UNIVERSIDADE FEDERAL DO CEARÁ

***CAMPUS* DE SOBRAL**

PROGRAMA DE PÓS - GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

JOSÉ MÁRIO DOS SANTOS PACHÊCO

O PAPEL DOS RECEPTORES OPIÓIDES CENTRAIS NO EFEITO ANTINOCICEPTIVO DE UM POLISSACARÍDEO SULFATADO ISOLADO DA ALGA MARINHA *SOLIERIA FILIFORMIS* NA HIPERNOCICEPÇÃO INDUZIDA PELA FORMALINA NA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATOS

SOBRAL-CE
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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Ceará – UFC, *Campus* Sobral, como requisito parcial para obtenção do Título de Mestre em Ciências da Saúde. Área de concentração: Saúde.

Orientadora: Profª Dra. Hellíada Vasconcelos Chaves

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2015

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Curso de Medicina de Sobral

P12p Pachêco, José Mário dos Santos.
O papel dos receptores opióides centrais no efeito antinociceptivo de um polissacarídeo sulfatado isolado da alga marinha *solieria filiforms* na hipernocicepçao induzida pela formalina na articulação temporomandibular de ratos. / José Mário dos Santos Pachêco. – 2015.
51 f. : il. color., enc. ; 30 cm.

Dissertação (mestrado) – Universidade Federal do Ceará, Curso de Medicina *Campus* de Sobral, Programa de Pós-Graduação em Ciências da Saúde, Sobral, 2015.
Área de Concentração: Clínica odontológica.
Orientação: Profa. Dra. Hellíada Vasconcelos Chaves.
Coorientação: Profa. Dra. Mirna Marques Bezerra.

1. Receptores opióides. 2. Medição da dor. 3. Articulação temporomandibular. I. Título.

CDD 617.6

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APROVADO EM: __/__/2015
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DEDICATÓRIA

Aos meus pais, Cosma Pachêco e Mário Pachêco, por serem meus exemplos;

Às minhas avós, Emília Fontenele e Norma Pachêco, a base de nossas famílias;

Aos meus irmãos, Emília Pachêco e Gabriel Pachêco, meus melhores amigos.

AGRADECIMENTOS ESPECIAIS

A minha orientadora Profª Drª,

Hellíada Chaves, pela paciência, exigência e disposição para ajudar e querer sempre o melhor;

A Minhas amigas,

Lorena Vasconcelos e Alice Ramos, sem vocês não conseguiria chegar até aqui;

A Profª Drª,

Mirna Marques, pela ajuda e ensinamentos ao longo desta caminhada.

Aos amigos,

Que compõem o LaFS, e que nos momentos de dificuldades sempre se mostram mais unidos em prol da pesquisa.

AGRADECIMENTOS

Aos professores do Programa de Pós-graduação em Ciências da Saúde que muito contribuíram para minha formação acadêmica.

À Coordenação de Aperfeiçoamento de Pessoal de nível Superior (Capes) pela concessão de bolsa de mestrado.

Ao Laboratório de Carboidratos e Lectinas (CarboLec) pela disponibilização da molécula extraída a partir da alga marinha *Solieria* filiformis.

A todos que, mesmo não citados aqui, contribuíram de alguma forma para a realização deste trabalho.

EPÍGRAFE

Conhecer o homem – esta é a base de todo o sucesso.

Charles Chaplin

RESUMO

Introdução: O presente estudo foi desenhado para investigar o efeito de um polissacarídeo sulfatado da alga marinha *Solieria filiformis* (fração FII) na hipernocicepção inflamatória na articulação temporomandibular (ATM) de ratos. **Métodos**: Foram utilizados 5 ratos machos Wistar (180-240 g) por grupo. Os ratos foram pré-tratados (30 min) com uma injecção subcutânea (s.c.) de salina ou de FII (0.03, 0.3 ou 3.0 mg/kg), seguido por injecção intra-ATM de 1,5% de formalina ou 5-hidroxitriptamina (5-HT, 225 µg/ATM). Em outro conjunto de experimentos, os ratos foram pré-tratados (15 min) com uma injecção intratecal dos receptores opióides não-selectivos de naloxona, ou antagonista do receptor opióide-µ CTOP, ou antagonista do receptor opióide-δ Naltridole, ou antagonista do receptor opióide-κ- Nor-binaltorfimina (nor-BNI) seguido por injecção de FII (s.c.). Após 30 min, os animais foram induzidos a hipernocicepção com uma injecção intra-ATM de 1,5% de formalina. Após a indução na ATM, a resposta nocicptiva comportamental foi avaliada durante um período de observação de 45 ou 30 minutos para exeperimentos com formalina ou serotonina, respectivamente. Em seguida, os animais foram eutanasiados e coletados tecido periarticular, gânglio trigeminal e subnúcleo caudal (SC) para dosagem de opióides endógenos e citocinas por ELISA e, extravasamento de plasma. **Resultados**: O pré-tratamento com FII reduziu a nocicepção induzida por formalina e serotonina na ATM ($P < 0,05$: ANOVA, teste de Bonferroni). O pré-tratamento com FII inibiu o extravasamento de plasma e liberação de citocinas inflamatórias (TNF-α e IL-1β) induzida por formalina na ATM ($P < 0,05$). O pré-tratamento com injecção intratecal de naloxona, CTOP, Naltridole ou nor-BNI bloqueou o efeito antinociceptivo de FII na nocicpeção induzida por formalina na ATM ($P < 0,05$). Além disso, FII foi capaz de aumentar significativamente a liberação de β-endorfina no subnúcleo caudal. **Conclusões**: Os resultados sugerem que FII tem um potente efeito antinociceptivo e anti-inflamatório na ATM mediada pela ativação de receptores opióides no subnúcleo caudal e inibição da liberação de mediadores inflamatórios nos tecidos periarticulares.

