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INTRA-ARTICULAR BOTULINUM TOXIN A
IN TREATMENT OF OSTEOARTHRITIC JOINT PAIN IN DOGS

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine, University of Helsinki, for public examination in Lecture Room 5, University Main Building, on 8th of December 2017, at 12 noon.

HELSINKI 2017
Osteoarthritis (OA) is considered to be the leading cause of chronic pain in dogs. Oral medication is the mainstay of pain treatment in canine OA, but it can provide insufficient pain relief and intolerable adverse events. Therefore, new treatment modalities are needed for dogs suffering from osteoarthritic pain. Intra-articularly injected botulinum toxin A (IA BoNT A) has shown efficacy in treatment of joint pain in arthritic human patients. The mechanism of the antinociceptive action of IA BoNT A has not been studied in vivo and its adverse effects have not been thoroughly investigated.

Our aim was to study the antinociceptive efficacy of IA BoNT A in osteoarthritic dogs and to investigate the effect it has on their pain mediators. Also, we wanted to compare the pain mediators between osteoarthritic and non-osteoarthritic joints and reveal any associations with osteoarthritic pain. Our aim was also to investigate the adverse effects of IA BoNT A and to study whether the toxin spreads from the joint.

We investigated the antinociceptive efficacy of IA BoNT A in a randomized, double-blinded, placebo-controlled, 12-week clinical trial in 35 client-owned osteoarthritic dogs after an injection of either IA BoNT A or placebo into the stifle, hip, or elbow joint. We detected significant improvement in the ground reaction forces and Helsinki Chronic Pain Index in the IA BoNT A group. There was also a significant difference in the improvement of the ground reaction forces between the IA BoNT A and placebo groups.

We measured substance P (SP), prostaglandin E₂ (PGE₂), and tumour necrosis factor-alpha (TNF-α) from the synovial fluid (SF) and serum of the 35 osteoarthritic dogs and also analysed SF SP and PGE₂ from 13 non-osteoarthritic control joints. IA BoNT A did not affect SF or serum SP or PGE₂. TNF-α was not detectable in our samples. SF PGE₂ correlated with osteoarthritic joint pain and was significantly higher in osteoarthritic than in non-osteoarthritic joints.

We investigated the adverse effects of IA BoNT A in a randomized, placebo-controlled, blinded trial in six healthy laboratory beagle dogs after IA BoNT A and placebo injections into the stifle joints. No significant clinical, cytological, or histopathological adverse effects were detected 12 weeks after the injections, but changes in the electrophysiological recordings in two dogs suggested possible spread of the toxin.

Our results indicate that IA BoNT A has efficacy in the treatment of osteoarthritic joint pain in dogs. The antinociceptive effect of IA BoNT A inside the joint seems not to be related to the inhibition in the release of SP or PGE₂. SF PGE₂ could be a marker of chronic OA and pain in dogs. IA BoNT A does not cause clinical, cytological, or histopathological adverse effects in healthy dogs, although the toxin may spread from the joint.

ABSTRACT

Osteoarthritis (OA) is considered to be the leading cause of chronic pain in dogs. Oral medication is the mainstay of pain treatment in canine OA, but it can provide insufficient pain relief and intolerable adverse events. Therefore, new treatment modalities are needed for dogs suffering from osteoarthritic pain. Intra-articularly injected botulinum toxin A (IA BoNT A) has shown efficacy in treatment of joint pain in arthritic human patients. The mechanism of the antinociceptive action of IA BoNT A has not been studied in vivo and its adverse effects have not been thoroughly investigated.

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This work was carried out at the Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki and in part at the Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease and School of Veterinary Science, University of Liverpool during 2010–2017.

I am grateful for the financial support provided by the Finnish Veterinary Association, the Finnish Veterinary Research Foundation, the Finnish Kennel Club, and the ANIWEL Graduate School in Animal Welfare.

I owe my deepest gratitude to my project leader and supervisor Professor Outi Vapaavuori. Her help has been invaluable in all aspects of this project, but first and foremost, she gave me the opportunity to develop my capabilities as a researcher and guided me through this process with an extremely encouraging attitude.

Also, I want to express my warmest thanks to my supervisor Docent Anna Hielm-Björkman for her contribution in planning the studies, finding solutions to problems encountered, and preparing the manuscripts. Her expertise in clinical research has been extremely useful and her innovative ideas and constant optimism have provided me with an inspiring example of an enthusiastic researcher.

My sincere thanks go to my supervisor Professor John Innes. I admire his profound expertise in canine arthritis and his extensive contribution to academia. I am extremely grateful for the opportunity to carry out an important part of the research in his laboratory in Liverpool and for his help and hospitality there. I am also deeply grateful to all the personnel in the laboratory who helped me in performing the analyses.

I warmly thank all my co-authors for their invaluable contribution in performing the studies and preparing the manuscripts. My special thanks go to Juhana Honkavaara for coming up with the idea of investigating the toxin in relieving joint pain in dogs in the first place. I also want to thank Mikael Morelius for helping me with the intra-articular injections and for evaluating the lameness by viewing the numerous videos. I want to express my gratitude to Professor Stig Larsen for his time and expertise in doing the statistical analysis of Study I. My warmest thanks go to Tarja Pääkkönen for performing the electrophysiological recordings and guiding me into the exciting world of electrophysiology. I am also very grateful to Pernilla Syrjä for the histopathology and for showing me the magnified beauty of cartilage and synovial structure. In addition, I would like to thank Jouni Junnila for the statistical analyses in Studies II and III.

I want to thank Professor Satu Sankari for finding solutions to the problems that emerged in the laboratory analyses. I am also very grateful to Merja Ranta and Lilia Jääskeläinen for evaluating the synovial fluid samples and for the personnel in the Clinical Research Laboratory for their dedicated work.
I want to thank Kirsi Piirainen, Heta Porola, and Marianne Haavisto for participating in this work as students. I would also like to express my gratitude to Johanna Roine and Laura Parikka in the practicalities of carrying out the studies.

I thank the pre-examiners of this thesis, Professor Peter Muir and Associate Professor Dennis Dykstra, for their insightful comments which helped me to improve this manuscript. I am also deeply grateful to Dylan Clements for accepting the task as my opponent.

I thank all my colleagues at the Department of Equine and Small Animal Diseases and the Veterinary Teaching Hospital for the moral support during these years. Also, I am deeply grateful to my best friends Linda, Liina, and Laura for the priceless friendship which has supported me during the ups and downs.

My deepest gratitude goes to my parents who have supported me in my career as in all other sectors of my life. Dad, you have taught me persistence and common sense and Mum, from you I have learned to believe in myself and go after my dreams. Arvi, my dear brother, and Laura, thank you for taking care of my horses and helping me to maintain a life outside work. I am also deeply grateful to Sinikka, the world’s best nanny, and to my dear parents-in-law for taking care of Veikko and Jaakko when I have been busy.

Thank you, Henna, for your invaluable contribution in getting everything done and all problems solved. From you I have received the most honest criticism and the strongest encouragement. It is a privilege to have such a devoted academic, skilled veterinarian, and inspiring mother as a twin sister. My thanks go also to Teemu for participating in the language check and especially for formulating answers to the reviewers’ questions regarding physics.

Thank you, dear Veikko, Jaakko, and Tuuli, for the love and joy you bring into my life with your presence. Thank you for guiding my thoughts away from work and showing me that there is much more to life than veterinary medicine.

Finally, I would like to thank my beloved husband Jukka. I admire your perfectionism. I would not have finished a single manuscript without you correcting the spelling, drawing the figures, editing the tables, and finalizing the layout. Thank you for proof reading everything, even when you were busy and tired. Thank you for believing in me.
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# Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADSC</td>
<td>adipose-derived stem cell</td>
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<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BONT</td>
<td>botulinum toxin</td>
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<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<td>CMAP</td>
<td>compound muscle action potential</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<tr>
<td>CS</td>
<td>corticosteroids</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EMG</td>
<td>electromyography</td>
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<td>HA</td>
<td>hyaluronic acid</td>
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<tr>
<td>HCPI</td>
<td>Helsinki Chronic Pain Index</td>
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<tr>
<td>IA</td>
<td>intra-articular</td>
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<td>IL</td>
<td>interleukin</td>
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<td>IM</td>
<td>intramuscular</td>
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<td>IQR</td>
<td>interquartile range</td>
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<td>IV</td>
<td>intravenous</td>
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<td>MNCV</td>
<td>motor nerve conduction velocity</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<td>OA</td>
<td>osteoarthritis</td>
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<td>PVF</td>
<td>peak vertical force</td>
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<td>PGE₂</td>
<td>prostaglandin E₂</td>
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<td>PRP</td>
<td>platelet-rich plasma</td>
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<td>RM</td>
<td>repeated-measures</td>
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<td>RNS</td>
<td>repetitive nerve stimulation</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SF</td>
<td>synovial fluid</td>
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<td>SFEMG</td>
<td>single-fibre electromyography</td>
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<td>SI</td>
<td>symmetry index</td>
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<td>SP</td>
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<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
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<td>VAS</td>
<td>visual analogue scale</td>
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<td>VI</td>
<td>vertical impulse</td>
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1 INTRODUCTION

Botulism was first described in the 1820s, when Justinius Kerner published his observations on the fatal food poisonings that tormented the city of Württemberg. He reported that the cause of these deaths was a toxin formed in spoiled sausages and described how this toxin acts on the motor and autonomic nervous systems. He even suggested that this lethal toxin, when administered in very small amounts, could be a useful treatment for patients with muscular overactivity (Erbguth, 2004). That goal was reached one and a half centuries afterwards, when Scott (1980) reported the experimental use of botulinum toxin serotype A (BoNT A) in the treatment of strabismus in human patients (Scott, 1980a; 1980b). After that, various therapeutic applications were investigated for the toxin and today the toxin is widely used in medicine. The most common applications for BoNT worldwide are the subdermal injections used for cosmetic purposes (Guo et al., 2015). However, the paralytic effects of BoNT are exploited in various conditions associated with painful and undesired muscle overactivity such as spasticity, dystonias, and spasms (Rosales and Chua-Yap, 2008; Dressler, 2010; Hallett et al., 2013).

The direct antinociceptive properties of BoNT were described by Cui and Aoki (2000) in a rat model of pain. They reported pain relief after BoNT injections without effects on the muscle tone of the animals. This led to further studies investigating BoNT's antinociceptive action in pain models (Cui et al., 2004; Aoki, 2005; Bach-Rojecky and Lacković, 2005; Park et al., 2006; Luvisetto et al., 2007; Gazerani et al., 2009) and eventually to case series reporting pain relief after intra-articularly injected (IA) BoNT A in human patients with painful arthritis (Mahowald et al., 2006; Singh and Mahowald, 2009; Singh et al., 2009a). After this, the antinociceptive efficacy of IA BoNT A in the treatment of joint pain was investigated in clinical trials with control groups with promising results (Mahowald et al., 2009; Singh et al., 2009b; Boon et al., 2010; Singh, 2010; Joo et al., 2012; Sun et al., 2014; Hsieh et al., 2016).

In contrast to medicine, the paralytic effects of BoNT have been of little value for veterinary clinicians. Conditions with constant painful muscle overactivity such as spasticity and dystonias are rarely treated in animals and the toxin is mainly known as the cause of unwelcome incidents where spoiled foliage has led to the death of various individuals in an animal herd (Johnson et al., 2010; Payne et al., 2011). However, finding that IA BoNT A is effective in treating joint pain opens up new possibilities for the use of this toxin in veterinary medicine.

Osteoarthritis (OA) is considered to be the most common cause of chronic pain in dogs (Bell et al., 2014). Despite rigorous research, there is no known cure for OA to date. Therefore, pain medication, specifically the non-
steroidal anti-inflammatory drugs, is the backbone of OA treatment. However, their use can be related to intolerable adverse events (MacPhail et al., 1998; Duerr et al., 2004; Lomas et al., 2013) and clinical trials have shown that their antinociceptive efficacy might be insufficient in advanced OA in dogs (Lascelles et al., 2008; Malek et al., 2012). New treatment modalities are needed for canine patients with painful OA.

For this reason, encouraged by the findings in human patients, we wanted to investigate the efficacy of IA BoNT A in the treatment of joint pain in osteoarthritic dogs. In addition, our purpose was to study whether the antinociceptive action of the toxin is related to a decrease in pain mediator concentrations in synovial fluid and serum. This would enlighten the mechanism of the antinociceptive action of the toxin, which would also benefit human patients. Also, our purpose was to gain knowledge on canine osteoarthritic pain and investigate whether substance P, prostaglandin E2, or tumour necrosis factor-alpha could serve as markers of pain in canine OA. To be able to consider this novel IA therapy as a treatment option for osteoarthritic pain in our patients, we also wanted to investigate the clinical, cytological, and histopathological adverse effects of this strong toxin after IA injection.
2  REVIEW OF THE LITERATURE

2.1  BOTULINUM TOxin A

2.1.1  GENERAL
Botulinum toxins (BoNTs) are considered to be the most poisonous toxins known to man (Smith et al., 2014; Peck et al., 2017). They are neurotoxins produced by the bacterial species Clostridium botulinum, but also by some strains of C. baratii and C. butyricum. At least seven serotypes of the toxin are recognized (A–G), and various subtypes exist in each serotype (Peck et al., 2017). A characteristic feature of all the serotypes is that they inhibit neurotransmission by preventing acetylcholine release in the neuromuscular junction and cholinergic nerve terminals (Wheeler and Smith, 2013). However, the serotypes differ substantially in potency and duration of action, and they have different intracellular target proteins and serological profiles (Dressler and Saberi, 2005; Wheeler and Smith, 2013; Peck et al., 2017).

Among the serotypes, the serotype A is the most potent one (Sugiyama, 1980) and it is also most investigated (Kessler and Benecke, 1997; Aoki, 2003). Serotypes A and B have been developed into pharmaceutical products (Turton et al., 2002; Wheeler and Smith, 2013). This literature review will focus mainly on serotype A (BoNT A).

2.1.2  MECHANISM OF ACTION

2.1.2.1  Effect on acetylcholine release
The mechanism of action of BoNT A in the neuromuscular junction and cholinergic nerve terminals has been thoroughly investigated. BoNT A consists of an inactivated single-chain polypeptide with a molecular weight of 150 kDa (the toxin itself) and a mixture of non-toxic proteins, which protect the polypeptide chain (Frevert, 2015). The toxin is activated when the polypeptide chain is proteolytically cleaved into a di-chain molecule consisting of a heavy chain (100 kDa) and a light chain (50kDa) linked by a single disulphide bond. The cleavage can be accomplished by several bacterial and tissue proteases. (Turton et al., 2002)

In the target tissue, the heavy chain of the toxin binds to a binding protein (Dong et al., 2006) and a receptor (Rummel et al., 2004) on the surface of the neuron and is internalized into the cell in a vesicle (Figure 1). Inside the cell, the light chain of the toxin is released into the cytoplasm, where it cleaves the SNARE protein SNAP-25, a protein essential for the
docking and fusion of acetylcholine-containing vesicles into the synaptic membrane (Turton et al., 2002). The cleavage of SNAP-25 blocks the fusion and inhibits the exocytosis of acetylcholine into the synaptic cleft (Turton et al., 2002).

The result is flaccid muscle paralysis, which provides secondary pain relief in conditions with painful muscle hyperactivity. If the target tissue is a gland, the result is inhibition in the secretion. (Dressler and Saberi, 2005) The clinical effect of the toxin lasts approximately 3 months (Marsh et al., 2014). The effect is terminated when the SNARE protein complex is restored (Dressler and Saberi, 2005). However, motor nerve endings sprout new processes that re-establish synaptic contacts with the denervated muscle, which is also an important factor in the clinical recovery (Turton et al., 2002).

![Diagram of the molecular mechanism of action of botulinum toxin A in the neuromuscular junction.](image)

**Figure 1.** Illustration of the molecular mechanism of action of botulinum toxin A in the neuromuscular junction. The heavy chain of botulinum toxin A binds to the surface of a neuron and the toxin is internalized into the cell inside a vesicle. The light chain of the toxin is released into the cytosol and it cleaves the SNAP-25 protein. This prevents the fusion of vesicles containing acetylcholine into the cell membrane thus inhibiting its release from the nerve ending. ACTH, acetylcholine; BoNT A, botulinum toxin A.

### 2.1.2.2 Effect on neurotransmitter release

BoNT A has direct antinociceptive effects, which are independent of its effects on the neuromuscular junction (Aoki, 2003). These antinociceptive effects have been detected in various models of pain (Cui et al., 2004; Aoki, 2005; Bach-Rojecky and Lacković, 2005; Park et al., 2006; Luvisetto et al., 2007; Gazerani et al., 2009) and in clinical trials on human patients with a variety of painful conditions (Zhang et al., 2010). However, the mechanism of the antinociceptive action is not fully understood.
BoNT A has been shown to inhibit the release of the neurotransmitters substance P (SP) (Ishikawa et al., 2000; Purkiss et al., 2000; Welch et al., 2000; Lucioni et al., 2008) and calcitonin gene-related peptide (CGRP) (Durham et al., 2004; Lucioni et al., 2008) in tissue and cell cultures, and glutamate in an animal model of pain (Cui et al., 2004). In addition, BoNT A reduced the expression of c-fos in the dorsal horn of the spinal cord and decreased excitation in the wide dynamic range neurons in a rat formalin pain model (Aoki, 2005). Thus, the clinical antinociceptive effect of the toxin is believed to be related to the inhibition in the release of the neurotransmitters SP, CGRP, and glutamate from the primary sensory afferent neurons, which directly decreases peripheral sensitization and indirectly leads to a reduction in the central sensitization as well.

However, except for glutamate, the association between clinically detected pain relief and a decrease in the neurotransmitter concentration has not been proven in a clinical setting and results which contradict the neurotransmitter hypothesis have also been published (Sycha et al., 2006; Bach-Rojeccky et al., 2008). In addition, the effects of the toxin may not be confined to the peripheral nerve endings. There is evidence indicating that BoNT A can undergo retrograde transport from the peripheral nerve endings into the central nervous system (CNS), undergo transcytosis over synapses, and remain active in the CNS (Mazzocchio and Caleo, 2015).

**2.1.3 USE OF BOTULINUM TOXIN A IN MEDICINE**

The paralytic effects of BoNT A have been widely exploited in medicine since the 1980s (Wheeler and Smith, 2013). Intramuscular (IM) BoNT A is used in treatment of various neuromuscular disorders associated with painful and undesired muscle hyperactivity such as cervical dystonia, spasticity, blepharospasm, and overactive bladder (Balash and Giladi, 2004; Simpson et al., 2008a; 2008b; Dong et al., 2017). In addition, BoNT A is extremely widely used to reduce facial wrinkles for cosmetic purposes (Guo et al., 2015). Finding the antinociceptive properties of the toxin has led to studies on the use of the toxin in various other painful conditions such as trigeminal neuralgia, tennis elbow, plantar fasciitis, myofascial pain syndrome, whiplash injury, and joint pain (Zhang et al., 2010).

