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SEGURANÇA DE USO E EFEITO CLÍNICO DA APLICAÇÃO INTRA-ARTICULAR DE TOXINA BOTULÍNICA TIPO A PARA DOR ARTICULAR EM EQUINOS

Santa Maria, RS
2022

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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Cirurgia e Clínica Veterinária.**

Orientador: Prof. PhD. Flávio Desessards De La Côrte

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Aprovado em 09 de setembro de 2022:

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Santa Maria, RS
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“Dificuldades preparam pessoas comuns para destinos extraordinários”
(C.S. Lewis)

RESUMO

SEGURANÇA DE USO E EFEITO CLÍNICO DA APLICAÇÃO INTRA-ARTICULAR DE TOXINA BOTULÍNICA TIPO A PARA DOR ARTICULAR EM EQUINOS

AUTOR: Antônio Alcemar Beck Júnior
ORIENTADOR: Flávio Desessards De La Côrte

O complexo sinovite-osteoartrite é a principal causa de claudicação em equinos, afetando diretamente sua qualidade de vida. A toxina botulínica tipo A (TBA) tem sido explorada em medicina humana e veterinária como uma opção promissora para pacientes que apresentam dor articular crônica. Nesta busca por opções terapêuticas, o primeiro estudo objetivou investigar se uma aplicação intra-articular de TBA promoveria efeitos adversos em parâmetros físicos, na avaliação de claudicação, e, sua potencial resposta inflamatória no fluido sinovial (FS). Uma articulação radiocarpiana selecionada aleatoriamente foi tratada com 50 UI de TBA em oito equinos, tendo a articulação contralateral recebido solução salina (SAL). Todos os equinos receberam aplicações no dia 0 e foram reavaliados duas vezes ao dia, durante sete dias para os parâmetros frequência cardíaca (FC), frequência respiratória (FR), temperatura retal (TR), coloração de membranas mucosas, tempo de preenchimento capilar, motilidade intestinal, apetite, consumo de água, defecação, micção e atitude. Nos mesmos momentos, dor e circunferência articulares foram avaliadas. Avaliações objetivas de claudicação também foram realizadas diariamente por sete dias e amostras basais de FS foram coletadas e também 24 e 168 horas após a aplicação (HPA), sendo avaliadas para parâmetros celulares no LS. Os parâmetros clínicos FC e TR permaneceram inalterados, apresentando oscilações durante o estudo ($p=0,001$). Os demais parâmetros clínicos foram inalterados pelo tratamento e pelo tempo ($p>0,05$). Dor articular não foi induzida pela flexão e palpação em ambos os membros, assim como a circunferência carpiana não foi alterada ($p=0,88$). Foi observada claudicação apenas em membros tratados com SAL. Os parâmetros celulares avaliados no FS em amostras de ambos os membros aumentaram significativamente dos valores basais até a HPA 24, diminuindo na HPA 168. Em conclusão, a aplicação IA de 50 UI de TBA foi sugerida como uma terapia segura para uso IA em equinos. No segundo estudo, o objetivo foi avaliar o efeito da aplicação IA de TBA em equinos com osteoartrite társica distal crônica. Nove equinos foram selecionados para o estudo após completa avaliação física e radiográfica. Os animais também foram submetidos à avaliação objetiva de claudicação e foram incluídos se apresentassem claudicação de impacto em dos membros pélvicos (variável Pmin $\geq$ 3mm), com resposta positiva ($\geq$50%) ao bloqueio anestésico das articulações tarsometatarsiana e intertarsiana distal. A presença de sinais radiográficos de osteoartrite também foi um critério de inclusão. Após, os equinos receberam aleatoriamente a aplicação IA de 50 UI de TBA ou volume equivalente de SAL. Cinco equinos foram incluídos no grupo TBA, enquanto quatro foram alocados no grupo placebo. Para avaliar as medidas de efeito, os equinos foram reavaliados nos dias pós-aplicação (DPA) 1, 7, 15, 30, 60, 90, 120, 150 e 180. Os critérios de sucesso incluíram: diminuição dos valores de Pmin (($\leq$ 3mm) e abolição da claudicação basal com surgimento de claudicação no membro contralateral, em casos de indivíduos afetados bilateralmente pela condição. Adicionalmente, um percentual de melhora na claudicação foi calculado para todos os animais nos diferentes momentos. Melhora significativa foi observada em equinos do grupo TBA quando comparados ao placebo nos DPA 90 ($p<0,05$), 120 ($p<0,001$), 150 ($p<0,001$) e 180 ($p<0,05$). Individualmente, 40% (2/5) dos equinos do grupo TBA apresentaram melhora absoluta (100%) na claudicação em todos os momentos avaliados. No mesmo grupo, 80% (4/5) dos equinos demonstraram melhora absoluta em pelo menos quatro momentos avaliados. Apenas um equino desse grupo não apresentou melhora absoluta durante o estudo, com média ± e.p. 51,53±19,36%. Maiores percentuais de melhora na claudicação foram observados no DPA 60 (95,92±9,13%). Em contraste, nenhum equino do grupo placebo demonstrou melhora absoluta na claudicação após o tratamento. Os resultados deste estudo mostraram que a aplicação IA de 50 UI de TBA foi efetiva em reduzir a claudicação em equinos com osteoartrite társica distal crônica, sobretudo após 90 dias após administração.

Palavras-chave: Equinos. Toxina botulínica tipo A. Osteoartrite. Dor articular

ABSTRACT

SAFETY AND CLINICAL EFFECT OF INTRA-ARTICULAR INJECTION OF BOTULINUM TOXIN TYPE A IN THE MANAGEMENT OF JOINT PAIN IN HORSES

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The complex synovitis-osteoarthritis is the main cause of lameness in horses, directly impacting their quality of life. Botulinum toxin type A has been explored in human and canine medicine as a very promising option for patients suffering from chronic joint pain. The first study aimed to investigate whether a single injection of botulinum toxin type A (BoNT-A) would produce adverse effects on physical parameters, on lameness evaluation and potential synovial fluid inflammatory response. One randomly selected radiocarpal joint was treated with 50 U of BoNT-A in eight horses, and the contralateral joint received saline solution. All horses received injections at day 0 and were re-evaluated twice daily for seven days for heart rate (HR), respiratory rate (RR), rectal temperature (RT), mucous membrane color, capillary refill time, intestinal motility, appetite, water intake, defecation, urination, and attitude. At these same time points, joint pain and circumference were assessed. Objective lameness evaluations were performed once daily for seven days and synovial fluid samples were collected at baseline, post-injection hour (PIH) 24 and PIH 168 and evaluated for synovial fluid parameters. HR and RT remained clinically unaltered, despite oscillations over time ($p=0.001$). The remaining clinical parameters were unaltered by treatment or time ($p>0.05$). Joint pain was not elicited by flexion and palpation in both limbs as well as carpal circumference was unaltered ($p=0.88$). Lameness was observed only on saline injected limbs. Cellular parameters evaluated in synovial fluid samples from both carpi had significantly increased from baseline to PIH 24, decreasing at PIH 168. In conclusion, intra-articular (IA) injection of 50 U of BoNT-A is suggested to be a safe therapy for IA use in horses. In the second study, the objective was to evaluate the clinical effect of intra-articular (IA) injection of BoNT-A in horses with chronic distal tarsal osteoarthritis. Nine horses were selected for the study after a complete physical and radiographic assessment. Horses also underwent an objective lameness examination and were included if they had a hind limb impact lameness (Pmin variable ≥ 3mm), which positively responded (≥50%) to the tarsometatarsal (TMT) and centrodistal (CD) joints anesthetic block. Presence of radiographic signs of osteoarthritis also was an inclusion criterion. After, horses randomly received IA injection with 50 U of BoNT-A or equivalent volume of saline solution. Five horses were included in the BoNT-A group, whereas four individuals were allocated in the placebo group. The horses were re-evaluated at post-injection days (PID) 1, 7, 15, 30, 60, 90, 120, 150 and 180. Success criteria included: decrease of Pmin values (≤ 3mm) and abolishment of lameness on the baseline lame limb with lameness shifting to the contralateral limb, in cases of individuals bilaterally affected by the condition. Additionally, a percentage of lameness improvement was calculated for all horses at all timepoints. Significant improvement was observed in horses from TBA group when compared with placebo at PID 90 ($p<0.05$), 120 ($p<0.001$), 150 ($p<0.001$) and 180 ($p<0.05$). Individually, 40% (2/5) of horses from the TBA group had a complete improvement (100%) in lameness at all the timepoints. In the same group, 80% (4/5) of horses demonstrated complete improvement for at least four timepoints evaluated. Only one horse from this group did not present complete improvement during the study, with mean ± s.e. 51.53±19.36%. Higher percentages of lameness improvement were observed at the PID 60 (95.92±9.13%). In contrast, no horses from the placebo group demonstrated complete lameness improvement after treatment. Results of this study suggested that the IA injection with 50 U of BoNT-A was effective in reducing lameness in horses with chronic distal tarsal osteoarthritis, mainly after 90 days post-injection.

Keywords: Horse. Botulinum toxin type A. Lameness. Osteoarthritis. Joint Pain.

SUMÁRIO

1 INTRODUÇÃO

Seja para o conforto de éguas e garanhões em meio à temporada reprodutiva ou em animais de alta exigência atlética, é constante a preocupação com o desenvolvimento e aperfeiçoamento de terapias para osteoartrite (OA) em equinos. Nesse sentido, inúmeras modalidades terapêuticas têm sido desenvolvidas ao longo das décadas, no intuito de reduzir os efeitos deletérios promovidos pela dor e inflamação articulares.

Classicamente, o uso sistêmico dos anti-inflamatórios não-esteroidais (AINEs) apresenta efetividade na redução de claudicação proveniente de doenças articulares (CARON, 2005). Entretanto, além de frequentemente representar apenas uma terapia paliativa para o controle da dor, quando utilizados por períodos prolongados ou em altas doses, suscitam importantes efeitos adversos, incluindo ulcerações gastrointestinais e nefrotoxicidade (McCKAY et al., 1983; MAY & LEES, 1996). Devido ao seu potente efeito anti-inflamatório, a aplicação intra-articular (IA) de corticosteróides, associados ou não ao hialuronato de sódio, é ainda considerada pela comunidade veterinária um dos tratamentos de eleição para animais afetados por essa condição. No entanto, além de seus benefícios, efeitos adversos ao ambiente articular são bem estabelecidos (McILWRAITH, 2010) e podem incluir necrose de condrócitos, efeitos sobre a estrutura das fibras de colágeno e inibição da síntese de proteoglicanos presentes na cartilagem articular (CHUNEKAMRAI et al., 1989; TODHUNTER et al., 1996). Adicionalmente, respostas clínicas parciais ou mesmo casos refratários a esses tratamentos tradicionais motivam a constante busca por alternativas que promovam conforto aos pacientes.

Sabidamente, os estímulos dolorosos são carreados por receptores e nociceptores localizados em variados tecidos articulares. Ao chegar no corno dorsal da medula espinhal, o sinal é enviado ao cérebro por neuromoduladores e neurotransmissores, onde ocorre a modulação e percepção da dor (RAFFA, 2003). O entendimento desse complexo mecanismo permite o desenvolvimento de terapias direcionadas ao controle nociceptivo como alternativa às tradicionais terapias anti-inflamatórias. Nesse cenário, descobertas datadas do início da década de 2000 apontam o potencial uso terapêutico da toxina botulínica tipo A (TBA) para o controle da dor.

Inicialmente a TBA era conhecida apenas por ser o agente causador do botulismo, doença potencialmente letal aos animais e humanos, e caracterizada paralisia muscular flácida, decorrente de inibição da acetilcolina na fenda sináptica em terminações nervosas colinérgicas. No entanto, desde o final dos anos de 1970, esse mecanismo tem sido explorado em medicina

humana para o tratamento de doenças caracterizadas por hiperatividade muscular dolorosa ((BALASH & GILADI, 2004; SIMPSON et al., 2008a; DONG et al., 2017). Posteriormente, estudos em roedores demonstraram efeitos anti-nociceptivos que precediam o relaxamento muscular, os quais foram atribuídos à inibição de neurotransmissores secretados sob estimulação nociceptiva, tais como a substância P (ISHIKAWA et al., 2000), o peptídeo relacionado ao gene da calcitonina (DURHAM et al., 2004) e o glutamato (CUI et al., 2004). Essa descoberta foi fundamental no entendimento das ações clínicas dessa neurotoxina e estimulou estudos clínicos em humanos, voltados para o tratamento da dor crônica em casos, por exemplo, de OA e artrite reumatóide MAHOWALD et al., 2006; SINGH et al., 2009). Resultados positivos foram também observados em cães que receberam aplicações IA de TBA para o tratamento de dor relacionada à OA (HADLEY et al., 2010; HEIKKILA et al., 2014).

Dessa forma, estimulado pelos resultados promissores do uso IA de TBA em pacientes humanos e em animais de companhia, nosso grupo de pesquisa desenvolveu os dois estudos clínicos contidos nesta tese. O primeiro objetivando atestar a segurança de uso da IA da TBA através da avaliação clínica de potenciais efeitos adversos sistêmicos e da resposta inflamatória local induzida pela aplicação IA da TBA. Adicionalmente, o segundo estudo, teve como objetivo avaliar o efeito clínico do uso IA da toxina em equinos com osteoartrite társica distal.

2 REVISÃO BIBLIOGRÁFICA

2.1 OSTEOARTRITE

2.1.1 Aspectos gerais

Osteoartrite (OA) ou doença articular degenerativa (DAD) é uma condição crônica e progressiva das articulações móveis, caracterizada por degeneração e perda da cartilagem articular, além de proliferação óssea na superfície e margens articulares (CARON, 2011). Esse processo complexo pode se manifestar clinicamente com claudicação de intensidade variável e figura entre as principais causas responsáveis pela incapacidade de competir, queda de rendimento, perdas econômicas e comprometimento definitivo da função atlética em cavalos de esporte (JEFFCOT et al., 1992; FRISBIE, 2006; GOODRICH & NIXON, 2004).

Em pacientes humanos, a OA é considerada uma das maiores preocupações em saúde, devido ao considerável número de pessoas afetadas e o severo impacto causado por esse processo degradante na qualidade de vida (VAN WEEREN & de GRAUW, 2010). Sob a ótica financeira, somente nos Estados Unidos (EUA), os custos com medicamentos e serviços médicos para tratar essa condição foram estimados em US$ 89,1 bilhões, somente em 2001 (LEIGH et al., 2001)

Na realidade da indústria equina, como já referido, a DAD tem impacto destacado no desempenho de animais atletas. Em um estudo desenvolvido na Grã-Bretanha, foi observado que o principal fator de desgaste em cavalos de corrida em campanha atlética foi a claudicação, sendo que, dentre esses animais, a dor relacionada às articulações foi predominante (ROSSDALE et al., 1985). Entre os anos de 1983 e 1987 na Universidade de Cornell (EUA), aproximadamente 20% dos animais recebidos no Hospital de Grandes Animais foram examinados para claudicação, dos quais, em torno de 40% tinham doença articular como causa primária (TODHUNTER & LUST, 1990).

