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FERNANDA BARCHESI ZANELATTO

EFEITOS ANTI-INFLAMATÓRIOS DO PROPRANOLOL NA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATAS E SUA CONTRIBUIÇÃO NA AÇÃO ANTINOCICEPTIVA

ANTI-INFLAMMATORY EFFECTS OF PROPRANOLOL IN THE TEMPOROMANDIBULAR JOINT OF RATS AND ITS CONTRIBUTION TO ITS ANTINOCICEPTIVE ACTION

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RESUMO

Foi demonstrado recentemente que os β-bloqueadores reduzem a dor da articulação temporomandibular. Perguntamos se eles também reduzem a inflamação da articulação temporomandibular e, em caso afirmativo, se este efeito anti-inflamatório pode contribuir para a sua ação analgésica. Para determinar se os β-bloqueadores reduzem a inflamação da articulação temporomandibular medimos alguns parâmetros de respostas inflamatórias após a co-administração do β-bloqueador propranolol com o agente inflamatório carragenina, na articulação temporomandibular de ratos fêmeas. Para determinar se o efeito anti-inflamatório de um β-bloqueador pode contribuir para a sua ação antinociceptiva, temos a hipótese de que a ativação de β-adrenoceptores na articulação temporomandibular induz nocicepção mediada, pelo menos em parte, pela resposta inflamatória. Para testar esta hipótese, foi examinada a resposta nociceptiva induzida pela ativação dos β-adrenoceptores na articulação temporomandibular em ratos fêmeas pré-tratados com talidomida e fucoidan, que inibe a migração de neutrófilos e a síntese da citocina pro-inflamatória TNF-α, respectivamente. Descobrimos que a administração do propranolol β-bloqueador com carragenina na articulação temporomandibular de ratas reduziu significativamente vários parâmetros da resposta inflamatória induzida pela carragenina, tais como extravasamento plasmático, a migração de neutrófilos e a libertação de citocinas pró-inflamatórias TNF-α, IL-1β e CINC-1. Além disso, a nocicepção induzida pela injeção do agonista do receptor β-adrenérgico, isoproterenol, na articulação temporomandibular, foi significativamente reduzida pela talidomida, fucoidan e pela co-administração do propranolol, mas não pelo antagonista de receptores α-adrenérgicos, fentolamina. Concluímos que o propanolol tem efeitos anti-inflamatórios que podem contribuir para a sua ação antinociceptiva na articulação temporomandibular de fêmeas.

Palavras-chave: propranolol, articulação temporomandibular, antinocicepção, anti-inflamatório

ABSTRACT

It has been recently demonstrated that β-blockers reduce temporomandibular joint pain. We asked whether they also reduce temporomandibular joint inflammation and, if so, whether this anti-inflammatory effect may contribute to its analgesic action. To determine whether β-blockers reduce temporomandibular joint inflammation, we measured many parameters of the inflammatory response after co-administration the β-blocker propronolol with the inflammatory agent carrageenan in the temporomandibular joint of female rats. To determine whether the anti-inflammatory effect of a β-blocker may contribute to its antinociceptive action, we hypothetized that the activation of β-adrenoceptors in the temporomandibular joint induces nociception mediated, at least in part, by the inflammatory response. To test this hypothesis, we examined the nociceptive response induced by the activation of the β-adrenoceptors in the temporomandibular joint in female rats pretreated with thalidomide and fucoidan, which inhibits neutrophil migration and the synthesis of the pro-inflammatory cytokine TNF-α, respectively. We found that the administration of the β-blocker propranolol with carrageenan in the temporomandibular joint of female rats significantly reduced several parameters of the inflammatory response induced by carrageenan such as plasma extravasation, neutrophil migration and release of the pro-inflammatory cytokines TNF-α, IL-1β, and CINC-1. Furthermore, the injection of the β-adrenergic receptor agonist isoproterenol in the temporomandibular joint induced nociception that was significantly reduced by thalidomide, fucoidan and by the co-administration of propranolol but not of the α-adrenergic receptor antagonist phentolamine. We conclude that propranolol has anti-inflammatory effects that may contribute to its antinociceptive action in the temporomandibular joint of females.

Keywords: propranolol, temporomandibular joint, antinociception, anti-inflammatory

LISTA DE ABREVIATURAS E SIGLAS

AINEs = Anti-inflamatórios não esteroidais

ATM = Articulação temporomandibular

DTM = Disfunção temporomandibular

h = hora

kg = quilograma

mg = miligrama

mL = mililitro

mM = milimolar

ng = nanograma

TMD = Temporomandibular disorder

TMJ = Temporomandibular joint

µg = micrograma

µL = microlitro

SUMÁRIO

1. Introdução

A dor pode ser definida como uma percepção desagradável associada à nocicepção. A percepção de dor pode ser modulada por condições motivacionais, emocionais, psicológicas e pela história pregressa individual, tendo uma função integrativa (Mersky, 1986). A nocicepçãoé resultado da ativação de uma população especifica de neurônios aferentes primários que transmitem informação nociceptiva para o sistema nervoso central (Milan, 1999; Julius & Basbaum, 2001). Uma parte dos episódios dolorosos é desencadeada por lesão tecidual, sendo uma resposta ao trauma para proteger-se através da inflamação e, também, alertar o cérebro do perigo iminente através da sensação dolorosa, impedindo a ocorrência de uma destruição ainda maior do tecido (Cooper, 1990). Após uma lesão tecidual, uma resposta inflamatória é gerada por macrófagos locais e amplificada por células sanguíneas migratórias, como os neutrófilos (Van Furth et al., 1985; Laskin & Pendino, 1995) que durante esse processo vão liberar mediadores inflamatórios (TNF-α, IL-1β e IL-6) que estimulam a síntese de prostaglandinas e aminas simpatomiméticas, que sensibilizam diretamente os nociceptores aferentes primários (Cunha et al., 1992; Ferreira et al., 1993; Gold et al., 1996; Rush & Waxman, 2004).

Embora a dor seja essencial para a preservação da vida, em muitas circunstâncias ela perde o seu caráter protetor e passa a constituir a própria doença como por exemplo, a dor proveniente da região orofacial (Sessle, 1987; Coderre et al., 1993; Loe, 1993). A captação e transmissão das informações sensoriais dolorosas provenientes da região orofacial são realizadas por fibras nervosas aferentes primárias trigeminais localizados no gânglio trigeminal e por receptores sensoriais nociceptivos (Machado, 1993).