Intra-articular (IA) BoNT A has shown antinociceptive efficacy in the treatment of joint pain in controlled clinical trials in human patients with osteoarthritis (OA) and rheumatoid arthritis (Mahowald et al., 2009; Singh et al., 2009b; Boon et al., 2010; Sun et al., 2014; Hsieh et al., 2016), and in human patients with painful prosthetic knees (Singh, 2010) and with adhesive capsulitis of the shoulder joint (Joo et al., 2012). IA BoNT A has been found to be as effective as IA corticosteroids (CS) (Boon et al., 2010; Joo et al., 2012) and IA hyaluronic acid (HA) (Sun et al., 2014).
2.1.4 USE OF BOTULINUM TOXIN A IN DOGS

BoNT A is not licensed for veterinary use and it is not commonly used in dogs. However, the antinociceptive efficacy of the toxin reported in human patients has led to studies investigating its efficacy also in dogs.

Recently, the antinociceptive efficacy of BoNT A was investigated in treating post-operative pain in dogs in a placebo-controlled, blinded, randomized study (Vilhegas et al., 2015). In this study, 7 IU/kg of BoNT A was administered subcutaneously into the mammary glands of dogs 24 h before bilateral radical mastectomy due to mammary gland tumours. The treatment response was evaluated using a visual analogue scale (VAS), the modified Glasgow Composite Measure Pain Scale, and the requirement for rescue analgesia up to 72 h after extubation. A significant reduction in the post-operative pain was detected in all variables compared to the placebo group. No adverse events were detected.

The antinociceptive efficacy of an IA injection of BoNT A has been studied in five osteoarthritic dogs (Hadley et al., 2010). In this study, 25 IU of BoNT A was injected into either hip or elbow joints of the five dogs and the response to treatment was followed by gait analysis and owner evaluation over three months. Ground reaction forces improved in all the dogs for a variable time period and owners reported improvement in four out of the five dogs. Two dogs developed short-term redness and swelling over the injected joint, but no other adverse events were reported.

There are also case reports describing the use of BoNT A as a paralytic agent in dogs. One case report has been published on the treatment of functional gastric outflow obstruction with intragastric and intraduodenal BoNT A injections (Rinaldi et al., 2014) and another describing the successful treatment of blepharospasm with subcutaneous injections of BoNT A (Meyer-Lindenberg et al., 2003). Additionally, the effects of BoNT A injections on specific organs have been studied for implications for human therapy. BoNT A has been found to be effective in producing temporary ptosis (Bittencourt et al., 2013) and paralysis of cricothyroid muscles (Cohen et al., 1989). BoNT A has also reduced prostatic contractility (Lin et al., 2007) and parasympathetic activation in the hearts of dogs (Tsuboi et al., 2002). BoNT A was effective in inhibiting the leakage of bile in a model where bile ducts were transected (Brodsky et al., 2002) and it has been found effective in decreasing salivary gland (Shaari et al., 1998) and nasal secretion (Shaari et al., 1995).
2.1.5 ADVERSE EFFECTS OF BOTULINUM TOXIN A

2.1.5.1 Generalized botulism and local weakness

BoNTs are feared as the cause of a life-threatening, neuroparalytic disease, botulism (Sugiyama, 1980). It is a rare disease and usually the consequence of ingestion of the toxin in poorly preserved food. Also, a wound or the gut can be colonized with the bacteria producing the toxin. (Sugiyama, 1980; Cherington, 1998) In dogs, botulism has been caused by ingestion of spoiled food or a carrion (van Nes and van der Most van Spijk, 1986; Bruchim et al., 2006; Uriarte et al., 2010; Lamoureux et al., 2015).

In human patients, botulism is manifested as a descending flaccid paralysis, which typically begins with abnormalities in the cranial nerve muscles and is followed by descending weakness first affecting the upper and then the lower limbs, and in severe cases, also the respiratory muscles. The function of the sensory system and the mental status of the patient remain unaffected. (Cherington, 1998) The patient may also have signs of dysfunction of the autonomic nervous system such as constipation and a dry mouth (Cherington, 1998; Kotan et al., 2013). Even with intensive care, botulism can be fatal in human patients.

In contrast to humans, the typical manifestation of botulism in a dog is an ascending paralysis, which starts with weakness in the hindlimbs and progresses into tetraparalysis, cranial nerve dysfunction, and possibly to respiratory failure and death (Barsanti et al., 1978; Bruchim et al., 2006; Uriarte et al., 2010; Lamoureux et al., 2015). The reason for the different manifestations of botulism in dogs and human patients is currently not known, but species differences exist regarding target proteins of the toxin and their aminoacid sequences (Peng et al., 2014).

Botulism is also a concern in the medical use of the toxin. Symptoms resembling generalized botulism have been reported in human patients after IM BoNT A treatment (Bakheit et al., 1997; Bhatia et al., 1999; Wyndaele and Van Dromme, 2002; Duffey and Brown, 2006; Howell et al., 2007; Crowner et al., 2010). Despite this, IM and subdermal BoNT A injections are considered safe in human patients (Naumann and Jankovic, 2004; Dong et al., 2017; Morra et al., 2016). Local muscle weakness is the most commonly reported adverse effect after IM and intradermal BoNT A treatment (Naumann and Jankovic, 2004). It is believed to be a consequence of diffusion of the toxin into adjacent tissues (Wheeler and Smith, 2013). It is not known whether the toxin can spread into adjacent structures after an IA injection.
2.1.5.2 Electrophysiological changes

Electrophysiological recordings detect disturbances in the neuromuscular transmission and are therefore used in the diagnostics of botulism in human (Preston, 2013) and veterinary patients (Anor, 2014). Electrophysiological recordings can also be used to investigate the spread of the toxin in patients without clinical signs (Olney et al., 1988; Lange et al., 1991).

The typical findings in patients with botulism are low compound muscle action potentials (CMAPs) after a single stimulation, a decrease in the CMAPs in repetitive nerve stimulation (RNS) at low frequency rates, and an increment in the CMAPs in RNS at high frequency rates. Electromyography (EMG) may show spontaneous activity or a normal measurement. Motor nerve conduction velocity (MNCV) is normal. (Cherington, 1998; Preston, 2013) Single-fibre EMG (SFEMG) may be a more sensitive method to diagnose botulism than conventional electrophysiological testing in human patients. It typically shows increased jitter and blocking. (Padua et al., 1999)

There are three case reports on dogs with botulism, which describe the findings of electrophysiological recordings (Barsanti et al., 1978; van Nes and van der Most van Spijk, 1986; Uriarte et al., 2010). As in humans, the reported findings include low CMAPs, a decrease in the CMAPs in RNS at low frequency rates, and normal EMG or spontaneous activity. In contrast to human patients, decreased MNCV and an inconsistent decrease of the CMAPs in RNS at high frequency rates have been reported. SFEMG has been described in healthy dogs (Hopkins et al., 1993), but to the best of the author’s knowledge, there are no reports on SFEMG on dogs with botulism.

2.1.5.3 Histopathological changes

BoNT injections cause histopathological changes in the injected muscles and adjacent structures. These changes can be used to assess the spread of the toxin, but their clinical relevance is not known.

IM BoNT A leads to denervation atrophy of the injected muscle. The atrophy is dose-dependent and long-lasting, persisting at least for a year after a single injection. (Rosales et al., 1996; Choi et al., 2007; Schroeder et al., 2009) Interestingly, denervation atrophy has also been detected in the contralateral muscles (Rosales et al., 1996) and in muscles very distant from the injection site (Ansved et al., 1997) after repeated IM administration, suggesting the spread of the toxin and a cumulative effect. Atrophy in the contralateral muscles has not been observed after a single IM injection (Schroeder et al., 2009).

The effects of IM BoNT A injection on the nerve innervating the injected muscles have been examined in mice (Rosales et al., 1996) and rabbits (Elmas et al., 2007). Sprouting of nerve terminals is an expected finding after IM BoNT A injection (Rosales et al., 1996), but increased rates for Wallerian degeneration in the nerve have also been reported (Elmas et al.,
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2007). The reason for the Wallerian degeneration after IM BoNT A is not known.

Studies on the histopathological effects of BoNT A inside a joint are scarce. DePuy et al. (2007) reported no pathological changes in the synovium of two healthy horses after IA BoNT A, and Henzel et al. (2008) reported no increase in the rate of cell death in a chondrocyte culture when administration of BoNT A was compared to administration of saline.

Additionally, the effects of IA BoNT A inside a joint have been studied in animal models of OA and inflammatory arthritis (Namazi, 2006; Namazi and Torabi, 2007; Yoo et al., 2014). Repeated injections of IA BoNT A had slowed down the degeneration of articular cartilage and decreased scar formation in rabbits 12 weeks after the induction of OA (Namazi, 2006; Namazi and Torabi, 2007). In a rat model of acute inflammatory arthritis, IA BoNT A reduced significantly inflammation and interleukin-1β (IL-1β) immunoreactivity in the synovium and articular cartilage (Yoo et al., 2014).

2.2 OSTEOARTHRITIS

2.2.1 AETIOLOGY

OA is a chronic, slowly progressive condition of the synovial joints. It is characterized by cartilage degradation, subchondral bone remodelling, osteophyte formation, and synovial inflammation (Johnston, 1997; Bijlsma et al., 2011). OA is presumably very common among dogs, although the data on its prevalence are scarce. Davies (2012) reported that 24% of 45 dogs brought to a geriatric screening had clinical signs associated with OA. In a survey among veterinary practitioners and specialists, it was found that OA was considered the leading cause of chronic pain in dogs (Bell et al., 2014). OA is also one of the most common reasons for euthanasia in dogs (Bonnett et al., 2005), especially in working animals (Moore et al., 2001).

OA can be classified as primary (idiopathic) or secondary depending on whether an initiating cause can be identified (Clements et al., 2006). In contrast to humans, canine OA is most commonly a secondary consequence of alterations in cartilage homeostasis and biomechanics due to osteochondrosis, hip or elbow dysplasia, or cranial cruciate ligament rupture (Clements et al., 2010; Innes, 2012).

OA is a multifactorial polygenetic disease (Sandell, 2012). In addition to the hereditary component, its aetiology involves a complex interplay of biochemical, biomechanical, and environmental factors (Glyn-Jones et al., 2015). Despite rigorous research, the aetiology of OA is not completely understood. Breed, increased age, obesity, high body weight, male sex, and joint laxity have been identified as risk factors for OA development in dogs (Smith et al., 2001; 2006; Hays et al., 2007). In addition to these, it is recognized that several other factors such as birth during winter or spring,
high birth weight, housing on a slippery surface as a puppy, living in a warmer climate, female sex, neutering, high fat intake, and certain types of exercise, increase the risk of joint dysplasias (van Hagen et al., 2005; Salg et al., 2006; Sallander et al., 2006; Loder and Todhunter, 2017). However, these risk factors may vary among breeds.

A specific quantitative trait locus associated with acetabular osteophyte formation due to hip dysplasia has been identified in Portuguese water dogs (Chase et al., 2005) and several quantitative trait loci have been related to hip dysplasia in various breeds (Todhunter et al., 2005; Marschall and Distl, 2007; Phavaphutanon et al., 2009). In addition, various candidate genes have been suggested for canine joint dysplasias and OA (Salg et al., 2006; Clements et al., 2010), but to date an association has only been found between one candidate gene, FBN2, and canine hip dysplasia (Friedenberg et al., 2011).

2.2.2 PATHOPHYSIOLOGY

The ends of articulating bones are covered with hyaline cartilage, which allows optimal force distribution and smooth gliding of joint surfaces on each other during limb loading and motion (Johnston, 1997). Articular cartilage consists of scattered chondrocytes that are surrounded by extracellular matrix (ECM) made up mainly of type II collagen, proteoglycans, and water (Craig et al., 2016). Type II collagen fibrils form a supporting meshwork in which proteoglycans are embedded drawing water into the cartilage. ECM is produced and maintained by chondrocytes. The unique structure in ECM provides articular cartilage its tensile strength and resistance against compressive forces.(Glyn-Jones et al., 2015)

The hallmark feature of OA is the progressive destruction of articular cartilage. In OA, the structure of ECM is gradually destroyed. Its water content increases, the proteoglycan content decreases, and the collagen network is broken down (Bollet and Nance, 1966; Inerot et al., 1978; Grynpas et al., 1994; Guilak et al., 1994), which leads to a weaker cartilage that is more susceptible to damage during normal limb loading (Guilak et al., 1994). In the early stages of OA, the chondrocytes proliferate and try to compensate for the changes in the ECM by increasing its synthesis (Lorenzo et al., 2004). However, chondrocytes become apoptotic and are eventually lost from the matrix. Macroscopically, OA begins with superficial fibrillations in the articular cartilage and progresses into thinned, necrotic cartilage with deep erosions and ulcers that reach the subchondral bone (Grynpas et al., 1994; Craig et al., 2016). In the end stage of OA the articular cartilage is completely lost from the joint surfaces and the underlying bones are exposed, rubbing painfully against each other during motion (Craig et al., 2016).

The cartilage degradation is driven forward, and possibly initiated by chronic, low-grade inflammation inside the joint (Sokolove and Lepus, 2013). Synoviocytes, chondrocytes, and synovial inflammatory cells produce
proinflammatory cytokines, specifically IL-1β and tumour necrosis factor-alpha (TNF-α) (Todhunter et al., 1996; Benito et al., 2005; Fujita et al., 2006), which induce the overexpression of other cytokines as well as cartilage-degrading collagenases and proteinases, such as matrix metalloproteinases and proteinases of the ADAMTS family (Clegg et al., 1997; Fernandes et al., 2002; Kevorkian et al., 2004; Sokolove and Lepus, 2013). Cartilage degradation products released into the synovial fluid (SF) increase the expression of these enzymes, leading into a vicious cycle, which drives forward the cartilage degeneration and inflammatory cascade inside the joint (Homandberg et al., 1998). Prostaglandin E2 (PGE2), nitric oxide, and various other cytokines, in excess to their inhibitors, are also involved in the pathogenesis of OA by inhibiting the synthesis and exacerbating the degradation of ECM, promoting chondrocyte apoptosis, and stimulating bone resorption (Martel-Pelletier et al., 1999; 2003; Sokolove and Lepus, 2013). Neuropeptides, which are released from the nerve endings of the primary afferent neurons into the joint, also contribute to this complex puzzle by promoting inflammation and affecting chondrocyte function (Sutton et al., 2009). It is not known whether the inflammation in the synovium is the primary phenomenon initiating the cartilage degeneration or whether the inflammation is a consequence of the changes in the cartilage (Sutton et al., 2009; Sokolove and Lepus, 2013).

Sclerosis and microfractures of the subchondral bone and new bone outgrowth in the joint margins (osteophytosis) are also prominent features in OA (Strom and Svalastoga, 1993; Havdrup et al., 2009; Craig et al., 2016). The changes in the subchondral bone are a consequence of a modelling and remodelling process mediated by osteoclasts and osteoblasts as an adaptation to biomechanical and biochemical changes in the bone tissue (Goldring and Goldring, 2010). The sclerotic subchondral bone is less compliant, which increases loading on articular cartilage (Craig et al., 2016). In addition, the alterations in the subchondral bone change the contour of the articular surfaces, which leads to incongruity of the joint contributing to abnormal loading (Goldring and Goldring, 2010). There is no consensus on the chronological order of the changes detected in the subchondral bone and cartilage (Dieppe and Lohmander, 2005; Goldring and Goldring, 2010).

### 2.2.3 OSTEOARTHRITIC PAIN

Pain is the most debilitating symptom in OA. Osteoarthritic pain is chronic and mainly of a nociceptive type, although a neuropathic pain component might also be involved (Lamont et al., 2000; Schaible et al., 2009). Human patients often describe osteoarthritic pain as dull, aching, and poorly localized (Schaible et al., 2009). In dogs, OA has been associated with lameness, stiffness, difficulties in jumping, getting up or lying down, and subtle changes in demeanour and behaviour, such as loss of general activity and unwillingness to play (Brown et al., 2007; Hercock et al., 2009; Hielm-
Björkman et al., 2009). The intensity of osteoarthritic pain varies. Typically, the pain is relieved at rest and worsens during exercise (Beraud et al., 2010). Episodic pain may become constant when OA progresses. Joint pain leads to a vicious cycle, in which diminished locomotion causes muscle atrophy, which further increases the loading on the weakened articular cartilage, thereby exacerbating its degeneration (Schaible et al., 2002).

2.2.4 PHYSIOLOGY OF JOINT PAIN

Conscious sensations can be provoked from all joint structures except for the aneural articular cartilage (Lamont et al., 2000; Schaible et al., 2009). The nociception of joints is mediated via primary afferent neurons. The primary afferent neurons contain Aβ-, Aδ-, and C-fibres (Schaible et al., 2009). The myelinated Aβ-fibres have a low impulse threshold and they serve as mechanoreceptors, which respond to innocuous stimuli and provide information on the position and movement of joints (Dorn et al., 1991). The thinly myelinated Aδ- and unmyelinated C-fibres have high thresholds and respond to noxious stimuli providing information on imminent tissue damage. A part of the C-fibres are silent nociceptors, which do not respond to noxious stimuli under normal conditions but become nociceptive during inflammation (Lawson, 2002).

The cell bodies of the primary afferent neurons are located in the dorsal root ganglia, from where they extend axons into the dorsal horn of the spinal cord. There, in the simplest of situations, they synapse with the second-order neurons, which transmit the impulse to the thalamus and synapse with the third-order neurons, which transmit the stimulus into higher brain centres for pain perception (Lamont et al., 2000). However, in reality, the noxious impulse is modified by various interneurons during its course from the joint into the brain. In addition, some of the second-order neurons receive additional input from other structures such as skin and also inhibitory mechanisms modulate the pain impulse in the spinal cord. (Schaible et al., 2009)

2.2.4.1 Peripheral and central sensitization

In OA, normal movement is painful for the patient. This is due to mechanical hyperalgesia, which is the dominant feature of osteoarthritic pain. In mechanical hyperalgesia, normal joint movement elicits a sensation of pain and noxious stimuli produce an abnormally strong pain response (Lamont et al., 2000; Schaible et al., 2002).

