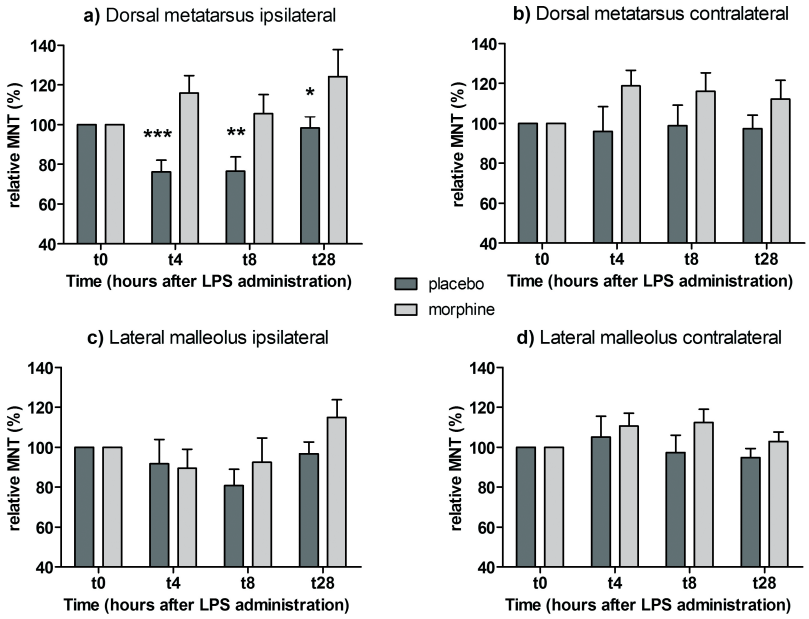

Figure 5 Mean ± SEM Mechanical Nociceptive Treshold (MNT) for dorsal aspect of proximal metatarsus of ipsilateral (synovitis) limb (A), dorsal aspect of proximal metatarsus of contralateral limb (B), lateral malleolus of ipsilateral (synovitis) limb (C) and lateral malleolus of contralateral limb(D) at 0, 4, 8 and 28 hours following intra-articular administration of 0.17 ng lipopolysaccharide (LPS). Horses (n = 8) were treated epidurally with morphine (100 mg) or placebo at one hour after administration of LPS. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Effects of LPS and morphine administration

Significant differences in MNTs between morphine and placebo treated horses were found at the dorsoproximal metatarsus of the injected limb at T4 ($P < 0.001$), T8 ($P = 0.006$) and T28 ($P = 0.013$) (Figure 5). No time effects or differences between morphine and placebo treatment at the other anatomical landmarks were found.

Inflammatory markers

No statistical differences between placebo and morphine treated animals were found for SF white blood cell counts, total protein content and PGE_2 concentrations (Figure 6).

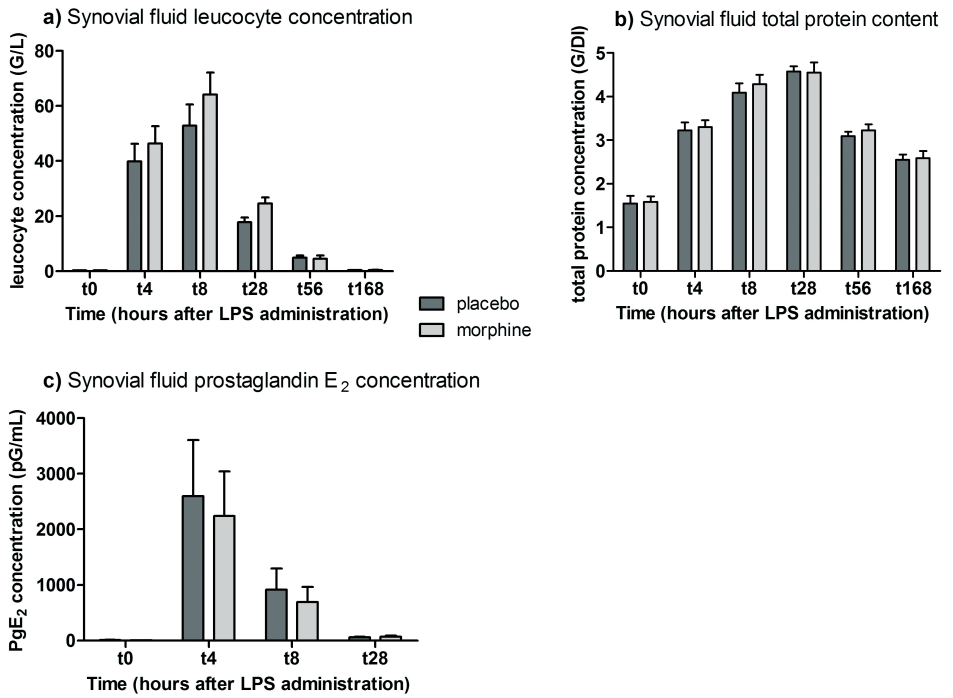

Figure 6 Mean ± SEM inflammatory synovial fluid markers at 0, 4, 8, 28, 56 and 168 hours following intra-articular administration of 0.17 ng lipopolysaccharide (LPS). Horses (n = 8) were treated epidurally with morphine (100 mg) or placebo at one hour after administration of LPS.

Correlation coefficients

There were significant negative correlations between MNTs and SF leukocyte counts, SF PGE_2 concentrations and SF total protein concentrations (Table 1).

Table 1 Pearson's correlations between MNTs and synovial fluid parameters. Correlations between changes in variables between T0 and T4, between T4 and T8 and between T8 and T28 for placebo treatment (n = 24).

	r	*P-value*
MNT - SF leukocyte count	-0.53	< 0.01
MNT – SF PGE_2 concentration	-0.49	0.014
MNT – SF total protein concentration	-0.49	0.015

Discussion

This study showed that epidural morphine significantly reduced lameness and analgesia, as evidenced by decreased lameness scores, increased ranges of articular motion and improved weight-bearing at rest, compared to placebo treatment in an LPS-induced acute equine synovitis model. Epidural morphine also counteracted decreases in MNTs close to the dorsal aspect of the inflamed talocrural joint, preventing secondary hyperalgesia. However, the peripheral anti-inflammatory effects of epidural morphine could not be determined.

Epidural morphine significantly reduced subjective clinical lameness scores at trot by 4 h after administration, while objective kinematical analysis was even more discriminative for the detection of differences between placebo and morphine treatment. Significant differences between treatment and placebo were demonstrated until 8 h after morphine administration for the talocrural joint and until 28 h for the metatarsophalangeal joint. At rest, weight-bearing of the LPS-injected limb was significantly reduced in placebo-treated animals at 8 h after treatment.

Analysis of the behavioural videos for activity budgets and event occurrence (Price et al., 2003; Pritchett et al., 2003) did not reveal any increase in pain related behaviour in placebo or treatment groups. In contrast, we had previously shown that the same LPS-induced synovitis model, but using a higher dosage of LPS, induced changes in individual pain-related behavioural variables, such as increased recumbency and reduced foraging behaviour (van Loon et al., 2010). Although composite pain scale analysis has been described to be very useful in detecting subtle or complex pain behaviours or small changes in degree of pain (van Dijk et al., 2001; Lerche, 2009), we were not able to find significant differences between placebo and morphine treatment at single time points, despite the fact that placebo-treated animals did show significant increases in CPS over time and morphine-treated animals did not. This was possibly due to the subtle inflammatory model used in the current study, the wide spread in the pain scores and the relatively small number of animals in our experiment. With the low dose of LPS used in this study, we were able to induce obvious lameness in the placebo group, without significant systemic LPS effects or behavioural pain-related signs, which was considered more ethically acceptable.

Although the analgesic effects of epidural morphine have been described extensively in horses previously (Natalini and Robinson, 2000; Goodrich et al., 2002), this is the first study that not only assessed morphine-induced analgesia, but also addressed the effects on hyperalgesia and peripheral inflammation. Morphine has been reported to prevent peripheral hyperalgesia in a rat model (Boettger et al., 2010), with strong antinociceptive effects of spinal morphine in the acute phase of experimental arthritis. Spinal morphine counteracted primary hyperalgesia (at the site of the inflamed joint) and reduced joint swelling. Morphine also prevented secondary hyperalgesia at a remote side of the inflamed joint, which indicated that central sensitisation occurred in the placebo group. Boettger et al. (2010) applied morphine intrathecally, pre-emptively and as a continuous infusion, whereas epidural morphine was administered as a single dose 1 h after induction of synovitis in the current study.

The volume of epidural injectable used (15 mL) was intended to reach the lumbosacral plexus to modulate the (sensory) innervation of the hindlimb in general and of the talocrural joint in particular. The lumbosacral plexus is formed by the spinal nerves of L4-S4 (although individual variation does exist), from which the peripheral nerves are formed to innervate the hindlimb (Fintl, 2009). Epidural morphine had a significant anti-hyperalgesic effect at the dorsal proximal metatarsus. Central sensitisation could not be determined at the axial pressure algometry landmarks (gluteus muscles and spinal process L6), although there was a non-significant tendency to decreased MNTs in the placebo treated animals at these locations. A possible explanation could be that the synovitis model used in the current study did generate acute phase central sensitisation, leading to secondary hyperalgesia, as shown by increased sensitivity to pressure stimuli of neighbouring deep structures of the joint, but should have been longer lasting or more intense to lead to late or disinhibition stage of central sensitisation (Woolf, 2007). These late stages of central sensitisation could lead to more diffuse and widespread pain sensitivity that can be quantified at more remote locations (Schaible et al., 2002). Alternatively, pressure algometry may not be sensitive enough to detect central sensitisation at more remote locations in these early stages.

Centrally administered morphine has been reported to induce peripheral anti-inflammatory effects (Schmitt et al., 2003; Tsai et al., 2009), although this was not found in the current study following analysis of SF leucocytes, total protein and prostaglandin (PG) E_2 levels. However, earlier studies administered the epidural morphine pre-emptively, whereas epidural morphine was administered after the induction of synovitis in the current study.

Baseline-MNTs measured at the axial skeleton in the current study were similar to a previous study (Haussler and Erb, 2006). Haussler et al. (2007) described MNTs of the thoracic limb in horses with induced osteoarthritis of the middle carpal joint and found baseline values that were much higher than the baseline MNTs in the current study at the dorsal aspect of the talocrural joint of the hind limb. This

difference in baseline values could possibly be caused by the fact that applying pressure to the dorsal aspect of the carpal joint with an algometer impedes a proper withdrawal reflex, whereas pressure on the dorsal aspect of the tarsal joint does not. Although we were able to demonstrate secondary hyperalgesia at the dorsoproximal metatarsus at close proximity of the talocrural joint, MNTs at the lateral aspect of the talocrural joint (lateral malleolus) did not show hyperalgesia. This may possibly be due to the fact that pressure on the lateral malleolus does not exert direct pressure on the articular capsule, which can be supposed to be the most sensitive structure of the joint and its related structures.

A limitation of the current study was the fact that we obtained relatively low post-hoc power (55.5-57%) with respect to statistical analysis of the parameters clinical lameness and ROM of the talocrural joint. This was mainly due to the relatively small sample size, variation in parameters and the relatively subtle lameness and the consequent low changes in ROM that were obtained using low dose intra-articular LPS. However, since changes in ROM of the metatarsophalangeal joint and weight-bearing at rest showed a post-hoc power of 72-79%, it was likely that epidural morphine did have beneficial analgesic effects on locomotion and weight-bearing. Furthermore, MNTs showed very high post-hoc power (97%) and strong statistical evidence for the anti-hyperalgesic effects of epidural morphine.

Conclusions

The current study showed that the analgesic effects of epidural morphine in an acute equine synovitis model decreased lameness, improved weight-bearing at rest and improved ranges of motion in both talocrural and metatarsophalangeal joints during locomotion. Furthermore, epidural morphine was shown to counteract secondary hyperalgesia as quantified by mechanical nociceptive thresholds, although peripheral anti-inflammatory effects of epidural morphine could not be confirmed. The results indicated the potential of epidural morphine in multimodal analgesic treatment of acute pain in the hindquarters of the horse.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors thank Janny de Grauw and Chris van de Lest for practical assistance with processing of synovial fluid samples and for help with statistical procedures and Annemarie Baars and Marie-Jose van‘t Klooster for their help during behavioural data collection.

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6

Intra-articular opioid analgesia is effective in reducing pain and inflammation in an equine LPS induced synovitis model

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Equine Veterinary Journal (2010) 42 (5) 412-419

Summary

Reasons for performing study Intra-articular administration of morphine as a local analgesic and anti-inflammatory drug is widely used in human medicine. In equids, little is known about its clinical analgesic and anti-inflammatory efficacy.

Objectives To use an inflammatory orthopaedic pain model to investigate the analgesic and anti-inflammatory effects of intra-articularly administered morphine as a new treatment modality in horses with acute arthritis.evj_77 41.419

Methods In a crossover study design, synovitis was induced in the left or right talocrural joint by means of intra-articular injection of 0.5 ng lipopolyssacharide (LPS). The effect of 120 mg morphine, intra-articularly administered at 1 h after induction of synovitis, was evaluated using both physiological and behavioural pain variables. Synovial fluid was sampled at 0, 4, 8, 28 and 52 h after induction of synovitis and analysed for total protein concentration, leucocyte count and for prostaglandin E_2, bradykinin and substance P concentrations by ELISA. Ranges of motion of metatarsophalangeal and talocrural joints were measured as kinematic variables with the horses walking and trotting on a treadmill under sound and lame conditions. Clinical lameness scores and several behavioural variables related to the perception of pain were obtained.

Results LPS injection caused marked transient synovitis, resulting in increased concentrations of inflammatory synovial fluid markers, clinical lameness, joint effusion and several behavioural changes, such as increased time spent recumbent, decreased limb loading at rest and decreased time spent eating silage. Intra-articular morphine resulted in a significant decrease in synovial white blood cell count, prostaglandin E_2 and bradykinin levels and improvement in clinical lameness, kinematic and behavioural parameters, compared to placebo treatment.

Conclusions Intra-articular morphine offers potent analgesic and anti-inflammatory effects in horses suffering from acute synovitis.

Potential relevance Local administration of opioids may be useful for horses with acute inflammatory joint pain and offers possibilities for multimodal analgesic therapies without opioid-related systemic side effects.

Introduction

In the last decade, an increase in interest in local analgesic procedures in equine veterinary medicine has evolved (Robinson and Natalini, 2002; Muir, 2005). Although the clinical use of intraarticularly (IA) administered local anaesthetic solutions and steroidal anti-inflammatory drugs is widespread in horses (Schumacher et al., 2003; Kristiansen and Kold, 2007), the IA administration of opioids has not received much attention in the equine species. There is abundant scientific and clinical evidence for the analgesic and anti-inflammatory properties of IA administered opioids in man, both in acute and chronic joint pain (Likar et al., 1997; Gupta et al., 2001; Kalso et al., 2002).

In dogs, IA morphine has been described to produce effective analgesia following arthrotomy (Day et al., 1995; Sammarco et al., 1996). In horses, however, literature on IA use of morphine is very limited. Sheehy et al. (2001) showed synovial membranes of horses to contain μ-opioid receptors and Raekallio et al. (1996) described disposition and local effects of IA morphine in normal ponies. In a recent study, Santos et al. (2009) investigated the effects of IA ropivacaine and morphine on lipopolysaccharide (LPS)-induced synovitis in horses by means of subjective pain scales only. To completely understand the mechanism of action of new treatments for joint disorders, not only functional effects (quality of locomotion) but also physiological effects at tissue level and effects on pain perception should be evaluated comprehensively and quantitatively. Several studies have evaluated orthopaedic pain in horses (Price et al., 2003; Bussières et al., 2008) and various approaches to the assessment of pain have been used, but composite evaluation of

pain related parameters, including both behavioural and physiological elements, has produced the most accurate and reliable results so far (Raekallio et al., 1997). Because orthopaedic pain resulting from synovitis is mostly accompanied by a certain level of lameness in horses (Caron, 2003), lameness evaluation needs to be incorporated when assessing acute orthopaedic pain in horses. Objective lameness evaluation can be performed by means of kinetic and kinematic gait analysis (Khumsap et al., 2003; Ishihara et al., 2009). At the physiological level, inflammatory synovial fluid markers, such as substance P, prostaglandin E_2 (PGE_2) and bradykinin, have been related to inflammatory pain in horses, dogs and man (Suzuki et al., 2003; Trumble et al., 2004; de Grauw et al., 2006). Further evaluation of these markers in relation to acute orthopaedic pain in horses is needed. Besides these physiological parameters, behavioural elements are indispensible when evaluating (orthopaedic) pain in horses (Raekallio et al., 1997; Price et al., 2003).

The present study was designed to serve a dual purpose. Firstly, to test the hypothesis that IA morphine significantly attenuates joint pain in a previously validated equine model of LPS induced synovitis (Palmer and Bertone, 1994; de Grauw et al., 2009a). Secondly, to develop a composite evaluation method for orthopaedic pain in horses that would deepen our insight into nociceptive mechanisms in equine inflammatory arthropathies. To this end, the analgesic and anti-inflammatory effects of IA morphine in this model were comprehensively assessed by means of quantitative gait analysis, behavioural observations and biomarker analysis in synovial fluid.

Materials and methods

Animals

The study design was approved by the institutional Ethics Committee on the Care and Use of Experimental Animals in compliance with Dutch legislation on animal experimentation. The experiment was performed with 8 Dutch Warmblood mares (age mean ± s.d. 7.1 ± 3.7 years, weight 589.1 ± 61.6 kg, height at the withers 166.4 ± 7.6 cm), which were all clinically nonlame.

Experimental set-up

A 2 period randomised placebo-controlled crossover design was adopted, in which left or right talocrural joints and order of treatment (placebo vs. morphine) were assigned randomly for each animal, with a 3 week washout period between the first and second experimental periods. In the weeks preceding the experiment, all horses were trained to walk and trot on a treadmill[1] for a minimum of 8 training sessions (Buchner et al., 2001). Furthermore, horses were habituated to the behavioural observation stables for several days. The day before synovitis was induced, baseline (BL) kinematics were measured and video recordings for clinical lameness scoring on the treadmill, as well as video recordings and direct clinical tests for behavioural assessment were performed. At T0, BL synovial fluid (SF) and plasma samples were collected, after which synovitis was induced by injection of LPS. One hour after induction of synovitis (T1), a 120 mg dose of morphine[2], diluted in 20 ml of saline[3] or a similar volume of saline without morphine as placebo treatment was administered IA. At 4, 8 and 28 h after LPS administration (T4, T8 and T28) blood was collected by jugular venipuncture and SF was obtained via routine arthrocentesis, after which hindlimb kinematics were measured and video recordings were made for clinical lameness evaluation on the treadmill. At these same time points, video recordings for behavioural variables were made and clinical behavioural testing was performed. Fiftytwo hours after LPS administration (T52), only SF samples were collected and video recordings for clinical lameness evaluation were made.

Synovitis induction

At T0, the left or right tarsus of each horse was clipped and surgically prepared for dorsomedial arthrocentesis of the talocrural joint. Sterile LPS from *Escherichia coli* O55:B5 (catalogue number L5418)[4] was diluted to a final concentration of 0.625 ng/ml in sterile saline solution. Horses were twitched and arthrocentesis was performed with a 21 gauge x 40 mm needle.

After withdrawal of the T0 synovial fluid sample, 0.8 ml LPS solution (containing 0.5 ng LPS) was delivered aseptically into the talocrural joint.

Subjective and objective lameness evaluation

For subjective lameness evaluations, video recordings were made for retrospective visual scoring of the lameness by a board certified equine orthopaedic surgeon, who was blinded for treatment and time after LPS-administration and scored all horses both at walk and trot in random order, using a standardised 0–5 scale (Ross and Dyson, 2003). Subjective lameness evaluations were performed after each period of the crossover trial. For objective lameness evaluation, kinematic gait analysis was performed with the horse walking (1.7 m/s) and trotting (3.3 m/s) on a treadmill. Spherical retroflective markers 1.5 cm in diameter were attached to the skin of both hindlegs over the following anatomical landmarks: lateral surface of the distal portion of the metatarsal condyle (for metatarsophalangeal joint), lateral malleolus of the tibia (for talocrural joint), distal attachment of the lateral collateral ligament on the lateral aspect of the proximal portion of the tibia (proximal fibula head; for femorotibial joint), proximal part of the major trochanter of the femur (for coxofemoral joint), and *tuber coxae*. Additional markers were placed on the lateral surface of the hoof wall (hoof) and on the dorsal midline between the right and left *tubera sacrale* (for sacrum). Six infrared video cameras linked with an automatic digitising system[5] were placed on 2 sides along the treadmill to record the horses' movements at 240 Hz in a precalibrated measuring volume of approximately 3 x 2 x 1 m.

Synovial fluid and blood sampling and inflammatory mediators

Blood was collected in EDTA tubes from the jugular vein for routine haematology and analysed immediately. After arthrocentesis, part of each SF sample was placed in EDTA tubes for macroscopic evaluation, routine SF white blood cell count with differentiation and total protein measurement (refractometer), while the remainder was centrifuged in plain tubes at 3400 *g* for 15 min, aliquotted and stored at -80°C for further analysis. PGE_2 concentration was measured by a commercial ELISA[6] following RP-18 extraction of SF samples (de Grauw et al., 2006). Substance P and bradykinin were measured using commercially available enzyme immunoassay kits[7,8] in the presence of 1 mmol/l (final concentration) phenylmethylsulphonylfluoride (an inhibitor of serine proteases). Before each

arthrocentesis, joint distension scores were determined using a simple descriptive scale (SDS) ranging 0–3 (0 meaning no distension, 3 meaning severe distension).

Behavioural variables

During the experiment, horses were housed individually in box stalls where they received water and silage *ad libitum*. Ninety minute video recordings of behaviour were conducted at BL and 12 h (T12), while between 5 and 6 h (T5–6) and between 29 and 30 h (T29–30) after LPS administration, 30 min video recordings were conducted. Twenty-two predefined behaviours (including elements in the categories eating, walking, standing still, lying down, rolling, shifting weight) were evaluated for either their frequency of occurrence or total duration. These behavioural videos were scored using Observer, version 5.03[9]. At BL, T4, T8 and T28 the horses were videotaped while several clinical tests were performed. The first was a 5 min human approach test in which the reaction to approach of an unknown person could be assessed and limb loading was evaluated. Five minutes later a palpation test of both talocrural joints (10 s pressure with the help of an extended artificial hand on lateral, medial and dorsal sites) was performed to detect hyperalgesia. Reactions to palpation were scored using an SDS ranging 0–7 (0 meaning no reaction, 7 meaning continuous lifting of the entire hindlimb, i.e. severe hyperalgesic reaction).

Statistical analysis

Data are presented as mean ± SEM Statistical analysis was performed using a linear mixed model for repeated measures, with horse as a random effect, and time and treatment as fixed effects and repeated factors. When significant treatment effects were found, univariate *post-hoc* tests were used for comparisons at each time point, using Bonferroni's correction for multiple comparisons. Categorical clinical variables (lameness score, joint distension score and reaction to palpation of the injected joint) were analysed using the Wilcoxon signed ranks test and are presented as median ± 25 and 75 percentiles. Correlations between various parameters (inflammatory markers, kinematic variables, hyperalgesia scores and clinical lameness scores) were assessed using parametric Pearson

and nonparametric Spearman correlation analysis. Statistical analyses were performed using computer software (SPSS version 16.0)[10] and significance was set at $P<0.05$.

Results

Clinical evaluation of lameness and other clinical variables

No changes in haematological variables (white blood cell count, percentage granulocytes, packed cell volume), heart rate or respiratory rate were found, all values remaining within physiological limits. Rectal temperature remained within physiological limits, except for a statistically but not clinically significant transient increase at 16 h after LPS administration in the placebo treatment group (mean ± s.e. temperature = 38.3 ± 0.37°C.). Figure 1 shows the results of clinical lameness scores. Morphine significantly decreased the lameness scores both at walk and trot at T4 ($P = 0.039$, $P = 0.027$) and T8 ($P = 0.034$, $P = 0.026$), and reduced joint distension scores at T4 ($P = 0.005$), T8 ($P = 0.014$) and T28 ($P = 0.025$) (Table 1). At T28, lameness scores had returned to BL level in all horses while joint distension showed a more gradual decline over time.

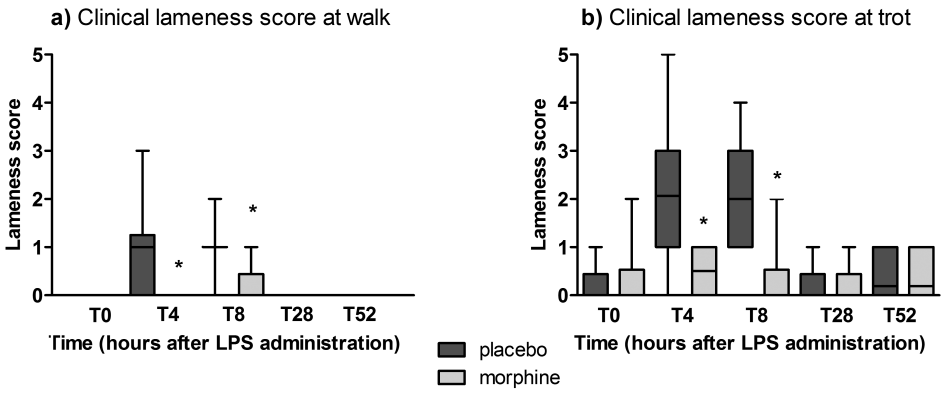

Figure 1 Median clinical lameness scores with 25th-75th percentiles (boxes) and ranges (whiskers) at walk (A) and at trot (B) at 0, 4, 8, 28 and 52 hours following intra-articular administration of 0.5 ng lipopolyssacharide (LPS). Horses (n = 8) were treated intra-articularly with morphine (0.2 mg/kg) or placebo at one hour after administration of LPS. * = $p < 0.05$.

