Botulinum neurotoxin type A does not exert concentration-dependent effects on equine articular cartilage in vitro

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OBJECTIVE
To determine whether Botulinum neurotoxin type A (BoNT-A) ameliorates the effects of interleukin 1 (IL-1) on equine articular cartilage, or exerts negative effects on normal equine articular cartilage homeostasis in vitro.

SAMPLE
Articular cartilage explants from 6 healthy femoropatellar joints of 3 adult horses.

METHODS
Explants were allocated to the IL-1 challenged or unchallenged group, then exposed to 1 of 6 concentrations of BoNT-A (0, 1, 10, 50, 100, or 500 pg/mL) for 96 hours. To assess BoNT-A’s effects on inflammation, prostaglandin E₂ (PGE₂) was measured in media via ELISA. Matrix degradation was determined as the percentage of sulfated glycosaminoglycans (sGAG) released from explants via dimethylmethylene blue assay. Aggrecan synthesis was estimated using CS846 ELISA and collagen type II degradation was estimated using C2C ELISA on media. Chondrocyte apoptosis was assessed via in-situ TUNEL assay. Generalized linear mixed models were fitted to determine treatment effects using α = 0.05.

RESULTS
The challenge with IL-1 resulted in increased concentrations of PGE₂ and CS846 in media and increased release of sGAG from explants. BoNT-A did not significantly impact PGE₂ or CS846 concentration in media, percentage of sGAG released, or chondrocyte apoptosis in IL-1 challenged or unchallenged cartilage explants. The concentration of C2C in media was below the quantifiable limit of the ELISA in all samples.

CLINICAL RELEVANCE
BoNT-A did not show chondroprotective effects or have negative effects on cartilage homeostasis in vitro at the concentrations tested. While chondroprotective effects were not observed, BoNT-A may be safe for intraarticular use. In vivo testing is warranted before clinical use.

Keywords: botulinum, equine, osteoarthritis, cartilage, Botox

As of the year 2000, approximately one-third of domestic equids in the United States suffered from lameness due to osteoarthritis (OA) and owners were spending around $145 million annually on veterinary care related to this disease. The goal of treating OA is not only to manage symptoms but also to slow the progression of the disease. Currently, the ideal therapy does not exist. Of the available therapies, all eventually fail to produce adequate results, and some treatments exert detrimental effects both locally and systemically.

Recently, Botulinum neurotoxin type A (BoNT-A) has been introduced in humans for the management of refractory joint pain related to OA. BoNT-A is the most potent of the 7 neurotoxins produced by Clostridium botulinum. After ingestion or exposure through wounds, BoNT-A binds to peripheral cholinergic terminals, inhibiting the release of acetylcholine, and causing flaccid paralysis. It was first used in human medicine in the late 1970s for the treatment of strabismus, and since then has been used to manage a multitude of conditions, from urinary incontinence to migraines. After intraarticular injections in humans with OA, there are reports of decreased pain scores and improved range of motion with a similar duration of action as corticosteroids and a decreased...
need for rescue medications. In a study of 4 horses with experimentally induced synovitis, BoNT-A was shown to attenuate lameness in 3 of 4 horses. More recently, intraarticular BoNT-A was compared with saline controls in healthy horses. A single injection resulted in no adverse clinical effects and cellular parameters in the synovial fluid were similar to those measured in saline control joints. In lab animals with experimentally induced arthritis, studies suggested that intraarticularly administered BoNT-A had both anti-inflammatory effects and reduced peripheral sensitization, but to the authors’ knowledge, nothing is known about the direct effects of BoNT-A on articular cartilage.

Thus, the objective of this study was to determine whether BoNT-A exerts any effects on equine articular cartilage in vitro. Effects of equine recombinant interleukin 1 (rEq IL-1) on cartilage matrix homeostasis and chondrocyte metabolism in cartilage explants in vitro are commonly utilized as a model for osteoarthritis, on which new treatments can be assessed for disease-modifying effects. Increased matrix degradation can be evaluated via measurement of breakdown products during the release of proteoglycans and collagen type II, increased proteoglycan production can be estimated through assessment of aggrecan synthesis and the inflammatory response can be evaluated by measurement of prostaglandin E₂ (PGE₂). We hypothesized that BoNT-A would ameliorate the catabolic and inflammatory effects of rEq IL-1 challenge on articular cartilage explants in a concentration-dependent manner, based on the previous study suggesting anti-inflammatory effects in rats. We further wanted to test the null hypothesis that BoNT-A would have no concentration-dependent effects on cartilage matrix homeostasis and chondrocyte viability in rEq IL-1 unchallenged, normal equine articular cartilage explants.