Mechanical hyperalgesia is a consequence of peripheral and central sensitization in the nervous system. In peripheral sensitization, the sensitivity of the primary afferent nociceptive neurons is increased due to a decrease in their impulse thresholds. This is caused by the action of various
inflammatory mediators and neurotransmitters in the joint, such as cytokines, prostaglandins, neuropeptides, and bradykinin (McDougall, 2006; Schaible, 2014). When they bind to their receptors in the afferent nerve ending the sensitivity of the ion channels in the cell membrane is increased, leading to easier depolarization of the cell. In peripheral sensitization, the receptors for the inflammatory mediators are upregulated, thereby enhancing this phenomenon (Schaible et al., 2002). In addition, some neurotransmitters, such as the proinflammatory neuropeptide SP, are not only released into the synaptic cleft in the dorsal horn but also antidromically (in the opposite direction to the nerve impulse transmission) released back into the joint after a nociceptive stimulus (Yaksh, 1988). This promotes further inflammation in the joint and facilitates peripheral sensitization (Garrett et al., 1992). Finally, the mechano-insensitive silent nociceptors also begin to respond to mechanical stimuli (Lamont et al., 2000).

Thus, the sensitized primary afferent neurons are more frequently depolarized and release increased amounts of both peripheral and intraspinal neurotransmitters, which leads to changes also in the neurons in the spinal cord (Schaible et al., 2009). The excitability of the second-order neurons is increased and their receptive fields are enlarged to include not only the joint but also adjacent tissues (Schaible et al., 1987; Neugebauer et al., 1993). The most important intraspinal neurotransmitter/receptor mechanism associated with central sensitization is glutamate and activation of its N-methyl-D-aspartate (NMDA) receptors (Schaible et al., 2002; Lee et al., 2011). However, SP, CGRP, enzymes such as cyclooxygenase-2 (COX-2), and inflammatory mediators such as PGE₂ are also associated with initiating and maintaining the central sensitization (Schaible et al., 2009).
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Figure 2. Illustration of the effect of mechanical stimulus on a normal (left) and sensitized (right) nociceptive neuron. Nerve endings of nociceptive Aδ- and C-fibres are insensitive to mechanical stimuli under normal conditions (left). In peripheral sensitization (down right), Aδ- and C-fibres have become mechanosensitive and are more easily depolarized as a result of the action of various inflammatory mediators and neuropeptides. Thus, mechanical stimulus leads to the depolarization of the nerve ending and transmission of a nerve impulse into the spinal cord. In central sensitization (up right), the presynaptic primary afferent neurons in the dorsal horn of the spinal cord release increased amounts of glutamate and neuropeptides into the synaptic cleft. The post-synaptic second-order neurons express an increased number of receptors to these excitatory neurotransmitters and are thus more easily depolarized. In addition, their receptive fields (not shown) are enlarged.

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid; BK, bradykinin; Cy, proinflammatory cytokines; Glu, glutamate; NMDA, N-methyl-D-aspartate; NP, neuropeptides; PG, prostaglandins; R, receptor.
2.2.5 OSTEOARTHRITIC PAIN MEDIATORS

2.2.5.1 Substance P
SP has been associated with OA and joint pain in various species. In horses, the concentration of SF SP is increased both in naturally occurring OA (Caron et al., 1992; Kirker-Head et al., 2000) and in induced inflammatory arthritis (de Grauw et al., 2009). In human patients, an increased number of SP-positive nerve fibres is associated with painful OA (Saxler et al., 2007). The concentration of SF SP correlates with joint pain both in horses (de Grauw et al., 2006) and in humans (Gotoh et al., 1998).

In dogs, SP-containing nerve fibres have been found in the carpal, shoulder, and stifle joints (Nakayama et al., 1995; Tamura et al., 1998; Karahan et al., 2002). Cranial cruciate ligament rupture leads to an increased concentration of SP in the spinal cord of dogs (Rialland et al., 2014) and the concentration of SP in cerebrospinal fluid is associated with cervical hyperaesthesia in dogs with syringomyelia (Schmidt et al., 2013). However, the association of SF SP with joint pain and OA has not been previously studied in this species.

2.2.5.2 Prostaglandin E<sub>2</sub>
PGE<sub>2</sub> has an important role in inflammatory pain in arthritis (Lee et al., 2013). It is produced from arachidonic acid by the action of COX enzymes in a variety of cells (Ganesh, 2013). In OA, PGE<sub>2</sub> is associated with both peripheral and central sensitization (Lee et al., 2013; Natura et al., 2013) as well as cartilage degradation by stimulating the synthesis of proteolytic enzymes from chondrocytes (Trumble et al., 2004; Lee et al., 2013). SP stimulates the release of PGE<sub>2</sub> from bovine synoviocytes (Halliday et al., 1993) and the concentrations of PGE<sub>2</sub> and SP correlate in equine SF (Kirker-Head et al., 2000).

The concentration of SF PGE<sub>2</sub> correlates negatively with ground reaction forces and positively with pain in dogs with transected cranial cruciate ligaments (Trumble et al., 2004). A positive correlation with pain and lameness or dysfunction has also been found in horses and osteoarthritic human patients (Brenner et al., 2004; Hogberg et al., 2006). The concentration of SF PGE<sub>2</sub> and its association with pain has not been studied in dogs with naturally occurring OA.

2.2.5.3 Tumour necrosis factor-alpha
TNF-α is one of the principal proinflammatory cytokines involved in the pathophysiology and pain in OA (Martel-Pelletier et al., 1999; Kapoor et al., 2011). It is produced by various cells in the articular cartilage and synovium
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(Kapoor et al., 2011). TNF-α has a major role in initiating the inflammatory cascade characteristic of OA. It increases the production of other cytokines and PGE2, which leads to pain and cartilage degradation (Lee et al., 2013). In addition, TNF-α acts directly on the nerve endings of the primary afferent neurons generating nociceptive nerve impulses (Lee et al., 2013; Schaible, 2014).

Both higher (Venn et al., 1993) and lower (Hay et al., 1997) concentrations of SF TNF-α have been reported in osteoarthritic dogs compared to healthy dogs. In the study by Venn and colleagues (1993), the concentration of TNF-α correlated with that of glycosaminoglycans, degradation products of articular cartilage in dogs with early experimental OA. In osteoarthritic horses, the expression of TNF-α is increased in the synovium and cartilage and its concentration is elevated in the SF (Kamm et al., 2010). In human patients, the concentration of SF TNF-α correlates with osteoarthritic pain (Orita et al., 2011). The correlation between SF TNF-α and osteoarthritic pain has not been previously investigated in dogs.

2.2.6 CONSERVATIVE TREATMENT OF OSTEOARTHRITIC PAIN IN DOGS

2.2.6.1 Oral pain medication

Oral non-steroidal anti-inflammatory drugs (NSAIDs) are the mainstay in pain treatment in osteoarthritic dogs. They inhibit COX-1 and COX-2 isoenzymes, decreasing the production of prostaglandins, prostacyclin, and thromboxane A2 from arachidonic acid (Day and Graham, 2013). Newer NSAIDs are COX-2 selective, which is associated with fewer gastrointestinal and haematological adverse events due to sparing of the constitutive physiological functions of COX-1 (Xu et al., 2016).

The evidence to support the use of NSAIDs in pain relief in canine OA is strong. Among the various NSAIDs, meloxicam, carprofen, and firocoxib had the strongest evidence regarding their efficacy in the two systematic reviews on the management of canine OA (Aragon et al., 2007; Sanderson et al., 2009). However, these reviews pre-date studies on some newer molecules such as robenacoxib (Edamura et al., 2012; Reymond et al., 2012; Bennett et al., 2013) and mavacoxib (Walton et al., 2014; Lees et al., 2015; Payne-Johnson et al., 2015) in the treatment of osteoarthritic pain in dogs. It is not known which NSAID would be the most effective in relieving joint pain in dogs (Johnston et al., 2008). For dogs with OA, long-term use of NSAIDs is recommended over short-term administration due to the chronicity of the disease and the benefits gained from keeping the dog active (Innes et al., 2010). In addition, some NSAIDs may produce cumulative antinociceptive effects over time during long-term treatment (Walton et al., 2014).
Diarrhoea and vomiting are the most commonly reported adverse events related to NSAID use in osteoarthritic dogs, but more serious events such as gastrointestinal perforation and hepatotoxicity have also been reported. The incidence of adverse events is estimated to be very low. (Monteiro-Steagall et al., 2013) In addition, long-term use of NSAIDs did not show an increase in the adverse event rate compared to shorter-term use (Innes et al., 2010). Despite this, NSAID-related adverse events are a concern for veterinarians and dog owners and NSAIDs are not considered suitable for every osteoarthritic dog (Belshaw et al., 2016a). In addition, their antinociceptive efficacy may not be strong enough in advanced OA.

Combining NSAIDs with other analgesics may produce greater analgesia than NSAIDs alone. However, only amantadine has been investigated in the peer-reviewed literature as an adjunct to NSAIDs in dogs with OA (Lascelles et al., 2008). When used as an adjunct to meloxicam and compared to placebo, amantadine significantly increased the physical activity of osteoarthritic dogs measured with an owner-specific questionnaire after 42 days of therapy. Amantadine inhibits NMDA-mediated responses in the central nervous system (Blanpied et al., 2005) and it might therefore decrease the central sensitization associated with OA pain (Lascelles et al., 2008).

Tramadol is commonly prescribed as an alternative to NSAIDs for osteoarthritic dogs with intolerable side effects (Belshaw et al., 2016a). It is a weak opioid analgesic, which mediates its effect via agonism of μ-opioid receptors and inhibition on serotonin and noradrenaline uptake (Schütter et al., 2017). The antinociceptive efficacy of tramadol on osteoarthritic pain in dogs has been studied in one placebo-controlled, randomized clinical trial in dogs with hip OA. In this study, oral tramadol led to significant improvement in mobility assessed with a validated owner questionnaire, but no improvement was detected in the ground reaction forces or accelerometer measurements (Malek et al., 2012). In human patients, tramadol has shown the same potency in relieving osteoarthritic pain as NSAIDs (Smith et al., 2016). However, in dogs, the short elimination half-life of the drug might require unpractically frequent dosing (KuKanich and Papich, 2004).

Gabapentin is an antiepileptic drug with antinociceptive activity in neuropathic pain due to effects on calcium channel α2δ subunits in the CNS (Patel and Dickenson, 2016). It is currently recommended for human patients with chronic neuropathic pain (Finnerup et al., 2015). Gabapentin is commonly used as a part of multimodal analgesia for dogs with OA. However, there are no studies investigating its efficacy in dogs with chronic pain. The short-term efficacy of gabapentin as an add-on analgesic to opioids has been studied in dogs undergoing surgery due to intervertebral disc disease (Aghighi et al., 2012) and front limb amputation (Wagner et al., 2010). In both studies, gabapentin did not produce clinically relevant additive effects to post-operative opioids when compared to placebo.
2.2.6.2 **Nutraeuticals**

Various nutraceutical products are available for osteoarthritic dogs, but the evidence regarding their efficacy in pain relief or disease modification in dogs is limited and difficult to assess due to the different formulations, salt forms, dosages, treatment regimens, and outcome measures used in the clinical trials (Beale, 2004; Vandeweerd et al., 2012; Comblain et al., 2016).

The antinociceptive efficacy of fish oil, green-lipped mussel, glucosamine hydrochloride and chondroitin sulfate, elk velvet antler, undenatured type II collagen, milk protein concentrate, calcium pentosan polysulphate, Indian and Javanese turmeric extract P54FP, a multivitamin antioxidant, hydroxycitric acid, dietary beta-1.3/1.6-glucans, gelatin hydrolysate, and boswellia resin has been studied in controlled trials in osteoarthritic dogs using various subjective and objective evaluation methods (Beale, 2004; Moreau, et al., 2004; Aragon et al., 2008; Vandeweerd et al., 2012; Comblain et al., 2016).

From these, fish oil had enough good-quality evidence to support its use in alleviating clinical signs in osteoarthritic dogs in a systematic review by Vandeweerd et al. (2012). Although some conflicting evidence existed, green-lipped mussel (*Perna canaliculus*) also showed potency in pain relief in dogs with OA. Fish oil and green-lipped mussel contain omega-3 fatty acids, especially eicosapentaenoic acid and docosahexaenoic acid, which compete with arachidonic acid as substrates for COX enzymes in the cell membranes and provide less inflammatory metabolic end products than arachidonic acid, thereby decreasing inflammation in the joint (Wann et al., 2010). They also decrease the release of degradative enzymes and cytokines providing a chondroprotective effect (Wann et al., 2010; Schwager et al., 2015; Buddhachat et al., 2017).

Chondroitin sulfate and glucosamine are also commonly recommended for osteoarthritic dogs. Chondroitin sulfate is a glycosaminoglycan and glucosamine is a precursor for glycosaminoglycans in the ECM of articular cartilage. In cell cultures, glucosamine and chondroitin sulfate have shown anti-inflammatory and anabolic synergistic effects on articular cartilage and synovium (Gouze et al., 2006; Uitterlinden et al., 2008; Calamia et al., 2014; Stoppoloni et al., 2015). Crystalline glucosamine sulfate has the greatest efficacy in human OA (Kucharz et al., 2016), but this glucosamine salt form has not been studied in dogs.

In a recent review, no recommendations could be given on the use of glucosamine or chondroitin sulfate in osteoarthritic dogs due to conflicting evidence on their efficacy (Bhathal et al., 2017). While two studies reported pain relief in subjective outcome measures (McCarthy et al., 2007; Gupta et al., 2012), no statistically significant improvement from baseline was detected in two other studies (Moreau et al., 2003; D’Altilio et al., 2007). Conflicting evidence on glucosamine and chondroitin sulfate in symptom relief and disease modification in osteoarthritic human patients has led to
contradictory guidelines on their use in medicine (Hochberg et al., 2012; McAlindon et al., 2014).

2.2.6.3 Intra-articular treatment

IA treatment is directly targeted into the painful joint, which can produce greater pain relief and reduce the systemic adverse effects of pain therapy (Nguyen et al., 2016). However, the scientific evidence on the clinical efficacy and adverse effects of IA treatment in canine OA is scarce.

In osteoarthritic human patients, conventional IA therapy consists of injections of CS or HA. However, despite decades of use in medicine, controversy exists regarding their efficacy, adverse effects, and guidelines for use (Hochberg et al., 2012; McAlindon et al., 2014).

CS has produced degradation and chondrocyte apoptosis in canine cartilage \textit{in vitro} (Murphy et al., 2000; Hossain et al., 2008; Euppayo et al., 2016), but only positive effects have been reported in the canine models of OA (Vandeweerd et al., 2015). Treatment with IA CS has been shown to slow down the development of cartilage lesions and osteophytes (Pelletier and Pelletier, 1989; Pelletier and Martel-Pelletier, 1991; Pelletier et al., 1994) and to decrease the synthesis of proteolytic enzymes and cytokines in the articular cartilage of dogs with experimental OA (Pelletier et al., 1995). However, there are no controlled clinical trials on the efficacy of IA CS on clinical signs of dogs with OA.

HA is responsible for the viscoelasticity of SF (Johnston, 1997). In OA, the quality and quantity of SF HA are altered due to decreased production and fragmentation (Venable et al., 2008). HA injections aim to restore the viscoelasticity of SF in the osteoarthritic joint, but IA HA also has anti-inflammatory, antinociceptive, and chondroprotective properties (Boettger et al., 2011; Bauer et al., 2016).

The evidence on the efficacy of IA HA on clinical signs and cartilage pathology in osteoarthritic dogs is mixed. Brandt et al. (2004) reported that IA HA did not improve the ground reaction forces or cartilage pathology in dogs with experimental OA. However, in a more recent study, IA HA significantly increased limb loading compared to placebo-treated limbs in dogs with experimental OA, although no difference was detected in cartilage pathology between groups (Pashuck et al., 2016). In addition, IA HA treatment provided more pain relief measured with validated owner questionnaires than IA saline and oral carprofen or a nutraceutical in a study by Carapeba et al. (2016). The discrepancy in the results may be due to different dosages, treatment regimens, and different products used.

Stem cells and other regenerative therapies such as platelet-rich plasma (PRP) have attracted attention in the treatment of OA recently. Instead of palliative pain treatment, the aim of regenerative therapies is tissue repair (Sherwood et al., 2017). Stem cells have an affinity for damaged tissue, such as articular cartilage, and they contribute to tissue regeneration (Agung et al.,
In addition, stem cells have shown anti-inflammatory effects in the articular cartilage in dogs with experimental OA (Yun et al., 2016). Platelets contain various growth factors that promote regeneration of cartilage in the osteoarthritic joint when given in supraphysiological amounts (Mascarenhas et al., 2015).

An IA injection of autologous adipose-derived stem cells (ADSCs) had positive benefits in clinical trials in osteoarthritic dogs assessed by subjective owner and veterinary evaluations (Black et al., 2007; Cuervo et al., 2014; Harman et al., 2016) and ground reaction forces (Vilar et al., 2014) in dogs with naturally occurring OA. In addition, IA PRP was shown to produce significant improvement from baseline in owner pain evaluation (Upchurch et al., 2016) and in ground reaction forces in osteoarthritic dogs (Fahie et al., 2013). IA ADSCs might be more effective in relieving osteoarthritic pain in dogs than IA PRP (Cuervo et al., 2014) and combining these two treatments may produce longer pain relief than the stem cells alone (Vilar et al., 2013; 2014).

Also, an injection of local irritants can be considered regenerative treatment inside a joint. IA-injected concentrated dextrose leads to osmotic tissue trauma which is thought to enhance tissue repair (Sherwood et al., 2017). However, an IA injection of concentrated dextrose did not produce any improvement in subjective or objective outcome variables when investigated in dogs with knee or elbow OA and compared to placebo (Sherwood et al., 2017).

### 2.2.6.4 Exercise modification and physiotherapy

Exercise modification is considered important in canine OA management and veterinarians commonly recommend it for osteoarthritic dogs (Bound et al., 2011). Although moderate, routine, and controlled exercise is recommended for osteoarthritic dogs, there is a lack of evidence on the best exercise regimen or on the benefits of exercise in general in canine OA. The ground reaction forces of osteoarthritic dogs decreased when measured immediately after a run of 1.2 km, which was interpreted as an increase in joint pain after exercise (Beraud et al., 2010). However, the long-term effects of exercise have not been studied in dogs.

Land- and water-based exercise and strength training are recommended for osteoarthritic human patients (Hochberg et al., 2012; McAlindon et al., 2014). In human patients, meta-analyses have shown positive effects of exercise on pain and function in OA without favoring any specific exercise regimen (McAlindon et al., 2014).

Physiotherapy is less often recommended for osteoarthritic dogs than exercise modification (Bound et al., 2011). There is currently limited scientific evidence on the benefits of physiotherapy in osteoarthritic dogs. Crook et al. (2007) showed that passive stretching of joints leads to improvement in the range of motion in osteoarthritic joints in dogs. Mueller
et al. (2007) and Souza et al. (2016) reported an improvement in the ground reaction forces in osteoarthritic dogs after radial shockwave therapy, while Dahlberg et al. (2005) did not detect any significant improvement from baseline in the ground reaction forces or in an owner questionnaire in dogs treated with extracorporeal shockwave therapy, although there was a trend for improvement in the range of motion of the treated joint. Combining diet restriction with intensive physiotherapy seems to improve both weight loss and mobility in lame and overweight dogs (Mlacnik et al., 2006).