2.1.2 Anatomia e fisiologia das articulações móveis

Para entender a manifestação clínica da OA, é imprescindível compreender a anatomia e fisiologia articulares. As articulações diartrodiais são formadas pelas extremidades de dois ossos, recobertas por uma fina cartilagem hialina, sob a qual repousa o osso subcondral, uma

fina placa osso cortical responsável por dar suporte à cartilagem e auxiliar na absorção de choques mecânicos (FRISBIE, 2012). Revestindo a articulação, existe uma estrutura bicamada conhecida como cápsula articular, formada por uma camada fibrosa externa dedicada à estabilidade articular e uma camada interna de natureza secretória e fagocitária denominada membrana sinovial ou sinóvia (FRISBIE et al., 2012). A população celular da sinóvia é baseada em dois tipos de células, os sinoviócitos tipos A e B, envolvidos, respectivamente, nos processos de fagocitose ou pinocitose e secreção de proteínas (HENDERSON & PETTIPHER, 1985). Esse tecido apresenta um padrão viloso, capaz de aumentar sua superfície de contato com o fluido sinovial, regulando a aparência deste através de permeabilidade seletiva. Dessa forma, a membrana sinovial funciona como uma espécie de barreira biológica, permitindo que apenas pequenas moléculas proteicas, oxigênio, dióxido de carbono e glicose ganhem acesso ao fluido sinovial (FRISBIE, 2012)

O referido fluido sinovial é um ultrafiltrado do plasma depositado na cavidade sinovial com a função de nutrir a cartilagem articular e auxiliar no processo de lubrificação, diminuindo a fricção entre tecidos moles e extremidades ósseas decorrentes da locomoção (TODHUNTER, 1996). A cartilagem articular é uma estrutura central na constituição da superfície e função articulares (FRISBIE, 2012) e cuja degradação é um evento *sine qua non* no processo da osteoartrite (McILWRAITH, 1982). Avascular e não-inervada, a cartilagem é composta por aproximadamente 2% de células especializadas (condrócitos), dispersas em uma matriz extracelular formada majoritariamente por água (75%), colágeno (15%) e proteoglicanos (10%) (POOLE, 2001). O colágeno tipo II constitui aproximadamente 90 a 95% das fibras colágenas da matriz e desempenha importante papel na absorção de forças de tensão e compressão impostas à cartilagem (FOX et al., 2009; FRISBIE, 2012). Por sua vez, os proteoglicanos (PG) são moléculas formadas pela combinação de proteínas e glicosaminoglicanas, sobretudo o 4-sulfato de condroitina, 6-sulfato de condroitina e o sulfato de queratan. Associados ao ácido hialurônico, os PG formam um monômero denominado agrecan, cujo equilíbrio de suas moléculas e da tensão das fibras de colágeno são fundamentais para a capacidade da matriz extracelular em resistir e distribuir a compressão decorrente das cargas mecânicas (HALL et al., 2003).

Por fim, a estabilidade articular pode ser definida como a habilidade da articulação em manter uma posição funcional adequada quando de variação pelo movimento (BURNSTEIN & WRIGTH, 1994). Esta função é desempenhada basicamente pelos ligamentos, tendões e músculos periarticulares. Nos membros de equinos, a maioria das articulações móveis é cercada

por ligamentos colaterais, os quais proporcionam a elas estabilidade nos aspectos medial e lateral.

2.1.3 Fisiopatologia da osteoartrite

Com o decorrer das décadas, análises anatômicas, histopatológicas e de diagnóstico por imagem têm auxiliado a definir a natureza dos eventos que culminam com a perda da cartilagem articular em casos de OA. Entretanto, essas vêm demonstrando que a OA não é uma desordem limitada à cartilagem, mas inclui eventos que afetam adversamente os múltiplos componentes articulares, tais como a inflamação da membrana sinovial e remodelações na camada subcondral (GOLDRING & GOLDRING, 2006). Dessa forma, alterações simultâneas na estrutura e/ou atividade da membrana sinovial, fluido sinovial, cartilagem articular e osso subcondral contribuem para a perpetuação e irreversibilidade do quadro de OA.

A inflamação da membrana sinovial pode ser definida como sinovite primária ou secundária, conforme a natureza do dano a ela causado (FRISBIE, 2012). Inicialmente, uma vez ocorrida inflamação no interior da articulação e consequente dano à cartilagem, debris celulares são acumulados no fluido sinovial, os quais requerem a produção de um maior número de sinoviócitos macrofágicos (tipo A) para sua remoção. Frente a esse aumento na sua demanda, as vilosidades sinoviais sofrem hiperplasia e subsequente hipertrofia, que favorecem a síntese de uma quantidade maior de líquido sinovial para prover mais nutrientes aos novos sinoviócitos e à cartilagem danificada. Clinicamente, a associação desses eventos produz a efusão sinovial observada em casos de OA (GOLDENBERG et al., 1982).

Com o progresso da OA, a permeabilidade dos capilares sinoviais aumenta, permitindo a passagem de moléculas maiores pela membrana. Assim, moléculas de ácido hialurônico e lubricina saem mais facilmente do ambiente articular, aumentando o conteúdo de água e diminuindo a viscosidade do fluido sinovial (KOHLHOF et al., 2016). Por consequência, ao experimentar um aumento na fricção dos tecidos, a cartilagem articular começa a ser degradada e sofrer degeneração, com posterior esclerose óssea subcondral e formação de osteófitos. Uma vez danificada, a membrana sinovial se torna um tecido fibroso, perdendo gradativamente sua elasticidade e criando um processo de limitação no ângulo de movimento articular (FRISBIE, 2012), um dos principais sinais de inflamação crônica.

Uma teoria amplamente aceita é a de que o ambiente articular normal possui um estado de homeostase, que é caracterizado por um equilíbrio nas atividades metabólicas e catabólicas. A partir da instauração do processo de OA, a inflamação altera a consonância dessas rotas,

predominando os efeitos da via catabólica, os quais levam à falhas estruturais e funcionais dos tecidos (FRISBIE, 2012). Ao detectar alterações IA, os condrócitos são estimulados a liberar proteínas regulatórias denominadas citocinas, as quais podem ser classificadas como anabólicas, modulatórias ou catabólicas. As citocinas catabólicas mais notáveis são a interleucina 1 (IL-1) e o fator de necrose tumoral alfa (TFN-α), ambas ativas no processo de OA ao promover a produção de metaloproteinases (MMPs), óxido nítrico e prostaglandina E_2 (PGE_2), bem como ao inibir a síntese de agrecan e colágeno tipo II (McILWRAITH, 1996). Enzimas modulatórias como a IL-4, IL-10 e IL-13 são envolvidas na modulação de enzimas pró-inflamatórias. Portanto, têm demonstrado a capacidade benéfica de inibir a liberação de IL-1 e de promover a síntese de moléculas antiartríticas naturais, como o tecido inibidor de matriz (TIMP) das MMPs e o antagonista do receptor da IL-1 (IL-1Ra) (MARTEL-PELETTIER et al., 1999). Citocinas que participam da cascata anabólica do metabolismo da cartilagem articular, tais como o fator de crescimento semelhante à insulina (IGF) e o fator de transformação do crescimento (TGF) têm sido alvo de estudo em pesquisas recentes para o tratamento da OA (STEINMEYER, 2004), devido à sua capacidade de estimular a produção condrócitos, PGs e colágeno tipo II (FRISBIE, 2012).

Da mesma forma, os sinoviócitos também são capazes de produzir uma variedade de enzimas anabólicas e catabólicas, como as prostaglandinas e metaloproteinases (MMPs). As prostaglandinas, com destaque para a PGE_2, têm apresentado concentrações elevadas no fluido sinovial de cavalos com OA, além de estarem correlacionadas com sinovite e claudicação clínica, e serem produzidas após estimulação *in vitro* com enzimas catabólicas como a IL-1 e o TNF-α (KIRKER-HEAD et al., 2000). Por sua vez, as MMPs são uma família de enzimas capazes de degradar componentes da matriz extracelular, com destaque especial para a destruição de fibras colágenas tipo II, proteoglicanos, elastinas e moléculas de agrecan (McILWRAITH, 1996; LITTLE et al., 1999; TRUMBLE et al., 2001).

Existem ainda evidências de que o osso subcondral é submetido a um processo de constante remodelação em resposta ao exercício ou em decorrência de sobrecarga. Ao sofrer trauma direto ou microdanos em sua arquitetura, o osso aumenta sua densidade a um nível patológico, diminuindo, portanto, sua habilidade em absorver cargas mecânicas e prejudicando a unidade osso-cartilagem (RADIN & ROSE, 1986; FRISBIE, 2012).

Em suma, frequentemente a etiologia base da OA varia desde sobrecarga de exercícios a defeitos de conformação, os quais predispõe um cavalo atleta aos efeitos de forças biomecânicas inapropriadas à cartilagem (GOODRICH & NIXON, 2006). Entretanto, apesar do papel preponderante da cartilagem nesses eventos, o processo inflamatório pode se originar

em qualquer das estruturas articulares e desencadear rapidamente uma cascata de mediadores inflamatórios a partir do dano tecidual primário. A consequência é um “efeito dominó” de inflamação dos demais tecidos, os quais, por sua vez, também liberam mediadores inflamatórios. Essa cascata, caso não interrompida, culmina com degradação da cartilagem, proliferação óssea peri-articular, danos aos tecidos moles adjacentes e, por fim, com perda da função articular.

2.1.4 Manifestação clínica e diagnóstico em osteoartrite

Muitos cavalos convivem por boa parte de sua campanha atlética com lesões de natureza articular e, consequentemente, com algum grau de claudicação. A fim de não negligenciar a ação desse processo, o objetivo primário em equinos com OA é a realização de um diagnóstico preciso e precoce, além da instituição de um tratamento capaz de prevenir o início e a progressiva deterioração dos tecidos articulares.

Por esse motivo, o exame deve ser completo e sistemático a fim de identificar e localizar sinais de dor, efusão e aumento de temperatura. Como resultado da degeneração dos componentes articulares, a dor é geralmente atribuída ao envolvimento dos tecidos moles periarticulares, osso e periósteo (CARON, 2003). Nos tecidos capsulares e ligamentares, fibras nervosas sensitivas conduzem a sensação dolorosa de terminações nervosas amplamente distribuídas (DEE, 1978). Com o processo inflamatório, tais fibras exibem aumento de sensibilidade.

A maior parte das condições que resultam em OA produz um acúmulo excessivo de fluido sinovial (FS), comumente referido como efusão sinovial. Essa característica marcante ocorre devido à perda de proteína no interior do espaço sinovial, resultante do efeito de diluição pelo aumento na permeabilidade no endotélio capilar. O mesmo não coincide com aumento compensatório na absorção linfática, levando ao aumento progressivo da pressão coloidosmótica e, por conseguinte, do volume de FS (CARON, 2003). Limitação na amplitude de movimentos é uma característica comum em casos de OA e é causada por uma combinação de fatores, incluindo auto-proteção devido à dor, efusão sinovial e fibrose peri-articular progressiva.

A avaliação do FS proporciona valiosas informações em adição aos achados clínicos e radiográficos. A redução de sua viscosidade é um achado frequente em animais com OA, atribuída à redução na concentração ou despolimerização do ácido hialurônico presente em sua

composição (TROTTER & McILWRAITH, 1996). A inspeção visual do FS no momento da coleta pode sugerir, através de avaliação subjetiva de sua aparência, a presença de inflamação no ambiente articular. Amostras de FS amarelo-escuras ou amarelo-alaranjadas (xantocromia) podem representar hemorragia prévia e são frequentemente associadas com processos articulares degenerativos crônicos (STEEL, 2008). A estimativa de proteínas totais (PPT) no FS é uma análise de rotina para diagnóstico de alterações articulares. Valores de PPT acima de 2,5 g/dl são considerados anormais, sendo concentrações acima de 4 g/dl referidos em casos de inflamação severa, sobretudo de caráter séptico, e abaixo desse valor em condições inflamatórias não infecciosas (TROTTER & McILWRAITH, 1996). Em OA, a contagem de células nucleadas totais (CCNT) está geralmente dentro dos limites de referência ou moderadamente aumentada, dependendo do grau de sinovite aguda, com valores usualmente abaixo de 5×10^9/L (TROTTER & McILWRAITH, 1996). A análise citológica é uma das fases mais importantes da análise do FS. Eritrócitos não são considerados constituintes normais do fluido, entretanto, sua presença em pequenas quantidades é atribuída à contaminação da amostra por sangue no momento da coleta. A contagem de leucócitos de um FS normal em equinos apresenta limite superior de 87 células/mm^3 (VAN PELT, 1974), com pequenas variações dependendo da literatura consultada. Neutrófilos, linfócitos e células mononucleares grandes são observados, com porcentagem de neutrófilos geralmente inferior a 10%. Em casos de OA a contagem celular varia muito conforme a presença de sinovite ativa. Nesses casos, devido à liberação de células pela sinóvia inflamada, contagens de 5000 a 10000 células/mm^3, com aumento proporcional da contagem de neutrófilos (TROTTER & McILWRAITH, 1996).

O exame radiográfico tem sido uma forma tradicional de avaliar as alterações estruturais de OA. Entretanto, cabe ressaltar que os achados radiográficos tendem a refletir eventos crônicos do processo patológico e não evidenciam consistentemente processos iniciais em progresso (CARON, 2003). Adicionalmente, em equinos com OA existe uma baixa correlação entre claudicação ou perda de rendimento e alterações ósseas estruturais evidentes em radiografias (DYSON, 1991; ROSS et al., 1991). Portanto, as alterações radiográficas de OA nem sempre estão diretamente correlacionadas com a manifestação clínica e estágio da doença, fator que enfatiza a importância de o exame físico do animal não ser negligenciado. Em geral, as características radiográficas refletem as mudanças patológicas ocorridas na articulação afetada. Inicialmente, ocorrem diminuição do espaço articular, esclerose subcondral e formação de osteófitos. Com o avanço do processo, lise óssea subcondral, fragmentação osteocondral e, eventualmente, anquilose podem ser observadas (CARON, 2003).

2.1.5 Tratamento clínico da osteoartrite

A rápida resolução da dor é a principal preocupação de proprietários e treinadores e o motivo maior de cavalos atletas serem apresentados para avaliações veterinárias. Por esta razão, inúmeras terapias foram desenvolvidas e aprimoradas a fim de reduzir a dor relacionada à doença articular, bem como para retardar a progressão dos efeitos degenerativos da OA (GOODRICH & NIXON, 2004).

