The potential role of *Clostridium botulinum* toxin in the treatment of equine laminitis

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The potential role of *Clostridium botulinum* toxin in the treatment of equine laminitis

De potentiële rol van *Clostridium botulinum* toxine in de behandeling van hoefbevangenheid bij het paard

(met een samenvatting in het Nederlands)

Proefschrift

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door

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Chapter I

General introduction

Introduction

In this introductory chapter, a brief overview is given of the characteristic aspects of laminitis in the horse, including pathogenesis, clinical signs and treatment. However, laminitis is an extremely complex disorder with many different possible causes, various pathogenetic mechanisms and a multitude of treatment options. This thesis focuses on one of the aspects of treatment, which is the reduction of the tensile loading of the distal phalanx by the tendon of the deep digital flexor muscle. For this reason, the deliberate choice was made to focus in this introduction on these mechanical aspects and present information on the general and other aspects of laminitis very succinctly.

Equine laminitis

Laminitis can be defined as a painful, aseptic inflammation of the lamellae of the hoof that may lead to loosening of the attachment of the distal phalanx to the horny hoof capsule and a displacement of the bone with respect to the hoof capsule. In most cases this loosening of the laminar bond, which is called “founder” in colloquial terms, is partial, leading to backwards rotation of the tip of the distal phalanx under the influence of the tensile force exerted by the deep digital flexor tendon. In very severe cases the laminar bond will loosen entirely and the distal phalanx will travel distally with respect to the horny capsule, which cases are called “sinkers”. The disease has been recognized long ago and has remained an item of interest through the ages. Heymering (2010) investigated the history of laminitis and recommended treatments from ancient history until present. Xenophon appears to have been the first to have written about laminitis as a result of barley surfeit in 380 BC. The first described therapy to treat laminitis was bleeding by Pliny in 50 AD, which was followed by many others over the millennia. Some of them are more than exquisite, such as the one given by Conrad Heresbach in 1586: *‘Use the skin of the weasel cut up in small pieces together with butter, rotten egg and vinegar’.* Ever since these first descriptions, laminitis has been extensively studied by many researchers and practitioners.

Laminitis is a severe and extremely painful disease. Apart from the effect on welfare, the prognosis for return to athletic soundness of a horse or pony suffering from laminitis deteriorates rapidly if rotation or even sinking of the distal phalanx occurs (Cripps and Eustace, 1999). The prevalence of the disease is influenced by breed and a variety of environmental circumstances, and can be rather high. A survey in the UK reported a prevalence of 7.1% (Hinckley and Henderson, 1996). The United States Department of Agriculture (USDA) documented that 13% of U.S. horse operation-owners reported a horse suffering from laminitis in the previous year (Anonymous, 2000). However, in a meta-analysis of studies reporting laminitis prevalence Wylie et al. (2011) noted that publications estimating laminitis frequency were generally of poor quality and concluded that high-quality, evidence-based studies still had to be performed. A recent study of this same group showed in a cohort of nearly 600 horses a prevalence of veterinary-diagnosed active (clinically apparent) laminitis of only 0.5% and an incidence of 0.5 cases per 100 horse years at risk, which is much lower than previously published estimates. The authors attributed this discrepancy to differences in geographical setting, study period, case definition, study design and study populations (Wylie et al., 2013).

Pathogenesis

The lamellae of the hoof connect the horny hoof capsule to the distal phalanx, which lamellar bond is called the Suspensory Apparatus of the Distal Phalanx (SADP) (Van Eps 2010; Pollitt, 2010). In case of laminitis structural failure of the SADP can occur, which may be caused by different mechanisms. The SADP consists of the dermal and epidermal lamellae that in the normal situation show strong interdigitation with the basement membrane (which forms the dermoepidermal junction) as interface (Van Eps et al., 2010). Although the pathogenesis of the various types of laminitis is quite different and an extensive description is beyond the scope of this introduction, they are all characterised by degradation of this basement membrane (Katz and Bailey, 2012). The deep digital flexor muscle-tendon unit inserts on the palmar or plantar side of the distal phalanx. In the healthy horse, the deep digital flexor exerts a tensile force on this insertion side, when loaded. In the normal situation, this force is resisted by the tight lamellar junction, but in case of laminitis this junction is weakened, allowing rotation and displacement of the distal phalanx within its hoof capsule (Morrison, 2004).

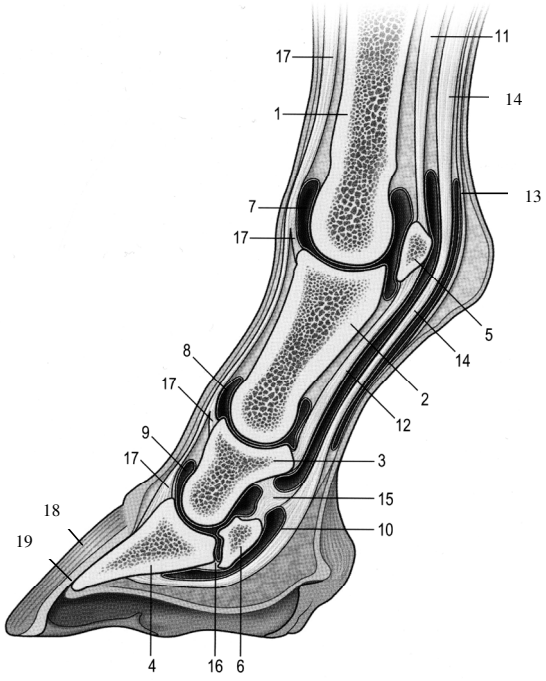

Fig. 1. *Sagittal section of the equine distal forelimb. 1. Metacarpal bone, 2. proximal phalanx, 3. middle phalanx, 4. distal phalanx, 5. proximal sesamoid bone, 6. navicular bone, 7. fetlock joint, 8. pastern joint, 9. coffin joint, 10. navicular bursa, 11. suspensory ligament, 12. straight sesamoidean ligament, 13. superficial digital flexor tendon, 14. deep digital flexor tendon, 15. synovial membrane of coffin joint, navicular bursa and tendon sheath with connective tissue, 16. distal navicular 'impar' ligament, 17. attachments of common digital extensor tendon, 18. hoof wall, 19. lamellae*
Modified from Back, W., Pille, F., 2013. The role of the hoof and shoeing. In: Equine Locomotion, Second Ed. Elsevier, Oxford, United Kingdom.

Whereas in many forms of laminitis it is the primary cause that leads to failure of the SADP with lessened resistance to mechanical loading by the deep digital flexor as a consequence, mechanical overload can also be a primary cause of laminitis on its own. Several forms of laminitis due to overloading of the feet have been identified, of which Supporting Limb Laminitis (SLL) is the most important one. In this case, one limb is affected by some kind of injury (*e.g.* a fracture), causing severely increased loading of the contralateral limb. This unaffected supporting limb can then be overloaded to such extent that structural failure of the lamellar tissue occurs (Van Eps et al., 2010). The most likely pathogenesis in case of SLL is inadequate perfusion of the lamellar tissue as a sequel to reduced blood flow in the supporting limb, caused by excessive compensatory weight-bearing (Orsini et al., 2009; Van Eps et al., 2010). Additional to direct load-induced arterial occlusion, the pulling of the deep digital flexor might also play a role in the impediment of the circulation and the suggested resulting deprivation of oxygen and glucose of the tissue (Orsini et al., 2009; Orsini, 2012). The diminished blood flow is thought to result in repeated micro-damage in the supporting foot and its germinal centres, eventually leading to SLL (Orsini, 2012). Excessive mechanical overload of the feet as a result of high-intensity work has also been mentioned to cause laminitis in the horse. This form of traumatic laminitis has been reported in historical reports as 'road founder' in horses frequently covering large distances on the road, but it is supposed that a similar form of laminitis may be the result of high-intensity work in modern sport horses (Van Eps et al., 2010).

Categorisation

Categorisation of patients with laminitis can be a tool to assess the severity of the disease and to determine prognosis (Cripps and Eustace, 1999). In acute laminitis, horses are very lame, hooves are warm and painful and digital pulses can be strong or bounding. Once the distal phalanx has rotated or sank within the hoof capsule (chronic laminitis), a supra-coronary depression is palpable (Eustace, 2010). In these cases the position of the distal phalanx with respect to the horny hoof capsule and the degree of rotation or sinking can be determined. To categorize patients based on the severity of pain, Obel introduced a four-point grading scale in 1948 (Obel, 1948). Nowadays, this grading scale is still widely used. However, a recent study of Menzies-Gow et al. (2010) concluded that repeatability of the Obel grading was only moderate. It is, therefore, advisable to base the determination of diagnosis, prognosis and evaluation of treatment effects not on Obel grading only but to use a more comprehensive panel of parameters including clinical parameters, pain scoring and radiographic measures.

Treatment

Since the first descriptions of laminitis several thousands of years ago, a large variety of treatment options has come and gone. Bleeding has been used for almost 2000 years whereas exercise has been recommended in case of laminitis for 1700 years (Heymering, 2010). Unfortunately, the ultimate therapy still has to be found. Large progress in the treatment of equine laminitis has been made in the last decades, as there is now more scientific evidence with regard to background and pathogenesis of laminitis (Wells-Smith, 2015). Still, many often used medications or interventions are not or barely evidence-based (Orsini, 2014). There is consensus nowadays that the best treatment for horses or ponies

suffering from laminitis is a multi-modal therapy that includes both medication, interventions through farriery, and management adaptations (Oosterlaan-Mayer et al., 2002; Moore, 2008; Orsini et al., 2009; Durham, 2010).

Administering Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) might be considered the most important part of the pharmacological therapy. Their use in case of laminitis is indicated for their anti-inflammatory as well as their analgesic properties (Belknap, 2010b; Van Eps, 2010). If NSAIDs would not provide enough analgesia in severe cases, opiates can be used as well (Orsini et al., 2009; Van Eps, 2010). As there are dramatic changes in the vascularisation of the digits in case of laminitis, several drugs have been used in attempts to increase the lamellar blood flow (Belknap, 2010b). Acepromazine has been reported to raise the lamellar blood flow when administered intra-muscularly compared to baseline levels before administration (Leise et al., 2007). Heparin and carbasalatum calcium have been used for their anticoagulant properties (Stokes et al., 2004).

The patient suffering from laminitis should be stabled and absolutely not walked. Sedatives and tranquilizers can be used to encourage recumbency, but deep comfortable bedding might be enough to stimulate the horse or pony to lie down in order to reduce physical load (Van Eps, 2010).

Corrective farriery and foot position

Farriery is an important aspect of treatment in both acute and chronic cases of laminitis, as it influences foot position and hence indirectly the biomechanical force patterns within the distal limb. The main goal in the acute phase is to prevent the distal phalanx from rotating or even sinking within the hoof capsule. Therefore, axial support is used to transfer the load from the contact area of the hoof wall with the surface to the sole, frog and bars, thereby unloading the lamellae (Morrison, 2004). Also, heel elevation can be an important aspect in the acute phase. The elevation of the heels intends to reduce the tensile force of the deep digital flexor tendon on the solar surface of the distal phalanx, thereby reducing the stress within the dorsal lamellae (Morrison, 2004; O'Grady, 2010).

In chronic laminitis, the three main goals of shoeing are: 1) to place the break-over point as far backwards (in palmar or plantar direction) as possible, 2) to provide support for frog and sole in order to make them weight-bearing and take load from the hoof wall, and 3) to elevate the heels to decrease the tensile force on the lamellae and transfer load to the sole, frog and bars (Morrison, 2004; O'Grady, 2010). These three steps aim at stabilizing and protecting the digit as well as possible, decrease discomfort to the horse and, ultimately, create conditions that enable returning the foot toward a normal conformation (Taylor et al., 2002). It should be emphasized that therapeutic shoeing, particularly in case of chronic laminitis, is a long-term process and large improvements should not be expected in the first period. Further, a great number of shoes have been developed over time to treat laminitis cases, *e.g.* egg-bar shoes, heart-bar shoes, reverse shoes, aluminium rail shoes and wooden shoes, but it should be noted that no single way of shoeing, shoe type or device will benefit all horses suffering from laminitis (Taylor et al., 2002; Morrison, 2004; O'Grady, 2010). Experience and clinical judgement of the treating veterinarian and farrier play an important role here.

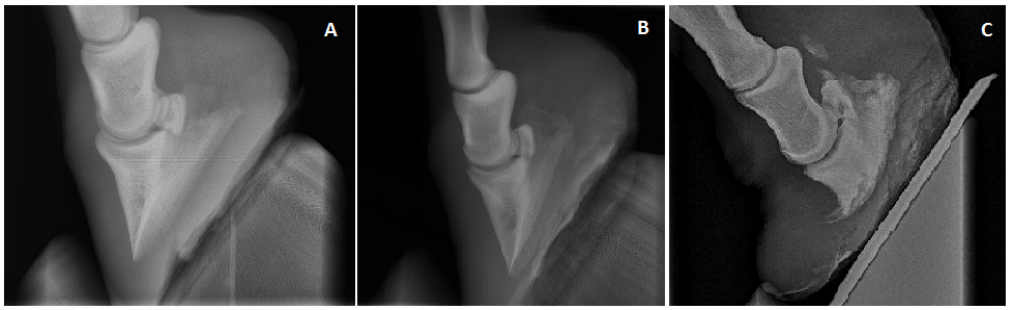

Fig. 2. *Lateromedial radiographs of the distal phalanx and its position towards the hoof wall. A. Normal position, B. Case of "founder" with backwards rotation of the tip of the distal phalanx, C. Severe deformation of the distal phalanx and hoof wall with osteolysis of the distal phalanx caused by chronic laminitis.*
Courtesy of Ms Timmer, Ms Wetzels and Stal Franke

The role of the deep digital flexor muscle

The deep digital flexor muscle is the largest flexor of the equine forelimb and has a humoral, radial and ulnar head (Dyce et al., 2010). The deep digital flexor tendon passes through the carpal canal and is joined by its accessory ligament toward the middle of the metacarpus and eventually inserts on the solar surface of the distal phalanx. The deep digital flexor flexes the lower limb but also limits carpal and fetlock extension, facilitates proximal interphalangeal joint flexion and stabilizes the distal interphalangeal joint (Dyce et al., 2010; Dyson, 2011b).

Another important feature of the deep digital flexor tendon is its energy storing capacity. In the locomotion cycle of the horse, kinetic energy is transformed into elastic energy during the first half of the stance phase of the limb, which is principally stored in three anatomical structures: the deep and superficial digital flexor tendons and the suspensory ligament. During the second half of the stance phase, this energy is used to propel the horse forward and converted again into kinetic energy. This system strongly decreases the energetic costs of equine locomotion and hence the need for muscular work (Wilson et al., 2001; McGuigan and Wilson, 2003; Harrison et al., 2010). This mechanism of storing and returning elastic strain is optimized in the horse by musculoskeletal adaptations like the short pennate architecture of muscle fibers and long, thin tendons (Biewener, 1998; Wilson et al., 2001; McGuigan and Wilson, 2003). The muscle bellies of the digital flexors are well developed. It has been shown that their main function is not to contribute to or regulate significantly the approximately 2.5-Hz cycle of movement, as might be intuitively supposed, but to function as dampers of limb vibration which might reduce fatigue damage (Wilson et al., 2001).

Manipulation of the deep digital flexor muscle-tendon unit as a treatment option in laminitis

When the deep digital flexor is loaded in case of weight bearing, a tensile force is exerted on the insertion site on the distal phalanx. This is normally resisted by the strong bond between the dorsal lamellae (Morrison, 2004; O'Grady, 2010). If the lamellae are weakened due to

laminitis, this resistance is decreased, which may lead to (partial) detachment of the distal phalanx from the horny hoof capsule and subsequently rotation or even sinking of the bone (Morrison, 2004; McGuigan et al., 2005). To halt or prevent such events tenotomy of the deep digital flexor tendon has been proposed as a treatment option (O'Grady, 2010; Morrison, 2011). The transection of the deep digital flexor tendon releases its pull on the distal phalanx and the inflamed lamellae, probably resulting in substantial pain relief (Orsini et al., 2009). The tenotomy can be performed at the mid-cannon or at the mid-pastern level, of which the first site is preferred (Eastman, 2010; Morrison, 2011). Opinions on the usefulness of the procedure vary. Whereas some veterinarians do perform a tenotomy quite regularly as part of laminitis treatment (Eastman, 2010), clinicians often consider it a salvage procedure (O'Grady, 2010; Morrison, 2011). It is clear that transection of the deep digital flexor will severely damage the tendon, but some believe that the resulting gap will be bridged quickly within a few months (Eastman, 2010). Next to the tendon damage, the procedure might result in subluxation of the distal interphalangeal joint (O'Grady, 2010; Morrison, 2011). Reports on outcomes are variable (Hunt et al., 1991; Eastman et al., 1999). In a large scale study, Morrison (2011) investigated the long-term prognosis of deep digital flexor tenotomy in 245 cases and concluded an overall success rate of 51%. However, only 6.5% of the total group returned to some form of athletic soundness (Morrison, 2011). These rather disappointing results of deep digital flexor tenotomy might be related to the treating veterinarian waiting too long to perform the procedure and not using it until there is already severe damage to the lamellar bond, vasculature and distal phalanx (Orsini et al., 2009; Eastman, 2010; Morrison, 2011).

Given that epidemiological studies indicate that approximately 50% of all acute laminitic patients progress into chronic laminitis characterized by distal phalanx displacement (Pollitt and Collins, 2011) and that once dislodging of the distal phalanx has occurred the prognosis for survival strongly decreases (Cripps and Eustace, 1999), there is certainly a thus far unmet need for therapies that aim at preventing such dislodgement or at creating favourable conditions for healing of the lamellar damage by reducing the tensile force by the deep digital flexor tendon on the solar surface of the distal phalanx and that are less invasive and coarse than transection of the tendon.

Is there a role for Clostridium botulinum toxin type A in the supportive treatment of laminitis?

In the early 19th century, an outbreak of poisoning caused by the ingestion of black pudding was described (Erbguth and Naumann, 1999). Although not recognised as such at the time, this is seen as the first report on what is now called 'botulism'. It took until the end of the 19th century to postulate a neurotoxic mechanism and to identify the bacterial origin (Erbguth and Naumann, 1999; Ney and Joseph, 2007). *Clostridium botulinum* produces several neurotoxins, typed A-G. The illnesses caused by *Clostridium botulinum* can be divided into food-borne, infantile or wound botulism based on the way of toxin intake (Davis, 1993). The toxin of *Clostridium botulinum* impedes acetylcholine release at the neuromuscular junction (Davis, 1993; Ney and Joseph, 2007; Oh and Chung, 2015). In short, the heavy chain of the toxin binds to a receptor of the pre-synaptic membrane allowing entry of the toxin into the axon terminal. Once in the axon terminal, the toxins interfere with parts of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein-

complex, thereby inhibiting fusion of neurotransmitter-containing intra-axonal vesicles with the presynaptic membrane. This results in a reduction of acetylcholine extrusion into the synaptic cleft. As a result, there is a diminution of muscle contraction. After a few weeks, recovery begins by the generation of new nerve sprouts emerging from the nodes of Ranvier that form new neuromuscular junctions and endplates with the adjacent muscle (Davis, 1993; Ney and Joseph, 2007). Eventually, there is regeneration of the SNARE-protein complex and acetylcholine is released from the original nerve terminal as the nerve sprouts start to regress (Ney and Joseph 2007, de Paiva et al., 1999). From human and equine studies it is known that the effect of *Clostridium botulinum* toxin lasts for at least 12 weeks (Ney and Joseph, 2007; Wijnberg et al., 2009).