Palavras-chave: alga marinha; polissacarídeo sulfatado; nocicepção; inflamação; receptores opióides

ABSTRACT

Background: The current study was designed to investigate the effect of sulfated polysaccharides from red seaweed *Solieria filiformis* (Fraction FII) in the inflammatory hypernociception in the temporomandibular joint (TMJ) of rats. **Methods:** Male Wistar rats (180–240 g). Rats were pretreated (30 min) with a subcutaneous injection (s.c.) of vehicle or FII (0.03, 0.3 or 3.0 mg/kg) followed by intra-TMJ injection of 1.5% Formalin or 5-hydroxytriptamine (5-HT, 225 µg/TMJ). In other set of experiments rats were pretreated (15 min) with an intrathecal injection of the non-selective opioid receptors Naloxone, or µ-opioid receptor antagonist CTOP, or δ-opioid receptor Naltridole hydrochloride, or κ-opioid receptor antagonist Nor-Binaltorphimine followed by injection of FII (s.c.). After 30 min, the animals were treated with an intra-TMJ injection of 1.5 % formalin. After TMJ treatment, behavioral nociception response was evaluated for a 45-min observation period, animals were terminally anesthetized and periarticular tissue, trigeminal ganglion and subnucleus caudalis (SC) were collected plasma extravasation and ELISA analysis. **Results:** Pretreatment with FII significantly reduced formalin- and serotonin-induced TMJ nociception ($P<0.05$: ANOVA, Bonferroni's test). Pretreatment with FII significantly inhibit the plasma extravasation and inflammatory cytokines (TNF-α and IL-1β) release induced by 1.5% formalin in the TMJ ($P<0.05$). Pretreatment with intrathecal injection of Naloxone, CTOP, Naltridole or Nor-BNI blocked the antinociceptive effect of FII in the 1.5% formalin-induced TMJ nociception ($P<0.05$). In addition, FII was able to significantly increase the β-endorphin release in the subnucleus caudalis. **Conclusions:** The results suggest that FII has a potential antinociceptive and anti-inflammatory effect in the TMJ mediated by activation of opioid receptors in the subncucleus caudalis and inhibition of the release of inflammatory mediators in the periarticular tissue.

Key words: marine algae; sulfated polysaccharide; nociception; inflammation; opioid receptors

LISTA DE ABREVIATURAS

Disfunção temporomandibular	DTM
Articulação temporomandibular	ATM
Polissacarídeo sulfatado	F II
Nor-binaltorfimina	nor-BNI
Subcutânea	s.c.
Gânglio trigêminal	GT
Subnúcleo caudal	SC
Anti-inflamatórios não esteroidais	AINES

SUMÁRIO

1. RELEVÂNCIA E JUSTIFICATIVA

As desordens temporomandibulares (DTM) envolvem uma etiologia multifatorial que resulta em dor na articulação temporomandibular (ATM) levando a dor orofacial que frequentemente limita a fala, mastigação e outras atividades diárias básicas com altos níveis de incapacidade relacionadas à dor inflamatória (CAIRNS, 2010). O alívio da dor na ATM é um desafio já que as DTMs envolvem tecidos profundos, o que torna difícil direcionar o sistema neural trigeminal (CAIRNS, 2010; CHIANG, 2011). Quando as estratégias tradicionais para controle da dor relacionadas às DTM são insatisfatórias, é imperativo para o clínico geral adotar uma abordagem baseada em evidências para o tratamento da dor orofacial (CAIRNS, 2010; DEROSSI, 2013).

Para desenvolver ferramentas potenciais através de novas terapias na melhora da dor inflamatória tem havido um aumento contínuo na utilização de produtos naturais, o que estimula estudos científicos na procura de novas substâncias com ação terapêutica (VAL *et al.,* 2014). Sendo assim, os organismos marinhos podem servir como fontes de compostos bioativos estruturalmente diversos com potenciais biomédicas (JIANG *et al.,* 2010;. YASUHARA-BELL E LU, 2010). Entre as substâncias biossintetizadas por algas, os polissacarideos sulfatados têm diversas atividades biológicas, incluindo a antioxidante (MELO *et al.,* 2013;. CÂMARA *et al.,* 2011;. MAGALHÃES, *et al.,* 2011;. COSTA *et al.,* 2010;. SOUZA *et al.,* 2007), imunomoduladora (AHN *et al.,* 2008; ZHOU *et al.,* 2004), antiviral (YASUHARA-BELL E LU, 2010), anti-inflamatória (ALBUQUERQUE *et al.,* 2013;. ANANTHI et al., 2010), anti-nociceptiva (ALBUQUERQUE *et al.,* 2013;. ASSREUY *et al.,* 2008; VIANA *et al.,* 2002), anti-tumoral (LINS *et al.,* 2009), e anti-proliferativa (MAGALHÃES *et al*., 2011). Por isso, nosso grupo mostrou previamente que os polissacarídeos sulfatados a partir de algas vermelhas e verdes têm potencial como drogas anti-inflamatórias e antinociceptivas (RODRIGUES *et al.,* 2014; QUINDERÉ *et al.,* 2013; COURA *et al.,* 2012;. VANDERLEI *et al*., 2011;. ARAÚJO *et al.,* 2011; CARNEIRO *et al.,* 2014; RODRIGUES *et al.,* 2012).

A alga marinha vermelha *Solieria filiformis* foi escolhida para realizar este estudo por ser uma espécie que já vem sendo cultivada ao longo da costa

do Estado do Ceará, apresentando uma biomassa considerável para a maricultura (RODRIGUES *et al*., 2010), visando a produção de carragenanas, que é um ficocoloide de grande interesse para indústria farmacêutica (CAMPO *et al.,* 2009). Além disso, existem relatos na literatura do efeito de seus polissacarídeos sulfatados na atividade analgésica em modelos clássicos de nocicepção (ARAÚJO *et al*., 2011) e na hiperalgesia da ATM no modelo de artrite induzida por zymosan na ATM de ratos (ARAÚJO, 2012). No entanto, o mecanismo de ação do efeito antinociceptivo dos polissacarídeos sulfatados de *S. filiformis* na dor orofacial ainda não foram elucidados, sendo este o primeiro relato da utilização de polissacarídeos sulfatados de algas marinhas no modelo de hipernocicepção na ATM.