Dentre as terapias mais populares, destaca-se a utilização dos anti-inflamatórios não-esteroidais (AINEs), drogas que inibem alguns componentes do sistema de enzimas que convertem o ácido araquidônico em prostaglandinas e tromboxanos, importantes moduladores inflamatórios (MAY & LEES, 1996). Em medicina equina, a fenilbutazona possui uso destacado, devido ao seu baixo custo e alta efetividade em reduzir a claudicação em animais afetados por diversas condições musculoesqueléticas, incluindo a OA (CARON, 2005). Entretanto, seu uso nem sempre é permitido para indivíduos competindo em eventos regulados por ordem de controle de dopagem. Somado a isso, o uso indiscriminado desses agentes possui efeitos deletérios bem caracterizados, tais como ulcerações gastrointestinais, necrose papilar renal e necrose perivascular, este último quando aplicado incorretamente pela via intravenosa (McCKAY et al., 1983). Mais recentemente, foi lançada no mercado farmacêutico uma formulação anti-inflamatória à base de diclofenaco sódico 1% para aplicação tópica. Após resultados clínicos promissores em humanos, seu uso foi testado em equinos sob a interessante ideia de reduzir os efeitos sistêmicos dos AINEs tradicionais. O uso desse creme induziu alívio da claudicação e dos sinais de inflamação em um estudo clínico (LYNN et al., 2004) e em um modelo de indução de osteoartrite a partir da produção de um fragmento osteocondral (FRISBIE et al., 2009). Outro estudo provou que a aplicação tópica dessa droga promoveu níveis sinoviais mais altos que aqueles necessários para inibir as enzimas do grupo das cicloxigenases (COX) (AZEVEDO et al., 2013). Apesar dos benefícios observados com seu uso, é frequentemente necessária a associação de outra terapia, tais como corticoides IA ou AINEs sistêmicos, a fim de otimizar sua efetividade anti-inflamatória. Além disso, a necessidade de repetidas aplicações e a até controvérsias em seu alívio sintomático (VILLARINO et al., 2003) contribuem para torná-lo muito mais uma alternativa do que uma solução para o problema.

A aplicação IA de corticosteroides permanece como uma das alternativas mais eficazes no tratamento dos sinais clínicos da OA, devido ao seu potente efeito anti-inflamatório. Além de reduzir a dilatação capilar, migração e acúmulo de células inflamatórias, os corticoides inibem a síntese e liberação de diversos mediadores envolvidos na cascata das prostaglandinas,

bem como interferem na síntese de IL-1 e TNF-α, importantes mediadores da degradação da cartilagem (LAUFER et al., 2002). O alívio da dor por essas drogas é atribuído à inibição da síntese de prostaglandinas, especificamente pelo bloqueio da enzima fosfolipase A2 e da expressão da COX 2 na cascata do ácido araquidônico (CARON, 2005). No entanto, estudos *in vivo*, têm demonstrado que além dos referidos benefícios, efeitos deletérios sobre o metabolismo dos condrócitos também são produzidos por essas drogas. Sobretudo em altas doses, os corticoides inibem a síntese de proteoglicanos e influenciam desfavoravelmente a organização estrutural das fibras colágenas no interior da cartilagem (CHUNEKAMRAI et al., 1989; FUBINI et al., 1993; TODHUNTER et al., 1996). Em particular, o acetato de metilprednisolona produz alterações morfológicas de cartilagem associadas ao seu uso IA (CARON, 2005).

Os chamados glicosaminoglicanos polissulfatados (GAGs), representados principalmente pelo sulfato de condroitina, pertencem a um grupo de polissacarídeos polissulfatados que incluem ainda o polissulfato de pentosan. Essas drogas são referidas como condroprotetoras e, portanto, tradicionalmente utilizadas em casos onde há a presença de danos à cartilagem articular, ao invés do tratamento da sinovite aguda. Dessa forma, acredita-se que, ao promover a prevenção, o retardo ou a reversão de danos morfológicos à cartilagem, essas drogas produzam algum efeito analgésico indireto e limitado, sendo úteis como adjuvantes, por exemplo, em animais submetidos à artroscopias onde há grande perda de cartilagem (McILWRAITH, 2010).

O hialuronato de sódio, droga sintética análoga ao AH, possui efeitos analgésicos modestos (GOTOH et al., 1993), possuindo mais ênfase seus efeitos anti-inflamatórios, os quais podem ser físicos (impedimento estérico) ou farmacológicos (inibição de células e mediadores inflamatórios) (HOWARD & McILWRAITH, 1996). Além disso, é necessário reconhecer que, baseado em evidências clínicas em humanos, os efeitos clínicos imediatos são menos notáveis, havendo, entretanto, efeitos anti-inflamatórios cumulativos em longo prazo (GOLDBERG & BUCKWALTER, 2005). Portanto, clinicamente, o hialuronato de forma isolada é mais útil em casos de sinovite sutil ou moderada, sendo na maioria dos casos necessária sua combinação com um corticosteroide para melhor efetividade. Como já observado, sinais de OA requerem terapias de longo prazo, o que pode oferecer efeitos indesejados pelas modalidades anteriormente citadas. Por isso, nos últimos anos o uso oral de nutracêuticos tem ganhado popularidade, devido a sua fácil administração e efeitos benéficos (TRUMBLE, 2005). Essa categoria de compostos é formada por nutrientes, suplementos dietéticos, alimentos funcionais e fitoquímicos, que visam além de seus efeitos próprios, diminuir a dosagem e consequentes

riscos do uso prolongado das demais classes farmacológicas (TRUMBLE, 2005). No entanto, é amplamente convencionado que o alívio sintomático promovido por esse grupo é insuficiente em atender a demanda em animais participando de corridas ou outras disciplinas de alto desempenho (CARON, 2005). Além disso, essa classe ainda carece de comprovação científica de sua efetividade, isto porque poucos estudos afirmam sua real validade terapêutica.

Nas últimas décadas, soluções para dor articular em equinos ganharam foco principal na pesquisa mundial. Somado a isso, a similaridade com alguns aspectos da fisiopatologia da OA equina com aqueles observados em humanos, permitiu aos pesquisadores veterinários utilizarem a base de conhecimento da pesquisa médica, bem como arrecadar fundos para o desenvolvimento de terapias que beneficiem ambas as espécies. Nessa linha, com a melhora no entendimento de alguns mediadores chave na OA equina, foram identificados alguns alvos para novas terapias, tais como a IL-1 e as MMPs. A partir dessas descobertas, foi iniciada a "Era das terapias celulares e medicina regenerativa" na clínica de cavalos de esporte. Extensamente explorado para tratar a inflamação articular, o soro autólogo condicionado, também conhecido como proteína antagonista do receptor da interleucina-1 (IRAP), inibe a atividade da IL-1 e diminui a progressão da doença (FRISBIE et al., 2007). Ainda segundo observações de Frisbie e colegas (2007), aplicações seriadas de IRAP no carpo de equinos com OA induzida melhoraram clinicamente a claudicação, além de reduzirem a fibrilação de cartilagem e danos à membrana sinovial. Todavia, apesar de todas essas impressões clínicas promissoras, essa terapia ainda apresenta limitações de uso ligadas, sobretudo, ao seu alto custo.

Notadamente, o plasma rico em plaquetas (PRP) é mundialmente uma das terapias regenerativas mais usadas em equinos, devido principalmente à sua natureza autóloga, rápida preparação e coleta não invasiva (TEXTOR, 2011). Sabidamente, as plaquetas são fontes ricas em fatores de crescimento, citocinas e quimiocinas, todos liberados durante os estágios iniciais de reparação tecidual. Dentre os efeitos mais notáveis de sua aplicação IA, destacam-se a estimulação da produção de condrócitos, proteoglicanos e colágeno tipo II (AKEDA et al., 2006), aumento da produção e secreção de AH (ANITUA et al., 2007) e diminuição clínica de claudicação e efusão sinovial (CARMONA et al., 2007; PICHEREAU et al., 2013). Dito isso, ainda não há um consenso a respeito da concentração ideal de leucócitos e plaquetas nessas preparações capazes de induzir efeitos anti-inflamatórios e resposta anabólica efetiva em tecidos articulares. Portanto, apesar de algumas comprovações científicas, seu uso IA ainda se baseia muito em experiências clínicas empíricas, carecendo de estudos controlados que atestem seu real valor terapêutico.

Outra terapia regenerativa que tem ganhado notoriedade na última década é o uso de células tronco mesenquimais (CT) derivadas de medula óssea ou tecido adiposo. Acredita-se que essas células possuam propriedades anti-inflamatórias, contribuindo também para a cicatrização de tecidos musculoesqueléticos. Em um estudo, o acompanhamento de 40 cavalos que receberam aplicação IA de CT derivadas de medula óssea revelou um retorno atlético em 72% dos animais com lesões articulares, independente do grau (FERRIS et al., 2009). Resultados como esses colocam as CT entre potenciais terapias intra-sinoviais para o futuro. Entretanto, a literatura científica até o presente momento não apresenta subsídios capazes de recomendar as CT para o tratamento de OA em equinos como sendo uma terapia totalmente segura e eficaz.

Uma das mais modernas opções terapêuticas para OA em equinos é a aplicação IA do hidrogel de poliacrilamida (HGP). Comercialmente é apresentado como um polímero gel não tóxico e não imunogênico formado por 97,5% de água estéril e 2,5% de poliacrilamida (ZARINI et al., 2004; CHRISTENSEN et al., 2003). Sua biocompatibilidade com tecidos moles tem sido aproveitada em medicina para procedimentos urológicos, oftalmológicos, dermatológicos e em cirurgias reconstrutivas (LLOYD et al., 2001; FERNÁNDEZ-COSSIO et al.,2006; CHRISTENSEN et al., 2008). Considerando que a diminuição da capacidade visco-elástica do fluido sinovial é parte do complexo da DAD, a viscosuplementação com moléculas de alto peso molecular, como o hialuronato, tem sido implementada como parte do tratamento para OA em humanos e equinos. Dessa forma, o uso IA de HGP surgiu inicialmente em humanos (ZAR et al., 2012a; ZAR et al., 2012b) como uma alternativa para cumprir essa função. Em equinos, um estudo multicêntrico revelou que o uso de HGP 2,5% IA aliviou a claudicação e diminuiu a efusão sinovial em 43 cavalos por um período de até 24 meses (TNIBAR et al., 2015). Outro estudo, que utilizou nova formulação comercial com HGP 4%, observou aos 45 dias após injeção IA, melhoras na claudicação e na mobilidade articular, além de diminuição da efusão sinovial em 82% dos animais tratados. Dos mesmos 28 animais desse estudo, 21 ainda apresentavam sinais de melhora transcorridos 75 dias de tratamento (McCLURE & WANG, 2017). Os resultados desses dois estudos clínicos, apesar de limitados pela ausência de um grupo controle, descrevem a aplicação de HGP como segura e muito promissora para o alívio dos sinais de OA. Entretanto, do ponto de vista prático, cabe ressaltar que sua utilização ainda apresenta considerável restrição, ligada principalmente à disponibilidade do produto e seu ao alto custo.

Por todas as limitações descritas acima para as opções terapêuticas já existentes, se acredita na necessidade de alternativas ao uso crônico de drogas para conforto e melhora na

funcionalidade, tanto para animais em campanha atlética como para garanhões e éguas reprodutoras que careçam de alívio na dor para ter um desempenho adequado na estação reprodutiva. Ademais, novas drogas que cumpram esse objetivo beneficiariam, ainda, animais em que o tratamento cirúrgico para OA não é uma opção, seja por estarem em atividade ou por limitações financeiras.

2.2 DE VENENO À OPÇÃO TERAPÊUTICA: O USO DA TOXINA BOTULÍNICA TIPO A NA MEDICINA MODERNA

2.2.1 Aspectos gerais

O botulismo foi inicialmente descrito na década de 1820 por Justinus Kerner, que publicou suas observações acerca de uma intoxicação alimentar fatal ocorrida em moradores de uma pequena cidade da Alemanha (ERBGUTH & NAUMANN, 1999). Decorridos mais de dois séculos, o mecanismo de ação das neurotoxinas envolvidas naqueles eventos foi elucidado com sucesso. Essa intoxicação alimentar letal é causada pelas toxinas botulínicas, as quais são produzidas por bactérias da espécie *Clostridium botulinum*, sendo consideradas as mais potencialmente tóxicas ao homem (SMITH et al., 2014; PECK et al., 2017). Pelo menos sete sorotipos são reconhecidos e nomeados ordenadamente por letras do alfabeto (A-G) (PECK et al., 2017). A principal característica de todos os sorotipos é o bloqueio da neurotransmissão pela inibição da liberação de acetilcolina na junção neuromuscular e terminais nervosos colinérgicos (WHEELER & SMITH, 2013).

Dentre os sorotipos, o sorotipo A é o mais potente (SUGIYAMA, 1980) e, por consequência, o mais investigado (KESSLER & BENECKE, 1997; AOKI, 2003). Os sorotipos A (TBA) e B (TBB) têm seu uso explorado ao longo dos anos pela indústria farmacêutica, visando seus potenciais efeitos benéficos (TURTON et al., 2002).

2.2.2 Mecanismo de ação

A TBA consiste em um polipeptídeo de cadeia simples com um peso molecular de 150 kDa (FREVERT, 2015), que é ativada quando a cadeia polipeptídica é clivada proteoliticamente em uma molécula de cadeia dupla, consistindo em uma cadeia longa (100 kDa) e uma cadeia curta (50 kDa) ligadas por uma ligação bissulfureto simples (TURTON et al., 2002).

No tecido alvo, a cadeia longa da toxina se liga a uma proteína ligante (DONG et al., 2006) e a um receptor (RUMMER et al., 2004) na superfície do neurônio e é internalizada na célula como uma vesícula. No interior da célula, a cadeia curta é liberada no citosol, onde cliva a substância SNAP-25, uma proteína essencial para o acoplamento e fusão das vesículas de acetilcolina na membrana sináptica (TURTON et al., 2002). A clivagem dessa proteína bloqueia a fusão e inibe a exocitose da acetilcolina na fenda sináptica (TURTON et al., 2002). Portanto, na ausência da acetilcolina na fenda pré-sináptica, o resultado é uma paralisia muscular flácida, a qual promove alívio da dor em condições de hiperatividade muscular dolorosa (DRESSLER & SABERI, 2005) e cujo efeito clínico dura aproximadamente três meses (MARSH et al., 2014).