**Methods**

**Animals**

All procedures were approved by the Institutional Animal Care and Use Committee. Three horses (aged 7, 12, and 15 years old) were euthanized with an overdose of pentobarbital (86 mg/kg IV; Fatal Plus, Patterson Veterinary) or pentobarbital and phenytoin (86 mg/kg and 11 mg/kg, respectively; Euthasol, Virbac) for reasons unrelated to the study. None of the horses had a previous history of lameness or musculoskeletal conditions. Within 8 hours of euthanasia, synovial fluid was collected from both femoropatellar joints for determination of PGE₂ concentration as described below and joints were grossly examined for signs of OA.

**Tissue collection**

Near full-thickness articular cartilage was collected steriley from the medial and lateral trochlear ridges and medial femoral condyles using sharp dissection with a scalpel blade. Cartilage was placed into Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 5% fetal bovine serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and gentamicin (50 µg/mL) and kept on ice until further processing in the lab.

**Cartilage explant culture and experimental design**

A previously described in vitro model was used to simulate the effects of OA on articular cartilage. Under aseptic conditions, harvested cartilage was cut into 5 X 5 X 1 mm explants totaling 36 explants per joint (72 total explants). The wet weight of the explants was recorded. Explants were placed into individual wells of 12-well tissue culture plates with 2 mL of DMEM, supplemented as described above, for a 48-hour equilibration period at 5% CO₂ and 37°C. After the equilibration period, culture media was replaced with the treatment culture media of 1°C of the 12 respective treatment groups (Table 1). Half of the explants were challenged with equine recombinant interleukin 1 (rEq IL-1; 0.1 ng/mL) to simulate osteoarthritis, and the other half remained unchallenged to simulate healthy joint conditions. Explants were exposed to 1 of 6 concentrations of research-grade BoNT-A (0, 1, 10, 50, 100, or 500 pg/mL; List Labs). Explants remained in culture for 96 hours with a media change at 48 hours.

**Sample collection and processing**

Culture media was collected at 48- and 96-hour treatment timepoints, for determination of prostaglandin E₂ (PGE₂), aggrecan synthesis, and collagen type II degradation at 96 hours, and sulfated glycosaminoglycans (sGAG) at 48 and 96 hours and frozen at −80°C until further processing. Explants were collected at 96 hours of treatment exposure. One explant from each treatment group was frozen at −80°C until further processing for sGAG determination and the other explant was placed in neutral-buffered 10% formalin for 48 hours, then paraffin-embedded and sectioned for analysis of apoptosis. The third explant was frozen for future gene expression analysis.

![Table 1—Allocation of explants from individual horses to treatment groups.](attachment:image1.png)

<table>
<thead>
<tr>
<th>Animals</th>
<th>Joints (n = 6) [number of explants]</th>
<th>Modeling of osteoarthritis [number of explants]</th>
<th>BoNT-A concentration in pg/mL [number of explants]</th>
</tr>
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</table>

BoNT-A = Botulinum neurotoxin type A, IL-1 = Interleukin-1.
performing the DMMB assay, culture media aliquots, and frozen explants were papain digested using equal volumes of culture media and papain digestion solution (0.5 mg/mL of papain in 3.45 g of monobasic sodium phosphate, 0.163 g of N-acetylcysteine, and 0.83 g of EDTA tetrasodium salt dissolved in H₂O to 500 mL; pH adjusted to 6.5) in a shaker incubator at 65 °C for 4 hours. Frozen explants were digested using 1 mL papain digestion solution per 10 mg wet weight in a shaker incubator at 65 °C for a minimum of 18 hours, or until the explant appeared completely digested.

**Assays**

The synovial fluid concentration of PGE₂ was determined to evaluate preexisting inflammation in the cadaver joints, whereas PGE₂ concentration in the media at 96 hours was used to determine the proinflammatory effects of BoNT-A on articular cartilage. A commercially available monoclonal PGE₂ ELISA kit (Cayman Chemical) was used to determine synovial fluid and culture media PGE₂ concentrations. The assay was performed according to manufacturer instructions and the manufacturer’s standards were used to generate the standard curve, positive and negative controls. All samples were run in duplicates.

