UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Odontologia de Piracicaba

VICTOR RICARDO MANUEL MUÑOZ LORA

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BONT-A REDUCES INFLAMMATORY HYPERNOCICEPTION INDUCED BY ARTHRITIS IN THE TEMPOROMANDIBULAR JOINT OF RATS

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Clínica Odontológica, na Área de Prótese Dental.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Dental Clinic, in Dental Prosthesis Area.

Orientadora: Profa. Dra. Celia Marisa Rizzatti Barbosa

Este exemplar corresponde à versão final da dissertação defendida pelo aluno Victor Ricardo Manuel Muñoz Lora e orientado pela Profa. Dra. Celia Marisa Rizzatti Barbosa

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RESUMO

A Toxina botulínica A (TxB-A) tem sido utilizada satisfatoriamente no tratamento de diferentes desordens motoras devido a sua capacidade de inibir a liberação de acetilcolina na junção neuromuscular. Recentemente, foram sugeridos efeitos da TxB-A nos terminais nervosos sensitivos, inibindo neurotransmissores como a substancia P (SP), glutamato (Glu) e o gene relacionado ao peptídeo da calcitonina (GRPC). Contudo, o presente estudo investigou os efeitos da TxB-A sobre a hipernocicepção inflamatória induzida pela artrite na ATM de ratos. Para atingir o objetivo, ratos Wistar foram induzidos a hipernocicepção inflamatória na ATM esquerda. Depois, os animais foram tratados com TxB-A utilizando doses de 3.5, 7 e 14U/kg, administradas intra-articularmente na ATM comprometida. Solução salina foi utilizada como grupo controle. Foram aplicados testes comportamentais para avaliar o efeito da TxB-A na hipernocicepção inflamatória. Posteriormente, os animais foram mortos e coletadas amostras de tecido peri-articular e gânglio trigeminal para serem submetidas a testes de ELISA e Western Blot. A TxB-A reduziu a hipernocicepção inflamatória persistente induzida pela artrite na ATM e inibiu a liberação periférica dos neurotransmissores substância P e o gene relacionado ao peptídeo da calcitonina; assim como da citosina pro-inflamatória IL1-β. Por outra parte, a TxB-A não teve efeito na liberação periférica do glutamato e da citosina TNF-α.

Keywords: Toxinas botulínicas tipo A, nociceptividade, neurotransmissores.

ABSTRACT

BoNT-A has been successfully used in the treatment of several motor disorders because of its capacity for inhibiting acetylcholine release in the neuromuscular junction. In the last decades, it has been suggested BoNT-A effects on sensory nerve endings, inhibiting other neurotransmitters such as substance P, glutamate and CGRP. This study aimed to investigate the effects of BoNT-A on inflammatory hypernociception induced by arthritis in TMJ of rats. To achieve this goal, Wistar rats were induced to inflammatory hypernociception in the left TMJ. Then, animals were treated with BoNT-A, using doses of 3.5, 7 and 14U/kg, administered intra-articular in the compromised TMJ. Saline was used as control group. Behavioral tests were applied to evaluate the effect of BoNT-A in inflammatory hypernociception. Then, animals were euthanized and samples from peri-articular tissues and trigeminal ganglia were obtained to apply ELISA and Western Blot tests. BoNT-A reduced the persistent inflammatory hypernociception induced by arthritis in rats TMJ, and it also inhibited the peripheral release of neurotransmitters SP and CGRP; and pro-inflammatory cytokine IL1-β. On the other side, BoNT-A has no effect on Glu and TNF-α peripheral release.

Keywords: Botulinum toxins type A, nociception, neurotransmitters.

SUMÁRIO

INTRODUÇÃO

As Toxinas botulínicas são um grupo de neurotoxinas produzidas pela bactéria gram positiva *Clostridium botulinum*. Atualmente, são conhecidos sete sorotipos da neurotoxina, sendo os sorotipos A e B os mais estudados nas últimas décadas. (Dressler *et.al*, 2005)

A TxB-A apresenta um peso molecular de 150k Da, e está composta por uma cadeia leve (50k Da) considerada a porção catalítica/proteolítica da neurotoxina, e uma cadeia pesada (100 kDa) encarregada da sua ligação e translocação à célula alvo (Poli *et.al*,2002; Turton *et. al*, 2002). Ambas as cadeias, estão unidas a proteínas auxiliares encarregadas da estabilização e preservação da neurotoxina, formando um complexo macromolecular de aproximadamente 900 kDa denominado de complexo proteico (Aoki *et.al,* 2004).

O mecanismo de ação da TxB-A tem sido amplamente descrito na literatura. Basicamente, consiste na inibição da liberação de acetilcolina nos terminais nervosos periféricos da junção neuromuscular (Dolly *et.al,* 2006; Wheeler *et.al,* 2013). Esse mecanismo de ação da neurotoxina é representado por três fases: 1) fase de ligação e internalização: mediada pela cadeia pesada da neurotoxina, a qual se une à membrana externa da célula alvo para depois ser transportada mediante endocitose no citosol celular; 2) fase de translocação: na qual é liberada a cadeia leve no citosol celular para exercer sua ação catalítica; e 3) fase de inibição da liberação de neurotransmissores: na qual a cadeia leve da TxB-A se une às proteínas do complexo SNARE, inibindo a liberação da acetilcolina na fenda sináptica da junção neuromuscular (Dolly *et.al,* 2006; Aoki *et.al,* 2004; Pellizzari *et.al,* 1999; Rossetto *et.al,* 2004; Wheeler *et.al,* 2013). A inibição na liberação de

acetilcolina impede a contração muscular do local onde foi aplicada a neurotoxina, gerando uma debilidade muscular prolongada. Este efeito torna útil seu uso em diversas condições onde se apresenta uma excessiva ou inapropriada contração muscular (Dolly *et.al,* 2006).

Estudos clínicos prospectivos têm demostrado a eficácia no uso da TxB-A para o alívio da dor em distúrbios associados com hiperatividade dos músculos esqueléticos (Dressler *et al., 2005)* surgindo como uma nova ferramenta para o tratamento da dor presente nas desordens de origem neuromuscular (Song *et.al,* 2007; Guarda-Nardini *et al,* 2012).

Clinicamente, quando a TxB-A é usada para tratar uma hiperatividade muscular dolorosa, frequentemente um considerável alívio da dor, atribuído à redução da hiperatividade muscular, é reportado (Colhado *et.al,* 2009, Dressler *et.al*, 2005). Entretanto, observa-se que essa redução da dor acontece antes da redução das contrações musculares, sugerindo um mecanismo de ação mais complexo do que o hipoteticamente indicado (Dressler *et.al*, 2005, Aoki *et al,* 2011; Cui *et. al,* 2004; De Almeida *et al,* 2014). Dados recentes apontam a um efeito anti-nociceptivo da TxB-A, independente do neuromuscular, e aparentemente com um mecanismo bioquímico de ação semelhante ao seu efeito no nervo motor, porém, inibindo a liberação de neurotransmissores diferentes à acetilcolina (Aoki *et al,* 2003).