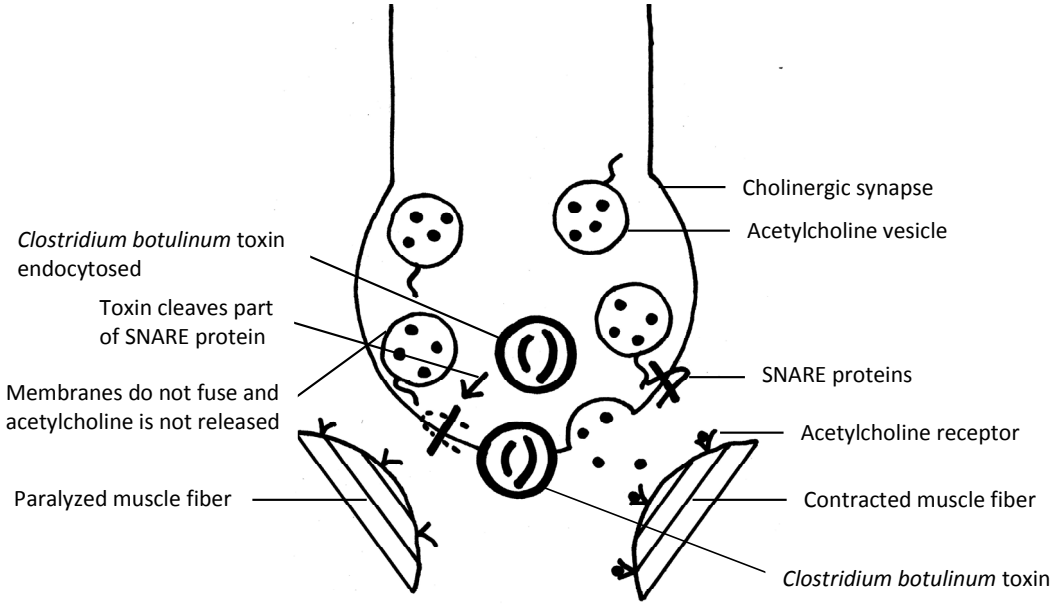

Fig. 3. *Mechanism of action of Clostridium botulinum toxin*

Since the 1970s, *Clostridium botulinum* toxin has been used in human medicine for its paralyzing effect to treat several disorders of muscle over-activity such as spasticity, limb and neck dystonia and, more recently, to attain a cosmetic effect (Davis, 1993; Ney and Joseph, 2007; Olver et al., 2010). Nowadays, it is also used in the management of painful disorders like migraine (Ravenni et al., 2013; Oh and Chung, 2015). Although it was initially believed that these analgesic effects were caused by muscle relaxation, it has now been proven that *Clostridium botulinum* toxin type A also inhibits the release of neurotransmitters regulating pain and inflammation, thereby decreasing peripheral sensitization and indirectly reducing central sensitization (Oh and Chung, 2015). Next to this, it is suggested that

Clostridium botulinum toxin type A can be axonally transported to the CNS resulting in central anti-nociceptive effects (Oh and Chung, 2015).

There are three types of *Clostridium botulinum* toxin type A available in the form of medication: Botox® (Allergan Inc., USA, approved in the Netherlands for use in human medicine in 1993), Dysport® (Ipsen Ltd., UK, approved in the Netherlands for use in human medicine in 1993) and Xeomin® (Merz Pharmaceuticals GmbH, Germany, approved in the Netherlands for use in human medicine in 2014). The biological activity and the amount of complexing proteins differ per product (Ravenni et al., 2013) and conversion rates should be used when the different formulations are interchanged (Sampaio et al., 2004). Although *Clostridium botulinum* produces one of the most potent toxins affecting humans, its therapeutic use is considered to be quite safe (Osborne et al., 2007). Nevertheless, some adverse events, such as fever, flu-like malaise, injection site reactions and even a single case of spontaneous death have been reported (Ney and Joseph, 2007). Additionally, *Clostridium botulinum* toxins can induce the formation of antibodies which can cause antibody-associated treatment failure if repeated treatments are necessary (Ney and Joseph, 2007; Benecke, 2012). The presence of complexing proteins (in the products Botox® and Dysport®) increases the total bacterial protein load and hence the risk of formation of neutralizing antibodies against the neurotoxin. This is not the case with Xeomin® that does not contain complexing proteins (Benecke, 2012).

As the *Clostridium botulinum* toxin acts upon the presynaptic membrane of the muscle endplate at the neuromuscular junction, injecting the toxin to the motor endplate-zone would be most optimal (Van Campenhout and Molenaers, 2011). The location of the motor endplate-zone is generally unknown, but electromyography (EMG) can be used to locate motor end-plates. This procedure has indeed proven to potentiate the effect of *Clostridium botulinum* toxin injections in a canine model (Childers et al., 1998). Apart from this, EMG can be useful to evaluate *Clostridium botulinum* toxin effects, as the decrease in muscle activity can be measured using several EMG techniques. These include motor unit action potential analysis, but also interference pattern analysis (Fuglsang-Frederiksen, 2000; Çakmak et al., 2006; Dunne et al., 2010).

In veterinary medicine, the use of *Clostridium botulinum* toxin is on the increase. In a preliminary study aiming at the development of techniques that might reduce incisional dehiscence after repair of perineal lacerations it was shown that *Clostridium botulinum* toxin type B could be used to decrease muscle tone of the anal sphincter in healthy horses (Adam-Castrillo et al., 2004). More recently, it has been successfully applied for its analgesic effects to temporarily alleviate chronic lameness after injections of 3.8-4.5 IU/kg bodyweight of *Clostridium botulinum* toxin type B into the navicular bursa (Gutierrez-Nibeyro et al., 2014). Hadley et al. (2010) investigated the use of *Clostridium botulinum* toxin type A in dogs suffering from osteoarthritis and concluded there was a beneficial effect after injections of 25 IU, based on improved ground reaction forces and positive dog-owner reports. A recent randomized, placebo-controlled clinical trial by Heikkilä et al. (2014) confirmed this efficacy in reducing osteoarthritic pain in dogs after an intra-articular injection of 30 IU of *Clostridium botulinum* toxin type A. The muscle relaxing effects of *Clostridium botulinum* toxin type A have now been tested experimentally in dogs for the induction of ptosis (injection of 15 IU) in order to temporary cover the cornea (Bittencourt et al., 2013) and to reduce bronchial

hyperreactivity in healthy dogs to test its potential for treating asthma patients (Al-Halfawy et al., 2012). In a preliminary study, Wijnberg et al. (2009) monitored the muscle activity reducing effect of *Clostridium botulinum* toxin type A administration in several skeletal muscles of healthy ponies (maximum of 400 IU per pony) and did not observe any negative side effects. As a proof of principle, in the same study the toxin (700 IU per case) was applied in two horses suffering from stringhalt, which showed significant reduction of the hypermetric movement after the injections. Notwithstanding these promising results, the use of *Clostridium botulinum* toxin in veterinary medicine is still largely experimental and limited, which may also be related to the elevated costs.

Because *Clostridium botulinum* toxin has proven to produce an excellent muscle tone reduction in human medicine and has been experimentally used in equine medicine without signs of side-effects, it can be conjectured that the toxin, when used to reduce the muscle tone of the equine deep digital flexor muscle, could have potential to reduce the traction of the deep digital flexor tendon on the distal phalanx. Unlike tenotomy of the deep digital flexor tendon, injection of *Clostridium botulinum* toxin is minimally invasive and not irreversible. It is therefore no salvage procedure and could be used in a much earlier stage of the development of laminitis than deep digital flexor tenotomy, which will always remain a last resort treatment. As mentioned earlier, *Clostridium botulinum* has a prolonged effect and, when given early, management and treatment regimes could be optimized to stimulate healing and prevent new flare-ups during its span of action. The basic hypothesis of this thesis is therefore that the use of *Clostridium botulinum* toxin injections into the deep digital flexor muscle is a safe and potentially useful supportive therapy in case of equine laminitis.

Outline of the thesis

The first research question that was addressed was whether *Clostridium botulinum* toxin indeed would reduce motor unit activity of the deep digital flexor muscle without substantial side effects and what would be the degree and course in time of such a reduction in muscle activity. To answer this question, needle electromyography recordings were performed before and after *Clostridium botulinum* toxin injections into the deep digital flexor muscle of healthy horses (**Chapter II**).

As a direct follow-up, **Chapter III** investigates the influence of *Clostridium botulinum* toxin injection on the locomotion of the horse. This is paramount because, if the reduced muscle activity would induce (or in case of a laminitic horse perhaps aggravate) lameness, this might impede the clinical application of the agent. For this purpose, locomotion was quantified in the same group of healthy horses as used in Chapter II before and after *Clostridium botulinum* toxin injections using an inertial sensor system to determine the range of motion of joints and body segments and a dynamically calibrated pressure plate to assess ground reaction forces and pressure patterns.

In **Chapter IV** the question is addressed to what extent deep digital flexor muscle tone is affected by laminitis. It has been suggested that laminitis may result in increased muscle tone or even contracture of the deep digital flexor muscle, which would be pain-induced and most obvious at a chronic stage of the disease (Pollitt, 1995b; Parks, 2003). This clinical impression has never been proven by evidence-based research, but, if true, would further

Chapter I

support the use of *Clostridium botulinum* toxin therapy. To verify this assumption, electromyography Interference Pattern Analysis (IPA) was used to determine muscle force of the deep digital flexor in a group of healthy and a group of laminitic horses and ponies.

If *Clostridium botulinum* toxin is to be used in future clinical trials or at some stage as regular therapy, correct dosing will be crucial. Obviously, in real life various breeds and sizes of horses and ponies will suffer from laminitis. For the best possible estimation of correct dosages, a method to determine the volume of the target-muscle can therefore be very helpful to extrapolate human dosages as long as information on pharmacokinetics and dose-response curves of *Clostridium botulinum* toxin in the horse are not available. In **Chapter V** cadaveric forelimbs are used to develop a formula for the estimation of the volume of the deep digital flexor muscle in the living animal based on distances between anatomical landmarks.

In the last experimental chapter, **Chapter VI**, the hypothesis that *Clostridium botulinum* toxin injections into the deep digital flexor muscle are able to give substantial pain relief in case of acute laminitis and prevent the distal phalanx from displacing is tested. An extensive objective evaluation protocol was developed to determine the effects of the treatment on pain-status related parameters, indicators of inflammation, locomotion and the radiographic position of the distal phalanx.

Finally, in **Chapter VII** the results as presented in the preceding chapters are discussed against the background of existing knowledge. A preliminary balance is drawn up and suggestions are given how to proceed from here.

Chapter II

The effect of *Clostridium botulinum* toxin type A injections on motor unit activity of the deep digital flexor muscle in healthy sound Royal Dutch Sport horses

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Abstract

Therapeutic reduction of the activity of the deep digital flexor muscle may play a role in the treatment of laminitic horses. *Clostridium botulinum* toxin type A induces reduced muscle activity and has a spasmolytic effect in horses. In this study, the effectiveness of 200 IU *Clostridium botulinum* toxin type A on reduction of the deep digital flexor muscle activity was measured in seven healthy, sound, adult Royal Dutch Sport horses. *Clostridium botulinum* toxin type A was injected using ultrasound and electromyographic (EMG) guidance. The effectiveness was assessed by Interference Pattern Analysis (IPA) and Motor Unit Action Potential (MUAP) analysis. All needle EMG MUAP variables, along with IPA amplitude/turn and turns/s, were significantly reduced after *Clostridium botulinum* toxin type A injections. The strongest effect occurred within the first three days after injection. The reduced muscle tone induced by *Clostridium botulinum* toxin type A may have benefits in the treatment of horses with laminitis.

Introduction

Laminitis and navicular disease, and their treatment options, have been studied intensively in the last few decades (Bailey et al., 2004; Moore, 2008; Orsini et al., 2009; Heymering, 2010). The deep digital flexor muscle originates from the humerus, ulna and radius proximally, continuing at the level of the palmar carpus in a tendon that crosses the navicular bursa and navicular bone distally, finally inserting on the distal phalanx. The suspensory apparatus of the distal phalanx (SADP), formed by the dermal and epidermal lamellae, connects the distal phalanx to the hoof wall. In a sound horse, the deep digital flexor is under tension when the hoof is fully weight bearing (Morrison, 2004). In horses with laminitis, the structural failure of the SADP enables the distal phalanx to be displaced within the capsule of the hoof (Van Eps et al., 2010; Pollitt, 2011).

The use of multimodal therapy, including anti-inflammatory, anti-coagulant and blood pressure reducing medication, multimodal pain management and farriery, are currently the favoured options for treatment of laminitis (Bailey et al., 2004; Morrison, 2004; Moore, 2008; Orsini et al., 2009; O'Grady, 2010). However, the rate of success is still low and it has been suggested that 50% of all acute laminitic horses eventually suffer from permanent sinking of the distal phalanx ('foundering'), thereby becoming chronic (Pollitt and Collins, 2011). Once the distal phalanx is displaced within the hoof, the prognosis for a successful long term outcome in the laminitic horse is poor (Cripps and Eustace, 1999).

Clostridium botulinum toxin type A (Botox®, Allergan Inc.) has been used in human medicine since the 1970s to alter muscle tone and to treat spasticity and disorders of muscle over-activity (Davis, 1993; Mancini et al., 2005; Ney and Joseph, 2007; Olver et al., 2010). *Clostridium botulinum* toxin type A has also been successfully used in equine medicine to reduce the muscle tone of several skeletal muscles (Adam-Castrillo et al., 2004; Carter and Renfroe, 2009; Wijnberg et al., 2009). In healthy ponies and horses with stringhalt, *Clostridium botulinum* toxin type A reduces muscle tone in the absence of side-effects (Wijnberg et al., 2009).

Injecting the deep digital flexor muscle with *Clostridium botulinum* toxin type A theoretically would result in reduced muscle tone and, in the case of acute laminitis, could prevent the distal phalanx from rotating or sinking by reducing the pull of the deep digital flexor muscle and tendon on the distal phalanx, and may be useful as a supportive therapy. However, the effectiveness of *Clostridium botulinum* toxin type A injections in reducing the tone of the deep digital flexor muscle has not been quantified.

Electromyography (EMG) can be a useful tool in the diagnosis of equine neuromuscular locomotor problems (Wijnberg et al., 2002b, 2004). Surface EMG has been used to monitor the effect of *Clostridium botulinum* toxin type A injections into the long digital extensor, lateral digital extensor and vastus lateralis muscles in horses (Wijnberg et al., 2009). However, the deep digital flexor muscle is covered with other muscles, so surface EMG is not a suitable technique for measuring the tone of this muscle.

In human medicine, needle EMG has been used to evaluate the efficiency of *Clostridium botulinum* toxin type A in muscles (Çakmak et al., 2006; Dunne et al., 2010). In addition,

needle EMG guidance is used to inject *Clostridium botulinum* toxin type A near to the muscle endplate zone to refine injection sites and minimise the required dosages (Barbano, 2001). Although there is some discussion regarding the need for EMG guidance, its usefulness has been demonstrated in animal models (Childers et al., 1998; Barbano, 2001). EMG guidance is preferred in the horse to minimise the dose of *Clostridium botulinum* toxin type A, thereby increasing safety and reducing costs.

The main aim of this study was to quantify the effectiveness of *Clostridium botulinum* toxin type A in reducing muscle tone in the deep digital flexor muscle of the horse following injection using needle EMG. An additional aim was to test if *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle of horses would cause side effects that would make its application in equine patients inadvisable.

Materials and methods

Horses
Seven healthy female Royal Dutch Sport horses (mean age ± standard deviation, age 11 ± 4 years, weight 539 ± 28 kg) were used in this study. All horses were part of the teaching herd of Utrecht University. The experiment was approved by the Animal Welfare Committee of Utrecht University (approval number 2010.III.09.105). Prior to the study, all horses underwent a standard orthopaedic examination and all were evaluated as being sound at the walk and trot. All horses were sedated and received analgesia prior to EMG examination and *Clostridium botulinum* toxin type A (Botox®, Allergan Inc.) injections using 0.3–0.5 mL 10 mg/mL detomidine hydrochloride (Domosedan®, Janssen Pharmaceutica), 0.3–0.5 mL 10 mg/mL butorphanol tartrate (Dolorex®, Intervet) and 10 mL 50 mg/mL flunixine meglumine (Bedozane®, Eurovet Animal Health). After EMG and *Clostridium botulinum* toxin type A injections, the injected forelimb was cooled with running cold water for ten minutes to reduce a potential inflammatory response. Simultaneously with the experiment described in the present study, a second study was performed to determine the effect of unilateral *Clostridium botulinum* toxin type A injections on several locomotion parameters of the sound horse using the same group of treated horses (Hardeman et al., 2013).

Treatment protocol
Clostridium botulinum toxin type A (200 IU) was injected into various sites of the deep digital flexor muscle of the left forelimb (Annex 1). Every 100 IU of *Clostridium botulinum* toxin type A was diluted with 2.5 mL sterile 0.9% NaCl solution. The dosage was extrapolated from the studies of Wijnberg et al. (2009) and Carter and Renfroe (2009), as well as human protocols (Mancini et al., 2005). The right (untreated) forelimb served as a control limb.

The caudal part of the forelimb was clipped of hair and cleaned. The distance between the top of the olecranon and the accessory carpal bone was measured. Based on a preliminary study on cadaveric forelimbs, this distance was used to determine five points in the widest part of the muscle belly based on proportional distances (**Fig. 1**). The forelimb was scrubbed and disinfected using alcohol, then five injections of 1 mL diluted *Clostridium botulinum* toxin type A were administered into the groove between the flexor carpi ulnaris and superficial digital flexor muscles of each limb. To distribute the *Clostridium botulinum* toxin type A evenly among the deep digital flexor muscle, one injection was placed in the

superficial part, two injections in the middle part and two in the deep part of the deep digital flexor muscle (**Fig. 1**). Injections were performed under ultrasonographic guidance to guarantee the correct position of the injection needle.

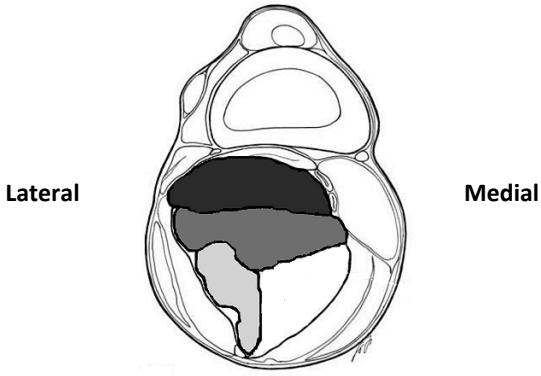

Fig. 1. *Diagram showing a cross-section of the forelimb of the horse, indicating muscles used for injection of Clostridium botulinum type A toxin. Black shaded area, deep part of deep digital flexor muscle; dark grey shaded area, middle part of the deep digital flexor muscle; light grey shaded area, superficial part of the deep digital flexor muscle.*

EMG guidance was used to inject the *Clostridium botulinum* toxin type A solution as close as possible to the endplate zone. The solution was injected through an EMG Myoject needle electrode (length 75 mm, diameter 0.71 mm, recording area 0.89 mm2; MedCat) using a monopolar needle (length 75 mm, diameter 0.46 mm, recording area 0.34 mm2; MedCat) with a portable EMG apparatus (Viking Quest EMG System®, Nicolet Biomedical, Viasys Health Care). The motor endplate zones were identified by the presence of endplate spikes or fibrillations.

Electromyographic analysis

Quantitative needle electromyography (QEMG) was performed on day 0 just before injection and on days 3 and 14 after *Clostridium botulinum* toxin type A injections. Examination sites and depths were the same as the *Clostridium botulinum* toxin type A injection sites. Details of materials and methods of QEMG examination have been described by Wijnberg et al. (2002a,b). Electromyographic examination, including Motor Unit Action Potential (MUAP) analysis, was accomplished using a 23 G concentric EMG needle (length 75 mm, diameter 0.60 mm, recording area 0.068 mm2; MedCat) and portable EMG apparatus. Bandpass was 5–10 kHz, sweep speed was 10–20 ms/division and amplifier gain was 100–500 lV/division. Selected MUAPs had a rise time <0.8 ms and occurred repeatedly at least four times (Wijnberg et al., 2004).

Ten MUAPs were analysed from each horse and EMG interference pattern analysis (IPA) was performed (Wijnberg et al., 2011). The low frequency filter was set at 20 Hz, the high frequency filter at 10 kHz and the sampling frequency at P25 Hz. Five contractions at random force per injection site were recorded using a concentric needle electrode (length 75 mm, diameter 0.60 mm, recording area 0.068 mm2; MedCat). IPA recordings, expressed as

number of turns/s (T) and amplitude/turn (M), were measured to determine *Clostridium botulinum toxin* type A effectiveness (Finsterer, 2001). These variables were calculated automatically by the EMG software.

Statistical analysis
Statistical analysis was performed using R-statistics (The R-Project). Needle EMG data was transformed into natural logarithms. The outcome variables were the MUAP amplitude, MUAP duration, MUAP phases, MUAP turns, IPA amplitude/turn (M) and IPA turns/s (T). The outcome variables were analysed using a linear model with random effects, with 'day' and a quadratic term for 'day' (day^2) as explanatory variables. This quadratic term for 'day' (day^2) was used to determine if there was a straight linear decrease of the parameters from days 0 to 14. Adding the quadratic term determined if the model fulfilled the requirements for a straight linear correlation ($y = ax + b$). For all outcomes, 'horse' was used as random effect to take the correlation between measurements into account. Akaike's information criterion was used in a backward selection method to obtain the best model.