2. PROPOSIÇÃO

2.1 Objetivo Geral:

Avaliar o papel dos receptores opióides centrais no efeito antinociceptivo de um polissacarídeo sulfatado isolado da alga marinha *Solieria filiformis* na hipernocicepção induzida pela formalina na articulação temporomandibular de ratos

2.2. Objetivos Específicos:

Avaliar o efeito antinociceptivo de um polissacarídeo sulfatado da alga marinha *S. filiformis* (F II) através do modelo comportamental de hipernocicepção induzido por formalina na ATM de ratos;

Investigar o papel dos receptores opióides endógenos na resposta antinociceptiva de F II após injeção intratecal de antagonistas dos receptores Mu (μ), Kappa (κ) e Delta (δ) no modelo comportamental de hipernocicepção induzido por formalina na ATM de ratos;

Avaliar o envolvimento de opioides endógenos na resposta antinociceptiva de F II através da dosagem dos opióides endógenos (β-endorfinas e dinorfina) em amostras do tecido periarticular, gânglio trigeminal e subnúcleo caudal.

Avaliar os efeitos do polissacarídeo sulfatado da alga *S. filiformis* (F II) no extravazamento plasmático do modelo comportamental de hipernocicepção induzido por formalina na ATM de ratos;

Avaliar os efeitos do de F II sobre as citocinas primárias TNFα e IL-1β em amostras do tecido periarticular da ATM no modelo comportamental de hipernocicepção induzido por formalina na ATM de ratos;

Avaliar o efeito antinociceptivo de F II através do modelo comportamental de hipernocicepção induzido por serotonina na ATM de ratos.

3. CAPÍTULO 1

PAPER

Title: Central opioid receptors on temporomandibular joint antinociception from seaweed *Solieria filiformis*

Running head: *Solieria filiformis* reduces hypernociception in the TMJ.

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The category for which the manuscript is being submitted: Original articles

Funding sources: This work was supported by Brazilian grants from Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), Conselho Nacional de Pesquisa (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Instituto de Biomedicina do Semi-Árido Brasileiro (INCT-IBISAB).

Conflicts of Interest: There are no conflicts of interest.

Answers to each of the following questions in 2 or 3 bulleted statements (not exceeding 70 words): 'what's already known about this topic?' and 'what does this study add?'.

Studies have shown the antinociceptive properties of the sulfated polysaccharide isolated from the red seaweed *Solieria filiformis* (Fraction FII). The FII induces an antinociceptive effect in the inflammatory hypernociception in the TMJ as a result of the inhibition of inflammatory mediators suggesting an anti-inflammatory effect. In addition, FII stimulated the release of endogenous opioids peptides and activation of μ- κ- δ-opioid receptors in the subnucleus caudalis.

Abstract

Background: The current study was designed to investigate the effect of sulfated polysaccharides from red seaweed *Solieria filiformis* (Fraction FII) in the inflammatory hypernociception in the temporomandibular joint (TMJ) of rats. **Methods:** Male Wistar rats (180–240 g). Rats were pretreated (30 min) with a subcutaneous injection (s.c.) of vehicle or FII (0.03, 0.3 or 3.0 mg/kg) followed by intra-TMJ injection of 1.5% Formalin or 5-hydroxytriptamine (5-HT, 225 µg/TMJ). In other set of experiments rats were pretreated (15 min) with an intrathecal injection of the non-selective opioid receptors Naloxone, or µ-opioid receptor antagonist CTOP, or δ-opioid receptor Naltridole hydrochloride, or κ-opioid receptor antagonist Nor-Binaltorphimine followed by injection of FII (s.c.). After 30 min, the animals were treated with an intra-TMJ injection of 1.5 % formalin. After TMJ treatment, behavioral nociception response was evaluated for a 45-min observation period, animals were terminally anesthetized and periarticular tissue, trigeminal ganglion and subnucleus caudalis (SC) were collected plasma extravasation and ELISA analysis. **Results:** Pretreatment with FII significantly reduced formalin- and serotonin-induced TMJ nociception ($P<0.05$: ANOVA, Bonferroni's test). Pretreatment with FII significantly inhibit the plasma extravasation and inflammatory cytokines (TNF-α and IL-1β) release induced by 1.5% formalin in the TMJ ($P<0.05$). Pretreatment with intrathecal injection of Naloxone, CTOP, Naltridole or Nor-BNI blocked the antinociceptive effect of FII in the 1.5% formalin-induced TMJ nociception ($P<0.05$). In addition, FII was able to significantly increase the β-endorphin release in the subnucleus caudalis. **Conclusions:** The results suggest that FII has a potential antinociceptive and anti-inflammatory effect in the TMJ mediated by activation of opioid receptors in the subncucleus caudalis and inhibition of the release of inflammatory mediators in the periarticular tissue.

Key words: marine algae; sulfated polysaccharide; nociception; inflammation; opioid receptors

1. Introduction

Temporomandibular disorders (TMD) involve multifactorial etiology and might result in temporomandibular joint (TMJ) pain leading to orofacial pain which frequently limits talking, chewing and other basic daily activities with high levels of inflammatory pain-related disability (Cairns, 2010). Relieving Temporomandibular Joint (TMJ) pain is a challenge since TMDs involve deep tissues, making it difficult to target the trigeminal neural system (Cairns, 2010; Chiang, 2011). Since traditional strategies to control TMD-related pain are unsatisfactory, it is imperative for the general clinician to adopt an evidence-based approach to the management of orofacial pain (Cairns, 2010; DeRossi, 2013).