A disfunção temporomandibular (DTM) é uma condição dolorosa comum da articulação temporomandibular (ATM) e dos músculos associados (Winocur et al., 2003). Ela afeta cerca de 7-15% da população, e sua incidência é maior em mulheres em idade reprodutiva (De Felício et al., 2012), com uma proporção de cinco mulheres para um homem (Biasotto-Gonzalez, 2005). Dados epidemiológicos demonstram que em uma amostra de 894 pacientes brasileiros, portadores de disfunções temporomandibulares, 87,9% apresentavam dor orofacial (Luz et al., 1997; Irving et al., 1999). Essas condições resultam principalmente

de trauma agudo, desarranjo interno ou artrites, e são comumente associadas à inflamação aguda ou crônica (Alstergren & Kopp, 2000; Suzuki et al., 2003).

A inflamação da ATM sensibiliza neurônios nociceptivos centrais do complexo sensório-nuclear trigeminal do tronco encefálico (Iwata et al., 1999; Sessle, 2000; Dubner & Ren, 2004) e de nociceptores periféricos desta região (Raja et al., 1988; Alstergren & Kopp, 2000; Nordahl et al., 2000; Kopp, 2001; Oliveira et al., 2005). Essas sensibilizações aumentam a excitabilidade da membrana neuronal em decorrência da liberação de mediadores inflamatóriosno local da lesão (Alstergren & Kopp, 2000; Kopp, 2001; Suzuki et al., 2003) e pela liberação de neuropeptídeos e aminoácidos excitatórios no complexo sensório-nuclear trigeminal do tronco encefálico (Bereiter & Benetti, 1996; Yu et al., 1996; Bakke et al., 1998; Cairns et al., 2001). Alguns dos mediadores inflamatórios liberados no local da lesão estão presentes em alta concentração no fluido sinovial de pacientes que apresentam dor na ATM, diminuindo o limiar nociceptivo, contribuindo para a alodínia e hiperalgesia (De Laat et al., 1998; Alstergren & Kopp, 2000; Kopp, 2001).

Atualmente, as alternativas farmacológicas para o tratamento da dor na ATM são analgésicos, agentes anti-inflamatórios não esteroidais, corticosteróides, ansiolíticos, relaxantes musculares e anestésicos locais, dependendo do estado clínico de cada paciente (Hersh et al., 2008). No entanto, sua eficácia e segurança são limitadas. Drogas anti-inflamatórias não esteroidais (AINEs) são frequentemente utilizadas no controle de dores inflamatórias (Dionne, 1997; List et al., 2003; Ta & Dionne, 2004). A ação analgésica dessas drogas resulta do bloqueio da síntese das prostaglandinas, prevenindo assim a sensibilização periférica dos nociceptores (Ferreira, 1972; Ferreira, 2002).

A dor da ATM tem um componente simpático importante, que está de acordo com a inervação simpática abundante da ATM (Widenfalk & Wiberg, 1990; Yoshino et al., 1998;. Kido et al., 2001), de onde aminas simpátomiméticas podem ser liberadas. Assim, a manipulação dos β-adrenoceptores tornou-se um alvo terapêutico promissor para o tratamento da dor na ATM. Por exemplo, em estudos em humanos, o propranolol, um antagonista de β-adrenoceptores não seltivo, melhora o quadro clínico de dor na ATM em mulheres (Light et al., 2009), especialmente sob condições de baixa atividade da enzima catecol-O-metiltransferase que metaboliza catecolaminas, e eleva a biodisponibilidade desses neurotransmissores (Tchivileva et al., 2010). Em estudos realizados em animais, o bloqueio de receptores β-adrenérgicos reduz significativamente a nocicepção na ATM induzida por

formalina (Fávaro-Moreira et al., 2012) e a hiperalgesia induzida por carragenina na ATM (Rodrigues et al., 2006). Além disso, em um estudo *in vitro* foi demonstradoque a ativação de β-adrenoceptores localizados nos macrófagos aumenta a expressão de IL-1β e IL-6 (Tan et al., 2007), contribuindo para o processo inflamatório.

É digno de nota que o sexo feminino parece ser mais sensível do que o masculino ao efeito anti-nociceptivo dos β-bloqueadores na ATM de ratos (Fávaro-Moreira et al. 2012), o que é de relevância clínica uma vez que a dor da ATM é mais prevalente em mulheres do que nos homens (Dworkin et al., 1990). No entanto, não se sabe até então se os β-bloqueadores reduzem a resposta inflamatória na ATM. Também são desconhecidos os mecanismos envolvidos na sua ação antinociceptiva. Assim, neste estudo, avaliamos se o β-bloqueador propranolol reduz a inflamação da articulação temporomandibularde ratas e, em caso afirmativo, se este efeito anti-inflamatório pode contribuir para a sua ação analgésica.

O presente estudo está em formato alternativo, conforme deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 002/2013.

II. Capítulo I

Anti-inflammatory effects of propranolol in the temporomandibular joint of rats and its contribution to its antinociceptive action

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Abstract

It has been recently demonstrated that β-blockers reduce temporomandibular joint pain. We asked whether they also reduce temporomandibular joint inflammation and, if so, whether this anti-inflammatory effect contributes to its analgesic action. To determine whether β-blockers reduce temporomandibular joint inflammation, we measured many parameters of the inflammatory response after co-administration of the β-blocker propronolol with the inflammatory agent carrageenan in the temporomandibular joint of female rats. To determine whether the anti-inflammatory effect of propranolol contributes to its antinociceptive action, we hypothesized that the activation of β-adrenoceptors in the temporomandibular joint induces nociception mediated, at least in part, by the inflammatory response. To test this hypothesis, we examined the nociceptive response induced by the activation of the β-adrenoceptors in the temporomandibular joint in female rats pretreated with thalidomide and fucoidan, which inhibits neutrophil migration and the synthesis of the pro-inflammatory cytokine TNF-α, respectively. We found that the co-administration of propranolol with carrageenan in the temporomandibular joint of female rats significantly reduced several parameters of the inflammatory response induced by carrageenan such as plasma extravasation, neutrophil migration and the release of the pro-inflammatory cytokines TNF-α, IL-1β, and CINC-1. Furthermore, the injection of the β-adrenergic receptor agonist isoproterenol in the temporomandibular joint induced nociception that was significantly reduced by thalidomide, fucoidan and by the co-administration of propranolol but not of the α-adrenergic receptor antagonist phentolamine. We conclude that propranolol has anti-inflammatory effects that contribute to its antinociceptive action in the temporomandibular joint of females.