Table 1 Results of behavioural tests following intra-articular administration of 0.5 ng Lipopolyssacharide (LPS). Mean (SEM) relative time (%) with injected limb non-weight bearing during clinical approach test (total duration: 5 min.), median (25^{th} – 75^{th} percentile) joint palpation scores (0-7 SDS scale) and joint distension scores (0-3 SDS scale) (n = 8 horses). * = $p < 0.05$, ** = $p < 0.001$.

		Time after LPS administration			
	treatment	T_0	T_4	T_8	T_{28}
Non-weight bearing (% time)	Placebo	0 (0)	54.3 (13.15)	74.2 (11.80)	19.5 (12.78)
	Morphine	2.1 (2.07)	11.1 (8.73) *	12.3 (8.46) **	0 (0)
Response to joint palpation	Placebo	1 (1-1)	3 (1-3.5)	1 (1-1.25)	1 (1-1)
	Morphine	1 (1-1)	1 (1-1) *	1 (1-1)	1 (1-1)
Joint distension score	Placebo	0 (0-0.25)	3 (3-3)	3 (3-3)	3 (2.75-3)
	Morphine	0 (0-0)	2 (2-2)*	2 (2-2)*	2 (2-2)*

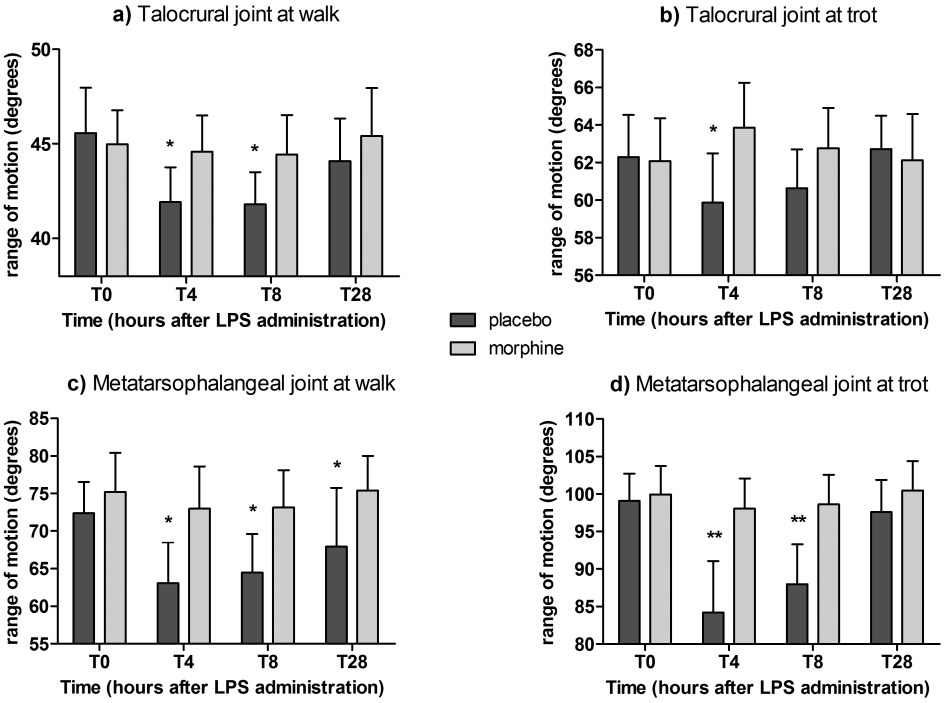

Figure 2 Mean ± SEM Range of Motion (ROM) for tarsus at walk (A), tarsus at trot (B), metatarsophalangeal joint at walk (C), and metatarsophalangeal joint at trot (D) at 0, 4, 8 and 28 hours following intra-articular administration of 0.5 ng lipopolyssacharide (LPS). Horses (n = 8) were treated intra-articularly with morphine (0.2 mg/kg) or placebo at one hour after administration of LPS. * = $p < 0.05$, ** = $p < 0.001$.

Kinematic gait analysis variables

Significant effects of morphine treatment were found in the range of motion of the tarsus, which showed a decrease during walk (T4: P = 0.045, T8: P = 0.048) and during trot (T4: P = 0.022), and in the range of motion of the fetlock joint, which also decreased during walk (T4: P = 0.009, T8: P = 0.021, T28: P = 0.044) and during trot at T4 and T8 (P<0.001) (Figure 2).

Synovial fluid variables

Significant effects of morphine compared to placebo treatment included a decreased SF leucocyte count (T8: P<0.001), decreased total protein level (T4: P = 0.033), decreased PGE_2 (T4: P<0.001) and decreased bradykinin concentration (T8: P = 0.026). Substance P levels did not differ between morphine and placebo groups at any time point (Figure 3).

Behavioural variables

Morphine compared to placebo treatment led to significant decreases in time horses spent lying (sternal and/or lateral) and to significant increases in time eating silage between 5 and 6 h after LPS administration (P = 0.001, P = 0.039)

Table 2 Correlations between various pain-related variables

Correlations between changes in variables between T0 and T4, both with placebo and morphine treatment (n = 16). ROM = range of motion, SF = synovial fluid.

* = p < 0.05, ** = p < 0.01, *** = p < 0.001.

	r
Clinical lameness score trot - ROM tarsus trot	-0.47
Clinical lameness score trot - ROM fetlock trot	-0.70 **
Clinical lameness score trot – SF PGE_2 levels	0.68 **
Clinical lameness score trot – SF Bradykinin levels	0.55 *
Clinical lameness score trot – SF Substance P levels	0.49
Hyperalgesia on palpation talocrural joint – SF PGE_2 levels	0.51 *
ROM fetlock trot – SF PGE_2 levels	-0.80 ***
ROM tarsus trot– SF PGE_2 levels	-0.70 **
ROM fetlock trot – SF Substance P levels	-0.60 *
ROM tarsus trot– SF Substance P levels	-0.72 **

(Figure 4). Horses were bearing less weight on the injected limb during the human approach test with placebo treatment at T4 ($P = 0.001$) and at T8 ($P<0.001$). Reaction to palpation of the injected talocrural joint evoked exaggerated responses in the placebo group at T4 ($P = 0.044$) compared to morphine treatment (Table 1).

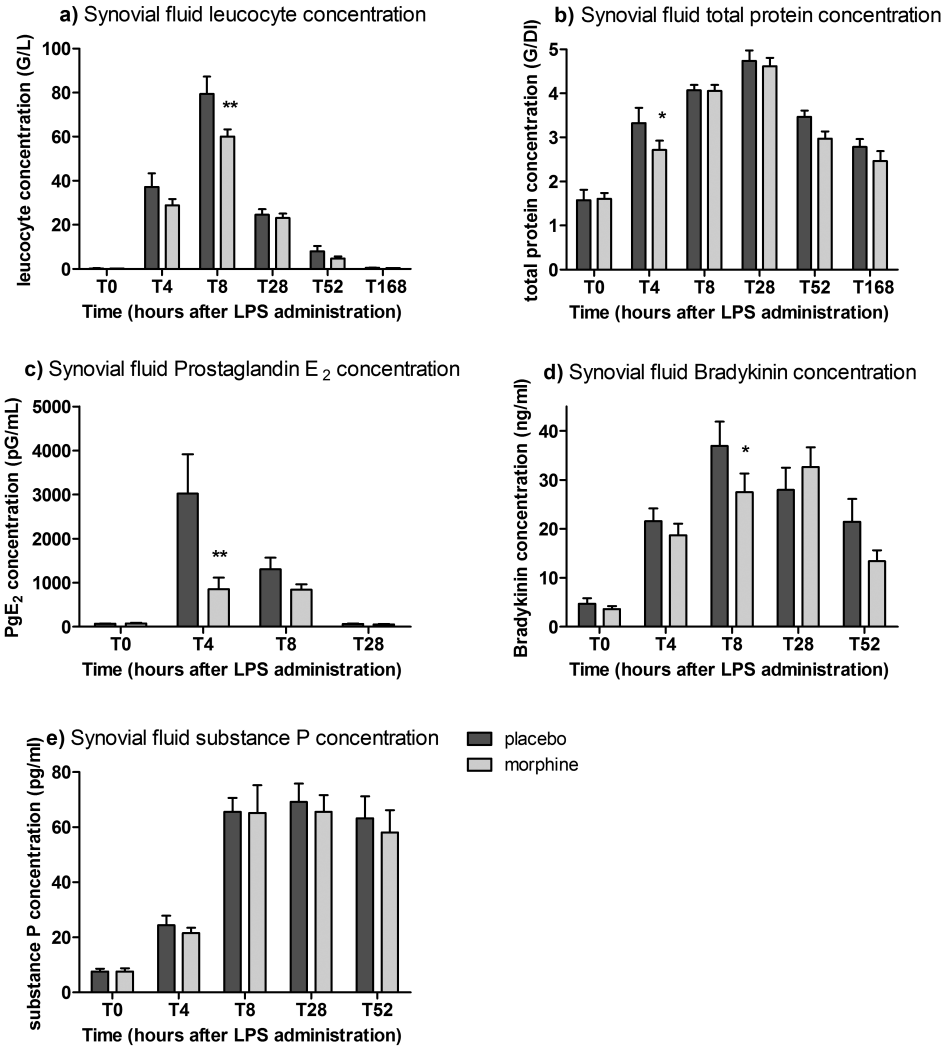

Figure 3 Mean ± SEM synovial fluid (SF) concentrations of leukocytes (A), total protein (B), prostaglandin E_2 (C), bradykinin (D) and substance P (E) at 0, 4, 8, 28, 52 and 168 hours following intra-articular administration of 0.5 ng lipopolyssacharide (LPS). Horses (n = 8) were treated intra-articularly with morphine (0.2 mg/kg) or placebo at one hour after administration of LPS. * = $p < 0.05$, ** = $p < 0.001$.

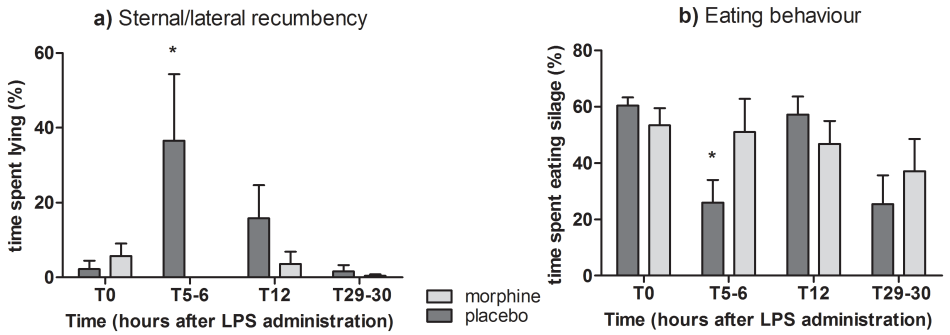

Figure 4 Mean ± SEM relative time spent lying in the box (A) and time spent eating silage (B) during 30 minutes (T5-6 and T29-30) and 90 minutes (T0 and T12) video observations at 0, 5-6, 12 and 29-30 hours following intra-articular administration of 0.5 ng lipopolyssacharide (LPS). Horses (n = 8) were treated with morphine (0.2 mg/kg intra-articularly administered) or placebo at one hour after administration of LPS. * = $p < 0.05$.

Correlation analysis

Significant correlations were found between various inflammatory SF markers, clinical lameness scores, hyperalgesia scores at palpation and kinematic variables (Table 2).

Discussion

We have demonstrated the relationship and correlations between several SF inflammatory markers, objective gait analysis and behavioural parameters, aiming to obtain a panel of objective parameters for assessment of acute orthopaedic pain in horses. By means of these objective parameters, we have assessed the analgesic and anti-inflammatory effects of IA administered morphine in an acute LPS-induced equine synovitis model.

The analgesic effects of IA morphine in man have already been described by various authors (Gupta et al., 2001; Kalso et al., 2002). Opioid receptors appear to be located on the peripheral nerve terminals in the synovium and are up-regulated under inflammatory conditions (Stein, 1995). The analgesic effects of opioids are mediated by depression of cyclic-AMP formation, activating inwardly directed potassium currents and leading to cell hyperpolarisation. The end result

is decreased neuronal excitability at peripheral sensory nerve endings (Yaksh, 1997). Peripheral anti-inflammatory effects are mediated through inhibition of calciumdependent release of excitatory, pro- inflammatory mediators from peripheral sensory-nerve endings (Stein, 1995). The anti-inflammatory effects of morphine that we found are in agreement with previous reports by other authors (Andjelkov et al., 2005; Anz et al., 2009). Although morphine significantly reduced SF leucocyte count and total protein concentrations, as well as PGE_2- and bradykinin-concentrations, we found only subtle, nonsignificant effects on SF substance P concentrations. Several authors have described depressive effects of opioids on substance P release (Yaksh, 1988; Aimone and Yaksh, 1989), but other reports (Kuraishi et al., 1983; Trafton et al., 1999) contradicted these, showing a similar lack of effect of opioids on substance P release to that in the present study. Our finding that IA administered morphine attenuated the inflammatory response in the joint without influencing SF substance P levels, suggests that opioid analgesia predominantly involves presynaptic control of nonsubstance P containing primary afferent nociceptors. This is in accordance with findings of other authors (Trafton et al., 1999; Kondo et al., 2005). In contrast, meloxicam (a nonsteroidal anti-inflammatory analgesic) did influence substance P release during LPS-induced inflammatory conditions in horses (de Grauw et al., 2009b). This shows that NSAIDs and opioids have different effects on the inflammatory response and can have synergistic effects when combined in clinical therapies.

Opioids possibly mediate presynaptic release of the pro-inflammatory neuropeptide calcitonin-gene-related peptide (CGRP), which is also found to play an important role in LPS induced inflammation in rats (Hou and Wang, 2001). Furthermore, opioids antagonise the release of arachidonic acid and block the excitatory effects of PGE_2 on nociceptors, preventing the development of peripheral hyperalgesia and allodynia (Levine and Taiwo, 1989). This agrees with the fact that palpation of the injected joint under placebo conditions led to hyperalgesia, as evidenced by an exaggerated and more pronounced response compared to BL, while treatment with morphine abolished this exaggerated response. In addition to the effects on SF inflammatory markers, we found significant effects of morphine on

clinical lameness scores and on objective kinematic variables during movement on the treadmill. The observed correlation between subjective clinical lameness evaluation and kinematic evaluation from angular motion patterns confirms and extends previous reports (Back et al., 1993; Buchner et al., 2001). Several authors have previously described kinematics in horses with LPS-induced distal intertarsal lameness, which leads to a mixed clinical lameness (both swinging and supporting components), but not with synovitis induced in the talocrural joint. The decreases in range of motion of both talocrural and fetlock joints in our lameness model are more pronounced, compared to LPS-induced distal intertarsal lameness (Khumsap et al., 2003).

Behavioural assessment showed that we did not induce opioid related side-effects such as dysphoria and locomotor behaviour, while behavioural signs of pain and discomfort could be detected in the placebo-treated horses. They spent less time eating silage and more time lying in the box and when standing were bearing weight on the injected limb for a shorter period of time, compared to morphine treatment. This agrees with previous behavioural studies in horses (Price et al., 2003; Bussieres et al., 2008) and stresses the importance of these parameters in orthopaedic pain assessment in horses.

A strong correlation was found between SF inflammatory marker levels on the one hand and clinical lameness scores, ranges of motion and hyperalgesia scores on palpation of the injected limb on the other. This indicates that these quantitative SF parameters may be useful biomarkers for objective quantification of acute orthopaedic pain in horses, although in more chronic naturally acquired lameness confounding factors may obscure or alter the relation between individual mediators and joint pain (de Grauw et al., 2006). Other authors also described the relation between SF PGE_2 and pain in man (Hogberg et al., 2006) and dogs (Trumble et al., 2004).
The analgesic effects of IA morphine are described to last up to 24 h (Kalso et al., 2002; Santos et al., 2009). Because of the shortlasting lameness that we induced with IA LPS administration (from 8 h after induction of synovitis, there was no

difference in lameness between placebo and morphine treated horses) we could not evaluate the duration of analgesia produced by IA morphine. In addition, we did not evaluate the effect of repeated morphine injections or the use of IA catheters in this study. Another limitation of our study is that we did not study joint pain in naturally occurring synovitis, but in an LPS induced synovitis model. This model reliably allows the study of severe acute transient joint inflammation, but is less likely to mimic joint pain due to naturally occurring, more chronic conditions.

In conclusion, we found potent analgesic as well as anti-inflammatory effects of IA morphine in an LPS-induced equine synovitis model. These results provide a rationale for IA opioid treatment of horses with acute inflammatory joint pain, for instance in patients with acute septic arthritis or post operative synovitis. This is of special interest in those patients that remain very painful despite NSAID treatment and/or in patients that are at increased risk for NSAID-related side-effects, and offers possibilities for multimodal analgesic therapies, combining different classes of analgesics and different routes of administration. Because of the local IA administration of opioids, the systemic side-effects of this therapy are likely to be negligible.

Acknowledgements

The authors would like to thank Renske Hammink, Eline van den Oever and Francisca Hilbers for their help during the experimental procedures.

Manufacturers' addresses

[1] Graber AG, Fahrwangen, Switzerland.

[2] Centrafarm, Etten-Leur, The Netherlands.

[3] BBraun, Melsungen, Germany.

[4] Sigma-Aldrich, St. Louis, Missouri, USA.

[5] Qualisys AB, Gotheburg, Sweden.

[6] RnDsystems, Minneapolis, Minnesota, USA.

[7] Cayman Chemical, Ann Arbor, Michigan, USA.

[8] Bachem, Bubendorff, Switzerland.

[9] Noldus information technologies, Wageningen, the Netherlands.

[10] SPSS Inc., Chicago, Illinois, USA.

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7

Upregulation of intra-articular μ-opioid receptors in an acute equine synovitis model

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Submitted

Abstract

Reasons for performing the study Intra-articular injection of opioids provides analgesia in painful equine joints and μ-opioid receptors have been demonstrated in equine synovial membranes, but it remains unknown whether up-regulation of μ-opioid receptor expression occurs in inflamed equine synovial tissue.

Objectives To evaluate whether acute inflammatory conditions will lead to up-regulation of μ-opioid receptors in equine synovial membrane, and if so, whether anti-inflammatory treatment would prevent this from happening.

Methods In a two-period, blinded, placebo-controlled randomised cross-over design, lipopolysaccharide (LPS, 1.0 ng) was injected into the L or R middle carpal joint of seven healthy ponies. Arthroscopy and synovial membrane biopsy was performed at baseline, T48 and T672 hours after LPS injection, with ponies assigned to receive either NSAID (phenylbutazone, 2.2 mg/kg PO BID) or placebo from 2 hours post-LPS. Ponies were scored clinically for pain and lameness, repeated synovial fluid (SF) samples were obtained, and the degree of synovitis was scored both macroscopically and microscopically. The density and staining pattern of μ-opioid receptors in synovial membrane biopsies over the course of the synovitis with or without NSAID treatment was evaluated using immunohistochemical techniques.

Results LPS injection consistently induced a severe transient synovitis. The clinical signs (pain, lameness) were significantly attenuated by treatment with phenylbutazone. With immunohistochemistry, up-regulation of μ-opioid receptor protein in the inflamed equine synovial membrane could be demonstrated in the placebo treated animals.

Conclusions and potential relevance Up-regulation of μ-opioid receptors in equine synovial tissue during acute inflammatory conditions could be demonstrated in this experimental set-up. In phenylbutazone (PBZ)-treated

animals this up-regulation was not seen, but there was no significant difference at any time point between PBZ- and placebo-treated animals. Our results could indicate increased efficacy of intra-articular treatment with opioids during acute inflammatory conditions.

Introduction

Local analgesic procedures have gained popularity in equine medicine in the last decade as they allow potent analgesic effects to be achieved with low plasma concentrations of drugs and thus a reduced risk of systemic side effects (Robinson and Natalini, 2002; DeRossi et al., 2004; Muir, 2005). The opioids constitute a class of drugs that is well-suited for use in such local analgesic techniques, due to the peripheral tissue expression of opioid receptors. Various studies have described the presence of peripheral opioid receptors in both humans and rodents (Levine and Taiwo, 1989; Parsons et al., 1990; Stein, 1995) and the analgesic and anti-inflammatory efficacy of intra-articularly (IA) administered opioids has been documented in several studies in humans, both in acute and chronic joint pain (Likar et al., 1997; Gupta et al., 2001; Kalso et al., 2002). The presence of peripheral opioid receptors in equine joints has been reported by Sheehy et al. (2001), who demonstrated μ-opioid receptor (MOR) expression in equine synovial membranes. In addition, several recent studies have reported substantial anti-inflammatory and analgesic effects of intra-articular morphine in equine joints with experimentally induced acute synovitis (Santos et al., 2009; Lindegaard et al., 2010a, 2010b; van Loon et al., 2010).

Under normal (unstimulated) conditions, only limited numbers of active opioid receptors are present in peripheral tissues (Stein et al., 1993). Several hypotheses exist to explain enhanced peripheral opioid effectiveness in inflammatory conditions: First, there could be an increase in *de novo* synthesis and peripheral axonal transport of opioid receptors; second, opioid receptors that are pre-existent on sensory nerves, but inactive or inaccessible by virtue of a perineural barrier under normal conditions ('silent receptors') may be activated (Antonijevic

et al., 1995). These processes need not be mutually exclusive and may even be complementary over time, with inflammation-induced disruption of the perineural barrier and facilitated access for agonists likely to occur in earlier stages of inflammation, while at later stages true receptor up-regulation may take place. Up-regulation of peripheral MORs during acute inflammation has been demonstrated in various rodent models (JeanJean et al., 1994; Hassan et al., 1993; Mousa et al., 2001), but it remains unknown whether this phenomenon also occurs in the horse.

The aim of this study was to assess whether acute inflammatory conditions will lead to up-regulation of μ-opioid receptors in equine synovial membrane, and if so, whether anti-inflammatory treatment would prevent this. To this end, we employed the LPS-induced synovitis model used in previous studies (de Grauw et al., 2009; van Loon et al., 2010). We evaluated the effect of inflammation and treatment with phenylbutazone (PBZ) or placebo on expression of μ-opioid receptors, and on microscopic and macroscopic synovitis scores in middle carpal joint synovial membranes.

Materials and Methods

Animals

The study design and experimental protocols were approved by the institutional Ethics Committee on Animal Experimentation, in accordance with Dutch national legislation on experimental animal use. For this study, n=7 sound Shetland pony geldings (mean ± SD age: 11.1 ± 3.1 yr; body weight 205.6 ± 38.5 kg) with no history of orthopaedic disease and with normal baseline carpal radiographs were used.

Experimental set-up

A randomised placebo-controlled blinded cross-over design was used, in which left and right middle carpal joints and order of treatment (placebo versus NSAID) were randomly allocated for each animal, with a three week wash-out period

between the first and second experimental period. Three weeks before induction of synovitis (T-504), arthroscopy was performed under general anaesthesia. Baseline synovial fluid was collected and a synovial membrane biopsy was taken with a 6 mm dermal biopsy punch using a dorsal approach. Arthroscopy images were recorded on video for blinded evaluation at study conclusion. At the time of synovitis induction (T0), synovial fluid (SF) was first collected by aseptic arthrocentesis, after which sterile synovitis was induced by lipopolysaccharide (LPS) injection. Sterile LPS from *Escherichia Coli* O55:B5 (catalogue number L5418)[1] was diluted to a final concentration of 1.25 ng/ml in sterile saline solution. Ponies were sedated with 10 mcg/kg detomidine[2] IV and arthrocentesis was performed with a 21Gx40 mm needle, after which 0.8 ml LPS solution (containing 1.0 ng LPS) was delivered aseptically into the middle carpal joint. Two hours after induction of synovitis (T2), 2.0 mg/kg phenylbutazone (PBZ) powder[3] or a similar volume of placebo (chalk powder) was dissolved in 10 ml of applesauce and administered orally. This treatment was repeated at 12 hour intervals for a total of 7 days. At 8, 24, 168 and 336 hours after LPS administration (T8, T24, T48, T168 and T336) SF was obtained by means of aseptic arthrocentesis. At T48 and T672, arthroscopy was performed under general anaesthesia during which a SF sample was taken and the synovial membrane biopsy (at a site just distal to the previous, avoiding biopsy of scar tissue) and video recordings were repeated. At T0, T8, T24 and T48 the ponies were walked and trotted on a straight line for lameness evaluation. Video recordings were taken, which were evaluated by a board certified equine orthopaedic surgeon (A.B.), who was blinded for treatment and time after LPS-administration and who scored all ponies in random order, using a standardised 0-5 scale (Ross and Dyson, 2003). Pain scores were assigned using the composite pain scale according to Bussières et al. (2008). Joint circumference was determined using a tape measure at T0, T8, T24 and T48.

General anaesthesia and surgical protocol

After fasting overnight with *ad lib* availability of water, ponies were prepared for general anaesthesia. The ponies were sedated with 10 mcg/kg detomidine IV and a 14 gauge catheter[4] was placed aseptically into the jugular vein and sutured to

the skin. The ponies received 10 mg/kg ampicillin[5] IV before induction of general anaesthesia and 1.1 mg/kg flunixin[6] IV before baseline arthroscopy and at T672; at T48, no NSAIDs were administered because of interference with the treatment protocol. The mouth was rinsed with water and anaesthesia was induced with ketamine[7] (2 mg/kg IV) and midazolam[8] (0.1 mg/kg IV) after which the ponies were placed in dorsal recumbency on soft mattresses. Intubation was achieved with a 16 mm silicone cuffed orotracheal tube[9] that was subsequently connected to a circle system. Anaesthesia was maintained with 1.1-1.3% end tidal isoflurane[10] in O_2. The ponies were mechanically ventilated with Intermittent Positive Pressure Ventilation (IPPV), using a tidal volume of 8-10 ml/kg and a breathing frequency of 8-10 breaths/min, in order to maintain end tidal CO_2 between 4.5 and 5.5 kPa. Heart rate and rhythm were monitored by means of ECG. Arterial oxygenation and peripheral pulse were monitored by means of pulsoxymetry. Lactated Ringer's solution[11] was infused at 5 ml/kg/hour and rectal temperature was monitored during surgery.