In order to develop potential tools for new therapies to improve inflammatory pain, there has been a continuous increase in the use of natural products, which has encouraged scientific studies to search for new substances with therapeutic action (Val et al., 2014). In this regard, marine organisms may serve as sources for structurally diverse bioactive compounds with biomedical potentials (Jiang et al., 2010; Yasuhara-Bell and Lu, 2010). Among the substances biosynthesized by seaweed, the sulfated polysaccharides have diverse biological activities, including antioxidant (Melo et al., 2013 ;Camara et al., 2011; Magalhaes, et al., 2011; Costa et al., 2010; Souza et al., 2007), immunomodulatory (Ahn et al., 2008; Zhou et al., 2004), antiviral (Yasuhara-Bell and Lu, 2010), anti-inflammatory (Albuquerque et al., 2013; Ananthi et al., 2010), antinociceptive (Albuquerque et al., 2013; Assreuy et al., 2008; Viana et al., 2002), antitumor (Lins et al., 2009), and antiproliferative (Magalhaes, et al., 2011). Therefore, our group has previously shown that sulfated polysaccharides from red and green seaweeds have potential as novel anti-inflammatory and analgesic drugs (Rodrigues et al., 2014; Quinderé el al. 2013; Coura et al., 2012; Vanderlei et al., 2011; Araújo et al., 2011; Carneiro et al., 2014; Rodrigues et al., 2012).

The cell wall of red seaweed is composed of sulfated galactans (agaranas and/or carrageenan). The best known types of commercial carrageenans are ι-carrageenan, κ-carrageenan and λ-carrageenan. Araújo et al. (2011) observed by infrared spectra the total extract of sulfated

polysaccharides from *Solieria filiformis* contains both κ- and ι-carrageenan, and when the sulfated polysaccharides were fractionated by ion exchange chromatography, fractions I and II (F I and F II) are identified as κ- and ι-carrageenan, respectively. These authors showed F I antinociceptive and anti-inflammatory effects in rats (Araújo et al., 2011). The structural components of these polysaccharides are mainly a 3,6-anhydrogalactose 2-sulfate→galactose 4-sulfate (DA2S-G4S)-type structure which can be characteristic of gelling carrageenans with a dominant *iota* repeating structure. Furthermore, a typical resonance from *kappa*-carrageenan was also detected in *S. filiformis*, although with a very low intensity (Murano et al., 1997).

Therefore, the current study was designed to investigate the effect of sulfated polysaccharides from seaweed *Solieria filiformis* (FII) in the inflammatory hypernociception induced in the TMJ of rats.

2. Methods

2.1. Animals

This study was carried out with male Wistar rats (180–240 g) from the Animal Care Unit of the Federal University of Ceará in Fortaleza, Brazil. The animals were housed in a temperature-controlled room (23 ± 2 ºC) with free access to water and food on a 12:12 light cycle. For each experiment, groups of five animals were segregated and handled separately. All animal experimental procedures and protocols were approved by the Ethics Committee of the Federal University of Ceará, Fortaleza, Brazil (CEPA nº 76\12) and are in accordance with guidelines of National Council for Control of Animal Experimentation (CONCEA) and International Association for the Study of Pain (IASP) guidelines for the study of pain in conscious (Zimmermann, 1983).

2.2. Drugs

Formalin was prepared from commercially (Sigma Chemicals, Perth, Australia) available stock formalin (an aqueous solution of 37% of formaldehyde) further diluted in 0.9% NaCl to a concentration of 1.5%. Indomethacin – non-steroidal anti-inflammatory drug – used as positive control; naloxone - a non selective opioid antagonist; CTOP - antagonist of Mu (μ)

opioid receptor; naltridole hydrochloride - antagonist of Delta (δ) opioid receptor; and Nor-Binaltorphimine (Nor-BNI) - antagonist of Kappa (κ) opioid receptor; and 5-Hidroxytryptamine (5-HT) were purchased from Sigma Aldrich, St. Lois, MO, USA. Morphine sulfate (Dimorf®) was purchased from Cristália (Itapira, SP, Brazil).

2.3. Isolation of sulfated polyssacharides from the red seaweed *Solieria filiformis*

Solieria filiformis was obtained from the Atlantic coast of Brazil (Flecheiras Beach, Trairí-Ceará). After collection, specimens were transported to the Carbohydrates and Lectins Laboratory (CarboLec), Department of Biochemistry and Molecular Biology, Federal University of Ceará and then cleaned of epiphytes, washed with distilled water and stored at -20°C until further use. A voucher specimen (nº. 35682) was deposited in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil.

The extraction procedure of polysaccharides was performed according to Araújo et al. (2011) and Farias et al. (2000). The total sulfated polysaccharide obtained from *Solieria filiformis* (50 mg) was dissolved in 25 ml of 50 mM sodium acetate buffer (pH 5.0) and applied to a DEAE-cellulose column (26×2.0 cm) equilibrated with the same buffer. The elution was performed by a stepwise gradient of 0 - 1.5 M NaCl in the same solution with of intervals of 0.25M between each concentration. The flow rate of the column was 2.3 ml/min. The fraction FII was eluted with 0.75 M NaCl, and analyzed using the metachromatic assay (A_{525} nm) with DMB as described by Araújo et al. (2011) and Famdale et al. (1986). The biological protocols were performed with the fraction F II (ι-carrageenan).

2.4. Temporomandibular Joint Injection

Animals were briefly anesthetized by inhalation of isoflurane and a 30-gauge needle was introduced into the left TMJ at the moment of injection. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 µl). Intra-TMJ injections volumes were 50 µl in all

cases. Each animal regained consciousness approximately 30 s after discontinuing the anesthetic.

2.5. Testing Procedure for TMJ Pain

Testing sessions took place during light phase (between 9:00 am and 5:00 pm) in a quiet room maintained at 23 ± 2 °C (Rosland et al., 1991). Each animal was manipulated for 7 days to be habituated to the experimental manipulation. After this period, the 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. Each animal immediately recovered from anesthesia after TMJ injection and was returned to the test chamber for counting nociceptive responses during the following 45 min observation period. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period. Since head flinches followed a uniform pattern of 1 second of duration, each flinch was expressed as 1 second (Roveroni et al., 2001; Clemente et al., 2004). Results are expressed as the duration time of nociceptive behavior. At the conclusion of the nociceptive behavioral testing, animals were terminally anesthetized by isoflurane and their periarticular tissue, trigeminal ganglion and subnucleus caudalis were removed for further analyses.