Since the first horse showed some minor lameness after *Clostridium botulinum* toxin type A injections, the second measurement on this horse was performed on day 7 instead of day 3 and EMG IPA measurements were not performed in this horse on day 0. Day was not treated as a factor, but as a continuous variable in the linear model, since not all measurements were performed at the same time points. This enabled the admittance of the day 7 data for the first horse and also permitted the omission of the day 0 EMG IPA data for the first horse. QQ-plots were used to check the data for normality. P values <0.05 were considered to be significant.

Results

There were no signs of systemic toxicity in any horses used in the study. There was minimal swelling at the injection sites. All horses exhibited minor lameness (≤ 1/5) of the injected leg at the trot on a hard surface one day after *Clostridium botulinum* toxin type A injections. The first horse exhibited minor lameness for two days after the injections and therefore EMG re-examination was performed on day 7 instead of day 3.

There was a significant decrease in the number of MUAP phases from day 0 just before *Clostridium botulinum* toxin type A injections to day 14 after injections ($P = 0.009$; **Fig. 2a**). The addition of 'day^2' to the fixed part of the model was not significant ($P = 0.10$), indicating that there was a straight linear decrease. There was a significant decrease in IPA amplitude/turn (M) from days 0 to 14 ($P < 0.001$). The data for this parameter showed a straight linear decrease; the addition of 'day^2' to the fixed part of the model was not significant ($P = 0.56$; **Fig. 2b**).

There was a significant decrease in MUAP duration from days 0 to 14 ($P = 0.006$). This parameter did not exhibit a straight linear decrease; the addition of 'day^2' to the fixed part of the model was significant ($P = 0.03$; **Fig. 2a**). There was a significant decrease in MUAP amplitude from days 0 to 14 ($P = 0.0014$). The addition of 'day^2' to the fixed part of the model was significant ($P = 0.02$), indicating that there was no straight linear decrease. There was a significant decrease in MUAP turns from days 0 to 14 ($P = 0.003$). The addition of 'day^2'

to the fixed part of the model was significant (P = 0.006), indicating that there was no straight linear decrease in the parameter. There was a significant decrease in IPA turns/s (T) from days 0 to 14 (P = 0.003), but there was no straight linear decrease; the addition of 'day^{2}' to the fixed part of the model was significant (P = 0.004; **Fig. 2b**).

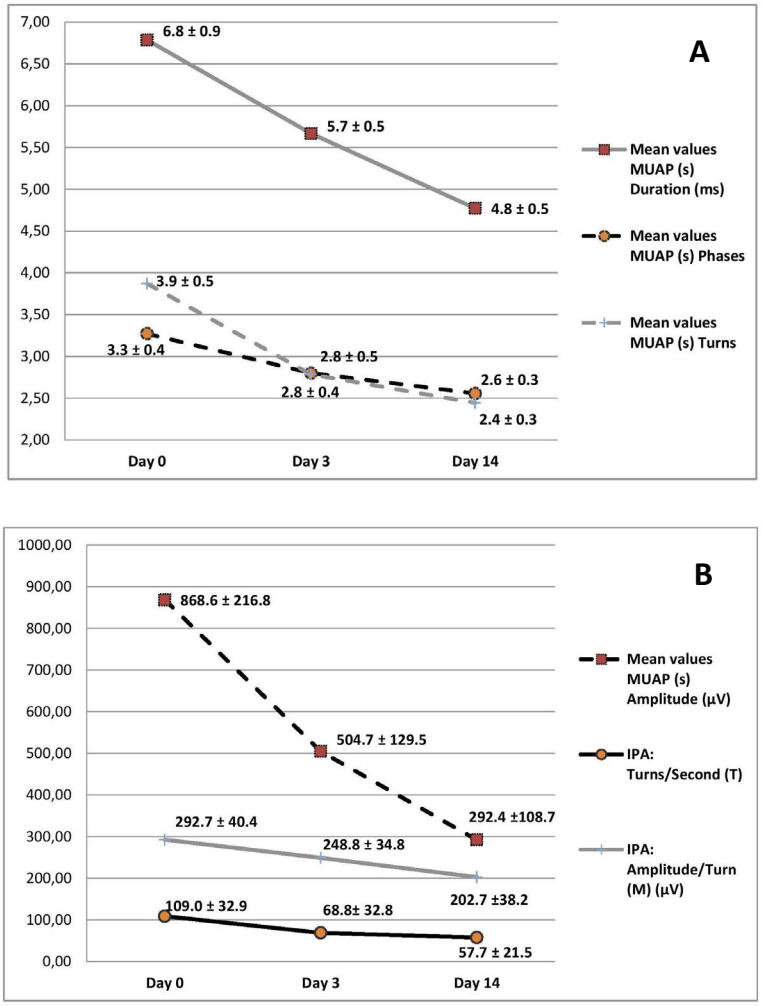

Fig. 2. *Graphs showing responses to injection of Clostridium botulinum type A toxin. (a) Black dotted line, motor unit action potential (MUAP) amplitude (µV); black line, interference pattern analysis (IPA; turns/s, T); grey line, IPA (amplitude/turn, M); day 0, before Clostridium botulinum type A toxin injections; days 3 and 14, 3 and 14 days after Clostridium botulinum type A injections, respectively (± standard deviation). (b) Black dotted line, motor unit action potential (MUAP) phases; grey line, MUAP duration (ms); grey dotted line, indicates MUAP Turns; day 0, recordings taken before Clostridium botulinum toxin type A injections; days 3 and 14, recordings taken 3 and 14 days after Clostridium botulinum type A toxin injections, respectively (± standard deviation).*

There was no straight linear decrease in MUAP duration, MUAP amplitude, MUAP turns or IPA T from days 0 to 14. These parameters decreased sharply from days 0 to 3, then levelled off between days 3 and 14.

Discussion

The main aim of this study was to quantify the effectiveness of *Clostridium botulinum* toxin type A injections in reducing the muscle tone of the equine deep digital flexor muscle using needle EMG IPA analysis and MUAP analysis. A secondary aim was to test if *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle resulted in lameness or signs of toxicity. It appeared that *Clostridium botulinum* toxin type A injections into the equine deep digital flexor muscle are safe, since no severe short term side effects were observed, similar to previous findings (Wijnberg et al., 2009). Since *Clostridium botulinum* toxin type A was injected near the motor endplate in our study, the most optimal effect should have been induced.

The number and location of motor endplates forming the motor end plate zone in the equine deep digital flexor muscle is unknown; however, even in human studies, the exact anatomy of innervation and localisations of the motor end plate zones is a subject of ongoing investigation (Van Campenhout and Molenaers, 2011). *Clostridium botulinum* toxin type A diffuses through muscle and fascia and is thus likely to reach most of the muscle with only a limited number of injections (Barbano, 2001; Van Campenhout and Molenaers, 2011).

The *Clostridium botulinum* toxin type A dosage used in our study was extrapolated from the studies of Carter and Renfroe (2009) and Wijnberg et al. (2009). There would be advantages in extrapolating dosages on the basis of the results of double-blind control studies on *Clostridium botulinum* toxin type A dosages, as have been performed in human beings (Mancini et al., 2005).

The aim of this study was not to determine the duration of reduced muscle tone after *Clostridium botulinum* toxin type A injection. Wijnberg et al. (2009) reported that the decreased muscle activity of several equine skeletal muscles following *Clostridium botulinum* toxin type A injections lasted for ≥90 days. In human beings, functional paralysis following injection of *Clostridium botulinum* toxin type A usually lasts for 14–18 weeks (Ney and Joseph, 2007).

Our data are consistent with previous studies using needle EMG to determine the effects of *Clostridium botulinum* toxin type A injections on skeletal muscles (Çakmak et al., 2006; Dunne et al., 2010). The outcome of the EMG IPA analysis is also consistent with the use of EMG IPA in human beings treated with *Clostridium botulinum* toxin type A (decreased amplitude/turn and turns/s), indicating a loss of functional muscle fibres (Buchman et al., 1993; Finsterer, 2001).

In our study, only 10 representative MUAPs were analysed, whereas our other studies examined 20 representative MUAPs (Wijnberg et al., 2002a, 2004). The number of injections into the superficial and deeper muscles was reduced in the present study in order to limit side effects, such as swelling and transient mild lameness.

Our finding that most EMG parameters do not show a straight linear decrease indicates that the neuromuscular blockage caused by *Clostridium botulinum* toxin type A is strongest in the first three days, levelling off thereafter. This is relevant to the use of *Clostridium botulinum* toxin type A in case of equine laminitis, since it implies that the reduction in muscle tone and the expected corresponding release of pull of the deep digital flexor on the distal phalanx are likely to decrease several days after treatment with *Clostridium botulinum* toxin type A. According to Morrison (2011), decreasing the tension of the deep digital flexor tendon is one of the aims of rehabilitation of laminitic horses with rotational displacement.

Conclusions

Clostridium botulinum type A injections into the equine deep digital flexor muscle can be used safely to reduce tone in this muscle. *Clostridium botulinum* toxin type A may have a place in the treatment of equine laminitis, without the risks involved with tenotomy, such as contracture of the deep digital flexor.

Acknowledgements

The authors would like to thank Hans Vernooij and Jan van den Broek for statistical advice and Augusta Lamers for technical assistance.

Annex

Injection sites in the deep digital flexor muscle.

Distance from the top of the olecranon to the accessory carpal bone (cm)	Distance from the top of the olecranon to the accessory carpal bone (cm)				
	1	2	3	4	5
20	6.00	7.25	8.50	9.75	11.00
21	6.30	7.61	8.93	10.24	11.55
22	6.60	7.98	9.35	10.73	12.10
23	6.90	8.34	9.78	11.21	12.65
24	7.20	8.70	10.20	11.70	13.20
25	7.50	9.06	10.63	12.19	13.75
26	7.80	9.43	11.05	12.68	14.30
27	8.10	9.79	11.48	13.16	14.85
28	8.40	10.15	11.90	13.65	15.40
29	8.70	10.51	12.33	14.14	15.95
30	9.00	10.88	12.75	14.63	16.50
31	9.30	11.24	13.18	15.11	17.05
32	9.60	11.60	13.60	15.60	17.60
33	9.90	11.96	14.03	16.09	18.15
34	10.20	12.33	14.45	16.58	18.70
35	10.50	12.69	14.88	17.06	19.25
36	10.80	13.05	15.30	17.55	19.80
37	11.10	13.41	15.73	18.04	20.35
38	11.40	13.78	16.15	18.53	20.90
39	11.70	14.14	16.58	19.01	21.45
40	12.00	14.50	17.00	19.50	22.00
41	12.30	14.86	17.43	19.99	22.55
42	12.60	15.23	17.85	20.48	23.10
43	12.90	15.59	18.28	20.96	23.65
44	13.20	15.95	18.70	21.45	24.20
45	13.50	16.31	19.13	21.94	24.75
46	13.80	16.68	19.55	22.43	25.30
47	14.10	17.04	19.98	22.91	25.85
48	14.40	17.40	20.40	23.40	26.40
49	14.70	17.76	20.83	23.89	26.95
50	15.00	18.13	21.25	24.38	27.50

Chapter III

Effect of *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle on the range of motion of the metacarpus and carpus, and the force distribution underneath the hooves, of sound horses at the walk

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Abstract

In the treatment of laminitis, reducing deep digital flexor muscle activity might diminish its pull on the distal phalanx, thereby preventing displacement and providing pain relief. Injection of *Clostridium botulinum* toxin type A into the deep digital flexor muscle of horses is potentially therapeutic. However, the effects of *Clostridium botulinum* toxin type A on the gait characteristics of sound horses at the walk are not known. The aim of this study was to test if a reduced deep digital flexor muscle activity would lead to (1) alterations of the sagittal range of motion of the metacarpus (SROM) and range of motion of the carpal joint (CROM); (2) changes in the force distribution underneath the hoof (toe vs. heel region: balance index); and (3) changes in the force distribution between the treated and untreated limb (symmetry index). The deep digital flexor muscles of the left forelimbs of seven sound Royal Dutch Sport Horses were injected with 200 IU *Clostridium botulinum* toxin type A using electromyography and ultrasound guidance. Measurements using an inertial sensor system and dynamically calibrated pressure plate were performed before and after injections. The SROM and CROM of the treated limb were significantly increased after *Clostridium botulinum* toxin type A injections. No significant changes were detected in the balance index or in the symmetry index, indicating that no lameness was induced. *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle of sound horses do not appear to result in substantial gait alterations at the walk.

Introduction

The suspensory apparatus of the distal phalanx of the horse, formed by the dermal and epidermal lamellae, connects the distal phalanx to the hoof wall. The deep digital flexor muscle, originating from the humerus, ulna and radius, inserts on the flexor surface of the distal phalanx. The accessory ligament of the deep digital flexor tendon arises from the palmar carpal ligament and joins the deep face of the deep digital flexor tendon towards the middle of the metacarpus (Dyce et al., 2002). It prevents overstretching of the proximal part of the deep digital flexor tendon and facilitates carpal extension when the limb is loaded (Dyson, 2011c).

In equine laminitis, structural failure of the junction between the hoof capsule and the distal phalanx results in displacement of the distal phalanx (Van Eps et al., 2010; Pollitt, 2011). In the sound horse, the deep digital flexor muscle has a degree of rest tone that can be quantified by electromyography including interference pattern analysis. The deep digital flexor tendon is placed under tension when the hoof is fully weight bearing (Morrison, 2004). If the lamellae are weakened due to laminitis, it is hypothesised that the tension of the deep digital flexor muscle on the distal phalanx results in displacement of the distal phalanx within the hoof. One of the aims of rehabilitation of rotational displacement of the distal phalanx in laminitis is to decrease the tension exerted by the deep digital flexor tendon, for example by deep digital flexor tenotomy (Eastman et al., 1999; Morrison, 2004). Morrison (2011) suggested that deep digital flexor tenotomy should be performed before the horse experiences advanced distal phalanx disease. The rate of success in the treatment of laminitis is low and ~50% of acute laminitic patients eventually suffer from sinking of the distal phalanx ('foundering') (Pollitt and Collins, 2011).

Clostridium botulinum toxin type A has been used widely in human medicine to treat disorders of muscle overactivity (Davis, 1993; Ney and Joseph, 2007). Needle electromyography (EMG) guidance is often used to inject *Clostridium botulinum* toxin type A near to the muscle endplate zone to refine injection sites and minimise required dosages (Barbano, 2001). *Clostridium botulinum* toxin type A has been used in horses to reduce the activity of several skeletal muscles without major side effects (Adam-Castrillo et al., 2004; Carter and Renfroe, 2009; Wijnberg et al., 2009). Injection of *Clostridium botulinum* toxin type A into the deep digital flexor muscle to reduce muscle activity may have a role in the treatment of laminitis without the risks associated with tenotomy (Eastman et al., 1999; Morrison, 2011). Reducing deep digital flexor muscle activity might reduce the pull of the deep digital flexor tendon on the distal phalanx, which may help in preventing displacement of the distal phalanx within the hoof capsule and reducing pain.

However, before embarking on the application of *Clostridium botulinum* toxin type A in laminitic horses to test this hypothesis, the effects in sound horses should be explored, since any adverse effect on gait characteristics might impair the clinical application of this agent. Wijnberg et al. (2013) demonstrated that *Clostridium botulinum* toxin type A decreases the activity of the deep digital flexor muscle in horses. The aim of the present study was to test if *Clostridium botulinum* toxin type A could be injected into the deep digital flexor muscle without inducing gait asymmetries, as assessed by (1) alterations of the sagittal range of motion of the metacarpus (SROM) and range of motion of the carpal joint (CROM) at the

walk; (2) changes in the force distribution underneath the hoof (toe vs. heel region: balance index) at the walk; and (3) changes in the force distribution between the treated and untreated limb (symmetry index) at the walk.

Materials and Methods

Horses
This study used the same horses and experimental design as the study by Wijnberg et al. (2013). The experimental subjects were seven healthy female Royal Dutch Sport horses (mean age ± standard deviation, age 11 ± 4 years, weight 539 ± 28 kg). The experiment was approved by the Animal Welfare Committee of Utrecht University (approval number 2010.III.09.105). All horses were sedated and received analgesia prior to EMG examination and *Clostridium botulinum* toxin type A (Botox®, Allergan Inc.) injections using 0.3–0.5 mL 10 mg/mL detomidine hydrochloride (Domosedan®, Janssen Pharmaceutica), 0.3–0.5 mL 10 mg/mL butorphanol tartrate (Dolorex®, Intervet) and 10 mL 50 mg/mL flunixine meglumine (Bedozane®, Eurovet Animal Health).

Treatment protocol
Clostridium botulinum toxin type A (200 IU) was injected into various sites of the deep digital flexor muscle of the left forelimb, as described by Wijnberg et al. (2013). Every 100 IU of *Clostridium botulinum* toxin type A was diluted with 2.5 mL sterile 0.9% NaCl solution. The right (untreated) forelimb served as a control limb. Five injections of 1 mL diluted *Clostridium botulinum* toxin type A were administered into the groove between the flexor carpi ulnaris and superficial digital flexor muscles of each limb. To distribute the *Clostridium botulinum* toxin type A evenly among the deep digital flexor muscle, one injection was placed in the superficial part, two injections in the middle part and two in the deep part of the deep digital flexor muscle. Injections were performed under ultrasonographic guidance. EMG guidance was used to inject *Clostridium botulinum* toxin type A solution as close as possible to the endplate zone. The solution was injected through an EMG Myoject needle electrode (length 75 mm, diameter 0.71 mm, recording area 0.89 mm2; MedCat) using a monopolar needle (length 75 mm, diameter 0.46 mm, recording area 0.34 mm2; MedCat) with a portable EMG apparatus (Viking Quest EMG System®, Nicolet Biomedical, Viasys Health Care).

Forelimb angle alterations
SROM and CROM (**Fig. 1**) were measured using a standardised walk protocol on day 0 before *Clostridium botulinum* toxin type A injections and on days 3, 7 and 14 after *Clostridium botulinum* toxin type A injections using an inertial sensor system (ISS). After warm-up, this protocol consisted of the directions: (1) walk at hand in a straight line; (2) walk on the lunge in a clockwise circle; and (3) walk on the lunge in an anticlockwise circle; on (1) hard; and (2) soft surfaces, resulting in a walk protocol of six permutations. Four ISS sensors were applied using custom-made sleeves on a horse boot (metacarpus) or wrap (radius) and used to measure angular and linear acceleration and angular rotation throughout a complete stride. A stride was determined by carrying out auto-correlation on the z-axis gyro signal. From all different parts of the described protocol, the steadiest sections of at least ten strides in the middle of a recording were selected to ensure consistency of data. From this selection, the software calculated the typical stride, defined as being the stride with a minimal error from

the other selected strides. All data was analysed using the Pegasus Stride System® (European Technology for Business) gait analysis and joint angle software.

Balance index

Measurements using a dynamically calibrated pressure plate were recorded on day 0 before *Clostridium botulinum* toxin type A injections and on days 3, 7 and 14 after *Clostridium botulinum* toxin type A injections. The measurement system consisted of a pressure plate (Footscan 3D 1 m-system, RsScan International), which was dynamically calibrated using a force plate (Z4852C, Kistler) mounted on top of the pressure plate (Van Heel et al., 2004; Oosterlinck et al., 2010; Oomen et al., 2012). The measurement system was embedded in the middle of a runway and covered with a 5 mm rubber mat. With the aim to record five valid measurements, a minimum of seven runs were performed and, on average, 6.7 valid measurements (range 5-9) of each forehoof were recorded at all time points. A recording was accepted if at least one forehoof fully contacted the plate and the horse was looking forward and walking at a visually constant velocity. The velocity of the horse was measured using two pairs of photoelectric-sensors forming two gates perpendicular to the runway and connected to an electronic timing box. Dedicated software (Footscan Scientific Gait® and Kistler Bioware 4®) was used to divide hoof prints into a toe and a heel region at the level of the widest part of the hoof. The balance index of the Peak Vertical Force (PVF) was calculated using the following formula: (PVFtoe - PVFheel)/[0.5 * (PVFheel + PVFtoe)] * 100% (Oomen et al., 2012). A negative balance index indicates a higher maximal force in the heel region, whereas a positive balance index indicates a higher maximal force in the toe region (range -200 to 200).