Modelos de indução à dor e/ou inflamação que associando terapias farmacológicas como variáveis independentes experimentais foram desenvolvidos e validados com sucesso. Por meio destes modelos podem-se estudar os mecanismos envolvidos nas condições de dor craniofacial superficial e profunda nas alterações do sistema nervoso central e periférico, e no seu envolvimento com diversos mediadores inflamatórios (Bonjardin *et.al,* 2009; Gameiro *et.al,* 2005, Roveroni *et.al,* 2001;

Fiorentino *et. al,* 1999). Desta maneira, estudos *in-vitro* são comumente utilizados *para* avaliar a eficácia da TxB-A sobre alvos celulares específicos, porém, as amostras empregadas correspondem a tecidos animais, que a pesar de, na maioria dos casos, apresentarem respostas parecidas com os tecidos humanos, não pode-se assumir sempre um comportamento similar, devido a diversos fatores não considerados neste de tipo de estudos (Durham *et al,* 2004). É por este motivo, que os estudos *in-vitro* devem ser complementados com estudos em animais (*in-vivo*), com o propósito de avaliar de forma mais abrangente os efeitos da neurotoxina.

Assim, estudos realizados em gânglios da raiz dorsal de ratos e na musculatura do íris de coelhos (Welch *et al,* 2000; Ishikawa *et al,* 2000), demonstraram que a liberação da sustância P (SP), um neuropeptídio envolvido na percepção da dor, vasodilatação e inflamação neurogênica parece ser bloqueada pela TxB-A. Ademais, a liberação do glutamato (Glu) e do peptídeo relacionado com o gene da calcitonina (PRGC) também poderiam ser suprimidos pela TxB-A, segundo estudos desenvolvidos em gânglios trigeminais de ratos (Durham *et al,* 2004; Welch *et al,* 2000; Ishikawa *et al,* 2000; Meng *et al,* 2007) e modelos de indução à dor por formalina (Cui *et al,* 2004). Isto sugere a presença de mecanismos adicionais da toxina sobre a transmissão da dor.

Baseando-se nas informações acima descritas, este estudo avaliou o efeito anticociceptivo da TxB-A na hipernocicepção inflamatória persistente induzida por artrite na Articulação Temporomandibular (ATM) de ratos.

CAPÍTULO I

Highlights

- Botulinum toxin type A (BoNT-A) reduces inflammatory hypernociception in the TMJ
- Intra-articular injection of BoNT-A reduces peripheral release of SP, CGRP and IL-1β in TMJ tissues.
- Intra-articular injection of BoNT-A have a potencial antinociceptive effect mediated by the reduction of neurons function.

Botulinum toxin type A reduces inflammatory hypernociception induced by arthritis in the temporomadibular joint of rats

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ABSTRACT

Objective: This study aimed to investigate the antinociceptive effects of Botulinum toxin type A (BoNT-A) on persistent inflammatory hypernociception induced by arthritis in the TMJ of rats.

Material and methods: Wistar rats were induced to persistent inflammatory hypernociception in the left TMJ. Then, animals were treated with intra-TMJ injections of BoNT-A, using doses of 3.5, 7 and 14 U□kg. Saline was used as control group. Behavioral tests were applied to evaluated the effect of BoNT-A in the inflammatory hypernociception. After that, animals were euthanized and samples from peri-articular tissues and trigeminal ganglia were obtained for further analyses.

Results: BoNT-A reduced the persistent inflammatory hypernociception induced by arthritis in the TMJ of rats. BoNT-A significantly reduced the peripheral release of the neurotransmitters Substance P and Calcitonin gene related peptide; and the pro-inflammatory cytokine IL-1β. Otherwise, BoNT-A had no effect in the peripheral release of glutamate and the cytokine TNF-α.

Conclusion: These results demonstrate that intra-articular injection of BoNT-A reduces the albumin-induced arthritis persistent hypernociception in TMJ of rats by peripheral inhibition of neuropeptides release.

Keywords: Botulinum toxins type A, nociception, neurotransmitters.

INTRODUCTION

Botulinum toxin type A (BoNT-A) has been commonly used to treat muscle disorders. However, clinical evidences strongly support the use of BoNT-A as a new therapeutic perspective to treat sensory pain nerve dysfunction (Poli and Lebeda, 2002; Aoki, 2011; Pellizzari et al., 1999).

BoNT-A mechanism of action is based on the inhibition of acetylcholine release in cholinergic nerve terminals, producing a temporary chemical denervation at the neuromuscular junction (Aoki, 2011; Dolly and Aoki, 2006; Wheeler and Smith, 2006). Due to this, the use of BoNT-A reported very positive outcomes when controlling different motor disorders like bruxism and painful muscle hyperactivity, decreasing muscle contraction and easing pain (Tinastepe, Kuçuk and Oral, 2014; Song, Schwartz and Blitzer, 2007; Dressler, Saberi and Barbosa, 2005). Besides that, clinical studies have shown that pain alleviation often occurs before the reduction of muscle contractions and it also may last for a longer period of time, leaving muscle relaxation as a simple secondary effect and suggesting a more complex mechanism of action than the one mentioned above (Dolly and Aoki, 2006; Aoki, 2005; Cui and Aoki, 2000; Freund and Schwartz, 2003).

The treatment of different headache conditions using BoNT-A, supports an independent analgesic effect of the neurotoxin (Dressler, Saberi and Barbosa, 2005; Aoki, 2005; Silberstein, 2000), especially when it is used to treat migraine. Considering that, it has been suggested that BoNT-A could inhibit the activation of primary nociceptive afferents. It's possible to suggest that BoNT-A was able to inhibit the release of neurotransmitters such as substance P (SP), glutamate (Glu) and calcitonin gene related peptide (CGRP); that in turn reduce

the peripheral sensitization and prevent a central sensitization (Dressler, Saberi and Barbosa, 2005; Aoki, 2003; Cui et al.,2004; Favre- Guilmard, Auguet and Chabrier, 2009; Cui and Aoki, 2004).

Inflammation of orofacial tissues is the most common cause of many orofacial pain conditions including Temporomandibular Disorders (TMDs), and the most common cause of chronic orofacial pain (Cairns, 2010; Sessle, 2011). Thus, the aim of this study was to investigate the peripheral effect of an intra-articular injection of BoNT-A in persistent inflammatory hypernociception in the temporomandibular joint (TMJ) of rats.

2. MATERIAL AND METHODS:

2.1. Animals

The present study was approved by the Ethics Committee in Animals Research of the State University of Campinas (CEUA UNICAMP #3452-1), following the National Council for Control of Animal Experimentation (CONCEA) and the International Association for the Study of Pain (IASP) guidelines (Zimmermann, 1983).

Male Wistar rats (250-500g) obtained from the University of Campinas Multidisciplinary Center for Biological Research (CEMIB-UNICAMP, São Paulo, Brazil) were housed in standard clear plastic cages with a maximum of four animals per cage and maintained in a temperature-controlled room (23 ± 1°C) with a 12h dark-light cycle (lights on at 06:00 a.m.) with free access to food and water.

2.2. Albumin-induced arthritis in the TMJ of rats

Animals were sensitized with subcutaneous injections of 500 μg of methylated bovine serum albumin (mBSA) (Sigma-Aldrich, St. Louis, MO, USA) diluted in an emulsion containing 100 μl of phosphate-buffered saline (PBS) and 100 μl of Freund's complete adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) administered in the back (Quintero et al., 2014). Booster injections of mBSA with Freund's incomplete adjuvant (IFA) were applied in different sites of the back 7 and 14 days after the first immunization. TMJ-arthritis was induced in the immunized animals by an intra-articular injections of 10 μg of mBSA dissolved in 15 μl of PBS (challenge) into the TMJ 7, 14 and 21 days after the last immunization (21st, 28th and 35th day of experiment). Non- immunized rats (control group) were treated by intra-articular injections of mBSA. Arthritis-induced TMJ inflammatory hypernociception was assessed by measuring behavioral nociceptive responses induced by intra-articular injection of a low dose of formalin (0.5%) into the TMJ 7 days after the last challenge in immunized rats (Quintero et al., 2014) (Figure 1).