2.6. Experimental protocols

2.6.1. Effect of FII on formalin-induced TMJ nociception. Rats were pretreated (30 min) with a subcutaneous injection (s.c.) of FII (0.03, 0.3 or 3.0 mg/kg; s.c.; n=5/group) followed by intra-TMJ injection of 1.5 % formalin in a final volume of 50 µl. At the conclusion of the nociceptive behavioral testing, animals were terminally anesthetized by isoflurane and their periarticular tissue, trigeminal ganglion and subnucleus caudalis were removed for further analyses.

2.6.2. Role of opioid receptors on FII antinociceptive effect in the TMJ. Rats were pretreated (15 min) with an intrathecal injection of the non-selective antagonist of opioid receptors naloxone (15 µg/10 µl; n=5/group) (Eisenberg et

al., 1996); or inhibitor of μ-opioid receptor CTOP (0.2 or 10 μg/10 μl; n=5/group) (Picolo et al., 2000); or the inhibitor of δ -opioid receptor Naltrindole (10 or 30 μg/10 μl; n=5/group) (Picolo et al., 2000); or the selective κ-opioid receptor antagonist Nor-BNI (15 or 90 μg/10 μl; n=5/group) (Clemente et al., 2004) followed by s.c. injection of FII (0.3 mg/kg /s.c / n=5) 30 min prior intra-TMJ injection of 1.5 % formalin (50 μl /TMJ). followed by F II (0.3 mg/kg/s.c). After 30 min, animals received an intra-TMJ injection of 1.5 % formalin (50 μl). Behavioral nociception response was evaluated for 45 min observation period. All animals received a final volume of 50 μl of solutions into TMJ.

2.6.3. Effect of FII in formalin-induced plasma-protein extravasation in the TMJ. Different group of rats were pretreated (30 min) with a subcutaneous injection (s.c.) of FII (0.03, 0.3 or 3.0 mg/kg; s.c.; n=5/group) followed by intra-TMJ injection of 1.5 % formalin in a final volume of 50 μl. Immediately after formalin 1.5 % (50 μg/TMJ) injection, the Evan's Blue dye (1%, 5 mg/kg) was injected into the left femoral vein and 45 min later TMJ inflammation was assessed by the extravasation of Evan's Blue dye bound to plasma protein (Haas et al., 1992; Fiorentino et al., 1999). Each rat was then sacrificed under deep anesthesia and perfused with saline. TMJ tissues were dissected, weighed and stored at -20 °C. Evans' Blue dye was extracted by immersing the joint tissue in 1 ml of formamide at 60 °C for 24 h. The samples absorbance was then determined in a spectrophotometer (Genesys) at 620 nm, and the Evan's Blue dye concentration determined by comparison to a standard curve of known amounts of Evan's Blue dye in extraction solution, which was assessed within the same assay. The amount of Evan's Blue (micrograms) was then calculated per gram weight of tissue.

2.6.4. Effect of FII on formalin-induced release of inflammatory cytokines Tumor Necrosis Factor–α (TNF-α) and Interleukin-1β (IL-1β) in the TMJ. Periarticular tissues were homogenized in a solution of RIPA Lysis Buffer System (Santa Cruz Biotechnology, USA). The samples were centrifuged at 10.000 rpm for 15 min at 4 °C. The supernatants were stored at −80 °C for later use to evaluate the protein levels of inflammatory cytokines TNF- α and IL-1 β in the TMJ tissues. The cytokines were quantified by the following kits: TNF-α–Rat TNF-

alpha/TNFSF1A Quantikine ELISA Kit (R&D Systems, catalog number RTA00) and IL-1β–Rat IL-1 beta/ IL-1F2 Quantikine ELISA Kit (R&D Systems, catalog number DY501). All procedures followed the instructions of the manufacturer R&D Systems. The absorbance was measured at 450 nm. IL-1β and TNF-α concentrations were expressed as pg/mL.

2.6.5. Effect of FII on the release of opioid peptides in the periarticular tissues, trigeminal ganglion and subnucleus caudalis. Samples of periarticular tissues, trigeminal ganglion and subnucleus caudalis were homogenized, separately, in a solution of RIPA Lysis Buffer System (Santa Cruz Biotechnology, USA). After homogenization, the samples were centrifuged (3000 rpm / 10 min), and the supernatant was used to evaluate the expression of the endogenous opioid β-endorphin (Phoenix Pharmaceuticals, Inc., code: EK-RAB-022-33) and dinorphin (Phoenix Pharmaceuticals, Inc., code: EK-021-03) according to all procedures in the instructions of the manufacturer. Add 50 µL\weel of standard sample, or positive control, 25 µL primary antibody and 25 µL biotinylated peptide. After Incubation at room temperature (20-23 ºC) for 2 hours, plates were washed immunoplate 4 times with 350 µL\well of assay buffer. It was added 100 µl\well of SA-HRP solution. After incubation at room temperature (20-23 ºC) for 1 hour, plates were washed 4 times with 350 µl\well of assay buffer. Then, add 100 µL\well of TMB substrate solution (Pharmaceuticals, Inc., code:EK-SS). After incubation at room temperature (20-23 ºC) for 1 hour, terminate reaction with 100 µL\well of 2N HCI and the absorbance O.D. at 450 nm was determined. β-endorphin concentration was expressed as ng/mL.

2.6.6. Effect of the FII on 5-HT-induced TMJ nociception. Rats were pretreated (30 min) with a subcutaneous injection (s.c.) of FII (3 mg/kg; n=5/group) followed by intra-TMJ injection 5-hydroxytriptamine (5-HT) (225 µg; 50 µl) (Oliveira-Fusaro et al., 2012). Behavioral nociception response was evaluated for 30 min observation period.

2.7. Statistical Analysis

To determine if there were significant differences ($P<0.05$) among treatment groups, the data were analyzed using the one-way ANOVA. If there was a significant between-subjects main effect of treatment group following one-way ANOVA, post-hoc contrasts, using the Bonferroni test, were performed to determine the basis of the significant difference. Data are presented in figures as means ± SEM.