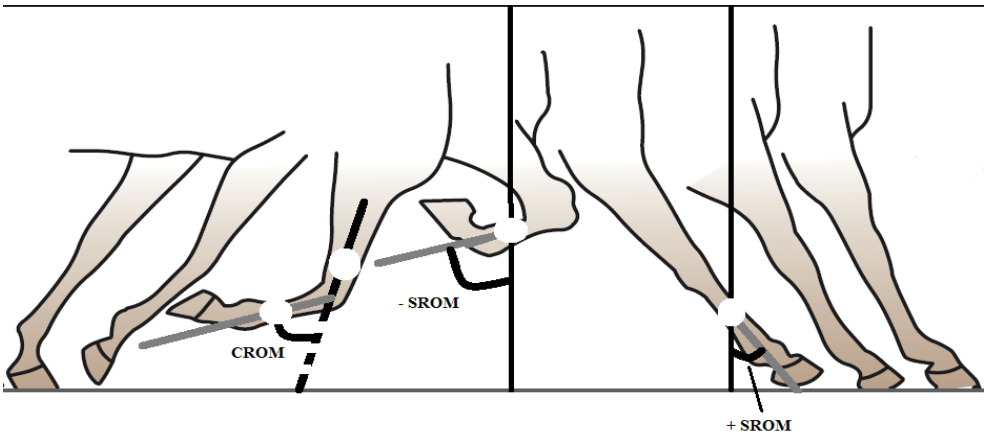

Fig. 1. *Range of motion of the carpal joint (CROM) of the forelimb and the sagittal range of motion of the metacarpus (SROM). White circle, sensor of the inertial sensor system; CROM, zero angle defined as radius and metacarpus being aligned, carpal flexion generates a positive angle; SROM, angle positive during limb protraction, negative during retraction (adapted from Back and Pille, 2013).*

Symmetry index

The symmetry index between the left and right limbs was calculated to determine if the horses loaded both forelimbs equally using the following formula: (PVFleft - PVFright)/[(0.5 * (PVFright + PVFleft)] * 100%. A negative symmetry index indicates a higher loading of the

right limb, whereas a positive symmetry index indicates a higher loading of the left limb (range -200 to 200).

Statistical analysis

Statistical analysis was performed using R-statistics (The R-Project). The outcome variables were the SROM and CROM, the balance index and the symmetry index. The outcome variables were analysed using a linear model with random effects, with 'day', 'limb' and the interaction between 'day' and 'limb' as explanatory variables. 'Limb' was not used as an explanatory variable for the analysis of the symmetry index, which measures the symmetry between the limbs. The variables 'direction' and 'surface type' were not used as explanatory variables for the analysis of the SROM and CROM. For all outcomes, 'horse' was used as a random effect to take the correlation between measurements into account. Akaike's information criterion (AIC) was used in a backward selection method to select the best model (Burnham and Anderson, 2004). The velocities of the horses recorded during pressure plate measurements of the left (treated) and right (control) limb were analysed using an analysis of variance (ANOVA) model. Since 'direction' and 'surface type' were not used as explanatory variables for the analysis of the SROM and CROM, standard error of the mean instead of standard deviation was chosen to show the variation of the ISS-data. QQ-plots were used to check the data for normality. P values <0.05 were considered to be significant.

Results

Forelimb angle

1. Range of motion of the carpal joint

The right (control) limb had a significantly larger CROM on day 3 compared to day 0 (+1.84°, 95% confidence interval, CI, 0.62-3.05°; P = 0.004). After day 3, the control limb showed no significant difference between time points. The left (treated) limb had a significantly smaller CROM compared to the control limb on day 0 (before *Clostridium botulinum* toxin type A injection; -2.75°, 95% CI -3.97 to -1.54°; P < 0.001). After day 3, the treated limb had a significantly higher CROM on day 7 (+3.21°, 95% CI 1.99-4.42°) and on day 14 (+2.74°, 95% CI 1.52-3.95°) compared to the control limb (P < 0.001; **Fig. 2a**). An example of the CROM angle-time diagram of the treated and control limbs on day 14 is shown in **Fig. 2b**.

2. Range of motion of the metacarpus

The right (control) limb showed no significant differences in SROM among days 0, 3, 7 and 14. The SROM of the left (treated) limb was significantly lower than that of the control limb on day 0 (before *Clostridium botulinum* toxin type A injections; -1.21°, 95% CI -2.30 to -0.13°; P = 0.03). SROM was significantly higher after *Clostridium botulinum* toxin type A injections in the treated limb than the control limb on days 3 (+1.12°, 95% CI 0.04-2.21°; P = 0.04) and 14 (+1.27°, 95% CI 0.18-2.35°; P = 0.02) (**Fig. 3a**). An example of the SROM angle-time diagram of the treated and control limbs on day 14 is shown in **Fig. 3b**.

Balance index

There was no significant difference in balance index between pre-treatment and post-treatment measurements, indicating that the toe-heel force distribution did not alter after *Clostridium botulinum* toxin type A injections. The balance index of the hoof of the left

(treated) limb was significantly higher at all measurements (mean + 18.13, 95% CI 12.17-24.08; P < 0.001) compared to the right (control) limb (**Fig. 4**).

Symmetry index

There were no significant differences in symmetry index among time points, indicating an absence of asymmetric limb loading (i.e. lameness) after *Clostridium botulinum* toxin type A injections (**Fig. 5**).

Velocity

There were no significant differences between the velocities recorded during measurements of the left (treated) and right (control) limbs before (day 0) and after *Clostridium botulinum* toxin type A injections (days 3, 7 and 14) (**Table 1**).

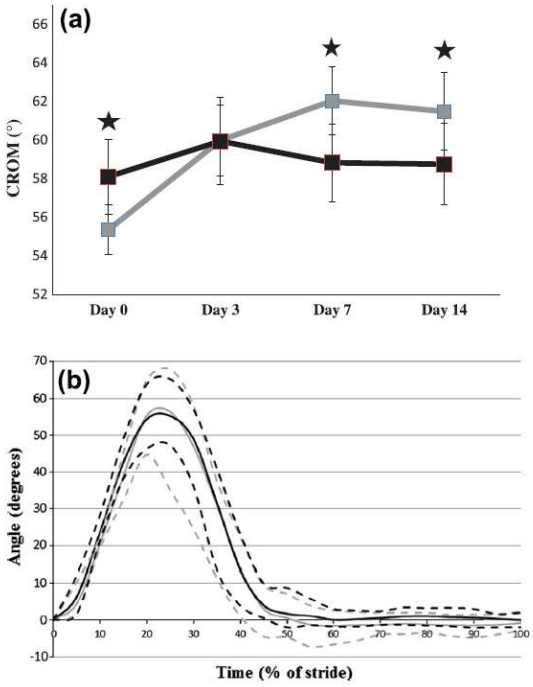

Fig. 2. *(a) Range of motion of the carpal joint (CROM) of the treated limb (grey line) and control limb (black line) before (day 0) and after (days 3, 7 and 14) injection of Clostridium botulinum toxin type A into the deep digital flexor muscle of horses. Error bars, standard error of the mean. *P < 0.05. (b) Mean angle-time diagram of the range of motion of the carpal joint (CROM) of the treated limb (grey line) and control limb (black line) at day 14 on a straight line on soft surface. Range of angle-time diagrams is indicated (treated limb, grey dotted lines; control limb, black dotted lines; number of horses, n = 7); range of motion is maximum minus minimum. The individual graphs were adapted so they all start at the same phase of the stride on the x-axis (t = 0%), namely when the carpus starts to flex again at the end of stance phase, determined from the moment the metacarpal and radial sensors were out of longitudinal alignment again; at that same time point the joint excursions were normalised to cross the y-axis at the same relative angle (angle = 0°). Note that the diagram is an example of one part of the protocol used in this study (straight line on soft surface).*

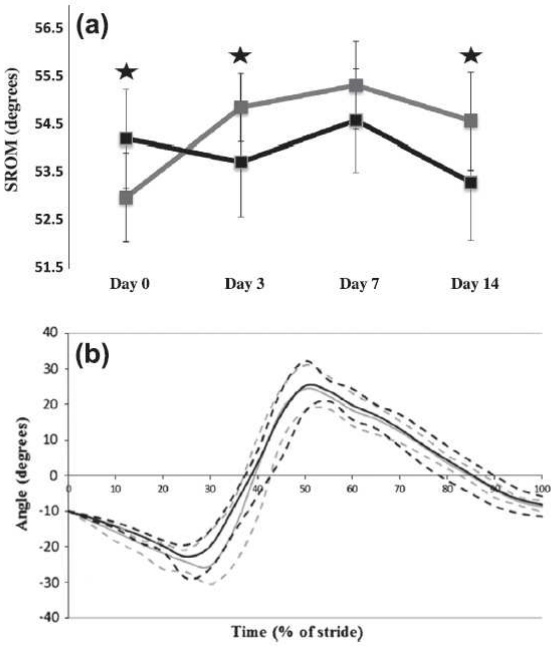

Fig. 3. *(a) Sagittal range of motion of the metacarpus (SROM) of the treated limb (grey line) and control limb (black line) before (day 0) and after (days 3, 7 and 14) injection of Clostridium botulinum toxin type A into the deep digital flexor muscle. Error bars, standard error of the mean. *P < 0.05. (b) Mean angle-time diagram of the sagittal range of motion of the metacarpus (SROM) of the treated limb (grey line) and control limb (black line) at day 14 on a straight line on soft surface. Range of angle-time diagrams is indicated (treated limb, grey dotted lines; control limb, black dotted lines; number of horses, n = 7); range of motion is maximum minus minimum. The individual graphs were adapted so they all start at the same phase of the stride on the x-axis (t = 0%), namely when the carpus starts to flex again at the end of stance phase, determined from the moment the metacarpal and radial sensors were out of longitudinal alignment again; at that same time point the joint excursions were normalised to cross the y-axis at the same relative angle (angle = 0°). Note that the diagram is an example of one part of the protocol used in this study (straight line on soft surface).*

Table 1. Velocity of the horses during pressure plate measurements.

Limb	Day 0	Day 3	Day 7	Day 14
Right (control)	0.88 ± 0.10	0.90 ± 0.09	0.90 ± 0.10	0.88 ± 0.08
Left (treated)	0.90 ± 0.10	0.87 ± 0.10	0.89 ± 0.11	0.87 ± 0.10

Mean ± standard deviation (SD) velocity (m/s) of the horses recorded during measurements of the right (control) and left (treated) limb using the dynamically calibrated pressure plate before (day 0) and after (days 3, 7 and 14) Clostridium botulinum toxin type A injections into the deep digital flexor muscle.

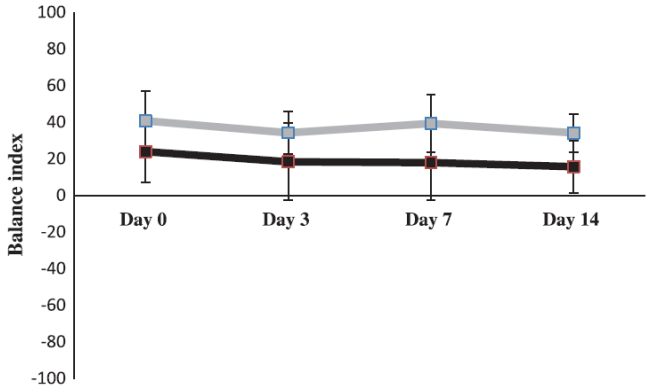

Fig. 4. *Balance index of the treated limb (grey line) and control limb (black line) before (day 0) and after (days 3, 7 and 14) injections of Clostridium botulinum toxin type A into the deep digital flexor muscle. The range of the balance index is -200 to 200. Error bars, standard deviation. *P < 0.05.*

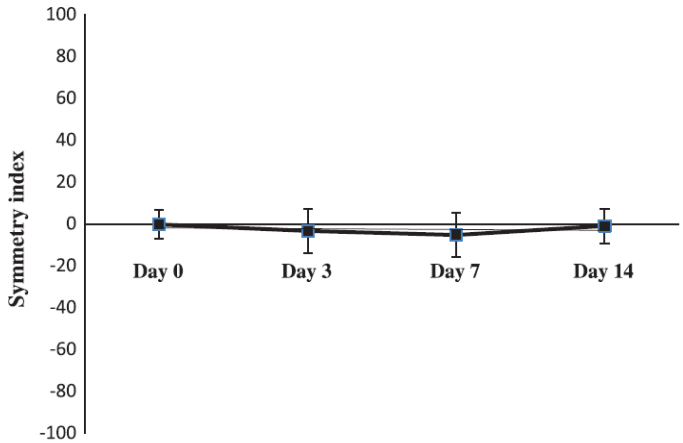

Fig. 5. *Symmetry index before (day 0) and after (days 3, 7 and 14) injection of Clostridium botulinum toxin type A into the deep digital flexor muscle. The range of the symmetry index is -200 to 200. Error bars, standard deviation.*

Discussion

After unilateral injections with *Clostridium botulinum* toxin type A into the deep digital flexor muscle, the SROM and CROM of the treated limb were significantly increased. No significant changes were detected in the balance index or in the symmetry index, indicating that no lameness was induced. This observation that intramuscular injection of *Clostridium botulinum* toxin type A into the deep digital flexor muscle did not induce lameness is consistent with our earlier study (Wijnberg et al., 2009). Since *Clostridium botulinum* toxin type A reduces the activity of the deep digital flexor muscle (Wijnberg et al., 2013), the increase in SROM and CROM is most likely to be a result of the reduced tone of this muscle.

Although the deep digital flexor is the largest of the carpal and digital flexor muscles of the horse (Zarucco et al., 2004), the contribution of the deep digital flexor to the total work performed during a locomotion cycle appears to be small (Wilson et al., 2001; Harrison et al., 2010). The spring like mechanism of muscle tendon units, loaded mainly by gravitational and inertial forces, creates an efficient and energysaving system of locomotion in horses that relies on conversion of kinetic energy to elastic energy and back again. The muscles mainly serve to dampen the springs (Wilson et al., 2001; Harrison et al., 2010). This may explain the relatively minor kinematic changes after *Clostridium botulinum* toxin type A injections.

The significant left-right differences in SROM and CROM on day 0 (before *Clostridium botulinum* toxin type A injections) is probably due to motor laterality (Hardeman et al., 2011), but may also be due to the effect of the handler. Our results show that both SROM and CROM increased most strongly from days 0 to 3 (**Figs. 2a and 3a**). This time frame is in agreement with the simultaneously sharply decreasing muscle activity over the first three days demonstrated by needle-EMG (Wijnberg et al., 2013). After day 3, SROM and CROM both increased until day 7, levelling off thereafter.

The left-right differences in balance index observed in this study may be due to several reasons, including (but not limited to) hoof and limb conformation, the effect of the handler being on the left side of the horses, or motor laterality of the horses in this study group (Van Heel et al., 2004; Oosterlinck et al., 2013).

For this study, we used a protocol that could be used to determine ROM in outdoor laminitic patients so as to include this in future clinical trials. Inertial sensor systems are a promising new technology in locomotion analysis in horses (Pfau et al., 2005; Olsen et al., 2012). Since individual variation (e.g. motor laterality, preference for surface type) may have unequally influenced the SROM and CROM during the six different parts of the ISS walk protocol used in our study, the variables 'direction' and 'surface type' were not used as explanatory variables.

Since the main aim of the present study was to assess whether a reduction in deep digital flexor muscle activity due to *Clostridium botulinum* toxin type A injection would lead to locomotor modifications, the experimental design was simplified and a limited two-dimensional analysis was performed. As a consequence of this limitation, we were not able to assess the effect of reduced deep digital flexor activity on the motion of the limb at walk or trot in detail. Another weakness of the study is a lack of randomisation of the side of the injected limb (left vs. right), which could have reduced the effect of motor laterality in the horses and the influence of the handler.

Conclusions

Injection of *Clostridium botulinum* toxin type A into the deep digital flexor muscle has an effect on the range of motion of the metacarpus in the sagittal plane and the range of motion of the carpal joint, probably through the reduction in muscle tone, without causing asymmetric limb-loading, i.e. lameness. This suggests that injections of *Clostridium botulinum* toxin type A into the deep digital flexor muscle can be safely used in the laminitic patient without aggravating lameness.

Acknowledgements

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Chapter IV

The use of electromyography interference pattern analysis to determine muscle force of the deep digital flexor muscle in healthy and laminitic horses

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Abstract

In equine laminitis, the deep digital flexor muscle appears to have increased muscle force, but evidence-based confirmation is lacking. The purpose of this study was to test if the deep digital flexor muscle of laminitic equines has an increased muscle force detectable by needle electromyography Interference Pattern Analysis (IPA). The control group included six Royal Dutch Sport horses, three Shetland ponies and one Welsh pony [10 healthy, sound adults weighing 411 ± 217 kg (mean ± SD)]. The laminitic group included three Royal Dutch Sport horses, one Friesian, one Haflinger horse, one Icelandic horse, one Welsh pony, one miniature Appaloosa and six Shetland ponies (14 adults, weight 310 ± 178 kg) with acute/chronic laminitis. The electromyography IPA measurements included firing rate, turns/second (T), amplitude/turn (M) and M/T ratio. Statistical analysis used a general linear model with outcomes transformed to geometric means. The firing rate of the total laminitic group was higher than the total control group. This difference was smaller for the ponies compared to the horses; in the horses, the geometric mean difference of the laminitic group was 1.73 [geometric 95% confidence interval (CI) 1.29-2.32], and in the ponies this value was 1.09 (geometric 95% CI 0.82-1.45). In human medicine, an increased firing rate is characteristic of increased muscle force. Thus, the increased firing rate of the deep digital flexor muscle in the context of laminitis suggests an elevated muscle force. However, this seems to be only a partial effect as in this study the unchanged turns/second and amplitude/turn failed to prove the recruitment of larger motor units with larger amplitude motor unit potentials in laminitic equids.

Introduction

Laminitis, which causes the lamellae of the hoof to become inflamed and weakened, has a severe impact on horse welfare. The traction of the deep digital flexor muscle–tendon unit on the distal phalanx might cause it to rotate because there is no opposition because of lamellar failure (Morrison, 2004). The traction of the deep digital flexor on the distal phalanx in a founder is thought to be very painful as the descended phalanx places pressure on the sole (Pollitt, 1995a). To reduce this traction on the distal phalanx, a tenotomy of the deep digital flexor can be performed (Cripps and Eustace, 1999; Carter and Renfroe, 2009; Eastman, 2010; Morrison, 2011). However, Morisson (2011) described the outcome of 245 cases of deep digital flexor tenotomy and found a 51% success rate. Among the successful cases, only 13% returned to some form of athletic soundness (Morrison, 2011). Some clinicians, researchers and horse owners have suggested that increased muscle tone or even contracture of the deep digital flexor muscle arises from laminitis. This symptom would be most obvious at a chronic stage of the disease when the pain induces contracture of the deep digital flexor muscle (Pollitt, 1995b; Parks, 2003). However, to our knowledge, this increased muscle tone has never been proved by evidence-based research.

Clostridium botulinum toxin type A has been used as a therapeutic agent in human medicine for several years to treat muscle over-activity, among other disorders. In addition, *Clostridium botulinum* toxin has been successfully used to reduce the muscle tone of several equine skeletal muscles (Adam-Castrillo et al., 2004; Wijnberg et al., 2009). *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle of healthy, sound horses significantly reduced muscle activity without inducing lameness (Hardeman et al., 2013; Wijnberg et al., 2013). The hypothesis is that laminitis weakens the lamellae due to inflammation and the tension of the deep digital flexor on the distal phalanx displaces it. Relieving the pull of the deep digital flexor might give results similar to a tenotomy, but the *Clostridium botulinum* toxin type A action would only last for three or four months and would not damage the deep digital flexor tendon (Ney and Joseph, 2007; Wijnberg et al., 2009). If this study supports the hypothesis that laminitis causes the deep digital flexor muscle to have increased muscle force, then treating equine laminitis with *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle could be substantiated.

Electromyography (EMG) has been successfully used in horses for over a decade (Wijnberg et al., 2003; Wijnberg et al., 2004; Westermann et al., 2007). However, the use of electromyography Interference Pattern Analysis (IPA) in the horse is nascent (Wijnberg et al., 2011; Jose-Cunilleras and Wijnberg, 2015). In human medicine, IPA has been an important diagnostic tool for several years (Fuglsang-Frederiksen, 2000; Finsterer, 2001). The interference pattern is composed of motor unit potentials representing the electric activity of the motor units, and it is based on the recruitment pattern of the potentials (Kimura, 2001). The interference pattern can be analysed subjectively or objectively by using quantitative computer-based methods like Turn/Amplitude Analyses (TAA) which measures turns/second (T) and amplitude/turn (M) and permits calculating the M/T ratio. Amplitude can be described as the mean voltage difference between turns, while a turn is a change in polarity occurring at each positive or negative peak (Sanders et al., 1996; Farrugia and Kennett, 2005). Several IPA parameters are influenced by muscle force (Sanders et al., 1996; Fuglsang-Frederiksen, 2000; Finsterer, 2001). If force increases, the first motor unit to be

activated is a small one with a low frequency. If force increases, the frequency of the small motor unit increases and once it hits a certain frequency, a larger motor unit will be activated (Sanders et al., 1996). With additional force enlargement, both motor units increase their firing rate and another motor unit becomes activated (Sanders et al., 1996). The activation of motor units is based upon a 'size principle' described by Henneman in 1965: the motor units that are recruited first are small and fatigue resistant (type I muscle fibre), while the motor units activated later are larger and can fatigue earlier (type II muscle fibre) (Henneman and Olson, 1965; Sanders et al., 1996; Kimura, 2001). Thus, the firing rate will increase as the force becomes greater. Moreover, if the force becomes greater, several mechanisms cause the interference pattern amplitude to increase. Specifically, there is a larger chance of recruiting a motor unit close to the electrode, and larger motor units with larger territories and muscle fibre diameter (type II muscle fibres) are recruited later when force increases. In addition, there is more summation of motor unit potentials (Sanders et al., 1996; Finsterer, 2001; Farrugia and Kennett, 2005). The number of turns increases as force grows because the number of motor unit discharges becomes higher. The number of turns increases until approximately 50% of maximum force and levels off thereafter due to summation of motor unit potentials (Christensen et al., 1984; Sanders et al., 1996).