Keywords – propranolol, temporomandibular joint, antinociception, anti-inflammatory

Introduction

Temporomandibular disorder (TMD) is a common painful condition in the temporomandibular joint (TMJ) and associated muscles (Winocur et al., 2003). It affects about 7-15% of the population, and its incidence is higher in adult women of reproductive age (De Felício et al., 2012), with a ratio of five women for one man (Biasotto-Gonzalez, 2005).

Currently, the pharmacological alternatives for the treatment of TMD-associated pain are analgesics, non-steroidal anti-inflammatory agents, corticosteroids, anxiolytics, muscle relaxants, and local anesthetics, depending on the clinical status of each patient (Hersh et al., 2008). However, their efficacy and safety are limited.

TMD-associated pain has an important sympathetic component, which is in agreement with the abundant sympathetic innervation of the TMJ (Widenfalk & Wiberg, 1990; Yoshino et al., 1998; Kido et al., 2001) from where sympathetic amines may be released. Thus, the β-adrenoceptor has emerged as a promising therapeutic target for the treatment of TMD-associated pain. For example, in human studies, the β-adrenoceptor antagonist propranolol improves clinical TMJ pain report in women (Light et al., 2009) especially under conditions of low activity of the enzyme catechol-*O*-methyltransferase which metabolizes catecholamines, and high catecholamine bioavailability (Tchivileva et al., 2010). In animal studies, the blockade of β-adrenoceptors in the TMJ significantly reduces formalin-induced TMJ nociception (Fávaro-Moreira et al., 2012 and 2015) and carrageenan-induced TMJ hyperalgesia (Rodrigues et al., 2006). Additionally, an *in vitro* study showed that the activation of β-adrenoceptors located in macrophages increases the expression of IL-1β and IL-6 (Tan et al., 2007), contributing to the inflammatory process. It is known that after a tissue injury, an inflammatory response is generated by local macrophages and amplified by migrating blood cells, such as neutrophils (Van Furth et al., 1985; Laskin & Pendino, 1995) that during this process will release inflammatory mediators (TNF-α , IL-1β and IL-6) that stimulate the synthesis of prostaglandins and sympathomimetic amines, which directly sensitize the primary afferent (Cunha et al., 1992; Ferreira et al., 1993; Gold et al., 1996; Rush & Waxman, 2004).

However, whether β-blockers reduce TMJ inflammation is unknown. Also unknown are the mechanisms involved in their antinociceptive action. Thus, in this study we asked

whether the β-blocker propranolol reducestemporomandibular joint inflammation in female rats, and if so, whether this anti-inflammatory effect contributes to its analgesic action.

Materials and Methods

Animals:

This study was carried out in female Wistar rats (HanUnib/WH, eight weeks old, 220-280g), obtained from the Multidisciplinary Center for Biological Research (CEMIB – University of Campinas, SP, Brazil). The animals were housed in plastic cages with soft bedding (five rats/cage) in the animal care facility of the Institute of Biology of the State University of Campinas on a 12-hour dark-light cycle (lights on at 6:00 A.M.), ±23ºC with food and water *ad libitum*. The animals were handled for at least one week prior to the experiments. Each animal was used once and the number of animals per group was kept to a minimum. The Committee on Animal Research of the University of Campinas (protocol number: 3776-1) approved the experimental protocols, which adhered to the guidelines set by IASP for the study of pain in animals (Zimmermann, 1983).

General Procedures:

Testing sessions took place during the light phase (between 09:00 A.M. and 5:00 P.M.) in a quiet room maintained at 23°C (Rosland, 1991). Each animal was placed in a test chamber (30x30x30 cm mirrored wood chamber with a glass at the front side) for a 15 min habituation period to minimize stress (Abbott et al. 1986). The animal was removed from the test chamber to allow the TMJ injection.

TMJ Injections:

TMJ injections were performed via a 30-gauge needle introduced into the right TMJ of rats under brief inhalation of isoflurane anesthesia. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μL). The postero inferior border of the zygomatic arch was palpated and the needle was inserted immediately inferior to this point and advanced in an anterior direction until reach the posterolateral aspect of the condyle. Volume per injection was 25 μL.

Measurement of nociceptive responses:

After the TMJ injection, the animals recovered from anesthesia and immediately returned to the test chamber for a 30 minutes observation period. The time evaluation was divided into 10 x3-minutes blocks and a pain score was determined for each block by measuring the number of seconds of two types of nociceptive behavior: rubbing the orofacial region asymmetrically with the ipsilateral fore and hind paw and/or flinching the head in an intermittent and reflexive way characterized by high frequency shakes of the head (Roveroni et al. 2001). The sum of these nociceptive behaviors was used as a quantitative measurement of isoproterenol-induced TMJ nociception. During the tests, the animals had no access to water or food.

Drugs and doses:

The following drugs were injected into the TMJ: λ-carrageenan (100 μg/TMJ, (Oliveira et al., 2005), propranolol (2.25 μg/TMJ, Rodrigues et al. 2006), isoproterenol (1.0, 1.5, 3.0 and 6.0 μg/TMJ) (Khasar et al., 1999) and phentolamine (1 μg/TMJ) (Khasar et al., 1999). The injection volume was 25 μL. Fucoidan (20 mg/kg in 200 μL) was injected into the gingival vein 20 minutes before isoproterenol administration (Finley et al., 2013). Thalidomide (30 mg/kg in 1mL) was injected intraperitoneally (i.p.) 30 minutes before isoproterenol administration and was dissolved in 200 μL of DMSO (dimethyl sulfoxide) and 800 μL of 0.9% NaCl (Ribeiro et al., 2000). All other drugs were dissolved in sterile 0.9% NaCl. All drugs were obtained from Sigma-Aldrich (MO, USA), except Thalidomide which was obtained from RBI - Research Biochemicals International (MA, USA).

Enzyme-linked immunosorbent assay (ELISA) procedure:

An adaptation of ELISA (Safieh-Garabedian et al., 1995) was used to determine whether propranolol was able to reduce the carrageenan-induced local increase in pro-inflammatory cytokines concentration (TNF-α, IL-1β and cytokine-induced neutrophil chemoattractant-1 (CINC-1). The TMJ surrounding tissue was collected 60 min post-TMJ injections of drugs or its vehicle (0.9% NaCl). TMJ surrounding tissues were weighed and homogenized in the same weigh/volume proportion in a solution of phosphate-buffered saline

(PBS) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin (BSA), 0.1 mM phenyl-methyl-sulfonyl fluoride, 0.1 mM benzotonic chloride, 10 mM EDTA, and 2 ng/mL aprotinin (Sigma, USA). The samples were centrifuged at 10,000 rpm for 15 min at 4 ºC. The supernatants were stored at -80 °C for posterior use to evaluate the protein levels of TNF-α, IL-1β and CINC-1 in the TMJ tissues. The cytokines were quantified by the following DuoSet ELISA Kits: TNF-α: Rat TNF-α/TNFSF1A (DY510); IL-1β: Rat IL-1β/IL-1F2 (DY501) and CINC-1: Rat CXCL1/CINC-1 (DY515). All procedures followed the instructions of the manufacturer (R&D Systems, Minneapolis, MN, USA). All procedures were repeated twice to guarantee the accuracy of the results.