2.3. Effect of the BoNT-A in the arthritis-induced persistent hypernociception in the TMJ of rats

To test the effect of BoNT-A on arthritis-induced TMJ persistent hypernociception, the animals were treated with an intra-TMJ of BoNT-A (Botox®, Allergam, Inc. Irvine, CA; a hundred units were reconstituted in 2 ml of 0,9% saline solution) 7 days after the last challenge in the TMJ (mBSA 10 mg/TMJ) (Cui et al., 2004). After 24 hours or 14 days of BoNT treatment, an intra-articular injection of formalin (0,5%) was administered. Immediately after the formalin injection, the behavioral nociceptive response was evaluated for 45

min observation period. For that each animal was placed individually into a mirrored-wood chamber (30x30x30cm) with glass at the front side for a habituation period of 30 minutes in order to minimize stress. The nociceptive behavior, as previously described in other studies (Roveroni et al., 2001; Clemente et al., 2004), was evaluated during 45 minutes divided into 9 blocks of 5 minutes. During this time, the behavior characterized by rubbing the orofacial region was quantified by the amount of time animals exhibited and the behavior characterized by flinching the head was quantified by its occurrence. The nociceptive response score was the sum of seconds each animal spent rubbing the orofacial region plus the number of times the animal flinched the head, considering each head flinch as 1 second since head flinches followed a uniform pattern for 1s. The evaluations were made by a researcher blind to the group assignment (Roveroni et al., 2001).

After behavioral evaluation, animals were terminally anesthetized and periarticular tissue of the left TMJ region and trigeminal ganglia were removed and stored at -80°C for analyses.

2.4. Protein extraction from TMJ tissues

The periarticular tissue from TMJ and the trigeminal ganglia were homogenized separately in 500 µl of the appropriate buffer containing protease inhibitors (Sigma- Aldrich) followed by a centrifugation of 10 min/10,000g. The total amount of extracted proteins was measured by BCA protein kit (Thermo, Rockford, IL, USA). The supernatants were used for ELISA and Western Blot analyses.

2.5. Effect of the BoNT-A in the release of neuropeptides SP and CGRP; Glu and the inflammatory cytokines IL1-β and TNF-α in the TMJ tissues.

The release of Glu, SP and CGRP was measured in the supernatant obtained from the trigeminal ganglia; and the release of IL1-β and TNF-α were measured in the supernatant obtained from TMJ periarticular tissue by Enzyme-linked immunossorbent assay (Elisa) using protocols supplied by the manufacturers. Briefly, a 96-well plate for Elisa was incubated overnight at 4°C with 100μl of capture antibody. Then the plate was washed and blocked for 1 hour at room temperature with 300 ml of reagent diluent. Fifty microliters standard and samples were added on the plate and incubated for 2h at room temperature. Then, the plate was washed and incubated for 2h with 100 μl of detection antibody. After this, the plate was washed again and incubated for 20 min. with Streptavidin-HRP solution. After a new wash, the TMB one-step substrate reagent was added to each well. The reaction was stopped with the Stop Solution and read immediately in a spectrophotometer (Epoch, Biotek, Winoosky, VT, USA) at 490 nm. The results were expressed as pictograms per milligram of tissue.

2.6. Effect of BoNT-A in the expression of AMPA and NMDA receptors

The expression of AMPA and NMDA receptors were measured in the supernatant obtained from trigeminal ganglia by western blotting analysis. For that, equals amounts of protein (80 μg) from the trigeminal ganglia homogenized were separated by electrophoresis into 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). A

molecular mass standard (Bio-Rad, Hercules, CA, USA) was run in parallel to estimate molecular mass. Membranes were blocked overnight at 4°C in TBST (20 nM Tris-HCl [pH 7.5], 500 nM NaCl, and 0,1% Tween 20) containing 5% of nonfat dried milk. After blocking, the membranes were incubated with specific antibodies: Anti-Glutamate Receptor1 (AMPA Subtype) Antibody (Abcam, Cambridge, MA, USA), and anti-NMDAR1 Antibody-Neuronal Marker (Abcam, Cambridge, MA, USA) diluted in TBST containing 5% of nonfat dried milk at room temperature for 120 min. Then the membranes were rinsed again and incubated for 1h with anti-rabbit IgG peroxidase-conjugated (Vector Laboratories, Burlingame, CA, USA). Membranes were visualized using ECL solution (Pierce) and exposed to X-ray film (Kodak) in a dark room. To confirm uniform protein loading, membranes were stripped and blocked overnight at 4°C, and then incubated for 160min. with mouse monoclonal to GAPDH (Novus Biologicals, Littleton CO, USA) followed by incubation for 1h with anti-mouse IgG peroxidase-conjugated (Vector Laboratories, Burlingame, CA, USA). Banding specificity was determined by omission of primary antibody from the western-blotting protocol. Finally, the bands recognized by the specific antibodies were visualized using chemiluminescence-based ECL system (Amersham Biosciences, Piscataway, NJ, USA) and exposed to an X-ray film (Eastman Kodak, Rochester, NY, USA) for 30 s. A computer-based imaging system (Imagej; National Institutes of Health, Bethesda, MD, USA) was used to measure the optical density of the bands.

3. RESULTS

3.1. BoNT-A reduced the inflammatory hypernociception induced by arthritis in TMJ of rats.

The intra-articular injection of BoNT-A (3.5, 7 and 14U/Kg) significantly reduced the persistent hypernociception induced by arthritis and this effect was observed 7 days after BoNT-A treatment ($P<0.05$; ANOVA: Tukey's test) (Figure 2). These findings indicate that BoNT-A was able to reduce the inflammatory episode evoked by albumin- induced arthritis into TMJ that results in the progression of the hypernociception. Nociceptive behavioral response was observed in all animals that receive an intra- articular injection of low concentration of formalin (0.5%); however, formalin-induced nociception of immunized animals challenged with mBSA was significantly higher than that non-immunized group. There no difference among groups treated with BoNT-A ($P>0.05$; ANOVA: Tukey's test), thus we established the dose of the BoNT-A at 7 U/Kg for the next experiments.

3.2. BoNT-A inhibited the release of neuropeptides SP and CGRP of albumin- induced arthritis in TMJ of rats.

The intra-articular injection of BoNT-A 7 U/Kg inhibited the release of the neuropeptides SP and CGRP of albumin-induced arthritis in the TMJ of rats ($P<0.05$; ANOVA: Tukey's test) (Figure 3A and B).

3.3. BoNT-A had no effect in the release of glutamate of albumin-induced arthritis in TMJ.

The intra-articular injection of BoNT-A 7 U/Kg had no effect in the release of glutamate of albumin-induced arthritis in the TMJ of rats ($P>0.05$; ANOVA:

Tukey's test) (Figures 4A). Western blotting analyses demonstrate no difference among groups in the expression of AMPA and NMDA receptors (Figure 4B and C).