Articular cartilage matrix degradation was determined as a percentage of sGAG released from each explant via a colorimetric dimethylmethylene blue (DMMB) assay. Digest buffer was used as a negative control and chondroitin-6-sulfate (MP Biomedical) was used to generate a standard curve ranging from 20 µg/mL to 60 µg/mL. Samples were assayed in duplicates. The percent of sGAG released from the explant was calculated as: ([sGAG in culture media collected 48 hours after initial treatment + sGAG in culture media collected 96 hours after initial treatment]) / [sGAG in culture media collected 48 hours after initial treatment + sGAG in culture media collected 96 hours after initial treatment + sGAG in cartilage explant]) X 100%

For estimation of aggrecan synthesis, a commercially available chondroitin sulfate 846 epitope (CS846) ELISA (IBEX Pharmaceuticals) was used. The assay was performed according to manufacturer instructions on undigested culture media and the manufacturer’s standards were used to generate the standard curve and positive, as well as negative controls. All samples were run in duplicates.

Collagen type II degradation was estimated using a commercially available collagen type II cleavage (C2C) ELISA (IBEX Pharmaceuticals). The assay was performed according to manufacturer instructions on undigested culture media and the manufacturer’s standards were used to generate the standard curve and positive, as well as negative controls. All samples were run in duplicates.

Chondrocyte apoptosis was quantified using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method with a commercially available in situ cell death detection kit (Roche Applied Science). Briefly, formalin-fixed, paraffin-embedded cartilage explants were sectioned before being deparaffinized and rehydrated individually as previously described. Sections were then treated with the TUNEL reaction mixture according to manufacturer instructions. Positive controls were treated with DNase I and negative controls were treated with label solution in place of the TUNEL reaction mixture. Slides were stored protected from light until brightfield and fluorescent images were obtained (BZ-X series, Keyence) within 24 hours of staining using excitation wavelengths from 440–470 nm and detecting emission wavelengths of 525–550 nm (green). Areas in which cells were counted included superficial through deep layers of cartilage; 200 total chondrocytes were counted on the brightfield image. Next, the fluorescent image was overlaid on the brightfield image, and the green fluorescent cells in the same locations were counted to obtain a percentage of apoptotic cells (number of fluorescent cells/200 X 100). Analyses were performed by a single investigator (MBM) who was blinded to treatment group allocation.

**Statistical analysis**

To quantify the effects of IL-1 challenge and treatment with BoNT-A on the measured responses, generalized linear mixed models (GLMM) were fitted to the PGE₂, sGAG, CS846, and TUNEL data using Proc GLIMMIX (SAS/STAT 15.2) with IL-1, BoNT-A and their interaction as fixed effects, horse as a random effect and BoNT-A as repeated measurement factor. Conditional lognormal distributions were assumed for PGE₂ and CS846 data, beta distribution for sGAG data, and binomial distribution assumed for TUNEL data. For PGE₂ and CS846 data, an identity link function was used and for sGAG and TUNEL data, a logit link function was used. The main and interaction fixed effects of IL-1 and BoNT-A were evaluated with F tests based on Type 3 Wald tests. Contrasts of the least squares mean of interest were constructed to determine the effect of IL-1 challenge at each BoNT-A concentration and to determine the effect of different BoNT-A concentrations on response variables where the overall effect was significant. All results are reported as means and a P-value of < .05 or less was considered statistically significant.

**Results**

**Prostaglandin E₂ in synovial fluid**

Synovial fluid PGE₂ levels from all 6 joints were < 250 pg/mL (range = 74.3–200.2 pg/mL), indicating all joints were free of subclinical disease.

**Prostaglandin E₂ in culture media**

Prostaglandin E₂ in culture media was used to evaluate the inflammatory response to the rEq IL-1 challenge and to assess if BoNT-A ameliorates any of these effects. There was a significant overall main effect of the IL-1 challenge (P = .0004), indicating that the challenge with rEq IL-1 significantly increased PGE₂ concentrations in media when compared with unchallenged explants. Results from
contrasts showed that this was true for all concentrations of BoNT-A treatment (all \( P < .001 \)). However, neither the main effect of BoNT-A nor the interaction between IL-1 challenge and BoNT-A treatment were significant (\( P = .0908, P = .3410 \), respectively), indicating that treatment with BoNT-A did not significantly impact concentrations of PGE\(_2\) in media (Figure 1).