Measurement of myeloperoxidase activity (MPO):

The measurement of MPO activity was evaluated by the myeloperoxidase (MPO) kinetic colorimetric assay (Bradley et al., 1982) and used as a marker of neutrophil migration in the TMJ of rats after the injection of drugs. Myeloperoxidase (MPO) is one of the enzymes released from neutrophils and directly associated with tissue injury. Although monocytes/macrophages and fibroblasts also contain myeloperoxidase, neutrophils show the highest intracellular levels of this enzyme, that represents up to 5% of neutrophil proteins (Klebanoff, 1991).

Approximately 0.5 cm² of TMJ surrounding tissue was harvested 60 min after the TMJ injections, weighed, quickly frozen in liquid nitrogen and stored at -70 ºC. In the assay procedure, each sample was homogenized in 500 µL of buffer 1 (0.1 M NaCl, 0.02 M NaPO4, 1.015 M NaEDTA, pH 4.7) followed by centrifugation at 3000 rpm for 15 min at 4 ºC. The pellet was resuspended in 500 µL of buffer 1 and subjected to hypotonic lysis by the addition of 500 µL of 0.2% NaCl followed 30 s later by the addition of 500 µL of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 500 µL of 0.05 M NaPO4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10,000 rpm for 15 min at 4 ºC. The supernatant was used in the assay.

Fifty microliters of each sample and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate. Twenty-five microliters of 1.6 mM 3,3´,3,3´-tetramethylbenzidine (TMB) was added to each well and the reaction was initiated by the addition of 100 µL of 0.5 mM

H_2O_2. The reaction was stopped 5 minutes later by the addition of 50 µL of 4M H_2SO_4. The optical density was read at 450 nm using an Asys UVM340. The results were calculated by comparing the optical density of TMJ tissue supernatant with a standard curve of neutrophil (>95% purity), as previously described (Torres-Chávez, 2012). The results were presented as a number of neutrophils 10^6/mg tissue. All procedures were repeated twice to guarantee the authenticity of the results.

Plasma extravasation measurement:

Immediately after the TMJ injection, the Evans blue dye (Sigma Chemicals; 50 mg/kg (Haas et al., 1992), was injected into the gingival vein. The Evans blue dye binds to plasma protein extravasated in the inflammatory site and therefore was used as a marker for plasma extravasation (Haas et al., 1992). Sixty minutes after the Evans blue dye injection, animals were perfused transcardially with saline (NaCl 0.9%) to flush the dye from the vasculature. Joint tissues were dissected to a standardize size (30±2 mg) and stored at -30 °C until analysis. The dye was extracted by immersing the samples in 1 ml of formamide at 60 °C for 24 h. The amount of blue dye (mg) in the tissue sample was determined using a spectrophotometer that measured the absorbance of the formamide solution (620 nm). The concentration of dye was then calculated per gram weight of tissue.

Statistical analysis:

Statistical analysis was performed using Prism v5 (GraphPad, La Jolla, CA, USA). All data were tested for normality before statistics tests. The sum of the behavioral responses measured for 30 min was used for statistical analysis. Data with the homogeneity of variance were analyzed using the One-way analysis of variance (ANOVA) and multiple post hoc comparisons were performed using Tukey test. A probability level of less than 0.05 ($P<0.05$) was considered statistically significant. Group data are presented as means ± S.E.M.

Results

The local blockade of β-adrenoceptors reduces the carrageenan-induced plasma extravasation in the TMJ

To verify whether the blockade of TMJ β-adrenoceptors reduces the carrageenan-induced plasma extravasation in the TMJ region, the non-selective β-adrenoceptor antagonist propranolol was co-administered with carrageenan and the plasma extravasation was quantified 1 hour later.

Plasma extravasation was significantly higher in carrageenan (100 µg/TMJ) treated animals than in controls (naive and vehicle groups) (Figure 1, $F_{4,24}$=11.86; P<0.0001, one-way ANOVA, Tukey test). Co-administration of propranolol (2.25 µg/TMJ) with carrageenan (100 µg/TMJ) significantly reduced the carrageenan-induced plasma extravasation (Figure 1, $F_{4,24}$ =11.86; P<0.0001, one-way ANOVA, Tukey test). The administrations of either 0.9% NaCl with propranolol (2.25µg/TMJ) or 0.9% NaCl alone did not affect the plasma extravasation since it was similar to that of the naive group (Figure 1, $F_{4,24}$ =11.86; P<0.0001, one-way ANOVA, Tukey test).

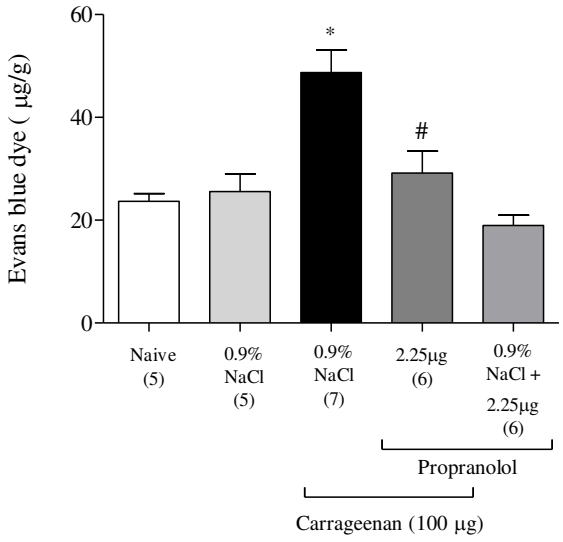

Figure 1. Effect of the co-administration of propranolol on carrageenan-induced plasma extravasation. The administration of carrageenan (100 µg/TMJ) increased plasma extravasation 1 hour after administration. The co-administration of propranolol (2.25 µg/TMJ) significantly reduced the carrageenan-induced plasma extravasation, as indicated by the

symbol "#" (ANOVA, Tukey test, $F_{4,24}$ =11.86; P<0.0001). The symbol "*" indicates a plasma extravasation significantly higher than that of the naive, 0.9% NaCl alone and 0.9% NaCl plus propranolol groups (2.25 µg/TMJ) (ANOVA, Tukey test, $F_{4,24}$ =11.86; P<0.0001). In this and in subsequent figures, the number of rats used per group is in parentheses.