3.4. BoNT-A reduced the release of IL-1β of albumin-induced arthritis in TMJ of rats

The intra-articular injection of BoNT-A 7 U/Kg significantly reduced the release of IL- 1β but not TNF-α of albumin-induced arthritis in TMJ of rats ($P<0.05$; ANOVA: Tukey's test) (Figure 5A and B).

4. DISCUSSION

The present study demonstrated that intra-articular injection of BoNT-A reduced the persistent hypernociception of albumin-induced arthritis in TMJ of rats. The results suggested that the antinociceptive effect of BoNT-A involves the reduction of sensory neuron activation.

Previous studies investigated the antinociceptive action of BoNT-A. *In-vitro* models, using cultures of embryonic rat dorsal root ganglion, demonstrated BoNT-A inhibition of SP release (Welch, Purkiss and Foster, 2000) and reduction of stimulated CGRP (Durham and Cady, 2004; Meng et al., 2007). Despite these studies provided important contributions to the better understanding of the antinociceptive mechanism of BoNT-A, it was necessary more experiments to elucidate this effect. Considering that, the aim of the present study was to evaluate the effect of BoNT-A on persistent induced pain through the study of some specific inflammatory mediators. For that, we chose the pain induction model designed by Quintero et al., adding some modifications, to induce a persistent hypernociception in the TMJ of rats, and by

this way, study the antinociceptive mechanism of BoNT-A. The model success was confirmed by a behavioral test and biochemical analyses. Animals with albumin-induced arthritis presented a significantly higher nociceptive behavioral response associated with a higher release of neuropeptides Glu, SP, and CGRP, involved in inflammatory conditions (Carozzi, Marmiroli and Cavaletti, 2008; O´Connor et al., 2004; Yu et al., 2009). In addition, it was found that albumin-induced arthritis increased the release of the pro-inflammatory cytokines IL1-β and TNF-α.

BoNT-A has been subject of innumerous studies to confirm its antinociceptive effect. In the present study, intra-articular injection of BoNT-A significantly reduced the persistent inflammatory hypernociception induced by arthritis in the TMJ of rats. This reduction of inflammatory hypernociception may be due to an inhibition in the release of certain pain related neurotransmitters and pro-inflammatory cytokines (Cui et al., 2004; Welch, Purkiss and Foster, 2000; Durham and Cady, 2004; Meng et al., 2007; Quinteiro et al., 2014). In addition, we could see that BoNT-A already had an antinociceptive effect 24 hours after its application, in accordance with the hypothesis that this possible antinociceptive effect begins faster than the neuromuscular one (Tsui et al., 1986; Brin, Fahn and Moskowitz, 1987; Aoki, 2005; Dolly and Aoki, 2006).

The substance P is a neuropeptide synthesized in neurons of the dorsal root and transported both centrally and peripherally, taking an important part in pain neurotransmission (Welch, Purkiss and Foster, 2000). According to that, it has been shown that BoNT-A inhibited the secretion of SP in cultures of rat dorsal root ganglia (Welch, Pukiss and Foster, 2000) and trigeminal nerve terminals of the iris sphincter muscle of rabbits (Ishigawa et al., 2000).

Corroborated with the literature, our results demonstrated that intra-articular injection BoNT-A significantly reduced the release of substance P in the trigeminal ganglia. This reduction can be observed 24 hours after the BoNT-A treatment in the TMJ of rats and this effect was maintenance during 14 days.

Durham et al. and Meng et al., demonstrated that BoNT-A can directly decrease the amount of CGRP released from trigeminal sensory neurons in cultures of rat trigeminal ganglia. CGRP is a multifunctional regulatory neuropeptide (Van Rossum, Hanisch and Quirion, 1990) strongly related in the underlying pathology of migraine. The present study showed that BoNT-A decreased the quantity of peripherally released CGRP, in agreement with the studies mentioned above. BoNT-A inhibition of CGRP suggests an analgesic effect of the neurotoxin, and it also help us to understand the mechanism in which the toxin may prevent the initiation and spread of migraine due to the relationship between the amounts of CGRP and this disease.

Glutamate (Glu) is considered one of the principal excitatory neurotransmitters involved in central and peripheral nervous system (Danbolt, 2001). Despite being demonstrated its active participation in peripheral sensory transduction (Carozzi, Marmiroli and Cavaletti, 2008), it is known that Glu levels are higher at CNS. Cui et al., showed that BoNT-A inhibited the release of Glu in a formalin-induced pain model, on the other hand, our results revealed no significant differences in Glu quantity between the BoNT- A group and the induced group. This result does not mean that BoNT-A has no effect on sensory neurons or Glu, and we could also see that the non-iduced group presented no significant differences against BoNT-A group. Complementing these results, western blot analysis showed no alterations in the expression of Glu receptors NMDA

and AMPA. These results have to be interpreted carefully, and one possible explanation could be due to the existence of lower levels of glutamate at the periphery, causing a reduction in Glu levels, however, with no significant differences as observed in Figure 4. In addition, current scientific evidence suggests that BoNT-A clinical effects cannot be explained just by a peripheral mechanism of action (Aoki, 2011). This means that peripheral applications of the neurotoxin could directly or indirectly reach the CNS (Aoki, 2003). Based on this hypothesis, BoNT-A mechanism of action could not be restricted to a peripheral mechanism but also to a central action on these three neurotransmitters (SP, CGRP and Glu), where the inhibition of Glu release would take a more important role that the on earned peripherally.

As expected, there was an increase in the release of pro-inflammatory cytokines TNF-α and IL1-β, central mediators in rheumatoid arthritis (Brennan and McInnes, 2008; Di Paola and Cuzzocrea, 2008; Sachs et al., 2011). To corroborate IL1-β hypernociceptive effect in peripheral tissue, it was demonstrated that intracerebroventricular injection of IL1-β enhances the nociceptive responses of neurons in the trigeminal nucleus caudalis of rats (Hori et al., 1998). Thus, data suggests that IL1-β has an important peripheral role as an intermediate hypernociceptive mediator, placing it as a target to control inflammatory pain (Verri et al., 2006). TNF-α is recognized as a potent, pro-inflammatory cytokine, and it was confirmed as a thermal and mechanical hypernociception mediator via IL1-β (Woolf et al., 1997). The present study showed no reduction of TNF-α quantity, suggesting no anti-inflammatory effects of BoNT-A; thus, reduction of IL1-β quantity after BoNT-A application may be due to the inhibition of neurotransmitters SP and CGRP, which in turn reduced

the inflammatory chemotaxis and consequently reduced the release of this cytokine.

4. CONCLUSSION

In conclusion, intra-articular injections of BoNT-A reduced the albumin-induced arthritis persistent hypernociception in the TMJ of rats by peripheral inhibition of SP and CGRP release. These results strongly suggest the hypothesis that BoNT-A have a potential antinociceptive effect mediated by the decreased of neurons function.