A TBA possui ainda efeitos antinociceptivos diretos, os quais independem de suas ações na junção neuromuscular (AOKI, 2003). Essas propriedades foram descritas por Cui & Aoki (2000) em um modelo de dor em ratos, os quais relataram alívio da dor após aplicações de TBA sem efeitos no tônus muscular dos animais. Estas observações levaram à exploração dos efeitos antinociceptivos dessa nova classe terapêutica em outros modelos de dor (CUI et al., 2004; BACH-ROJECKY & LACKOVIC, 2005; PARK et al., 2006) e em estudos clínicos em pacientes humanos com uma variedade de condições dolorosas (ZHANG et al., 2010).

Apesar de o mecanismo antinociceptivo da TBA ainda não ser totalmente compreendido, este mecanismo parece inibir a liberação de neurotransmissores como a substância P (SP) (ISHIKAWA et al., 2000, WELCH et al., 2000; LUCIONI et al., 2008) e o peptídeo relacionado ao gene da calcitonina (CGRP) (DURHAM et al., 2004) em culturas celulares, e do glutamato em um modelo de dor em animais (CUI et al., 2004). Dessa forma, o efeito antinociceptivo clínico é creditado à inibição desses neurotransmissores dos neurônios aferentes sensoriais primários, os quais diminuem diretamente a sensibilidade periférica e indiretamente levam à redução da sensibilidade nervosa central.

2.2.3 Uso da TBA em medicina humana

Os efeitos clínicos da paralisia flácida causada pela TBA têm sido amplamente explorados em medicina desde a década de 1980 (DAVIS, 1993). A aplicação intramuscular de TBA é usada no tratamento de diversas enfermidades neuromusculares associadas com dor e hiperatividade muscular indesejada, tais como espasticidade, distonia cervical, blefaroespasmo e síndrome da bexiga hiperativa (BALASH & GILADI, 2004; SIMPSON et al., 2008a;

SIMPSON et al., 2008b; DONG et al., 2017). Outro campo no qual a aplicação local de TBA é bastante explorada é a cosmética, usando técnicas de harmonização facial (GUO et al., 2015).

A partir da descoberta da eficácia antinociceptiva da TBA, inúmeros relatos de casos e estudos clínicos controlados utilizaram a aplicação IA de 100 UI de TBA em pacientes humanos com OA e artrite reumatoide (MAHOWALD et al., 2009; SINGH et al., 2009; BOON et al., 2010; SUN et al., 2014) e em indivíduos com dor pós-operatória após colocação de próteses de joelho (SINGH et al., 2010) ou mesmo com capsulite adesiva no ombro (JOO et al., 2012). Adicionalmente, a injeção IA com 100 UI de TBA no ombro, joelho ou cotovelo em pacientes com OA foi comprovadamente tão efetiva no alívio da dor quanto as aplicações IA de corticosteroides (BOON et al., 2010; JOO et al., 2012) e de hialuronato de sódio (SUN et al., 2014), por períodos de duração que variaram de dois a seis meses.

Um interessante estudo clínico pioneiro foi desenvolvido por Mahowald e colaboradores (2006) investigando os efeitos em longo prazo da aplicação IA de TBA em 15 articulações de 11 pacientes com OA e artrite reumatoide refratárias a outras terapias. O sucesso do tratamento foi avaliado através de um acompanhamento dos pacientes por 12 meses. Foi observado alívio da dor em todos os pacientes por períodos que variaram de três a 12 meses, dependendo da articulação e do número de repetições do procedimento.

2.2.4 Uso intra-articular de TBA em cães

De posse dos resultados supracitados, alguns pesquisadores foram motivados a investigar os efeitos da aplicação IA de TBA em cães. Em um primeiro estudo piloto, Hadley et al. (2010) aplicaram 25 UI de TBA nas articulações coxofemoral ou no cotovelo de cinco cães com OA crônica. Em análise objetiva de marcha por plataforma de forças, houve melhora significativa da dor nos membros tratados com TBA em comparação ao membro contralateral não-tratado. Da mesma forma, quatro dos cinco tutores dos animais relataram melhora clínica em análise subjetiva. Dois cães do mesmo estudo apresentaram em curto prazo eritema e efusão sinovial nas articulações tratadas, sem efeitos adversos adicionais.

Um estudo finlandês dedicado a investigar possíveis efeitos colaterais da aplicação IA de 30 UI de TBA utilizou seis cães sadios, não observando alterações clínicas, neurológicas, citológicas ou histopatológicas decorrentes do tratamento (HEIKKILA et al., 2017). Em outro estudo em que 36 cães foram aleatoriamente tratados com TBA ou placebo, a melhora clínica foi observada em análise objetiva de marcha e escala analógica de dor nas articulações tratadas

(HEIKKILA et al., 2014). Um experimento mais recente em cães osteoartríticos investigou os efeitos da aplicação IA de TBA sobre os níveis de SP, PGE_2 e TNF-α, biomarcadores relacionados à dor articular (HEIKKILA et al., 2017). Não houve alteração significativa nos níveis de SP e PGE_2 pré e pós-tratamento, sugerindo que os efeitos antinociceptivos da aplicação da toxina nessa espécie não estão relacionados à inibição desses biomarcadores.

2.2.5 Estudos com toxinas botulínicas em equinos

Um número limitado de estudos utilizando toxinas botulínicas em equinos foi realizado até o momento. As aplicações clínicas das toxinas botulínicas tipos A e B têm sido investigadas para redução do tônus muscular em algumas condições comuns em equinos, tais como laminite (CARTER & RENFOE, 2009; WIJNBERG et al., 2013), harpejamento (WIJNBERG et al., 2009), lacerações perineais (ADAM-CASTRILLO et al., 2004) e síndrome podotroclear (GUTIERREZ-NIBEYRO et al., 2014).

Nos primeiros estudos, foi confirmada a hipótese de que a injeção com 100 (CARTER & RENFOE, 2009) ou 200 UI (WIJNBERG et al., 2013) de TBA no ventre do músculo flexor digital profundo resulta em uma diminuição do tônus muscular, o que, em casos de laminite aguda, pode prevenir a rotação da falange distal pela redução da tração exercida pelo tendão desse músculo sobre o referido osso. Em pôneis sadios e cavalos com harpejamento, a aplicação de TBA nos músculos extensor digital longo, extensor digital lateral e vasto lateral diminuiu o tônus muscular, reduzindo, portanto, sinais de espasticidade, sem a presença de efeitos adversos (WIJNBERG et al., 2009). Por sua vez, Adam-Castrillo et al. (2004) relataram que a aplicação de TBB reduziu a pressão do esfíncter anal em equinos saudáveis, inferindo que a utilização dessa técnica pode ser útil em éguas para a redução de deiscência incisional após o reparo de lacerações perineais. Enquanto isso, a injeção intra-bursal de TBB (3,8 – 4,5 U/kg) foi testada por Gutierrez-Nibeyro et al. (2014) em animais com doença degenerativa relacionada ao osso navicular. Os autores utilizaram 10 cavalos da raça quarto de milha, sendo três para testar a segurança da aplicação e sete para avaliação da resposta clínica. Os resultados da avaliação subjetiva de claudicação dos animais – ao trote em linha reta e ao círculo nos sentidos horário e anti-horário – após sete e 14 dias de tratamento apontaram alívio da dor, sobretudo no último momento.

Ao conhecimento do autor desta tese, somente um estudo investigou os efeitos da aplicação IA de TBA em equinos, destinado a usar o cavalo como modelo experimental de avaliação dos efeitos da TBA, extrapolando-os para a medicina humana (DePUY et al., 2007). Trata-se de um estudo piloto, onde somente quatro animais sadios foram selecionados. Os

equinos receberam aleatoriamente tratamento prévio com 50 UI de TBA ou volume equivalente de solução salina em ambas as articulações intercarpianas, de maneira a formar dois grupos com dois animais cada (GTBA e GC). Posteriormente, decorridos 14 dias, uma articulação de cada animal foi submetida à indução de sinovite com IL-1β. Um dos animais do grupo experimental (GTBA) não apresentou claudicação após a indução do processo inflamatório. Dessa forma, os autores ressaltam a validade da aplicação IA de TBA como terapia emergente e a necessidade de mais estudos que confirmem sua eficácia.

3 ARTIGO

3.1 ARTIGO 1

Safety and synovial inflammatory response after intra-articular injection of botulinum toxin type A in healthy horses

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Abstract

Botulinum toxin type A (BoNT-A) is a promising alternative for patients suffering from chronic joint pain. The aim of this study was to investigate whether a single injection of BoNT-A would produce adverse effects on clinical parameters and synovial parameters as well as lameness. One randomly selected radiocarpal joint was treated with 50 U of BoNT-A in eight horses, and the contralateral joint received saline solution. All horses received injections at day 0 and were re-evaluated twice daily for seven days for heart rate (HR), respiratory rate (RR), rectal temperature (RT), mucous membrane color, capillary refill time, intestinal motility, appetite, water intake, defecation, urination, and attitude. At these same time points, joint pain and circumference were assessed. Objective lameness evaluations were performed once daily for seven days and synovial fluid samples were collected at baseline, post-injection hour (PIH) 24 and PIH 168 and evaluated for synovial fluid parameters. HR and RT remained clinically unaltered, despite oscillations over time ($p=0.001$). The remaining clinical parameters were unaltered by treatment or time ($p>0.05$). Joint pain was not elicited by flexion and palpation in both limbs as well as carpal circumference was not altered ($p=0.88$). Lameness was observed only on saline limbs. Cellular parameters evaluated in synovial fluid samples from both carpi had significantly increased from baseline to PIH 24, decreasing at PIH 168 ($p<0.05$). It was concluded that the injection of 50 U BoNT-A is suggested to be a safe therapy for intra-articular use in horses and must be verified by further investigation.

Keywords: osteoarthritis; intra-articular therapy; joint pain; botulinum toxin; horse.

1. Introduction

Management of chronic joint pain is an important concern for owners of older and retired horses [1]. Over the past decades, a plethora of medical treatments for joint disease were developed aiming to reduce pain and inflammation, with maintained or improved function. Among them, new systemic nonsteroidal anti-inflammatory drugs (NSAIDs) and joint injections of corticosteroids combined or not with sodium hyaluronate have gained popularity over time. However, despite their cost-effectiveness and efficacy in reducing pain [2], several studies have documented some well-established side effects caused by prolonged systemic use or high doses of NSAIDs, in particular gastric ulcerations, colitis and kidney toxicity [3-5].

Corticosteroids are the most potent and commonly used anti-inflammatory drugs, responsible for pain relief attributed to the inhibition of the enzyme phospholipase A2, cyclooxygenase expression in the aracdonic acid cascade and, hence, inhibiting prostaglandin synthesis [2, 6]. Beyond the actions of corticosteroids over these pro-inflammatory components, controlled studies also have demonstrated deleterious effects produced by their repeated use, including chondrocyte necrosis and inhibition of proteoglycan synthesis [7, 8]. In turn, sodium hyaluronate is used to restore the viscoelasticity, lubrication and steric hindrance of articular components [9], which were negatively affected by the inflammatory process. Nevertheless, its analgesic effect was considered modest in a rat pain model [10] and reached cumulative effects only at long-term use in human patients [11]. Because of all these limitations encountered for the already tested medications to treat joint pain, researchers and clinicians have concentrated their efforts to develop new pharmacological therapies that could achieve good clinical response with minimal adverse effects.

Botulinum toxins (BoNTs) are biological agents produced by *Clostridium botulinum*, a gram-positive, anaerobic spore-forming bacterium. These products are considered the most

potent neurotoxins known, causing a life-threatening flaccid paralysis in humans and animals, so-called botulism [12]. BoNTs exert their effects at the neuromuscular junction, inhibiting the release of acetylcholine in the synaptic cleft, causing muscle relaxation [13]. Since the late 1970s, BoNTs type A (BoNT-A) and B (BoNT-B) have been applied in human medicine to treat several disorders characterized by muscle spasticity, such as strabismus [14], cervical dystonia [15], blepharospasm [16], limb spasticity [17] and overactive bladder syndrome [18], among others. Later, further investigations demonstrated additional mechanisms of action exerted by BoNTs, which include pain relief promoted by direct action on peripheral sensory nerves, blocking the release of neurotransmitters [19-21]. Indeed, encouraged by these findings, many open-label [22-24] and randomized-controlled trials [25-27] were published reporting experiences after intra-articular injections of BoNT-A to treat articular pain in human patients. Promising results with minimal adverse effects were described. In veterinary medicine, pain alleviation was also reported in dogs receiving intra-articular deposition of BoNT-A both in a pilot study without a placebo-controlled group [28] and in a placebo-controlled trial [29]. While no adverse events were observed in two randomized, blinded, and placebo-controlled trials [29, 30], Hadley and coworkers (2010) observed only the development of redness and swelling at the injection site in two out of five dogs enrolled in their study, without systemic or lasting local side effects [28]. Only one small pilot study using an equine model of experimentally induced synovitis investigated clinical and potential undesirable effects of intra-articular BoNT-A application in four horses [31]. Despite recognizing the small sample as an important limitation of their study, the authors reported no apparent adverse effects on physical examination and synovial fluid analysis.

Considering the BoNT-A as a promising therapy for selected cases of chronic joint pain and the importance to warrant its safety, the purpose of this study was to investigate potential adverse effects on general health and lameness, as well as the synovial fluid inflammatory

response after a single intra-articular injection of BoNT-A in sound horses. We hypothesized that a single intra-articular injection of BoNT-A would not produce clinical side effects or asymmetries compatible with lameness and that there would be no significant synovial fluid inflammatory response when compared with saline solution.

2. Materials and Methods

2.1 Animals and selection criteria

The Ethics Committee on Animal Research Use at Federal University of Santa Maria approved all experimental procedures (Protocol number CEUA 3627290920).

Eight mature crossbred horses from our Veterinary College teaching herd (five mares and three geldings; age: 11.25±7.95 years; body mass: 420±52.44 kg), were enrolled in the study. Horses were included in the study if they were sound based on objective lameness evaluation (LL; Lameness Locator, Equinosis, Columbia, MO, USA) and no effusion of the radiocarpal joints were detected nor pre-existing radiographic changes consistent with joint disease as assessed by a clinician experienced in radiology. After two days of habituation before the study was started, the animals were maintained in individual stalls, were fed with alfalfa hay twice daily, and had free access to water during the experimental protocol.