The purpose of this study was to test if the deep digital flexor muscle shows an increased muscle force in horses with laminitis that is detectable by needle electromyography IPA.

Materials and Methods

Horses

The control group included six Royal Dutch Sport horses, three Shetland ponies and one Welsh pony [adults weighing 411 ± 217 kg (mean ± SD) and aged 10 ± 5 years]. All horses were found to be clinically healthy and sound upon orthopaedic examination. The study was approved by the Animal Welfare Committee, Utrecht University (approval number 2008.III.07.061 and 2013.III.01.012).

The laminitic group included three Royal Dutch Sport horses, one Friesian, one Haflinger horse, one Icelandic horse, one Welsh pony, one miniature Appaloosa and six Shetland ponies (adults weighing 310 ± 178 kg and aged 13 ± 6 years) with acute or chronic laminitis. All horses were privately owned, and owners provided written consent. To enable distinguishing the different severities of laminitis, all horses and ponies underwent a clinical examination that included orthopaedic examination before electromyography. Patients in this study had laminitis with some degree of characteristic lameness, and symptoms included strong digital pulses, toe relieving stance and weight-shifting; some cases were complicated by acute/chronic founder or sinker (Eustace, 2010). Patients were classified on a four-point scale as described by Obel (1948) and Dutton et al. (2009). Some horses or ponies of the laminitic group were receiving medication (different dosages, mostly based on severity of the laminitis as judged by the owner or owner's veterinarian): six were receiving nonsteroidal anti-inflammatory drugs (NSAIDs; meloxicam or fenylbutazone), three were receiving anticoagulants (salicylic acid-acetate), one of the three was also receiving acepromazin and tramadol.

Preparations for EMG recordings

In preparation for EMG recording, the caudal part of the left forelimb between the olecranon and the carpal joint was shaved and cleaned on each horse or pony. Measurements were performed at three different sites in the widest part of the deep digital flexor muscle belly. To determine the sites, the distance between the top of the olecranon and the os carpi accessorium was measured using a tape measure. Then, data from an earlier study on cadaveric limbs were used to determine the specific sites in the widest part of the muscle belly (**Table 1**). The skin and subcutis were anaesthetised at each with an injection of 10 mg of lidocaine hydrochloride with 5 µg of adrenaline (Alfacaine 2% + adrenaline®, Alfasan). Afterwards, the limb was disinfected using 70% alcohol. As previously mentioned, some laminitic patients were receiving NSAIDS or anticoagulants to treat laminitis. All control horses and all laminitic patients who were not already receiving NSAIDs, were injected with 1 mg/kg BW flunixin meglumine (Bedozane®, Eurovet Animal Health) intravenously. The NSAIDS and lidocaine were administered in order to allow for the deep intramuscular insertions. After EMG measurements, the forelimb was cooled using cold water for 10 min.

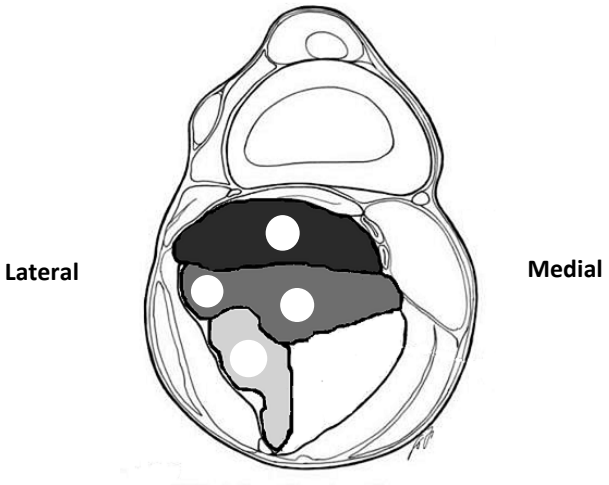

Fig. 1. *Different depths of the deep digital flexor muscle used for electromyography recordings (white points). Black shaded = deep part of deep digital flexor muscle. Dark-grey shaded = middle part of the deep digital flexor muscle. Light-grey shaded = superficial part of the deep digital flexor muscle. Medial, medial aspect of forelimb; Lateral, lateral aspect of forelimb. Adapted from Zarucco et al. (2004) with permission of American Journal of Veterinary Research.*

EMG recordings

A portable electromyography apparatus (Viking Quest EMG system®, Nicolet Biomedical, Viasys Healthcare) and a 23 G concentric needle (length 75 mm; diameter 0.60 mm; sampling area 0.068 mm^2; MedCat) was used to record electromyographic signals. The ground electrode consisted of a surgical pad attached to the horse with a girdle and connected to the preamplifier.

The electromyography needle was inserted and measurements were performed at three different depths of the deep digital flexor muscle as shown in **Fig. 1**. At the middle insertion

site, a fourth measurement in the middle part of the deep digital flexor muscle was performed. The needle was inserted in the groove between the Musculus flexor carpi ulnaris and the Musculus flexor digitorum superficialis.

For the analysis of the firing rate, the sensitivity was set at 200 µV. Measurements were performed at three sites in the deep digital flexor muscle at three or four different depths as already described. At all measurement points, three recordings with an epoch length of 100 ms were performed, and firing rates per epoch (Hz) were manually recorded. The threshold level was set beneath 100 µV (Fuglsang-Frederiksen, 2000) based on macroscopic evaluation of the contraction pattern to exclude the influence of background noise.

For IPA, sensitivity was again set at 200 µV. The epoch length was maintained at 100 ms. At each insertion site, 3 contractions of the deep digital flexor muscle were measured, resulting in the measurement of 30 contractions in total. The electromyography software calculated turns/second (T) (Hz), amplitude/turn (M) (µV) and M/T ratio (%) automatically (Finsterer 2001) .

Table 1. M. flexor digitorum profundus insertion sites based on data from an earlier study on cadaveric limbs.

	Insertion sites: distances from os carpi accessorium (cm)			
Distance top of the olecranon – os carpi accessorium (cm)	1	2	3	
20	7.3	8.5	9.8	
21	7.6	8.9	10.2	
22	8.0	9.4	10.7	
23	8.3	9.8	11.2	
24	8.7	10.2	11.7	
25	9.1	10.6	12.2	
26	9.4	11.1	12.7	
27	9.8	11.5	13.2	
28	10.2	11.9	13.7	
29	10.5	12.3	14.1	
30	10.9	12.8	14.6	
31	11.2	13.2	15.1	
32	11.6	13.6	15.6	
33	12.0	14.0	16.1	
34	12.3	14.5	16.6	
35	12.7	14.9	17.1	
36	13.1	15.3	17.6	
37	13.4	15.7	18.0	
38	13.8	16.2	18.5	
39	14.1	16.6	19.0	
40	14.5	17.0	19.5	
41	14.9	17.4	20.0	
42	15.2	17.9	20.5	
43	15.6	18.3	21.0	
44	16.0	18.7	21.5	
45	16.3	19.1	22.0	
46	16.7	19.6	22.4	
47	17.0	20.0	22.9	
48	17.4	20.4	23.4	
49	17.8	20.8	23.9	
50	18.1	21.3	24.4	

Statistical analysis
Data were analysed using R version 3.1.1. All outcome variables were transformed to natural logarithm, and normality was checked using QQ-plots. A general linear model was used with the outcome variables firing rate, turns/second, amplitude/turn and M/T ratio, respectively. The independent factors were group (control or laminitis), breed (horse or pony) and the interaction between group and breed. Likelihood ratio tests were used to select the best model. A 95% confidence interval instead of p-values was used to interpret the data (Cumming 2008). Outcomes were back-transformed to geometric means and geometric confidence intervals in order to describe the data more comprehensible.

Results

All patients showed clinical signs of laminitis, and Obel scores varied from grade 1 to grade 3. Duration of the laminitic episode varied from 0.5 to 312 weeks. Among the horses, the mean duration was 6 weeks, and among the ponies it was 75 weeks. The mean (± SD) firing rates of the control and laminitic group were 53 ± 11 and 72 ± 22 Hz, mean turns/second values were 112 ± 57 and 102 ± 40 Hz, mean amplitude/turn values were 284 ± 51 and 249 ± 33 µV and mean M/T ratios were 0.39 ± 0.17 and 0.41 ± 0.16 %, respectively.

According to the likelihood ratio tests, significant independent factors for firing rate were group, breed and the interaction between group and breed. For turns/second, breed was the significant independent factor. For amplitude/turn and M/T ratio, there were no significant differences between the means of the groups, resulting in a model with no significant independent factors.

Fig. 2 shows the boxplots of the EMG parameters subdivided into control or laminitic and horse or pony. The firing rate of the total laminitic group was higher than the total control group. This difference was smaller for the ponies compared to the horses; in the horses, the geometric mean difference of the laminitic group was 1.73 [geometric 95% confidence interval (CI) 1.29-2.32], and in the ponies this value was 1.09 (geometric 95% CI 0.82-1.45). The number of turns/second of the ponies was slightly lower compared to the horses (geometric mean difference 0.70, geometric 95% CI 0.50-0.99). Other differences were not significant.

Discussion

The objective of this study was to test if increased muscle force detectable by needle electromyography IPA occurs in the deep digital flexor muscle in association with equine laminitis. Our study data show an increased firing rate in the laminitic group compared to the control group. This increased firing rate is a characteristic of increased muscle force that has been demonstrated in human medicine (Sanders et al., 1996; Finsterer, 2001). Thus, the increased firing rate of the deep digital flexor muscle in the context of laminitis suggests an elevated muscle force. However, this seems to be the only evidence for altered muscle force as the turns/second and amplitude/turn did not change and thus provided no evidence for the recruitment of larger motor units with larger amplitude motor unit potentials. The discrepancy in firing rate between the control and laminitic groups of the horses and ponies could be caused by a difference in bodyweight or physique. In addition, the dissimilar

duration of laminitis (6 and 75 weeks mean duration in horses and ponies, respectively) might have influenced the muscle force of the deep digital flexor.

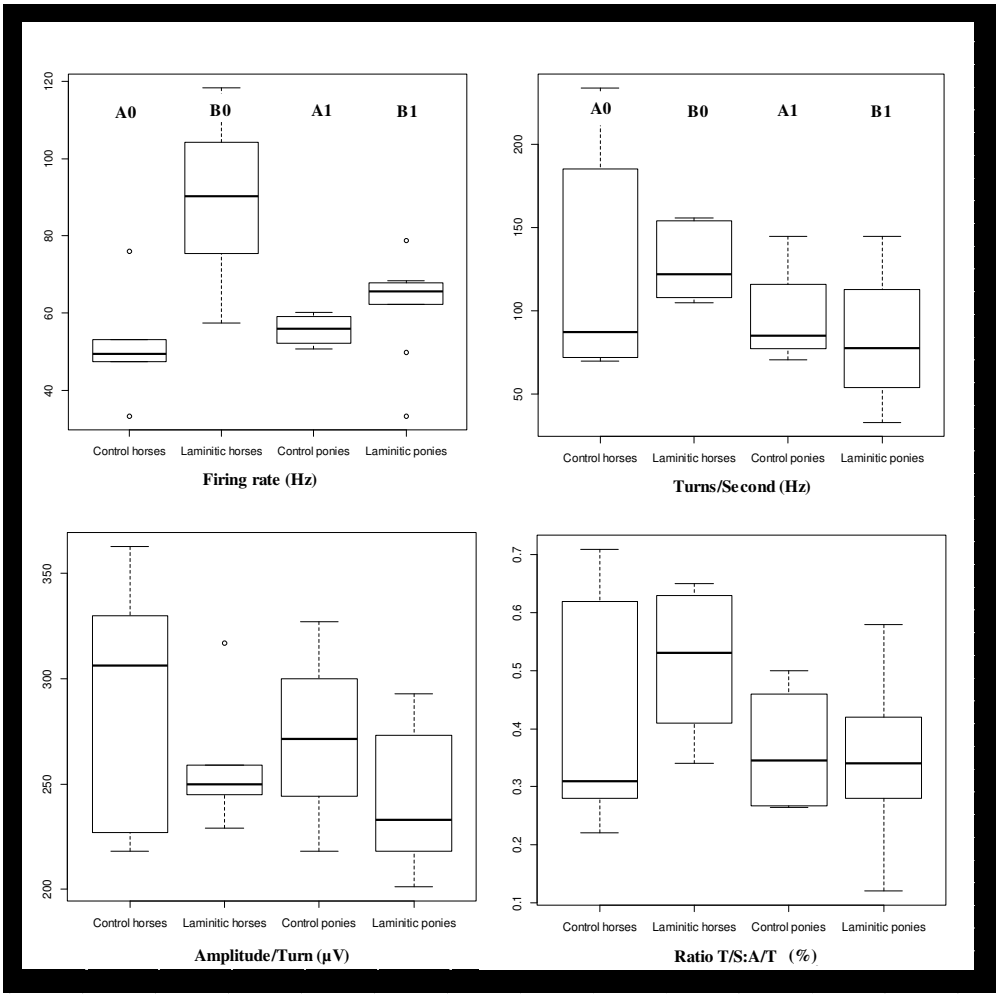

Fig. 2. *Boxplots of the electromyography interference pattern firing rate (Hz), turns/second (T) (Hz), amplitude/turn (M) (µV) and M/T ratio (%) of the deep digital flexor muscle of the horses and ponies of the control group (n = 10) and the laminitic group (n = 14). The firing rate of the laminitic group (B0 + B1) was higher compared to the control (A0 + A1). This difference was smaller for ponies (A1 + B1) compared to horses (A0 + B0). The number of turns/second of the ponies (A1 + B1) was slightly lower compared to the horses (A0 + B0). Other differences were not significant.*

As described by Finsterer (2001), muscle temperature influences the outcomes of electromyography IPA. However, in clinically healthy humans, the need for temperature measuring was unnecessary (Finsterer and Mamoli, 1996). Because none of our patients showed a body temperature outside the reference values upon clinical examination, the outcomes of our study can be compared without muscle temperature monitoring data. Next

to muscle temperature, the age of the subject might influence electromyography IPA outcomes (Fuglsang-Frederiksen, 2000; Finsterer, 2001). In human medicine, test results within an age range of 20-65 years were not age dependent (Haridasan et al., 1979; Sanders et al., 1996). Extrapolated from these results, only adult horses, ages ranging from 4 to 20 years, were included in our study. In our study, results of horses and ponies of all types of breeds were combined. Back et al. (2007) found a difference in load distribution of the front and hind limbs between Quarter horses and Warmbloods. If front limbs are loaded with a larger part of the bodyweight in a particular breed, it might result in more muscle force of the deep digital flexor, resulting in higher IPA values. However, the measurements by Back et al. (2007) were performed in trotting horses and extrapolation of these data to standing horses is questionable.

The IPA in horses has only been investigated twice for the purpose of comparing normative data of several skeletal muscles using among others IPA cloud analysis (Wijnberg et al., 2011; Jose-Cunilleras and Wijnberg, 2015). However, study designs differed from this study, impeding the possibility of comparing data. Although the use of interference pattern TAA has been shown to be useful in determining the effect of *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle (Wijnberg et al., 2013), the variance of these parameters in this study led to non-significant results. This high variance might have been influenced by the personality and the corresponding behaviour of the horses and ponies during the test. In human medicine, increased muscle tension appeared to be a uniform finding related to anxiety (Hoehn-Saric et al., 1997; Pluess et al., 2009). This notion might partly explain the high variance in our data, given that we were measuring horses and ponies of different ages and personalities.

In general, this study's results provide some evidence of an increased muscle force in the deep digital flexor associated with laminitis based on the increased firing rate. This finding might provide some support to the hypothesis that reducing the muscle tone of the deep digital flexor by using *Clostridium botulinum* toxin type A might be a successful supportive therapy in treating equine laminitis. As the number of turns/second and amplitude/turn of the control and laminitic groups were not dissimilar, larger motor units with larger amplitude motor unit potentials were apparently not recruited. Therefore, it can be concluded that there is only a partial increase in muscle force. A larger test group in a future study would narrow the 95% CI, thereby estimating the mean of the population more precisely. Furthermore, a larger group would enable subdividing the laminitic group based on, for example, duration and severity of the laminitis.

Acknowledgments

The authors would like to thank Hans Vernooij for statistical advice and Cornelia Mijs for her help collecting the data.

Chapter V

Determination of equine deep digital flexor muscle volume based on distances between anatomical landmarks

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Abstract

In equine medicine the use of *Clostridium botulinum* toxin is experimental. Dosages are determined from human treatment protocols and limited numbers of equine studies. Determination of target-muscle volume can be helpful to extrapolate human dosages. The aim of the study was to calculate a formula enabling the estimation of the deep digital flexor muscle volume based on distances between anatomical landmarks. Nineteen cadaveric limbs were collected and distance A (top of olecranon to Os carpi accessorium) and B (circumference of limb) were measured. Converting mathematical formulas, C was calculated: $\pi \times (((0.5B)/\pi)2) \times A$. Deep digital flexor muscle volume was determined by water displacement. Linear Regression Analysis was used to analyse data. The line best fitting the observed points was: $\text{Ln}(\text{volume}[\text{ml}]) = -1.89 + 0.98 \times \text{Ln}(\text{value C}[\text{cm}^3])$. Correlation was highest when natural logarithm was applied to both variables and was 0.97. The calculated formula enables estimating deep digital flexor muscle volume of a living horse. This estimated volume can be useful to apply human *Clostridium botulinum* toxin treatment-protocols.

Introduction

In case of laminitis, the structural failure of the suspensory apparatus of the distal phalanx enables the distal phalanx to displace within its hoof capsule (Van Eps et al., 2010; Pollitt, 2011). If the lamellae are weakened due to inflammation, it is hypothesized that the tension of the deep digital flexor on the distal phalanx results in the displacement of the distal phalanx. Also, the traction of the deep digital flexor on the distal phalanx in case of foundering is thought to be painful (Pollitt, 1995a). Morrison (2011) claims that a deep digital flexor tenotomy should be performed before the patient experiences advanced coffin bone disease which suggests that a rapid decrease of tension of the deep digital flexor might be beneficial.

Clostridium botulinum toxin type A (Botox®, Allergan Inc.) has been used in human medicine to alter muscle tone and treat different kinds of spasticity and disorders of muscle overactivity (Davis, 1993; Ney and Joseph, 2007). Recently, *Clostridium botulinum* toxin has been successfully used in equine medicine to reduce the activity of several skeletal muscles without side effects (Adam-Castrillo et al., 2004; Carter and Renfroe, 2009; Wijnberg et al., 2009). *Clostridium botulinum* toxin injections into the deep digital flexor muscle of healthy, sound horses have proven to significantly reduce the activity of the deep digital flexor muscle without inducing lameness (Hardeman et al., 2013; Wijnberg et al., 2013). The hypothesis as described in Hardeman et al. (2013) and Wijnberg et al. (2013), is that the diminished activity of the deep digital flexor muscle prevents the distal phalanx from displacing within its hoof capsule and gives pain relief by reducing its pull on the distal phalanx. Based on the formerly mentioned results of *Clostridium botulinum* toxin on the reduction of muscle activity and the described potential benefits of deep digital flexor tenotomy (Eastman et al., 1999; Morrison, 2011), the use of *Clostridium botulinum* toxin might have a place in the treatment of laminitis without the risks involved with tenotomy such as contracture of the deep digital flexor (Morrison, 2011).

In human medicine, *Clostridium botulinum* toxin treatment protocols and administered dosages differ widely. The recommended dosages in human clinical practice are suggested to be often personalized (Mancini et al., 2005) and differ between muscles and types of illness (Olver et al., 2010). Because the use of *Clostridium botulinum* toxin in equine medicine is still experimental, the rationale behind choosing dosages is not always clear other than based on human treatment-regimes. In earlier studies, the normal average volume of the target muscles was used to apply this knowledge of human *Clostridium botulinum* toxin dosages (Hardeman et al., 2013; Wijnberg et al., 2009, 2013). In these studies, the chosen *Clostridium botulinum* toxin dosage proved to result in a significantly reduced muscle activity. However, in real life various breeds and sizes of horses and ponies will suffer from laminitis. Until placebo-controlled dose–response studies using *Clostridium botulinum* toxin in horses are performed, target-muscle volume can be helpful in comparing human dosages per muscle. Therefore, the aim of the study was to calculate a formula to estimate the volume of the deep digital flexor muscle of different breeds and sizes of horses and ponies using distances between anatomical landmarks.