5. ACKNOWLEDGEMENTS

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REFERENCES

1. Aoki KR, & Francis J. (2011). Updates on the antinociceptive mechanism hypothesis of botulinum toxin A. Parkinsonism Relat Disord., 17(1):28-33.
2. Aoki KR. (2003). Evidence for antinociceptive activity of botulinum toxin type A in pain management. Headache, 43(1):9-15
3. Aoki, KR. (2005). Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A. Neurotoxicology, 26(5):785–793.
4. Brennan FM, & McInnes IB. (2008). Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest, 118(11): 3537–45.
5. Brin MF, Fahn S, Moskowitz C, et al. (1988). Localized injections of botulinum toxin for the treatment of focal dystonia and hemifacial spasm. Mov Disord, 50: 599-608
6. Cairns BE. (2010). Pathophysiology of TMD pain--basic mechanisms and their implications for pharmacotherapy. J Oral Rehabil, 37(6): 391-410.
7. Carozzi V, Marmiroli P, & Cavaletti G. (2008). Focus on the role of Glutamate in the pathology of the peripheral nervous system. CNS Neurol Disord Drug Targets, 7(4): 348-60.
8. Clemente JT, Parada CA, Veiga MC, Gear RW, & Tambeli CH. (2004). Sexual dimorphism in the antinociception mediated by kappa opioid receptors in the rat temporomandibular joint. Neurosci Lett, 372(3):250-5
9. Cui M, & Aoki KR. (2000). Botulinum toxin type A (BTX-A) reduces inflammatory pain in the rat formalin model. Cephalalgia, 20:414.
10. Cui M, & Aoki KR. (2004). Mechanisms of antinociceptive effect of subcutaneous Botox: inhibition of peripheral and central nociceptive processing. In: Olesen J,

Silberstein SD, Tfelt-Hansen P. Preventive pharmacotherapy of headache disorders. (pp.158-62). Oxford: Oxford University Press.

11. Cui, M., Khanijou, S., Rubino, J., & Aoki, K.R. (2004). Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. Pain, 107(1-2), 125–133.
12. Danbolt NC. (2001). Glutamate uptake Review. Prog Neurobiol, 65(1): 1-105..
13. Di Paola R, & Cuzzocrea S. (2008).Predictivity and sensitivity of animal models of arthritis. Autoimmun Rev; 8(1): 73–5.
14. Dolly JO, & Aoki KR. (2006). The structure and mode of action of different botulinum toxins. European Journal of Neurology, 13(4): 1–9.
15. Dressler D, Saberi FA, & Barbosa ER. (2005). Botulinum toxin: mechanisms of action. Arq Neuro-Psiquiatr, 63(1): 180-5.
16. Durham PL, Cady R, & Cady R. (2004). Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: implications for migraine therapy. Headache, 44(1): 35-42.
17. Favre-Guilmard C, Auguet M, & Chabrier PE. (2009). Different antinociceptive effects of botulinum toxin type A in inflammatory and peripherial polyneurophatic rat models. , 617(1-3): 48-53
18. Freund, B., & Schwartz, M. (2003). Temporal relationship of muscle weakness and pain reduction in subjects treated with botulinum toxin A. J. Pain, 4(3): 159–65.
19. Hori, T., Oka, T., Hosoi, M., & Aou, S. (1998). Pain modulatory actions of cytokines and prostaglandin E2 in the brain. Ann N Y Acad Sci, 840, 269–81.
20. Ishikawa, H., Mitsui, Y., Yoshitomi, T., Mashimo, K., Aoki, S., Mukuno, K., & Shimizu, K. (2000). Presynaptic effects of botulinum toxin type A on the

neuronally evoked responses of albino and pigmented iris sphincter and dilator muscles. Jpn. J. Ophthalmol.; 44: 106–09.

21. Matak, I., Stracenski, I., & Lackovic, Z. (2013). Comparison of analgesic effects of single versus repeated injection of botulinum toxin in orofacial formalin test in rats. J. Neural Transm, 120: 141–44.

22. Meng J, Wang J, Lawrence G, & Dolly JO. (2007). Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential. J Cell Sci, 120(16): 2864-74.

23. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, & Shanahan F. (2004). The role of substance P in inflammatory disease. J Cell Physiol, 201(2): 167-80.

24. Pellizzari R, Rossetto O, Schiavo G, & Montecucco C. (1999). Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. , 354(1381):259- 68

25. Poli MA, & Lebeda FJ. (2002). An overview of clostridial neurotoxins. In Massaro EJ. Handbook of neurotoxicology. (pp.293-304). Totowa: Human Press

26. Roveroni RC, ParadaCA, Cecília M, Veiga FA, & Tambeli CH. (2001). Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain, 94(2): 185 – 91.

27. Sachs D, Coelho FM, Costa VV, Lopes F, Pinho V, & Amaral FA et al. (2011).

Cooperative role of tumour necrosis factor-a, interleukin-1b and neutrophils in a novel behavioural model that concomitantly demonstrates articular inflammation and hypernociception in mice. Br J Pharmacol, 162(1): 72–83.

28. Sessle BJ. (2011). Peripheral and central mechanisms of orofacial inflammatory pain. Int Rev Neurobiol, 97: 179-206.

29. Silberstein, S., Mathew, N., Saper, J, & Jenkins, S. (2000) Botulinum toxin type A as a migraine preventive treatment. Headache, 40: 445–450.

30. Silva Quinteiro M, Henrique Napimoga M, Gomes Macedo C, Furtado Freitas F, Balassini Abdalla H, Bonfante R, & Trindade Clemente-Napimoga J. (2014). 15- deoxy-Δ12,14-prostaglandin J2 reduces albumin-induced arthritis in temporomandibular joint of rats.. Eur J Pharmacol, 740:58-65.

31. Song PC, Schwartz J, & Blitzer A. (2007). The emerging role of botulinum toxin in the treatment of temporomandibular disorders. Oral Diseases, 13(1): 253-60.

32. Tinastepe N, Küçük BB, & Oral K. (2015). Botulinum toxin for the treatment of bruxism. Cranio, 33(4): 291-8

33. Tsui JK, Eisen A, Stoessl AJ, Calne S, & Calne DB. (1986). Double-blind study of botulinum toxin in spasmodic torticollis. Lancet, 2(8501): 245–7.

34. Van Rossum D, Hanisch UK, & Quirion R. (1990). Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev, 21: 649-678.

35. Verri WA Jr, Cunha TM, Parada CA, Poole S, Cunha FQ, & Ferreira SH. (2006).

Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development. Pharmacol Ther, 112(1): 116-38.

36. Welch MJ, Purkiss JR, & Foster KA. (2000). Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. Toxicon, 38(2): 245-58.

37. Wheeler A, & Smithb H. (2013). Botulinum toxins: Mechanisms of action,

antinociception and clinical applications. Toxicology, 5(306):124-46.

38. Woolf, C. J., Allchorne, A., Safieh-Garabedian, B. & Poole, S. (1997). Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br J Pharmacol, 121(3), 417–424.

39. Yu LC, Hou JF, Fu FH, & Zhang YX. (2009). Roles of calcitonin gene-related peptide and its receptors in pain-related behavioral responses in the central nervous system. Neurosci Biobehav Rev, 33(8): 1185-91.

40. Zimmermann M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. Pain, 16: 109–110.

LEGENDS

Figure 1: Experimental design of the effect of BoNT-A in the hypernociception of albumin-induced arthritis in TMJ.

Figure 2: BoNT-A reduce the persistent hypernociception of albumin-induced arthritis in TMJ of rats. Intra-articular injections of BoNT-A (3.5, 7 and 14 U/kg) significantly reduced nociceptive behavioral responses induced by arthritis in the TMJ. The symbol (+) indicates values significantly higher than that non-immunized group. (P<0,05: ANOVA, Tukey´s test). The symbol (∗) indicates values significantly lower than that of the immunized + group (P<0,05: ANOVA, Tukey´s test).