The local blockade of β-adrenoceptors reduces the carrageenan-induced neutrophil migration in the TMJ

To verify whether the blockade of β-adrenoceptors reduces the carrageenan-induced neutrophil migrationin the TMJ, propranolol was co-administered with carrageenan and the activity of the myeloperoxidase enzyme (MPO - neutrophil migration indicator) was quantified 1 hour later.

MPO enzyme activity was significantly higher in carrageenan (100 µg/TMJ) treated animals than in controls (naive and vehicle groups) (Figure 2, $F_{4,26}$= 9.902;P<0.0001, one-way ANOVA, Tukey test). The co-administration of propranolol (2.25 µg/TMJ) with carrageenan (100 µg/TMJ) significantly reduced the carrageenan-induced increase of MPO enzyme activity (Figure 2, $F_{4,26}$= 9.902; P<0.0001, one-way ANOVA, Tukey test). The administrations of either 0.9% NaCl with propranolol (2.25µg/TMJ) or 0.9% NaCl alone did not affect the MPO enzyme activity since it was similar to that of the naive group (Figure 2, $F_{4,26}$= 9.902; P<0.0001, one-way ANOVA, Tukey test).

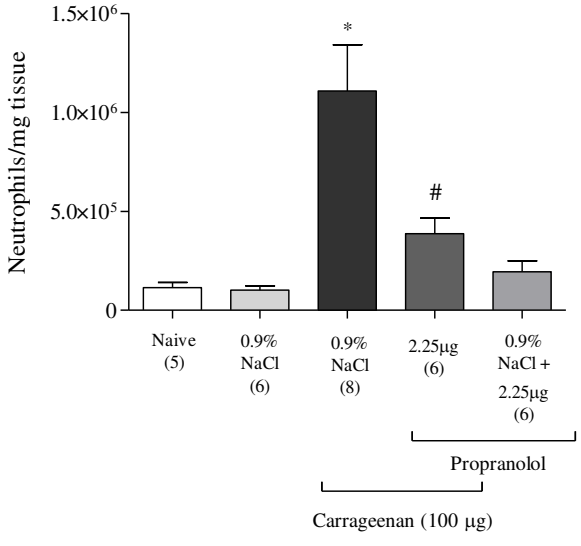

Figure 2. Effect of the co-administration of propranolol on carrageenan-induced increase of MPO enzyme activity. The administration of carrageenan (100 µg/TMJ) significantly increased the MPO enzyme activity 1 hour after administration. The co-administration of propranolol (2.25 µg/TMJ) with carrageenan (100 µg/TMJ) significantly reduced the carrageenan-induced MPO enzyme activity, as indicated by the symbol "#" (ANOVA, Tukey test, $F_{4,26}$= 9.902; p <0.0001). The symbol "*" indicates a response significantly higher than that of the naive, 0.9% NaCl alone and 0.9% NaCl plus propranolol groups (2.25 µg/TMJ) (ANOVA, Tukey test, $F_{4,26}$= 9.902; p <0.0001).

The local blockade of β-adrenoceptors reduces the carrageenan-induced local increase in pro-inflammatory cytokines concentration in the TMJ

To verify whether the blockade of β-adrenoceptors reduces the carrageenan-induced local increase in pro-inflammatory cytokines concentration in the TMJ, propranolol was co-administered with carrageenan and the concentration of the pro-inflammatory cytokines TNF-α, IL-1β and CINC-1 were quantified 1 hour later by enzyme-linked immune sorbent assay (ELISA).

The local concentration of the pro-inflammatory cytokines TNF-α (Figure 3A – $F_{4,25}$=9.437; P<0.0001, one-way ANOVA, Tukey test), IL-1β (Figure 3B – $F_{4,25}$=5.257; P<0.0033, one-way ANOVA, Tukey test) and CINC-1 (Figure 3C – $F_{4,25}$=6.005; P=0.0016, one-way ANOVA, Tukey test) was significantly higher in carrageenan (100 µg/TMJ) treated animals than in controls (naive and vehicle groups). The co-administration of propranolol (2.25 µg/TMJ) with carrageenan (100 µg/TMJ) significantly reduced the carrageenan-induced local increase in pro-inflammatory cytokines TNF-α (Figure 3A -$F_{4,25}$=9.437; P<0.0001, one-way ANOVA, Tukey test), IL-1β (Figure 3B - $F_{4,25}$=5.257; P<0.0033, one-way ANOVA, Tukey test) and CINC-1 (Figure 3C -$F_{4,25}$=6.005; P=0.0016, one-way ANOVA, Tukey test) concentration. The administrations of either 0.9% NaCl with propranolol (2.25 µg/TMJ) or 0.9% NaCl alone did not affect the local concentration of pro-inflammatory cytokines TNF-α, IL-1β and CINC-1 since it was similar to that of the naive group (Figure 3A, B and C, one-way ANOVA, Tukey test).