3. Results

3.1. Sulfated polysaccharide (FII) from seaweed *Solieria filiformis* induced antinociception in the TMJ.

The treatment with FII (0.03, 0.3 and 3.0 mg/kg; s.c.) significantly reduced ($P<0.05$: ANOVA, Bonferroni's test) the nociceptive behavioral response induced by formalin in the TMJ of rats (Figure 1). Considering this result, all further experiments were stablished the dose of 0.3 mg/kg for the next experiments.

3.2. The antinociceptive effect of Sulfated polysaccharide (FII) from seaweed *Solieria filiformis* in the TMJ is mediated by opioid receptors activation in the subnucleus caudalis.

Figure 2A shows that pretreatment with intrathecal application of naloxone (15 µg/10 µl) blocked the antinociceptive effect of FII (0.3 mg/kg) induced in the TMJ ($P<0.05$: ANOVA, Bonferroni's test). Pretreatment with intrathecal injection of the antagonist of Mu (µ) opioid receptor CTOP (0.2 and 10 µg/10 µl) (Figure 2B), or the antagonist of Delta (δ) opioid receptor Naltridole (10 and 30 µg/10 µl) (Figure 2C) or the antagonist of Kappa (κ) opioid receptor Nor-Binaltorphimine (15 and 90 µg/10 µl) (Figure 4D) significantly inhibit the FII antinociceptive effect in the TMJ ($P<0.05$). Taken together, these results suggest that the antinociceptive effect of FII in the TMJ is mediated by µ-, δ-, and κ- opioid receptors activation in the subnucleus caudalis.

3.3. The antinociceptive effect of sulfated polysaccharide (F II) from seaweed *Solieria filiformis* induced the release of the opioid peptide β-endorphin in the subnucleus caudalis.

The treatment with FII (0.3 mg/kg) induced a significantly release of β-endorphin in the subnucleus caudalis (Figure 3C) (P<0.05: ANOVA, Tukey's test) but not in the periaricular tissue or trigeminal ganglion (Figures 3A and 3B) (P>0.05). Dynorphin levels in periarticular tissue, trigeminal ganglion and subnucleus caudalis were not affected by pretreatment with F II (0.3 mg/kg) (Figure 3C, D, E).

3.4. Sulfated polysaccharide (FII) from seaweed *Solieria filiformis* induced anti-inflammatory effect in the TMJ.

Formalin is a noxious stimulus used in animal behavioral experiments that have an inflammatory mechanism. It is well been established that formalin induces tissue damage and the release of inflammatory mediators (Fisher et al., 2008; Macpherson et al., 2007; MacNamara et al., 2007). Considering that we test with the FII has anti-inflammatory properties in the formalin-induced inflammation in the TMJ. The treatment with FII (0.3 mg/kg) significantly reduced plasma extravasation (*P*<0.05) induced by 1.5% formalin in the TMJ (Figura 4A) (P<0.05: ANOVA, Bonferroni's test). In addition, the treatment with FII (0.3 mg/kg) significantly reduced the release of the inflammatory cytokynes TNFα and IL-1β induced by 1.5% formalin in the TMJ of rats (Fig. 4B and C). These results suggested that FII has a potential anti-inflammatory effect in the TMJ.

3.5. Sulfated polysaccharide (F II) from seaweed *Solieria filiformis* inhibits TMJ serotonin (5-HT)-induced nociception

Inflammatory hypernociception is mediated by an interdependent network of pro-inflammatory cytokynes, such as TNF-α and IL-1β, that trigger the release of prostanoids or sympathetic amines that act directly on the nociceptors to cause hypernociception (Verri et al., 2006). Data from literature demonstrates that 5-HT induced a hypernociception mediated by an indirect release of prostanoids and sympathetic amines (Oliveira et al., 2007). Thus, to confirm the anti-inflammatory effect of FII, animals were pretreated (30 min) with a FII (3 mg/Kg) followed by an intra-TMJ injection of 5-HT (225 µg). The intra-TMJ injection of 5-HT significantly increased the nociceptive behavior (*P*<0.05:ANOVA, Bonferroni's test). The treatment with FII significantly reduced

the nociceptive behavior induced by 5-HT (P<0.05). These findings corroborated a potential anti-inflammatory effect of FII.

4. Discussion

In this work, we demonstrated the antinociceptive and anti-inflammatory effect of sulfated polysaccharide (FII) from the red seaweed *Solieria filiformis* in formalin-induced hypernociception in the TMJ of rats. The mechanisms that mediated the antinociceptive and anti-inflammatory effect of FII is mediated, at least in part, by the activation of the release of opioid peptides and activation of opioid receptors in the subnucleus caudalis. In addition, FII was able to inhibit the plasma extravasation and release of inflammatory cytokines in the periarticular tissues induced by formalin.

Sulfated polysaccharides from seaweed are a source of numerous biological activities that may find therapeutic benefit (Jiao et al., 2011). In fact, our group showed previously that the efficacy of sulfated polysaccharide (FI) from the red seaweed *Solieria filiformis* in models of nociception occurs *via* a peripheral mechanism (Araújo et al., 2011), and Sousa et al. (2011) showed anti-inflammatory and antinociceptive effects of marine alga. In agreement with the present work, it was demonstrated that polysaccharides and lectins from green seaweeds have a potential biological compound for the treatment of TMJ inflammatory nociception (Rodrigues et al. 2014; Rivanor et al. 2014). Following that, it was demonstrated that lectin of the green seaweed *Caulerpa cupressoides* (CcL) induces antinociceptive and anti-inflammatory effect of in a model of zymosan-induced inflammatory hypernociception in the TMJ of rats by the inhibition of TNFα and IL-1β release (Rivanor et al., 2014). Taken together these studies demonstrated the potential antinociceptive and anti-inflammatory effect of sulfated polysaccharides from red and green seaweeds (Vanderlei et al., 2011; Coura et al., 2012; Rodrigues et al., 2012; Quinderé et al. 2013; Carneiro et al., 2014; Rodrigues et al. 2014)