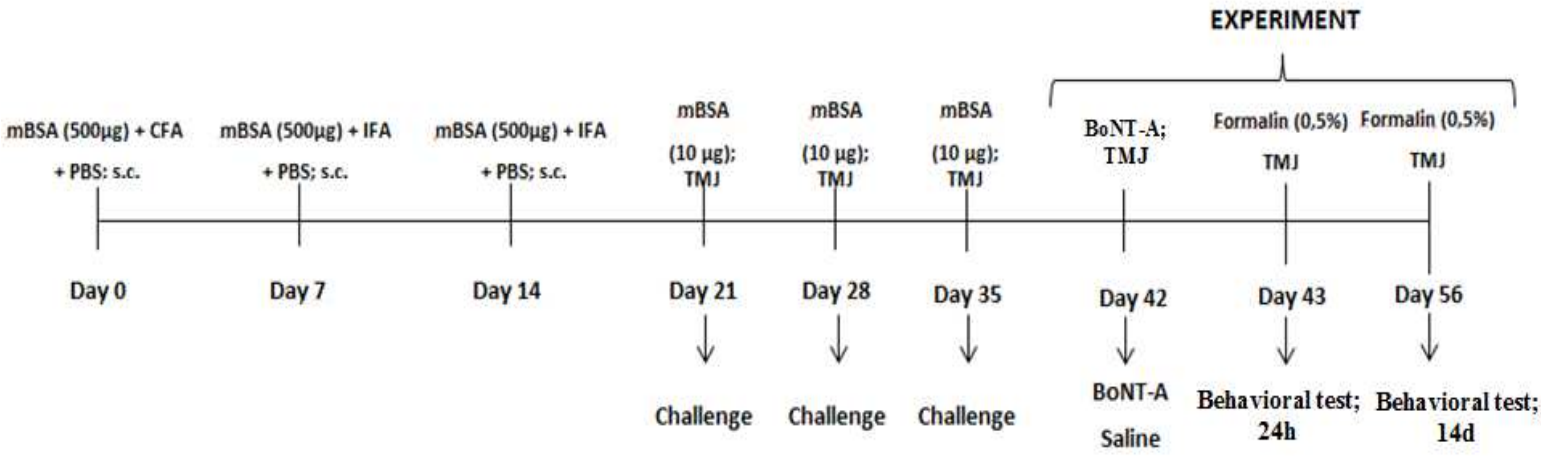
Figure 3: Intra-articular injection of Botulinum toxin A (BoNT-A) in the TMJ of rats inhibit the release of neuropeptides Substance P (SP) and Calcitonin Gene Related Peptide (CGRP) induced by arthritis. (A) BoNT-A (7 U/kg) intra-articular injection significantly reduced the release of SP induced by arthritis in the TMJ. The symbol (+) indicates values significantly higher than the non-immunized group. (P<0,05: ANOVA, Tukey test). The symbol (∗) indicates values significantly lower than that of immunized + group (P<0,05: ANOVA, Tukey´s test). (B) BoNT-A (7U/kg) intra-articular injection significantly reduced the release of CGRP induced by arthritis in the TMJ. The symbol (+) indicates values significantly higher than the

non-immunized group (P<0,05: ANOVA, Tukey test). The symbol (∗) indicates values significantly lower than that of immunized + group (P<0,05: ANOVA, Tukey´s test).

Figure 4: Intra-articular injection of Botulinum toxin A (BoNT-A) in the TMJ of rats had no effect in the release of glutamate and the expression of AMPA and NMDA receptors. (A) BoNT-A (7U/kg) intra-articular injection did not affect the release of Glu quantity induced by arthritis in the TMJ of rats. (P>0,05: ANOVA, Tukey´s test). (B) and (C): There is no difference among groups in the expression of AMPA and NMDA receptors (P<0,05: ANOVA, Tukey´s test).

Figure 5: Intra-articular injection of Botulinum toxin A (BoNT-A) in the TMJ of rats reduced the release of IL-1β inflammatory cytokine. (A) BoNT-A (7 U/kg) intra-articular injection did no affect the release of TNF-α induced by arthritis in the TMJ of rats. (P<0,05: ANOVA, Tukey´s test). (B) BoNT-A (7 U/kg) intra-articular injection significantly reduced the release of IL1-β induced by arthritis in the TMJ. The symbol (+) indicates values significantly higher than the non-immunized group (P<0,05: ANOVA, Tukey test). The symbol (∗) indicates values significantly lower than that of the immunized + group (P<0,05: ANOVA, Tukey´s test).

Figure 1:


EXPERIMENT
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+ PBS: s.c.
mBSA (500µg) + IFA
+ PBS; s.c.
mBSA (500µg) + IFA
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mBSA
(10 µg);
TMJ
mBSA
(10 µg);
TMJ
mBSA
(10 µg);
TMJ
BoNT-A;
TMJ
Formalin (0,5%)
TMJ
Formalin (0,5%)
TMJ
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
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Challenge
Challenge
Challenge
BoNT-A
Saline
Behavioral test;
24h
Behavioral test;
14d

Figure 2:

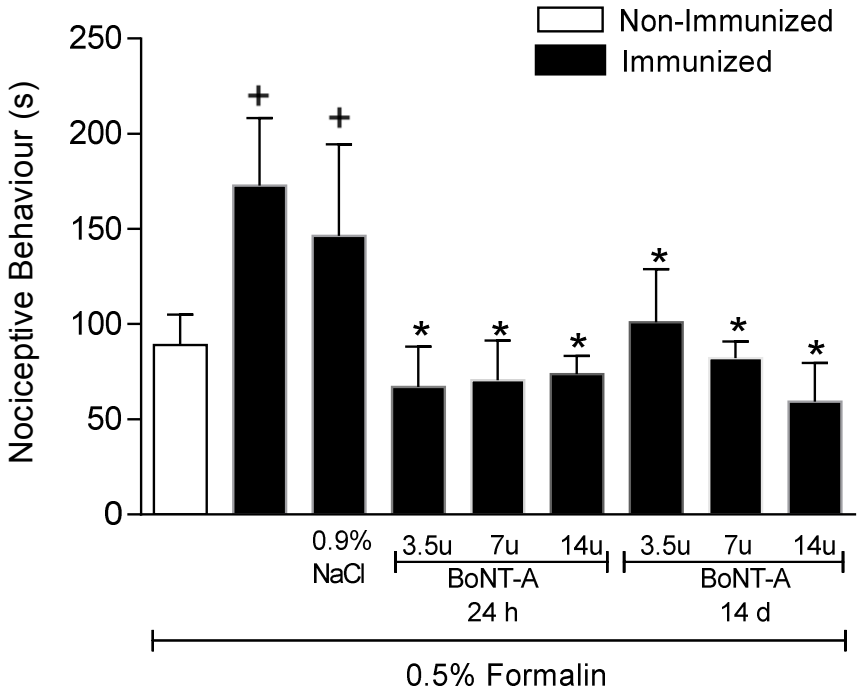


Figure 3

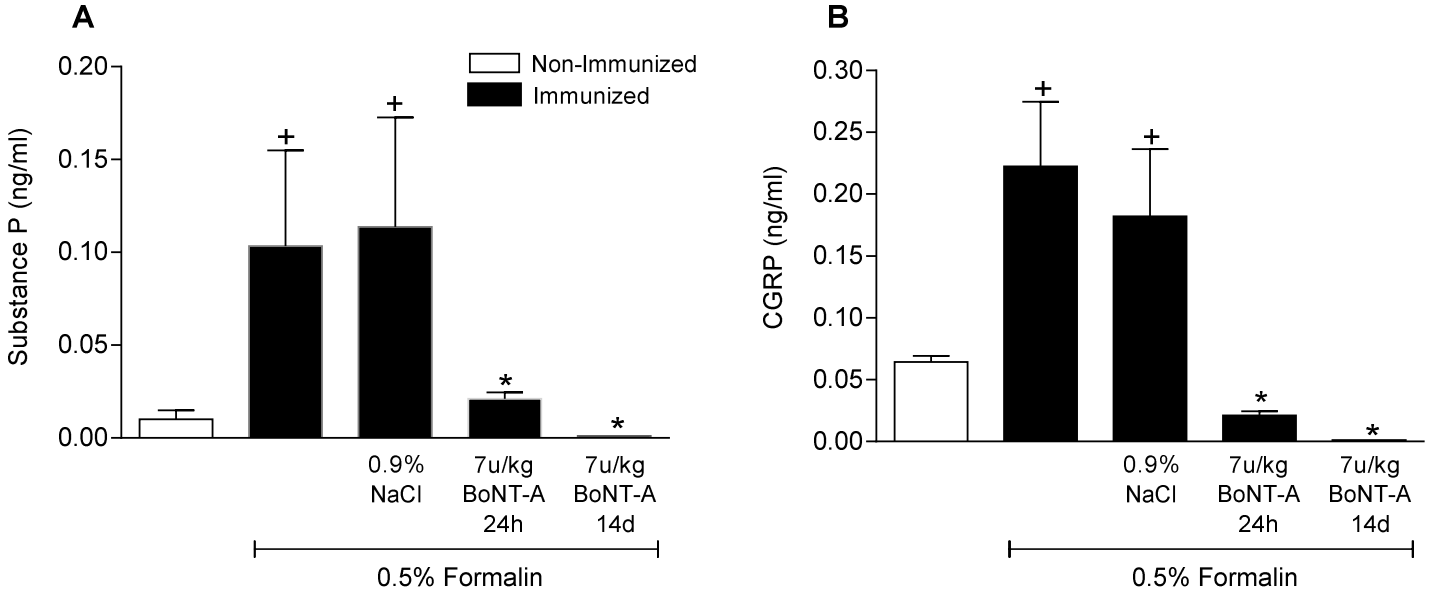


Figure 4:

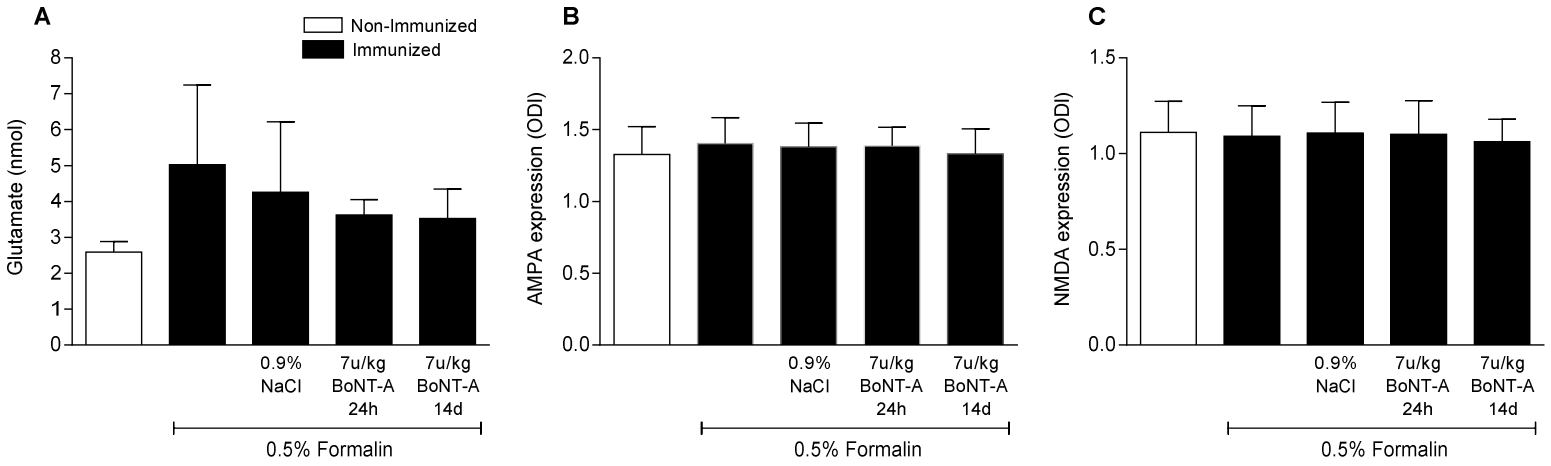
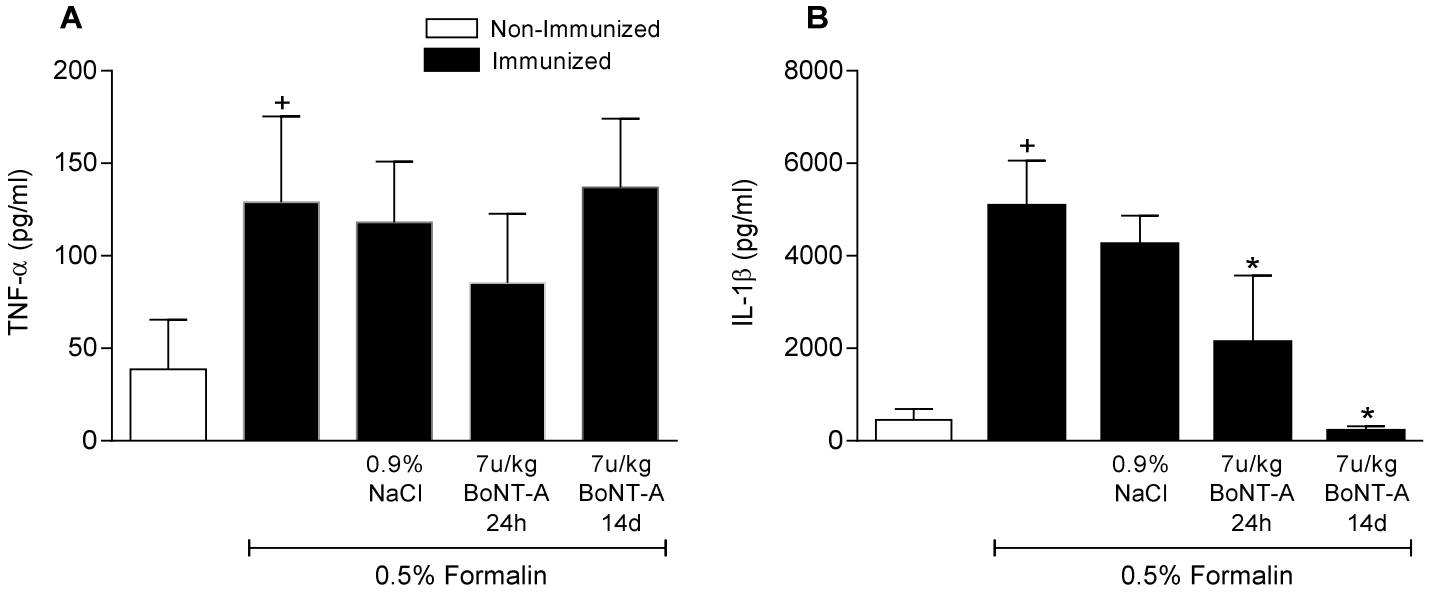


Figure 5:



CONCLUSÃO

Baseados nos resultados do estudo, podemos concluir que a aplicação de Toxina botulínica A (TxB-A) reduziu a hipernocicepção inflamatória induzida na ATM de ratos, mediante a inibição na liberação periférica da Substancia P, do Gene relacionado ao peptídeo da Calcitonina, e da citosina pro-inflamatória IL1-β. A pesar que a liberação do Glutamato não foi significantemente inibida pela neurotoxina, se recomenda o desenvolvimento de estudos complementares avaliando o efeito analgésico da TxB-A a nível central.