2.2.6.5 Bodyweight management
There is scientific evidence to support bodyweight management for osteoarthritic dogs and for dogs in general. Limited food intake and thus normal body condition decreases the incidence and severity of OA in dogs (Kealy et al., 2000) and shifts the onset of OA-related clinical signs to later in life (Kealy et al., 2002). In addition, limited food intake is associated with a longer life span in dogs in general (Kealy et al., 2002). Obesity has been shown to affect the gait of dogs. Compared to lean dogs, the peak vertical forces (PVFs) of obese dogs are greater, which leads to larger stresses on the surfaces of joints (Brady et al., 2013) and could predispose them to OA.

There is also some evidence to support weight loss in the management of painful OA in obese dogs (Impellizeri et al., 2000; Marshall et al., 2010). In these studies, a significant improvement in lameness began when overweight and obese osteoarthritic dogs had lost approximately 6% of their bodyweight. The improvement was seen in subjective lameness scores and VAS evaluated by veterinarians. No control group was enrolled in these studies. Weight loss is also recommended for overweight human patients with knee and hip OA in the guidelines provided for medical professionals (Hochberg et al., 2012; McAlindon et al., 2014).

2.2.7 EVALUATION OF OSTEOARTHRITIC PAIN IN DOGS

2.2.7.1 Dynamic weight-bearing
Measuring ground reaction forces with a force plate is an objective and quantitative method to evaluate lameness and dynamic weight-bearing in dogs (Voss et al., 2007; Volstad et al., 2016). It is widely used for evaluating treatment outcomes in various orthopaedic conditions in veterinary medicine. From the vertical, horizontal, and mediolateral forces obtained with a force plate, PVFs and vertical impulses (VIs) are the most commonly reported.

A force plate can detect even very subtle changes in the weight-bearing of a dog, i.e. lameness not visible to the naked eye. However, the
measurements are affected by many factors, such as the characteristics of the dog including its conformation, bodyweight, size, and stride frequency (Bertram et al., 2000; Mölsä et al., 2010; Kano et al., 2016; Volstad et al., 2016), which makes it difficult to compare the results between studies. There are also factors affecting the measurements, when the results of an individual dog are followed over time, such as the velocity of the dog (Riggs et al., 1993; Voss et al., 2010; Volstad et al., 2016), inter-week variation (Nordquist et al., 2011), exercise before the measurements (Beraud et al., 2010) and especially the number of trials (stance phase measurements) collected (Volstad et al., 2016). Therefore, measures to limit the variance in these factors such as narrow ranges for acceptable velocity and acceleration are commonly used, although this has been questioned recently (Volstad et al., 2016).

To reduce the effect of velocity between different examination time points, a symmetry index (SI) can be calculated instead of using the absolute values of the ground reaction forces (Fanchon and Grandjean, 2007). An index of 0 indicates moving in perfect symmetry. Ideally, the weight-bearing between contralateral limb pairs should be symmetrical in a healthy dog at trot. However, mean values of 2.4–6.6 have been reported for healthy trotting dogs (Voss et al., 2007; Souza et al., 2016; Volstad et al., 2016). The magnitude of the values may affect the precision of the SI. Therefore, alternative methods, such as calculating the percentage of bodyweight distribution for each limb, have recently been suggested for the evaluation of symmetry in weight-bearing (Kano et al., 2016).

### 2.2.7.2 Static weight-bearing

Static weight-bearing measurements provide information on limb functionality in dogs (Hyytiäinen et al., 2012; 2013). A simple method for measuring the static weight-bearing with two bathroom scales has been validated in dogs with stifle OA (Hyytiäinen et al., 2012). The use of pressure plate systems has also been reported (Brydges et al., 2012).

Static weight-bearing measures the ground reactive force of the dog while the dog is standing still (Meadows et al., 1990). In healthy dogs, approximately 60% of bodyweight is borne on the front limbs and 40% on the hindlimbs with equal distribution between the limb pairs while standing. In the study by Hyytiäinen et al. (2012), a cut-off value of 6% of bodyweight was proposed for the difference in the static weight-bearing between the hindlimbs in healthy dogs. Dogs with stifle OA shifted weight from the affected limb to the contralateral limb and had less variance in repeated measurements in the OA limb than healthy dogs, which was suggested to be a consequence of avoiding pain in the OA limb.

Static weight-bearing measurements have been used to evaluate limb function in canine models of bone healing (Meadows et al., 1990; Aro et al., 1991) and for evaluating surgical outcome in dogs with cranial cruciate ligament rupture (Mölsä et al., 2014). A reduction in the static weight-
bearing in dogs with cranial cruciate ligament rupture is associated with increased sensory sensitivity in the affected limb, a finding supposed to reflect chronic pain in dogs (Brydges et al., 2012). In rats with experimental OA, pain medication significantly and dose-dependently increases the static weight-bearing in the osteoarthritic limb (Bove et al., 2003). Thus, it can be speculated that measuring static weight-bearing provides information not only on functionality but also on joint pain in the limb.

2.2.7.3 Goniometry

Goniometry is an objective and reliable method for evaluating passive range of motion in a joint and thus joint function in healthy and osteoarthritic dogs (Jaegger et al., 2002; Hyytiäinen et al., 2013). A technique with a universal plastic goniometer has been validated for obtaining the measurements both in healthy dogs (Jaegger et al., 2002) and in dogs with OA (Hyytiäinen et al., 2013).

The range of motion in a joint is obtained by measuring the angle between bones with the joint in extension and flexion and subtracting the latter measurement from the former (Jaegger et al., 2002). In an osteoarthritic joint, the range of motion is limited by mechanical factors such as fibrosis of the joint capsule and periarticular osteophytes and a sensation of pain due to mechanical hyperalgesia (Johnston, 1997). Repeated goniometric measurements have been used to evaluate treatment outcomes in osteoarthritic dogs (Imhoff et al., 2011; Upchurch et al., 2016). The range of motion in the hip correlated negatively with the severity of OA and positively with dynamic weight-bearing in a dog model of experimental OA (Little et al., 2016). In osteoarthritic human patients, the range of motion in the hip and stifle joints correlates negatively with pain (van Baar et al., 1998). The correlation between joint pain and range of motion has not been studied in dogs.

2.2.7.4 Veterinary pain evaluation

There are no validated methods for a veterinarian to subjectively evaluate chronic pain in dogs (Belshaw et al., 2016b). Despite this, various subjective scoring systems have been used in the evaluation of treatment outcomes in canine OA.

One subjective scoring system commonly used by veterinarians consists of various items, which are individually scored using a descriptive scale and then summed up to form a total score (Bierer and Bui, 2002; Moreau et al., 2003; Trumble et al., 2004; Budsberg et al., 2007; McCarthy et al., 2007; Fritsch et al., 2010). However, using scores with different ranges, having different descriptions for each score, and combining different items to give a total score makes it impossible to compare results between trials (Belshaw et
al., 2016b). In addition, it is challenging to evaluate the magnitude of an improvement in this kind of scoring system when the distance between each score does not necessarily signify a similar change in the magnitude of pain (Sharkey, 2013).

A subjective pain score correlated with the ground reaction forces in dogs with transected cranial cruciate ligaments in a study by Trumble et al. (2004). In another study, no agreement was found, when three experienced veterinarians scored lameness on a five-point scale, the scores were compared to the ground reaction forces of lame dogs and very lame dogs were excluded (Quinn et al., 2007). This suggests that the scoring of lameness does not correlate with the ground reaction forces unless the lameness is severe. In addition, the agreement between the observers scoring the lameness was low (Quinn et al., 2007).

A VAS has also been used for veterinary evaluation of pain, lameness, and overall improvement in clinical trials on osteoarthritic dogs (Marshall et al., 2010; Cuervo et al., 2014; Souza et al., 2016). With a VAS, the pain of the dog is measured by marking a point in a 100 mm straight line, in which the ends represent “no pain” and “most severe pain” (Sharkey, 2013). In the study by Quinn et al. (2007), the VAS for lameness did not correlate with the ground reaction forces of lame dogs when very lame dogs were excluded.

Specific instruments have been validated for owners to evaluate chronic pain in their dogs. Also, the sensitivity and specificity of various outcome measures have been investigated for evaluating dogs with stifle OA in physiotherapy (Hyytiäinen et al., 2013). There is a demand for developing and validating similar instruments for the veterinary evaluation.

Pain evaluation in non-verbal patients is often based on the absence of normal behaviour (Sharkey, 2013). It is difficult for a person unfamiliar with the dog to evaluate the normal behaviour of a canine patient in the hospital environment. In addition, during the hospital veterinary evaluation the dog does not necessarily show the effects the pain has on its every day life. Therefore, the importance of an owner assessment that the owner can complete at home, in the dog’s natural environment, has been recognized when evaluating treatment outcomes in osteoarthritic dogs.

To date, several instruments have been developed for dog owners to assess their pets’ chronic pain and quality of life. The Glasgow University Veterinary School Questionnaire (GUVQuest) (Wiseman-Orr et al., 2004; 2006), the Helsinki Chronic Pain Index (HCPI) (Hielm-Björkman et al., 2009), the Canine Brief Pain Inventory (CBPI) (Brown et al., 2007; 2008; 2013), the Liverpool Osteoarthritis in Dogs (LOAD) questionnaire (Hercock et al., 2009; Walton et al., 2013), and the Canine Orthopedic Index (COI) (Brown, 2014a; 2014b; 2014c) are questionnaires validated for this purpose. From these, the HCPI, LOAD, and the CBPI have been tested against the
reference standard in lameness evaluation, i.e. the ground reaction forces, for
criterion validity (Hercock et al., 2009; Brown et al., 2013; Walton et al.,
2013). While Hercock et al. (2009) and Brown et al. (2013) reported no
correlation between LOAD and the CBPI and the ground reaction forces in
20 and 68 osteoarthritic dogs, respectively, a weak but significant correlation
was reported between these instruments and the SI calculated from the PVFs
of 222 osteoarthritic dogs in the study by Walton et al. (2013). The HCPI did
not correlate with the SI in that study. Finding at most a mild correlation
between the instruments for owner evaluation and the ground reaction forces
suggests that canine osteoarthritic pain as assessed by owners has more
dimensions than just lameness and a decrease in limb function.

The construct validities of the HCPI, LOAD, and the CBPI have been
tested against each other, with significant moderate correlations being found
between these instruments, which indicates that they measure similar
aspects of osteoarthritic pain in dogs (Walton et al., 2013).

In a survey among veterinarians in the UK, the difficulty in assessing
chronic pain in dogs was found to be the limiting factor for prescribing pain
medication to those patients (Bell et al., 2014). Currently, there is no
consensus on which is the best method to evaluate osteoarthritic pain in dogs
(Sharkey, 2013; Belshaw et al., 2016b). This emphasizes the fact that for
covering the different aspects of OA pain in dogs, several different evaluation
methods should be used.
3 AIMS OF THE THESIS

This work originates from studies reporting promising findings on IA BoNT A in joint pain relief in osteoarthritic human patients. We hypothesized that IA BoNT A would produce significant relief in joint pain in osteoarthritic dogs and conducted a placebo-controlled clinical trial to study the antinociceptive efficacy of IA BoNT A in this species. Also, we wanted to investigate the mechanism of the antinociceptive action of BoNT A inside the joint and to study in detail the adverse effects of this potent toxin after IA administration.

The detailed aims and hypotheses were as follows:

1. To investigate the efficacy of IA BoNT A in the treatment of chronic joint pain in osteoarthritic dogs and report possible adverse events in osteoarthritic dogs (Study I). The hypothesis was that IA BoNT A would produce significant pain relief compared to placebo and that the number of adverse events would be similar to those for the placebo group.

2. To explore the mechanism of the antinociceptive action of IA BoNT A by studying its effect on the concentration of SP, PGE$_2$, and TNF-$\alpha$ in the SF and serum of osteoarthritic dogs (Study II). The hypothesis was that the concentration of these pain mediators would decrease significantly after an IA BoNT A injection, which could explain the antinociceptive effect of the toxin inside a joint.

3. To investigate whether the SF concentration of SP, PGE$_2$, and TNF-$\alpha$ differs in osteoarthritic compared to non-osteoarthritic joints and to evaluate the associations between these pain mediators and the signalment of dogs (Study II). The hypothesis was that the concentration of these pain mediators would be higher in the SF of osteoarthritic joints than in non-osteoarthritic joints.

4. To study the clinical, cytological, and histopathological adverse effects of IA BoNT A in the stifle joints of healthy dogs and to study whether the toxin spreads from the joint (Study III). The hypothesis was that IA BoNT A would not produce significant clinical, cytological, or histopathological adverse effects, but it might spread from the joint.
4 MATERIALS AND METHODS

4.1 APPROVAL OF STUDY PROTOCOLS

The studies were approved by the Animal Experiment Board (ESAVI-2010-04178/Ym-023) and the Finnish Medicines Agency. All studies were performed at the Veterinary Teaching Hospital of the University of Helsinki, Finland, during the period 2011–2013.

The owners of the osteoarthritic dogs signed an informed consent form having received information on the study. The dog owners were free to discontinue the trial at any time during the study.

4.2 DOGS

4.2.1 OSTEOARTHRITIC DOGS (I, II)

Privately owned osteoarthritic dogs were recruited for Study I by advertising the study in journals and at dog events and by contacting various breed associations.

The inclusion criteria were chronic lameness present for at least three months, radiographic diagnosis of OA in the stifle, hip, or elbow joint, and pain on palpation of the joint. The exclusion criteria were lameness due to reasons other than OA, a neurological, systemic, or infectious disease, age less than one year, weight less than 15 kg, any IA treatment, CS or pentosane polysulphate injection less than one month before the study, and NSAIDs or tetracycline treatment less than one week before the study.

The screening for inclusion and exclusion criteria included physical, orthopaedic, and neurological examinations, analysis of serum biochemistry and haematology, and evaluation of radiographs taken from the lumbal spine and from the stifle and hip joints in dogs with stifle or hip OA and from the elbow and shoulder joints in dogs with elbow OA.

The SF and serum samples obtained from the dogs in Study I were used in Study II.

4.2.2 NON-OSTEOARTHRITIC CONTROL DOGS (II)

In Study II, SF samples were also taken from the stifle, hip, and elbow joints of non-osteoarthritic control dogs. These dogs were privately owned and brought to euthanasia to the Small Animal Teaching Hospital of the University of Helsinki for various reasons during year 2012. Their owners donated them for research after euthanasia. The exclusion criteria were a
Materials and methods

history of joint disease, age less than one year, bodyweight less than 15 kg, and the medications mentioned in the previous section on osteoarthritic dogs. Additional exclusion criteria were macroscopical alterations or considerable abnormal findings in the histopathological examination of the cartilage and synovium of the sampled joint, a total SF cell count of more than 2.0 x 10⁹/L, and a SF neutrophil percentage over 6% (Innes, 2012).

The dog owners were briefly interviewed regarding the history of their dog. The sampled joints were investigated by macroscopical evaluation, by obtaining SF total and differential cell counts, and performing histopathological examination of biopsies taken from the synovium and the cartilage and subchondral bone of the weight-bearing areas of the joints. The histopathological evaluation was performed as described in the OARSI Histopathology Initiative (Cook et al., 2010).

4.2.3 LABORATORY DOGS (III)

Six laboratory beagle dogs assigned for euthanasia due to discontinuation of the use of beagles in research were examined for inclusion in Study III. Before the study, the dogs underwent clinical, orthopaedic, and neurological examinations to confirm their health status. In addition, their serum biochemistry and haematology were analysed and radiographs were taken from the stifle and hip joints and from the lumbal spine.

4.3 STUDY DESIGN

4.3.1 STUDIES I AND II

Study I was performed as a placebo-controlled, randomized, double-blinded clinical trial with a stratified parallel group design. The dogs, described in Section 4.2.1, were stratified into six groups based on the administration of treatment into the stifle, hip, or elbow joint and on moderate or severe joint pain evaluated by the HCPI. The grouping was based on the median of the HCPI results acquired from the screening visit. Randomization was performed with randomly permuted blocks.

The dogs were randomized within each stratum in blocks of two in a 1:1 ratio to receive an IA injection of either 30 IU of BoNT A (Botox, Allergan Inc., Irvine, CA, USA) or an equivalent volume of placebo (sterile 0.9% saline) into the osteoarthritic joint. The randomization list was generated using SAS/Proc Plan (SAS for Windows version 9.2, SAS Institute, Cary, NC, USA). A statistician gave the list to the research technician, who performed the injections for each dog by following the list. The dog owners and the veterinarians performing the trial were blinded to the treatment allocation.
The minimum sample size required for Study I (34 subjects) was determined by a power analysis, in which the PVF was used as the primary efficacy variable. The power was set at 0.8 for a sample size of 17 per treatment group using two-tailed tests with alpha set at 0.05, to detect a difference equal to a standard deviation (SD) of 8 for a change in PVF from baseline to the follow-up visits in each treatment group. This SD was estimated from a previous study (Hielm-Björkman et al., 2012).

In Study II, the number of the non-osteoarthritic control dogs, described in Section 4.2.2, was planned to be half of the number of the osteoarthritic dogs. The stifle, hip, and elbow joints of the control dogs were sampled in such proportions that the number of different joints sampled would not differ significantly between the non-osteoarthritic and osteoarthritic dogs.

4.3.2 STUDY III
The study was performed as a longitudinal, placebo-controlled, randomized, blinded trial. The dogs, described in Section 4.2.3, were randomized in a 1:1 ratio to receive an IA injection of 30 IU of BoNT A (Botox, Allergan Inc., Irvine, CA, USA) into either the right or left stifle joint. An equivalent volume of placebo (sterile 0.9% saline) was injected into the contralateral joint. The research technician performed the randomization of the joints by drawing lottery tickets. The veterinarians performing the study and the pathologist performing the histopathological evaluation were blinded to the treatment allocation.

4.4 INJECTIONS AND SAMPLING (I–III)

4.4.1 INJECTIONS (I, III)
The dogs were sedated for the IA injection with IM dexmedetomidine or medetomidine and butorphanol, or only IM butorphanol, depending on the age of the animal. Intravenous (IV) propofol was used to effect, if necessary.

The joint was aseptically prepared before the IA injection. A research nurse prepared the injections and covered the syringes with non-transparent tape. Before the injection, SF was aspirated to collect SF for analysis and to verify the correct location of the needle inside the joint.

4.4.2 SAMPLES (I–III)
In Studies I and III, arthrocentesis was performed immediately before and at specific intervals after the IA injection to collect SF for further analysis.
Materials and methods

(Tables 1 and 2). The dogs were sedated for the procedure as described above. In addition, serum samples were obtained from the osteoarthritic dogs in Study I. In Study II, SF from the non-osteoarthritic control dogs was collected immediately after euthanasia.