2.2 Study design and synovial fluid analysis

The skin over both radiocarpal joints from each horse was clipped, aseptically prepared and approached from their dorsal aspect [31]. Using 23-gauge, 1.5-inch hypodermic needles attached to 5 mL syringes, synovial fluid aliquots were aspirated and immediately transferred

to EDTA-containing tubes to determine total protein (TP) concentration, total nucleated cell count (TNCC), percentage of neutrophils (PN) and red blood cells count (RBCC). Immediately after, for each horse, pairs of radiocarpal joints were randomly assigned, by coin toss, to receive 50 U of BoNT-A (Botulift, Laboratório Bergamo, São Paulo, Brazil), a previously described dose [32], or equivalent volume of saline solution (5 mL). To turn the arthrocentesis into a safe procedure in uncooperative horses, chemical restraint with xylazine hydrochloride (0.3 mg/kg IV) (J.A. Animal Health, Patrocínio Paulista, SP, Brazil) was performed as required. Synovial fluid collection was repeated at PIH 24 and at PIH 168 and the samples evaluated for the same cytological parameters. Total protein concentration was determined by refractometry (Ionlab Laboratory Equipment, Araucária, PR, Brazil). For TNCC, 20 μL of synovial fluid were transferred into 400 μL of saline solution (NaCl 0.9%), loaded onto a hemocytometer (Kasvi Laboratory Products, São José dos Pinhais, PR, Brazil) and evaluated by a blinded experienced clinical pathologist. For differential cell counts, direct smears of synovial fluid samples were made. The smears were air-dried, stained with a Diff-Quick rapid panoptic stain [33] and cells microscopically evaluated on the monolayer of the smears by the blinded assessor at all time points. Then, cells were classified as neutrophils, small mononuclear cells (lymphocytes) and large mononuclear cells (including blood monocytes, macrophages and synoviocytes) [34]. A hundred nucleated cells were classified and percentage of each recorded. A remaining sample of synovial fluid was placed into propylene conical tubes (Eppendorf, Hannover, Germany) without anticoagulants, evaluated for the mucin precipitate quality (MPQ), and scored by the same blinded evaluator. A subjective scale already described by Mills et al. (2000) was used to graduate the MPQ: (1) minimal clot; (2) minimal to moderate clot (3); moderate to maximal clot; and (4) maximal clot formation with flocculation [35]. MPQ was accomplished by adding one part of the synovial fluid sample to four parts of 2% glacial acetic acid.

2.3 Clinical response to treatment injection

Horses were objectively examined for heart rate (HR), respiratory rate (RR) and rectal temperature (RT). Observational subjective parameters were also monitored by a blinded evaluator during the entire study, and included mucous membrane color, capillary refill time, intestinal motility, appetite, water intake, defecation, urination and attitude. All these parameters were recorded prior to the first synovial fluid collection and treatment injection (baseline). For comparisons over time, all parameters were re-evaluated at post-injection hour (PIH) 12 and 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156 and 168.

2.4 Joint pain and circumference assessment

Joint pain was subjectively evaluated pre- and post-treatment by a descriptive scale of resistance to flexion and palpation [36]. An investigator unaware of which limb received the injection of BoNT-A or saline elevated and palpated the carpi and scored the horses' reaction: (0) no pain; (1) mild pain; (2) moderate pain; (3) severe pain. The same evaluator assessed the carpal circumference before the treatment and at the same post-injections time points used during the clinical monitoring. Thus, a measuring tape was positioned at the point of greatest circumference over the radiocarpal joints of each horse, as an adaption to the previously described by Colbath et al. (2018) [37].

2.5 Objective lameness evaluation

Lameness examinations were performed using the LL, a wireless inertial sensor-based system, which consists of two accelerometers (one positioned on the top of the head between

the ears, and other one on the dorsal midline between the *tuber sacrale*) and a uni-axial gyroscope attached on the dorsal aspect of the right front pastern. The first assessment was done prior to experimental interventions (baseline), in order to enroll only horses considered free of lameness on their fore- and hindlimbs. Therefore, horses included in the study showed head and pelvic asymmetries below the threshold values, represented by the variables vector sum (**VS**) (head amplitude) < 8.5 mm and Pmax and Pmin (pelvic amplitude) < 3 mm, respectively. Then, after joint injections with the study drug and saline solution, horses were trotted by hand in a straight line on a concrete surface for at least 25 strides at PIH 12 and 36, 60, 84, 108, 132, 156 and 168h post-injection. The LL system records the measurement of head (Hmax and Hmin) vertical movement asymmetries, documented as mean ± standard deviation. Hmax and Hmin are related with the differences in the maximum and minimum head heights for each forelimb. These variables cannot be assessed separately, thus VS of these two components is the single variable for forelimb lameness, and it was considered if VS values, calculated as $\sqrt{Hmax^2 + Hmin^2}$, were outside the threshold (> **8.5 mm**) [38]. Side of lameness is defined by sign of Hmin, which positive values indicates right forelimb and negative indicates left forelimb lameness. Thus, depending on the sign of Hmin, it was possible to identify whether lameness was present on BoNT-A- or saline-treated limbs.

2.6 Statistical methods

Data were examined by use of a D'Agostino-Pearson omnibus test for normality. To compare the continuous variables HR, RR and RT before and after treatment, data were examined by analysis of variance (ANOVA) with repeated measures adjusted for comparisons with the *post hoc* Tukey's test. Alternatively, Friedman test with Dunn's post-test to analyze the specific sample pairs for stochastic dominance was performed as required. Two-way

ANOVA with repeated measures was applied to compare the effect of both treatments on TP, TNCC, PN, RBCC and carpal circumference. Tukey's or Bonferroni's adjustment for comparisons was also applied as required. The categorical variable MPQ was examined by the Mann-Whitney test to compare the effects between BoNT-A and placebo groups. The same subjective score was evaluated by Friedman test for effects over time. All results were considered significant if $p \leq 0.05$ (GraphPad Prism Software 6.0, San Diego, CA, USA).

3. Results

3.1 Synovial fluid analysis

Treatment did not affect the TP concentration ($p=0.381$) nor TNCC ($p=0.179$) on the synovial fluid in comparison with saline solution. Similarly, PN on synovial fluid was not different between saline- and BoNT-A-treated joints ($p=0.79$) (Table 1). Significantly higher TP concentrations were found in synovial fluid at PIH 24 ($p<0.05$) and PIH 168 ($p<0.05$) when compared to baseline levels. In addition, TP concentration significantly decreased ($p<0.05$) at PIH 168 when compared with PIH 24. Considering the TNCC, time strongly affected the cell counts ($p=0.001$). Higher TNCC were found at PIH 24 when compared to the baseline ($p<0.05$). Significant decrease of TNCC was observed as from PIH 24 to the PIH 168 in both front limbs ($p<0.05$). In turn, TNCC recorded from the synovial fluid samples collected at PIH 168 were comparable to those observed at the baseline ($p>0.05$). Similar pattern was observed in both groups when considered the effect of time on PN ($p<0.0001$). Thus, significant increase of PN was found at PIH 24 when compared to the baseline ($p<0.05$). After that, samples collected at PIH 168 revealed significant decrease of NP ($p<0.05$) when compared with PIH 24 and values comparable to those observed at the baseline ($p>0.05$). Both small and large mononuclear cells

did not differ between groups at the different time points ($p>0.05$), and alterations in cell morphology were not observed in both groups. Similarly, platelets were not observed. Red blood cells were found in samples from all time points. Time affected the RBCC ($p=0.042$), but RBCC from BoNT-A-treated limbs did not differ from those obtained from saline-injected limbs ($p=0.317$). In fact, for limbs injected with BoNT-A, superior RBCC was observed at baseline ($p<0.05$), with substantial reduction at PIH 24 and PIH 168. In contrast, saline-treated limbs demonstrated higher RBCC at PIH 24 and PIH 168 when compared to the baseline ($p<0.05$). There was not observed presence of erythrophagocytosis, hemosiderin granules in macrophages and hematoidin crystals in the samples from both groups at the different time points. Analyzing the MPQ, scores attributed for both BoNT-A- or saline-treated samples did not vary significantly over time ($p>0.05$). Furthermore, neither BoNT-A nor saline interfered with the MPQ scores ($p=0.938$).

3.2 Clinical response to treatment injection

The HR significantly decreased ($p<0.001$) from PIH 12 to PIH 24, between PIH 12 and PIH 168 and from PIH 120 to PIH 168 (Figure 1). Rectal temperature also varied ($p<0.001$) between time points. However, these clinical parameters remained within normal limits during the entire experiment. Throughout the same period, RR was unaffected by treatments ($p=0.065$) (Figure 2), and horses remained bright, alert and responsive to the external stimuli. All horses maintained good appetite and water intake when receiving hay and water during the day. Capillary refill time, mucous membrane color and urination were considered normal throughout this observational period. During the study, 4/8 (50%) horses experienced transient periods of soft-formed feces, accompanied by moderate increases in intestinal motility. For these subjects, no other alterations in clinical or behavioral parameters were observed.

3.2 Joint pain and circumference assessment

No painful reactions were appreciable after the stimuli elicited by both flexion and palpation of carpal joints of all horses. Thus, all individuals received score zero for both treated and saline joints at all time points evaluated. Additionally, neither BoNT-A nor saline injections resulted in enlargement of the carpal circumference ($p=0.884$) and there was no influence of time on carpal circumference either ($p=0.215$) (Figure 3).

3.3 Objective lameness evaluation

One horse out of eight (12.5%) developed a moderate to severe lameness on the saline-injected limb at PIH 84. The horse positively responded (90% on the objective evaluation) to a palmar digital nerve block with lidocaine hydrochloride 2% and, after physical and radiographic examination, the lameness was attributed to a negative palmar angle. Thus, this horse was excluded from the lameness evaluation. From the remaining sample, head asymmetries (**VS > 8.5mm**) were observed and interpreted as mild lameness on the objective evaluation. Four horses (57.1%) demonstrated mild lameness for at least one time point evaluated. The maximum mean head asymmetries (Table 2) were observed at PIH 36 (8.6±1.64 mm) and were recorded by horses and time points as follow: 1/7 (14.3%) PIH 12; 3/7 (42.8%) PIH 36, 1/7 (14.3%) PIH 60, 1/7 (14.3%) PIH 84, 3/7 (42.8%) PIH 108, 1/7 (14.3%) PIH 132; 3/7 (42.8%) PIH 156; and 2/7 (28.6%) PIH 168. Interestingly, all these subtle asymmetries were only observed on limbs that received the placebo injection (Table 2). Nevertheless, lameness was not linearly influenced by time ($p= 0.373$) and all, but one horse was lameness-free at the end of the experimental trial.

4. Discussion

Results of the present study indicated that a single intra-articular injection of 50 U of BoNT-A does not provoke a significant synovial inflammatory response in horses. These findings led us to accept our hypotheses that in a seven-day period (168h) this biologic neurotoxin does not produce adverse effects in the radiocarpal joint as reflected on the unaltered clinical parameters and absence of asymmetries compatible with lameness. In addition, the injection of BoNT-A resulted in a similar synovial inflammatory response when compared with saline solution.

Conventional management of joint pain in horses is mainly based on the use of systemic NSAIDs and intra-articular injection of corticosteroids alone or associated with hyaluronic acid. Considering their well-documented effectiveness as well as the side effects of NSAIDS and intra-articular corticosteroids [3-5, 7, 8], judicious use is recommended. Additionally, some cases of chronic joint pain are refractory to their use, partially responding or demanding frequent dosing to maintain their therapeutic effect. In selected patients with chronic pain due to arthritis, alternatives must be required to maximize comfort for the horse, including therapeutics that target nociceptive pathways rather than inflammatory pathways. In this context, temporary analgesia by continuous perineural block with known local anesthetics via subcutaneous catheter placement could be an option [39, 40], although their short half-life and potential subcutaneous irritation are limiting factors. Add to that some already documented toxic effects of intra-articular local anesthetics to the chondrocytes [41, 42] are important concerns of their prolonged use. In this scenario, the antinociceptive properties offered by BoNTs could also find a place in the equine medicine arena.

Allegedly, persistent pain, as the one observed in cases of chronic osteoarthritis, may produce neurogenic inflammation and increased pain perception, mainly by the release of a

variety of neurotransmitters in the peripheral neurons [42, 43]. Botulinum toxin type A binds to nociceptor C-fibers present in joints, undergoes endocytosis, and blocks vesicle release of substance P [19], calcitonin gene-related peptide [20] and glutamate [21]. Therefore, decreasing pain generation, transmission and neurogenic inflammation. Thus, studies in people have recommended therapies directed against sensory nerve functions, as a new approach to treat some patients with chronic joint pain [22-27]. Similarly, if proven safe and effective in horses, treatment could be considered for patients refractory to traditional therapies that target synovial inflammation. A human uncontrolled clinical trial indicated that pain relief was sustained for three to 12 months in 11 patients with chronic arthritis [22]. After, randomized-controlled trials supported this information, reporting at least six months of reduction in pain [45]. In veterinary medicine, two studies conducted in dogs receiving BoNT-A demonstrated that pain relief may last up to 12 weeks [28, 29].

Currently used to treat several spastic and painful conditions in human patients, the benefits, and adverse effects of BoNTs have scarcely been investigated in equine medicine. Given the risk of potential systemic spread of the toxin and, thus, the development of clinical signs of botulism, we conducted this study to evaluate the effects of BoNT-A at a previously described dose [32]. Local signs of inflammation were also investigated here, since synovitis and hypersensitivity response to the protein component of BoNTs were previously reported in human and canine patients [28, 44].

BoNT-B was firstly experimentally used in normal horses to decrease anal sphincter tone, with the interesting idea that the reduction in anal muscle activity would reduce the incidence of dehiscence in mares that underwent surgical repair of perineal lacerations [46]. In that limited study, local injection of this serotype in one horse at the dose 2,500 U resulted in clinical signs of botulism, such as lethargy, generalized weakness and dysphagia. Gutierrez-Nibeyro and colleagues (2014) [47] also investigated the same serotype as an alternative drug

to alleviate pain arising from the podotrochlear apparatus. In their study, the authors described BoNT-B as a short-term palliative option, with promotion of transient exacerbation in lameness observed in two out of three horses receiving intra-bursal injection of 4.5 U/kg of the toxin, with no systemic effects observed during the evaluation. Opposing the findings of these previous studies with BoNT-B, horses from our trial did not experience systemic signs of botulism or lameness after receiving joint injection of BoNT-A, because they remained bright, alert, comfortable while walking and with no asymmetries indicating lameness on the treated limb. Corroborating our results, some studies using BoNT-A as a therapy for acute induced synovitis [32], stringhalt [48] and laminitis [49, 50] at different doses did not report signs of systemic toxicity.

The only remarkable clinical alteration in this study was the development of transient soft-formed to watery feces in four out of eight horses. Although diarrhea was already described as a complication of BoNT-B injections [46], the loose manure output observed here was not accompanied by other systemic signs compatible with botulism. Therefore, it seems inconsistent to assert that it was a complication of intra-articular deposition of BoNT-A. Soft-formed manure was previously associated with stressful conditions, such as transportation [51] or housing [52]. Additionally, high quality leafy alfalfa hay may induce gastrointestinal upset, including diarrhea, in horses not previously adapted to it [53]. Considering that before this experimental study the horses were living free at a paddock and grazing a native pasture, it is plausible to infer that sudden stabling and changes in food management could be responsible for this finding.