The deep digital flexor muscle consists of several muscle bellies and includes three heads: a humeral (HH), ulnar (UH) and radial head (RH) (Dyce et al., 2002). Volume of the equine

deep digital flexor muscle has been determined by anatomical studies (Zarucco et al., 2004) but also by Computed Tomography (CT) and Magnetic Resonance Images (MRI) (Zarucco et al., 2006). The radial (RH) and ulnar (UH) muscle-tendon units are small and their contribution to the total muscle volume of the deep digital flexor is minor: 4% and 13% respectively (Zarucco et al., 2004). Besides, the radial and ulnar muscle-tendon units are not easily accessible for *Clostridium botulinum* toxin injections. As a result of these characteristics, the radial and ulnar muscle-tendon units were not included in the present study.

Materials and Methods

To detect the volume of the deep digital flexor muscle, 19 cadaveric forelimbs were collected from eight Royal Dutch Sport horses, two Friesians, four Shetland ponies, two Welsh ponies, one Irish Tinker, one Icelandic horse and one Haflinger horse. All animals were euthanized for reasons unrelated to this study. Exact data of the sexes and ages of the used horses and ponies are not available.

All limbs were collected as soon as possible after euthanasia. First, the distance between the top of the olecranon and the Os carpi accessorium was measured using a tape measure (Value A) (**Fig. 1**). Second, the circumference of the limb just above the chestnut was measured (Value B). Third, the deep digital flexor muscle was dissected free from fascia and tendons were removed. The RH and UH muscle-tendon units were removed. The volume of the blotted muscle (HH) was determined by water displacement (Zarucco et al., 2004) using a measuring cup.

The deep digital flexor muscle has a cylinder-like shape. The content and the circumference of a cylinder can be calculated using the formulas $\pi \times$ squared radius $\times$ height and $2 \times \pi \times$ radius respectively. Thus, Values A and B were used in a mathematical formula to best estimate the volume of the deep digital flexor musle: $\pi \times (((0.5B)/\pi)2) \times A$. The outcome of this formula was called Value C. Value C was compared to the true deep digital flexor muscle volume to calculate a formula to determine deep digital flexor muscle volume in the individual living animal based on Value C.

The formula was calculated using R-statistics (The R-project) Linear Regression Analysis with Ln (value C) as a dependent variable and Ln (volume) as an independent variable. Adding the factor Breed (horse or pony) was checked for significance. The Royal Dutch Sport horses and Friesians were considered horses, the others were considered ponies. All parameters were transformed to natural logarithm and normality was checked using QQ-plots. Values are given ± SD.

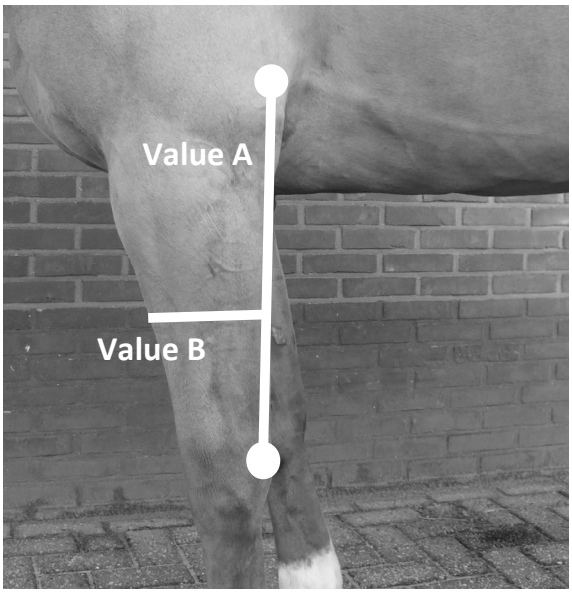

Fig. 1. *Distance between the top of the olecranon and the Os carpi accessorium (Value A) and the circumference of the limb just above the chestnut (Value B) used to estimate the volume of the deep digital flexor muscle.*

Results

The mean of Value A and Value B was 38.4 cm (±7.34 cm) and 29.4 (±4.4 cm) respectively. The mean of Value C was 2824.1 cm^3 (±1147.2 cm^3). The average deep digital flexor muscle volume of all horses and ponies was 376.3 ml (±155.3 ml), of the horses was 480.0 ml (±87.9 ml) and of the ponies was 261.1 ml (±131.1 ml). All determined C values and corresponding muscle volumes are plotted in **Fig. 2**. The line best fitting the observed points was: Ln(volume [ml]) = −1.89 + 0.98 × Ln(value C [cm^3]). Correlation was highest when natural logarithm was applied to both variables and was 0.97. A factor Breed (horse or pony) was added to the statistical model but was not significant (P = 0.49) implying that the relation between Ln (value C) and Ln (volume) is the same for both horses and ponies. The formula can be mathematically converted to: muscle volume (ml) = $e^{-1.89 + 0.98 \times \ln C.}$

Discussion

The determined deep digital flexor muscle volumes of the horses in our study are consistent with earlier studies concerning the determination of volume of the equine deep digital flexor muscle (Brown et al., 2003; Zarucco et al., 2004, 2006). However, the average muscle volume in our study is somewhat lower. This might be due to the variable breeds of horses used in the present study (Royal Dutch Sport horses and Friesians) while other studies used only Thoroughbreds or Thoroughbred-cross indicating that individual deep digital flexor muscle volume is highly variable (Brown et al., 2003; Zarucco et al., 2004, 2006). The level of training of the horses might also have influenced the deep digital flexor muscle volume.

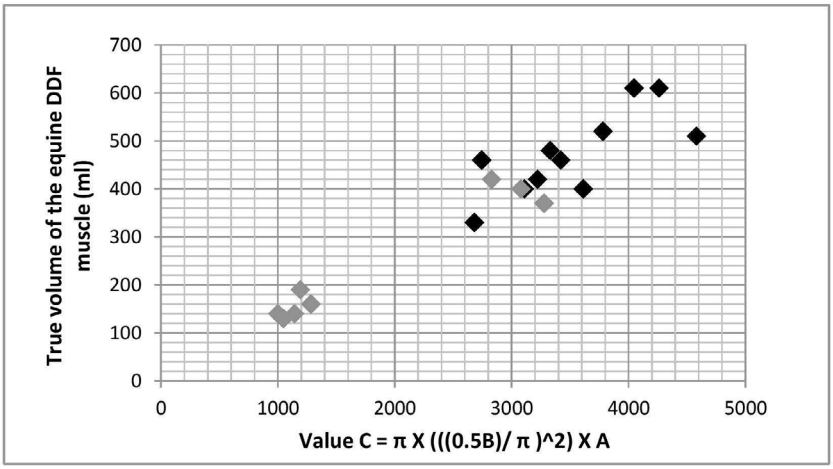

Fig. 2. *Black (horses) and grey (ponies) points representing the determined Values C (cm^3) and corresponding true volume (ml) of the deep digital flexor muscle of the forelimbs used in the present study (total number = 19).*

It should be noted that predicting the muscle volume based on the formula will never be as accurate as MRI or CT. In this study, the formula for volume calculation of a cylinder with perpendicular sides is used and although the deep digital flexor muscle has a cylinder-like shape it is not exact. Also, the chosen landmarks do not exactly match the origin and insertion of the deep digital flexor muscle. Next to this, to the authors' knowledge the size of the chestnut varies and the exact location of the chestnut to any other anatomical landmark is unknown. However, the formula enables estimating muscle volume quite accurately in the living animal.

Data of human muscle volumes are abundantly available (Elliott et al., 1997; Albracht et al., 2008; Seynnes et al., 2008). The volumes of the deep digital flexor muscle of the laminitic patient calculated by the presented formula can be compared with the volume of human muscles. Thereby, dosages for the individual patient can be optimized which benefit the patient's safety and reduce costs. However, it should be noted that the target-muscle volume is only an aid in calculating the required *Clostridium botulinum* toxin dosage. Information on i.a. pharmacokinetics and dose-response curves of *Clostridium botulinum* toxin in the horse is not available. Also, in human medicine chosen dosages differ between i.a. muscles and types of illness (Olver et al., 2010). However, as long as more detailed information on the use of this drug in equine medicine is lacking, the target-muscle volume provides a useful tool in case of a future clinical-trial. If in the future *Clostridium botulinum* toxin becomes an often used therapeutic agent in equine medicine, further research on dosages and treatment regimes would be valuable.

Acknowledgements

The authors would like to thank Hans Vernooij and Jan van den Broek for statistical advice.

Chapter VI

Evaluation protocol in two acute laminitic horses treated with *Clostridium botulinum* toxin injections into the deep digital flexor muscle

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Submitted

Abstract

An Irish Tinker horse and an Oldenburger horse suffering from acute laminitis were treated with *Clostridium botulinum* toxin injections into the deep digital flexor muscle. An objective evaluation protocol including cortisol concentrations in blood and saliva was developed and applied to test the outcome. The evaluation protocol consisted of pain-status related parameters, local inflammation-indicators, locomotion assessment and radiographs. Cortisol concentrations were also determined in a control group (n=10). Both laminitic horses showed an improvement from Day 0-7 and radiographs showed no distal phalanx displacement. Mean ± SD cortisol concentrations in blood and saliva of the control group were 88 ± 20 and 12 ± 12 nmol/L, respectively. Measuring cortisol concentrations particularly in saliva seemed to be an useful and accurate part of this evaluation protocol. The current uniform evaluation protocol enables judging treatment effects more objectively but recommendations can be made to improve the protocol used in the present report.

Introduction

Carter and Renfroe (2009) investigated the use of *Clostridium botulinum* toxin injections into the deep digital flexor muscle in case of laminitis. Their study included laminitic patients in various stages and subjected to different medication protocols. *Clostridium botulinum* toxin injections into the deep digital flexor muscle of healthy, sound horses have proven to significantly reduce muscle activity without inducing lameness (Hardeman et al., 2013; Wijnberg et al., 2013). The hypothesis is that, in case of laminitis, the lamellae are weakened due to inflammation and the tension of the deep digital flexor on the distal phalanx results in displacement of the latter. A reduced deep digital flexor muscle tone caused by *Clostridium botulinum* toxin injections might help in reducing the pull of the deep digital flexor on the distal phalanx. This release of tension might be able to prevent the distal phalanx from displacing within its hoof capsule. In addition, the reduced tension potentially provides pain relief. Morrison (2011) claims that a deep digital flexor tenotomy should be performed before the patient experiences advanced distal phalanx disease which suggests that a rapid decrease of tension of the deep digital flexor might be beneficial.

To be able to objectively evaluate the effects of treatment, a protocol was developed and the practical applicability was evaluated. The purpose of this protocol was to objectively measure the effects of treatment on the clinical progress, welfare and locomotion of the acute laminitic patient and to monitor distal phalanx displacement. The protocol included the determination of cortisol concentrations in blood and saliva. Cortisol concentrations were also determined in a control group (n=10).

Case history, clinical findings and diagnosis

Horse 1 (H1) was a castrated male Irish Tinker horse (age 8 years, weighing 534 kg). Six days after colic-surgery, acute laminitis developed. Horse 2 (H2) was a castrated male Oldenburger horse (age 12 years, weighing 455 kg). After several weeks suffering from chronic weight-loss for unknown reasons, acute laminitis developed. Diagnosis of laminitis was made and treatment was started within 48 hours after onset of clinical symptoms. Both horses showed no signs of distal phalanx displacement on lateromedial views. Both horses were privately owned and owner consent was obtained before treatment was started.
The monitoring tests were performed on day 0 before *Clostridium botulinum* toxin injections and on day 3, 7 and 14 thereafter. The following parameters were determined:

1. Pain-status related parameters:

- Determination of cortisol concentrations in blood and saliva. Sample collection and processing as in the control group, samples taken between 11 AM and 1 PM.
- Two composite pain scores (CPS) (Annex 1 and 2): 1) CPS described by Dutton et al. (2009) including components of the Obel-scoring system (Obel, 1948) and 2) parts of the CPS as described by Bussières et al. (2008)
- Manually counted weight shifting frequency (WSF) during 5-7 minutes.
- Clinical parameters (i.a. breathing frequency, pulse frequency and rectal temperature).

2. Indicators of local inflammation:

- Clinical parameters (i.a. breathing frequency, pulse frequency and rectal temperature).
- Scoring of digital pulse amplitude of the fore hoofs (1=very weak/undetectable, 2=weak, 3=moderate, 4=strong/lightly bounding, 5=very strong/bounding).

3. Locomotion parameters:

- Obel-scoring as described above.
- Gait kinematical analysis: stride duration, range of motion of the carpal joint (CROM) and sagittal range of motion of the metacarpus (SROM) as described in an earlier study (Hardeman et al., 2013). Only walk at the hand on a straight line on hard surface was performed as a more extensive protocol was considered unethical.

4. Radiographic position of the distal phalanx on lateromedial views of the foot on day 7.

Cortisol concentrations were also determined in ten healthy Royal Dutch Sport horses (one gelding and nine mares, mean ± SD age 12 ± 4 years). All horses were part of the teaching herd of Utrecht University (Animal Welfare Committee of Utrecht University approval number 2008.III.07.061). Blood and saliva samples were collected between 11 AM and 1 PM on the same day to minimalize the effect of the circadian rhythm (Van Der Kolk et al., 2001)

Treatment

Bilateral *Clostridium botulinum* toxin (Botox®, Allergan Inc.) injections into the deep digital flexor muscle were performed as described in Wijnberg et al. (2013) In short, *Clostridium botulinum* toxin (200 IU per deep digital flexor muscle) was injected into various sites of the deep digital flexor muscle. Electromyographic guidance was not available in the practice of both cases. Advised additional medication consisted of 1) NSAIDs: 1^{st} day meloxicam (Metacam®, Boehringer Ingelheim) 0.6 mg/kg bwt, IV, q 24 h, 2^{nd}-5^{th} day phenylbutazone (Pro-dynam®, Dechra) 4.4 mg/kg bwt, q 12 h, afterwards 2.2 mg/kg bwt, PO, q 12 h, 2) salicylic-acid-acetate (Carbasalaatcalcium®, Pharmachemie) 1^{st} day 12 mg/kg bwt, PO, q 24 h, afterwards 6 mg/kg bwt, PO, q 24 h and 3) acepromazin (Tranquigel®, AST Farma) 0.1 mg/kg bwt, PO, q 8 h. Management and dietary strategies were the following: the front hooves had to be cooled using cold water or ice q 12 h. Deep beddings of straw/shavings were advised to improve the patient's comfort. Diet had to consist of hay supplemented with vitamins and minerals.

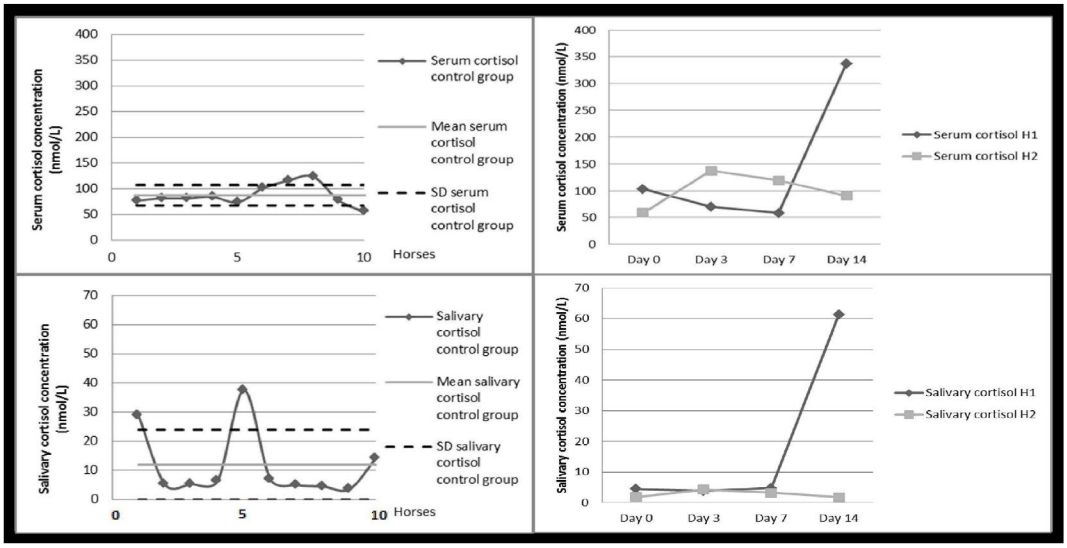

Fig. 1. *Cortisol concentrations in serum and saliva including mean and SD of the control group (n=10, no timeframe) and of H1 and H2 on Day 0 before Clostridium botulinum toxin injections and on day 3, 7 and 14 after injections.*

Outcome

H1 showed a warm, not painful swelling (approximately 10x5 cm) on the injection site on the right limb for 7 days, whereas H2 showed no side effects. H1 showed an overall improvement from day 0 until day 7 based on a decreased Obel-score, breathing frequency, WSF, blood cortisol, CPS1, digital pulse amplitude and an increased CROM and SROM (**Fig. 1, 2 and 3**). H2 showed a comparable improvement in the same period although the cortisol concentrations in blood and saliva increased from 59 to 137 nmol/L and 2 to 4 nmol/L, respectively, in the first three days, decreasing thereafter. Radiographs of both horses on day 7 showed no displacement of the distal phalanx as judged by the attending equine veterinarian.

From day 7 to day 14, H1 showed an increased pulse frequency and a sharp rise in blood and salivary cortisol concentrations as shown in **Fig. 1 and 3**. This deterioration appeared to be caused by severe duodenal ulcers of which he died spontaneously about two weeks after our last control on day 14. The digital pulse amplitude and cortisol concentrations of H2 further reduced between day 7-14, CROM simultaneously increased. The increased Obel-score, pulse frequency, breathing frequency, CPS1 and CPS 2 on day 14 were presumably the result of a hind limb laminitis which H2 developed after day 7. This might also have negatively influenced the SROM. Stride duration increased in H1 but decreased in H2. The increase in CROM and SROM of both horses is in line with earlier research (Hardeman et al., 2013).

Cortisol concentrations in blood and saliva of the control group were (mean ± SD) 88±20 and 12±12 nmol/L, respectively. Cortisol concentrations in blood and saliva of the control group and the two laminitic horses are shown in **Fig. 1**.