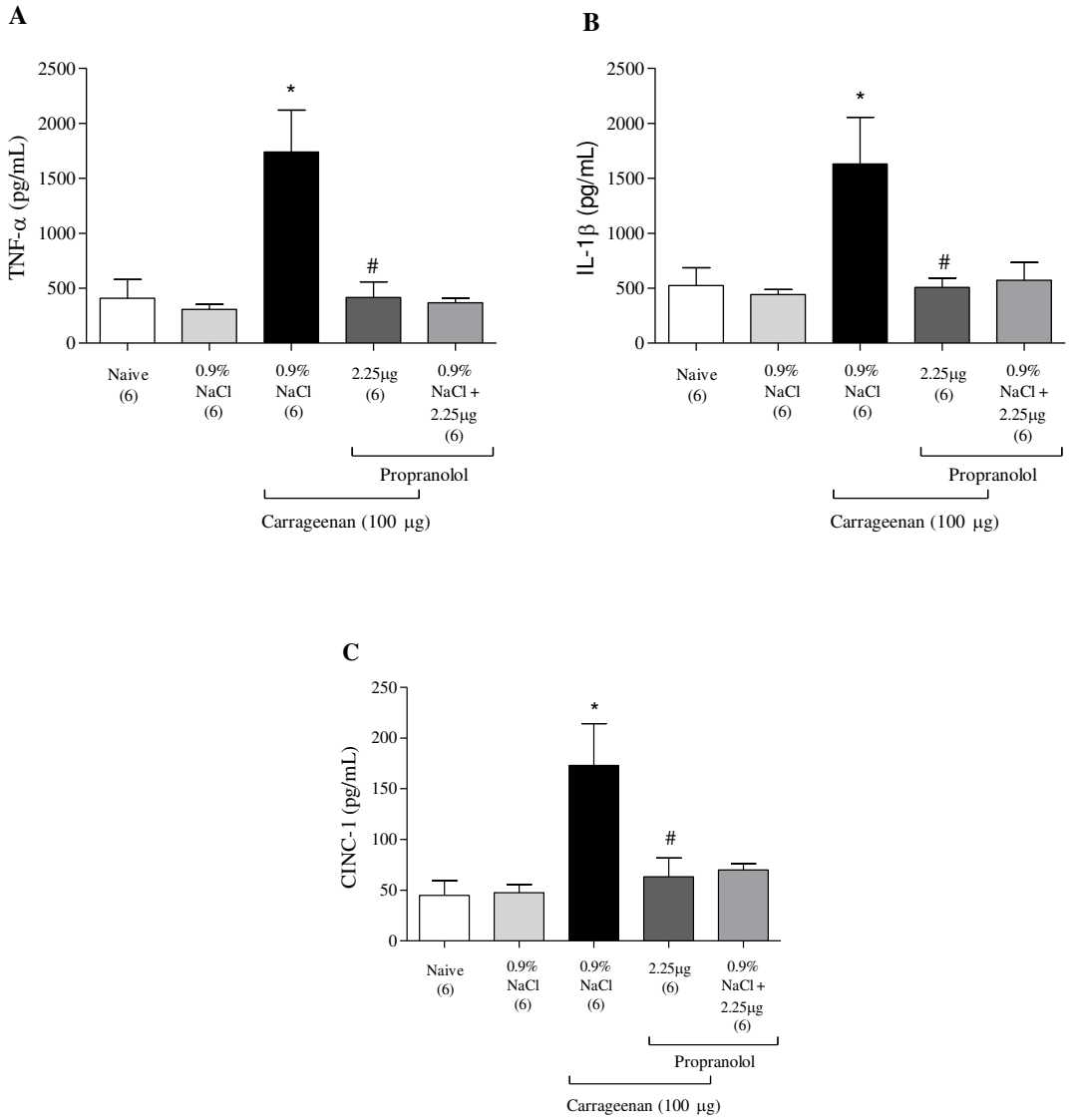

Figure 3. Effect of propranolol on carrageenan-induced local concentration increase of pro-inflammatory cytokines in the TMJ. The administration of carrageenan (100 µg/TMJ) significantly increased the local concentration of pro-inflammatory cytokines TNF-α, IL-1β and CINC-1. Co-administration of propranolol (2.25 µg/TMJ) with carrageenan (100 µg/TMJ) significantly reduced the carrageenan-induced local concentration increase of TNF-α (A) IL-1β (B) and CINC-1 (C), as indicated by the symbol "#" (A - ANOVA, Tukey's test;$F_{4,25}$ = 9.437; p<0.0001) IL-1β (B – ANOVA, Tukey's test; $F_{4,25}$ =5.257; p=0.0033) and CINC-1 (C – $F_{4,25}$=6.005; p=0.0016), as indicated by the symbol "#". The symbol "*" indicates a response significantly higher than that of the naive, 0.9% NaCl and 0.9% NaCl plus propranolol (2.25µg/TMJ) groups (ANOVA, Tukey's test).

TMJ β-adrenoceptors activation induces nociception mediatedby the inflammatory response

To confirm previous findings that the activation of β-adrenoceptors in the TMJ induces nociception, the non-selective agonist of β-adrenoceptors isoproterenol was administered in the TMJ region. The TMJ administration of isoproterenol (3 and 6 µg/TMJ, but not 1 and 1.5 µg/TMJ) induced a significant increase in the nociceptive behavior (rubbing the orofacial region and/or flinching the head) (Figure 4A, $F_{4,25}$=21.09; P<0.0001, one-way ANOVA, Tukey test), when compared to the vehicle group (0.9% NaCl).

To confirm that the isoproterenol-induced TMJ nociception was mediated by the activation of β-adrenoceptors, propranolol was co-administered with isoproterenol. The co-administration of propranolol (2.25 µg/TMJ) blocked the nociceptive effect induced by isoproterenol (3 µg/TMJ) (Figure 4B, $F_{2,15}$=12.73; P=0.0006, one-way ANOVA, Tukey test). To rule out the possibility of the involvement of α-adrenoceptors in the isoproterenol-induced TMJ nociception, in another set of experiments, the non-selective antagonist of α-adrenoceptors phentolamine was co-administered with isoproterenol. The co-administration of phentolamine (1 µg/TMJ) did not affect TMJ isoproterenol-induced nociception (3 µg/TMJ) (Figure 4B, $F_{2,15}$=12.73; P=0.0006, one-way ANOVA, Tukey test).

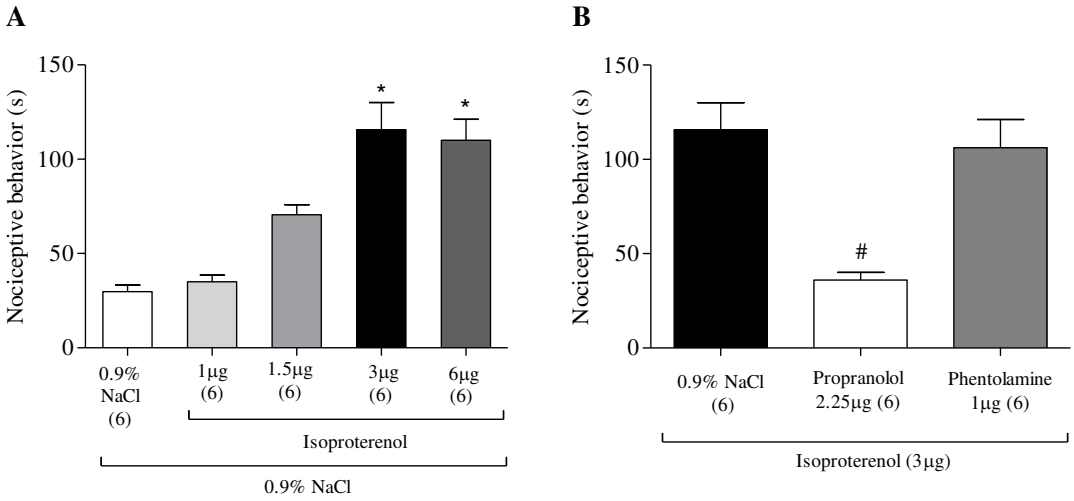

Figure 4. Nociceptive effect of isoproterenolon the TMJ. The administration of isoproterenol into the TMJ at doses of 3 µg and 6 µg/TMJ, but not 1 µg and 1.5 µg/TMJ, induced a nociceptive behavior significantly greater than that of the vehicle group (0.9% NaCl) (A - ANOVA, Tukey test, $F_{4,25}$=21.09; P<0.0001), as indicated by the symbol "*". The co-administration of propranolol (2.25 µg/TMJ) with isoproterenol (3 µg/TMJ) significantly