In the present study, it was demonstrated that treatment with FII was able to induce an antinociceptive effect in the formalin-induced inflammatory hypernociception in the TMJ of rats. Considering that the systemic administration of FII could activated central opioid systems to control pain

conditions, we test the role of opioid system in the antinociceptive effect induced by FII in the TMJ. To distinguish between central and peripheral antinociceptive actions, it was evaluated the role by central opioid system in the antinociceptive effect of FII using intrathecal injections of the opioids antagonists. These injections evaluated a possible specific central action, in which the opioids agents exert their analgesic effects *via* supraspinal and spinal receptors. In the present study, naloxone, a competitive antagonist of μ-, κ- and δ-opioid receptors (MOR, DOR, KOR, respectively) but with higher affinity for the μ-opioid receptors (Tamaddonfa rd et al., 2011), significantly reduced the antinociceptive effect of FII. Additionally, specific receptor opioid antagonists including: Naltridole (a selective δ-opioid receptor receptor antagonist), Nor-BNI (a selective κ-opioid receptor antagonist), and CTOP (a selective μ -opioid receptor antagonist) were able to reverse the antinociceptive effect of FII in the TMJ of rats. These findings suggest that FII induced-central antinociceptive response in the TMJ may result from the activation of more than one neuronal receptor. Similarly to other studies (Tamaddonfard et al., 2011; Bodnar, 2012), we showed that the antinociceptive effect of FII is similar than that morphine in the orofacial tissues of rats.

It is well known that opioid antinociception is mediated by the activation of central and peripheral receptors (Sánchez, et al., 2010; Kumar, et al., 2013). Further, there is evidence that endogenous opioids are involved in the control of inflammatory pain (Napimoga et al., 2007; Chicre-Alcântara et al, 2011). One theory to explain the generalized hypersensitivity in the TMJ is a reduced capacity to recruit endogenous opioid on pain inhibitory control (Tashiro et al., 2008). This is clearly observed in our study when occur the reduction of the synthesis of endogenous opioid β--endorphin on formalin-induced TMJ nociception. On the other hand, the increased pain threshold might be due to the activation of an endogenous opioid system (Arthuri et al., 2005; Suzuki et al., 2007). In this regard, it was observed in the present study that F II increased β-endorphin levels in the subnucleus caudalis (SC).

Studies utilizing in situ hybridization and reverse-transcription–polymerase chain reaction (RT-PCR) techniques demonstrated that μ, δ, κ opioid receptors are expressed in dorsal root ganglia (DRG) (Nunez et al., 2007). Clemente-Napimoga et al. (2009) showed that kappa opioid receptor has

been found distributed in the axons and cell bodies of adult mouse trigeminal nerve and trigeminal ganglion. In addition, κ-opioid receptor has also been detected in the dorsal root ganglia and trigeminal ganglia (TG) of humans (Xie et al., 1999). Patwardhan et al. (2006) and Nunez et al. (2007) also demonstrated the expression of DOR and MOR in TG, respectively. Our present results demonstrated that all the three opioid receptors are involved in the antinociceptive action of F II in the TMJ hypernociception of rats.

The μ-opioid receptors are presynaptically located on C fibers and inhibit the release of neurotransmitters via blockage of calcium channels of the presynaptic terminal, thereby providing analgesic action (Matsuura et al., 2008). The TMJ region is innervated mainly by small-diameter sensory fibers that project to the trigeminal subnucleus caudalis／upper cervical cord junction, a region that also expresses a high density of nuclear estrogen receptors and μ-opioid receptors (Tashiro et al., 2008).

Nunez et al. (2007) provided the genetic, proteomic and behavioral evidence for the involvement of peripheral MORs in alleviating inflammatory pain arising from craniofacial muscle tissue, and suggested that the all three opioid receptors subtypes are involved in inflammatory responses, but they underwent different transcriptional regulations. Pena-dos-Santos et al. (2009) showed that peripheral κ / δ opioid receptors mediated antinociception in rat temporomandibular joint. Zubrzycka, et al. (2011); Zubrzycka and Janecka, (2011) demonstrated that tooth pulp stimulation significantly up-regulated the mRNA levels for a number of neuropeptides and all the three types of opioid receptors in the rat brain, which would result in more potent antinociception.

Considering the inflammatory events, Chicre-Alcântara et al. (2011) provided evidence that activation of kappa opioid receptors located in the TMJ region reduces two important parameters of inflammation, plasma extravasation and neutrophil migration. Intra-TMJ administration of the selective kappa-agonist blocked formalin-induced plasma protein extravasation and neutrophil migration in a dose dependent manner (Chicre-Alcântara et al., 2011). In the present study, we also demonstrated the antiiflammatory effect of FII by the decreased of plasma extravasation, and the release of inflammatory mediators induced by formalin in the TMJ.

Cytokines have emerged as the major controlling the degradation of joint tissue in osteoarthritis and models of inflammatory arthritis in humans. TNFα has an enormously detrimental effect on bone and cartilage (Gunson et al., 2012). Animal models have shown that induction of IL-1β expression in the TMJ adult mice leads to the pathology development and pain (Oakley and Vieira, 2008). Also, a positive correlation was found between cytokines in synovial fluid and osteoarthritis. It has been suggested that the presence of IL-1β and TNFα in the TMJ synovial fluid affect the treatment outcome of patients with osteoarthritis (Hamada et al. 2008).

Opioid analgesics, such as fentanyl and morphine, are used for the treatment of moderate to severe pain (Fukuda et al., 2009). They are general analgesics that are widely used, but they also induce several side effects, including dependence, nausea, vomiting, constipation, and respiratory depression (Moriyama et al., 2013). The most common adverse effect of these opioids is pruritus (itch), which is usually generalized, but is more likely to be localized to the face, neck, or upper thorax (Andoh et al., 2008). Hence, in order to develop potential tools for new therapies to improve inflammatory pain, there has been a continuous increase in the use of natural products in the past decades, which has encouraged scientific studies to search for new substances with therapeutic action (Val et. al., 2014).