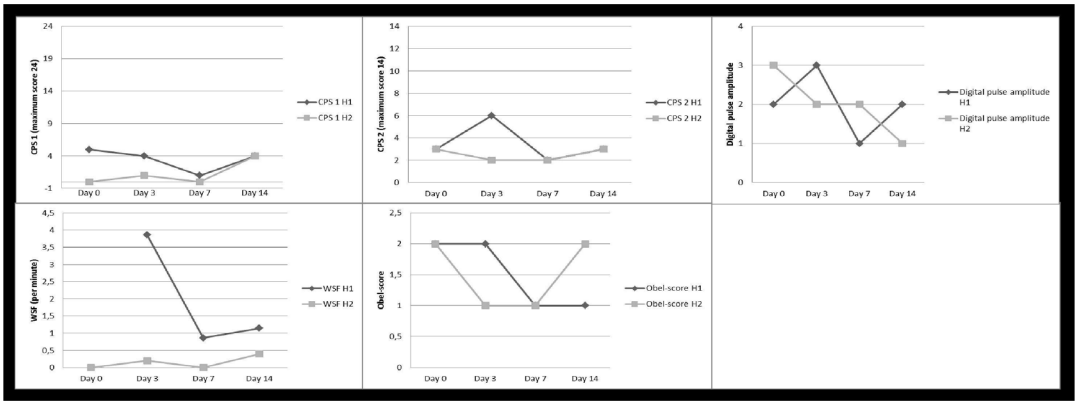

Fig. 2. *Outcomes of CPS 1 and 2 (see Annex), weight shifting frequency (WSF) per minute, Obel-score and digital pulse amplitude of the fore hoofs (1=very weak/undetectable, 2=weak, 3=moderate, 4=strong/lightly bounding, 5=very strong/bounding) of H1 and H2 on day 0 before Clostridium botulinum toxin injections and on day 3, 7 and 14 after injections.*

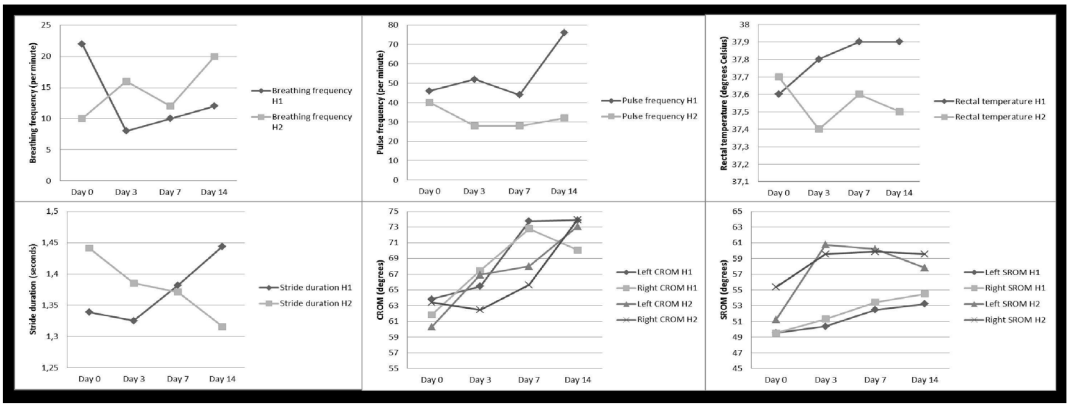

Fig. 3. *Breathing frequency, pulse frequency, rectal temperature, stride duration, CROM and SROM of H1 and H2 on day 0 before Clostridium botulinum toxin injections and on day 3, 7 and 14 after injections.*

Discussion

The effect of *Clostridium botulinum* toxin starts directly after injecting and maximum-change occurs before day 7 (Wijnberg et al., 2009; Wijnberg et al., 2013). Therefore, the initiation of effects can be expected within this timeframe. Although the pulse frequency of H1 showed an obvious increase after day 7 (multiplication factor 1.7) probably caused by the development of severe duodenal ulcers, the increase in blood and salivary cortisol concentrations (multiplication factor 5.8 and 12.7, respectively) was much more explicit. As shown in **Fig. 1**, the blood and salivary cortisol concentrations of H1 on day 14 were also clearly elevated compared to the control group. As described earlier, the cortisol concentrations in both blood and saliva of H2 increased from day 0-3 and decreased

thereafter. This decrease in cortisol concentrations after day 3 could be the result of an improvement of the state of laminitis probably caused by the *Clostridium botulinum* toxin treatment and additional medication and management. This would be in line with the earlier discussed timeframe of *Clostridium botulinum* toxin effects. As the correlation between serum and salivary cortisol concentrations proved to be good (Van Der Kolk et al., 2001; Bohák et al., 2013), it might be warranted to determine cortisol concentrations in saliva only in a future study, because the procedure is less invasive and measures the bioactive free cortisol.

Recommendations for the objective evaluation protocol after this trial study includes the following: pain scoring and WSF determination is not time-consuming and is therefore recommended to be performed daily in order to enhance sensitivity. The assessment of digital pulse amplitude is subjective and is suggested to vary between different breeds (Dyson, 2011a) but to the authors' knowledge a more objective method is not available. Obel-scoring in the present protocol was based on walking only (Dutton et al., 2009). Thus, sound walking horses were scored Obel-score 1. A more detailed grading-scale might therefore be valuable. Gait kinematical analysis equipment proved to be easy to use as all components were transportable. Counting the number of strides per day using pedometers might give information about the willingness to move and is therefore recommended to be included in a future study. Less feasible options would be adding force-plate data including load distribution profile (Taylor et al., 2002) and digital venography to determine digital pulse amplitude more objectively (Redden, 2001) but this is difficult in a clinical setting when horses cannot be transported.

The improvement of the two laminitic horses treated with *Clostridium botulinum* toxin injections is in line with the earlier study of Carter and Renfroe (2009). Unfortunately, only two patients met our inclusion criteria in which the limiting factor was the absence of rotation of the distal phalanx at the beginning of the study and this low number prevents evidence-based conclusions. A double blinded study should further differentiate between applied therapies.

The developed evaluation protocol enables judging treatment effects more objectively. Furthermore, multicentre uniformity would lead to the possibility to share data on research in laminitic patients and would contribute to the improvement of horses' welfare. Strictly following protocols on managing and feeding remains difficult, especially when dealing with privately-owned animals. However, objective monitoring remains essential if we want to progress in evidence based veterinary medicine.

Annex 1

CPS as described by Dutton et al. (2009)

Dynamic score: Obel laminitis pain scale	
Criteria	**Score**
Frequent shifting of weight between the feet with no discernible lameness at the walk	1
Does not resist having a foreleg lifted, is not reluctant to walk, but does show lameness at the walk	2
Resists having a foreleg lifted and is reluctant to walk	3
Walks only if forced	4

Static score: Modified from Glasgow composite scale	
Criteria	**Score**
No pain or distress: normal behaviour	1
Mild pain: irritable, restless, decreased appetite	2
Mild pain: 2 plus resists handling	3
Mild-moderate pain: 3 plus standing in back of stall or with back to stall door	4
Moderate pain: 4 plus camped-out legs, increased digital pulses	5
Moderate-severe pain: 5 plus frequent recumbency, HR >44 beats/min, and/or RR >24 breaths/min	6
Moderate-severe pain: 6 plus sweating, muscle fasciculation, head-tossing	7
Severe pain: 7 plus unwilling to move.	8
Severe-extreme pain: 8 plus nonweightbearing when standing	9
Extreme pain: 9 or entirely recumbent, bordering on agonal	10

Total maximum score	14

Annex 2

Used parts of CPS as described by Bussieres et al. (2008)

Physiologic data	**Criteria**	**Score**
1 Heart rate	24-44 beats/min	0
	45-52 beats/min	1
	53-60 beats/min	2
	>60 beats/min	3
2 Respiratory rate	8-13 breaths/min	0
	14-16 breaths/min	1
	17-18 breaths/min	2
	>18 breaths/min	3
3 Rectal temperature	36.9-38.5 °C	0
	36.4-36.8 or 38.6-39.0 °C	1
	35.9-36.3 or 39.1-39.5 °C	2
	35.4-35.8 or 39.6-40.0 °C	3
Response to treatment	**Criteria**	**Score**
4 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
Behaviour	**Criteria**	**Score**
5 Appearance	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
6 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
7 Posture	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
8 Appetite	Eats hay readily	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
Total CPS		24

Chapter VII

Summarizing discussion

Introduction

Laminitis has a severe impact on horse welfare. As described in **Chapter I**, this disease has been investigated by many researchers but unfortunately, the ultimate therapy has not been elucidated yet. In preliminary studies, *Clostridium botulinum* toxin has proven to be effective and safe in equine medicine (Adam-Castrillo et al., 2004; DePuy et al., 2007; Carter and Renfroe, 2009; Wijnberg et al., 2009; Gutierrez-Nibeyro et al., 2014). If in case of laminitis *Clostridium botulinum* toxin injections would be able to decrease the muscle tone of the deep digital flexor muscle, it might help in reducing the pull of the deep digital flexor on the distal phalanx. This release of tension might then be able to prevent the distal phalanx from displacing within its hoof capsule. Furthermore, the reduced pull on the distal phalanx and the inflamed lamellae might give substantial pain relief and hence result in an increase in comfort for the patient suffering from laminitis. With this background, the main hypothesis of this thesis was that the use of *Clostridium botulinum* toxin injections into the deep digital flexor muscle would be a successful supportive therapy in case of equine laminitis.

First, the effectiveness and safety of *Clostridium botulinum* toxin injections into the deep digital flexor muscle had to be tested in the healthy horse. Obviously, if the toxin would not significantly reduce the muscle tone or induce severe side-effects, it would strongly impede the clinical application of the agent. Second, the suggested increased muscle tone or contraction of the deep digital flexor in case of laminitis (Pollitt, 1995b; Parks, 2003) was investigated, as the outcome could further substantiate the hypothesis. Third, although the dosage used in the first studies proved effective, the horses used were all Royal Dutch Sport horses and in real life various breeds and sizes of horses and ponies will suffer from laminitis. Based on target-muscle volume, an attempt was made to calculate a formula that would allow for the individualization of dosages in case of future clinical trials. Last, the hypothesis of the effectiveness of *Clostridium botulinum* toxin in the acute laminitic horse was tested.

Clostridium botulinum toxin injections in the healthy horse

Seven healthy, sound Royal Dutch Sport horses were unilaterally injected with *Clostridium botulinum* toxin into the deep digital flexor muscle. Using electromyography Motor Unit Action Potential (MUAP) analysis and Interference Pattern Analysis (IPA), the muscle tone of the deep digital flexor was determined. All MUAP variables (amplitude, duration, phases and turns) and IPA variables (amplitude/turn and turns/second) were significantly reduced after multiple injections with a total of 200 IU of *Clostridium botulinum* toxin (**Chapter II**). The strongest effect occurred within three days after *Clostridium botulinum* toxin injections, levelling off thereafter. This implies that the muscle tone of the deep digital flexor will be reduced quickly after injections. Although in this study the last tests were performed on Day 14, from earlier equine and human studies it is known that the effect of *Clostridium botulinum* toxin lasts for at least 12 weeks (Ney and Joseph, 2007; Wijnberg et al., 2009). In case of a laminitic horse/pony, this timeframe would enable the owner, veterinarian and farrier to optimize management and treatment regimes in order to stimulate healing and prevent new laminitic flare-ups.

Simultaneously with the study described in Chapter II, the effect of the unilaterally reduced muscle tone of the deep digital flexor on the sagittal range of motion of the metacarpus

(SROM) and carpus (CROM), and the force distribution underneath the hooves (balance index and symmetry index) was tested (**Chapter III**). The balance index and symmetry index showed no significant changes, indicating that no lameness was induced, but the SROM and CROM increased. These parameters increased most strongly from Day 0 to Day 3, which is in line with the timeframe described in Chapter II, indicating that this effect was caused by the quick reduction in muscle tone of the deep digital flexor**.** From these studies, it could be concluded that *Clostridium botulinum* toxin can be safely used to significantly reduce the muscle tone of the deep digital flexor without causing severe side effects or inducing lameness.

The muscle force of the deep digital flexor

From human medicine it is known that several IPA parameters are influenced by muscle force: the firing rate, amplitude and number of turns increase with increasing force (Sanders et al., 1996; Finsterer, 2001). It has been suggested that laminitis may result in increased muscle tone or even contracture of the deep digital flexor muscle, which would be pain-induced and most obvious at a chronic stage of the disease (Pollitt, 1995b; Parks, 2003). The study described in **Chapter IV** using electromyography IPA indeed provided some evidence of an increased muscle force of the deep digital flexor in laminitic horses, as it showed an increased firing rate. However, there seems to be only a partial effect as the number of turns/second and the amplitude/turn were not dissimilar, indicating that larger motor units with larger-amplitude motor unit potentials were not recruited. Therefore, although the finding of an increased firing rate still provides some support for the claim that reducing the muscle tone of the deep digital flexor by using *Clostridium botulinum* toxin might be a successful supportive therapy in equine laminitis, the suggested increased muscle tone or contraction of the deep digital flexor muscle in laminitic horses, that would further substantiate such a claim, could only partially be confirmed. A larger laminitic group in a future study would narrow the 95% confidence interval and lead to a more precise estimation of the mean of the population, thereby enabling the subdivision of the laminitic horses/ponies based on severity and/or duration of the laminitis episode and making a more differentiated approach possible.

Electromyography IPA has been successfully used in human medicine for several years for making the distinction between several muscle and nerve diseases (Sanders et al., 1996; Fuglsang-Frederiksen, 2000; Finsterer, 2001), but this technique had not been used before in equine medicine to determine muscle force. Interestingly, there was a subjective clinical impression that the personality and corresponding behaviour of the horses seemed to influence muscle force, which is in line with human literature (Pluess et al., 2009). In future studies using this technique, this aspect can be taken into account.

Refining Clostridium botulinum toxin dosage

The chosen dosage in the earlier studies (Chapters II and III) proved to result in a significantly reduced muscle activity in Royal Dutch Sport horses, but in real life various breeds and sizes of horses and ponies will suffer from laminitis. Means to estimate the target-muscle volume correctly can therefore be helpful to provide the best possible basis for the extrapolation of human dosages as long as information on pharmacokinetics and dose-response curves of

Clostridium botulinum toxin in the horse is not available. During preparatory work aiming at determining the optimal injection (Chapters II, III and VI) or insertion (Chapter IV) sites in the widest part of the muscle belly, several deep digital flexor muscles were dissected. This material was used to develop a formula to estimate the muscle volume of the deep digital flexor based on distances between anatomical landmarks (**Chapter V**). The calculated muscle volumes based on this formula proved to have a high correlation with the real muscle volumes, making the formula suitable for the accurate determination of deep digital flexor muscle volume in the living animal.

Clostridium botulinum toxin in the acutely laminitic horse

The intention of the work described in **Chapter VI** was to provide clinical evidence for the use of *Clostridium botulinum* toxin therapy in case of acute laminitis. To achieve this, the original design of the investigation included three groups of patients suffering from acute laminitis with 21 patients in total. A uniform and objective evaluation protocol including pain-status related parameters, indicators of local inflammation, locomotion parameters and radiographic data was developed. Unfortunately, laminitic patients diagnosed within 48 hours after onset of clinical symptoms and without signs of distal phalanx displacement proved to be very scarce in a relatively large geographical area around our clinic. Only two patients meeting all inclusion criteria became available during the study period of six months. Therefore, the outcome remained restricted to two extensive case reports, forcibly precluding any statistical analysis and hence drawing of evidence-based conclusions on the potential of *Clostridium botulinum* toxin therapy to prevent distal phalanx displacement and/or give substantial pain relief. In those two cases, the treatment seemed to be beneficial and severe side-effects were not seen, confirming the results in sound animals reported elsewhere in this thesis. Therefore, despite its preliminary character, the beneficial outcomes of the study in these two cases underline the good perspective for further research in this area. The uniform evaluation protocol that was developed for this study, which enables more objective judging of treatment effects and easy harmonisation and sharing of data in multi-centre studies, may be of great help in subsequent studies.

There are several reasons for the failure to include a larger number of patients in the study in Chapter VI. As mentioned in Chapter I, the pathogenesis of laminitis can be highly variable. Although all pathways result in the structural failure of the Suspensory Apparatus of the Distal Phalanx (SADP), caused by degradation of the basement membrane, the mechanisms leading to this failure are different. As a result, the standardization of a naturally occurring patient group is extremely difficult. Furthermore, displacement of the distal phalanx can occur within a very short time period (Morrison, 2004). Performing a field study using horses or ponies suffering from naturally occurring acute laminitis, but still without distal phalanx displacement, is therefore difficult to accomplish.

Alternatively, there are various models to induce laminitis, which are regularly used in research. Laminitis can be induced by administering carbohydrate overload, black walnut extract or insulin (Van Eps and Pollitt, 2006; Asplin et al., 2007; Belknap, 2010a; de Laat et al., 2010). The use of such a model to induce laminitis would give the opportunity to test the potential of a *Clostridium botulinum* toxin therapy to prevent the distal phalanx from displacing in acute laminitis in a fairly standardized situation. This is very attractive from a

scientific viewpoint. However, the ethical aspects of such a study using a model to induce laminitis should be strongly considered since laminitis is an excruciatingly painful disease and, given the experimental set-up that would be needed to evaluate the treatment effect, animals cannot be euthanized in an early stage of the disease. Therefore, setting up an international multi-centre clinical trial on acute laminitis would be a much preferred approach. Perhaps, the effectiveness of *Clostridium botulinum* toxin injections in giving pain release and preventing further displacement of the distal phalanx in case of chronic laminitis should be tested first. Such a study will be easier to realize, as horses with signs of chronic laminitis are plentiful and because horse owners will most likely be more inclined to cooperate in a study with uncertain effect, as the prognosis for horses with chronic laminitis is poor anyhow.

Conclusions

The work in this thesis contributes to the development of a *Clostridium botulinum* toxin therapy in equine laminitis. Hopefully, such a therapy will one day find its way into practice and may help to reduce the mobility restriction and pain of horses and ponies suffering from laminitis. The initial steps in developing and testing such a therapy were made in the research described in this thesis, from which the following can be concluded:

- ✓ *Clostridium botulinum* toxin injections have been proven to reduce the muscle tone of the deep digital flexor muscle significantly without severe side-effects or inducing lameness in the healthy horse. Therefore, further research into the clinical application of the substance was justified.
- ✓ The increased firing rate of the deep digital flexor muscle in laminitic horses, as evidenced by EMG interference pattern analysis, suggests an increased muscle force which provides some support for the claim that reduction of the muscle tone by use of *Clostridium botulinum* toxin might be a successful supportive therapy. However, further in-depth research into this item is necessary, as two other parameters, the number of turns/second and amplitude/turn, were not dissimilar, indicating that larger motor units with larger-amplitude motor unit potentials were not recruited.
- ✓ It proved possible to determine the muscle volume of the deep digital flexor in the living animal quite accurately. This way of determining muscle volume can be used in future clinical studies to estimate individual *Clostridium botulinum* toxin dosages as precise as possible.
- ✓ Realization of a large-scale clinical study into the application of *Clostridium botulinum* toxin in cases of naturally occurring acute laminitis proved difficult. The main reason is that recruitment of a sufficiently large population of acutely laminitic horses and ponies without distal phalanx displacement and complying with strict inclusion criteria is problematic. However, the beneficial outcome as seen in two animals suggests that putting effort in the design and execution of such a (multi-centre and probably multinational) trial using a uniform and objective evaluation protocol, is certainly warranted and that perspectives are likely to be good.

Future research

As mentioned above, further research into the application of *Clostridium botulinum* toxin in acute and chronic laminitis seems to be justified. Xeomin® (Merz Pharmaceuticals GmbH) is now approved in the Netherlands for its use in human medicine. Given the advantage of the absence of complexing proteins, it might be advisable to use this type of *Clostridium botulinum* toxin formulation in future studies.

It may be worthwhile to test whether better results can be obtained if a *Clostridium botulinum* toxin therapy would be combined with desmectomy or desmotomy of the accessory ligament of the deep digital flexor. Obviously, the advantage of such an approach in comparison with total deep digital flexor tenotomy is the much less invasive character. It may have also added value to the *Clostridium botulinum* toxin therapy, as it addresses the possible role of the accessory ligament, which, as a non-muscular structure, is most probably not influenced by *Clostridium botulinum* toxin therapy.

From an orthopaedic point of view, it might be interesting to determine the long-term effects of a reduced deep digital flexor muscle tone. As described in Chapter I, the muscles of the digital flexors function as dampers of limb vibration. Impairing this function might have consequences for the bone and tendon as they would probably be more vulnerable to fatigue damage. It can be argued that the consequences of this possibly impaired function would most likely be minor as long as the laminitic horse is stabled. However, once the laminitic episode has ended within the 12-week duration of the *Clostridium botulinum* toxin effect, this aspect might become more important. Thus, future research into this aspect is indicated.

Furthermore, *Clostridium botulinum* toxin injections might have a potential role in the prevention of Supporting Limb Laminitis (SLL). Venography shows that relieving the tension of the deep digital flexor (by unlocking the carpus with a little pressure to the palmar side) is needed to fill the lamellae of the dorsal hoof wall (Redden, 2001; Redden, 2004). Redden (2004) developed a hoof support device that raises the heels and thereby decreases the tension in the deep digital flexor. However, the technique has some disadvantages. First, the application on the supporting limb can be challenging and sometimes requires the use of (general) anaesthesia or a sling (Redden, 2004). Second, although venography shows that the device preserves perfusion of the dorsal laminae, it also shows that the vascular perfusion of the heel diminishes (Redden, 2004), which might potentially cause damage to the foot. Also, it has been suggested that not all horses are comfortable with heel elevation (Orsini, 2012). Our studies show that the tone of the deep digital flexor muscle can be safely and significantly reduced by injecting *Clostridium botulinum* toxin into the muscle belly, thereby probably reducing the pull of the deep digital flexor. This relief of tension on the distal phalanx might also preserve the blood perfusion in the dorsal laminae and in this way act preventatively. The possible preventative effect of *Clostridium botulinum* toxin is therefore a potentially interesting other application that warrants further research.

A fourth possible avenue for further research is the potential analgesic effect of *Clostridium botulinum* toxin. In human medicine, the use of the product for this purpose increases rapidly, as mentioned in Chapter I. It could be speculated that the local application of

Clostridium botulinum toxin at the coronary band of the laminitic horse might be useful in reducing the extreme pains associated with laminitis.