4.5 EXAMINATION PROCEDURES (I–III)

The detailed schedules for the examinations performed in Studies I and III are presented in Tables 1 and 2, respectively.

Table 1. Schedule for procedures performed in Study I.

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<td>Serum sample</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Baseline, before the injections; IA injection, intra-articular injection of botulinum toxin A or placebo; W, week.
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Table 2. Schedule for procedures performed in Study III.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>IA injection</td>
<td></td>
</tr>
<tr>
<td>Arthrocentesis</td>
<td></td>
</tr>
<tr>
<td>Dynamic weight-bearing</td>
<td></td>
</tr>
<tr>
<td>Static weight-bearing</td>
<td></td>
</tr>
<tr>
<td>Goniometry</td>
<td></td>
</tr>
<tr>
<td>Veterinary pain evaluation</td>
<td></td>
</tr>
<tr>
<td>Neurological examination</td>
<td></td>
</tr>
<tr>
<td>Electrophysiological recordings</td>
<td></td>
</tr>
<tr>
<td>Histopathological examinations</td>
<td></td>
</tr>
</tbody>
</table>

Baseline, before the injections; IA injection, intra-articular injection of botulinum toxin A and placebo into stifle joints of each dog; h, hour; W, week.

4.5.1 DYNAMIC WEIGHT-BEARING (I–III)

Dynamic weight-bearing was measured by obtaining ground reaction forces with a piezoelectric force plate (Kistler force plate, type 9286, Kistler Instrumente AG, Winterhur, Switzerland) and a computer software program (Aquire 7.3, Sharon Software Inc., Dewitt, MI, USA). Velocity and acceleration were measured using three photoelectric cells placed 1m apart and a start-interrupt timer system (Sharon Software Inc., Dewitt, MI, USA).

The dogs were led over the plate at trot by an assistant or by their owners. PVFs (Studies I–III) and VIs were measured (Study I). All forces were normalized to bodyweight in kilograms.

For a valid trial, a front limb had to hit the centre of the plate and to be followed by the ipsilateral hindlimb. The dog had to run in a straight line without pulling on the leash. No target velocity was set beforehand, because dogs of different sizes with variable degree of lameness were included in Studies I and II. Instead, the dogs were led over the plate at a speed each dog felt comfortable with. The acceptable range for the velocity of a trial was ±0.5 m/s around the mean velocity of each dog and the acceptable range for acceleration was ±0.5 m/s². The mean velocity of each dog was calculated when each study had ended and all trials were obtained. Three to five valid trials were chosen for each dog at each examination time point and their mean was used for analysis.
The PVF and VI were used as primary outcome variables in Study I. The PVF and VI obtained at baseline in Study I were used as clinical variables of OA pain in Study II.

In Study III, a SI was calculated from PVFs using the equation $100 \left( \frac{\text{PVF}_{\text{BoNT}} - \text{PVF}_{\text{Pla}}}{0.5 \left( \text{PVF}_{\text{BoNT}} + \text{PVF}_{\text{Pla}} \right)} \right)$, where $\text{PVF}_{\text{BoNT}}$ is the mean of the PVFs of the IA BoNT A-injected limb and $\text{PVF}_{\text{Pla}}$ is the mean of the PVFs of the placebo-injected limb (Volstad et al., 2017). In this equation, an index of 0 signifies perfect symmetry, while a positive value indicates more weight-bearing on the IA BoNT A-injected limb and a negative value more weight-bearing on the IA placebo-injected limb.

4.5.2 STATIC WEIGHT-BEARING (III)

In Study III, the static weight-bearing of both hindlimbs of each dog was measured with two factory-calibrated bathroom scales (Hyytiäinen et al., 2012). For the measurement, the hindlimbs were symmetrically placed on the scales and the front limbs were placed on a custom-made platform of the same height. For a valid measurement, the dog had to stand straight and still in place until a fixed final value was obtained for both hindlimbs. Five measurements were recorded for both hindlimbs at each examination time point and their mean was used for analysis. The results were expressed as percentage of bodyweight.

4.5.3 VETERINARY PAIN EVALUATION (I–III)

The veterinarian evaluated the joint pain in the injected joints by palpating the joints and scoring the pain on a five-point scale. In Study I, the score for the pain on palpation of the joint was part of a subjective pain score, which also included five-point scoring of lameness, weight-bearing, and range of motion in the joint, each of which had equal weighting in the total score. This subjective pain score was used as a secondary outcome variable in Study I.

In Studies II and III, only pain on palpation of the joint was scored and used for analysis. The subjective scoring system is presented in detail in Table 3. It was modified from Moreau et al. (2003) and Budsberg et al. (2007).

4.5.4 GONIOMETRY (III)

The painless range of motion of both stifle joints of each dog was measured by goniometry with a universal plastic goniometer as described in Jaegger et al. (2002). To obtain the painless range of motion in the joint, the measurements were performed in awake dogs. A decrease in the range of motion would indicate pain in the joint. Three measurements were obtained...
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from both stifle joints at each examination time point and their mean was used for analysis.

**Table 3. Scoring system for subjective pain score evaluated by veterinarian (I–III).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Score</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lameness</td>
<td>0</td>
<td>Walks and trots normally</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight lameness at walk or trot</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate lameness at walk or trot</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe lameness at walk or trot</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Extreme lameness (non-weight-bearing) at walk or trot</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Weight-bearing</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>Normal weight-bearing at rest and at walk</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Normal weight-bearing at rest, favours affected limb at walk</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Partial weight-bearing at rest and walk</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Partial weight-bearing at rest, non-weighbearing at walk</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Non-weight-bearing at rest and walk</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pain on palpation of joint</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>No sign of pain</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild pain (dog turns head in recognition)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate pain (dog pulls limb away)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe pain (dog vocalizes or becomes aggressive)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Extreme pain (dog does not allow palpation)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Range of motion in joint</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>No limitation of movement, no crepitus</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild (10–20 %) decrease in range of motion, no crepitus</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild (10–20 %) decrease in range of motion with crepitus</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate (20–50 %) decrease in range of motion with crepitus</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Severe (&gt; 50 %) decrease in range of motion with crepitus</td>
</tr>
</tbody>
</table>

The subjective pain score consisted of the sum of the variables and was used in Study I. The score for pain on palpation of the joint was used for analysis in Studies II and III. The scoring system was modified from Moreau et al. (2003) and Budsberg et al. (2007).

**4.5.5 NEUROLOGICAL EXAMINATION (I, III)**

The neurological examination consisted of evaluation of mental status and gait, testing of postural reactions including evaluation of proprioception and wheel-barrowing, testing of spinal reflexes including myotatic and withdrawal reflexes, and evaluation of cranial nerves.
4.5.6 OWNER PAIN EVALUATION (I)

The owner pain evaluation in Study I consisted of the HCPI, which was used as a primary outcome variable, and the requirement for rescue analgesia, which was used as a secondary outcome variable. The detailed schedule for the owner pain evaluation is presented in Table 1.

4.5.6.1 Helsinki Chronic Pain Index

The owners of the dogs evaluated their dog’s pain by answering the HCPI questionnaire at home as described in Hielm-Björkman et al. (2009). Briefly, the questionnaire consisted of 11 questions regarding the dog’s demeanour and behaviour during the previous week. The answers were scored and the sum of the scores was used for analysis. The minimum was 0 and the maximum was 44 points. The bigger the total score the more severe pain the dog was experiencing. The same dog owner always filled in the questionnaire independently of the veterinary evaluation.

The HCPI score obtained at baseline in Study I was used as a clinical variable of OA pain in Study II.

4.5.6.2 Requirement for rescue analgesia

For ethical reasons, the owners of the dogs were provided with carprofen tablets to administer to their dogs at a dosage of 4 mg/kg once daily, if necessary. The dog owners recorded the requirement for the rescue analgesia on the following scale once a week: 0, not needed; 1, needed 1–2 times/week; 2, needed 3–4 times/week; 3, needed 5–6 times/week; and 4, needed every day.

4.5.7 ELECTROPHYSIOLOGICAL RECORDINGS (III)

In Study III, electrophysiological recordings were performed with a Nicolet Viking Quest (Nicolet Biomedical Inc., Madison, WI, USA) before and at specific intervals after the IA injections (Table 2). The rectal temperature of the dogs was monitored during the recordings to rule out temperature changes affecting the results.

4.5.7.1 Electromyography

EMG was performed in the paraspinal muscles and in the gluteus, vastus lateralis, biceps, gastrocnemius, tibialis cranialis, and interosseous muscles of both hindlimbs and the supraspinatus, infraspinatus, deltoideus, biceps, triceps, extensor carpi radialis, deep and superficial flexors, and interosseal muscles of both front limbs. A concentric needle electrode was used for the
EMG while a subdermal needle electrode served as a ground on the animal’s flank. Abnormalities detected included abnormal insertional activity and spontaneous activity such as fibrillations and positive sharp waves.

4.5.7.2 Motor nerve conduction velocity
MNCVs of the sciatic/peroneal nerves were measured from both hindlimbs of each dog. The measurements were performed with two stimulating monopolar needle electrodes and three subdermal needle electrodes as the recording, reference, and ground electrodes. In addition to MNCV, amplitudes of the CMAPs were recorded.

4.5.7.3 Repetitive nerve stimulation
RNS was evaluated from the peroneal nerves of both hindlimbs of each dog with distal stimulation sites. RNS was performed at a low frequency rate (3 Hz) with supramaximal stimulus, a stimulus duration of 0.2 ms, and train of 10 stimuli. CMAPs were measured. In the data analysis, the CMAP amplitudes and areas of subsequent potentials were compared to the initial one.

4.6 LABORATORY ANALYSES (II, III)
The detailed schedules for the laboratory analyses are presented in Tables 1 (Study II) and 2 (Study III).

4.6.1 PROCESSING OF SAMPLES (II, III)
SF was visually inspected for changes in viscosity, colour, and turbidity immediately after arthrocentesis. Then, SF samples were put into EDTA tubes for calculating the total and differential cell counts. In Study II, the rest of the SF sample was put into sterile Eppendorf-tubes, which were centrifuged at 10,000 rpm for 15 minutes. The supernatant was separated and stored at -80 °C.

The serum samples (Study II) were put into serum-separating tubes and left to stand for 30 minutes. After this, they were centrifuged at 3,500 rpm for 10 minutes. The serum was separated and stored at -80 °C.

4.6.2 SYNOVIAL FLUID CELL COUNT (III)
Total and differential cell counts of the SF samples were calculated within 30 minutes after arthrocentesis. If an insufficient amount of SF was obtained for
the total cell count, only the differential cell count was evaluated from a microscope slide. The differential cell count consisted of calculating the percentage of neutrophils and mononuclear cells. The percentage of neutrophils in the SF samples was used for analysis.

4.6.3 SUBSTANCE P (II)

The concentration of SP in SF and serum was measured with a commercial ELISA based on competitive binding technique (Substance P EIA Kit Cayman Chemical Company, Ann Arbor, MN, USA). The samples were analysed in duplicate according to the manufacturer’s instructions. The manufacturer reported a detection limit of 3.9 pg/mL for the assay.

The validation of the assay consisted of evaluating the dilution parallelism by analysing samples both undiluted and at dilutions of 1:2, 1:5, and 1:10. The concentration of SP increased in the diluted samples, which was explained by an increase in the ratio of high-affinity antiserum to the proteins binding SP (Corbally et al., 1990; Campbell et al., 2006). Because the SP concentration of the samples was in the low concentration range for the assay, the samples were assayed undiluted. No extraction was used for the samples to avoid inconsistent loss of SP, which has previously been reported after extraction procedures (Corbally et al., 1990; Campbell et al., 2006).

The intra-assay coefficient of variation (CV) was 11.4% for SF and 11.8% for serum. The inter-assay CV was 19.3%.

4.6.4 PROSTAGLANDIN E₂ (II)

The concentration of PGE₂ in SF and serum samples was measured with a commercial ELISA (PGE₂ ELISA Kit, Enzo Life Sciences Inc., Farmingdale, NY, USA). The assay was based on forward sequential competitive binding technique and the samples were assayed in duplicate according to the manufacturer’s instructions. The detection limit of the assay was 13.4 pg/mL, as reported by the manufacturer.

For validation of the assay, samples were analysed undiluted and at dilutions of 1:2, 1:5, 1:10, and 1:20. The results showed good linearity. To test for possible matrix interference, the linearity of the results from both diluted and undiluted samples with and without extraction was evaluated. No extraction was considered necessary based on the results.

The SF samples were assayed in 1:20 dilution, and the serum samples were assayed in either 1:20 or 1:50 dilution, depending on the concentration of PGE₂ in the serum sample. The samples were diluted in assay buffer. The intra-assay CV was 10.5% for SF and 9.1% for serum. The inter-assay CV was 18.0%.
4.6.5 TUMOUR NECROSIS FACTOR-ALPHA (II)

The concentration of TNF-α in SF and serum samples was measured with a commercial ELISA (Quantikine ELISA, Canine TNF-α, R&D Systems Inc., Minneapolis, Mn, USA), which is based on the quantitative sandwich enzyme immunoassay technique. The samples were analysed in duplicate according to the manufacturer’s instructions. The detecting limit of the assay was 2.4 pg/mL, as reported by the manufacturer.

The validation of the assay consisted of adding a canine TNF-α provided by the manufacturer to each plate and performing a spiking recovery test to determine the recovery rate of the control in SF and serum. The control and spiking recovery test yielded expected results, but the concentration of TNF-α was below the detection limits of all samples analysed. Therefore, no intra- or inter-assay CVs could be calculated.

4.7 HISTOPATHOLOGICAL EXAMINATION (III)

4.7.1 JOINTS

After 12 weeks, the laboratory beagle dogs in Study III were sedated with IV medetomidine, butorphanol, and MK-467 after which euthanasia was performed by IV propofol and pentobarbital.

Both stifle joints of each dog were histopathologically examined post mortem. The articular cartilage and synovium were macroscopically inspected for discoloration and changes in the thickness and surface contour immediately after euthanasia. After this, the stifle joints were dissected and immersed in 10% neutral buffered formalin for a minimum of 36 h. Biopsies were obtained sagittally from the cartilage of the weight-bearing areas of the medial and lateral femoral condyles and medial and lateral tibial plateau. Soft-tissue samples were taken from the synovium. Samples were embedded in paraffin wax, sectioned routinely, and stained with haematoxylin and eosin as well as toluidine blue. The samples from bone were decalcified in EDTA solution before embedding.

The histopathological evaluation of the cartilage and synovium biopsies was performed as described in the OARSI Histopathology Initiative (Cook et al., 2010) by two independent, blinded reviewers. The cartilage was evaluated by scoring its structural integrity and chondrocyte pathology. Synovium was evaluated by scoring the number of lining cell layers and the presence of villous hyperplasia and inflammatory cell infiltrates. The obtained scores were used for statistical analysis. The details of the scoring system are listed in Table 4.
The popliteal, vastus lateralis, and semimembranosus muscles, and the sciatic, tibial, and saphenous nerves, were sampled from both hindlimbs of each dog post mortem and fixed in 10% neutral buffered formalin. Samples were embedded in paraffin wax, sectioned routinely, and stained with haematoxylin and eosin.

A blinded veterinary pathologist performed the histopathological evaluation of the muscle and nerve biopsies. The muscle biopsies were evaluated for the presence of muscle cell size variation and angular and ring fibres. One cross and one longitudinal section were evaluated from each examined muscle.

The nerve biopsies were evaluated for the presence of Büngner bands, Wallerian degeneration, and for the presence of inflammatory cell infiltrates.

Table 4. Histopathological grading of cartilage and synovium (III).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Classification</th>
<th>Characteristics</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage structure</td>
<td>Structural integrity</td>
<td>Normal</td>
<td>None 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fissures in upper zone</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fissures to mid zone</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fissures to deep zone</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Full thickness loss of cartilage</td>
<td>0</td>
</tr>
<tr>
<td>Chondrocyte pathology</td>
<td>None</td>
<td>Loss of cells in upper zone</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small cell clusters</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large cell clusters</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell loss</td>
<td>0</td>
</tr>
<tr>
<td>Synovial structure</td>
<td>Cell layers</td>
<td>1–2 layers</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–6 layers</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 6 layers</td>
<td>0</td>
</tr>
<tr>
<td>Villous hyperplasia</td>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Short villi</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Finger-like</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Synovial infiltrates</td>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild to moderate</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Marked, diffuse</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

The scores for structural integrity and chondrocyte pathology were summed up and used as the score for cartilage structure. The scores for cell layers and villous hyperplasia were summed up and used as the score for synovial structure. The histopathological grading was modified from the OARSI Histopathology Initiative (Cook et al., 2010).

4.7.2 MUSCLES AND NERVES

The popliteal, vastus lateralis, and semimembranosus muscles, and the sciatic, tibial, and saphenous nerves, were sampled from both hindlimbs of each dog post mortem and fixed in 10% neutral buffered formalin. Samples were embedded in paraffin wax, sectioned routinely, and stained with haematoxylin and eosin.

A blinded veterinary pathologist performed the histopathological evaluation of the muscle and nerve biopsies. The muscle biopsies were evaluated for the presence of muscle cell size variation and angular and ring fibres. One cross and one longitudinal section were evaluated from each examined muscle.

The nerve biopsies were evaluated for the presence of Büngner bands, Wallerian degeneration, and for the presence of inflammatory cell infiltrates.
4.8 STATISTICAL ANALYSES

4.8.1 STUDY I

The response to treatment was assessed as the change from baseline to each examination time point. The Shapiro-Wilk test was used to test the variables for normality. Normally distributed variables were expressed as mean ± SD. Ninety-five per cent confidence intervals (CIs) were calculated using the standard student procedure for the main changes between the examination weeks and the mean differences between the IA BoNT A and placebo groups. The data from all six strata were pooled and analysed together.

All tests were two-tailed and the significance was set at $P \leq 0.05$. Differences between the IA BoNT A and placebo groups regarding treatment effect, baseline variables, and stratification factors were analysed using analysis of variance (ANOVA) model. The ground reaction forces, the HCPI, and subjective pain score within groups were measured using repeated-measures (RM) ANOVA model. The requirement for rescue analgesia was grouped into contingency tables and analysed using the Pearson chi-squared test. Statistical analysis was performed with JMP software, version 9 (SAS Institute, Cary, NC, USA).

4.8.2 STUDY II

All the continuously distributed variables were tested for normality using the Shapiro-Wilk test. Data were expressed as mean ± SD (normally distributed data) or as median and interquartile range (IQR) (non-normally distributed data). To normalize distributions, all the statistical modelling was conducted using logarithmic transformed data for all the pain mediators.