Synovial fluid analysis is an important diagnostic tool and provides an indication of the degree of synovitis. In this scenario, determination of TP, TNCC and PN are used in first intention as synovial parameters [33]. In all horses of this study, TP significantly increased from baseline to PIH 24, a common alteration observed in inflammatory conditions, which is

attributed to the increase of permeability of synovial vessels, determining protein leakage into the joint [35]. In these cases, TP values commonly are over 2g/dL [33]. The same pattern was observed in samples for the TNCC, in which significant increase was observed from baseline to PIH 24. Interestingly, both parameters decreased from the PIH 24 to PIH 168 collection, demonstrating the transitory nature of the inflammatory process observed here, resulting from the chemical insult. Chemical synovitis, also referred as a "joint flare", was already described after intra-articular injection of antibiotics, corticosteroids, local anesthetics and hyaluronan as well [2, 54-56]. Furthermore, in a previous study, intra-articular injection of saline solution also elevated TNCC in synovial fluid to a concentration of 41,425 cells/μL [57], while reference range is described as up to 30,000 cells/μL in most cases, demonstrating the severity of the inflammatory process [58]. In healthy horses, the proportion of synovial neutrophils is on average less than 10% [59]. In the horses of this study, both treated and saline joints showed neutrophilia 24 hours after injection, with most returning to normal reference range at the end of the experimental period. Previous studies demonstrated that a single injection of 0.9% saline solution into the tibiotarsal joint and repeated arthrocentesis elevated the PN to values up to 71% [56, 60], resembling proportions encountered in joints suffering from traumatic noninfectious synovitis [58]. Otherwise, red blood cells are not considered normal constituents of synovial fluid and their presence, as observed in samples from both treated and saline limbs in this study, is usually attributed to blood contamination during arthrocentesis [33]. Added to that, contamination with blood during collection is known for typically elevate the TNCC and number of neutrophils [33, 59]. In this scenario, blood contamination must be differentiated from pathologic hemorrhage into the joint space, so-called hemarthrosis. As such, typical microscopic signs of hemarthrosis, including erythrophagocytosis, hemosiderin granules in macrophages and hematoidin crystals, were not found in smears from both groups in this study. Thus, the pattern of similar increases in PT, TNCC and PN after treatments followed by

decreases toward the baseline values, led us to assume these findings as normal events, interpreted as a normal response from the synovial membrane to the deposition of substances into the joint and/or to the blood contamination during sample collection. However, these findings must be interpreted with caution, because in saline-injected samples were found far higher RBCC than BONT-A samples at PIH 24 and 168 post-injection, yet roughly comparable PN. Thus, blood contamination could be falsely increased PN in the saline-injected samples, which makes it very difficult to statistically compare the PNs between both groups at these time points. Mucin precipitate quality provides an estimate of hyaluronic acid concentration, degree of polymerization and, hence, the degree of viscosity of synovial fluid [33]. Since it decreases as the degree of inflammation within the joint increases, the good scores recorded over time associated with similar responses between both BoNT-A- and placebo-treated joints corroborate that only a mild and transient inflammatory response was present.

Noteworthy, the absence of lameness on BoNT-A-treated limbs also reinforces the safety of injecting this neurotoxin at the recommended dose into a normal joint. These findings, associated with no painful reactions noticed during limb palpation and passive flexion as well as no joint enlargement, warranty the safety necessary for further clinical investigations that would explore the potential anti-nociceptive properties of this new class of drugs for selected cases of chronic joint pain in horses.

Like any clinical trial, there are some limitations to our study that merit comment. It must be considered that this study included a small number of normal injected joints and, therefore, uncommon or rare local or systemic adverse effects probably would not have been detected, as well as we still do not know what response would be from joints with chronic disease. Additionally, results of this study reflect relatively short-term outcomes for a single injection of the drug investigated. Therefore, further research should be carried out to investigate potential adverse events induced by repeated injections of BoNT-A. Likewise, only

one toxin concentration was investigated here. Since while there is no consensus on the ideal concentration to produce maximal clinical response, the single one used on this study was based on a previous report [32]. Another major limitation of this study was the lack of a better method to evaluate joint effusion. Only an assessment of joint circumference was made, which despite informative could reflect other clinical entities, such as subcutaneous edema. In regard to synovial fluid analysis, we assume that the manual hemocytometer is not an optimal method for determining cell differentiation. Thus, for future studies, automated cell count would be a very good option to consider, because it seems a more accurate method for this purpose. Considering the wide range of commercial BoNT-A preparations with different biological characteristics, results of this study should not be extrapolated to products other than the one tested here. To better address possible joint inflammation induced by the drug, future studies could be focused on inflammatory biomarkers in synovial fluid, which certainly could contribute to elucidate the actual safety of the drug.

In conclusion, intra-articular injection of 50 U of BoNT-A produced a mild transient elevation in synovial fluid TP, TNCC and PN that peaked 24 hours, similar to the one induced on saline-treated joints, without producing joint enlargement or significant lameness. Nevertheless, no systemic adverse effects were observed on the clinical parameters evaluated during the experimental period. Thus, the results of our investigation suggest the BoNT-A at recommended dose as a safe drug for intra-articular use in horses. However, interpretation of its safety requires further studies. Much work remains to be done, and if proven safe, clinical trials could be performed to assess its efficacy in controlling arthritis pain.

Author's declaration of interests

All authors declare no financial or personal relationships with people or organizations that could inappropriately influence or bias this study.

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Tables

Table 1. Mean ± standard error of total nucleated cell count (TNCC), synovial fluid total protein (TP) concentration, percentage of neutrophils, red blood cells count (RBCC) and MPQ scores from the radiocarpal joints before and after treatments administration.

Parameters	Saline solution			Botulinum toxin type A		
	Baseline	PIH 24	PIH 168	Baseline	PIH 24	PIH 168
TNCC (cells/μL)	142±50.2[a]	26979±9538[b]	1244±439.9[ac]	228±80.65[a]	9049±3199[b]	1085±383.6[ac]
TP (g/dL)	1.17±0.41[a]	2.87±1.01[b]	1.6±0.56[c]	1.05±0.37[a]	1.72±0.61[b]	1.97±0.69[c]
Neutrophils (%)	1.25±0.44[a]	36.5±2.84[b]	3.5±0.86 [ac]	0.5±0.17[a]	35.6±1.96[b]	2.5±0.51[ac]
RBCC (cells/μL)	15806±17744[a]	382482±320635[b]	195577±18994[c]	50840±50024[a]	4155±1459[b]	1720±614[c]
MPQ scores	3.01±0.37[a]	3.87±0.12[a]	3.25±0.36[a]	3.25±0.36[a]	3.51±0.37[a]	3.12±0.44[a]

Different letters in superscript at the same line indicate statistical difference between time points ($p<0.05$).

Table 2. Severity of the lameness of seven horses was determined by calculating the vector sum (VS) obtained during the objective lameness assessment with the Lameness Locator system. To facilitate interpretation, VS values for saline-treated limbs are represented by negative values. Lameness was detected (*) only on saline-treated limbs and represented by values above the threshold (VS > 8.5 mm). Last row represents mean ± standard error of VS for each time point.

Horse	BoNT-A limb	Vector sum (amplitude) of lameness (mm)								
		Baseline	PIH 12	PIH 36	PIH 60	PIH 84	PIH 108	PIH 132	PIH 156	PIH 168
1	LF	-3.2	-4.5	-8.4	-2	5.8	-6.2	-6.9	-3.2	3.6
2	RF	-4.4	-8.1	-8.9*	-5.3	-6	-12.7*	-13.2*	-6.2	-7.1
3	LF	6.4	5.9	5.7	6.6	7.1	1.9	2.4	8.1	4.2
4	RF	3.3	5.3	2.4	6.7	2.3	2	1.9	2.4	1.6
5	LF	-7.2	-7.1	-10	-8.2	-10.9*	-8.2	-5.8	-10.8*	-6.2
6	LF	-6.9	-8.6*	-16.6*	-11	-7.3	-10.1*	-5.9	-9.9*	-9.5*
7	RF	-4.6	-4.3	8.2	4.2	5.5	4.3	4.7	2.4	4.5
Mean±s.e.	-	5.14±0.63	6.25±0.64	8.6±1.64	6.28±1.08	6.41±0.97	6.48±1.54	5.82±1.41	6.14±1.34	5.24±0.97

LF left forelimb; RF right forelimb

Figures

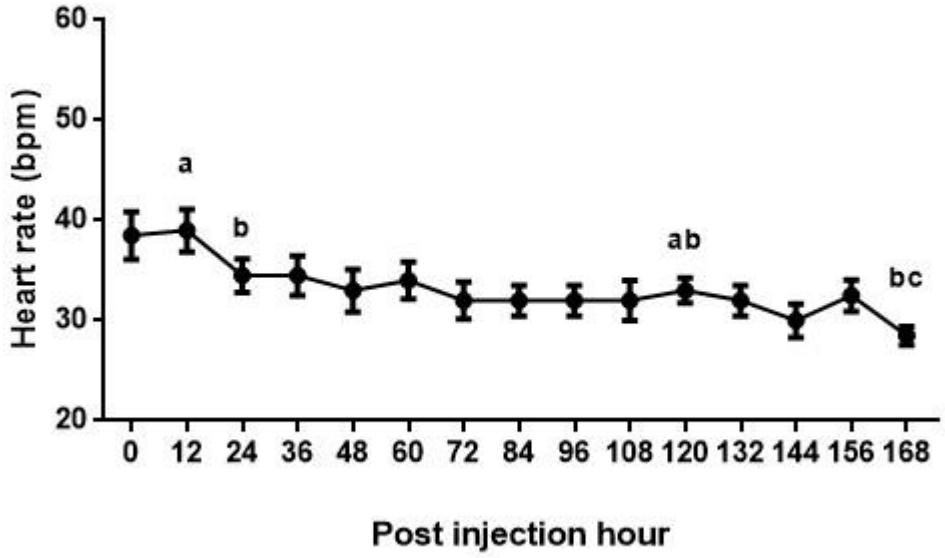

Figure 1 - Mean ± standard error heart rate evaluated by indirect auscultation. Different letters indicate statistical difference between time points ($p<0.001$). However, this parameter remains clinically within the refence interval.

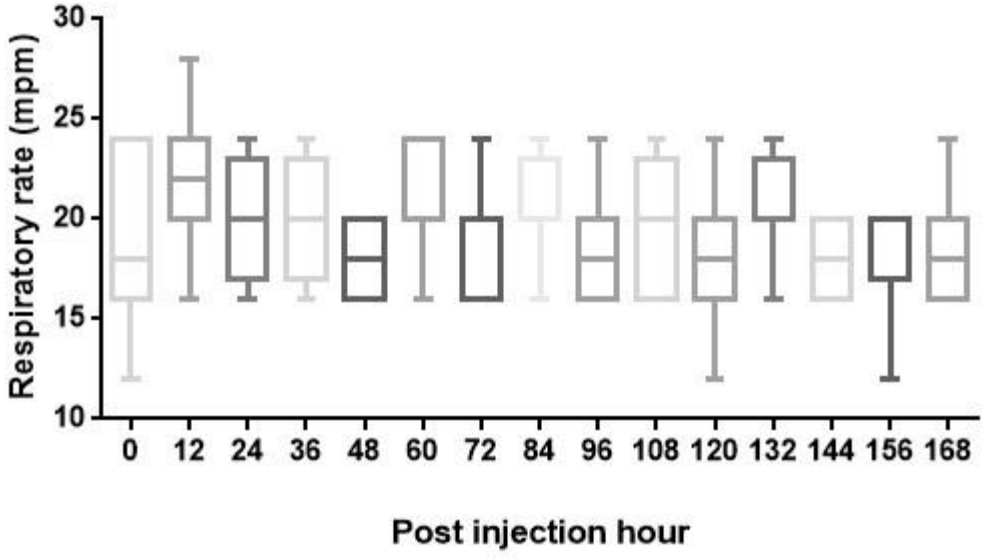

Figure 2 – Box-and-whisker plots representing the median ± interquartile range of the respiratory rate. There was no difference between time points after treatments ($p = 0.065$).

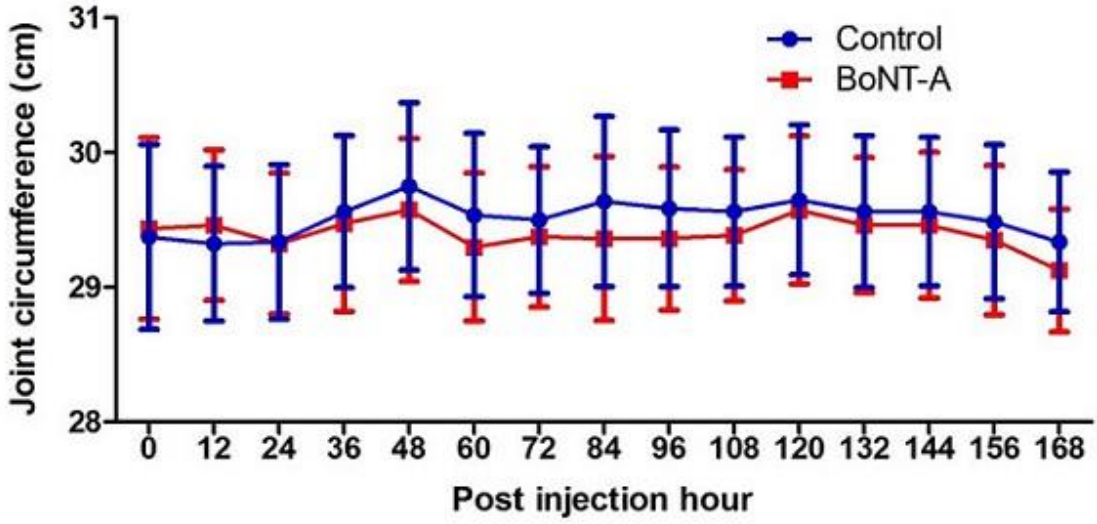

Figure 3 – Mean ± standard error circumference for botulinum toxin type A-injected joints compared with saline-treated ones. There was no influence of treatment ($p = 0.884$) or time ($p = 0.815$) on carpal circumference.