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Nederlandse samenvatting

Inleiding

Hoefbevangenheid (laminitis) bij het paard is een zeer ernstige en pijnlijke aandoening. Zoals beschreven in **Hoofdstuk I**, worden bij het paard het hoefbeen (distale falanx) en de hoefschoen verbonden door de hoeflamellen, ook wel het steunapparaat van het hoefbeen genoemd. In geval van hoefbevangenheid is deze verbinding ernstig aangetast. De diepe buiger hecht aan op de palmaire/plantaire zijde van het hoefbeen. Bij het gezonde paard oefent de diepe buiger een trekkracht uit op het hoefbeen als het been belast wordt. In geval van hoefbevangenheid kan het aangetaste steunapparaat van het hoefbeen deze trekkracht niet weerstaan waardoor het hoefbeen kan verplaatsen in de hoefschoen. Wanneer deze verplaatsing eenmaal heeft plaatsgevonden wordt de prognose veel slechter. De aandoening hoefbevangenheid werd enkele duizenden jaren geleden al beschreven en is sindsdien onderwerp geweest van veel wetenschappelijk onderzoek. Desalniettemin is er nog steeds geen optimale therapie die de verplaatsing van de hoefbeen in de hoornschoen kan voorkomen en/of betere condities voor genezing creëert.

Clostridium botulinum toxine is in de afgelopen decennia volop gebruikt in de humane geneeskunde. Ook in de paardengeneeskunde is het middel op experimentele basis succesvol ingezet om de spieractiviteit van verschillende skeletspieren te verlagen. Indien in geval van hoefbevangenheid de spieractiviteit van de diepe buiger succesvol zou kunnen worden verlaagd, dan zou de trekkracht van de diepe buiger aan het hoefbeen mogelijk kunnen worden verminderd. Hiermee zou mogelijk verplaatsing van het hoefbeen in de hoefschoen kunnen worden voorkomen. Daarnaast zou deze verminderde trekkracht de pijnlijkheid die met deze aandoening gepaard gaat mogelijk kunnen reduceren. De hypothese van dit proefschrift is dan ook dat *Clostridium botulinum* toxine een succesvolle ondersteunende therapie zou kunnen zijn bij hoefbevangenheid.

Clostridium botulinum toxine injecties bij het gezonde paard

Eerst is de toepassing bij het gezonde, niet kreupele paard getest om de effectiviteit vast te stellen en bijwerkingen te kunnen monitoren **(Hoofdstuk II en III)**. Het ontstaan van kreupelheid of andere ernstige bijwerkingen zou het middel immers ongeschikt maken voor een patiëntenstudie. De diepe buigspier van het linker voorbeen van zeven gezonde, niet kreupele KWPN paarden werd geïnjecteerd met 200 IU *Clostridium botulinum* toxine. De spiertonus van de diepe buiger werd vooraf en op verschillende tijdstippen na de injecties vastgesteld door middel van elektromyografische Motor Unit Actie Potentiaal (MUAP) analyse en Interferentie Patroon Analyse (IPA). Al deze variabelen daalden significant na de injecties met het toxine: het sterkst in de eerste drie dagen, daarna vlakte deze daling af.

Simultaan met de bovenvermelde studie werd het effect van de eenzijdige verminderde spiertonus van de diepe buiger op de locomotie van het paard getest. Hiervoor werd de sagittale range of motion van de metacarpus (SROM) en carpus (CROM) getest, evenals de verdeling van de kracht die door de hoeven op de bodem werd uitgevoerd (linker been vergeleken met rechter been = symmetrie-index en het teengedeelte van de hoef vergeleken met het hielgedeelte = balans-index). Zowel de balans-index als de symmetrie-index vertoonde geen significant verschil voor en de na de injecties met *Clostridium botulinum* toxine, hetgeen betekent dat er geen asymmetrische beenbelasting oftewel kreupelheid

optrad. De SROM en CROM namen na de injecties wel significant toe, waarschijnlijk door de gereduceerde spiertonus van de diepe buiger. De sterkste stijging werd waargenomen in de eerste drie dagen, dit komt overeen met de sterkste daling in spieractiviteit van de diepe buiger zoals hierboven beschreven.

Deze studies tonen aan dat *Clostridium botulinum* toxine injecties veilig kunnen worden toegepast om de activiteit van de diepe buigspier bij het paard significant te verminderen.

De spierkracht van de diepe buiger

Door verschillende dierenartsen en onderzoekers is gesuggereerd dat de spiertonus van de diepe buiger bij hoefbevangenheid verhoogd zou kunnen zijn en dat er mogelijk zelfs een contractuur zou kunnen optreden. Dit zou veroorzaakt worden door de pijn die gepaard gaat met hoefbevangenheid en zou het meest duidelijk zijn in een chronisch stadium. Deze suggestie is echter nooit wetenschappelijk onderzocht, maar zou het gebruik van een therapie met *Clostridium botulinum* toxine wel ondersteunen.

Uit de humane geneeskunde is bekend dat een aantal van de IPA parameters beïnvloed worden door spierkracht: bij een toenemende spierkracht verhoogt de vuurfrequentie, de amplitude en het aantal keerpunten in het interferentiepatroon. In het onderzoek beschreven in **Hoofdstuk IV** werd inderdaad een verhoging van de vuurfrequentie in het interferentiepatroon van de diepe buiger vastgesteld bij hoefbevangen paarden. Echter, dit lijkt een partieel effect te zijn gezien het feit dat de andere parameters niet stegen. Een grotere groep hoefbevangen paarden zou het 95% betrouwbaarheidsinterval versmallen waarmee een preciezere schatting van het gemiddelde van de populatie gemaakt zou kunnen worden. Bovendien zou het bij een grotere groep mogelijk zijn om de hoefbevangen paarden verder op te delen, bijvoorbeeld op basis van duur of ernst van de hoefbevangenheid.

Verfijning van de *Clostridium botulinum* toxine dosering

De gekozen *Clostridium botulinum* toxine dosering zoals beschreven in hoofdstuk twee en drie leidde tot een significante afname van de spieractiviteit van de diepe buiger zonder dat dit ernstige bijwerkingen teweeg bracht. Echter, hoefbevangenheid komt voor bij allerlei verschillende rassen en bij paarden en pony's van verschillende grootte. Het zou daarom nuttig zijn om het volume van de diepe buigspier bij het levende paard te kunnen bepalen. Hiermee zou informatie over doseringen uit de humane geneeskunde geëxtrapoleerd kunnen worden zolang er geen informatie beschikbaar is over de farmacokinetiek en dosis-respons curves van *Clostridium botulinum* toxine bij het paard. Voor de studie beschreven in **Hoofdstuk V** werden de voorbenen van dode paarden gebruikt om een formule te ontwikkelen die het mogelijk maakt om nauwkeurig het volume van de diepe buigspier bij het levende paard te bepalen aan de hand van afstanden tussen anatomische herkenningspunten.

Clostridium botulinum toxine bij het acuut hoefbevangen paard

De oorspronkelijke opzet van de studie beschreven in **Hoofdstuk VI** was het verzamelen van

wetenschappelijk bewijs voor het gebruik van *Clostridium botulinum* toxine bij het acuut hoefbevangen paard met als doel kanteling of verzakking van het hoefbeen te voorkomen en de met deze aandoening gepaard gaande pijn te reduceren. De originele studieopzet omvatte dan ook drie groepen hoefbevangen paarden of pony's met een totaal van 21 dieren. Helaas bleek het erg moeilijk om acuut hoefbevangen paarden te vinden die voldeden aan de zeer strenge inclusiecriteria, namelijk het opstarten van de experimentele behandeling inclusief röntgenfoto's binnen 48 uur na het ontstaan van de eerste symptomen en geen kanteling of verzakking van het hoefbeen bij aanvang van het onderzoek. Helaas voldeden maar twee patiënten aan deze eisen in het tijdsbestek van zes maanden waarin de studie liep. Daarom werden slechts twee acuut hoefbevangen paarden behandeld en gemonitord. Dit maakt het trekken van wetenschappelijk onderbouwde conclusies uiteraard onmogelijk. Echter, in deze twee casussen werd een positief effect waargenomen en bleven ernstige bijwerkingen uit. Het lijkt dus de moeite waard om te investeren in een grotere, multicenter, en mogelijk internationale studie naar het gebruik van *Clostridium botulinum* toxine bij acuut hoefbevangen paarden. Om het effect van de behandeling zo objectief en nauwkeurig mogelijk te kunnen beoordelen in plaats van alleen op basis van de klassieke Obel-score, werd een uitgebreid uniform en objectief evaluatieprotocol ontwikkeld. Dit protocol zal het in een vervolgstudie makkelijker maken om hoefbevangen paarden objectief te evalueren en te vergelijken.

Vervolgonderzoek

Zoals uit bovenstaande duidelijk is geworden lijkt het lonend om te investeren in vervolgonderzoek naar het gebruik van *Clostridium botulinum* toxine bij het hoefbevangen paard. Zoals beschreven in **Hoofdstuk VII**, is het naast de hierboven genoemde grotere studie naar de toepassing bij het acuut hoefbevangen paard, mogelijk ook zinvol om het gebruik van *Clostridium botulinum* toxine bij het chronische hoefbevangen paard te onderzoeken. Daarnaast kan bijvoorbeeld gedacht worden aan studies naar het gebruik van *Clostridium botulinum* toxine als preventie van eenzijdige hoefbevangenheid als gevolg van overbelasting of de lokale toepassing van het toxine ten behoeve van het analgetisch effect.

Deutsche Zusammenfassung

Einleitung

Hufrehe beim Pferd stellt eine sehr ernsthafte und schmerzhafte Erkrankung dar. Wie in **Kapitel I** beschrieben, sind das Hufbein und der Hufschuh durch die Huflamellen miteinander verbunden. Dieser Apparat wird auch als Unterstützungsmechanismus des Hufbeins beschrieben. Im Fall der Hufrehe ist dieser Mechanismus deutlich geschädigt. Die tiefe Beugesehne ist an der palmaren beziehungsweise plantaren Seite des Hufbeins befestigt. Bei einem gesunden Pferd übt die tiefe Beugesehne bei der Belastung des Beines Zugkraft auf das Hufbein aus. Im Rahmen der Hufrehe hält der Unterstützungsmechanismus des Hufbeins diesem Zug nicht stand, und das Hufbein wird verschoben. Bei Patienten, bei denen diese Verschiebung stattgefunden hat, verschlechtert sich die Prognose deutlich. Die Erkrankung der Hufrehe ist schon seit tausenden Jahren bekannt und intensiv erforscht. Leider ist zum jetzigen Zeitpunkt keine optimale Therapie bekannt, die die Verschiebung des Hufbeins verhindert und/oder bessere Optionen für die Heilung ermöglicht.

Clostridium botulinum-Toxin ist im letzten Jahrzehnt in der Humanmedizin in vielen Bereichen therapeutisch eingesetzt worden. Auch in der Pferdemedizin wurden sie experimentell mit Erfolg verwendet, um die Muskelaktivität von unterschiedlichen Bereichen der Skelettmuskulatur zu erniedrigen. Daraus resultierte die Überlegung, dass eine Erniedrigung der Muskelaktivität der Skelettmuskulatur zu einer verminderten Zugkraft auf das Hufbein führen könnte. So könnte eine Verschiebung des Hufbeins verhindert werden und außerdem könnte eine kleinere Zugkraft die mit der Erkrankung einhergehenden Schmerzen vermindern. Die Hypothese dieser Dissertation ist, dass *Clostridium botulinum*-Toxin deswegen als unterstützende Therapie bei der Hufrehe eingesetzt werden könnte.

Clostridium botulinum-Toxin-Injektion beim gesunden Pferd

Zuerst wurde die Anwendung beim gesunden, lahmfreien Pferd getestet, um die Wirksamkeit der Injektion und mögliche Nebenwirkungen zu testen (**Kapitel II und III**). So würde die Induktion einer Lahmheit durch die Injektion oder andere schwerwiegende Nebenwirkungen den Einsatz in Patientenstudien erschweren. Bei sieben gesunden, lahmfreien KWPN Pferden wurde die Musculus flexor digitorum profundus vorne links mit 200 IU *Clostridium botulinum*-Toxin injiziert. Der Muskeltonus des Musculus flexor digitorum profundus wurde vorher und zu unterschiedlichen Zeitpunkten nach der Injektion mittels elektromyographischer Motor Unit Action Potential (MUAP) Analyse und Interferention Pattern Analysis (IPA) gemessen. Alle gemessenen Variablen waren nach der Toxininjektion für drei Tage vermindert; danach ließ der vermindernde Effekt nach.

Gleichzeitig mit oben genannter Studie wurde der Effekt eines einseitig verminderten Muskeltonus des Musculus flexor digitorum profundus auf die Bewegung des Pferdes getestet. Dafür wurden die sagittale Bewegung (range of motion) von der Metakarpus (SROM) und die karpale Bewegung (CROM) getestet und zudem die Belastungsverteilung am Boden gemessen (linke Gliedmaße im Vergleich mit der rechten Gliedmaße = Symmetry-Index und der Zehenteil im Vergleich mit dem Trachtenteil = Balance-Index). Der Balance-index als auch der Symmetry-Index zeigten keine signifikanten Unterschiede vor und nach den Injektionen mit *Clostridium botulinum*-Toxin. So konnte gezeigt werden, dass keine asymmetrische Beinbelastung (Lahmheit) auftrat. SROM und CROM stiegen nach den

Injektionen signifikant an, wahrscheinlich aufgrund der reduzierten Muskelspannung im Musculus flexor digitorum profundus. Die größte Steigerung wurde in den ersten drei Tagen nach Injektion beobachtet, was mit der starken Abnahme an Muskelaktivität im Musculus flexor digitorum profundus übereinstimmt (siehe oben).

Diese Studien zeigen, dass Injektionen mit *Clostridium botulinum*-Toxin ohne Nebenwirkungen eingesetzt werden können, um die Muskelaktivität des Musculus flexor digitorum profundus beim Pferd zu vermindern.

Die Muskelkraft des Musculus flexor digitorum profundus

Forschungsergebnisse unterschiedlicher Tierärzte und Wissenschaftler suggerieren, dass der Muskeltonus des Musculus flexor digitorum profundus bei Hufrehe erhöht sein könnte und es möglicherweise auch zu einer Kontraktur des Muskels kommt. Ursächlich für den erhöhte Muskeltonus und die Kontraktur wird Schmerz angesehen. Der erhöhte Muskeltonus und die Kontraktur werden meist im chronischen Stadium beobachtet. Es gibt keine wissenschaftlichen Studien, die diese Hypothese untersuchen, aber im Falle einer erhöhter Muskeltonus oder Kontraktur sollte die Therapie mit *Clostridium botulinum*-Toxin unterstützend wirken. Das macht die Therapie mit *Clostridium botulinum*-Toxin besonders relevant.

Aus der Humanmedizin ist bekannt, dass einige der IPA Parameter durch Muskelkraft beeinflusst werden: bei zunehmender Muskelkraft erhöht sich die Signalfrequenz, die Amplitude und die Anzahl der Kehrpunkte in dem Interferentionpatron. In dieser Studie, beschrieben in **Kapitel IV**, wurde eine Erhöhung der Signalfrequenz in dem Interferentionpatron des Musculus flexor digitorum profundus bei Pferden mit Hufrehe festgestellt. Es wird vermutet, dass dies ein partieller Effekt ist, weil andere Parameters nicht gesteigert sind. Eine größere Gruppe von Pferden mit Hufrehe würde das 95% Konfidenzintervall verkleinern und so die Durchschnittspopulation besser repräsentieren. Außerdem wäre es möglich die Gruppe Pferden mit Hufrehe weiter zu unterteilen, beispielsweise nach Dauer und Schweregrad der Erkrankung.

Verfeinerung der Dosierung des *Clostridium botulinum*-Toxins

Die in Kapitel II und III beschriebene ausgewählte Dosierung des *Clostridium botulinum*-Toxins resultierte in einer signifikanten Abnahme der Muskelaktivität des Musculus flexor digitorum profundus ohne ernsthafte Nebenwirkungen. Da Hufrehe viele unterschiedliche Rassen und Pferde und Ponys von unterschiedlicher Größe betrifft, wäre es vorteilhaft, eine Möglichkeit zu haben, das Volumen des Musculus flexor digitorum profundus am lebenden Pferd zu bestimmen. So könnten weitere Information aus der Humanmedizin extrapoliert werden, bis Information über Pharmakokinetik und Dosis-Response Curves des *Clostridium botulinum*-Toxins beim Pferd bekannt sind. Für diese Studie, beschrieben im **Kapitel V**, wurden Vorderbeine von toten Pferden benutzt, um eine Formel zu entwickeln, die es ermöglicht mittels Abständen zwischen anatomischen Erkennungspunkten das genaue Volumen des Musculus flexor digitorum profundus am lebenden Pferd zu bestimmen.

Clostridium botulinum-Toxin beim Pferd mit akuter Hufrehe

Der ursprüngliche Plan der Studie beschrieben im **Kapitel VI**, war das Sammeln wissenschaftlicher Daten zur Nutzung von *Clostridium botulinum*-Toxin bei akuter Hufrehe des Pferdes mit dem Ziel, eine Hufbeinsenkung und -rotation zu vermindern und die gleichzeitig auftretenden Schmerzen zu reduzieren. Die ursprüngliche Planung ging von drei Gruppen mit insgesamt 21 Pferden aus. Es war nicht möglich, genügend Pferde mit akuter Hufrehe zu finden, die die strengen Inklusionskriterien erfüllten. Um in die Studie aufgenommen zu werden, musste die experimentelle Behandlung innerhalb 48 Stunden nach Anfang der Symptomatik beginnen und keine Hufbeinsenkung und –rotation zu Beginn der Untersuchung vorliegen. Leider erfüllten innerhalb der sechsmonatigen Studienphase nur zwei Patienten diese Kriterien. Deswegen wurden nur diese zwei Pferde behandelt und beobachtet. Aus diesem Grund ist es nicht möglich wissenschaftliche Schlüsse zu ziehen. Trotzdem wurde in diesen zwei Fällen ein positiver Effekt beobachtet, und es traten keine Nebenwirkungen auf. Aus diesem Grund ist eine größere, multizentrische Studie anzuraten, die die Therapie von Pferden mit akuter Hufrehe mit *Clostridium botulinum*-Toxin untersucht. Dabei sollte statt des klassischen Obel-Scores ein neuentwickeltes, umfangreiches und objektives Evaluationsprotokoll angewendet werden, um den Effekt der Behandlung so genau und objektiv wie möglich zu beurteilen.

Fortsetzungsstudien

Aus den Resultaten dieser Studien ist ersichtlich, dass weitere Untersuchungen zur Verwendung von *Clostridium botulinum*-Toxin bei Pferden mit Hufrehe erfolgversprechend sind. Wie in **Kapitel VII** beschrieben, ist es sinnvoll, nicht nur Studien zu der Therapie mit *Clostridium botulinum*-Toxin an Pferden mit akuter, sondern auch an Pferden mit chronischer Hufrehe durchzuführen. Zudem könnte eine Verwendung von *Clostridium botulinum*-Toxin zur Prävention von einseitiger Hufrehe in Folge einer Überbelastung und die lokale Anwendung des Toxins zum Nutzen des analgetischen Effekts untersucht werden.

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Lotte van Proosdij-Hardeman

List of publications

Published papers

Lotte C. Hardeman, Bram R. van der Meij, Maarten Oosterlinck, Stefanie Veraa, Johannes H. van der Kolk, Inge D. Wijnberg, Willem Back, 2013. Effect of *Clostridium botulinum* toxin type A injections into the deep digital flexor muscle on the range of motion of the metacarpus and carpus, and the force distribution underneath the hooves, of sound horses at the walk. The Veterinary Journal 198, e152-e156.

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Curriculum Vitae

Lotte Hardeman werd geboren op 19 mei 1988 te Barneveld. Na het behalen van haar diploma aan het Johannes Fontanus College te Barneveld, begon zij in 2006 aan de studie Diergeneeskunde aan de Universiteit Utrecht. Na de doctoraalfase besteedde zij een jaar aan het uitvoeren van wetenschappelijk onderzoek in het kader van een Excellent-Tracé traject. In dat jaar is het grootste deel van het wetenschappelijk werk zoals beschreven in dit proefschrift uitgevoerd. In 2013 studeerde zij 'met genoegen' af en begon zij als practicus bij De Klomp Dierenartsen in De Klomp waar ze tot op heden werkzaam is.

Lotte Hardeman was born on May 19, 1988 in Barneveld. After graduation from high school (Johannes Fontanus College in Barneveld) she started her studies in Veterinary Medicine at the Faculty of Veterinary Medicine, Utrecht University in 2006. In the fifth year of her study, she followed an honours program, during which the majority of the studies described in this thesis were conducted. She graduated 'with honours' in 2013 and started as a veterinarian at De Klomp Dierenartsen in De Klomp, where she is still working.