The differences in the signalment of the groups of dogs (IA BoNT A, placebo, and non-osteoarthritic controls) were analysed using ANOVA (continuous variables) or Fisher’s exact test (categorical variables). The differences in the change from baseline (at 2 and 8 weeks) in the mean SF and serum pain mediator concentrations between the treatment groups (IA BoNT A vs placebo) were analysed with linear mixed models for RM analysis of covariance (ANCOVA) model. The models included the treatment group, time point, and the interaction between treatment group and time point as fixed terms, the baseline value of the pain mediator as a covariate, and dog as a random term. An unstructured covariance structure was applied in the model. The Tukey-Kramer multiplicity adjustment method was used to correct the p-values of the multiple treatment comparisons.

The differences in the SF pain mediator concentrations at baseline between osteoarthritic (pooled treatment groups) and non-osteoarthritic control dogs were analysed using Mann-Whitney U tests. For the osteoarthritic dogs (pooled treatment groups) the differences in pain
mediator concentrations between SF and serum at baseline were analysed using Wilcoxon matched-pair signed-rank tests. The correlations among the clinical variables of osteoarthritic pain and SF and serum pain mediator concentrations in osteoarthritic dogs (pooled treatment groups) were assessed by analysing either the Pearson correlation coefficient (normally distributed data) or the Spearman’s rank correlation coefficient (non-normally distributed data) at baseline.

The associations between SF and serum pain mediator concentrations and signalment of the dogs (IA BoNT A, placebo, and non-osteoarthritic controls) were analysed by using Fisher’s exact test. All tests were two-tailed and significance was set at $P < 0.05$. Statistical analysis was performed using SAS System for Windows, version 9.3 (SAS Institute, Cary, NC, USA).

4.8.3 STUDY III

The data were summarized by treatment and examination time point using descriptive statistics and frequency tables. The SI, static weight-bearing, and the goniometric measurements were analysed with RM ANCOVA. In the SI, the examination time point and baseline measurement were used as fixed terms and dog was used as a random term. In the static weight-bearing and goniometric measurements the change from baseline was used as the response. Treatment, time point, injected limb (left or right), and the interaction between the treatment and examination time point were included as the fixed effects and the corresponding baseline measurement as a covariate. Dog within injected limb was used as a random term.

The neutrophil percentage in the SF samples after baseline was analysed with RM ANOVA where treatment, examination time point, injected limb, and the interaction between the treatment and examination time point were included as fixed effects and dog within injected limb as the random term.

The histopathological variables were analysed using the following approaches. The variables synovium structure and synovium infiltrates were analysed with cumulative logit models with treatment and injected limb as fixed terms and dog as the random subject effect. The variables muscle cell size variation, angular fibres, ring fibres, Büngner bands, Wallerian degeneration, and dichotomized cartilage structure (0 vs > 0) were analysed with logistic regression, where at least the treatment was included as fixed term and injected limb if feasible. Dog was used as a random subject effect. The number of inflammatory cells in the nerve biopsies was analysed with one-way ANOVA, where treatment and injected limb were included as fixed terms and dog within injected limb as a random term.

The differences between treatments and examination time points together with two-sided 95% CIs for the difference were estimated from the fitted RM ANCOVA, RM ANOVA, and ANOVA models using contrasts. In the logistic and cumulative logit-models the differences between treatments were
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quantified with odds ratios and CIs. Statistical significance was set at $P < 0.05$. The statistical analysis was performed using SAS System for Windows, version 9.3 (SAS Institute, Cary, NC, USA).
5 RESULTS

5.1 DOGS

The signalment of all dogs included in this thesis is presented in Table 5.

5.1.1 OSTEOARTHRITIC DOGS (I, II)

For Study I, 106 dogs were screened for inclusion and exclusion criteria and 36 were eligible for participation in the study. One dog was later excluded due to incorrect diagnosis (immune-mediated arthritis).

5.1.2 NON-OSTEOARTHRITIC CONTROL DOGS (II)

Thirteen privately owned dogs which donated for research after euthanasia proved eligible for participation in Study II as non-osteoarthritic control dogs during year 2012.

5.1.3 LABORATORY DOGS (III)

All the six laboratory beagle dogs were found healthy and were included in Study III.
Table 5. *Signalement of dogs (I–III).*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Classification</th>
<th>Osteoarthritic dogs (I, II)</th>
<th>Non-osteoarthritic Laboratory dogs control dogs (II)</th>
<th>Laboratory dogs (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IA BoNT A ( n = 19 )</td>
<td>IA Placebo ( n = 16 )</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>Mean (SD)</td>
<td>7.3 (3.0)</td>
<td>5.3 (3.1)</td>
<td>6.0 (3.2)</td>
</tr>
<tr>
<td></td>
<td>Total range</td>
<td>3–13</td>
<td>1–11</td>
<td>2–13</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>Mean (SD)</td>
<td>33.0 (7.9)</td>
<td>33.2 (10.0)</td>
<td>34.9 (10.9)</td>
</tr>
<tr>
<td></td>
<td>Total range</td>
<td>16.8–47.2</td>
<td>16.4–61.5</td>
<td>20.3–55.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.2–14.2</td>
</tr>
<tr>
<td>BCS</td>
<td>Thin</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>11</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Overweight</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Obese</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>2</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Neutered female</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Neutered male</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Investigated joint</td>
<td>Stifle</td>
<td>6</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Hip</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Elbow</td>
<td>8</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Duration of lameness</td>
<td>3–6</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6–12</td>
<td>2</td>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>&gt; 12</td>
<td>16</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>Breeds (number of dogs)</td>
<td>Labrador</td>
<td>Retriever (6)</td>
<td>German</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retriever (4)</td>
<td>Bernese Mountain Dog (2)</td>
<td>Shepherd (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shepherd (3)</td>
<td>Nova Scotia Duck Dog (1)</td>
<td>Bernese Mountain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Collie (2)</td>
<td>Nova Scotia Duck Dog (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Basset Hound (1)</td>
<td>Tolling Retriever (2)</td>
<td>Boxer (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beauceron (1)</td>
<td>Rottweiler (2)</td>
<td>Bracco Italiano (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Belgian</td>
<td>Black Russian Dog (1)</td>
<td>Dalmatian (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shepherd (1)</td>
<td>Terrier (1)</td>
<td>Dobermann (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cockerspaniel (1)</td>
<td>Catalan</td>
<td>Great Dane (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flat-Coated</td>
<td>Sheepdog (1)</td>
<td>Rottweiler (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retriever (1)</td>
<td>Central Asian Dog (1)</td>
<td>Siberian Husky (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Irish Setter (1)</td>
<td>Shepherd Dog (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rottweiler (1)</td>
<td>Mixed Breed (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mixed Breed (1)</td>
<td>Siberian Husky (1)</td>
<td>Welsh Springer</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spaniel (1)</td>
<td></td>
</tr>
</tbody>
</table>

BoNT A, botulinum toxin A; BCS, body condition score; IA, intra-articular; NA, not applicable; placebo, 0.9% saline; SD, standard deviation.
Results

5.2 EFFICACY IN TREATMENT OF OSTEOARTHRITIC JOINT PAIN (I)

5.2.1 DYNAMIC WEIGHT-BEARING

The VI and PVF were not statistically different between the IA BoNT A and placebo groups at baseline ($P = 0.749$ and $P = 0.963$, respectively) (Figure 3). There was a statistically significant increase from baseline in VI 2, 4, 8, and 12 weeks after the injection in the IA BoNT A group ($P = 0.037$, $P = 0.016$, $P = 0.016$, and $P = 0.001$, respectively), but not in the placebo group ($P = 0.534$, $P = 0.828$, $P = 0.273$, and $P = 0.515$, respectively). The difference in the increase from baseline between the IA BoNT A and placebo groups in VI was statistically significant 12 weeks after the injections ($P = 0.005$).

There was a statistically significant increase from baseline in PVF 12 weeks after the injection in the IA BoNT A group ($P = 0.054$), but not in the placebo group ($P = 0.571$). There was no statistically significant difference in the increase from baseline in PVF between the IA BoNT A and placebo groups during the study ($P = 0.093$).

We were not able to obtain valid trials in three dogs in the IA BoNT A group and in one dog in the placebo group due to short stride (n = 3) and continuous pacing (n = 1).

5.2.2 HELSINKI CHRONIC PAIN INDEX

The HCPI was not statistically different between the IA BoNT A and placebo groups at baseline (mean (SD) 15.7 (4.7) for IA BoNT A group; 15.0 (5.7) for placebo group; $P = 0.649$). The HCPI decreased from baseline to week 12 in both groups, but the decrease was statistically significant only in the IA BoNT A group (mean decrease (SD) 2.8 (6.0), $P = 0.053$ in IA BoNT A group; 2.0 (5.7), $P = 0.180$ in placebo group). There was no statistically significant difference in the change from baseline to week 12 between the groups ($P = 0.663$).

5.2.3 VETERINARY PAIN EVALUATION

The subjective pain score was not statistically different between the IA BoNT A and placebo groups at baseline (mean (SD) 6.3 (2.4) for IA BoNT A group; 6.7 (2.1) for placebo group; $P = 0.581$). The subjective pain score improved from baseline in both groups during the study but the change from baseline was not statistically significant in either group (mean decrease from baseline (SD) 0.6 (0.2), $P = 0.318$ for IA BoNT A group; 0.8 (0.3), $P = 0.301$ for placebo group) and there was no significant difference between the groups ($P = 0.721$).
Figure 3. Improvement from baseline in vertical impulses (A) and peak vertical forces (B) (mean and 95% CI) after intra-articular botulinum toxin A (n = 16) or intra-articular placebo (n = 15) in osteoarthritic dogs. Baseline, before the injections; IA BoNT A, intra-articular botulinum toxin A; placebo, 0.9% saline; PVF, peak vertical force; VI, vertical impulse; W, week.

*P ≤ 0.005 between groups; **P ≤ 0.05 within group.
5.2.4 RESCUE ANALGESICS
There was no statistically significant difference between the IA BoNT A and placebo groups in the requirement for rescue analgesia during the study.

5.2.5 ADVERSE EVENTS IN OSTEOARTHRITIC DOGS
Severe adverse events were not detected during the study. One dog got a superficial skin infection over the IA BoNT A-injected hip joint one week after the injection. The infection healed uneventfully with local treatment.

Another dog was reluctant to jump and developed a hypometric gait and hindlimb ataxia after receiving IA BoNT A into the left stifle joint. Spinal and cranial reflexes and proprioception were considered normal. Additionally, the dog showed pain in the lumbar spine and in the thoracolumbar junction. A mild disc protrusion was diagnosed using MRI in the T13–L1 region, which explained the ataxia. The lumbar pain was considered to be a consequence of painful stifle OA, because no other reason could be identified for the pain.

5.3 PAIN MEDIATOR ANALYSES (II)

5.3.1 SUBSTANCE P
SF and serum SP concentrations were not statistically different between the IA BoNT A and placebo groups at baseline ($P = 0.180$ and $P = 0.683$, respectively) (Figure 4). SF and serum SP concentrations did not change statistically significantly in the IA BoNT A and placebo groups during the study ($P = 0.119$ for overall change in SF SP and $P = 0.148$ for overall change in serum SP in the IA BoNT A group; $P = 0.230$ and $P = 0.613$ in the placebo group, respectively). There was no statistically significant difference in the change from baseline in SF and serum SP concentrations between the groups ($P = 0.952$ for SF and $P = 0.176$ for serum).
Figure 4. Synovial fluid (A) and serum (B) substance P concentrations (median and IQR) in osteoarthritic dogs after intra-articular botulinum toxin A or intra-articular placebo. No statistically significant difference was detected within or between groups. A: n = 12 for the IA BoNT A group, n = 7 for the placebo group; B: n = 17, n = 16, respectively. Baseline, before the injections; IA BoNT A, intra-articular botulinum toxin A; placebo, 0.9% saline; W, week.
Results

5.3.2 PROSTAGLANDIN E\(_2\)
SF and serum PGE\(_2\) concentrations were not statistically different between the IA BoNT A and placebo groups at baseline (\(P = 0.353\) and \(P = 0.052\), respectively) (Figure 5). SF and serum PGE\(_2\) concentrations did not change statistically significantly in either group during the study (\(P = 0.105\) for overall change in SF PGE\(_2\) and \(P = 0.907\) for overall change in serum PGE\(_2\) in the IA BoNT A group; \(P = 0.726\) and \(P = 0.863\) in the placebo group, respectively). There was no statistically significant difference in the change from baseline in SF and serum PGE\(_2\) concentrations between the groups during the study (\(P = 0.475\) for SF and \(P = 0.963\) for serum).

5.3.3 TUMOUR NECROSIS FACTOR-ALPHA
SF and serum TNF-\(\alpha\) concentrations were below the detection limit of the assay at baseline and remained below the detection limit during the study in all samples of the osteoarthritic dogs.

5.3.4 OSTEOARTHRITIC VERSUS NON-OSTEOARTHRITIC DOGS
SF PGE\(_2\) concentration was significantly higher in osteoarthritic dogs than in non-osteoarthritic control dogs (\(P = 0.001\)) (Figure 6). SF SP concentration was not statistically different between the osteoarthritic and non-osteoarthritic dogs (\(P = 0.204\)). SF TNF-\(\alpha\) was below the detection limit in all osteoarthritic dogs and it was therefore not measured in the non-osteoarthritic control dogs.
Results

Figure 5. Synovial fluid (A) and serum (B) prostaglandin E$_2$ concentrations (median and IQR) in osteoarthritic dogs after intra-articular botulinum toxin A or intra-articular placebo. No statistically significant difference was detected within or between the groups. A: $n = 12$ for the IA BoNT A group, $n = 7$ for the placebo group; B: $n = 17$, $n = 16$, respectively. Baseline, before the injections; IA BoNT A, intra-articular botulinum toxin A; placebo, 0.9% saline; W, week.
Results

Figure 6. Synovial fluid prostaglandin E₂ (A) and substance P (B) concentrations (median and IQR) in osteoarthritic and non-osteoarthritic dogs. The central horizontal line represents the median value and the boxes indicate the IQR. The top and bottom whiskers indicate the highest and lowest case within 1.5 times of the IQR, respectively. Values more than 1.5 times the IQR are labelled outliers and presented as dots. PGE, prostaglandin E₂; SP, substance P.

* \( P = 0.001 \)
5.3.5 **PAIN MEDIATORS AND OSTEOARTHRITIC PAIN**

A negative correlation was found between the SF PGE$_2$ concentration and the ground reaction forces PVF and VI and a positive correlation was found between the SF PGE$_2$ and the pain on palpation of the joint. Additionally, serum PGE$_2$ correlated negatively with the serum SP concentration. No other correlations were detected between the SF and serum pain mediators, and the clinical variables of osteoarthritic pain (Table 6).

No associations were detected between the pain mediators and the age, bodyweight, gender, duration of lameness, or the sampled joint.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SF SP</th>
<th>Serum SP</th>
<th>SF PGE$_2$</th>
<th>Serum PGE$_2$</th>
<th>PVF</th>
<th>VI</th>
<th>HCPI</th>
<th>Pain on palpation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF SP</td>
<td>1.00</td>
<td>0.09</td>
<td>0.10</td>
<td>-0.11</td>
<td>-0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>-0.09</td>
</tr>
<tr>
<td>Serum SP</td>
<td>0.09</td>
<td>1.00</td>
<td>-0.05</td>
<td>-0.48*</td>
<td>0.12</td>
<td>0.10</td>
<td>-0.03</td>
<td>-0.04</td>
</tr>
<tr>
<td>SF PGE$_2$</td>
<td>0.10</td>
<td>-0.05</td>
<td>1.00</td>
<td>0.23</td>
<td>-0.62*</td>
<td>-0.61*</td>
<td>0.05</td>
<td>0.45*</td>
</tr>
<tr>
<td>Serum PGE$_2$</td>
<td>-0.11</td>
<td>-0.48*</td>
<td>0.23</td>
<td>1.00</td>
<td>0.03</td>
<td>0.04</td>
<td>-0.08</td>
<td>-0.14</td>
</tr>
</tbody>
</table>

HCPI, Helsinki Chronic Pain Index; pain on palpation, pain on palpation in the joint; PGE$_2$, prostaglandin E$_2$; PVF, peak vertical force; SF, synovial fluid; SP, substance P; VI, vertical impulse.

*P < 0.05
5.4 CLINICAL ADVERSE EFFECTS (III)

5.4.1 DYNAMIC WEIGHT-BEARING

The dogs were moving symmetrically at baseline (mean SI -0.39 (SD 6.8)). The SIs changed from baseline during the study (mean estimated change from baseline at 12 weeks -4.27, 95% CI -9.40–0.86), but the change was not statistically significant ($P = 0.106$) (Figure 7).

![Symmetry Index Diagram]

**Figure 7.** Symmetry indices (mean and SD) of healthy dogs ($n = 6$) before and after intra-articular botulinum toxin A and intra-articular placebo. Symmetry indices were calculated from PVFs obtained with a force platform at trot. 0 = dog is moving in perfect symmetry; > 0 dog is bearing more weight on the IA BoNT A-injected limb; < 0 dog is bearing more weight on the IA placebo-injected limb. Baseline, before the injections: W, week.
### 5.4.2 STATIC WEIGHT-BEARING

The static weight-bearing was not statistically different between the IA BoNT A- and placebo-injected limbs at baseline ($P = 0.432$). The static weight-bearing decreased in the IA placebo-injected limbs and increased in the IA BoNT A-injected limbs during the study (Figure 8). The difference in the change from baseline was statistically significant between the limbs ($P = 0.013$), but the change from baseline was not statistically significant within the limb (mean estimated change in weight-bearing presented as percentage of body weight 1.31 %units, 95% CI -1.83–4.45 %units, $P = 0.406$ for IA BoNT A-injected limbs; -0.92 %units, -4.07–2.22 kg, $P = 0.556$ for IA placebo-injected limbs).

---

**Figure 8.** Static weight-bearing (mean and SD) of hindlimbs of healthy dogs ($n = 6$) presented as percentage of bodyweight before and after intra-articular botulinum toxin A or intra-articular placebo. Baseline, before the injections; IA BoNT A, intra-articular botulinum toxin A; placebo, intra-articular 0.9% saline; W, week.
5.4.3 GONIOMETRY

The IA BoNT A-injected stifle joints had a wider range of motion (mean 134.2° (SD 3.8°)) than the placebo-injected stifle joints (129.2° (4.9°)) at baseline, but this difference did not reach statistical significance \( (P = 0.076) \). The painless range of motion decreased in the BoNT A- and placebo-injected stifle joints during the study, but the decrease was statistically significant only in the placebo-injected joints (mean estimated decrease -3.5°, 95% CI -5.9°– -1.0°, \( P = 0.007 \) for placebo-injected joints; -1.1°, -3.5°–1.4°, \( P = 0.395 \) for BoNT A-injected joints) (Figure 9). The difference between the change from baseline was not statistically significant between the BoNT A- and placebo-injected joints \( (P = 0.150) \).