Synovial fluid parameters

Part of each SF sample was used for macroscopic evaluation, routine SF white blood cell count and total protein measurement (refractometer), while the remainder was centrifuged in plain tubes at 6000 rpm for 15 minutes, aliquotted and stored at -80^{0}C for further analysis.

Processing, histopathological and immunohistochemical evaluation of synovial membrane biopsies

Histopathological evaluation

Synovial membrane biopsies were fixed overnight in 10% neutral buffered formalin solution and thereafter stored in 70% alcohol. Tissue was then paraffin embedded and subsequently 3-5 μm tissue sections were cut on Poly-L-Lysine coated slides. Slides were stained with haematoxylin and eosin (H&E) for microscopic evaluation and semi-quantitative scoring of the degree of synovitis according to a modified grading scheme for the use with human synovitis (Krenn

et al., 2006). Briefly, our modified grading scheme included: 1) thickness of the synovioblast (lining) cell layer (by counting the average number of cell layers); 2) changes of the subintimal tissue and resident cells (oedema, hyperaemia, degeneration and necrosis in the acute phase (T48) and formation of granulation tissue in the chronic phase (T672)); 3) presence and extent of haemorrhage (numbers of extravasated erythrocytes at T48, numbers of hemosiderin-laden macrophages at T672); 4) numbers of inflammatory cells (mainly neutrophilic granulocytes at T48, mainly lymphocytes and plasma cells at T672) (Table 1).

Table 1: *Histological grading scheme for microscopic synovitis (Modification of the scoring scheme by Krenn et al., 2006).*

1) Thickness of the synovioblast (lining) cell layer		
Grade 0		1 layer of synovioblasts
Grade 1		2 – 3 layers of synovioblasts
Grade 2		4 – 5 layers of synovioblasts
Grade 3		more than 5 layers of synovioblasts
2) changes of the subintimal tissue and resident cells		
Grade 0		Normal numbers of resident cells (fibroblasts, macrophages), no oedema, hyperaemia, degeneration, necrosis or granulation tissue
Grade 1	T48	Slight hypercellularity, edema, hyperaemia
	T672	Slight hypercellularity, mild amounts of granulation tissue
Grade 2	T48	Moderate hypercellularity, oedema, hyperaemia, degeneration
	T672	Moderate hypercellularity, moderate amounts of granulation tissue
Grade 3	T48	Highly increased cell numbers, oedema, hyperaemia, degeneration, necrosis
	T672	Highly increased cell numbers, large amounts of granulation tissue
3) Presence and extent of haemorrhage		
Grade 0		No haemorrhage or haemosiderin-laden macrophages
Grade 1	T48	Focal mild hemorrhage
	T672	Few haemosiderin-laden macrophages
Grade 2	T48	Focal to multifocal moderate haemorrhage
	T672	Small numbers of haemosiderin-laden macrophages
Grade 3	T48	Multifocal marked haemorrhage
	T672	Moderate numbers of haemosiderin-laden macrophages
4) Numbers of inflammatory cells		
	T48	Neutrophils
	T672	Lymphocytes and plasma cells
Grade 0		No inflammatory infiltrates
Grade 1		Small numbers of inflammatory cells
Grade 2		Moderate numbers of inflammatory cells
Grade 3		Large numbers of inflammatory cells

Immunohistochemical evaluation

Tissue sections for immunohistochemistry were pre-treated with 10 mM citrate (pH 6.0) at 100^{0}C. Endogenous peroxydase activity was blocked by incubating the slides with freshly prepared 0.3% H_2O_2 in methanol. Tissue sections were incubated overnight at 4°C with rabbit polyclonal anti-MOR antibody (AB5511)[12] in 0.05 M PBS or with normal (non-immune) rabbit serum at the same dilution as a negative control. After washing, sections were incubated for 30 minutes at room temperature with a goat anti-rabbit/biotin secondary antibody (BA-1000)[13] diluted 1:125 in PBS. After rinsing 3 times in PBS the slides were incubated with ABC complex[14] for 30 minutes at room temperature, and allowed to react with DAB substrate[15] for 10 minutes. After rinsing with water, the slides were counterstained with Haematoxylin for 30-60 seconds, dehydrated with a series of alcohol from 70%, 96% to 100% and then xylene. Tissue sections of equine spinal cord stained concurrently using the same protocol were used as positive tissue controls. Staining of MOR was evaluated by means of a scoring system based on Lee et al. (2010).

A representative microscopic view was scored for A) intensity of staining with an SDS score from 0-3 (0 = negative control; 3 = intensity close to positive controls) and for B) proportion of cells stained (including synovioblasts, endothelial layer of blood vessels and other cells) from 0-3 (0 = no cells stained, 1 = small numbers, 2 = moderate numbers and 3 = large numbers).

In analogy with Lee et al. (2010), total μ-opioid receptor score was expressed as A*B. Scoring was performed by a resident in veterinary pathology (E.W.), supervised by a board-certified veterinary pathologist, who were blinded for treatment and time after LPS-administration.

Western blot analysis

Specificity of equine MOR detection by antibody AB5511 was verified using western blot analysis. In short, 2-3 g of equine dorsal horn, equine synovial membrane and mouse liver were obtained from donated cadavers and stored in liquid nitrogen. Tissue samples were disrupted for 90 seconds at 3000 rpm using a dismembrator[16]. To 100 mg of pulverized sample, 500 μl 2X NU-Page LDS

sample buffer (Invitrogen[17]) was added, after which samples were heated to 80 °C for 10 minutes and then centrifuged for 10 minutes at 13.000 rpm. Murine liver, equine synovial membrane and equine dorsal horn preparations as well as a 10 – 250 kDa protein ladder[18] were examined by SDS-PAGE (4% tris-glycine gel) under reducing conditions. Gels were transferred to nitrocellulose membranes and blocked overnight at 4°C with 1% BSA in 1x high-salt TBS with 0.05% Tween. Following blocking, membranes were rinsed 3 times with 1x TBS and incubated overnight at 4°C with primary antibody AB5511 (diluted 1:500 in PBS-T with 1% BSA). Membranes were then washed and incubated with a goat-anti rabbit HRP conjugated secondary antibody (diluted 1:5000; Thermo Fisher Scientific, Rockford, IL, USA) for 2 hours at room temperature. After further washing, membranes were treated with chemiluminencence reagent[19] for 5 minutes and proteins visualised radiographically. A band at 45 kDa was expected for the MOR protein.

Statistical analysis

All data are expressed as mean ± SEM, except for categorical parameters (CPS, lameness scores, microscopic and macroscopic synovitis scores), which are expressed as median (range). Normally distributed data were tested using a linear mixed model for repeated measures, with horse as a random effect, and time and treatment as fixed effects and repeated factors. When significant treatment effects were found, univariate *post-hoc* tests were used for comparisons at each time point, using Bonferroni's correction for multiple comparisons. Categorical data were tested by means of Friedman tests and post-hoc Wilcoxon signed rank tests. Statistical analysis was performed using SPSS version 16.0[20] Statistical significance was accepted at $P<0.05$.

Results

Composite pain scores

There was a significant effect of time after induction of synovitis on CPS in both placebo and NSAID treated ponies ($p = 0.013$ for NSAID treatment and $p = 0.001$ for placebo treatment). Placebo treated animals showed significantly higher composite pain scores at T8 ($p = 0.018$) and a tendency towards significance at T24 ($p = 0.058$) compared to the same ponies receiving placebo treatment (Figure 1).

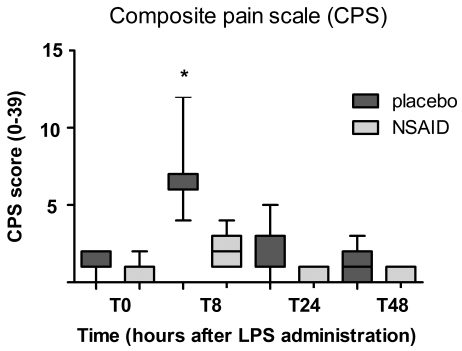

Figure 1 Median Composite Pain Scale (CPS) scores with 25th-75th percentiles (boxes) and ranges (whiskers) at 0, 8, 24 and 48 hours following intra-articular administration of 1.0 ng lipopolysaccharide (LPS). Ponies (n = 7) were treated with phenylbutazone (NSAID, 2.0 mg/kg orally administered twice daily for 7 days) or placebo, starting two hours after administration of LPS. * = $p < 0.05$.

Clinical lameness scores

Significant effects of time after induction of synovitis on lameness scores at walk and trot were found in both placebo and NSAID treated animals ($p < 0.001$ *placebo walk and trot*, $p < 0.05$ *NSAID walk and trot).* At T8 and T24, significantly lower lameness scores were observed in NSAID compared to placebo treated ponies at walk and trot (walk T8: $p = 0.017$, walk T24: $p = 0.034$, trot T8: $p = 0.018$, trot T24: $p = 0.038$) (Figure 2).

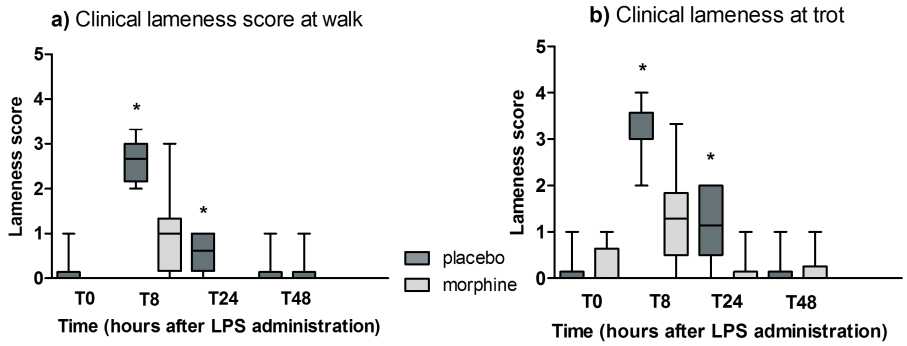

Figure 2 Median clinical lameness scores with 25th-75th percentiles (boxes) and ranges (whiskers) at walk (panel a) and trot (panel b) at 0, 8, 24 and 48 hours following intra-articular administration of 1.0 ng lipopolysaccharide (LPS). Ponies (n = 7) were treated with phenylbutazone (2.0 mg/kg orally administered twice daily for 7 days) or placebo, starting two hours after administration of LPS. * = $p < 0.05$.

Synovitis scores

Macroscopic synovitis scores in placebo treated animals showed a significant effect of time after LPS injection (*p = 0.015)*, while scores in NSAID treated animals did not (*p = 0.091*) (figure 3A). We did not observe significant differences between placebo and NSAID-treated animals at any individual time point. Microscopic synovitis scores were markedly variable. In most cases, the synovioblast cell layer

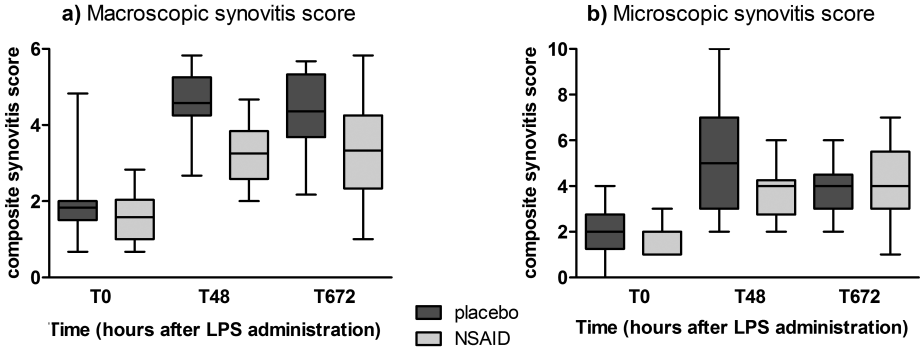

Figure 3 Median macroscopic (a) and microscopic (b) synovitis scores with 25th-75th percentiles (boxes) and ranges (whiskers) at 0, 48 and 672 hours following intra-articular administration of 1.0 ng lipopolysaccharide (LPS). Horses (n = 7) were treated with phenylbutazone (2.0 mg/kg orally administered twice daily for 7 days) or placebo, starting at two hours after administration of LPS. Placebo-treated animals showed significant increase in macroscopic synovitis score over time (p = 0.015).

was not more than five cells thick. Most abnormalities in the subintimal tissue were seen in the T48 placebo group and included mild to marked haemorrhage in several animals. At T48, small to large numbers of neutrophilic granulocytes, mainly in clusters, were present in both treatment groups. At T672, several inflammatory cell infiltrates consisting of lymphocytes and plasma cells appeared in the placebo group, but hardly any in the NSAID group. Composite microscopic synovitis scores showed a tendency to increase at T48 compared to baseline, but there were no statistically significant differences between individual time points or between treatments (Figure 3B).

SF leukocyte count and total protein concentration

Leukocyte count and total protein concentration in synovial fluid demonstrated marked increases over baseline both in PBZ and placebo treated ponies (Figure 4A and B). Leukocyte counts were not affected by phenylbutazone vs. placebo treatment, while SF total protein concentration showed a faster return to normal in the PBZ compared to the placebo-treated situation (Figure 4B).

Immunohistochemistry

Immunohistochemical analysis of MOR expression in the dorsal horn of the equine spinal cord (positive control) showed areas of intense staining particularly

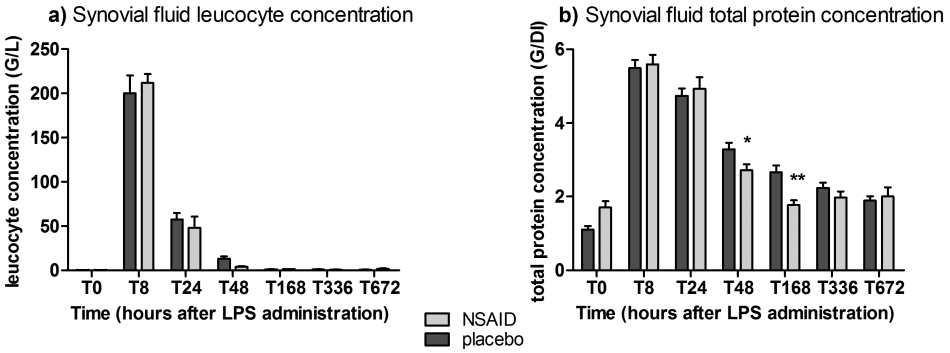

Figure 4 Mean ± SEM Synovial fluid leukocyte count (a) and total protein concentration (b) at 0, 8, 24, 48, 168, 336 and 672 hours following intra-articular administration of 1.0 ng lipopolysaccharide (LPS). Horses (n = 7) were treated with phenylbutazone (2.0 mg/kg orally administered twice daily for 7 days) or placebo, starting at two hours after administration of LPS. * = $p < 0.05$, ** = $p < 0.01$.

of neuronal axons and dendrites and no staining in negative control slides (Figure 5). Consistent with observations by Sheehy et al. (2001), the intensity of the specific immunohistochemical staining in synovial membranes was particularly high in endothelial cells of vessel walls and the synovioblast lining (Figure 7). Additionally, moderately dense staining of the cytoplasm and nuclei of fibroblasts and marked numbers of inflammatory cells (mainly lymphocytes, plasma cells and macrophages) were observed. In placebo treated animals there was a significant effect of time on MOR staining, indicating up-regulation after LPS injection ($p = 0.032$). This was not seen in NSAID treated animals ($p = 0.48$). We did not observe significant differences between placebo and NSAID-treated animals at any individual time point (Figure 6). Figure 7 shows typical examples of synovial membrane biopsies taken at baseline, 48 and 672 hours after LPS injection in both placebo- and NSAID-treated individuals.

Western blot

Western blot confirmed the presence of MOR in equine synovial tissue. Figure 8 shows the characteristic 45 kDa MOR protein band in equine synovial membrane (lane 1) and the 2 positive controls mouse liver (lane 3) and equine dorsal horn of the spinal cord (lane 2), where MOR is most abundant.

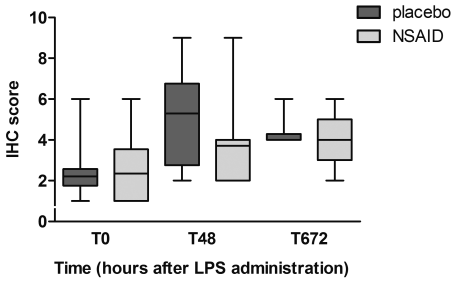

Figure 6 Median MOR Immunohistochemical (IHC) staining scores with 25th-75th percentiles (boxes) and ranges (whiskers) at 0, 48 and 672 hours following intra-articular administration of 1.0 ng lipopolysaccharide (LPS). Horses (n = 7) were treated with phenylbutazone (NSAID, 2.0 mg/kg orally administered twice daily for 7 days) or placebo, starting at two hours after administration of LPS. Placebo-treated animals show up-regulation of MOR staining (p = 0.032) from T0 to T48.

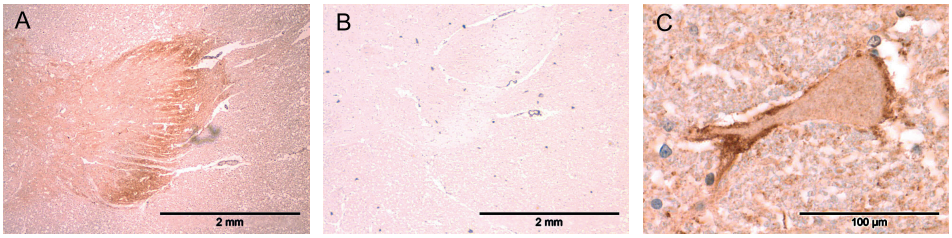

Figure 5 immunohistochemistry of MOR in equine dorsal horn spinal cord. A: overview, B: negative control (incubated without primary antibody), C: detailed view.

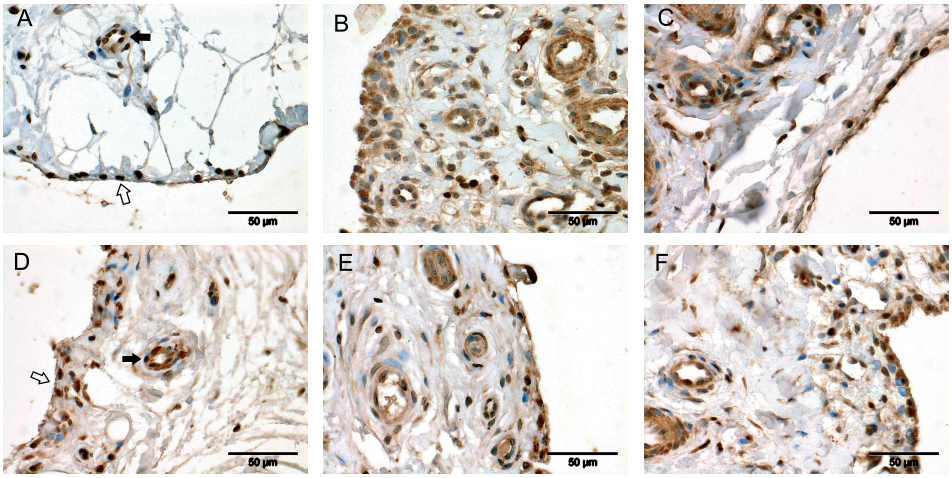

Figure 7 Immunohistochemistry of MOR in synovial membrane. A:T0 placebo, B: T48 placebo, C: T672 placebo, D: T0 NSAID, E: T48 NSAID, F: T672 NSAID. A+D: Open arrows indicate staining of synovioblasts, closed arrows of endothelial cells of vessel walls.

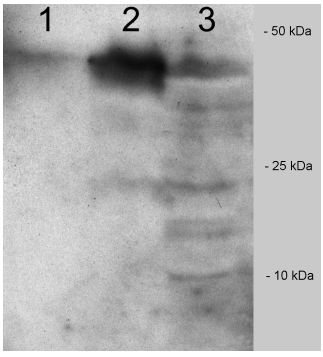

Figure 8 Western blot of MOR. Lane 1 = equine synovial membrane, Lane 2 = equine dorsal horn of spinal cord, Lane 3 = murine liver.

Discussion

This study aimed to investigate whether acute inflammatory conditions would lead to up-regulation of peripheral μ-opioid receptors (MORs) in equine synovial membrane, and if so, whether anti-inflammatory treatment with an NSAID (phenylbutazone) would abolish this effect. Up-regulation of the number of μ-opioid receptors in synovial membrane tissue was observed after the induction of acute synovitis in placebo-treated animals. This effect was not found in NSAID-treated animals, but there were no differences at any individual time point between placebo- and NSAID-treated animals.

The presence of peripheral opioid receptors in equine synovial membrane was previously reported by Sheehy et al. (2001) using immunohistochemical staining techniques. Similar to their findings, we found a relatively intense positive staining pattern for MOR protein in both the superficial synovioblast layer and the subsynovial intimal layers. Staining was predominantly associated with vessel structures, which corresponds with the proximity of nerve terminals to the synovial microvasculature (Illes, 1989; Sheehy et al., 2001). Furthermore, the MOR staining of the cytoplasm and nuclei of inflammatory cells (mainly lymphocytes, plasma cells and macrophages) confirmed work by Sibinga and Goldstein (1988), Stein (1993) and Chuang et al. (1995), whereas κ- and δ-opioid receptors have been described to be expressed in fibroblast-like synoviocytes (Shen et al., 2005; Sprott, 2008). Together with up-regulation of μ-opioid receptors, we found increased macroscopic synovitis scores on arthroscopy videos. The microscopic appearance of the synovial membrane depended on the time point, but these differences were not statistically significant. We did not find treatment effects of phenylbutazone (PBZ) on these scores.

The up-regulation of MOR during inflammatory conditions in horses corresponds with observations in rats and other rodents (Hassan et al., 1993; Mousa et al., 2001+2002). In rats, Freund's adjuvant-induced hindpaw inflammation leads to up-regulation of MORs in dorsal root ganglia (DRG), increased axonal transport

of MORs in the sciatic nerve and an accumulation of MORs in peripheral nerve terminals (Hassan et al., 1993). In the same species, Puehler et al. (2004) showed rapid up-regulation of μ-opioid receptor mRNA in dorsal root ganglia in response to peripheral inflammation to depend on neuronal conduction. Electrical stimulation of peripheral primary afferent nerves caused increased transcription of MOR mRNA in dorsal root ganglia, followed by a significant increase of MOR binding and by increased anterograde axonal transport of MOR toward the periphery in the sciatic nerve. The events in the DRG could not be evaluated in our study, as it would have required obtaining dorsal root ganglion tissue at several time points shortly after induction of synovitis; the invasiveness of the procedure and inevitable spinal dysfunction that would follow precluded any such sampling.

Several theories have been put forward to explain enhanced opioid analgesic efficacy in inflamed conditions compared to normal conditions (Stein et al., 1993), including an increase in *de novo* synthesis and peripheral axonal transport of opioid receptors, as well as activation of opioid receptors that are pre-existent on sensory nerves but that are normally inactive or inaccessible because of a perineural barrier (so-called 'silent receptors'; Antonijevic et al., 1995). Not much is known about how much time is needed to allow for increased de-novo MOR protein synthesis and anterograde axonal transport from the dorsal horn to the peripheral joint site, but 48-72 hours should be enough to allow for such a response, based on rodent studies (Hassan et al., 1993). Because we wanted, besides possible up-regulation of μ-opioid receptors, to assess acute phase alterations in the joint at the second arthroscopy as well, we did not want to wait until 72 hours after LPS-administration. The 48-hour time point for the first post-LPS arthroscopy was therefore chosen to on the one hand reduce the risk of complications (sepsis, wound dehiscence) from surgery performed during or shortly after the peak of a severe local inflammatory response and on the other hand allow for up-regulation of synovial membrane μ-opioid receptors.

It is possible that clinically 'silent' receptors that are pre-existent on nerve terminals in peripheral tissue are targeted with equal affinity by the specific

primary antibody as activated ones. In that case, anti-MOR staining will be unaffected by whether MORs are 'silent' or active, and hence IHC will detect equal amounts of MOR expression in synovial membrane regardless of whether these receptors have become activated by local inflammation or not.
We did not detect up-regulation of MOR in NSAID-treated animals, but we could neither find significant differences at individual time points between the two treatments. This means that PBZ may prevent or suppress MOR up-regulation, but such an effect cannot be unequivocally confirmed. Group size and the relatively large inter-individual variation may play a role. Further, immunohistochemical analysis and scoring of staining intensity for protein expression is a qualitative or semi-quantitative method at best with poor discriminative power. In this context, it is interesting to note that PBZ-treatment improved both clinical lameness and composite pain scores significantly, but did not affect synovial membrane macroscopic or microscopic scores. All these outcome parameters were scored on a categorical scale, with consequent reduction of discriminative power. Hence, the conclusion seems justified that the clinical analgesic efficacy of PBZ is more pronounced than the anti-inflammatory influence on the synovial membrane. This would agree with the lack of anti-inflammatory effects on synovial fluid leukocyte count and total protein concentration we found during the acute phase of inflammation.