REFERÊNCIAS

1. Dressler D, Saberi FA, Barbosa ER. Botulinum toxin: mechanisms of action. Arq Neuro-Psiquiatr. 2005; 63(1).

2. Poli MA, Lebeda FJ. An overview of clostridial neurotoxins. In: Massaro EJ. Handbook of neurotoxicology. Totowa: Human Press; 2002. p. 293-304

3. Turton K, Chaddock JA, Acharya KR. Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. Trends Biochem Sci. 2002; 27(11): 552-8.

4. Aoki KR. Pharmacology of Botulinum neurotoxins. Oper Tech Otolaryngol Head Neck Surg. 2004; 15(2): 81-5.

5. Dolly JO, Aoki KR. The structure and mode of action of different botulinum toxins. European Journal of Neurology 2006, 13(4): 1–9.

6. Wheeler A, Smithb H, Botulinum toxins: Mechanisms of action, antinociception and clinical applications. Toxicology. 2013; 5(306): 124-46.

7. Aoki KR. Botulinum toxin: a successful therapeutic protein. Curr Med Chem. 2004; 11(23): 3085-92.

8. Pellizzari R, Rossetto O, Schiavo G, Montecucco C. Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. Philos Trans R Soc Lond B Biol Sci. 1999; 28;354(1381):259-68.

9. Rossetto O, Rigoni M, Montecucco C. Different mechanism of blockade of neuroexocytosis by presynaptic neurotoxins Toxicol Lett. 2004; 149(1-3): 91-101

10. Wheeler A, Smithb H, Botulinum toxins: Mechanisms of action, antinociception and clinical applications. Toxicology. 2013; 5(306):124-46.

11. Song PC, Schwartz J, Blitzer A. The emerging role of botulinum toxin in the treatment of temporomandibular disorders. Oral Diseases. 2007; 13(1): 253-60.

12. Guarda-Nardini L, Stecco A, Masiero S, Manfredini D. Myofascial pain of the jaw muscles: comparison of the short-term effectiveness of Botulinum toxin injections and fascial manipulation technique. Cranio. 2012; 30(2): 95-102.

13. Colhado OC, Boeing M, Ortega LB. Toxina botulínica no tratamento da dor. Ver Bras Anestesiol. 2009; 59(3): 366-81.

14. Aoki KR, Francis J. Updates on the antinociceptive mechanism hypothesis of botulinum toxin type A. Parkinsonism. Relat Disord. 2011; 17(1): 28-33

15. Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain Pain. 2004;107(1-2):125-33

16. De Almeida AR, Montagner S. Botulinum toxin for axillary hyperhidrosis. Dermatol Clin. 2014; 32(4): 495-504

17. Aoki K. Evidence for antinociceptive activity of botulinum toxin type A in pain management. Headache 2003; 43(1): 9-15.

18. Bonjardin LR, da Silva AP, Gameiro GH, Tambeli CH, Veiga MCFA. Nociceptive behavior induced by mustard oil injection into the temporomandibular joint is blocked by a peripheral non-opioid anagesic and a central opioid analgesic. Pharmacol Biochem Behav. 2009; 91(3): 321-26.

19. Gameiro GH, Andrade Ada S, de Castro M, Pereira LF, Tambeli CH, Veiga MC. The effects of restarint stress on nociceptive responses induced by formalin injected in rat's TMJ. Pharmacol Biochem Behav. 2005; 82(2): 338-44.

20. Roveroni RC, Parada CA, Cecília M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: the TMJ formalin test. Pain. 2001; 94(2): 185 – 91.

21. Fiorentino PM, Cairns BE, Hu JW. Development of inflammation after application of mustard oil glutamate to the rat temporomandibular joint. Arch Oral Biol. 1999; 44(1): 27-32.

22. Durham PL, Cady R, Cady R. Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: implications for migraine therapy. Headache. 2004;44(1):35-42.

23. Welch MJ, Purkiss JR, Foster KA. Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. Toxicon. 2000;38(2):245-58.

24. Ishikawa, H., Mitsui, Y., Yoshitomi, T., Mashimo, K., Aoki, S., Mukuno, K., Shimizu, K., 2000. Presynaptic effects of botulinum toxin type A on the neuronally evoked responses of albino and pigmented iris sphincter and dilator muscles. Jpn. J. Ophthalmol. 44, 106–109.

25. Meng J, Wang J, Lawrence G, Dolly JO. Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential. J Cell Sci. 2007; 120(16): 2864-74.

CONFIRMAÇÃO DE SUBMISSÃO DE ARTIGO

ANEXOS

Elsevier Editorial System(tm) for Archives of Oral Biology

Manuscript Draft

Manuscript Number:

Title: Botulinum toxin type A reduces inflammatory hypernociception induced by arthritis in the temporomadibular joint of rats

Article Type: Research Paper

Keywords: Botulinum toxins type A, nociception, neurotransmitters

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Abstract: Objective: This study aimed to investigate the antinociceptive effects of Botulinum toxin type A (BoNT-A) on persistent inflammatory hypernociception induced by arthritis in the TMJ of rats.
Material and methods: Wistar rats were induced to persistent inflammatory hypernociception in the left TMJ. Then, animals were treated with intra-TMJ injections of BoNT-A, using doses of 3.5, 7 and 14 U/kg. Saline was used as control group. Behavioral tests were applied to evaluated the effect of BoNT-A in the inflammatory hypernociception. After that, animals were euthanized and samples from peri-articular tissues and trigeminal ganglia were obtained for further analyses.
Results: BoNT-A reduced the persistent inflammatory hypernociception induced by arthritis in the TMJ of ratos. BoNT-A significantly reduced the peripheral release of the neurotransmitters Substance P and Calcitonin gene related peptide; and the pro-inflammatory cytokine IL-1β. Otherwise, BoNT-A had no effect in the peripheral release of glutamate and the cytokine TNF-α.
Conclusion: These results demonstrate that intra-articular injection of BoNT-A reduces the albumin-induced arthritis persistent hypernociception in TMJ of rats by peripheral inhibition of neuropeptides release.

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Comissão de Ética no Uso de Animais
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CERTIFICADO

Certificamos que o projeto **"Estudo dos mecanismos de ação da toxina botulínica sobre a hipernocicepção persistente induzida pela artrite reumatoide na ATM de ratos"** (protocolo nº **3452-1**), sob a responsabilidade de **Profa. Dra. Celia Marisa Rizzatti Barbosa / Victor Ricardo Manuel Muñoz Lora**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **10 de julho de 2014**.

Campinas, 10 de julho de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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