![Painless range of motion of stifle joints (mean and SD) in healthy dogs (n = 6) before and after intra-articular botulinum toxin A or intra-articular placebo. Baseline, before the injections; h, hour; IA BoNT A, intra-articular botulinum toxin A; placebo, intra-articular 0.9% saline; W, week. ★ = Differs significantly from baseline, \( P = 0.001 \) for W4, \( P = 0.017 \) for W8.](image)

5.4.4 VETERINARY PAIN EVALUATION

No pain was detected in the palpation of the stifle joints in any of the dogs during the study.
5.5 SPREAD OF TOXIN (III)

5.5.1 NEUROLOGICAL EXAMINATION

Three out of six dogs (dogs 2, 3, and 5) had abnormal findings in the neurological examination at baseline and two additional dogs (dogs 1 and 4) developed abnormal neurological examination findings during Study III (Table 7).

5.5.2 ELECTROPHYSIOLOGICAL RECORDINGS

Three out of six dogs (dogs 1, 2, and 4) had abnormal findings in the electrophysiological recordings during Study III (Table 8).

Two dogs had abnormal findings in EMG. Dog 1 had increased insertional activity in the supraspinatus and infraspinatus muscles at baseline and dog 2 had fibrillation potential in the deep and superficial flexors of one front limb 12 weeks after the injections.

In three dogs, the CMAP amplitudes were lower than the calculated canine reference values (10.94 mV for proximal stimulation and 10.02 mV for distal stimulation of the peroneal nerve (Walker et al., 1979)) during the study. In dog 1, the CMAP amplitude was 8.66 mV in distal stimulation in the IA BoNT A-injected limb 12 weeks after the injection. In dog 2, the CMAP amplitudes were low in distal stimulation in the IA BoNT A-injected limb 8 and 12 weeks after the injection (9.15 mV and 8.65 mV, respectively). In dog 4, the CMAP amplitudes were low both in proximal and distal stimulation in both hindlimbs at baseline (6.20 mV and 7.13 mV, 6.80 mV and 5.12 mV for proximal and distal stimulation in the IA BoNT A- and placebo-injected limbs, respectively).

MNCVs were above the reference values published for dogs (Lee and Bowen, 1970; Walker et al., 1979) in all dogs at all examination time points. No abnormal response was detected in the RNS at 3 Hz (i.e. > 10% decrement (van Nes and van der Most van Spijk, 1986)) at any examination time point during the study. The rectal temperature of the dogs varied between 36.7 °C and 38.4 °C during the recordings, and thus did not affect the results.
**Table 7.** Neurological examination findings in healthy dogs \((n = 6)\) before and after intra-articular botulinum toxin A and intra-articular placebo (III).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Baseline</th>
<th>24h</th>
<th>72h</th>
<th>W1</th>
<th>W2</th>
<th>W4</th>
<th>W8</th>
<th>W12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>Patellar(^A)(^B) ↓ Patellar(^A) ↓ NO</td>
<td></td>
</tr>
<tr>
<td>Dog 2</td>
<td>Extensor carpi radialis(^C) ↓ Extensor carpi radialis(^C) ↓</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>Facial sensation ↓ Facial sensation ↓ NO</td>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 3</td>
<td>Menace response ↓</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>Menace response ↓ NO</td>
<td>Menace response ↓ Facial sensation ↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 4</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>Patellar (^B) ↓ NO</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Dog 5</td>
<td>Patellar(^B) ↓</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>Dog 6</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

\(^A\) in the limb injected with IA BoNT A; \(^B\) in the limb injected with IA placebo; \(^C\) in front limb ↓ = mildly decreased, \(↓\) = severely decreased
Baseline, before the injections; extensor carpi radialis, extensor carpi radialis reflex; h, hour; IA BoNT A, intra-articular botulinum toxin A; NO, no abnormal findings; patellar, patellar reflex; placebo, 0.9% saline; W, week.

**Table 8.** Electrophysiological examination findings in healthy dogs \((n = 6)\) before and after intra-articular botulinum toxin A and intra-articular placebo (III).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Baseline</th>
<th>W2</th>
<th>W4</th>
<th>W8</th>
<th>W12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 1</td>
<td>Insertional activity(^C)</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>CMAP amplitude(^A) ↓</td>
</tr>
<tr>
<td>Dog 2</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>CMAP amplitude(^A) ↓</td>
</tr>
<tr>
<td>Dog 3</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Dog 4</td>
<td>CMAP amplitudes(^A)(^B) ↓</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Dog 5</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Dog 6</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

\(^A\) in the limb injected with IA BoNT A; \(^B\) in the limb injected with IA placebo; \(^C\) in front limb ↓ = low
Baseline, before the injections; CMAP, compound muscle action potential; IA BoNT A, intra-articular botulinum toxin A; NO, no abnormal findings; placebo, 0.9% saline; W, week.
5.6 CYTOLOGICAL AND HISTOPATHOLOGICAL ADVERSE EFFECTS (III)

5.6.1 SYNOVIAL FLUID ANALYSIS

No abnormalities were detected in the macroscopical evaluation of the SF samples in Study III, except for one sample with severe blood contamination. The total and differential cell counts were in the reference range published for dogs (total cell count < 2.0 x 10^9/L, neutrophil percentage < 7% (Innes, 2012)) in all samples except for the sample contaminated with blood. This sample had 76% of neutrophils. The sample was not included in the statistical analysis. No significant difference was present in the SF neutrophil percentage between the IA BoNT A and placebo-injected joints during the study (P = 0.960). The total cell count was calculated from 25 out of 60 samples taken during the study, because of insufficient amounts of SF obtained from the joints.

5.6.2 HISTOPATHOLOGICAL EXAMINATIONS

5.6.2.1 Stifle joints

No pathological changes were detected in the macroscopical evaluation of the stifle joints. No statistically significant difference was detected in the scores for cartilage and synovial structure and synovial inflammatory cell infiltrates between the BoNT A- and placebo-injected joints (Table 9).

Table 9. Histopathological grading (median and IQR) of stifle joints of healthy dogs 12 weeks after an intra-articular injection of botulinum toxin A or intra-articular placebo (III).

<table>
<thead>
<tr>
<th>Variable</th>
<th>IA BoNT A n = 6</th>
<th>IA Placebo n = 6</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage structure</td>
<td>3 (0–3)</td>
<td>4 (2–7)</td>
<td>0.534</td>
</tr>
<tr>
<td>Synovial structure</td>
<td>3 (3–4)</td>
<td>3 (2–4)</td>
<td>0.593</td>
</tr>
<tr>
<td>Synovial infiltrates</td>
<td>1 (1–2)</td>
<td>2 (1–2)</td>
<td>0.638</td>
</tr>
</tbody>
</table>

The score for cartilage structure (0–24) consisted of grading of structural integrity (0–12) and chondrocyte pathology (0–12). The score for synovial structure consisted of grading of lining cell layers (0–6) and the presence of villous hyperplasia (0–6). Synovial infiltrates were scored by the amount of inflammatory cell infiltrates in the synovium (0–6). A higher score indicates a more severe pathology. BoNT A, botulinum toxin A; IA, intra-articular; placebo, 0.9% saline.
5.6.2.2 Muscles and nerves

The muscle and nerve biopsies had only few and scattered abnormal histopathological findings. No statistically significant difference was detected in the histopathological changes related to muscle and nerve pathology between the IA BoNT A- and IA placebo-injected limbs (Table 10).

The number of inflammatory cells in the nerve biopsies did not differ between the IA BoNT A- and IA placebo-injected limbs (median 4 cells per 5 HPF, IQR 3–6 in the IA BoNT A limbs; 4, 4–5 in the IA placebo limbs; $P = 1.000$).

Table 10. Histopathological changes in muscles and nerves of healthy dogs 12 weeks after an intra-articular injection of botulinum toxin A (n = 6) or placebo (n = 6) (III).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Variable</th>
<th>IA injection</th>
<th>Pathological findings</th>
<th>P-value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dogs present</td>
<td>Dogs absent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscles(^a)</td>
<td>Cell size variation</td>
<td>BoNT A</td>
<td>4</td>
<td>2</td>
<td>0.477</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Angular fibres</td>
<td>BoNT A</td>
<td>2</td>
<td>4</td>
<td>0.427</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ring fibres</td>
<td>BoNT A</td>
<td>2</td>
<td>4</td>
<td>0.477</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Nerves(^b)</td>
<td>Büngner bands</td>
<td>BoNT A</td>
<td>2</td>
<td>4</td>
<td>0.292</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wallerian degeneration</td>
<td>BoNT A</td>
<td>4</td>
<td>2</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) = Evaluated in popliteal, vastus lateralis, and semimembranosus muscles  
\(^b\) = Evaluated in sciatic, tibial, and saphenous nerves  
BoNT A, botulinum toxin A; IA, intra-articular; placebo, 0.9% saline.

5.6.2.3 Autopsy

All dogs had moderate splenic congestion. Three dogs (dogs 1, 2, and 3) had mild lymphocytic or lymphoplasmacytic nephritis and three dogs (dogs 2, 3, and 5) had scattered hypereosinophilic myocytes. Dog 1 additionally had mild, lymphoplasmacytic myocarditis. All dogs had scattered spheroids (median 5.5 spheroids/cross section, range 1–11) in the lumbar spinal cord.
Discussion

OA is a debilitating condition characterized by irreversible destruction of articular cartilage in the synovial joints leading to progressive discomfort and pain, and a decrease in the quality of life in affected dogs. Oral NSAIDs are currently the mainstay of pain therapy in canine OA; however, fear of NSAID-related adverse events limits their use in dogs (Belshaw et al., 2016a). In addition, osteoarthritic dogs can suffer pain despite NSAID treatment (Lascelles et al., 2008; Malek et al., 2012). Therefore, more treatment modalities are needed for dogs with painful OA refractory to conventional treatment.

BoNT A is a strong neurotoxin used in the treatment of various painful musculoskeletal conditions in human patients (Boyd and Hays, 2001; Simpson et al., 2008a; 2008b; Marsh et al., 2014). Conventionally, BoNT A is administered as IM and subdermal injections. However, the toxin has also shown direct antinociceptive efficacy in arthritic human patients when administered IA directly into the painful joint (Mahowald et al., 2009; Singh et al., 2009b; Boon et al., 2010; Sun et al., 2014; Hsieh et al., 2016). This encouraged us to investigate the pain-relieving efficacy of this novel IA therapy in osteoarthritic dogs in a randomized, placebo-controlled, double-blinded clinical trial.

The mechanism of the antinociceptive action of the toxin inside a joint is not known, but there is a prevailing hypothesis that IA BoNT A inhibits the release of neurotransmitters and inflammatory mediators inside the joint (Aoki, 2005; Mahowald et al., 2009). To test this hypothesis, we investigated the effect of IA BoNT A on SF and serum SP, PGE2, and TNF-α in osteoarthritic dogs.

To gain more knowledge on canine osteoarthritic pain, we studied whether the concentration of SP and PGE2 differs in the SF or serum of osteoarthritic dogs compared to non-osteoarthritic dogs. In addition, we investigated the associations between the pain mediators, clinical variables of osteoarthritic pain, and the signalment of dogs.

In addition, because the adverse effects of IA BoNT A have not been thoroughly investigated in any animal species in vivo, we studied the clinical, cytological, and histopathological adverse effects of IA BoNT A in healthy dogs and evaluated the spread of the toxin after the IA injection.

6.1 Efficacy in Treatment of Osteoarthritic Joint Pain

Our hypothesis was that IA BoNT A would produce significant pain relief compared to placebo in dogs with chronic OA and that no significant adverse
events would be detected related to the treatment. Our results support this hypothesis; however, the effect of the toxin seems to be mild.

We detected a statistically significant improvement from baseline to week 12 in our primary outcome variables, the ground reaction forces VI and PVF and the HCPI in the IA BoNT A-treated dogs ($P=0.001$, $P=0.054$, and $P=0.053$ for VI, PVF and the HCPI, respectively), while no improvement was detected in the placebo group ($P=0.515$, $P=0.571$, $P=0.180$). In VI, there was also a statistically significant difference in the improvement from baseline between the IA BoNT A and placebo groups 12 weeks after treatment ($P=0.005$).

Our findings are in accordance with the results of a pilot study by Hadley et al. (2010). They reported improvement in PVF and VI in five osteoarthritic dogs for a variable time period after IA BoNT A. Also, IA BoNT A has shown efficacy in controlled clinical trials on arthritic human patients and human patients with painful prosthetic knees leading to significant improvement in various indexes of pain, function, and quality of life (Mahowald et al., 2009; Singh et al., 2009b; Boon et al., 2010; Singh, 2010; Sun et al., 2014; Hsieh et al., 2016). In addition, antinociceptive efficacy of IA BoNT A has been reported in case series of patients with chronic pain due to OA, rheumatoid arthritis, and psoriatic arthritis (Mahowald et al., 2006; Singh and Mahowald, 2009). However, conflicting results have also been published. In a recent placebo-controlled clinical trial on IA BoNT A in human patients with chronic osteoarthritic pain in the knee, no difference was detected in the clinical outcome variables between the IA BoNT A and placebo groups during the 16-week trial (Arendt-Nielsen et al., 2016).

It is difficult to compare our results to other IA therapies investigated with the aim of reducing pain in osteoarthritic dogs. The results from studies performed in dogs with experimentally induced OA (Brandt et al., 2004; Smith et al., 2005; Pashuck et al., 2016) may not be extrapolated into the population of dogs with naturally occurring disease. In addition, many trials lack objective outcome measures (Black et al., 2007; 2008; Cuervo et al., 2014; Carapeba et al., 2016; Harman et al., 2016) and some lack a control group (Black et al., 2008). To the best of the authors’ knowledge, there have been three other studies in which an IA treatment has been compared to IA placebo using objective outcome variables in dogs with naturally occurring OA, to date. Fahie et al. (2013) reported improvement in the PVF of dogs with naturally occurring OA in the stifle, elbow, tarsus, or shoulder joint 12 weeks after an injection of PRP. The mean improvement in PVF as reported as a percentage change from baseline was 12%, which is bigger than the 5.7% improvement in our study. However, in our study, the biggest improvement was detected in the VI (mean improvement from baseline 10.7%), but VI was not assessed in the study by Fahie et al. (2013). Upchurch et al. (2016) reported no improvement from baseline in PVF or VI in dogs with hip OA after an IA injection of ADSCs and PRP and Sherwood et al. (2017) reported no statistically significant difference in the improvement from baseline in
PVF or VI of dogs with knee or elbow OA after an IA injection of concentrated dextrose.

Vilar and colleagues have studied the efficacy of IA ADSCs and PRP in dogs with naturally occurring hip OA and compared the results to a control group of healthy dogs with no treatment. They reported significant improvement from baseline in PVF and VI after an IA injection of ADSCs (Vilar et al., 2014) and an injection of ADSCs together with PRP (Vilar et al., 2013). In these studies, the improvement from baseline in the ground reaction forces was statistically significant 30 days, but no longer 90 or 180 days, after treatment, whereas in our study, the biggest improvement from baseline was detected 12 weeks (approximately 84 days) after IA BoNT A at the end of the trial.

The HCPI and the subjective pain score improved in both the IA BoNT A and placebo groups during our study. This improvement detected in the placebo group is typical of the caregiver placebo effect, in which a dog owner or a veterinarian tends to see improvement in the dog’s condition regardless of the treatment (Conzemius and Evans, 2012). The caregiver placebo effect emphasizes the importance of having at least one explicitly objective outcome measure, such as the ground reaction forces in our study, when evaluating a treatment effect in dogs with OA. In our study, no improvement was detected in the ground reaction forces in the placebo group (mean improvement from baseline in VI and PVF 0.0% and 0.3%, respectively). The improvement detected in the HCPI in our study was statistically significant only in the IA BoNT A group, which supports the improvement detected in the ground reaction forces.

The requirement for rescue analgesia did not differ between the IA BoNT A and placebo groups during the study, which does not support the idea of IA treatment diminishing the need for oral NSAIDs. However, not finding a difference in the need for rescue analgesia between the groups might be explained by the differences between the groups in the duration of lameness before the study. Despite using stratified randomization, 84% of the dogs in the IA BoNT A group had been lame for more than a year compared to 50% of the dogs in the placebo group. The difference in the duration of lameness did not reach statistical significance; however, it might have affected our results. In a study by Mansa et al. (2007), the duration of lameness before the trial significantly affected the rate of improvement in osteoarthritic dogs treated with oral NSAIDs. The response to treatment was less in dogs with a longer duration of lameness.

One dog developed a skin infection over the IA BoNT A-injected hip joint one week after the injection. It is possible that the skin was irritated when it was aseptically prepared for the IA injection as no joint infection was detected in SF analysis.
6.2 PAIN MEDIATOR ANALYSES

6.2.1 EFFECT ON PAIN MEDIATORS

In contrast to our hypothesis, we did not detect a difference in the concentrations of SF and serum SP and PGE$_2$ between the IA BoNT A and IA placebo groups during the study. The concentration of TNF-α remained under the detection limit of the assay in all serum and SF samples analysed.

It is possible that instead of inhibiting SP and PGE$_2$ inside the joint, IA BoNT A might produce pain relief via a different mechanism. The pain-relieving effect could be mediated via the inhibition of CGRP or glutamate, which are both considered important neurotransmitters in osteoarthritic pain (Ogbonna et al., 2013; Benschop et al., 2014; Bullock et al., 2014). BoNT A has been shown to inhibit the release of CGRP from rat neurons and rat bladder preparations (Durham et al., 2004; Lucioni et al., 2008) and glutamate from the primary afferent nerve endings in rat paws in a formalin pain model (Cui et al., 2004). The primary afferent neurons in the stifle joints of dogs contain CGRP (Tamura et al., 1998) but its association with osteoarthritic pain has not been studied in dogs. In addition, the role of glutamate in canine osteoarthritic pain is currently unknown.

Another possible explanation could be that the toxin produces its effects mainly in the spinal cord rather than peripherally in the joint. Instead of being internalized into the nerve cell in a vesicle, which leads to the toxin exerting its effects peripherally in the nerve ending, BoNT A has been shown to enter the nerve cell using an alternative pathway (Restani et al., 2012a). This alternative pathway leads to a retrograde transport via the axon into the cell soma in the CNS, where the toxin can undergo transcytosis and remain active cleaving SNAP-25 in sites various synapses away from the nerve cell in which the toxin first entered (Antonucci et al., 2008; Restani et al., 2012b). Central antinociceptive effects are supported by the finding that unilateral peripheral injection of BoNT A produces bilateral pain inhibition in rat models of pain (Bach-Rojecky and Lacković, 2009).