**Figure 1**—Concentration of Prostaglandin E\(_2\) (PGE\(_2\)) in media after explants were maintained in culture for 96 hours with or without interleukin-1 (IL-1). Increased levels of PGE\(_2\) indicate a proinflammatory effect of treatment group on articular cartilage. Error bars represent 95% confidence intervals. Asterisk (*) denotes significant difference between IL-1 challenged and unchallenged treatment groups.

**Sulfated glycosaminoglycans (sGAG) released from explants into culture media**

Measurement of sGAG in culture media and cartilage explants using the DMMB assay was used to determine the effects of rEq IL-1 challenge on cartilage matrix proteoglycan breakdown and release into the media and to determine if BoNT-A would ameliorate these effects. There was a significant overall main effect of the IL-1 challenge (\( P < .0001 \)), indicating that the challenge with rEq IL-1 significantly increased the release of sGAG into the media when compared with unchallenged explants. Results from contrasts showed that this was true for BoNT-A treatment concentrations of 0, 1, 100, and 500 pg/mL (\( P < .001, P < .001, P = .05, P < .01 \), respectively), but not for the BoNT-A concentrations of 10 pg/mL or 50 pg/mL (\( P = .31, P = .07, P = .31 \), respectively). Neither the main effect of BoNT-A nor the interaction between IL-1 challenge and BoNT-A treatment were significant (\( P = .05, P = .31 \), respectively), indicating that treatment with BoNT-A did not significantly impact aggrecan synthesis in cartilage explants with or without IL-1 challenge (Figure 2).

**Collagen type II neopeptide (C2C) in culture media**

Measurement of C2C in culture media was used to estimate the effects of rEq IL-1 challenge on aggrecan synthesis and to determine if BoNT-A would ameliorate these effects. There was a significant overall main effect of IL-1 challenge (\( P = .0013 \)), indicating that challenge with rEq IL-1 significantly increased concentrations of CS846 and aggrecan synthesis when compared with unchallenged explants. Results from contrasts showed that this was true only for BoNT-A treatment concentrations of 1, 50, and 100 pg/mL (\( P = .05, P = .02, P = .03 \), respectively), but not for the BoNT-A concentration of 0, 10, and 500 pg/mL (\( P = .31, P = .07, P = .31 \), respectively). Neither the main effect of BoNT-A nor the interaction between IL-1 challenge and BoNT-A treatment were significant (\( P = .4526, P = .8542 \), respectively), indicating that treatment with BoNT-A did not significantly impact aggrecan synthesis in cartilage explants with or without IL-1 challenge (Figure 3).
Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay on cartilage explants

An in-situ TUNEL assay was performed to estimate chondrocyte apoptosis cartilage explants. There was no significant main effect of IL-1 challenge ($P = .8817$), BoNT-A treatment ($P = .2244$), or interaction between them ($P = .9305$), indicating that neither challenge with rEq IL-1, nor treatment with BoNT-A affected chondrocyte apoptosis in our model. The fraction of apoptotic cells ranged from 0% to 28.5% (Figure 4).

![Figure 4](image-url)

**Figure 4**—Percentage of apoptotic cells measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The percentage of apoptotic cells was counted over a 200-cell field. Error bars represent 95% confidence intervals.

Discussion

In our study, challenging equine articular cartilage explants with rEq IL-1 increased levels of PGE2, indicating an inflammatory response. It also increased the percentage of sGAG released into the media, indicating cartilage matrix degradation, and increased CS846 concentration, indicating attempts at extracellular matrix production in the face of matrix breakdown. We chose this model because it is well-established, has been used successfully in our laboratory, and it allows for the determination of disease-modifying effects of BoNT-A downstream of IL-1 secretion. We used a >95% pure research grade, active BoNT-A (molecular weight 150 kDa), rather than the pharmaceutical grade BoNT-A product used in prior in vivo equine studies, because the use of the pharmaceutical grade product in the proposed study would have been financially unfeasible. Treatment with BoNT-A did not appear to ameliorate any changes due to the rEq IL-1 challenge. Thus, we can reject our first hypothesis that BoNT-A would ameliorate the catabolic and inflammatory effects of rEq IL-1 on articular cartilage explants in a concentration-dependent manner. With respect to our second hypothesis, that BoNT-A would have no concentration-dependent effects on cartilage matrix homeostasis and chondrocyte viability in rEq IL-1 unchallenged, normal equine articular cartilage explants, we were unable to disprove this null hypothesis.