In conclusion, we evaluated the effect of inflammation and treatment with phenylbutazone (PBZ) in an LPS-induced synovitis model on the expression of MOR, on microscopic and macroscopic synovitis scores of the synovial membrane of the middle carpal joint, and on clinical lameness and pain scores. Specific MOR protein expression was evident in native and inflamed equine synovial membrane, and we detected up-regulation of the number of MOR in synovial membrane of placebo-treated animals with inflammation. No up-regulation was seen in case of PBZ treatment, but there was no significant difference in MOR numbers between treatment groups at any time point.

Manufacturers' addresses

[1] Sigma-Aldrich, St. Louis, Missouri, USA.

[2] Pfizer Animal Health, Capelle a/d IJssel, The Netherlands.

[3] Dechra veterinary products, Shropshire, UK.

[4] Vygon, Valkenswaard, The Netherlands.

[5] Dopharma, Raamsdonkveer, The Netherlands.

[6] Eurovet Nederland, Heusden-Zolder, Belgium.

[7] Vetoquinol, Zurich, Switzerland

[8] Roche, Woerden, the Netherlands

[9] Cook, Chicago, USA.

[10] Abbott Animal Health, Zwolle, The Netherlands.

[11] BBraun, Melsungen, Germany.

[12] Millipore, Billerica, MA.

[13] Vector laboratories, Vector laboratories, Burlingame, CA, USA.

[14] Vector laboratories, Peterborough, UK.

[15] Vector laboratories, Peterborough, UK.

[16] Mikro-Dismembrator S, BBraun, Melsungen, Germany.

[17] Carlsbad, CA, USA.

[18] Pageruler Plus, Fermentas, St Leon-Roth, Germany.

[19] Supersignal west pico, Thermo Fisher scientific, Rockford, IL, USA.

[20] SPSS version 16.0, SPSS Inc. Chicago, USA.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

We would like to thank Ronald Molenbeek and Ronald Kisjes for their technical assistance with immunohistochemistry and Guy Grinwis for his help with microscopic evaluation of synovial membrane slides.

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8

Application of a composite pain scale to objectively monitor horses with somatic and visceral pain under hospital conditions

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Willem Back
Ludo hellebrekers
René van Weeren

Journal of Equine Veterinary Science (2010) 30 (11) 641-649

Abstract

Pain recognition and management in animals has advanced considerably in the last decade and currently animal welfare is receiving increasing public interest. However, the comprehensive assessment of pain has been inadequately addressed in horses and till recently composite pain scales (CPS) have hardly been validated for use in patients. We investigated the reliability and clinical applicability of a CPS, originally developed under experimental conditions for assessing pain in horses with various acute soft-tissue and orthopedic conditions before and after general anaesthesia and/or (non)elective surgery. These clinical cases ($n = 94$) were scored by means of the CPS twice daily. Horses without painful conditions and horses admitted for nonpainful diagnostic procedures under general anaesthesia were compared with those that were admitted with either acute or chronic surgical and nonsurgical painful conditions of both visceral and somatic origin. Scores of observer 1 were compared with observer 2 to study inter-observer reliability. Composite pain scores showed low baseline values in healthy animals with nonpainful conditions and were not affected when general anaesthesia was the only intervention. Inter-observer reliability was very high ($n = 23$ horses; weighted kappa correlation coefficient $\kappa = 0.81$). Horses with painful conditions responding well to analgesic treatment could be discriminated from horses that had to be euthanised on humane grounds because of painful nonresponsive conditions. We found the CPS to be a promising tool that has the potential to provide a good basis for direct day-to-day assessment of pain status in equine patients with various painful conditions in the future.

Keywords

Pain; Equine; Analgesia; Behaviour; Composite

Introduction

Pain recognition and management in animals has advanced considerably in the last decade (Vinuela-Fernandez et al., 2007; Flecknell, 2008; Lerche, 2009) and animal welfare is currently receiving increased public interest (Nolen, 2001; Stafford and Mellor, 2007). This has resulted in numerous publications on pain therapy in horses (Price et al., 2002+2003; Taylor, 2003; Ashley et al., 2005; Muir, 2005; Weary et al., 2006). However, pain assessment in all animal species remains to be highly subjective because objective and reliable assessment of pain is difficult in nonverbal individuals. Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such type of damage (Anonymous, 1983). This definition of pain highlights the fact that it is both a sensory and emotional experience. Experiencing emotional states is not restricted to higher levels of consciousness (as found in human beings and some species of animals), but is common to all vertebrates (Flecknell, 2008). There is compelling evidence that horses do experience pain in a manner similar to that seen in human beings and show highly emotional responses, suggesting that these animals have a definite affective component to their nociceptive experience (Taylor et al., 2002). The general definition of pain has been modified for animals by Molony and Kent (1997) as follows: Animal pain is 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.

As pain in any species is a complex multidimensional experience expressing itself in behavioural, physiological, and emotional variables, there is no single read-out parameter that specifically indicates the presence of pain. Therefore, pain assessment can be challenging and must rely for a large part on the appreciation of sometimes subtle changes in animal physiology and behaviour (Lerche, 2009). In pain assessment, simple descriptive scales classify pain as absent, mild, moderate, or severe. However, combined interactive and observational multifactor

pain behaviour rating scales, used in conjunction with physiological parameters, have been proposed as being more sensitive in identifying and documenting changes brought about by pain in animals (Dobromylskyj et al., 2000). Such composite pain scales (CPS) have been described and validated for dogs (Holton et al., 2001; Morton et al., 2005; Murrell et al., 2008). For horses, only a limited number of CPS has been described for use in clinical patients (Sellon et al., 2004; Sanz et al., 2009). Moreover, the CPS described by these authors do not contain physiological variables. Bussières et al. (2008) described and validated a CPS in an equine experimental model of acute pain (synovitis induced by amphotericin B). This pain scale, although only validated for equine orthopedic pain, contains various elements that could be applied equally to visceral pain.

The aim of the present study was to investigate the clinical applicability and inter-observer reliability of the CPS described by Bussières et al. (2008) for the assessment of pain in different equine patients with acute or chronic somatic or visceral pain caused by surgical and nonsurgical conditions. This pain scale would be useful for clinicians and researchers dealing with potential painful conditions in horses because it would allow for more evidence-based, objective decision making with respect to analgesic treatment, including euthanasia, and therefore would be beneficial for equine welfare.

Materials and methods

Animals

For this study, we used 94 cases that were admitted to the Department of Equine Sciences of the Faculty of Veterinary Medicine between April and July 2009. During this period, all admitted patients that presented with a painful condition as a main complaint were included, with the exception of mares with foals. Attending veterinarians decided whether the patient was suited for admittance to the study. Owners were informed about the application of the pain scoring system for their animals. In this cohort of patients, the pain score was used for documentary purposes only, and clinical decisions about the initiation of analgesic or other

treatments continued to be taken on the basis of subjective clinical criteria according to standing clinical practice. In total, we used 89 horses, comprising 94 clinical cases, as some horses were admitted for surgery on more than one occasion. Table 1 presents the reasons for admittance to the equine clinic.

Table 1 General overview of the cases (n=94) evaluated in the study and typical for an equine hospital population, differentiated on grounds of acute or chronic, elective or non-elective, visceral or somatic pain versus control horses.

	Number of horses	**stallions**	**Sex mares**	**geldings**	**Euthanised**
Group					
Healthy baseline I	14	6	8	0	0
General anaesthesia II	6	0	2	4	0
Castrations	20	20	0	0	0
Colic surgery	13	0	8	5	2
Orthopaedic surgery	13	2	11	0	1
Elective soft tissue surgery	15	2	11	2	1
Acute surgical and non-surgical patients	13	1	4	8	0
Total	**94**	**31**	**44**	**19**	**4**

I: healthy, clinically sound horses, not subjected to anaesthetic and/or surgical procedures
II: horses subjected to routine anaesthetic procedures for CT- or MRI-scans

Composite Pain Scale

For this study, we used the CPS described by Bussières et al. (2008). This pain scale is a multifactorial numerical rating scale incorporating physiologic data, responses to stimuli, and spontaneous behavioural data (Table 2). The range in general scores is between 0 (representing no signs of pain) and 39 (representing maximum pain score). Pain scoring was performed with the animals in their box stalls.

Experimental Set-up

All pain scoring in the 94 clinical cases was performed by one of the investigators (Judith Weening). This observer was not blinded for the reason of admittance to the study, but was unaware of current analgesic treatment. After careful

training with the CPS in horses that were not included, patients were evaluated twice daily, between 9 and 11 AM and between 2 and 4 PM for a minimum of 3 days and a maximum of 7 days, depending on their clinical situation. Timing of pain evaluation was not coupled to analgesic treatment. Postoperatively, the first pain scores in horses were performed at least 4 hours after recovery. The horses were observed after an acclimatisation period of several minutes; the observer stood in front of the box and was visible to the patient. To assess inter-observer variability, 23 horses were also scored at 120 time points by a second observer. This second observer was trained to use the CPS by the first observer. Pain scoring by the second observer was done independently but at same time points as the first observer. We also assessed healthy horses that were owned by the clinic and were being used for embryo transfer. These horses were acclimated to the hospital environment before CPS evaluation and did not undergo surgery (baseline group, n = 14). Furthermore, we assessed horses after general anaesthesia for noninvasive procedures, such as magnetic resonance imaging (MRI)- or computed tomography (CT)-scans (general anaesthesia group, n = 6), because we wanted to determine the effect of general anaesthesia on pain scores. Further, to determine the applicability of the pain score in different types of hospitalised patients, horses were scored in the postoperative phase after orthopaedic and soft-tissue surgery, as were horses that had been admitted for a variety of acute somatic and visceral pain conditions. Stallions that were admitted for surgical castration were analysed separately (n = 20), standard anaesthetic and analgesic protocols were used for these animals, comprising premedication with detomidine and butorphanol, induction with ketamine and midazolam, and maintenance with isoflurane. Perioperatively, an intratesticular block was achieved with lidocaine, and flunixin meglumine was administered for postoperative analgesia.

Data and Statistical Analysis

All data were expressed as mean ± SEM. We performed a weighted κ analysis between the two independent observers to assess inter-observer variability. We analysed possible changes over time for the various groups of patients by means

of repeated measures analysis of variance. Analysis was performed with the aid of Microsoft Excel 2000 and SPSS (Chicago, IL, USA) and significance was set at $p < 0.05$.

Results

Clinically healthy horses and horses after general anaesthesia

The CPS scores for the healthy, unanaesthetised horses (n = 14) and for those that underwent general anaesthesia for noninvasive procedures (CT- or MRI-scan, n = 6) are presented in Figure 1 (left panel).

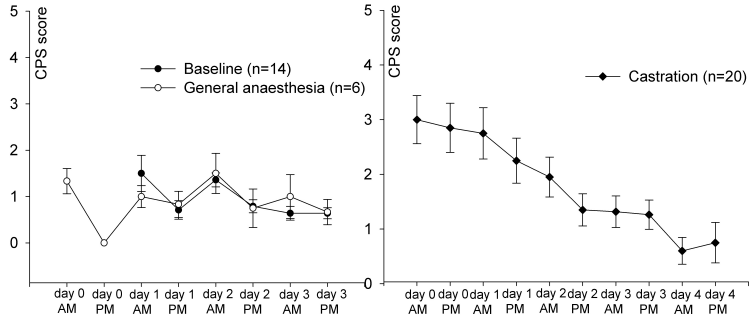

Figure 1 CPS values for healthy horses, general anaesthesia only and castration patients. The left panel shows baseline CPS values (mean ± SEM) of healthy horses (n =14) and of horses that underwent non-invasive procedures under general anaesthesia (n=6). The right panel shows CPS values (mean ± SEM) for horses that were castrated (n = 20) under general anaesthesia. General anaesthesia and castration were performed at day 0 after the morning (AM) CPS scores.

Horses admitted for castration under general anaesthesia

A total of 20 stallions that were admitted for surgical castration were assessed for CPS scores twice daily for 5 days (Figure 1, right panel).We did not find any significant changes over time in CPS scores after surgical castration (P = 0.40).

Horses admitted for emergency laparotomy

For 13 horses that underwent laparotomy because of surgical colic, CPS scores were obtained during the intensive care period twice daily for 7 days postoperatively. Two horses were euthanised postoperatively. CPS scores for all animals including those that were euthanised are shown in Figure 2A.

For horses that survived colic surgery, we did find a trend towards significant changes in CPS scores over time (P = 0.060).

Horses admitted for elective orthopaedic and soft tissue surgery

Ten horses that were admitted for soft-tissue surgery and 13 that were admitted for orthopaedic surgery were evaluated for CPS scores twice daily for 5 to 7 days

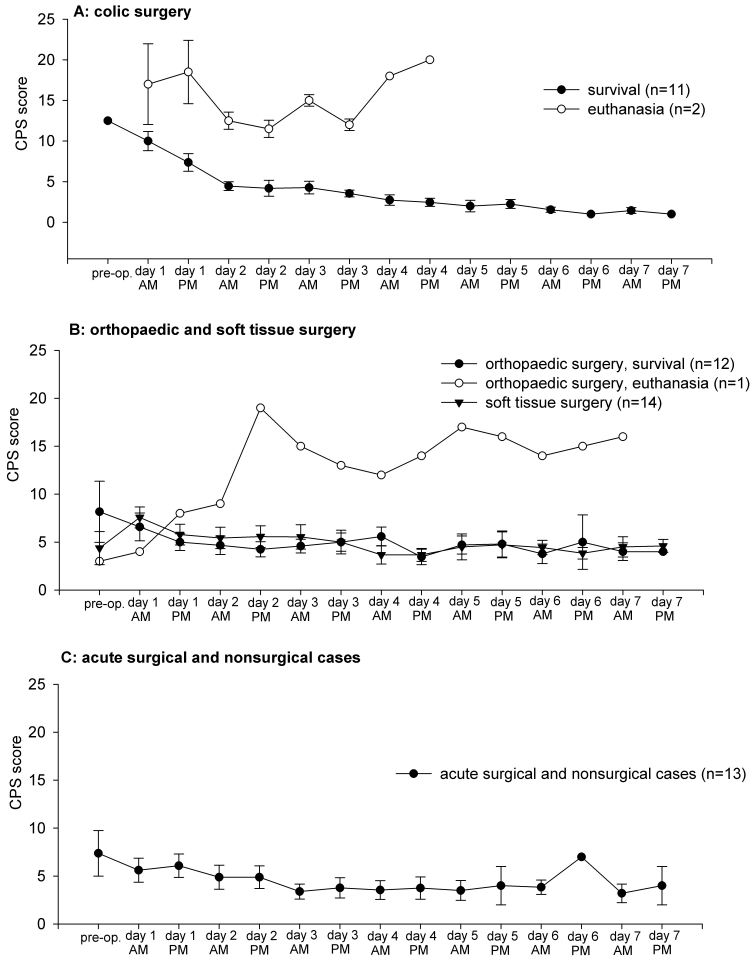

Figure 2 CPS values of horses undergoing laparotomy, elective orthopaedic and soft tissue surgery and acute surgical and non-surgical cases. A: CPS-values (mean ± SEM) of horses that underwent emergency soft tissue (colic) surgery (n = 13). B: CPS-values (mean ± SEM) of horses that underwent elective orthopaedic and soft tissue surgery (n=27). C: CPS-values (mean ± SEM) of horses admitted with acute painful non-surgical conditions (n=13). Surgery was performed at day 0 (after day 0 AM).

postoperatively. In the orthopaedic surgery group, one animal that suffered from hoof cancer was euthanised 7 days postoperatively because of untreatable severe pain (Figure 2B). For horses that survived orthopaedic surgery and those that were admitted for soft-tissue surgery, we did not find any significant changes in CPS scores over time ($P = 0.86$ and $P = 0.18$, respectively).

Horses admitted with acute surgical and nonsurgical painful conditions

Thirteen horses were admitted with acute surgical and nonsurgical painful conditions. This group comprised five horses with acute nonsurgical colic, four horses with acute orthopaedic pain, and four horses with acute soft-tissue injuries. CPS scores were evaluated twice daily for 7 days (Figure 2C).The left panel of Figure 3 shows a patient from this group that was admitted with severe soft-tissue trauma experienced at the head and front- and hindlimbs and was surgically treated under sedation and local anaesthesia. The right panel of Figure 3 shows the individual CPS scores of this patient during the first week of hospitalisation. Analgesic treatment consisted of nonsteroidal anti-inflammatory drugs (NSAIDs) and repeated epidural injections with morphine. Figure 4 shows a 21-year-old mare that was admitted with a complicated mandibular fracture. Under general anaesthesia, external fixation of the fractured mandible was performed. Perioperative analgesia consisted of local anaesthetic blocks with lidocaine and bupivacaine. Because of postoperative swelling of the head, these drugs could not be continued postoperatively. Postoperatively, the horse was treated with NSAIDs.

Inter-Observer Variability

Evaluation of the 120 pain scores in 23 animals from two independent observers resulted in a high weighted k correlation ($\kappa = 0.81$) (Figure 5).

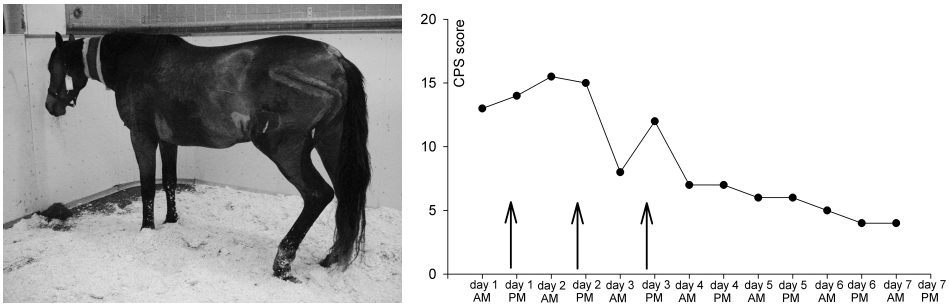

Figure 3 Horse that was admitted with severe soft tissue trauma at the head and front and hind limbs (left panel). Right panel shows individual CPS-scores of this patient that received multimodal analgesic treatment, including epidural morphine (marks) and systemic NSAIDs.

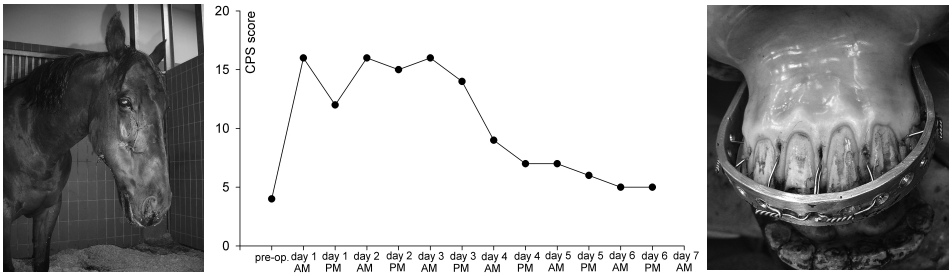

Figure 4 Horse that was admitted with a complicated mandibular fracture (left panel). Middle panel shows individual CPS-scores of this patient that received mandibular nerve blocks with lidocaine+bupivacaine pre-operatively and systemic NSAIDs for 7 days. Right panel shows external fixation of the mandibula.

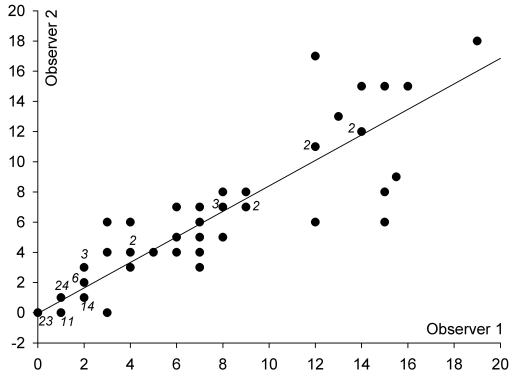

Figure 5 Inter-observer reliability in CPS-scores. Weighted kappa correlation between 2 observers, who scored the same horses (n = 23) independently at 120 time points; $\kappa = 0.81$. Numbers in italics show total counts for pain score-combinations per dot (if greater than 1).

Discussion

We have demonstrated that the CPS described by Bussières et al. (2008) can potentially become a useful, reliable, and easy-to-use tool for the assessment of pain in equine patients under different clinical conditions. With this score, the clinician has an objective means of assessing day-to-day evolution of the pain state in horses and can make evidence-based decisions on pain management and analgesic treatment. Moreover, using the CPS allows for discriminating objectively between horses that respond well to treatment and exhibit low levels of pain as opposed to those that suffer from severe painful conditions. This does not only hold for horses with acute orthopaedic pain, for which condition the pain scale was originally validated by Bussières et al. (2008), but also for those with nonorthopaedic surgical and nonsurgical acute visceral and somatic pain. We obtained low pain scores in healthy animals and found the influence of general anaesthesia to be insignificant, thus making the use of this CPS also suitable for scoring postoperative pain. Therefore, the use of this pain scoring system can help in optimising analgesic treatment and subsequently improve welfare of the equine patient.

Over the complete spectrum of pain scores obtained, the inter-observer reliability was high. This makes the CPS well-suited for clinical use in a situation involving hospitalisation because different observers can score the same animal at different time points without affecting the reliability of the outcome. An attractive aspect of this CPS is that it takes less than 10 minutes to completely score the patient, with a concrete pain score as an immediate outcome. In agreement with Lerche (2009), the CPS contains both behavioural and physiological elements. Behavioural elements are initially obtained by silent observation and later as a response to auditory and tactile stimuli. It is well known that both behavioural and physiological pain parameters lack sensitivity and specificity when used individually; therefore, combining these behavioural and physiological elements in a CPS leads to increased validity (Van Dijk et al., 2001). We decided not to use endocrinological variables (catecholamines, cortisol, β-endorphin), as they

have the disadvantage of always providing information in retrospect because of processing time (Taylor et al., 2002). Additionally, these variables lack specificity for pain (Raekallio et al., 1997) and can be influenced by concomitant use of drugs or by the autonomic status of the patient (fear, anxiety, stress) (Valverde and Gunkel, 2005).

This study illustrated that horses did not show increased CPS scores after being subjected to our standard anesthaetic and analgesic protocols during surgical castration. This was interpreted as the analgesic treatment being effective in these animals. Baseline CPS scores were somewhat increased at admittance in castration patients. This could probably be attributed to excitement after transport and the new stable conditions. This group of patients comprised mostly young, inexperienced, and excitable stallions.
From the first pain score after admittance, we saw decreasing CPS scores in these animals during the study, which was probably the consequence of adaptation to the new stable conditions and was not related to pain. Therefore, use of CPS scores in this category is probably not indicated before the subjects have settled in their new environment. In all other types of hospitalised patients, the CPS scores proved very useful for monitoring pain status. Major differences were found between patients that recovered well after surgery and responded positively to analgesic treatment and those that deteriorated after surgery and ultimately had to be euthanised on humane grounds.

Although the CPS of Bussières et al. (2008) was originally designed for assessment of acute orthopaedic pain, it contains several elements of pain behaviour that are expressions of acute visceral pain as well. Pritchett et al. (2003) described different behaviours in horses, such as pawing, sweating, looking at the flank, flehmen response, and repeatedly lying down and/or standing up, which were assessed for postoperative pain after exploratory coeliotomy for colic. We obtained CPS scores in a limited group of postsurgical colic patients that received intensive care. In this group, day-to-day 24-hour monitoring of the patient status is very important and is often performed by different clinicians and technicians, during

day and night shifts. The CPS provided an objective tool to assess the status of the patient and showed big differences between survivors and nonsurvivors. The two nonsurvivors (one horse with small intestinal impaction and one horse with entrapment of the large colon on the renosplenic ligament) were not endotoxemic, had normal body temperatures, and were clinically not dehydrated (pink and moist mucous membranes, normal packed cell volumes, and normal serum urea and creatinine levels). Increased CPS scores in these animals were therefore not caused by systemic inflammation or cardiovascular status and were more likely to be related to pain.

In the patients described in this study, the CPS scores we obtained were additional measurements and were deliberately not used as the basis for decisions on analgesic treatment. However, the outcome of this study led us to further explore and validate the CPS for a larger number of patients in the intensive care unit. More extended and more frequent CPS monitoring, coupled with subjective pain scores by attending clinicians and subsequent analgesic treatment, could potentially generate more accurate information for improved prognostication. The CPS could then ultimately be used more explicitly for decision making with respect to analgesic protocols.

There are several limitations to our study. First, observer bias cannot be totally ruled out because the observer was not blinded with regard to indications for pain assessment. However, the observer was unaware of analgesic treatment and uninvolved in clinical treatment of the patients. Second, there may be an effect of transport and hospitalisation and observer presence, which is inevitable in this kind of study. However, this effect will be comparable for all patients observed. Third, we used a CPS, which was originally designed for orthopaedic pain for various types of pain in a highly variable clinical population of horses. However, our study showed that the pain scale served well for the different categories of patients. Fourth, the numbers of patients in the subgroups were limited. For this reason, we plan to investigate the usefulness and applicability of the CPS in a more extended group of equine postsurgical intensive care patients. Finally, the lack of validation of the pain scale we used for measuring visceral

pain in horses is an important disadvantage. With the help of further and more extended studying of the CPS in visceral surgical pain and more direct coupling to subjective pain scores and analgesic treatment, we plan to further improve the clinical applicability and reliability of the CPS in equine intensive care unit. For patients other than those in intensive care unit, the CPS seems to be a very useful tool as well. The case report of Dutton et al. (2009), in which a modified canine CPS was used to evaluate multimodal analgesia in a horse with severe hoof pain, describes the usefulness of composite multifactor pain scoring in individual patients. The pain scores facilitated the early, objective recognition of the changing pain status and made subsequent timely adjustment of therapy possible. This led to the conclusion that composite pain scoring in horses, which includes observational, interactive, and physiological components, may prove very sensitive for quantifying the response to therapy when multiple types and stages of pain exist. We found the CPS scores to be very useful for the recognition of changing pain states, for instance in the horse that was surgically treated for hoof cancer and later developed severe, nontreatable postoperative pain (Figure 2B, orthopaedic surgeries, euthanasia), the horse that was admitted with severe soft-tissue trauma at the head, front- and hindlimbs and whose CPS scores gradually decreased after initial treatment (Figure 3), and in the horse that was admitted with a complicated mandibular fracture that was surgically treated (Figure 4).