reduced the isoproterenol-induced nociception (B - ANOVA, Tukey test, $F_{2,15}$=12.73; P=0.0006), as indicated by the symbol "#". The co-administration of phentolamine (1 µg/TMJ) with isoproterenol (3 µg/TMJ) did not a affect the isoproterenol-induced nociception (ANOVA, Tukey test).

To determine whether the activation of the β-adrenoceptors in the TMJ induces nociception mediated by the inflammatory response, we examined the nociceptive response induced by the administration of isoproterenol in the TMJ of female rats pretreated with thalidomide or a non specific inhibitor of selectins, fucoidan, which inhibits neutrophil migration and the synthesis of the pro-inflammatory cytokine TNF-α, respectively. The pretreatment with either fucoidan (25 mg/kg, 200 µL, i.v., 20 minutes before the isoproterenol administration; Figure 5A - $F_{2,15}$=26.99; P<0.0001, one-way ANOVA, Tukey test) or thalidomide (30 mg/kg, 1 mL, i.p., 30 minutes prior to administration of isoproterenol; Figure 5B – $F_{2,15}$=30.24; P<0.0001, one-way ANOVA, Tukey test), significantly reduced the isoproterenol-induced TMJ nociception (3 µg/TMJ) (P< 0.05, one-way ANOVA, Tukey test), indicating that the isoproterenol-induced TMJ nociception depends on neutrophil migrationand previous TNF-α local release. The pretreatment with either 0.9% NaCl solution (fucoidan vehicle, 200 µL, i.v.) or the vehicle of thalidomide (DMSO + 0.9% NaCl, i.p.) did not affect the isoproterenol-induced TMJ nociception (Figures 5A and B, one-way ANOVA, Tukey test). The pretreatment with fucoidan or thalidomide did not change the nociceptive responses induced by the administration of 0.9% NaCl in the TMJ either (Figures 5A and B, one-way ANOVA, Tukey test).

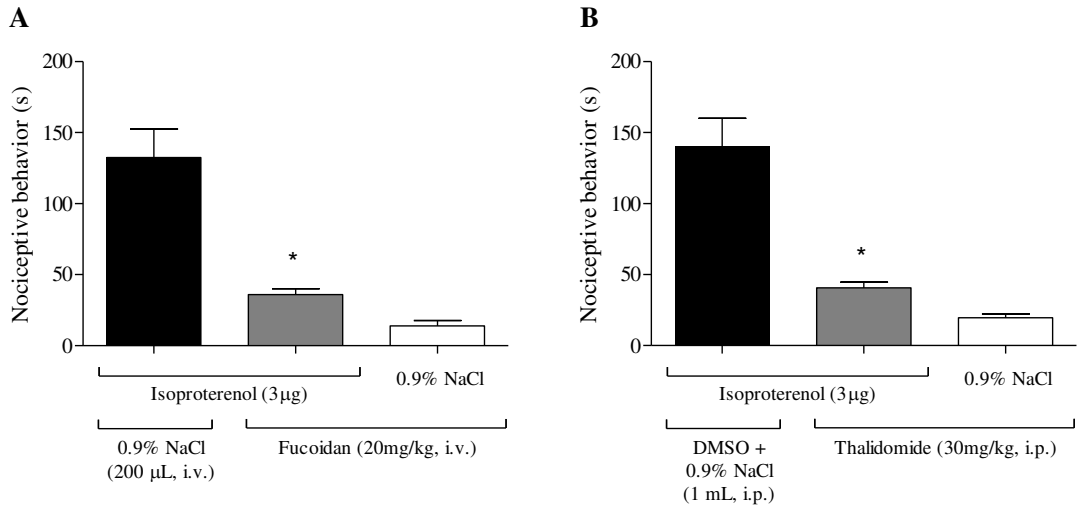

Figure 5. Effects of pretreatment with Fucoidan and thalidomide on isoproterenol-induced TMJ nociception. The pretreatment with fucoidan (A; 20mg/kg, 200µL, i.v. - ANOVA, Tukey test, $F_{2,15}$=26.99; P<0.0001) 20 minutes before the injection of isoproterenol (3 µg/TMJ) and with thalidomide (B; 30mg/kg, 1 mL, i.p. - ANOVA, Tukey test, $F_{2,15}$=30,24; P<0.0001) 30 minutes before the injection of isoproterenol (3 µg/TMJ) significantly reduced the isoproterenol-induced TMJ nociception, as indicated by the symbol "*".

Discussion

In this study we showed that the β-blocker propranolol has anti-inflammatory effects that contribute to its antinociceptive action in the TMJ of female rats. Evidence that propranolol has anti-inflammatory effects relies on the fact that co-administration with the inflammatory agent carrageenan into the TMJ of female rats significantly reduced carrageenan-induced plasma extravasation, neutrophil migration and the release of the pro-inflammatory cytokines TNF-α, IL-1β, and CINC-1. Evidence that the anti-inflammatory effects induced by propranolol in the TMJ contribute to its antinociceptive action in the TMJ is that the pretreatment with the TNF-α inhibitor thalidomide or with the nonspecific selectins inhibitor fucoidan used to inhibit neutrophil migration significantly reduced the TMJ nociception induced by the β-adrenergic receptor agonist isoproterenol.

Our results corroborate those obtained inprevious studies showing that the administration of carrageenan to the articular region induces plasma extravasation, the release of the pro-inflammatory cytokines TNF-α, IL-1β, and CINC-1 and neutrophil migration (Denadai-Souza et al., 2009, Pena-dos-Santos et al., 2009; Teixeira et al., 2016 a and b) However, to our best knowledge, this is the first demonstration that the blockade of β-adrenoceptors in the articular tissue reduces inflammation.