The concentration range of BoNT-A for the above experiment (0, 1.0, 10, 50, 100, or 500 pg/mL) was chosen to include the estimated clinically achieved synovial fluid concentration in the middle carpal joint in horses. Briefly, the commercially available pharmaceutical product Botox contains 7.3 pg of active 150 kDa BoNT-A per unit medication. The anecdotally and reportedly used intra-articular dose of Botox in horses is 50 U, which is equivalent to 365 pg of active BoNT-A per joint. The volume of synovial fluid of the middle carpal joint is estimated to be 15 mL for average-sized horses, resulting in a presumed clinically achieved synovial fluid concentration of 24 pg/mL of active BoNT-A, which is covered by the concentration range chosen for this study.

Given the lack of quantifiable levels of C2C in any of our media samples, it is impossible to ascertain whether or not BoNT-A alters collagen degradation. The most likely explanation for the readings below the lower end of the standard curve is that there were only very low concentrations of C2C in the culture media, independent of the rEq IL-1 challenge or BoNT-A treatment. This particular assay is optimized for synovial fluid, not for culture media that may have impacted the results. However, standard curves were run in both dilution buffer and culture media, and samples were run at dilutions of 1:1, 1:20, and 1:50, but the same results were obtained at all concentrations. Thus, it is unlikely that culture media was interfering with the results. It is also unlikely the assay was faulty given the standard curves read as expected. A significant increase in C2C was expected in rEq IL-1 challenged explants, with or without BoNT-A treatment, but this was not reflected in the results. There is a chance the explant size was too small to release detectable levels of C2C into the volume of culture media used.

Based on our results, BoNT-A may be safe for articular cartilage at the concentrations tested, as no detrimental effects on cartilage matrix homeostasis or chondrocyte viability were found. This, combined with results by Beck et al (2022), suggests that BoNT-A is likely to be safe for intraarticular use in horses.

Negative impacts of rEq IL-1 on cartilage matrix homeostasis in our experiment were not improved by BoNT-A treatment, making it unlikely to be disease-modifying at the concentrations tested. A previous study suggested anti-inflammatory effects in rats based on a decrease in IL-1β antibody and anti-ionized calcium-binding adaptor molecule 1 (Iba-1). Any possible disease-modifying effects of this therapeutic would have to occur upstream of IL-1, or by decreasing neurogenic inflammation. The authors found no additional evidence in the peer-reviewed literature suggesting disease-modifying effects of BoNT-A in the treatment of OA or other forms of arthritis. Thus, additional studies are warranted to elucidate if BoNT-A is in fact disease modifying in vivo, as well as to determine if a decrease in IL-1β antibody and Iba-1 is seen in equine patients, similar to what has been reported in rats.

While no disease-modifying or anti-inflammatory effects were noted at the concentrations tested in
this study, BoNT-A may still be a viable therapeutic for equids suffering from joint pain due to OA, as improvements in pain score and range of motion in humans with OA have been reported. In our opinion, BoNT-A is more likely to provide symptom-modifying effects than disease-modifying effects when used as a treatment for OA, but the in vitro model used did not allow us to investigate the symptom-modifying effects of BoNT-A. Thus, future investigations in models where symptom modification in equids with OA can be studied should be undertaken to determine BoNT-A’s mechanism of action in this disease. The major limitations of the current study are the in vitro design and the small number of horses used. The cartilage explant study used does not consider the effects of treatment on other components of the joint (synovium, subchondral bone, synovial fluid, etc) nor does it consider the effects of IL-1 on these components. As previously discussed, all joint structures play an important role in the incitement and progression of OA. Furthermore, our in vitro model did not allow us to evaluate any co-factors that may affect the effects of BoNT-A on OA symptoms. For example, the effects of pharmaceutical grade BoNT-A on variables measured in this study are unlikely to be different.

In conclusion, BoNT-A does not appear to ameliorate the effects of Eq IL-1 on articular cartilage, nor does it appear to have detrimental effects on articular cartilage in vitro at concentrations of up to 500 pg/mL. It is unknown if BoNT-A provides disease-modifying effects for joints with OA upstream of IL-1 and future studies in live equids are warranted before recommending BoNT-A in the management of this disease.

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