In conclusion, we found the CPS used in this study to be a promising tool for the immediate, adequate, and objective assessment of pain status in equine patients with acute surgical and nonsurgical painful conditions. Our preliminary results show that the CPS is capable of providing an objective basis for day-to-day assessment of clinical patients and can potentially be a very helpful tool for evaluating changes in pain status and for the objective assessment of the effect of analgesic treatment. After more clinical experience with this pain scale is obtained in horses with painful conditions, it can potentially be of use for better prognostication as well.

Acknowledgments

The authors thank Judith Weening for her assistance during data collection and Dr. Paul van Dijk for critical reading of the manuscript.

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Table 2 Data collection sheet of the Composite Pain Score (CPS, described by Bussières et al. 2008) used in this study to score pain in a group of 94 hospitalised equine patients.

Behaviour	***Criteria***	***Score***
Appearance	Bright, lowered head and ears, no reluctance to move	0
(reluctance to move,	Bright and alert, occasional head movements, no reluctance to move	1
restlessness, agitation and anxiety)	Restlessness, pricked up ears, abnormal facial expressions, dilated pupils	2
	Excited, continuous body movements, abnormal facial expression	3
Sweating	No obvious signs of sweat	0
	Damp to the touch	1
	Wet to the touch, beads of sweat are apparent over the horse's body	2
	Excessive sweating, beads of water running off the animal	3
Kicking at abdomen	Quietly standing, no kicking	0
	Occasional kicking at abdomen (1–2 times/5 min)	1
	Frequent kicking at abdomen (3–4 times/5 min)	2
	Excessive kicking at abdomen (>5 times/5 min), intermittent attempts to lie down and roll	3
Pawing on the floor	Quietly standing, no pawing	0
(pointing, hanging limbs)	Occasional pawing (1–2 times/5 min)	1
	Frequent pawing (3–4 times/5 min)	2
	Excessive pawing (>5 times/5 min)	3
Posture	Stands quietly, normal walk	0
(weight distribution,	Occasional weight shift, slight muscle tremors	1
comfort)	Non-weight bearing, abnormal weight distribution	2
	Analgesic posture (attempts to urinate), prostration, muscle tremors	3
Head movement	No evidence of discomfort, head straight ahead for the most part	0
	Intermittent head movements laterally or vertically, looking at flanks (1–2/5 min), lip curling (1–2/5 min)	1
	Intermittent and rapid head movements laterally or vertically, frequent looking at flank (3–4/5 min), lip curling (3–4/5 min)	2
	Continuous head movements, excessively looking at flank (>5 times/5 min), lip curling (>5 times/5 min)	3
Appetite	Eats hay readily or is not allowed to eat hay	0
	Hesitates to eat hay	1
	Shows little interest in hay, eats very little or takes hay in mouth but does not chew or swallow	2
	Neither shows interest in nor eats hay	3

Response to observer	***Criteria***	***Score***
Interactive behaviour	Pays attention to people	0
	Exaggerated response to auditory stimulus	1
	Excessive-to-aggressive response to auditory stimulus	2
	Stupor, prostration, no response to auditory stimulus	3
Response to palpation	No reaction to palpation	0
of the painful area	Mild reaction to palpation	1
	Resistance to palpation	2
	Violent reaction to palpation	3
Physiologic data	***Criteria***	***Score***
Heart rate	24-44 bpm	0
	45-52 bpm	1
	53-60 bpm	2
	> 60 bpm	3
Respiratory rate	8-13 breaths pm	0
	14-16 breaths pm	1
	17-18 breaths pm	2
	18 breaths pm	3
Digestive sounds	Normal motility	0
(bowel movements)	Decreased motility	1
	No motility	2
	Hypermotility	3
Rectal temperature	36.9-38.5 °C	0
	36.4-36.9 °C or 38.5-39.0 °C	1
	35.9-36.4 °C or 39.0-39.5 °C	2
	35.4-35.9 °C or 39.5-40.0 °C	3
Total composite pain score		***39***

9

Monitoring of equine visceral pain with composite pain scales after emergency gastrointestinal surgery

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Ludo Hellebrekers

Submitted

Summary

Reasons for performing study Recognition and management of equine pain has been studied extensively over the last decades, which has led to significant improvements. However, there is still room for improvement in our ability to identify and treat pain in horses that have undergone emergency gastrointestinal surgery.

Objectives To assess the validity and clinical applicability of the composite pain scale (CPS), initially described for orthopaedic pain, to determine pain levels in horses after emergency gastrointestinal surgery.

Methods Composite pain scores were determined every 4 hours during 3 days following emergency gastrointestinal surgery in 48 horses. Simultaneously with CPS scores, another composite visceral pain score (NRS) was determined to support clinical validity of the CPS and inter-observer reliability was determined.

Results Composite pain scores had high inter-observer reliability ($r = 0.84$, $p < 0.001$) and showed fair correlation with NRS ($r = 0.62$, $p < 0.001$), which was not affected when a condensed form of the CPS, consisting of 9 out of 13 variables, was used. Horses that survived without complications had significantly lower CPS values compared to horses that were euthanised or underwent relaparotomy ($p < 0.001$). There was no breed effect or an effect of location of the gastrointestinal lesion (small or large intestine).

Conclusions and potential relevance The use of the CPS improves objectivity of pain scoring in horses that have had emergency gastrointestinal surgery. Thanks to high inter-observer reliability over a range of CPS scores, comparison between different observers is possible. This last feature is of great benefit in larger veterinary hospitals where more attending clinicians are involved in a single patient to guarantee more transparent and persistent monitoring and treatment of pain.

Introduction

Pain recognition and management in animals are important in optimising animal welfare and have received increasing attention over the last decades. As a result, considerable progress has been made in this area (Viñuela-Fernández et al., 2007; Flecknell, 2008; Lerche, 2009). However, several surveys under veterinarians have shown that improvements in the recognition and management of pain can and need still be made in companion animals (Hugonnard et al., 2004; Williams et al., 2005; Hewson et al., 2006), farm animals (Hewson et al., 2007a+b; Laven et al., 2009) and horses (Price et al., 2002; Dujardin and van Loon, 2011). In the last study, a large proportion (40-60%) of veterinary practitioners classifies their own ability to recognise pain and knowledge of analgesic therapy 'moderate' in horses. Continuing education and research in pain management will contribute to the improvement of objective and reproducible pain assessment and newer analgesic drugs and techniques, which can lead to improved welfare (Valverde and Gunkel, 2005).

Animal pain is an aversive sensory and emotional experience representing 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 (Molony and Kent, 1997). As pain is a complex multidimensional experience expressing itself in behavioural, physiological, and emotional variables, there is no single read-out parameter that specifically indicates the presence of pain (Büttner and Finke, 2000; Lerche, 2009). Simple descriptive and thus subjective scales that usually classify pain as absent, mild, moderate or severe, are suboptimal and largely inadequate instruments for pain evaluation, not in the least because of poor inter-observer reliability (Lindegaard et al., 2010). Combined interactive and observational multifactor pain behaviour rating scales, used together with physiological parameters, have been proposed as more sensitive in identifying and documenting changes instigated by pain in animals (Abbott et al., 1995; Dobromylskyj et al., 2000). Such composite pain scales have been described in horses for orthopaedic pain (Lindegaard et al.,

2010; Bussières et al., 2008) and for visceral pain (Pritchett et al., 2003; Graubner et al., 2011). The disadvantage of both visceral pain scales is that they contain a subjective pain score element. The NRS by Pritchett et al. (2003) has not been subjected to inter-observer reliability assessment.

In a previous study (van Loon et al., 2010), we used the CPS described by Bussières et al. (2008) to assess pain in a cross-section of equine patients in a referral centre. This pain scale, although validated for equine orthopaedic pain only, contains various elements that could equally be applied to visceral pain. Because the CPS seemed particularly useful for pain evaluation in intensive care patients after gastrointestinal (colic) surgery in a previous study (van Loon et al., 2010), the aim of the current study was to further investigate the usefulness and applicability of the CPS for pain assessment and prognostication in a larger group of patients. In this case, one pain scale could be used both for orthopaedic and postoperative visceral pain assessment. The current lack of validation of the CPS scale by Bussières et al. (2008) for measuring visceral pain has been addressed by combined scoring of the CPS and the composite NRS by Pritchett et al. (2003). This latter scale has also been used in other studies (Sellon et al., 2004; Sanz et al., 2009).

Materials and Methods

Animals

We used 48 colic cases that were admitted for emergency laparotomy to our equine referral centre between September 2010 and July 2011 and were subsequently hospitalised in the Intensive Care Unit (ICU) (Table 1). The study population consisted of 22 mares, 21 geldings and 5 stallions. Breeds included Warmbloods (30), Friesians (2), Irish Cobs (2), Fjords (2), Haflingers (2), Standardbred (1) and ponies (9).

Composite pain scale (CPS)

The CPS described by Bussières et al. (2008) was used. This pain scale is a multifactorial numerical rating scale incorporating physiologic parameters,

Table 1 Number of horses that were included in the study.

	Survivors	Non-survivors
Number of horses	39	9
Small intestinal lesion	19*	7
Large intestinal lesion	21*	2
Mean (± sd) weight (kg)	526.3 (120.7)	568.0 (78.9)
Mean (± sd) age (years)	12.2 (7.4)	14.6 (5.4)

* *One horse had both small and large intestines involved. Non-survivors are horses that developed complications after surgery and were euthanised or were admitted for re-laparotomy.*

responses to stimuli, and spontaneous behavioural parameters (Table 2). Total pain scores range from 0 (no signs of pain) to 39 (maximal pain score). To support clinical validation of the CPS, we concurrently determined composite pain scores using the multifactorial NRS, described by Pritchett et al. (2003), which has been used in several studies related to visceral pain (Sellon et al., 2004; Sanz et al., 2009). Pain scoring was performed with the animals in their box stalls.

Experimental set-up

Observations were, due to practical constraints, performed by 5 different observers, which is representative of the working situation in a large clinic. This was deemed justifiable given the very high inter-observer reliability (correlation coefficient 0.94) determined earlier (van Loon et al., 2010). All assessments of a specific patient were performed by one observer. All observers were carefully trained previously, with applying the CPS and NRS in horses not included in the study. The observers were not blinded for the clinical diagnosis, but were unaware of the analgesic treatment. Patients were evaluated every 4 hours for 3 days after colic surgery. Timing of pain evaluation was not related to analgesic treatment. Postoperatively, the first pain scores were performed not earlier than 4 hours after recovery. To determine inter-observer variability, 10 horses were also scored at 40 time points by a second observer. Pain scoring by the second observer was done independently but at the same time points as the first observer.

Data processing and statistical analysis

All data are expressed as medians and quartiles. Median Area Under the Curve (AUC) values were calculated to analyse differences in CPS scores between groups of patients by using the Mann Whitney U test. Inter-observer reliability was assessed by using Pearson correlation coefficients. Bland-Altman plots were used to visually evaluate these correlations and limits of agreement (average difference ± 1.96 standard deviation of the difference) were calculated (Bland and Altman, 1986; Myles, 2007). Correlations between different individual CPS variables and total CPS scores were assessed using 261 CPS evaluations from 17 randomly selected animals. To assess correlations between CPS and NRS scores, CPS scores were converted from a 0-39 scale to a 9-36 scale (in accordance with Lindegaard et al., 2010) and correlations between complete and condensed CPS scores and NRS scores were calculated. Bland-Altman plots were used as described earlier. Statistical analysis was performed using SPSS version 16.0 (SPSS). Statistical significance was accepted at $p < 0.05$.

Results

Inter-observer reliability

There was a strongly significant correlation between CPS scores of two different observers ($r = 0.84$, $p < 0.001$) (Figure 1).

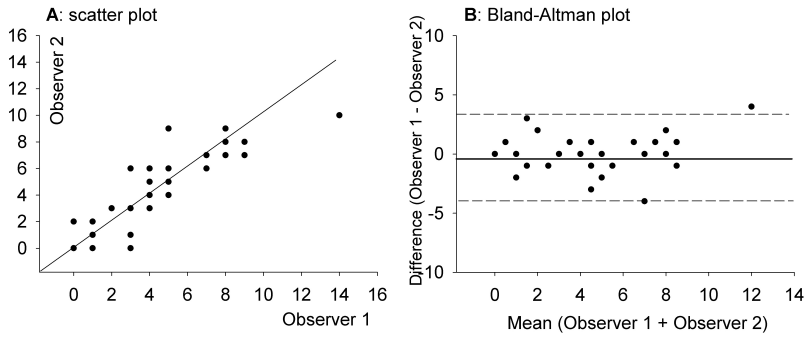

Figure 1 A: Scatter plot of CPS scores, assessed by 2 different observers at the same time (n = 40, some dots represent 2 or more combinations of scores), r = 0.84 (p < 0.001). B: Bland-Altman plot of same 40 CPS combinations (limits of agreement: -3.3 to +3.1).

CPS scores over time for survivors and non-survivors

Median CPS scores of horses that survived the intensive care unit (ICU) period showed a different pattern during the entire period, compared to horses that did not survive (Figure 2). The median AUC of the non-survivors was significantly higher than of the survivors ($p < 0.001$).

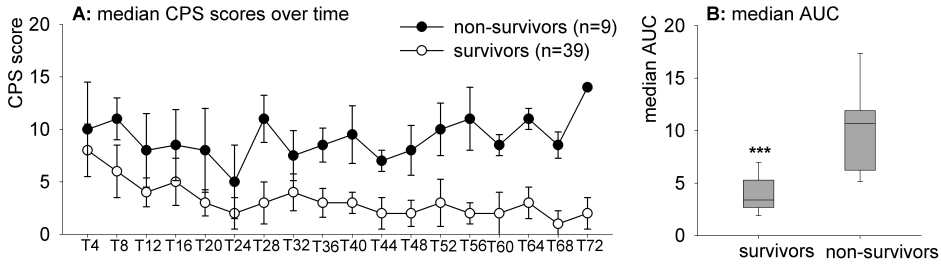

Figure 2 A: Median ± 25-75th percentiles of CPS scores of horses that left ICU alive (n = 39) and of horses that did not survive ICU hospitalisation (n = 9). B: Median AUC scores with 25th-75th percentiles (boxes) and ranges (whiskers) of survivors and non-survivors. *** = p < 0.001.

Influence of breed and type of gastrointestinal lesion

There were no differences in median AUC scores between Coldblood and Warmblood horses (Figure 3A), nor between horses with small intestinal and large intestinal lesions (Figure 3B). Combining survivors and non-survivors, no differences between strangulating and non-strangulating lesions were found (Figure 3C). However, when horses with strangulating (Figure 3D) and non-strangulating (Figure 3E) lesions were assessed separately, median AUC was significantly lower in survivors compared to non-survivors in both groups ($p < 0.05$ and $p < 0.001$ respectively).

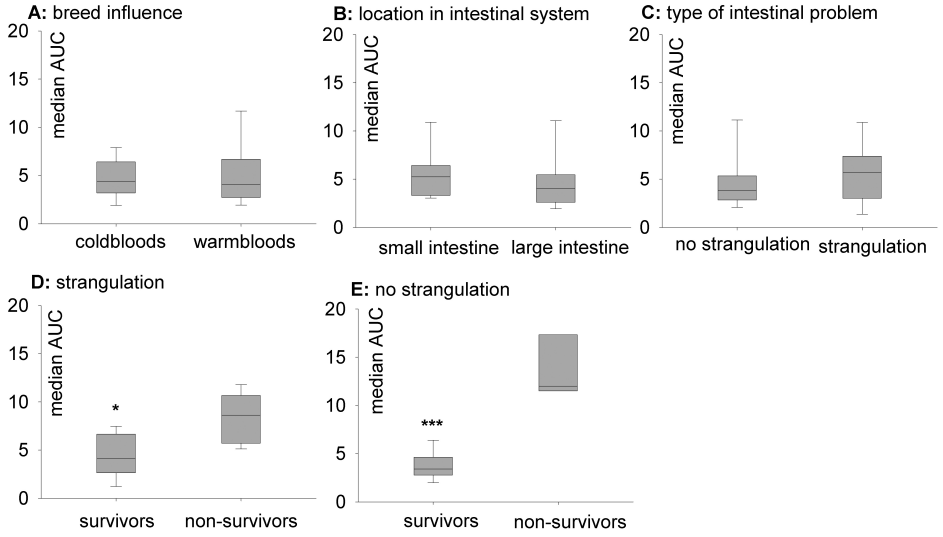

Figure 3 A: Median AUC values with 25th-75th percentiles (boxes) and ranges (whiskers) for Coldbloods (n = 18) and Warmbloods (n = 30), B: Median AUC values with 25th-75th percentiles (boxes) and ranges (whiskers) for horses with small intestinal (n = 25) and large intestinal lesions (n = 23), C: Median AUC values with 25th-75th percentiles (boxes) and ranges (whiskers) for horses with non-strangulating (n = 31) and strangulating intestinal lesions (n = 17), D: Median AUC values with 25th-75th percentiles (boxes) and ranges (whiskers) for horses that had a strangulating intestinal lesion (n = 11 survivors, n = 6 non-survivors). E: Median AUC values with 25th-75th percentiles (boxes) and ranges (whiskers) for horses that did not have a strangulating intestinal lesion (n = 28 survivors, n = 3 non-survivors). * = $p < 0.05$, *** = $p < 0.001$.

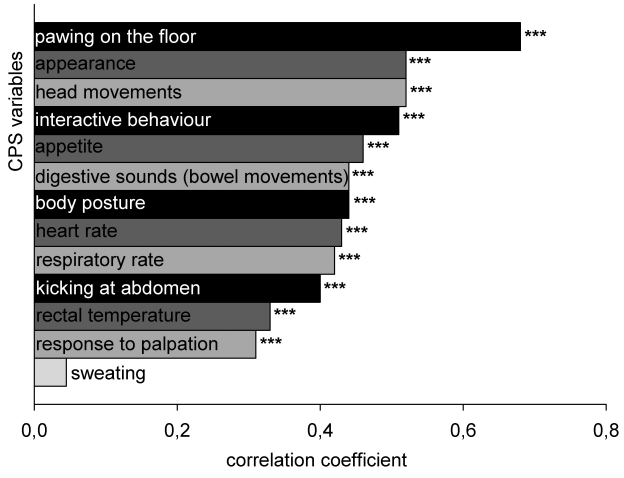

Figure 4 Correlation coefficients between 13 CPS individual parameters and the total composite pain score (n = 261 CPS evaluations from 17 animals), *** = $p < 0.001$.

Contribution of individual CPS components

Figure 4 shows the correlation coefficients between all 13 individual CPS components and the total CPS scores. All individual parameters, except for "sweating", showed significant correlations with total CPS score ($p < 0.001$). Table 3 presents correlation coefficients between complete (13 variables) and condensed (12, 8 and 5 variables with highest correlation with total CPS scores) CPS values and NRS values. Figure 5 (A, B) presents Bland-Altman plots for NRS scores in relation to CPS scores (with 9 respectively 5 best correlating variables). The CPS based on 9 variables had a higher correlation coefficient, compared to 5 variables (0.62 compared to 0.54, both with $p < 0.001$) with narrower limits of agreement (-7.4 to +6.0 compared to -7.4 to +8.8).

Table 3 Correlation coefficients between Numerical Rating Scale pain scores and complete or condensed Composite pain scores (n = 261 composite pain scale evaluations from 17 animals).

	correlation coefficient	p-value
CPS complete (13 variables)-NRS	0.62	$p < 0.001$
CPS condensed (12 variables)-NRS	0.62	$p < 0.001$
CPS condensed (9 variables)-NRS	0.62	$p < 0.001$
CPS condensed (5 variables)-NRS	0.54	$p < 0.001$

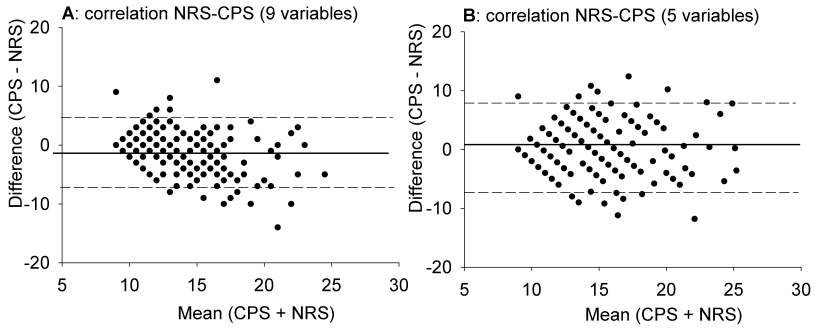

Figure 5 A: Bland-Altman plot of NRS and CPS (9 variables). Limits of agreement: -7.4 to +6.0; r = 0.62 ($p < 0.001$). B: Bland-Altman plot of NRS and CPS (5 variables). Limits of agreement: -7.4 to +8.8; r = 0.54 ($p < 0.001$) (based on n = 261 pain scale evaluations from 17 animals).

Discussion

This study demonstrates the usefulness of the CPS, validated for orthopaedic pain in horses by Bussières et al. (2008) and later applied to various clinical patients by van Loon et al. (2010), for the objective, reliable and repeatable evaluation of pain in horses after emergency gastrointestinal surgery. We found high inter-observer reliability and a fair correlation with another composite pain scale for visceral pain in horses (composite NRS by Pritchett et al., 2003, also used by Sellon et al., 2004 and Sanz et al., 2009) further supporting the value of the CPS for pain assessment in equines. Condensing the CPS to the nine individual variables that most strongly correlated to total CPS scores led to equal correlation values with NRS scores and decreased the time needed for pain assessment, thus increasing practical applicability.

The composite pain scale by Bussières et al. (2008) comprises physiological, behavioural and interactive variables. Although originally validated for use in orthopaedic pain, the scale contains several elements that could also be expressions of acute visceral pain. If this pain scale would also be applicable to visceral pain, it would be very interesting to have one pain scale that could be used in both orthopaedic and visceral pain in horses. Thus we hypothesised that the CPS could be a useful indicator of, not only orthopaedic pain, but also of visceral pain in horses. In the original study by Bussières et al. (2008) the key specific and sensitive behavioural indices were response to palpation of the painful area, posture and, to a lesser extent, pawing on the floor, kicking at the abdomen and head movements. For visceral postoperative pain, pawing on the floor, overall appearance, head movements and interactive behaviour were the most important elements. These different results show that the CPS can be used for the assessment of pain intensity in both orthopaedic and visceral types of pain, for each of which there may be a subset of the most significant parameters. The specificity and sensitivity of the CPS could along this line potentially be increased by introducing different weighting factors for individual variables used, but that was outside the scope of the current study.

As the CPS was not validated for use in visceral pain, we simultaneously assessed CPS scores and composite NRS scores. The composite NRS was originally described by Pritchett et al. (2003), and is calculated as the sum of scores of 9 individual behaviours, including 2 interactive elements. The scale was first used to assess pain in horses after exploratory celiotomy for colic. The NRS does not include physiological variables and one of the 9 variables is "gross pain behaviour", which is largely subjective. Furthermore, inter-observer reliability has not been assessed. Although never validated by experimental standardised conditions and never subjected to inter-observer reliability assessment, the NRS of Pritchett et al. (2003) is probably the best suitable composite pain scale in horses to compare the CPS with. We found fair correlations ($r = 0.62$) of our CPS with the NRS, which supports the conclusion that the CPS could be a useful tool to assess visceral pain in horses. There was only one variable (sweating) that did not correlate with the total CPS score. Bussières et al. (2008) earlier concluded that sweating was a non-sensitive parameter for orthopaedic pain.

By sequentially subtracting variables with lowest correlations to the total CPS, we found that using the nine best correlating variables led to a similar correlation with the NRS, compared to using the total CPS. With only the 5 best correlating variables, this correlation decreased. Therefore, the use of a condensed version of the CPS enables optimal pain intensity specificity, while decreasing observation time and possibly increasing inter-observer reliability.
Recently, Graubner et al. (2010) described a Post Abdominal Surgery Pain Assessment Scale (PASPAS) in horses. This combines behavioural (observational and interactional) with physiological parameters, as in the CPS. A disadvantage of this scale is that it contains a general subjective assessment of pain, as in the NRS. Comparison of the PASPAS-score to the NRS and CPS scores could possibly lead to further optimisation and fine-tuning of equine visceral pain assessment.

In surveys of equine veterinarians in the UK (Price et al., 2002) and in the Netherlands and Belgium (Dujardin and van Loon, 2011), heart rate was cited as one of the major criteria used to assess pain. There has, however, never

been an objective study that demonstrated a correlation between heart rate and pain in horses. Heart rate is likely to be influenced by factors other than pain, including endotoxaemia and hypovolemia (Sellon et al., 2004). Moreover, several studies have reported that increased heart and respiratory rates are not indicative of postoperative pain in cats and dogs (Hansen et al., 1997; Holton et al., 1998). Although it may not be very reliable as a single indicator, heart rate is nevertheless often incorporated in various composite pain scales. Bussières et al. (2008) found moderate specificity and sensitivity for heart rate and we found a moderate correlation between heart rate and total CPS scores in our study. This is in agreement with van Dijk et al. (2001) who described behavioural and physiological pain parameters in human infants after surgery. Although simple Visual Analogue Scale (VAS) scores have been described as reliable, sensitive and very suitable for pain assessment in verbal humans after day surgery (Coll et al., 2004), both in neonates and nonverbal children (Suraseranivongse et al., 2001 + 2006) and in nonverbal older adults with dementia (Herr et al., 2006; Ersek et al., 2011), behavioural and composite pain scales are better as they have been assessed and cross-validated for clinical use.