The concentration of TNF-α remained under the detection limit of the assay in all samples of osteoarthritic dogs. We ruled out a problem in the assay by analysing the recovery rate of a known amount of canine TNF-α in our SF and serum samples with consistent results. Not detecting TNF-α in the SF or serum of our osteoarthritic dogs is in line with a previous study, in which only two out of 80 SF samples from osteoarthritic dogs tested positive for TNF-α (Carter et al., 1999). The level of TNF-α in SF has been reported to be higher in acute severe joint disease than in joint disease in general (Bertone et al., 2001), and thus the chronicity of the disease might explain our results. Other studies have reported higher (Venn et al., 1993) and lower (Hay et al., 1997) concentrations of TNF-α in osteoarthritic than in normal joints of dogs. Different results in the previous studies have been explained by a loss of TNF-α during storage, the presence of inhibitors of TNF-α, peaks
Discussion

in TNF-α activity during the disease, and different sample preparation methods (Venn et al., 1993; Hay et al., 1997; Carter et al., 1999;).

6.2.2 Osteoarthritic versus non-osteoarthritic dogs

Our results indicate that SF PGE₂, but not serum PGE₂, could be a marker for chronic OA and pain in dogs. On the other hand, SF or serum SP appears not to be a marker of osteoarthritic pain in dogs.

SF PGE₂ concentration was significantly higher in the osteoarthritic dogs than in non-osteoarthritic control dogs in our study (P = 0.001). The concentration of SF PGE₂ correlated negatively with the ground reaction forces PVF and VI (r = -0.62, P = 0.001; r = -0.61, P = 0.001; respectively) indicating less weight-bearing on joints with higher concentrations of PGE₂. In addition, SF PGE₂ correlated positively with the palpatory pain in our dogs (r = 0.45, P = 0.017).

Our results are in accordance with the study by Trumble et al. (2004). They reported an increased concentration of SF PGE₂ in the stifle joints of dogs after cranial cruciate ligament transection. The concentration of SF PGE₂ correlated with the ground reaction forces and subjective evaluation of joint pain. In addition, elevated SF PGE₂ concentration has been reported in osteoarthritic horses (Caron et al., 1992; Kirker-Head et al., 2000). It has been speculated that the level of SF PGE₂ would peak in the early phase of OA (Kirker-Head et al., 2000; Trumble et al., 2004), but our results show that the SF PGE₂ concentration is also significantly increased in chronic OA in dogs. Despite this, we did not find an association between SF PGE₂ and the duration of lameness. Additionally, SF PGE₂ was not associated with age, bodyweight, gender, the breed of the dogs, or the sampled joint.

The serum concentration of PGE₂ was not different between the osteoarthritic and non-osteoarthritic dogs. Serum PGE₂ did not correlate with SF PGE₂ or with the clinical variables of osteoarthritic pain. This indicates that osteoarthritic pain is associated with local production of PGE₂, and serum PGE₂ is affected by other factors. The negative correlation between PGE₂ and SP in serum (r = -0.48, P = 0.004) was unexpected and might not be of clinical importance. A positive correlation between serum PGE₂ and SP has been reported in horses previously (Kirker-Head et al., 2000).

SF SP concentration did not differ between the osteoarthritic dogs and control dogs. In addition, we did not detect an association between SF or serum SP and the clinical variables of osteoarthritic pain in our study. This supports our finding that IA BoNT A does not affect the SF SP concentration despite the improvement in the ground reaction forces and the HCPI during the study.

In a study by Rialland et al. (2014), the spinal SP level correlated with VAS and central pain evaluated with electrodermal activity measurements in dogs with transected cruciate ligaments. However, spinal SP did not correlate
with the ground reaction forces or other variables used to evaluate peripheral and central sensitization. Conflicting findings have also been reported in human patients on the role of SP in osteoarthritic pain. Although a positive correlation has been reported between SF SP level and OA pain (Gotoh et al., 1998), no correlation (Holmlund et al., 1991; Sato et al., 2007), and a negative correlation (Appelgren et al., 1998) have also been published in human patients.

6.3 ADVERSE EFFECTS AND SPREAD OF TOXIN

6.3.1 CLINICAL ADVERSE EFFECTS

Our hypothesis was that BoNT A would not produce significant clinical adverse effects when administered IA in the stifle joints of healthy dogs. Our results support this hypothesis.

Our results are in line with the pilot study in dogs by Hadley et al. (2010) and with the controlled trials on IA BoNT A in human patients (Mahowald et al., 2009; Singh et al., 2009b; Boon et al., 2010; Singh, 2010; Joo et al., 2012; Arendt-Nielsen et al., 2016). In those studies, no severe clinical adverse events were reported related to IA BoNT A treatment. However, in contrast to our study, these studies did not focus specifically on examining the adverse effects. The monitoring of adverse events included telephone interviews in the case of osteoarthritic dogs, and interviews, muscle strength evaluation, and repeated neurosensory examinations in the human patients.

In a meta-analysis of the safety of IM and subdermal BoNT A injections in human patients, the only adverse event occurring significantly more often in the BoNT A than in the control groups was local weakness (Naumann and Jankovic, 2004). In an arthritic patient, local muscle weakness would be especially undesirable as it would counteract the muscle strengthening exercise, which is recommended for OA treatment in human patients (McAlindon et al., 2014).

We hypothesized that if IA BoNT A leads to local weakness in dogs this would be best measured by evaluating the dynamic and static weight-bearing of healthy dogs by a force platform and bathroom scales as manual muscle strength evaluation used in human studies is not feasible in our patients. We did not detect any significant change in the weight distribution between the IA BoNT A- and IA-placebo injected limbs while the dogs were running on the force platform. When the dogs were standing they bore more weight on the IA BoNT A-injected limbs compared to the IA placebo-injected limbs. This suggests that local weakness should not be a concern after an IA injection of 30 IU of BoNT A in healthy dogs.

We did not detect any palpatory pain in the injected joints of the laboratory dogs during Study III. However, a mild but significant decrease in
Discussion

the range of motion was seen in the placebo-injected joints. This, in addition to the weight shift away from the placebo-injected limbs during standing, might have been a consequence of discomfort caused by repeated arthrocentesis and electrophysiological recordings during the study. If this hypothesis is true, IA BoNT A might have alleviated this discomfort in the contralateral limbs.

Our results contrast with the injection site swelling, redness, and increased lameness reported by Hadley et al. (2010). The study did not have a control group, so it is not known whether the reported adverse events were related to the IA injection or to the toxin itself. In human patients, local weakness and injection site pain have been reported at similar frequencies in the IA BoNT A and control groups (Mahowald et al., 2009; Singh et al., 2009b; Boon et al., 2010; Singh, 2010; Arendt-Nielsen et al., 2016;).

6.3.2 SPREAD OF TOXIN

Our results support our hypothesis that the toxin may spread from the joint. However, its effect seems to be mild.

BoNT A can spread systemically after a single IM injection in human patients. Systemic spread has been detected as neurological deficits and generalized weakness resembling botulism (Bakheit et al., 1997) but also only as a block in the neuromuscular transmission in patients without any clinical signs (Olney et al., 1988). We investigated the spread of the toxin through repeated neurological examinations in Studies I and III and also through electrophysiological recordings in Study III.

We detected neurological abnormalities in one of the 35 osteoarthritic dogs in Study I and in four of the six laboratory dogs in Study III. In Study I, the cause of the hypometric gait and mild ataxia in the hindlimbs of one dog in the IA BoNT A group was a mild disc protrusion in the T13–L1 region detected by MRI. In Study III, one dog had decreased patellar reflexes four weeks after the injection, which might have resulted from the spread of the toxin. However, another dog developed a decreased patellar reflex during the study, but only in the placebo-injected limb. Therefore, we consider it more likely that the decreased patellar reflexes were a result of repeated manipulations performed on the joints during the study. The other neurological abnormalities detected in Study III, decreased extensor carpi radialis reflexes and facial sensation, may be an underestimation of those reflexes due to the shyness of the laboratory dogs.

As in our study, no abnormalities have been reported in repeated neurosensory testing in human patients treated with IA BoNT A (Mahowald et al., 2009; Singh et al., 2009b; Singh, 2010). However, generalized botulism-like symptoms have been described in human patients after an IM injection of the toxin (Bakheit et al., 1997; Bhatia et al., 1999; Crowner et al., 2010). The symptoms included generalized fatigue, instability, urinary incontinence, respiratory difficulties, weakness in the muscles of the limbs,
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neck, and trunk, difficulties in swallowing and speaking, drooping of eyelids, diplopia, nasal regurgitation, and various neurological deficits. The symptoms started 4 days to 4 weeks after the BoNT A injection and lasted for up to 6 months. In some patients, the symptoms developed after the first injection (Bakheit et al., 1997; Wyndaele and Van Dromme, 2002; Duffey and Brown, 2006; Crowner et al., 2010), but other patients had been injected regularly for several years before the event (Bakheit et al., 1997; Bhatia et al., 1999; Wyndaele and Van Dromme, 2002; Howell et al., 2007; Crowner et al., 2010). It has been suggested that the cumulative effect of the toxin after repeated injections and the total dosage of the toxin given contribute to these symptoms. However, there are patients in whom the same treatment regimen has been continued after recovery without further botulism-like events (Bhatia et al., 1999). Iatrogenic botulism has not been reported in dogs.

Although we did not detect neurological abnormalities related to IA BoNT A, the results of our electrophysiological recordings suggest subclinical spread of the toxin. The decrease in the CMAP amplitudes in the IA BoNT A-injected limbs of two dogs may be a consequence of local spread of the toxin. Low CMAP amplitudes have been described in dogs with botulism (Barsanti et al., 1978; van Nes and van der Most van Spijk, 1986; Uriarte et al., 2010) and low CMAP amplitudes are considered the most consistent finding in human patients with botulism (Cherington, 1998). In addition, one of these dogs developed abnormal EMG activity in one front limb during the study, which might indicate systemic spread of the toxin. An IM injection of BoNT A has been shown to cause electrophysiological changes far from the injection site in human patients despite them being asymptomatic, which has been interpreted as systemic subclinical spread of the toxin (Olney et al., 1988; Lange et al., 1991).

However, there is some inconsistency in our results. In the previous case reports of dogs with botulism, the low CMAPs have been reported to occur at the same time with marked abnormalities in the neurological examinations (Barsanti et al., 1978; van Nes and van der Most van Spijk, 1986; Uriarte et al., 2010), whereas in our study, the findings in the electrophysiological recordings did not coincide with neurological abnormalities. In addition, one dog had low CMAPs at baseline before the injections. However, the dog might have had a subclinical, transient neuromuscular disorder at the beginning of the study.

6.3.3 CYTOLOGICAL AND HISTOPATHOLOGICAL ADVERSE EFFECTS

Our results suggest that an IA injection of 30 IU of BoNT A does not have delayed histopathological effects on articular cartilage or synovium in a healthy canine joint, which supports our hypothesis. We did not detect any statistically significant difference in the scores for articular cartilage or synovial pathology between the BoNT A- and placebo-injected joints 12
weeks after the injections. In addition, we did not detect any difference in the synovial inflammatory cell infiltrates between the joints. Also, the SF total and differential cell counts were in the reference range published for dogs during the study except for the one sample with blood contamination. Our findings are in accordance with not detecting an increase in the rate of cell death in chondrocyte cell culture (Henzel et al., 2008) and with not finding abnormalities in the synovium of two horses 15 days after IA BoNT A (DePuy et al., 2007). Also, not detecting histopathological or cytological adverse effects is in line with not detecting clinical adverse effects in these dogs.

We detected mild muscle cell size variation and some angular and ring fibres in the muscles of the IA BoNT A- and placebo-injected limbs. In addition, we detected some Büngner bands and few changes associated with Schwann cell degeneration in the nerves we examined. Although changes associated with denervation atrophy and degeneration have been detected in the BoNT A-injected muscles and the nerves innervating the muscles previously (Choi et al., 2007; Elmas et al., 2007; Rosales et al., 2008; Schoroeder et al., 2009), our findings were mild and without a statistically significant difference or any obvious bilateral asymmetry between the BoNT A- and placebo-injected limbs. Therefore, we believe that the mild changes we detected were not related to IA BoNT A injection.

In the autopsy of a dog that died due to botulism, no lesions were reported in the nervous system or in the limb musculature (Barsanti et al., 1978). In a human patient that succumbed to foodborne botulism, gross muscular atrophy was reported but otherwise the autopsy findings were non-specific (Devers and Nine, 2010). We did not detect any significant findings in the autopsy of our dogs. We found spinal spheroids in the lumbal spinal cord of all six dogs, but we consider this an incidental finding. Spinal spheroids are local axonal swellings associated with degeneration of the axon and blockage of the axonal transport (Cantile and Youssef, 2016). They are commonly found in low numbers in adult and aged animals. No absolute cut-offs for a pathological number of spheroids exist. However, local increase in spheroids can occur with spinal cord compression and vitamin E deficiency (Cantile and Youssef, 2016). In our dogs, the spheroids were randomly scattered within the white and grey matter and the dogs did not show signs of spinal dysfunction.

The mild lymphoplasmacytic myocarditis detected in one dog may have been a consequence of an infection before the study, because no clinical signs indicating infection were detected in the clinical examinations performed during the study. Our findings support the conclusion that the possible spread detected in the electrophysiological recordings is of minor clinical significance.
6.4 LIMITATIONS

The major limitation in Studies I and II is the small sample size. We screened 106 dogs for inclusion and exclusion criteria in Study I, but only 36 dogs were proven eligible for the trial and one was later excluded due to erroneous diagnosis. In addition, we were not able to obtain valid trials for obtaining PVF and VI in three dogs in the IA BoNT A group and in one dog in the placebo group. However, owner compliance was excellent as no owner discontinued the trial and no dog was lost for follow-up. The small sample size may have resulted in a lack of power for detecting a significant difference in the improvement from baseline in PVF and the HCPI between the groups. In addition, the small sample size prevented us from performing a subgroup analysis to investigate which dogs had the greatest benefit from the treatment. In human patients, IA BoNT A has shown better antinociceptive efficacy in patients with more severe disability (Mahowald et al., 2009; Singh et al., 2009b). In addition, although Arendt-Nielsen et al. (2016) did not detect a difference in the improvement in joint pain between the IA BoNT A and placebo groups, in a subgroup analysis, IA BoNT A produced significant pain relief in patients with a nociceptive type of pain. No improvement was detected in patients with a neuropathic type of pain.

Although 35 dogs were included in Study I, we were not able to collect enough SF for analysis from every dog at every visit due to the small amount of SF in the joints of the dogs. Therefore, the number of samples in our pain mediator analyses in Study II was small (n = 12 in the IA BoNT A group and n = 7 in the placebo group). A lavage method has been described to increase the volume of SF collected (Kraus et al., 2002), but we decided not to use this method to avoid any interference in the pain mediator analysis and in the clinical effects of the toxin. Also, although the strongest effect of the toxin on osteoarthritic pain in Study I was seen at week 12, the serum and SF samples for the pain mediator analysis in Study II were collected 2 and 8 weeks after the injections. The schedule for collecting the samples was based on our estimate of the maximal effect of the toxin, which was derived from the duration of the effect of the toxin after IM injections.

Another limitation in Study II is that during the process of writing this thesis, the manufacturer of the commercial SP ELISA we used reported 100% cross reactivity between SP and a novel neuropeptide haemokinin-1 in human samples in their assay. Haemokinin-1 binds to the same receptors as SP with similar affinity and has similar biological activities (Zhang et al., 2000). However, unlike SP, it is mainly expressed in non-neuronal tissues (Zhang et al., 2000; Bellucci et al., 2002). The cross reactivity was reported in human samples, but it is currently not known whether the cross reactivity applies to dogs. Haemokinin-1 and other novel SP-like neuropeptides have been described in humans, rat, and rabbits (Page, 2004), but, to the best of our knowledge, not yet in dogs.
We used a standardized histopathological evaluation system for assessing the adverse effects of the toxin on the cartilage and synovium in Study III (Cook et al., 2010). Although the histopathological evaluation is currently the reference standard for evaluating OA, analysing the expression of biomarkers of cartilage degradation and synthesis or oxidative stress might lead to the detection of more subtle effects on the cartilage homeostasis. We did not detect significant cytological or histopathological adverse effects in healthy canine joints, but osteoarthritic cartilage might be more prone to toxic effects and injury.

6.5 FUTURE ASPECTS

The dosage of 30 IU of BoNT A we used in our studies was extrapolated from the previous trials on human patients (Mahowald et al., 2006; 2009; Singh and Mahowald, 2009; Singh et al., 2009b). However, species differences exist in the sensitivity to different BoNT serotypes (Peng et al., 2014). Although serotype A causes botulism in humans (McCallum et al., 2015), it has not been reported to cause botulism in dogs, which may indicate that dogs could be less sensitive to BoNT A. BoNT serotypes B (Lamoureux et al., 2015), C (Barsanti et al., 1978; van Nes and van der Most van Spijk, 1986; Bruchim et al., 2006; Uriarte et al., 2010), and D (Doutre, 1983; 1982) have been identified as the cause of canine botulism. BoNT B is also commercially available, and, in addition to a larger dosage of BoNT A, the antinociceptive efficacy of BoNT B might be an interesting subject for further studies in dogs.

In addition, a clinical trial with a longer follow-up period would be of interest, as the biggest improvement in the osteoarthritic dogs in Study I was detected after 12 weeks at the end of the trial. Interestingly, in a controlled trial on IA BoNT A in painful stifle OA in human patients, the pain relief lasted until the end of the study period of 6 months (Hsieh et al., 2016). In a case series of patients with rheumatoid arthritis and OA, the pain relief lasted for 3–17 months (Singh et al., 2009a).

As ELISA tests did not reveal any effect of IA BoNT A on the SF SP or PGE2 concentrations, more sophisticated techniques, such as SF metabolomics, could be considered in investigating the mechanism of the toxin’s antinociceptive action inside a joint.
7 CONCLUSIONS

Based on these studies, we conclude the following:

1. IA BoNT A has some efficacy in the treatment of osteoarthritic joint pain in dogs and it may be of clinical importance in dogs suffering from chronic, osteoarthritic pain. We did not detect any adverse events related to IA BoNT A in the osteoarthritic dogs.

2. Contrary to the prevailing hypothesis, the antinociceptive effect of IA BoNT A in the joint seems not to be related to the inhibition of the release of SP or PGE2. TNF-α was not detectable in the serum or SF of the osteoarthritic dogs.

3. SF PGE2, but not serum PGE2, could be a marker of chronic OA and pain in dogs. However, neither SF nor serum SP seems to be a marker of osteoarthritic pain in this species.

4. IA BoNT A does not produce clinical, cytological, or histopathological adverse effects when injected into a healthy stifle joint in dogs. The toxin may spread from the joint, but its effect seems to be mild.
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