One of the inflammatory parameters reduced by the co-administration of propranolol with carrageenan in the TMJ was the plasma extravasation. Because the plasma extravasation precedes the development of edema, this finding is in agreement with previous studies showing that propranolol decreases peripheral (Bhattacharya & Das, 1986) and traumatic brain injury edema (Kota et al., 2016). It has been previously shown that the activation of β2-adrenoceptors in normal skin leads to increased capillary permeability (Cassuto et al., 2005). Therefore, the effect of local propranolol in reducing carrageenan-induced TMJ plasma extravasation may result from the blockade of the β-adrenoceptors expressed on endothelial cells (Steinberg et al., 1984).

Another inflammatory parameter reduced by the co-administration of propranolol with carrageenan in the TMJ was the release of pro-inflammatory cytokines. It has been previously shown that carrageenan induces the release of pro-inflammatory cytokines in a cascade manner (Cunha et al., 1991; Cunha et al., 1992; Ferreira et al., 1993a). TNF-α triggers the

release of IL-1β and CINC-1 (Ferreira et al., 1993a; Ferreira et al., 1993b) which ultimately leads to the synthesis of the final inflammatory mediators prostaglandins and sympathomimetic amines (Cunha et al., 1991; Ferreira et al., 1993a). Therefore, our findings that propranolol significantly reduced the increased concentration of TNF-α, IL-1β and CINC-1 induced by the administration of carrageenan into the TMJ region suggests that activation of β-adrenoceptor in the TMJ region is particularly important for the TNF-α release. Our data are consistent with previous studies showing that the activation of β-adrenoceptors by norepinephrine and epinephrine leads to increased levels of pro-inflammatory cytokines in rat plasma (Nackley et al., 2007). They are also consistent with a previous report that the treatment of monocytic cells with a β2-adrenoceptor agonist leads to an increased production of the pro-inflammatory cytokines IL-1β and IL-6 (Tan et al., 2007).

In the articular tissue, the pro-inflammatory cytokines IL-1β, TNF-α, and CINC-1 are produced by synovial macrophages (Firestein et al., 2003). The interaction of macrophages with various pathogens is under tonic sympathetic control which involves the activation of β-adrenoceptors expressed on these cells (Abrass et al., 1985; Spengler et al., 1990; Vizi, 1998). Thus, the release of sympatomimetic amines during TMJ inflammation may activate the β-adrenoceptors expressed on macrophages, resulting in the release of pro-inflammatory cytokines, which in turn, contribute to the inflammatory response. Many studies have found increased levels of pro-inflammatory cytokines in the synovial fluid of patients with TMD pain and have suggested that it may play a role in this pain condition (Kaneyama et al., 2010; Kim et al., 2012). Therefore our findings that propranol reduced the release of pro-inflammatory cytokines in the TMJ may be of clinical relevance for the treatment of TMD pain.

The last inflammatory parameter reduced by the co-administration of propranolol with carrageenan in the TMJ was the neutrophil migration, a key event related to tissue injury (Bradley et al., 1982; Secco et al., 2003). This finding suggests that the activation of the β-adrenoceptors by sympatomimetic amines released during TMJ inflammation is critical for the neutrophils recruitment to the injured joint. Neutrophil migration can be induced by the pro-inflammatory chemokine CINC-1. Therefore, the effect of propranolol in reducing neutrophil migration may result from its effect in reducing carrageenan-induced increased concentration of CINC-1.

The analgesic action of propranolol in TMJ pain conditions has been previously reported (Rodrigues et al., 2006; Light et al., 2009; Tchivileva et al., 2010; Fávaro-Moreira et al., 2012 and 2015), but the mechanisms involved in propranolol-induced TMJ analgesia were not known.This study shows not only that propranolol has anti-inflammatory effect but also that this anti-inflammatory effect may contribute to its previously reported analgesic action in the TMJ.The evidence is that the activation of β-adrenoceptors in the TMJ region induced nociception that was significantly reduced by the TMJ pretreatment with thalidomide or fucoidan, which inhibits neutrophil migration and the synthesis of the pro-inflammatory cytokine TNF-α, respectively.

The β-adrenoceptor activation in the TMJ region was induced by the TMJ injection of the β-adrenoceptor agonist isoproterenol. The TMJ nociception induced by isoproterenol was blocked by the co-administration of propranolol. Because isoproterenol is a low potency agonist at α-adrenoceptors (Copik et al., 2015), our findings that isoproterenol-induced nociception was not affected by the co-administration of the non-selective α-adrenergic antagonist phentolamine at a dose that reduces formalin-induced nociception (Khasar et al. ,1999) confirms that isoproterenol-induced nociception was due only to β-adrenoceptor activation.

In the present study we used a dose of thalidomide (30mg/kg) lower than the usual dose (45mg/kg) because the usual dose caused mild sedation in the animals. In fact, this observation is in agreement with its dose dependent sedative effect on the central nervous system, reducing the voluntary activity of the animals and promoting sleep (Somers, 1960).

In summary, we have shown that blockade of β-adrenoceptorsin the TMJ region has an anti-inflammatory effect that contributes to the analgesic action of β-blockers in the TMJ. Therefore, the β-blockers can be used to treat TMJ pain conditions, especially those associated to inflammation.

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II. Conclusões

O bloqueio dos β-adrenoceptores na região da ATM reduz a inflamação induzida pela carragenina na articulação temporomandibular em ratas fêmeas. Portanto, além do efeito analgésico previamente descrito, os β-bloqueadores também possuem um efeito anti-inflamatório na ATM que contribui para seu efeito analgésico. Nesse contexto, os resultados deste trabalho sugerem que os β-bloqueadores podem ser utilizados para tratar as condições dolorosas da ATM, especialmente quando elas estão associadas a um processo inflamatório.

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V. Anexos

Certificado aprovação pelo Comitê em Pesquisa Animal da Universidade Estadual de Campinas:

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto **"Participação dos β adrenoceptores na inflamação da ATM e a relação entre o efeito anti-nociceptivo e anti-inflamatório"** (protocolo nº **3776-1**), sob a responsabilidade de **Profa. Dra. Claudia Herrera Tambeli / Fernanda Barchesi Zanelatto**, 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 **06 de abril de 2015**.

Campinas, 06 de abril de 2015.

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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Efeitos anti-inflamatorios do propranolol na articulação temporomandibular de ratas e sua contribuição na ação antinociceptiva**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 12 de Dezembro de 2016.

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