The ultimate aim of pain scales is to develop a reliable tool for guidance of analgesic treatment. To facilitate appropriate analgesic strategy, the pain scale not only needs to be specific for pain, but objective cut-off values need to be available as well. Gerbershagen et al. (2011) described 4 different methods for cut-off point analysis in humans with postoperative pain, using a numeric rating scale (0-10). These methods used verbal expression of pain state and included subjective tolerable pain intensity, satisfaction with pain therapy, pain-related interference with mood and estimation of pain thresholds. With nonverbal animals, determining such cut-off values for more intensified pain treatment is very difficult to objectify. A possibility could be to use the 25-75 percentiles of surviving horses as reference values for pain state after emergency gastrointestinal surgery.

In our study, the CPS allowed for the objective discrimination between horses that responded well after emergency gastrointestinal surgery and horses that developed complications and did not survive the ICU period. The use of the CPS certainly improves objectivity in pain management and, due to a high inter-observer reliability over a range of CPS scores, enables comparisons between different observers. This is of great benefit in larger veterinary hospitals where often several attending clinicians are involved in the treatment of a single patient. The optimal clinical applicability when using a condensed form of the CPS, consisting of 9 instead of 13 variables, makes the CPS useful for objectifying and monitoring of pain during the ICU period and could help guide decision making in analgesic treatment.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

We would like to thank Judith Weening, Astrid Rijckaert, Esther van Neste, Marjolijn Beugelsdijk and Marjolijn Heemskerk for their help with data collection.

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Table 2 Score sheet of the Composite Pain Score (CPS, Bussières et al., 2008) used to assess pain in a group of 48 equine intensive care patients

Behaviour	***Criteria***	***Score***
Appearance (reluctance to move, restlessness, agitation and anxiety)	Bright, lowered head and ears, no reluctance to move	0
	Bright and alert, occasional head movements, no reluctance to move	1
	Restlessness, pricked up ears, abnormal facial expressions, dilated pupils	2
	Excited, continuous body movements, abnormal facial expression	3
Sweating	No obvious signs of sweat	0
	Damp to the touch	1
	Wet to the touch, beads of sweat are apparent over the horse's body	2
	Excessive sweating, beads of water running off the animal	3
Kicking at abdomen	Quietly standing, no kicking	0
	Occasional kicking at abdomen (1–2 times/5 min)	1
	Frequent kicking at abdomen (3–4 times/5 min)	2
	Excessive kicking at abdomen (>5 times/5 min), intermittent attempts to lie down and roll	3
Pawing on the floor (pointing, hanging limbs)	Quietly standing, no pawing	0
	Occasional pawing (1–2 times/5 min)	1
	Frequent pawing (3–4 times/5 min)	2
	Excessive pawing (>5 times/5 min)	3
Posture (weight distribution, comfort)	Stands quietly, normal walk	0
	Occasional weight shift, slight muscle tremors	1
	Non-weight bearing, abnormal weight distribution	2
	Analgesic posture (attempts to urinate), prostration, muscle tremors	3
Head movement	No evidence of discomfort, head straight ahead for the most part	0
	Intermittent head movements laterally or vertically, looking at flanks (1–2/5 min), lip curling (1–2/5 min)	1
	Intermittent and rapid head movements laterally or vertically, frequent looking at flank (3–4/5 min), lip curling (3–4/5 min)	2
	Continuous head movements, excessively looking at flank (>5 times/5 min), lip curling (>5 times/5 min)	3

Appetite	Eats hay readily or is not allowed to eat hay	0
	Hesitates to eat hay	1
	Shows little interest in hay, eats very little or takes hay in mouth but does not chew or swallow	2
	Neither shows interest in nor eats hay	3
Response to observer	***Criteria***	***Score***
Interactive behaviour	Pays attention to people	0
	Exaggerated response to auditory stimulus	1
	Excessive-to-aggressive response to auditory stimulus	2
	Stupor, prostration, no response to auditory stimulus	3
Response to palpation	No reaction to palpation	0
of the painful area	Mild reaction to palpation	1
	Resistance to palpation	2
	Violent reaction to palpation	3
Physiologic data	***Criteria***	***Score***
Heart rate	24-44 bpm	0
	45-52 bpm	1
	53-60 bpm	2
	> 60 bpm	3
Respiratory rate	8-13 breaths pm	0
	14-16 breaths pm	1
	17-18 breaths pm	2
	18 breaths pm	3
Digestive sounds	Normal motility	0
(bowel movements)	Decreased motility	1
	No motility	2
	Hypermotility	3
Rectal temperature	36.9-38.5 °C	0
	36.4-36.9 °C or 38.5-39.0 °C	1
	35.9-36.4 °C or 39.0-39.5 °C	2
	35.4-35.9 °C or 39.5-40.0 °C	3
Total composite pain score		***39***

10

Summary and General Discussion

Summary

This thesis focuses on pain and nociception in horses. We have used a multidisciplinary approach to quantify nociception and inflammatory pain and as a means to objectively and quantifiably recognise clinical pain in horses. To this aim, neurophysiological techniques, kinematic gait analysis, pressure algometry, assessment of inflammatory biomarkers and composite pain scale analysis have all been used. Further, we have assessed clinical efficacy and analgesic, anti-inflammatory and anti-hyperalgesic effects of locoregional analgesic techniques in horses with acute inflammatory pain. The ultimate aims of this thesis are to contribute to fundamental and clinically applicable knowledge in the field of equine pain and analgesia research and thereby improve clinical analgesia in the horse.

Chapter 2 describes the initial approach to creating and validating an equine model for quantification of caudal nociception using spinal cord somatosensory evoked potentials (SSEPs). Caudal epidural electrodes (advanced from the first intercoccygeal junction towards the lumbosacral junction) were used in conscious ponies to measure spinal cord dorsal horn activity in response to electrical stimulation of the distal hindlimb. Simultaneously, dorsal epaxial muscle activity was assessed with surface electromyography (EMG). When evaluating the evoked potentials after propofol administration (which has muscle relaxant effects), we found these to be of limited value, as dorsal epaxial muscle activity interference disturbed our epidurally derived electrical tracings.

In **chapter 3**, the SSEP model was optimised by performing the experiments under general anaesthesia, thereby eliminating interference from possibly remaining dorsal epaxial muscle activity. SSEP recording electrodes were placed spinally at the lumbosacral junction, a position both closer to the dorsal horn of the spinal cord, and with better controllable positioning of the electrode in relation to the lumbosacral plexus compared to the approach from chapter 2. In this experiment, SSEP tracings showed increasing amplitudes following

increasing stimulus intensities. Further, the conduction velocities calculated for different SSEP components corresponded with the conduction velocity range for Aβ- and Aδ-afferent fibres. These Aδ-afferent mediated signals were significantly influenced by epidural methadone, a μ-agonist opioid, leading to significantly decreased SSEP amplitudes.

In **chapter 4,** the differential effects of a low concentration (0.15 mg/kg) of lumbosacral epidural ropivacaine are analysed in anaesthetised ponies with the SSEP model from chapter 3 and in conscious ponies by means of pressure algometry. Ropivacaine, due to sodium channel blockade, decreased both N1P1 Aβ-afferent related and N2P2 Aδ-afferent related SSEP complexes, showing different effects on SSEP complexes compared to methadone. Epidural ropivacaine produced subtle and transient ataxia with all animals remaining standing. Mechanical Nociceptive Thresholds (MNTs), as assessed by pressure algometry and providing another measure of (anti)nociception, were increased after ropivacaine administration, indicating hypoalgesia. The effect was most pronounced at the sacrococcygeal and lumbosacral level, compared to more cranial locations, with effects lasting for maximally 8 hours at the sacrococcygeal junction.

Chapters 5, 6 and 7 focus on clinical pain and treatment effects of various analgesics using an acute synovitis model based on the intra-articular injection of bacterial lipopolysaccharide (LPS) in healthy horses.

In **chapter 5** we describe the analgesic, anti-hyperalgesic and anti-inflammatory effects of epidural morphine using the LPS-induced synovitis model. Epidural morphine showed potent analgesic effects, as evidenced by improved clinical lameness scores, larger ranges of joint motion and improved weight bearing of the LPS-injected limb. Although epidural morphine did not lead to significant differences in synovial fluid PGE_2 levels, leukocyte count or total protein content and hence did not have peripheral anti-inflammatory effects, we did document peripheral anti-hyperalgesic effects, as determined by pressure algometry. It was concluded that epidural morphine possesses potent analgesic neuraxial

properties and can be a useful means of treatment of peripheral clinical pain.

In **chapter 6** the analgesic and anti-inflammatory effects of intra-articular morphine in the aforementioned LPS-induced synovitis model are described. In contrast to epidural morphine, intra-articular morphine did demonstrate peripheral anti-inflammatory effects, evidenced by changes in synovial fluid PGE_2, bradykinin, leukocyte and total protein levels. Morphine-treated animals showed less distension of the talocrural joints, milder reactions to palpation of the joint and more weight-bearing of the injected limb. Behavioural parameters of discomfort, such as time spent eating silage and time spent in lateral or sternal recumbency, improved as well. On the treadmill, morphine-treated horses had decreased clinical lameness scores and increased ranges of joint motion, compared to placebo-treated animals.

In **chapter 7** we have confirmed the presence of μ-opioid receptors in the equine synovial membrane of middle carpal joints of healthy ponies using immunohistochemistry and Western blotting. By means of the LPS-induced synovitis model, we induced inflammatory conditions and quantitatively evaluated possible up-regulation of these synovial membrane μ-opioid receptors 48 and 672 hours after synovitis induction. Up-regulation of these receptors could be demonstrated in placebo-treated animals. The analgesic and anti-inflammatory effects of treatment with phenylbutazone (NSAID), were assessed. Phenylbutazone significantly improved both clinical lameness and composite pain scores, but did not affect synovial membrane macroscopic or microscopic inflammatory scores. Furthermore, we could not demonstrate significant differences in μ-opioid receptor staining between placebo- and NSAID-treatment at single time points.

In **chapter 8** a composite pain scale (CPS) is introduced, based on a previously validated scale for acute inflammatory joint pain, to assess clinical pain in a cross-section of patients in an equine referral centre. The CPS proved to be an adequate measure to assess clinical pain, particularly in intensive care patients

and in patients with acute orthopaedic and/or soft tissue trauma. The CPS demonstrated good inter-observer reliability and it appeared possible to monitor daily progression of patients in an objective manner. With use of the CPS, it was possible to differentiate horses that subsequently survived the intensive care period from those that needed a second laparotomy or had to be euthanised. This feature may enable prognostic use in the future.

In **chapter 9**, the CPS was applied to a larger group of intensive care patients. CPS scores were compared to another composite pain scoring system, described for equine visceral pain and the influence of individual CPS parameters on the total pain score was assessed. CPS scores were not influenced by breed or location of gastrointestinal problem. Both for strangulating and non-strangulating intestinal problems, the CPS proved useful to differentiate between future survivors and non-survivors and a reduced number of parameters (the 9 best correlating parameters with total pain scores) proved sufficient to obtain optimal correlation with a previously published composite pain scoring system. The 25th to 75th interquartile-interval of surviving horses can be used as normal reference values for clinical application. The CPS is of special benefit in larger clinics, as the pain state of individual patients can be evaluated consistently by different attending veterinarians, because of its high inter-observer reliability.

Finally, **chapter 10** presents a comprehensive discussion of all results of this thesis, putting these in the perspective of the international literature and outlining potential clinical use.

General discussion

The experiments described in this thesis focussed on increasing knowledge on pain recognition and quantification of nociception in horses. Furthermore, clinical efficacy of locoregional analgesic techniques was determined in horses using various approaches.

The physiological nociceptive pathways describe the different levels and aspects of processing of painful stimuli, which include transduction, transmission, projection and perception (Willis, 2007) (Figure 1). Transduction comprises of peripheral nociceptive processing, while modulation covers spinal cord dorsal horn processing. Both at peripheral and central levels, sensitisation can occur. Up to the level of processing of information in the brain ("perception"), this complete process is called nociception. Only the subjective and emotional perception of painful stimuli can officially be called "pain". There are convincing indications to consider that animals are both able to experience nociception, and are also able to have motivational, affective and operant responses to pain (McMillan, 1999; McMillan and Rollin, 2001). This thus holds for horses since they show highly emotional responses to nociceptive stimuli, strongly suggestive for these animals having a definite affective component to their nociceptive experience (Taylor et al., 2002). Knowledge of the various underlying mechanisms of nociception and pain enables us to treat pain at these various levels. Multimodal analgesia is based upon a therapeutic strategy that combines different analgesic approaches, acting by way of different mechanisms and at different sites in the nervous system. This approach results in improved overall analgesia with reduced side-effects compared to the administration of individual analgesics used in a mono-drug approach (Kehlet and Dahl, 1993). This multimodal approach has now become widely accepted in human (Ashburn et al., 2004) and veterinary (including equine) anaesthesia (Lamont, 2008).

In this thesis, nociception and pain in the horse are studied at several levels of the physiological pain pathway. Transduction, transmission and modulation

of nociceptive stimuli are studied by means of a neurophysiologic approach, using Somatosensory Spinal cord Evoked Potentials (SSEPs) that were validated in an equine model (chapters 2, 3 and 4). Furthermore, we studied the effects of several systemic and local analgesic treatment modalities. The analgesic and anti-inflammatory effects of neuraxial administration of both opioids and low dose local anaesthetics are described in chapters 4 and 5 respectively. Chapter 6 describes the effects of intra-articular morphine. In chapter 7, the presence of μ-opioid receptors in the synovial membrane is described and the possible up-regulation of these receptors after an inflammatory stimulus is investigated. In the same chapter, the analgesic and anti-inflammatory effects of systemic NSAID treatment are described. Clinical pain assessment, which ultimately also covers perception of painful stimuli in the brain, has been studied by assessment

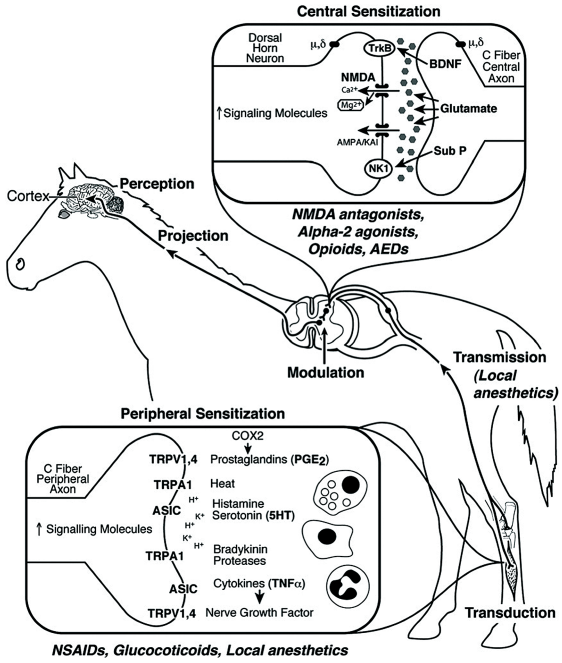

Figure 1 Nociception includes the transduction, transmission, modulation, projection, and perception of noxious stimuli. Peripheral and central sensitisation exaggerate and amplify the noxious stimulus. NSAIDs, glucocorticoids, local anaesthetics, opioids, alpha-2 agonists, N-Methyl-D-Aspartate (NMDA) receptor antagonists and antiepileptic drugs (AEDs) are effective in modifying these responses (Courtesy of Muir 2010b).

of behavioural and physiological pain parameters in clinical equine patients (chapter 8 and 9). The various analgesic modalities explored in this thesis can all be incorporated in multimodal analgesic strategies and hence provide valuable information that is of benefit to the horse, the equine practitioner and equine pain research in general. Therefore, these studies contribute to fundamental and clinically applicable knowledge in the field of equine pain and analgesia research, and help to improve clinical analgesia in the horse.

Neurophysiologic assessment of nociception

The anatomical substrate of pain, the so-called nociceptive system, consists of cutaneous and visceral nociceptors (innervated by Aδ- and C-fibres) and spinal cord neurons, which transmit the peripheral input to supraspinal structures like brainstem, thalamus, cortex and limbic system, and pass it to motor neurons which mediate withdrawal reflexes, or to autonomous efferents which modify cardiovascular and respiratory activity (Bromm and Lorenz, 1998; Willis, 2007). Nociceptors respond to chemical, mechanical and thermal stimuli and stimulation results in propagation of impulses along the afferent fibre, through the dorsal root ganglion cell body, which provides the first relay for structures that transmit nociceptive information from the periphery, towards the dorsal horn of the spinal cord (Siddal and Cousins, 1996). Primary afferent nociceptors terminate primarily in laminae I, II and V of the dorsal horn on several classes of neurons that can either transmit or modulate nociceptive signals. Nociceptive-specific projection neurons preferentially respond to noxious stimuli, while wide dynamic range neurons respond to a range of stimuli. The third class of neurons comprises excitatory and inhibitory interneurons (Siddal and Cousins, 1996). Tissue damage and inflammatory mediators can lead to peripheral sensitisation and increased responsiveness of the nociceptors (Julius and Basbaum, 2001) (Figure 2). Central sensitisation is a state of increased responsiveness of the dorsal horn of the spinal cord, in combination with a reduction in inhibitory transmission due to reduced action of inhibitory activity and to a loss of inhibitory interneurons, which may produce a persistent enhancement of pain sensitivity (Woolf, 2007). This state

of sensitisation can have impact on the severity of clinical pain and will lead to longer, more intense chronic pain states that are more difficult to treat (Curatolo et al., 2006).

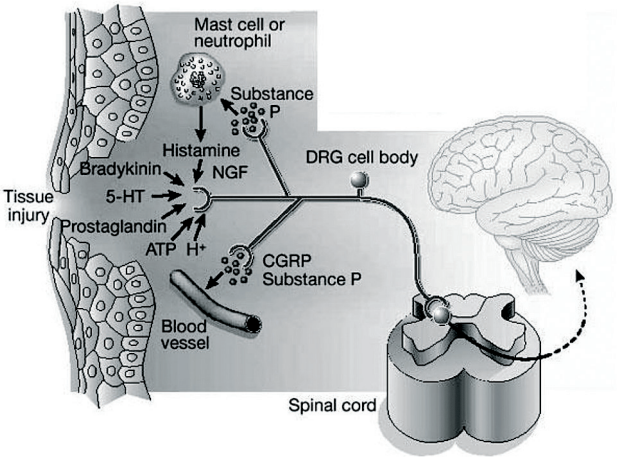

Figure 2 Some of the main components of the 'inflammatory soup' that sensitise (i.e. lower the threshold) or excite the terminals of the nociceptor by interacting with cell-surface receptors expressed by these neurons. NGF = Nerve Growth Factor, 5-HT = 5-hydroxy Tryptamine, CGRP = Calcitonin Gene Related Peptide, DRG=Dorsal Root ganglion (Modified from Julius and Basbaum, 2001).

As the neuronal system is the basic vehicle of pain, neurophysiological techniques have been developed for the evaluation of pain. Pain sensation has been correlated to neurophysiologic phenomena or to (electromyographic analyses of) withdrawal reflexes. Other approaches use spontaneous and evoked electro- or magneto-encephalography (Bromm and Lorenz, 1998). These neurophysiologic measurements assessing neuronal activity have been applied both at cerebral and spinal cord level. We concentrated on neurophysiologic techniques that assess spinal cord activity, as the focus of this thesis is on characterisation of local analgesic techniques, including epidural analgesia.

Neurophysiologic measurements at the spinal cord level

Evoked potential monitoring has become part of electrophysiological monitoring of selected neural pathways of the brain, brainstem, spinal cord and peripheral nervous system (Kumar et al., 2000). Evoked potentials are generated in response to stimulating the nervous system by sensory, electrical, magnetic or cognitive stimulation (Sloan, 1996). Somatosensory evoked potentials (SSEPs) are potentials that are produced by stimulation of the sensory system. These responses arise from action potentials or graded polysynaptic potentials during the propagation of an electrical impulse from the periphery to the brain, and can be recorded over the scalp, as well as at various sites along the anatomic pathway, using surface, subdermal or more invasively placed electrodes (Kumar et al., 2000). The SSEP waveform consists of a series of peaks and valleys presented as a graph of voltage over time and can be described in terms of amplitude, latency and morphology (Banoub et al., 2003) (Figure 3). Amplitude is often measured as a peak-to-peak voltage difference and latency can be measured from stimulus to peak response. It is well established that peaks and valleys arise from specific neural generators (Kumar et al., 2000).

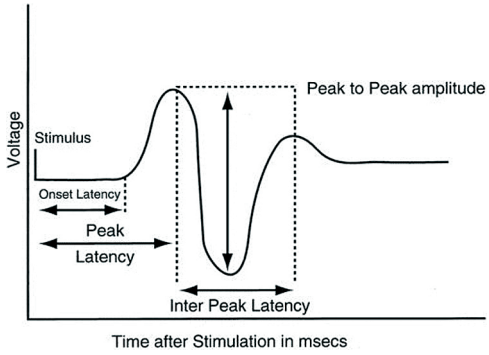

Figure 3 Schematic evoked potential described in terms of latency and amplitude (Courtesy of Banoub et al., 2003).

Our research group has performed numerous studies on cortically derived SSEPs, assessing SSEPs as a measure of anaesthetic depth (Haberham et al., 2000), as

a measure of nociceptive stimulation in rats (van Oostrom et al., 2007) and dogs (van Oostrom et al., 2009). Further, the analgesic and sedative effects of anaesthetic drugs in rats (Franken et al., 2008) and in dogs (van Oostrom et al., 2011) have been assessed.

As the aim of this thesis was to use SSEPs for quantification of spinal (anti) nociception, we will further focus on spinally derived SSEPs. Yates et al. (1982) and Jeanmonod et al. (1991) have published detailed descriptions of spinal cord field potentials from intra-operative spinal cord surface recordings in humans. Experimental studies in rats have been performed using spinal cord evoked potentials to evaluate the effects of traumatic spinal cord oedema, pathology and regeneration following trauma (Shanker Sharma et al., 1991; Shanker Sharma and Winkler, 2002). At present, somatosensory evoked potential monitoring of the spinal cord has come into common use to assess spinal cord function during spine surgery (Britt and Ryan, 1986; Seyal and Mull, 2002). In this way, SSEPs provide an intra-operative measure of the integrity of the spinal cord and the introduction of SSEP monitoring has reduced the incidence of post-operative neurological deficits in scoliosis surgery by approximately one half (Nuwer et al., 1995). Athayde and Franklin (2005) performed SSEP measurements via an epidural catheter in healthy obstetrical patients undergoing caesarean section in order to determine signal changes after an epidural block with lidocaine. They obtained reproducible and reliable signals in which analgesia appeared to correspond with a significant drop in the epidural SSEP amplitudes. Spinal cord-derived SEPs have not been previously described in horses. However, in dogs, several studies have been published (Meij et al., 2006; Cozzi et al., 1998; Cuddon et al., 1999). These showed that increased stimulus intensity did not affect latency of the first 3 peaks, but did increase amplitudes. Variability in amplitudes between different animals could be traced back to differences in distance between the tip of the recording electrode and the dorsal surface of the spinal cord. The closer the recording electrode was to the cord surface, the greater the amplitude and resolution of the evoked potential. This was in agreement with work by Ertekin (1979), who compared SSEPs recorded at intrathecal, epidural and cutaneous

levels. As we aimed to assess nociceptive processing of afferent stimulation of the spinal cord in equines, we have developed a SSEP model in Shetland ponies.

Our aim was to quantify dorsal horn related activity in response to peripheral electrical stimulation. To achieve this, we designed, optimised, validated and applied the SSEP model in several experiments (chapters 2, 3 and 4). There appeared to be an increase in evoked potential amplitude when signals were recorded from the spinal space and therefore closer to the spinal cord. We could not detect measurable SSEP signals, when recording took place from the epidural space (chapter 2). From this study, we proceeded with several adaptations to the initial model; these changes are described in chapter 3. In the further SSEP studies, animals were under general anaesthesia and the recording electrodes were introduced in the lumbosacral spinal (intrathecal) space, thus allowing for a closer approach of the spinal cord compared to the earlier used epidural route. Using this model we were able to record increased amplitudes of SSEP signals with increasing stimulus intensity without interference from muscle activity. By using different locations for electrical stimulation, we induced latency shifts in the SSEP tracings. From these latency shifts and differences in afferent distance to the spinal cord recording location between distal and proximal stimulation sites, conduction velocities of the different components of the SSEP tracings were calculated. In this way two different complexes in the SSEP tracings could be discerned; the N1P1 complex mediated by fast conducting Aβ-afferent fibres and the N2P2 complex mediated by slower conducting Aδ-afferent fibres. The next step in validating the SSEP model was to assess the effects of μ-opioids on the different components of the SSEP signals. The μ-agonistic opioid methadone was found to affect primarily the N2P2 (Aδ fibre–mediated) complex, while the early N1P1 (Aβ-fibre mediated) complex was not significantly influenced. These differential effects of opioids on Aβ- and Aδ-afferents are in accordance with results of Yeomans et al. (1995), who documented the influence of systemic morphine on various components of the flexion reflex in nonhuman primates. In chapter 4 we investigated the effects of low dose ropivacaine in the same SSEP model and found both the N2P2 complex and the N1P1 complex to be influenced.