3.2 ARTIGO 2

Lameness improvement in horses with distal tarsal pain after intra-articular injection of botulinum toxin type A

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Abstract

Botulinum toxin type A (BoNT-A) has been described as a therapeutic agent in the management of joint pain in human patients. Thus, the objective was to evaluate the effect of IA injection of BoNT-A in horses with chronic distal tarsal osteoarthritis. Nine horses were selected for the study after a complete physical and radiographic assessment. Horses also underwent an objective lameness examination and were included if they had a hind limb impact lameness (Pmin variable ≥ 3mm), which positively responded (≥50%) to the tarsometatarsal (TMT) and centrodistal (CD) joints anesthetic block. Presence of radiographic signs of osteoarthritis also was an inclusion criterion. After, horses randomly received IA injection with 50 U of BoNT-A or equivalent volume of saline solution. Five horses were included in the BoNT-A group, whereas four individuals were allocated in the placebo group. To evaluate outcome measures, horses were re-evaluated at post-injection days (PID) 1, 7, 15, 30, 60, 90, 120, 150 and 180. Success criteria included: decrease of Pmin values (≤ 3mm) and abolishment of lameness on the baseline lame limb with lameness shifting to the contralateral limb, in cases of individuals bilaterally affected by the condition. Additionally, a percentage of lameness improvement was calculated for all horses at all timepoints. Significant improvement was observed in horses from TBA group when compared with placebo at PID 90 ($p<0.05$), 120 ($p<0.001$), 150 ($p<0.001$) and 180 ($p<0.05$). Individually, 40% (2/5) of horses from the TBA group had a complete improvement (100%) in lameness at all the timepoints. In the same group, 80% (4/5) of horses demonstrated complete improvement for at least four timepoints evaluated. Only one horses from this group did not present complete improvement during the study, with mean ± se 51.53±19.36%. Higher percentages of lameness improvement were observed at the PID 60 (95.92±9.13%). In contrast, no horses from the placebo group demonstrated complete lameness improvement after treatment. Results of this study suggested that the IA injection with 50 U of BoNT-A was effective in reducing lameness in horses with chronic distal tarsal osteoarthritis, mainly after 90 days post-injection.

Keywords: joint disease; toxin; joint pain; intra-articular therapy; horse.

1. Introduction

The past decades have witnessed unparalleled advances in the management of equine osteoarthritis. In this scenario, joint pain relief is a cornerstone of therapeutic strategies. Some medications have been directed to reduce the compromising effects of pain and inflammation in these cases, including systemic nonsteroidal anti-inflammatory drugs (NSAIDs) and intra-articular injections with corticosteroids. Overall, despite effective, several studies have reported the side effects promoted by abusive use of systemic NSAIDs [1-3] and deleterious effects produced by repeated injections of corticosteroids, including chondrocyte necrosis and inhibition of proteoglycan synthesis [4-5]. Adds to that, some patients can be refractory to their use, needing complementary therapies to achieve good clinical response.

Joint pain is a hallmark of osteoarthritis and a leading cause of lameness attributable to the disease, directly impacting both performance and animal welfare. Thus, in a complex mechanism, pain stimuli can be generated by both direct damage to joint tissues and chemical stimuli resulting from tissue inflammation [6]. These stimuli are detected and forwarded by a variety of receptors, mechanoreceptors, and nociceptors located in different joint tissues. Finally, the signal is carried to the dorsal horn of spinal cord, where neuromodulators and neurotransmitters send the signal to the brain where it is processed, modulated and perceived [7]. This information raises question about the need for therapeutic alternatives to control joint pain that target nociceptive pathways rather than traditional medications that target inflammatory pathways.

Nowadays, botulinum toxin type A (BoNT-A) has emerged as one of the most versatile therapeutic agents, which have been used to treat several painful conditions affecting both humans and animals. Originally discovered as the most potent neurotoxins in nature, botulinum toxins (BoNTs) are produced by the endospore-forming anaerobic bacterium *Clostridium*

botulinum, the causative agent of botulism, a potentially lethal intoxication that causes paralysis of skeletal muscles and loss of parasympathetic function [8]. BoNT-A inhibit the release of acetylcholine into the synaptic cleft in cholinergic nerve terminals, producing muscle paralysis [9]. Thus, this property has been explored in human medicine to treat disorders characterized by constant painful overactivity, such as strabismus [10], blepharopasm [11], cervical dystonia [12], limb spasticity [13] and overactive bladder syndrome [14], among others. Additionally, some evidence from studies in rodent models demonstrated that BoNTs inhibit the release of neurotransmitters secreted upon nociceptive stimulation, such as substance P [15], glutamate [16] and calcitonin gene-related peptide [17]. These findings suggested that BoNT-A may be used in the management of inflammatory and chronic pain. Then, few studies in human patients were carried out to investigate the intra-articular use of BoNT-A to treat joint pain, which reported promising results and minimal side effects [18-22]. Growing clinical interest of BoNT-A in human medicine motivated its use also as a therapeutic agent in veterinary medicine. In this scenario, two clinical studies investigated the intra-articular injection of BoNT-A in dogs with osteoarthritis [23-24]. First, Hadley and colleagues (2010) in a pilot study reported consistent improvement in lameness based on the evaluation of ground reaction forces and also by the owners' perception following treatment [23]. After, Heikkila and coworkers (2014) performed a placebo-controlled trial, in which they reported significant improvement in lameness, detected in the ground reaction forces, while no changes were observed in horses receiving placebo injections [24]. In equine medicine, however, only one small pilot study using four healthy horses in an equine model of experimentally induced synovitis [25]. In that study, two horses received injections of BoNT-A into the midcarpal joint, while the remaining two horses serving as control received injections with saline solution. After, an acute synovitis was induced with intra-articular injection of interleukin-1β (IL-1β). The authors suggested that BoNT-A can attenuate lameness, considering that one out of two horses treated with the toxin

did not present lameness when assessed by a veterinary evaluation and by a computer-assisted kinematic analysis [25]. More recently, a study was published suggesting BoNT-A as a safe drug for intra-articular use in horses. After injection of 50 U of BoNT-A into the radiocarpal joint and saline solution into the contralateral joint, horses developed similar inflammatory response in both limbs, without development of lameness on the BoNT-A injected limb or any systemic adverse events [26].

Thus, the aim of this study was to evaluate the effect of a single intra-articular injection of BoNT-A in horses with naturally occurring chronic distal tarsal osteoarthritis. We hypothesized that BoNT-A injection would alleviate pain arising from the injected diseased joints.

2. Materials and Methods

2.1 Animals and clinical trial enrollment

The Ethics Committee on Animal Research Use at Federal University of Santa Maria approved all experimental procedures (Protocol number CEUA 3627290920).

Nine mature crossbred horses from a retired mounted police herd (8 mares and 1 gelding, mean ± se age 11.9±2.1 years and weighing mean 494.12±23.45 kg), were included in this study after a complete physical examination, including visual appraisal at rest, limb palpation, objective lameness evaluation at motion, joint flexion tests and diagnostic anesthesia. Horses were considered qualified for the study if they had a primary hind limb impact lameness, based upon the objective lameness evaluation using an inertial sensor-based system (LL; Lameness Locator, Equinosis, Columbia, MO, USA).

In brief, for collection of sensor data, horses were trotted in a straight line on a concrete surface for at least 25 strides. The sensor program recorded the Pmin and Pmax variables, which are measures of the degree of asymmetry of pelvis vertical movement over all collected strides. Thus, horses were considered eligible if Pmin (impact component) was ≥3mm and if intra-articular anesthesia of the tarsometatarsal (TMT) and centrodistal (CD) joints with lidocaine hydrochloride 2% gave a lameness improvement of 50 per cent or more when evaluated 5 minutes after the anesthetic block. All horses also underwent a complete radiographic assessment of the tarsus, including the lateromedial, dorsolateral-plantaromedial, dorsoplantar and dorsomedial-plantarolateral views, obtained by standard radiographic techniques [27]. Radiographic signs of osteoarthritis in each joint were observed in a blinded fashion, and included: presence of irregular and sclerotic subchondral bone, narrowed joint space, presence of osteophytes, and subchondral bone lysis. Then, horses that showed one or more of these signs, were included in the study. Horses were excluded from the experimental protocol if they had received any intra-articular medication in the previous 6 months or they had received systemic NSAIDs in the previous 7 days. Additionally, some horses that presented lameness in both hind limbs were considered affected by the condition in both limbs.

2. 2 Study design and definition of outcome

The study was designed as a randomized, placebo-controlled clinical trial. Horses that met the inclusion criteria were randomly assigned by coin toss to receive a joint injection of 50 U of BoNT-A (Botulift, Laboratório Bergamo, São Paulo, Brazil), a previously described dose [25, 26], or equivalent volume of saline solution on the TMT and CD joints, after aseptic preparation of the joints. Horses that had bilateral lameness received both treatment or placebo

injection on the more severally affected limb. Enrolled horses were re-evaluated by objective lameness examination at post-injection days (PID) 1, 7, 15, 30, 60, 90, 120, 150 and 180.

Treatment was considered successful if the horses had complete lameness abolishment on the injected limb as assessed by the objective assessment (Pmin ≤ 3mm) or if the lameness shifted to the contralateral untreated limb (in cases of bilateral lameness). Also, a percentual of lameness improvement was calculated by the formula √(Pmin before injection -P after injection)/P before injection [100], for all horses at the all evaluated timepoints, in order to verify absolute or partial lameness improvement.

2.3 Statistical methods

Data were examined by use of a D'Agostino-Pearson omnibus test for normality. To compare the variable Pmin before and after treatment, data were examined by analysis of variance with repeated measures adjusted for comparisons with the *post hoc* Bonferroni's test. In horses with bilateral lameness, Pmin values were multiplied by -1 before statistical analysis, indicating change of lameness to the contralateral limb. All results were considered significant if $p \leq 0.05$ (GraphPad Prism Software 5.0, San Diego, CA, USA).

3. Results

From the initial selected population, by randomization, five horses were allocated into the BoNT-A group, while the remaining four individuals were included in the placebo group. During the experimental protocol, no immediate or delayed signs of swelling or pain were observed after injections of the study drug or placebo on the TMT and CD joints.

The BoNT-A injection significantly decreased the Pmin variable at the PID 90 ($p<0.05$), 120 ($p<0.001$), 150 ($p<0.001$) and 180 ($p<0.05$) when compared to placebo (Figure 1), demonstrating substantial reduction in severity of lameness at these timepoints. Botulinum toxin type A injection resulted in higher percentages of lameness improvement at the PID 60 (95.92±9.13%). During the study, four out of five horses shifted lameness to the contralateral untreated limb for at least one timepoint. This phenomenon was considered as a complete improvement in lameness, due to the shifting lameness to the contralateral limb, in a bilateral lameness pattern. Considering individual responses (Table 1), 60% (3/5) of horses showed complete lameness alleviation on the limb treated with BoNT-A at the PID 1. Forty per cent (2/5) of horses that received BoNT-A injection demonstrated complete improvement (100%) at all PID evaluated. In the same group, 80% (4/5) of horses had complete alleviation in lameness for at least four timepoints evaluated. Interestingly, only 20% (1/5) of horses that received BoNT-A injection did not completely improve lameness at all PID, demonstrating mean ± se lameness improvement of 51.53±19.36% during the study. In contrast, no horses from the placebo group had completely improved lameness after injection.

4. Discussion

Results of the present study indicated that a single intra-articular injection of 50 U of BoNT-A alleviated pain related to the TMT and CD joints in horses. To the authors' knowledge, this is the first study evaluating the effect of this neurotoxin as an option for the management of selected cases of naturally occurring osteoarthritis pain. The findings of this clinical trial were interesting, which demonstrated significant long-lasting anti-nociceptive effects of BoNT-A at the PID 90, 120, 150 and 180, when compared to placebo. However, early responses to the drug administration were also observed considering that three out of five horses demonstrated

lameness abolishment at the PID 1. Also, it must be emphasized that all these subjects showed no adverse events during the clinical trial, corroborating the findings from a safety study recently published [26].

Distal tarsal osteoarthritis is the major hind limb musculoskeletal problem observed in middle-aged horses that perform a variety of athletic disciplines [28], the main reason that motivated the choice for including retired horses with this condition in our study. At least three horses from this study had bilateral lameness, which is often observed in performance horses [29]. In these particular subjects, predominant lame limb can switch over time depending on the severity of each lameness. This pattern of pain alleviation in the baseline lame limb and lameness shifting to the contralateral limb was attributed by the authors as a response to BoNT-A injection.

Chronic osteoarthritis is characterized by persistent pain, which may produce neurogenic inflammation and increased pain perception, due to the action of a variety of neurotransmitters located in peripheral neurons [30]. It is well-established that BoNT-A exerts anti-nociceptive effects, which are independent of its classical mechanism of blockade of acetylcholine at the neuromuscular junction. Earliest *in vivo* evidence of these alternative effects was reported in a study using a formalin-induced model of inflammation in rats [16]. Pre-treatment with BoNT-A reduced inflammation and nociceptive behaviors, without obvious muscle weakness [16]. In further studies in rats, BoNT-A proved to block the release of some of these substances, such as substance P [15], glutamate [16] and calcitonin gene-related peptide [17], decreasing pain generation and transmission. These findings motivated clinical studies in people suffering for chronic joint pain, reporting promising and variable results. A pioneer uncontrolled study was conducted by Mahowald et al. (2006), in which 15 joints from 11 human patients with chronic arthritis received injection of BoNT-A. Patients were followed in a one-year assessment, reporting pain relief during 3 to 12 months [18]. After, a systematic review

and meta-analysis compiled results from different clinical randomized-controlled trials that evaluated the effect of BoNT-A intra-articular injection in elderly patients with refractory joint pain to traditional therapies, such as systemic NSAIDs and intra-articular use of corticosteroids. The study reported clinical benefits, with at least 6 months of pain alleviation [31].

In dogs, promising but controversial results were found in different clinical studies. Hadley at al. (2010) conducted an uncontrolled study using 5 client-owned dogs with elbow or hip chronic osteoarthritis. Pain relief was observed for up to 12 weeks after intra-articular deposition of 25 U of BoNT-A, based on changes on ground reaction forces evaluated by a pressure platform gait analysis and by owner's perception of outcome [23]. Similar results were described in a randomized-controlled trial, in which 36 dogs with chronic pain due to stifle, elbow or hip osteoarthritis responded positively to intra-articular injection with 25 U BoNT-A for a 12-weeks period [24]. Further, contrasting these results, another randomized-controlled clinical trial reported that neither veterinarian nor owner assessments were able to detect relevant analgesic effects of BoNT-A at the same dose [32]. Interestingly, results from our study were in accordance with the majority of clinical studies performed in both human and canine medicine. Horses from this trial demonstrated substantial improvement in lameness, peaking 60 days in average after BoNT-A injection. Likewise, partial or complete lameness improvement was obtained for at least 6 months depending on individual responses, as observed in human patients.