This is in agreement with earlier work (Casati et al., 2001) and can be explained by the sodium-blocking effect of local anaesthetics that affects both Aβ- and Aδ-afferents. The SSEP model we validated in horses and the specific effects of local anaesthetics and opioids on the various components of the SSEP waveform form a basis for further study of spinal antinociceptive effects of anaesthetics and analgesics in horses.

Electrical (non-selective) versus selective stimulation

We used electrical intradermal stimulation of peripheral afferents using an intra-epidermal (0.2 mm in the epidermis) stimulation modality. In other studies in humans and in dogs (Inui et al., 2002; Mouraux et al., 2010; van Oostrom et al., 2009) this approach led to preferential stimulation of Aδ-fibres. However, this same approach appeared to be non-nociceptive specific in our model and we obtained both Aβ- and Aδ-mediated signals. Nevertheless, the relatively long afferent pathways (approximately 1 m) allow for a clear distinction between the different types of stimulated afferent fibres and, consequently, the separate evaluation of specific nociceptive (ie, primarily Aδ fibre-mediated) and non-nociceptive (tactile Aβ fibre-mediated) responses. It should be noted that the quoted studies based their conclusions on cortically evoked potentials; hence the results might not be comparable to ours. Furthermore, our lack of specificity in stimulating afferent fibres could also have resulted from differences between canine, human and equine skin. Differences in skin thickness could result in different relative distances from stimulating needles and skin nociceptors. Furthermore, differences in stimulus intensity could also have been responsible for differences in the effect of stimulation.

Another method to achieve more nociceptive-specific stimulation could be laser stimulation of peripheral afferents, allowing specific stimulation of thermal nociceptors, connected to thin myelinated Aδ- and unmyelinated C-fibres (Arendt-Nielsen and Chen, 2003). Laser-evoked potentials (LEPs) have been described by various authors (Treede et al., 2003; Arendt-Nielsen and Chen, 2003) and CO_2 laser stimulation was shown to stimulate Aδ- or C-fibres selectively by Tran et

al. (2001). A major disadvantage of the use of LEPs is the high cost of the device (Treede et al., 2003).

SSEPs versus reflex testing

Measuring SSEPs enables us to objectively and specifically quantify spinal cord dorsal horn activity and thereby specifically evaluate the afferent activity of the nervous system at the spinal cord level. In our SSEP model, ponies were anaesthetised by means of propofol and isoflurane, both of which are known to selectively suppress ventral horn motor neuron activity (Jinks et al., 2003; Mitsuyo et al., 2006; Kim et al., 2007) thus supporting undisturbed SSEP measurements. This was confirmed by us by way of concurrent EMG measurements (van Loon et al., 2010a) that demonstrated an absence of EMG-activity in our SSEP tracings, proving the true dorsal horn activity-character of the SSEPs. When using reflex testing (see the next paragraph), suppression of the reflex arch can be caused by suppression of the ventral horn motor neuron only, while nociceptive dorsal horn-activity and subsequent nociceptive brain centre activation remain (partially) intact (You et al., 2005). Reflex suppression can then be falsely interpreted as antinociception. As reflex testing is often used for quantitative assessment of (anti) nociception, two distinct forms, nociceptive withdrawal reflexes (NWR) and mechanical nociceptive threshold (MNT) testing, will be briefly described and discussed in the next section.

Nociceptive withdrawal reflexes

The Nociceptive Withdrawal Reflex (NWR) was first described by Sherrington (1910) and has since extensively been used for the study of experimental nociception in animals and humans. The NWR is a polysynaptic spinal reflex responsible for the escape (nocifensive) reaction that protects the body's integrity against damaging stimuli (Spadavecchia et al., 2002). In horses, thermal stimulation was the first method used to evoke a nociceptive withdrawal reflex (Pippi and Lumb, 1979). High variability and inconsistency in results have been observed in many studies. Electrical stimulation has also been used as a method to evoke a nocifensive

behavioural response in horses, in order to demonstrate the analgesic effects of alpha$_2$-adrenergic agonists (Moens et al., 2003). Spadavecchia et al. (2002) described surface electromyographic responses from several muscles in conscious horses following electrical stimulation of the distal front limb and characterised NWRs and their reflex thresholds. In subsequent studies, they described repeated electrical stimulations as a means to achieve temporal summation in conscious horses (Spadavecchia et al., 2004), evaluated the effect of isoflurane on NWRs in ponies (Spadavecchia et al., 2006) and the effects of butorphanol (Spadavecchia et al., 2007) and alpha$_2$-agonists (Rohrbach et al., 2009). Apart from assessing electromyographic responses, NWRs can also be quantified by other means. Pressure algometry, which is discussed in the next paragraph, is one of these techniques. Because the SSEP model proved useful in anaesthetised animals only and we wanted to assess (anti)nociception in conscious animals as well, we decided to perform pressure algometry in our experimental procedures.

Mechanical nociceptive threshold testing in horses

Mechanical nociceptive thresholds (MNTs), assessed by means of pressure algometry, have been used in various equine studies that aimed at quantifying the effects of certain analgesics and physiotherapeutic interventions, thus providing baseline values for several anatomical landmarks (Haussler and Erb, 2006a+b; Varcoe-Cocks et al., 2006; Haussler et al., 2007; Sullivan et al., 2008; de Heus et al., 2010). In a recent review article, Love et al. (2011) extensively describe both thermal and mechanical nociceptive threshold testing in horses and include other than pressure algometer-based techniques, such as monofilament-derived MNTs (von Frey or Semmes Weinstein filaments, which use flexible elements of varying thickness that produce a thickness-related force when pressed on the skin of an animal) or models using distensible balloons placed in the gastro-intestinal system. MNTs can be used to assess spinal reflexes (e.g. muscle fasciculations, cutaneous trunci reflex) or more complex behavioural reactions (e.g. active vertebral movement or stepping away). In both situations, efferent nociceptive pathway activation following afferent spinal cord dorsal horn

activation is assessed simultaneously. MNTs result from mechanical stimulation of nociceptive afferents in the epidermis and deeper layers, consisting of free nerve endings of A- and C-fibre nociceptors (Fig.4) (Treede et al., 2002). Given the mechanical character of stimulation with pressure algometry, afferents involved are thought to be primarily of Aβ-nature (Linden and Millar, 1988). Although classical theories dictate that nociceptors have only C- or Aδ-fibres and that all Aα/β-fibre afferents are low threshold mechanoreceptors (Doubell et al., 1999), a substantial proportion of A-fibre nociceptors conduct in the Aβ-conduction velocity range (Djouhri and Lawson, 2004).

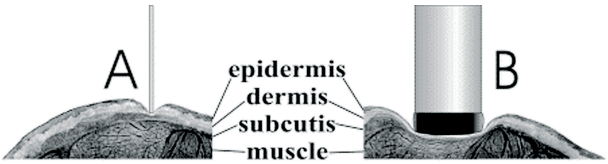

Figure 4 Differential effects of punctuate (A) or blunt (B) pressure to the skin.
The larger the probe size, the more force is needed to activate the intra-epidermal afferents. (A) Contact with a punctate probe that has pronounced contours, e.g. the tip of a needle or a small flat cylinder, causes a strong deformation within the epidermis. The epidermis is densely innervated by free nerve endings of A- and C-fibre nociceptors. If the punctate probe is small enough, nociceptive afferents can be activated by extremely low forces. (B) Contact with a large blunt probe induces deformations in both epidermis and deeper tissue. Rounded or cushioned tips minimize the deformation in the epidermis, and may enable a preferential activation of deep afferents (Modified from Treede et al., 2002).

We have used MNTs for several different purposes. We have quantified reference nociceptive thresholds in healthy animals and showed that low dose epidural ropivacaine can influence MNTs (chapter 5). This can be explained by the stabilising effects of local anaesthetics on neuronal membranes through inhibition of sodium channels. Most prominent effects were found at the sacrococcygeal and lumbosacral junctions where the skin was clipped because of epidural catheterisation. Apart from diffusion effects in the epidural space, differences in coat thickness might partly account for the observed differences in magnitude and duration of treatment with ropivacaine, as reviewed by Love et al. (2011). In future studies it would therefore be better to standardise coat

thickness by clipping all areas. Because of these coat thickness differences, it was not possible to compare all assessed anatomical locations. However, our results enabled us to study the spread of local anaesthetic in the epidural space and we were able to quantify effects on MNTs at the sacrococcygeal and lumbosacral junctions that lasted for 6-8 hours, which agrees with literature (Hall et al., 2001; Simpson et al., 2005).

MNTs can also be used to assess clinical pain. Peripheral hyperalgesia, as determined by pressure algometry, has been described in horses with induced osteoarthritis by Haussler et al. (2007). They described medium long-term hyperalgesic effects and did not consider the acute effects during the first 72 hours. In chapter 6, we have assessed secondary hyperalgesia with pressure algometry in the acute phase of LPS-induced synovitis in horses. Epidural morphine was able to counteract secondary hyperalgesia at the dorsoproximal metatarsus close to the talocrural joint and MNTs showed significant negative correlations with synovial fluid inflammatory markers (PGE_2- and leukocyte levels), indicating that peripheral inflammation will sensitise the peripheral nociceptors and increase their responsiveness. In the acute phase we were not able to induce late stage central sensitisation, as determined by axial skeleton MNTs at the level of the spinal process of L6, which could be explained by the relatively subtle or short lasting effects of the model. Baseline MNTs in the study of Haussler et al. (2007) for the dorsal aspect of the middle carpal joint were much higher than our baseline MNTs at the corresponding dorsal aspect of the talocrural joint. This may possibly be explained by the fact that applying pressure to the dorsal aspect of the carpus does not induce a proper withdrawal reflex, whereas pressure applied to the dorsal aspect of the talocrural joint does. MNTs at the level of the talocrural joints have not been described in horses before, thus our baseline MNTs provide reference values. Because of the different types of studies we performed using pressure algometry, our results add to the expanding knowledge on mechanical nociceptive threshold testing, both in studies regarding physiological nociception and regarding clinical pain. In both situations, MNTs can provide objective means of quantification of hypo- and hyperalgesia and may

enable the study of peripheral and central sensitisation and the possible effects of anaesthetics and analgesics on these processes.

Inflammatory joint pain in horses

Lameness associated with joint disease is the most common cause of early retirement of both pleasure and performance horses (Rossdale et al., 1985; Todhunter, 1992) with osteoarthritis (OA) being the most frequent single cause. Hence, much effort has been put into studying OA-related pain in this species with the underlying mechanisms including the role of inflammatory markers and neuropeptides and influences of peripheral and central sensitisation (van Weeren and de Grauw, 2010), as is the case in humans where OA is the most important musculoskeletal disorder (Schaible et al., 2002 + 2009; McDougall, 2006; Kidd et al., 2007). McIlwraith (2005) reviewed the use of synovial fluid and serum biomarkers in equine joint disease and this research group assessed changes in synovial fluid and serum biomarkers in horses with early induced osteoarthritis (Frisbie et al., 2008). Our research group at Utrecht University has also performed several studies on biomarkers in equine degenerative joint disease, focusing on inflammatory markers in relation to joint pain, both in naturally occurring and induced joint disease (de Grauw et al., 2006a + b, 2009a + b, 2011).

In this thesis we have continued this work by studying the relationship between inflammatory synovial fluid markers and joint pain in an LPS-induced synovitis model, investigating clinical efficacy and anti-inflammatory effects of intra-articular and epidural morphine (chapters 5 and 6). Mu-opioid receptors have been described in the synovial membranes of different joints in humans (Levine and Taiwo, 1989; Stein, 1995), rodents (Parsons et al., 1990) and dogs (Keates et al., 1999). In the horse there is only one single study showing their presence (Sheehy et al., 2001), nevertheless Raekallio et al. (1996) described the local effects of intra-articular morphine in normal ponies already much earlier. More recently, Santos et al. (2009) investigated the effects of intra-articular ropivacaine and morphine on LPS-induced synovitis in horses by means of subjective pain scales. In our

study (van Loon et al., 2010b), intra-articular morphine showed potent analgesic and anti-inflammatory effects. Analgesic effects were assessed by subjective lameness scoring, by objective kinematic evaluation and behavioural analysis. Anti-inflammatory effects were evaluated analysing synovial fluid leukocytes and PGE_2-, bradykinin- and substance P-levels. There were significant treatment effects on synovial fluid leukocyte, total protein, PGE_2 and bradykinin-levels, but not on Substance P. These anti-inflammatory effects of intra-articular morphine were in accordance with observations by Lindegaard et al. (2010b), who used synovial fluid leukocyte count, total protein level and serum amyloid A-levels as outcome parameters.

Opioid receptors appear to be located on the peripheral nerve terminals in the synovial membrane and are up-regulated under inflammatory conditions (Stein, 1995) (Figure 5). From immunohistochemistry and Western blotting tests, we have confirmed the presence of those receptors in equine synovial membrane, as first reported by Sheehy et al. (2001). Using the LPS-induced equine synovitis model, we induced acute inflammatory conditions in the middle carpal joint of healthy ponies and quantitatively evaluated possible up-regulation of these synovial membrane μ-opioid receptors 48 and 672 hours after synovitis induction. In placebo-treated animals, we found up-regulation of the number of μ-opioid receptors in synovial membrane biopsies during inflammatory conditions, whereas this effect was not found in NSAID-treated animals. We did not find differences between placebo- and NSAID-treated animals at individual time points. Along with up-regulation of μ-opioid receptors, we found increased macroscopic synovitis scores from arthroscopy videos. Microscopic appearance of synovial membrane tended to differ between samples taken at different time points, but these differences did not reach statistical significance. We did not find treatment effects of the NSAID (phenylbutazone) at single time points. The difference between active μ-opioid receptors and receptors that are pre-existent on sensory nerves but that are normally inactive or inaccessible by virtue of a perineural barrier (so-called 'silent receptors' (Antonijevic et al., 1995)) and which become activated by an inflammatory stimulus cannot be detected by means

of immunohistochemistry. This means that the difference between baseline and inflammatory conditions in active μ-opioid receptors is probably even larger than the difference we detected between baseline and inflammatory conditions.

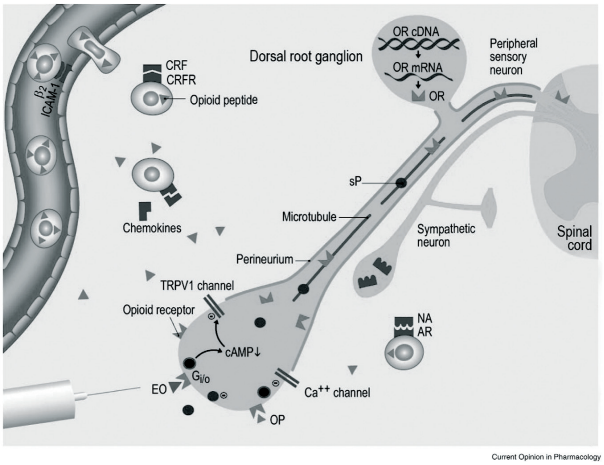

Figure 5 Opioid peptide-containing circulating leukocytes extravasate upon activation of adhesion molecules (e.g. intercellular adhesion molecule (ICAM)-1; beta2 integrin). Exogenous opioids (EO) or endogenous opioid peptides (OP, green triangles) bind to opioid receptors (OR) that are synthesized in dorsal root ganglia and transported along intra-axonal microtubules to peripheral (and central) terminals of sensory neurons. The subsequent inhibition of excitatory ion channels (e.g. transient receptor potential cation channel V- (TRPV) 1, Ca2+) and of substance P (SP) release results in antinociceptive effects (modified from Stein and Lang, 2009).

The analgesic effects of epidurally administered morphine have been described extensively in equines (Natalini and Robinson, 2000; Goodrich et al., 2002; Fischer et al., 2009), but only in healthy horses or in combination with detomidine in horses with acute orthopaedic pain. Peripheral anti-inflammatory effects of intrathecally applied morphine have been described in rodents (Zhang et al., 2005; Brock and Tonussi, 2008; Boettger et al., 2010). In analogy with these studies we hypothesised that epidural morphine would have both analgesic and peripheral anti-inflammatory effects in the LPS-induced synovitis model. Analgesic and anti-hyperalgesic effects were indeed detected by means of lameness evaluation, kinematic gait analysis and pressure algometry, but we were not able to detect

significant peripheral anti-inflammatory effects on SF leukocytes, total protein and PGE_2-levels. The differences with the earlier rodent studies can possibly be explained by differences in timing of morphine administration, as we administered epidural morphine shortly after synovitis induction and not pre-emptively.

De Grauw et al. (2009b) showed that meloxicam (a nonsteroidal anti-inflammatory analgesic) influenced leukocyte, substance P, bradykinin and PgE_2-release in synovial fluid in the same LPS-induced synovitis model that we used. Intra-articular morphine influenced all these SF inflammatory markers as well, except for substance P (chapter 6). In chapter 7, we have described the analgesic and anti-inflammatory effects of systemic treatment with phenylbutazone (another NSAID). We found significant analgesic effects, but could not detect peripheral anti-inflammatory effects in the acute phase, as assessed by means of SF leukocyte and total protein levels. These results show that NSAIDs and opioids have different effects on the inflammatory response. Combining opioids and NSAIDs in clinical therapies can possibly provide synergistic effects.

Clinical pain assessment in horses

As pain in any species is a complex multidimensional phenomenon expressing itself in behavioural, physiological, and emotional variables, there is no single read-out parameter that specifically indicates the presence of pain. Therefore, pain assessment in animals can be very challenging and must rely for a large part on the appreciation of sometimes subtle changes in animal physiology and behaviour (Lerche, 2009). In pain assessment, simple descriptive scales (SDS) classify pain as absent, mild, moderate, or severe. Dutton et al. (2009) used an SDS scale that ranged from 1 to 10. Visual Analogue Scales (VAS) have also been used in equine studies. Viñuela-Fernández et al. (2010) compared VAS scores to Obel scores (SDS scale specifically developed for horses with laminitis) and a clinical grading system in laminitic patients. These subjective scoring systems are characterised by moderate inter-observer reliability (generalizability (G) coefficient 0.5-0.75). Clinical use is reasonably reliable (intra-observer reliability

$G > 0.75$) if used by an experienced single observer. However, in clinical practice patient observation is often not restricted to one observer only, which reduces the reliability of these scores considerably.
Abbot et al. (1995) evaluated a well-defined combination of behaviours with or without weighting; these combinations appeared to correlate more closely with pain than evaluation of any single behaviour. Furthermore, combined interactive and observational multifactor pain behaviour rating scales, used in conjunction with physiological parameters, have been proposed as being more sensitive and more specific for the assessment of pain in both humans and animals (van Dijk et al., 2001; Dobromylskyj et al., 2000).

Lindegaard et al. (2010a) compared VAS scores and composite measure pain scores (CMPS) in an experimentally induced synovitis model in horses. Inter-observer reliability of the CMPS was good, of the VAS not more than fair. This CMPS was modified from an equine composite pain scale initially described by Pritchett et al. (2003) and consists of six behavioural variables including gross pain behaviour, weight-bearing, head position, location in stall (in front or in the back), response to open door and response to approach of the observer. The outcome is combined with an overall subjective pain score. However, both gross pain behaviour and the overall subjective pain score leave a substantial subjective component in the CMPS. No physiological variables are used. The original composite pain scale of Pritchett et al. (2003) is a composite numerical rating scale (NRS), calculated as the sum of scores of 9 behaviours and was used to assess pain in horses after exploratory celiotomy for colic. This NRS was also used by Sellon et al. (2004) and Sanz et al. (2009), who assessed pain after celiotomy and surgical castration respectively. Although never validated by experimental standardised conditions, the NRS of Pritchett et al. (2003) has proven beneficial for objective pain assessment in horses with visceral post-operative pain. The NRS does not contain physiological variables, but Sellon et al. (2004) assessed heart frequency, breathing frequency and plasma cortisol levels simultaneously with the NRS scores. Both heart and breathing frequency did not differ between

treated and control horses, but plasma cortisol levels were significantly different at several time points and were decreased in butorphanol treated horses.

Raekallio et al. (1997) were about the first to use the composite pain scale format, combining both predefined behavioural and physiological variables in a total post-operative pain severity index (TPPSI) in horses following arthroscopic surgery. In contrast to studies on visceral pain (Sellon et al., 2004) plasma β-endorphin and cortisol concentrations appeared to be not different between phenylbutazone and placebo treated animals, possibly partly due to the diurnal rhythm in horses. These authors concluded that β-endorphin and cortisol were not useful as indicators for acute orthopaedic pain in horses. Endocrinologic parameters (catecholamines, cortisol) are part of the equine stress response and can be elevated by painful stimuli, intense physical activity or psychological strain (Wagner, 2010). They have been evaluated for pain assessment in the horse (Pritchett et al., 2003; Raekallio et al., 1997; Rietmann et al., 2004; Sellon et al., 2004), but their disadvantages are that they are non-discriminatory, expensive to assess and they always provide information in retrospect because of processing time (Taylor et al. 2002). Additionally, these variables lack specificity for pain (Raekallio et al., 1997) and can be influenced by concomitant use of drugs or by the autonomic status of the patient (fear, anxiety, stress) (Valverde and Gunkel, 2005).

Price et al. (2003) used a comparable approach to study horses after arthroscopic surgery, determining activity budgets from video recordings and comparing this technique to direct observations. Assessment of activity budgets proved to be a more sensitive method for identifying behavioural changes indicative of equine discomfort, possibly because the influence of individual variability and variability of expression of certain behaviours can be reduced. However, assessment of activity budgets is not applicable in clinical situations and does not provide the clinician with real-time pain scores. The composite pain scale by Bussières et al. (2008), which has been validated for acute orthopaedic pain in horses, comprises both physiological and behavioural variables. This scale contains several elements

that are expressions of acute visceral pain as well, making the scale likely useful for more than orthopaedic pain alone.

In clinical practice, there is need for objective and reproducible assessment of clinical pain. As neurophysiologic and inflammatory pain assessment methods are not ready for clinical use as yet, we decided to evaluate equine pain through behaviour-based assessment using the composite pain scale by Bussières et al. (2008). We started with a pilot study that assessed pain in a cross-section of equine patients. Because in that study, our composite pain scale (CPS) seemed useful in postsurgical intensive care patients, we further investigated the suitability of the CPS in a larger group of these patients in a subsequent study. The lack of validation of the CPS scale by Bussières et al. (2008) for measuring visceral pain has been addressed by simultaneous scoring of the CPS and the composite NRS scoring system by Pritchett et al. (2003). We found fair correlations between both composite pain scores over the range of mild to severe post-operative visceral pain. A subset of 9 individual parameters from the CPS proved sufficient for optimal correlation with the NRS composite pain scale.

Recently, Graubner et al. (2010) described a Post Abdominal Surgery Pain Assessment Scale (PASPAS) in horses. It combines behavioural (observational and interactional) with physiological parameters, as in the study by Bussières et al. (2008). A disadvantage is that it contains a general subjective assessment of pain, like the composite pain score by Lindegaard et al. (2010a). In our study, the CPS allowed for the objective discrimination based on pain level between horses that responded well or unfavourably to analgesic treatment. Composite pain scales improve objectivity and, thanks to high inter-observer reliability, enable comparisons between different observers. This last feature is of great benefit in larger veterinary hospitals. Our ultimate aim is to develop the CPS as a reliable tool for prognostication in equine intensive care and as a guide for analgesic treatment.

Local anaesthetics for locoregional analgesia in horses

Chapter 4 of this thesis describes the epidural application of low dose local anaesthetics at the lumbosacral junction to produce antinociception in conscious standing animals. The lumbosacral application of ropivacaine in horses has been described earlier by Bussières et al. (2008), but they used it in a mixture with ketamine, detomidine and morphine, which did not allow for the assessment of the individual contribution of ropivacaine to the total analgesic outcome. Skarda and Muir (2003) used a very low dose (0.02 mg/kg) of ropivacaine for spinal anaesthesia in standing mares and encountered minimal behavioural changes and circulatory and respiratory disturbances.
In conscious horses the possible detrimental effects on motor function of epidurally applied local anaesthetics may limit their use for peri- and post-operative epidural analgesia of the hindlimb; hence they are mostly used for caudal perineal anaesthesia in horses (Robinson and Natalini, 2002). The side-effects depend on type of local anaesthetic, dosage, volume and concentration of the drug. In humans ropivacaine produces a marked differential blockade between sensory and motor fibres with relatively little motor side-effects, when administered at the low concentrations (0.2-0.5%) used for post-operative analgesia (Eledjam et al., 2001; Casati et al., 2001). In our study we found significant antinociceptive effects of epidural ropivacaine with only mild increases of ataxia. There was a significant increase in ataxia scores between ropivacaine and placebo treatment at 1 and 4 hours after epidural administration, but median ataxia scores were mild with 1.5 on a scale of 0-5. All animals remained in standing position unaided and recovery from general anaesthesia after SSEP measurements was uneventful without obvious signs of ataxia. DeRossi et al. (2010) described similar analgesic and mild ataxia-inducing effects in standing cattle after the use of low dose lumbosacral epidural hyperbaric bupivacaine.

Local anaesthetics are also commonly used for intra-articular administration. The analgesic effects of intra-articular ropivacaine in an LPS-induced equine synovitis model lasted for 2.5-3.5 hours, compared to 24 hours for morphine (Santos et al.,

2009). In a similar model mepivacaine effectively eliminated lameness within 45 minutes after injection (Kay et al., 2008). Combination of mepivacaine with triamcinolone did not alter the potent analgesic and anti-inflammatory effects of triamcinolone. The use of bupivacaine is questioned heavily at present. *In-vitro* studies describe cytotoxicity of bupivacaine in canine osteochondral explants (Hennig et al., 2010) and bovine articular chondrocytes (Chu et al., 2006). In equine articular chondrocytes bupivacaine and lidocaine exhibited marked chondrotoxicity, while mepivacaine did not (Park et al., 2011). Piper and Kim (2008) showed 0.5% ropivacaine to be significantly less toxic than 0.5% bupivacaine in both intact human articular cartilage explants and chondrocyte culture. In our study we did not assess analgesic or chondrotoxicity-related side-effects of intra-articular local anaesthetics, but our LPS-induced synovitis model, together with assessment of synovial fluid inflammatory markers, could be an excellent tool for this type of studies.

Future perspectives for equine pain research

Pain, comfort and well being are central topics in the debate on human interaction with animals, irrespective whether the animals serve as a food source or as working or companion animals (Pascoe, 2010). This has pushed studies on pain mechanisms, processes and therapies forward, which have increased our understanding of the neurobiological mechanisms behind pain (Muir, 2010a). Considerable advances in pain recognition and management in small animals have been made in the last decades (Taylor et al., 2002; Lerche, 2009). However, pain therapy in horses is still largely based on empiricism and clinical judgment and much effort is needed to improve equine pain management and recognition (Muir, 2010a). Therefore, there is an urgent need of both fundamental studies and clinical trials in horses to increase species-specific knowledge on pain therapy and pain recognition. This thesis hopes to contribute to this challenging task.

What can be implemented into current clinical strategies and standard operating procedures?

Epidural administration of morphine has been used in equine clinical practice for quite some time. The studies in this thesis help in extending the scientific basis for these existing therapies. The use of intra-articular morphine in horses is relatively new and several studies have emerged the last few years. These studies, including ours, provide fundamental evidence for the clinical use of intra-articular opioids in equine practice. The use of low-dose epidural local anaesthetics such as ropivacaine is promising and may increase our options for multimodal analgesic regimens in horses considerably. Finally, the availability in the near future of validated composite pain scales will be a big step forward in clinical practice.

Equine pain and analgesia research in the (near) future

Clinical 'validation' of composite pain scales through correlation analysis with other validated pain scales and clinical subjective VAS scores will extend their use for various types of pain, for instance for visceral post-operative pain in intensive care patients and for horses that present with orthopaedic or soft tissue trauma. Additionally, effects of administered analgesics can be assessed objectively. These studies will make composite pain scales into an invaluable tool for the evaluation of analgesic therapies.

Both the SSEP model and the LPS-induced synovitis model can be valuable tools to assess the effects of analgesic therapies in horses and to understand the mechanisms involved. The SSEP model can be used to evaluate the antinociceptive effects of new analgesics on spinal cord level and the LPS-induced synovitis model offers possibilities to gain more insight in possible beneficial effects of novel analgesic drugs or multimodal analgesic approaches. Clinical efficacy of potentially interesting new applications, for instance the use of intra-articular ketamine or alpha$_2$-agonists, the effects of new multimodal strategies (opioids

combined with alpha$_2$-agonists, ketamine or magnesium sulphate), or the effects of epidural or intra-articular constant rate infusions with various analgesics can be evaluated. These techniques have become available for clinical practice since the description of epidural (Martin et al., 2003) and intra-articular (Stewart et al., 2010) catheters in horses.

Synovial fluid biomarker analysis can be helpful to evaluate the anti-inflammatory or pro-inflammatory effects of intra-articularly administered drugs and to assess possible harmful effects on cartilage metabolism.
The combination of the SSEP and LPS models offers possibilities for induction and analysis of hyperalgesic states through the creation of peripheral and possibly central sensitisation induced by LPS-induced synovitis, and concurrent neurophysiologic assessment of the effects on spinal cord dorsal horn activity. In this way, antinociceptive effects of pre- or post-emptive administration of current and novel analgesics on transduction, transmission and modulation of nociceptive signals can be evaluated conveniently.

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Samenvatting

Dit proefschrift beschrijft onderzoek naar pijn en nociceptie in het paard. Bij dit onderzoek is een multi-disciplinaire aanpak gehanteerd om nociceptie en inflammatoire pijn te kwantificeren en om objectief klinische pijn in het paard te herkennen en meetbaar te maken. Om dit doel te bereiken hebben we neurofysiologische technieken, kinematische gangenanalyse, druk-algometrie, bepaling van inflammatoire bio-markers en het gebruik van samengestelde pijnschalen toegepast.
Daarnaast hebben we de klinische effectiviteit en pijnstillende, ontstekingsremmende en anti-hyperalgetische effecten van locoregionale analgetische technieken bij paarden met acute ontstekingspijn in kaart gebracht.
Het uiteindelijke doel van dit proefschrift is om een bijdrage te leveren aan zowel fundamentele als klinisch toegepaste kennis op het gebied van pijn en analgesie bij het paard en om hierdoor de klinische toepassingen voor pijnstilling bij het paard te verbeteren.

Hoofdstuk 2 beschrijft de eerste aanpak om een model in het paard te ontwikkelen en te valideren voor het kwantificeren van caudale nociceptie met behulp van ter hoogte van het ruggenmerg afgeleide somatosensorisch opgewekte potentialen (SSEPs). Caudaal geplaatste epidurale electroden (opgeschoven vanuit de eerste intercoccygeale ruimte richting lumbosacrale overgang) werden gebruikt bij ponies bij bewustzijn om de dorsale hoorn-activiteit van het ruggenmerg te meten in reactie op electrische stimulatie van het distale achterbeen. Tegelijkertijd werd de dorsale epaxiale spieractiviteit gemeten m.b.v. oppervlakte EMG. Bij het meten van de opgewekte potentialen na toediening van propofol (dat spierrelaxerende effecten heeft), vonden we een beperkte bruikbaarheid, omdat de dorsale epaxiale spieractiviteit interfereerde met onze epiduraal gemeten electrische signalen.

In **Hoofdstuk 3** hebben we het SSEP-model geoptimaliseerd door de experimenten onder algehele anesthesie uit te voeren, waardoor de invloed van mogelijk overblijvende dorsale epaxiale spieractiviteit werd uitgeschakeld. SSEP-electrodes werden spinaal geplaatst ter hoogte van de lumbosacrale overgang, waardoor de electrode dichter nabij de dorsale hoorn van het ruggenmerg geplaatst kon

worden en de uiteindelijke positie van de electrode t.o.v. de lumbosacrale plexus beter controleerbaar was t.o.v. de aanpak in hoofdstuk 2. In dit experiment werden EMG-vrije SSEP-signalen gemeten die een oplopende amplitude hadden bij toenemende stimulatie intensiteit. De geleidingssnelheden die berekend werden voor de verschillende componenten uit het signaal kwamen overeen met de range van geleidings- snelheden die past bij Aβ- en Aδ-afferente vezels. De Aδ-afferent gemedieerde signalen werden significant beïnvloed door epiduraal toegediend methadon, een μ-opiaat agonist, waardoor de SSEP amplitudes significant afnamen.

In **Hoofdstuk 4** worden de differentiële effecten van een lage concentratie (0.15 mg/kg) van het lumbosacraal epiduraal toegediend locaal anestheticum ropivacaine beschreven. Onder algehele anesthesie werden de effecten geanalyseerd m.b.v. het SSEP-model uit hoofdstuk 3 en bij het staande dier werden de effecten geanalyseerd m.b.v. druk algometrie. Ropivacaine zorgt, dankzij Na-kanaal blokkade, voor een afname in amplitude van zowel het Aβ-afferent gemedieerde N1P1-SSEP complex en het Aδ-afferent gemedieerde N2P2-SSEP complex. Dit patroon is anders t.o.v. het effect van methadon.
Epiduraal toegediend ropivacaine zorgt voor subtiele en tijdelijke ataxie als het wordt toegediend bij het staande dier, waarbij alle dieren wel konden blijven staan. Mechanische Nociceptieve Thresholds (MNTs), bepaald m.b.v. druk-algometrie en een maat voor (anti)nociceptie, nemen toe na toediening van ropivacaine, wat duidt op hypoalgesie. Het effect was het meest duidelijk op de sacrococcygeale en lumbosacrale overgang, in vergelijking met meer craniaal gemeten locaties, met een maximale duur van 8 uur op de sacrococcygeale overgang.

Hoofdstukken 5, 6 en 7 richten zich op klinische pijn en behandeleffecten van verschillende analgetica. Hierbij is het acute synovitis-model gebruikt dat gebaseerd is op de intra-articulaire toediening van bacterieel liopolysaccharide (LPS) bij gezonde paarden.

In **Hoofdstuk 5** beschrijven we de analgetische en anti-hyperalgetische effecten van epiduraal toegediend morfine met behulp van het LPS-geïnduceerd synovitis model. Epiduraal morfine laat potente analgetische effecten zien, die gekarakteriseerd worden door verbeterde klinische kreupelheidsscores, toegenomen ranges of motion en verbeterde belasting tijdens rust van het met LPS geïnjecteerde been. Ondanks het feit dat epiduraal morfine niet tot significante veranderingen in PGE_2-, leucocyten en totaal eiwit gehalten leidde in de synoviale vloeistof en dus geen perifere anti-inflammatoire effecten liet zien, vonden we wel perifere anti-hyperalgetische effecten, die werden vastgesteld met druk-algometrie. Uit dit hoofdstuk kan geconcludeerd worden dat epiduraal morfine potente neuraxiale analgetische eigenschappen heeft en dat het toegepast kan worden bij de behandeling van perifere klinische pijn bij het paard.

In **Hoofdstuk 6** worden de analgetische en anti-inflammatoire effecten van intra-articulair toegediend morfine beschreven met het eerder beschreven LPS-geïnduceerd synovitis model. In tegenstelling tot epiduraal morfine laat intra-articulair morfine wel perifere anti-inflammatoire effecten zien, gekarakteriseerd door veranderingen in PGE_2, bradykinine, leukocyten en totaal eiwit gehalten in de synoviale vloeistof. Met morfine behandelde dieren lieten minder zwelling van het talocruraal gewricht zien, mildere reacties op palpatie van het gewricht en meer belasting van het geïnjecteerde been. Gedragsparameters die duiden op ongemak, zoals de tijd die het paard besteedt aan het eten van ruwvoer en de tijd in zij- of borstbuikligging, verbeterden ook na behandeling met morfine. Op de tredmolen zorgde behandeling met morfine voor verminderde kreupelheidsscores en toegenomen ranges of motion, t.o.v. placebo-behandeling.

In **Hoofdstuk 7** hebben we de aanwezigheid van μ-opiaat receptoren in de synoviale membraan van het midcarpale gewricht van gezonde ponies aangetoond m.b.v. immunohistochemie en western blots. Met behulp van het LPS-geïnduceerde synovitis model hebben we inflammatoire condities gecreëerd en de mogelijke up-regulatie van μ-opiaat receptoren op 48 en 672 uur na het opwekken van synovitis gekwantificeerd. Deze up-regulatie kon worden

vastgesteld bij placebo-behandelde dieren. De analgetische en anti-inflammatoire effecten van behandeling met phenylbutazone (NSAID) zijn ook vastgesteld. Phenylbutazone gaf een significante verbetering van klinische kreupelheidsscores en samengestelde pijnscores, maar had geen effecten op de macroscopische en microscopische synoviaal membraan synovitis scores.
Er konden geen verschillen tussen placebo- en NSAID-behandeling in de aankleuring van het aantal μ-opiaat receptoren op individuele tijdspunten worden vastgesteld.

In **Hoofdstuk 8** is een composite pain scale (CPS) geïntroduceerd, die gebaseerd is op een eerder voor acute gewrichtspijn bij het paard gevalideerd systeem, om klinische pijn bij een dwarsdoorsnede van patiënten in een 2e lijns verwijskliniek voor paarden vast te stellen. Het CPS-systeem bleek bruikbaar en adequaat om klinische pijn meetbaar te maken, vooral bij intensive care-patiënten en dieren met acuut orthopedisch en/of weke delen-trauma. De CPS liet een goede inter-beoordelaar betrouwbaarheid zien en het bleek mogelijk om de dagelijkse voortgang van patiënten objectief te vervolgen. Met behulp van de CPS was het mogelijk om paarden die de intensive-care periode goed doorstonden te differentiëren van paarden die geïndiceerd werden voor een 2e laparotomie of die geëuthanaseerd moesten worden vanwege een slechte prognose en/ of onbehandelbare pijn. Deze laatste eigenschap van het CPS systeem zou het prognostisch gebruik in de toekomst mogelijk kunnen maken.

In **Hoofdstuk 9** is het CPS-systeem toegepast bij een grotere groep intensive-care patiënten. CPS scores werden vergeleken met een ander samengesteld pijnscorings-systeem dat beschreven is voor viscerale pijn bij het paard en de invloed van de individuele CPS parameters op de totale pijnscore werd vastgesteld. CPS-scores werden niet beïnvloed door ras of door de locatie in het maagdarmstelsel waar de aandoening aanwezig was. Zowel voor paarden met strangulerende als voor paarden met niet-strangulerende aandoeningen bleek het CPS-systeem bruikbaar om te differentiëren tussen toekomstige survivors en non-survivors. Een gereduceerd aantal CPS-parameters (de 9 best correlerende

parameters met de totale pijnscore) bleek voldoende om optimale correlatie te bereiken met het eerder beschreven samengestelde pijnscorings-systeem. Het 25-75 interkwartielen-interval van paarden die goed reageerden op behandeling in de IC zou gebruikt kunnen worden als normaalwaarde voor klinische toepassing. Het CPS-systeem kan vooral van nut zijn voor gebruik in grotere klinieken, omdat vanwege de hoge inter-beoordelaars betrouwbaarheid hiermee het evalueren van de pijnstatus van patiënten objectief door verschillende behandelend dierenartsen plaats kan vinden.

Ten slotte bevat **Hoofdstuk 10** een uitvoerige discussie van alle resultaten van dit proefschrift, waarbij deze in het perspectief van de internationale literatuur geplaatst worden en waarbij de potentiële klinische toepassingen worden samengevat.

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Dankwoord

Eindelijk is het zover!!!!! Het zit erop!
Het is dan ook hoog tijd om iedereen, die op welke manier dan ook zijn of haar bijdrage heeft geleverd aan dit proefschrift, eeuwige dankbaarheid te betuigen! Zonder al jullie hulp, ondersteuning, wijze raad en mentale steun was het nooit gelukt….. Een aantal mensen zou ik graag ook persoonlijk willen bedanken!

Allereerst mijn promotoren,
Professor Hellebrekers, beste Ludo. Vrij snel na mijn aanstelling als resident in de veterinaire anesthesiologie ben ik samen met jou en Paul (mijn beide mentoren van het eerste uur) gaan brainstormen over een richting voor de onderzoeksactiviteiten die toen in het kader van de opleiding op de rails gezet moesten worden. Ik kwam erachter dat het “iets met epiduraal” bij paarden moest worden. Met deze nog wat vage en ruim interpreteerbare richting zijn we aan de slag gegaan en hebben de richting van de neurofysiologie op spinaal niveau gekozen. In de begintijd van deze experimenten hebben we heel wat afgeploeterd met het zelf fabriceren van electroden (Arie dan vooral), door “trial and error” wegwijs worden in de (on)mogelijkheden van de epidurale ruimte (ikzelf dan vooral) bij de (soms te dikke) Shetlandponies en het verwerken en interpreteren van de 1^{e} meetresultaten (Peter dan vooral). Wat ik in deze begintijd vooral heel erg geleerd heb is, dankzij jouw nooit aflatende optimisme en niet te stuiten vertrouwen in de goede afloop, om de moed niet op te geven en ondanks tegenslagen toch altijd maar rustig door te blijven gaan! Ondanks het feit dat ik van nature redelijk optimistisch en positief ben, heb ik een aantal keren gedacht dat we nooit zouden slagen met ons project. Dankzij jouw peptalk kwam er toch een moment dat alles ging lopen en het model toch succesvol bleek! Dank voor deze levensles!

Professor van Weeren, beste René. Ergens gedurende het traject van DEC-aanvragen schrijven, pilots doen bij enkele ponies en met meer of minder succes sleutelen aan het SSEP-model van Ludo, kwam jij in beeld. Ondanks dat de neurofysiologische benadering van pijnprikkelverwerking bij de ponies op dat moment goed in de steigers stond, was het idee ontstaan om ook op

andere manieren naar pijnherkenning en pijnstilling bij het paard te kijken. Via de ervaringen van jou en Janny met het LPS-model werd er gewerkt aan de 2e tak van dit proefschrift en ontstond de samenwerking met jou, Janny en Chris rondom de synoviale biomarkers en hun relatie tot pijn bij het paard. Dit bleek een vruchtbare samenwerking, want we hebben samen een aantal mooie experimenten gedaan die goed voortborduurden op het werk van Janny. De kwantificering van acute orthopedische pijn bij het paard werd hiermee verder in kaart gebracht met allerlei objectieve parameters en de mogelijkheden voor locale toepassing van pijnstilling werden geëvalueerd. René, hartelijk dank voor alle hulp en richting die het project mede dankzij jou ook kreeg!

Mijn co-promotor doctor Back, beste Wim. Met het opzetten van de proeven om intra-articulaire toepassing van morfine bij paarden te gaan evalueren kwamen we al snel uit op de tredmolen en het gebruik van kinematica. Vandaar onze samenwerking in het performance lab, waarvoor jij hebt helpen denken, plannen en interpreteren. Samen met Jiske waren wij het nieuwe “qualisys-team” en ik ben jullie beiden dan ook zeer dankbaar voor het trekken van de kinematica-kar en het eruit persen van alle ranges of motion! Het waren gezellige sessies bij de tredmolen, zelfs als de computer weer eens een vastloper had of de paarden (te) enthousiast op de tredmolen vol gas gaven! Later hielp jij ook bij het schrijven aan de klinische pijnevaluatie-studies bij patiënten en niet in de laatste plaats bracht jij me in contact met de druk-algometrie! Dank hiervoor!

De volgende groep mensen die ik zou willen bedanken zijn mijn beste anesthesie-collega’s! Natuurlijk in de 1e plaats Paul en Daniëlle, trouwe bondgenoten van het (mijn) eerste uur! Aan jullie hand heb ik mijn intrede in de Utrechtse anesthesiologie gemaakt. Wij waren toen al van oudsher de gezelligste en leukste club binnen de UKP! Met z’n drieën op 1 kamer was er altijd wel een luisterend oor als het over patiënten, experimenten of andere dingen ging! Het was dan ook spijtig deze drie-eenheid te zien uiteenvallen met het vertrek van Daan en later het (welverdiende) terugtreden van Paul. Gelukkig hebben we nog regelmatig contact en gelukkig ook kwamen er nieuwe en ook hele fijne collega’s! Jiske en

recent ook Janny trekken nu mede heel hard aan de anesthesie-kar binnen onze afdeling en de samenwerking met Joost, Hugo, Ies en Rob is de laatste tijd steeds inniger en beter geworden! Dankzij jullie allemaal hebben we een heel goed en stevig fundament onder de Utrechtse veterinaire anesthesiologie liggen!

Voor het gedragswerk binnen de verschillende studies heb ik in eerste instantie veel samengewerkt met Machteld van Dierendonck. Beste Machteld, heel veel dank voor jouw expertise, gezelligheid en doorzettingsvermogen tijdens de 1e fase van onze experimenten. We hebben hard gewerkt aan het opzetten van verschillende experimenten en gelukkig ook het slagen van deze inspanningen bekroond zien worden in de intra-articulaire morfine studie! Later hebben we samengewerkt met Valerie Jonckheer, Annemarie Baars and Marie-Jose van 't Klooster. Beste Valerie, Annemarie en marie-Jose, ook jullie hartelijk dank voor alle inspanningen bij het uitvoeren van de epidurale morfine-proef en voor de begeleiding van de studenten in de CPS-studie!

Bij heel veel experimenten hebben studenten een steentje bijgedragen bij de praktische uitvoering en het uitwerken van data. Marjon van der Kraats, Renske Hammink, Eline van den Oever, Francisca Hilbers, Judith Weening, Suzanne Berlinski, Astrid Reijkaert, Esther van Neste, Marjolijn Beugelsdijk en Marjolijn Heemskerk, allen hartelijk dank voor jullie goede werk!

Alle collega's van het departement Paard en Gezelschapsdieren, heel hartelijk dank voor jullie interesse, mentale steun en praktische hulp tijdens de uitvoering van de experimenten! Speciale dank voor Arie Doornenbal voor jouw eindeloze geduld tijdens de vele SSEP-metingen en alle verwerking van bijbehorende data. Het was altijd heel fijn werken en ook gezellig om tijdens de proeven over schapen en klassieke muziek te kletsen! Peter Stienen heel veel dank voor de broodnodige technische ondersteuning tijdens de 1e SSEP-metingen. Dankzij Spongebob en jou kwamen we vaak tot een goed meetresultaat! Henk heel veel dank voor het eindeloos inpakken van spulletjes voor de gassterilisatie en de vele goede gesprekken die hier vaak aan vastgekoppeld werden! Alle kliniekbedienden en

dierverzorgers bedankt voor alle hulp tijdens het werk op de tredmolen, het verzorgen van de paarden in de LPS-proeven, het voorbereiden van ponies voor de SSEP-metingen en voor de arthroscopiën!

Mijn beide paranimfen Bart en Henk! Met zo'n grote en nog grotere broer achter me moet het wel een succes worden in het Academiegebouw! Allebei op jullie eigen manier hebben jullie veel voor mij betekend afgelopen periode; ook in tijden van crisis en tegenspoed waren er opbeurende woorden, veel bakjes koffie en heel veel kilometers op de fiets die allen mijn weerbaarheid vergroot hebben! Ik hoop ook na 27 maart nog vaak mooie dingen met jullie te kunnen beleven.

Heel veel dank aan alle familie en vrienden voor het altijd steunen en klaarstaan voor mij! In het bijzonder Jules en Jo, Renée en John, Leonie en Klaas, Bart en Adeline, Oom Peter, Jessie, Pieter en Monica en alle anderen die ik nu vergeten ben!!!!

Lieve Petra, met jou ging ik bijna 8 jaar geleden terug naar Utrecht. Beiden werken aan de Universiteit en uiteindelijk ook nog allebei in het promotie-onderzoek. Het cliché dat in elk promotie-onderzoek vele ups en downs zitten werd voor ons wel heel erg waar en ondanks alle moeilijke tijden hebben we elkaar toch altijd weten te blijven steunen! Ik hoop dat we altijd zo'n fijn contact kunnen blijven houden! Ook jij bent bijna aan de eindstreep, houdt moed want het einde is in zicht!!!!

Lieve papa en mama, heel veel dank voor jullie onvoorwaardelijke steun! Het begon al toen ik na 3 jaar medische biologie studeren toch besloot de uiteindelijk fel begeerde kans bij diergeneeskunde aan te grijpen, waardoor julie al wisten dat ik voor nog eens minstens 6 jaar zou tekenen voor studeren, studiefinanciering (gedeeltelijk) en ouderlijke bijdragen, zonder iets te verdienen! Jullie advies was altijd "volg je hart" en daar ben ik jullie heel erg dankbaar voor! In de moeilijke tijden die aanbraken stonden jullie altijd klaar met een hart onder de riem, heel veel wijze raad, praktische hulp, maar vooral veel onvoorwaardelijke liefde!

Lieve Eveline! Heel veel dank voor het in mijn leven verschijnen! Zonder dat Wim kon overzien wat hij indirect allemaal teweeg zou brengen, bracht hij mij met jou in contact om de druk-algometrie verder op te gaan zetten. Jij bracht veel meer dan dat en ondanks de moeilijke momenten hebben we al heel veel mooie dingen samen mogen beleven. Daarbij gaf jij altijd ruimte voor de onophoudelijke diensten, schrijfavonden, congressen, studie-weekenden, fietssessies en hardloopuurtjes die altijd maar van onze gezamenlijke tijd afgingen. Ik hoop dat het wat dat betreft ooit beter zal worden!!! Samen kregen wij onze lieve kleine Fiene, het mooiste wondertje van de heeeeele wereld, die ons voor altijd zal verbinden. Jullie zijn mijn zonnestraaltjes aan de hemel en brengen elke dag vreugde in mijn leven! Heel veel dank voor alles!!!!!!

Een heel groot woord van dank voor alle dieren die geheel belangeloos hebben meegewerkt en hebben gezorgd voor alle resultaten: Abeltje, Guus, Droopy, Snowwhite, Doc, Ieniemienie, Eeyore, Happy, Calimero, Barbabop, H'eppo, Floddertje, Sleepy, Frobiola, Rolindra, Wiske, Atesse, Ozora, Ostrona, Afrodiet, Verona, Trilarda, Amalia, Uro, Sidney, Viodessa, Wendy, Tosca, Adieu en alle patiënten uit de CPS studies.

Curriculum Vitae

Thijs van Loon was born on October 8, 1972 in Roosendaal, The Netherlands. He spent high school (Atheneum B) at the Gertrudis college in Roosendaal, where he graduated in 1991. He started studying Medical Biology at the Faculty of Medicine at Utrecht University after high school and decided to apply for Veterinary Medicine as well. After three years of studying medical biology, he was admitted to veterinary medicine as well through the *numerus fixus* system and was able to start his veterinary studies. During the first 2 years of this study he finished his medical biology studies and graduated in 1996. In 2000, he obtained the DVM degree. After graduation, Thijs started to work as an equine practitioner in "Dierenhospitaal Visdonk" in Roosendaal and he enjoyed his work as an equine vet in Brabant for 3 years. After this, he decided to return to University and applied for an internship at the Department of Equine Sciences at Utrecht University. After less then 6 months, this internship was stopped to start a residency in veterinary anaesthesia and intensive care at the same department. He finished his residency in 2010 and is planning to pass the board exams in 2012. Thijs lives in Amersfoort with his partner Eveline and their very sweet little daughter Fiene. He spends his time with his family, the dogs and horses while enjoying being outside on a horse or on his bicycle.