The scope of clinical use of BoNT-A in equine medicine seems to be ever expanding. Initially, the first serotype of BoNTs explored as therapeutic agent in horses was the Botulinum toxin type B (BoNT-B). A clinical study concluded that injections of BoNT-B in the external anal sphincter of mares may be useful to reduce dehiscence after surgical repair of perineal lacerations [33]. Further, Gutierrez-Nibeyro and coworkers (2014) verified short-term alleviation in lameness after intrabursal injection of BoNT-B in horses with pain related to the

podothroclear apparatus, representing a palliative option to manage this condition [34]. The reduction of muscle activity promoted by BoNT-A was also used to treat common clinical diseases in horses. Two studies investigated the injection of BoNT-A as a therapy for laminitis [35-36], a debilitating condition characterized by structural failure of the suspensory apparatus of the distal phalanx, formed by dermal and epidermal hoof lamellae. Both trials hypothesized that injecting the deep digital flexor muscle with BoNT-A would result in reduced muscle tone, preventing distal phalanx from rotating or sinking by reducing the pull of the muscle and tendon on the distal phalanx. It was concluded that BoNT-A was effective and may play a role in a multimodal therapy to control laminitis [35-36]. In healthy ponies and horses with stringhalt, injection of BoNT-A into the *extensor digitorum longus, digitorum lateralis* and *lateral vastus* muscles reduced the muscle tone and, hence, decreased signs of spasticity observed in clinical cases of stringhalt [37].

Only a pilot study with a small number of horses investigated potential benefits of intra-articular injection of BoNT-A in horses [25]. Fourteen days after injection with 50 U of BoNT-A into the midcarpal joint, two clinically healthy horses underwent induction of synovitis with IL-1β. Surprisingly, one horse developed lameness whereas the other remained sound. Two horses used as a control demonstrated changes in baseline evaluation compatible with lameness [25]. In this scenario, our investigation opened new horizons for the treatment of chronic osteoarthritis, mainly for horses with refractory joint pain. As observed in other species, despite remaining as the mainstay of joint pain therapy in horses, management with NSAIDs or intra-articular corticosteroids could not promote sufficient pain relief and may induce undesirable effects [1-5]. Results found in a retrospective study regarding intra-articular medication with corticosteroids with or without sodium hyaluronate into the distal tarsal joints reported an improvement for a median 56 days, and there was no significant difference between triamcinolone acetonide (TA) and methylprednisolone acetate (MPA) [38]. Similar results were

described by two studies using a carpal osteochondral fragmentation model of synovitis-induction. Injections with both TA and MPA were administered at post-surgical days 14 and 28 and effectiveness was still observed at day 70 post-injection [39-40]. Noteworthy, considering that the majority of horses from our study remained sound at PID 180, the effect of BoNT-A seems to be long-lasting than those obtained with corticosteroids. The anti-nociceptive effects of BoNT-A may help especially some animal categories, such as retired osteoarthritic horses to improve their quality of life, or even horses with advanced osteoarthritis which temporarily would not be candidates to undergo an arthrodesis, including stallions and broodmares during the breeding season. Thus, it is important to highlight that the intra-articular use of BoNT-A brings ethical concerns. Considering its property of suppressing pain, the off-label use of the toxin in athletic or racehorses would predispose to catastrophic injuries, emphasizing that its application should be reserved only to selected cases.

Our study has some important limitations that need to be commented. Firstly, the main limitation is the small sample size. Although outcome measures were objectively evaluated, we recognize that further studies using larger number of hoses might benefit the interpretation of BoNT-A effects. Likewise, only responses to the injection of TMT and CD joints were investigated here, therefore, future research must be conducted to investigate potential benefits of BoNT-A injection into other joints, especially the high-motion ones. Like any clinical trial using patients with naturally occurring osteoarthritis, it is possible the occurrence of misdiagnosis at the time of study enrollment, leading to selection of subjects that do not truly have the condition of interest. Regarding the horses of this study, horses were selected only if substantial improvement in severity of lameness was seen following diagnostic distal tarsal joints anesthesia. Additionally, it is possible that horses with proximal suspensory desmitis associated with lameness arising from distal tarsal joints could have been selected [39]. However, considering that horses were randomly assigned to treatment groups, the effect of

this should have been minimized. The concentration of the toxin tested in the present study was extrapolated from an early pilot study [25], which was further considered safe for intra-articular use in horses [26]. Therefore, results found here should not be extrapolated to other BoNT-A dose regimes. Considering the wide range of BoNT-A commercially available preparations, results of this study should not be extrapolated to products other than one used here. Finally, future studies could be focused on nociceptive biomarkers, which certainly would contribute to elucidate the promising use of BoNT-A for joint pain.

In conclusion, intra-articular injection of 50 U of BoNT-A produced a substantial improvement in lameness in horses with naturally occurring distal tarsal osteoarthritis that peaked 60 days after injection. When compared with placebo, significant pain relief was observed at PID 90, 120, 150 and 180. Interestingly, no immediate or delayed adverse effects were observed in patients included in the study. Thus, the antinociceptive properties offered by BoNT-A at recommended dose have some efficacy in alleviate pain for selected cases of osteoarthritis in horses, mainly for those refractories to traditional therapies. Future studies must be carried out to better explore the potential role of this neurotoxin as a therapy for this condition.

Author's declaration of interests

There is no conflict of interests that could inappropriately have influenced our work.

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Tables

Table 1: Percentage of lameness improvement (%) observed after intra-articular injection of BoNT-A. Asterisks represent complete abolishment in lameness

PID	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
1	100*	0	100*	40.1	100*
7	100*	25.3	100*	25.3	18.4
15	100*	47.7	100*	28.3	100*
30	100*	100*	100*	41.9	42.1
60	100*	100*	100*	79.6	100*
90	100*	37.3	100*	67.2	100*
120	100*	100*	100*	49.3	23.6
150	100*	32.8	100*	59.2	43.4
180	100*	100*	100*	72.8	57.8

PID = post-injection day

Figures

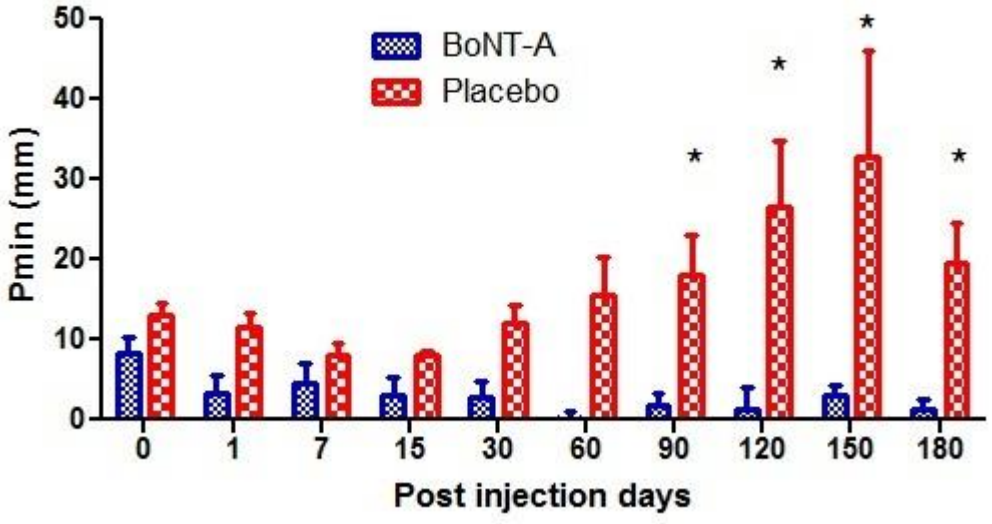

Figure 1: Comparison between Pmin values in the objective lameness evaluation after intra-articular injection of BoNT-A or placebo into the tarsometatarsal and centrodistal joints. Significant improvement ($p<0.05$) in severity of lameness, represented by asterisks, was observed from 90 up to 120 PID in horses from the BoNT-A when compared with placebo.

4 DISCUSSÃO

Por determinar importante impacto econômico, atlético e de bem estar animal, a OA necessita de terapias efetivas no que diz respeito à redução da dor e inflamação articulares (GOODRICH & NIXON, 2004; FRISBIE, 2006). Nesse cenário, o manejo convencional da dor articular em equinos é baseado sobretudo na utilização sistêmica de AINEs e aplicações IA de corticosteróides associados ou não ao hialuronato de sódio. Por apresentarem possíveis efeitos adversos, principalmente em médio a longo prazo (McCKAY et al., 1983; McILWRAITH, 2010) e devido a ocorrência de respostas refratárias ao seu uso, é justificável a busca por alternativas terapêuticas.

Nas últimas décadas, a TBA surgiu como um dos agentes terapêuticos mais versáteis da medicina moderna, sendo utilizada com sucesso clínico no manejo da dor crônica em pessoas e animais de companhia, incluindo casos de osteoartrite crônica (MAHOWALD et al., 2006; HEIKKILA et al., 2014). Oferecendo propriedades anti-nociceptivas, baseadas na inibição de neurotransmissores como a substância P, o peptídeo relacionado ao gene da calcitonina e o glutamato, a TBA parece uma alternativa para casos seletos de dor articular refratária às terapias tradicionais. A literatura científica revela poucos estudos utilizando a TBA em medicina equina, sendo destinada quase em sua totalidade à terapia de doenças que necessitam de redução do tônus muscular (CARTER & RENFOE, 2009; WIJNBERG et al., 2009; WIJNBERG et al., 2013). Somente um estudo piloto utilizando um modelo de indução experimental de sinovite sugeriu possível eficácia da TBA em reduzir a dor de origem articular. Sendo assim, encorajados pelos resultados promissores da aplicação IA de TBA em pacientes humanos e caninos, desenvolvemos dois estudos no intuito de atestar a segurança de uso e o efeito clínico após o uso de TBA em equinos com OA de ocorrência natural.

No primeiro estudo, os animais que receberam a aplicação de TBA não apresentaram efeitos adversos, contrariando achados de estudos clínicos utilizando a TBB, outro sorotipo dessa toxina. Dessa forma, sinais sistêmicos já relatados como letargia, fraqueza generalizada e disfagia não foram observados (ADAM-CASTRILLO et al., 2004). Adicionalmente, os animais se mantiveram livres de claudicação durante o período experimental, complicação observada em um estudo utilizando a injeção de TBB para o alívio da dor em casos de síndrome podotroclear (GUTIERREZ-NIBEYRO et al., 2014).

Considerando descrições prévias da ocorrência de sinovite química como resultado da aplicação de medicações IA (CARON, 2005), a resposta inflamatória após a injeção de TBA

também foi avaliada através da determinação de parâmetros no LS, incluindo PT, CCNT e percentual de neutrófilos. Em todos os equinos desse estudo foi observado o aumento significativo da concentração de PT e CCNT quando comparadas as amostras pré-tratamento e após 24 horas da aplicação. Interessantemente, ambos os parâmetros reduziram nas amostras coletadas 168 horas após os tratamentos. Esses achados demonstram o caráter transitório do processo inflamatório resultante da resposta aos tratamentos. Adicionalmente, neutrofilia no LS foi observada em amostras coletadas 24 horas após os tratamentos, tanto de membros tratados com TBA como naqueles que receberam SAL. Cabe ressaltar ainda, que em esses valores retornaram aos valores basais na última coleta do estudo. Os autores atribuíram as elevações da CCNT e do PN à possível contaminação por hemácias durante a coleta, a qual tipicamente costuma alterar esses parâmetros no LS (STEEL, 2008).

Ao final do estudo, sugerimos o desenvolvimento de novas pesquisas que continuem a elucidar possíveis efeitos adversos do uso IA de TBA, sobretudo com novas concentrações da toxina e àqueles voltados à avaliação de biomarcadores como parâmetros de avaliação de resposta inflamatória.

O segundo estudo investigou o efeito clínico da aplicação IA da TBA em equinos com OA társica distal crônica. À luz do conhecimento dos autores, esse foi o primeiro estudo dedicado a determinar a eficácia clínica dessa neurotoxina em casos de OA de ocorrência natural. Os resultados desse estudo trazem informações interessantes sobre o potencial terapêutico da TBA, sobretudo possíveis efeitos anti-nociceptivos duradouros, uma vez que os animais apresentaram melhora significativa após 90, 120, 150 e 180 dias de sua aplicação, quando comparada ao placebo. Comparando esses achados com resultados da literatura sobre o uso IA de corticosteroides, os quais demonstraram efeito clínico médio de até 70 dias (FRISBIE et al., 1997; FRISBIE et al., 1998), sugerimos ainda que a eficácia da TBA possa ser superior a esses em longo-prazo. No entanto, respostas individuais precoces também foram observadas, considerando que três dos animais do grupo tratado apresentaram melhora completa na claudicação no dia seguinte ao tratamento.

É fundamental ressaltarmos que a indicação de TBA para uso IA é recomendada para casos seletos. Isso porque, considerando sua propriedade de supressão da dor pode predispor a lesões catastróficas em animais atletas. Portanto, as propriedades anti-nociceptivas dessa neurotoxina poderiam beneficiar, por exemplo, animais aposentados ou mesmo garanhões ou éguas reprodutoras, os quais não sejam candidatos à outras modalidades terapêuticas.

Cabe enfatizar que reconhecemos o número reduzidos de animais selecionados para este estudo como uma importante limitação. Dessa forma, recomendamos que outras pesquisas

clínicas sejam conduzidas, utilizando um número maior de indivíduos e investigando os efeitos da TBA sobre a dor com fonte em outras articulações.

5 CONCLUSÃO

No primeiro artigo foi evidenciado que a aplicação IA de 50 UI de TBA produziu uma sutil e transitória elevação nas concentrações sinoviais de PT, CCNT e PN. Essa resposta inflamatória teve pico às 24 horas pós-tratamento e foi similar àquelas observadas em animais do grupo controle, sem a produção de aumento significativo de volume articular ou claudicação. Adicionalmente, nenhum efeito sistêmico adverso foi observado nos animais utilizados neste estudo. Em síntese, os resultados dessa pesquisa sugerem a TBA na dose recomendada como uma terapia segura para uso IA em equinos, garantindo que mais estudos clínicos possam ser realizados com essa neurotoxina biológica.

No segundo estudo foi concluído que a injeção IA com 50 UI de TBA produziu melhora substancial na claudicação em equinos com osteoartrite társica distal, com efeitos mais significativos aos 60 dias após o tratamento. Quando comparado ao grupo placebo, alívio significativo na dor foi observado nos dias 90, 120, 150 e 180 após sua aplicação. Ademais, nenhum efeito adverso imediato ou tardio foi observado nos pacientes deste estudo. Portanto, os efeitos anti-nociceptivos da TBA apresentam alguma eficácia em reduzir a dor para casos seletos de osteoartrite crônica.

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