5. Conclusion

The results suggest that FII has a potential antinociceptive and anti-inflammatory effect in the TMJ mediated by activation of opioid receptors in the subncucleus caudalis and inhibition of the release of inflammatory mediators in the periarticular tissue.

Acknowledgments: We thank Jordânia Marques de Oliveira for technical assistance.

Author Contributions

Induction of TMJ inflammatory hypernociception: Chaves HV; Pachêco JMS and Val DR;

Evaluation of inflammatory hypernociception: Pachêco JMS; Val DR; Vieira LV and Santos RS;

Evans Blue Extravasation Measurement: Val DR and Freitas, RS;

TMJ tissue TNF-α and IL-1β ELISA assays: Chaves HV; Clemente-Napimoga JT; do Val DR and Freitas, RS;

Evaluation of endogenous opioids by ELISA: Chaves HV; Clemente-Napimoga JT; do Val DR and Freitas, RS;

Statistical Analysis and manuscript redaction: Chaves HV; Bezerra MM; Pachêco JMS; Araújo IWF; Rivanor RLC; Monteiro VS; Clemente-Napimoga JT; Benevides NMB

Editorial support: Chaves HV; Bezerra MM; Clemente-Napimoga JT; Benevides NMB.

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Legends for illustrations and tables

Figure. 1. Sulfated polysaccharide (FII) from seaweed *Solieria filiformis* induced antinociception in the TMJ. Pretreatment with FII (0.03, 0.3 and 3.0 mg/kg) significantly reduced the magnitude of 1.5 % formalin-induced nociceptive response in the TMJ. Morphine (5 mg/kg) abrogated the formalin-induced nociceptive response. The symbol (*) indicates nociceptive response significantly higher than that vehicle group (0.9% NaCl) ($P<0.05$: ANOVA, Bonferroni's test). The symbol ($^{\#}$) indicates nociceptive response significantly lower than that formalin group ($P<0.05$: ANOVA, Bonferroni's test).

Figure 2. The antinociceptive effect of Sulfated polysaccharide (FII) from seaweed *Solieria filiformis* in the TMJ is mediated by opioid receptors activation in the subnucleus caudalis. Pretreatment with intrathecal injection of **(A)** the non-selective opioid receptors antagonist naloxone (Nlx; 15 µg/10 µl); **(B)** µ-opioid receptor antagonist CTOP (0.2 and 10 µg/10 µl); **(C)** δ-opioid receptor antagonist naltrindole (10 and 30 µg/10 µl); or **(D)** κ-opioid receptor antagonist nor-binaltorphimine (Nor-BNI; 15 and 90 µg/10 µl) significantly inhibit the antinociceptive effect of FII (0.3 mg/kg) in formalin-induced antinociception in the TMJ. The symbol (*) indicates nociceptive response significantly higher than that vehicle group (0.9% NaCl) ($P<0.05$: ANOVA, Bonferroni's test). The symbol ($^{\#}$) indicates nociceptive response significantly lower than that formalin group ($P<0.05$: ANOVA, Bonferroni's test). The symbol ($^{+}$) indicates nociceptive response significantly higher than that F II group ($P<0.05$: ANOVA, Bonferroni'test).

Figure. 3. The antinociceptive effect of sulfated polysaccharide (F II) from seaweed *Solieria filiformis* induced the release of the opioid peptide β-endorphin in the subnucleus caudalis. Pre-treatment with FII (0.3 mg/kg) significantly increased the release of opioid endogenous peptide β-endorphin in the subnucleus caudalis **(C)**, but not in the perianticular tissue **(A)** or trigeminal ganglion **(B)**. Pretreatment with FII did not affect the levels of opioid endogenous peptide dynorphin in the periarticular tissue **(D)**, trigeminal

ganglion **(E)** and subnucleus caudalis **(F)** (P>0.05: ANOVA, Bonferroni's test). The symbol ($^{\#}$) indicates response significantly higher than that other groups (P<0.05: ANOVA, Bonferroni' test).

Figure 4. Sulfated polysaccharide (FII) from seaweed *Solieria filiformis* induced anti-inflammatory effect in the TMJ. (A) Pretreatment with FII (0.3 mg/kg) significantly reduced the formalin induced plasma extravasation in the TMJ. **(B)** and **(C)** Pretreatment with F II (0.3 mg/kg) significantly reduced the release of inflammatory cytokines IL-1β and TNF-α induced by formalin in the TMJ. The symbol (*) indicates response significantly higher than that vehicle group (0.9% NaCl) (P<0.05: ANOVA, Bonferroni's test). The symbol ($^{\#}$) indicates response significantly lower than that formalin group (P<0.05: ANOVA, Bonferroni's test).

Figure 5. Sulfated polysaccharide (F II) from seaweed *Solieria filiformis* inhibits TMJ 5-HT-induced nociception. Pretreatment with FII (3.0 mg/kg) significantly reduced the magnitude of 5-HT-induced nociceptive responses. The symbol (*) indicates nociceptive response significantly higher than that vehicle (0.9% NaCl) (P<0.05: ANOVA, Bonferroni's test) The symbol ($^{\#}$) indicates nociceptive response significantly lower than that 5-HT group (P<0.05: ANOVA, Bonferroni' test).

ANEXO

Figures

Fig. 1

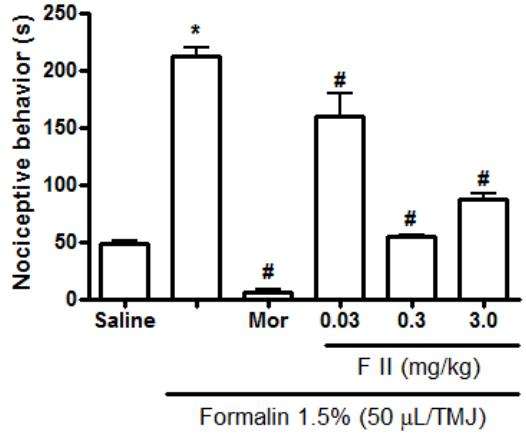


Fig. 2

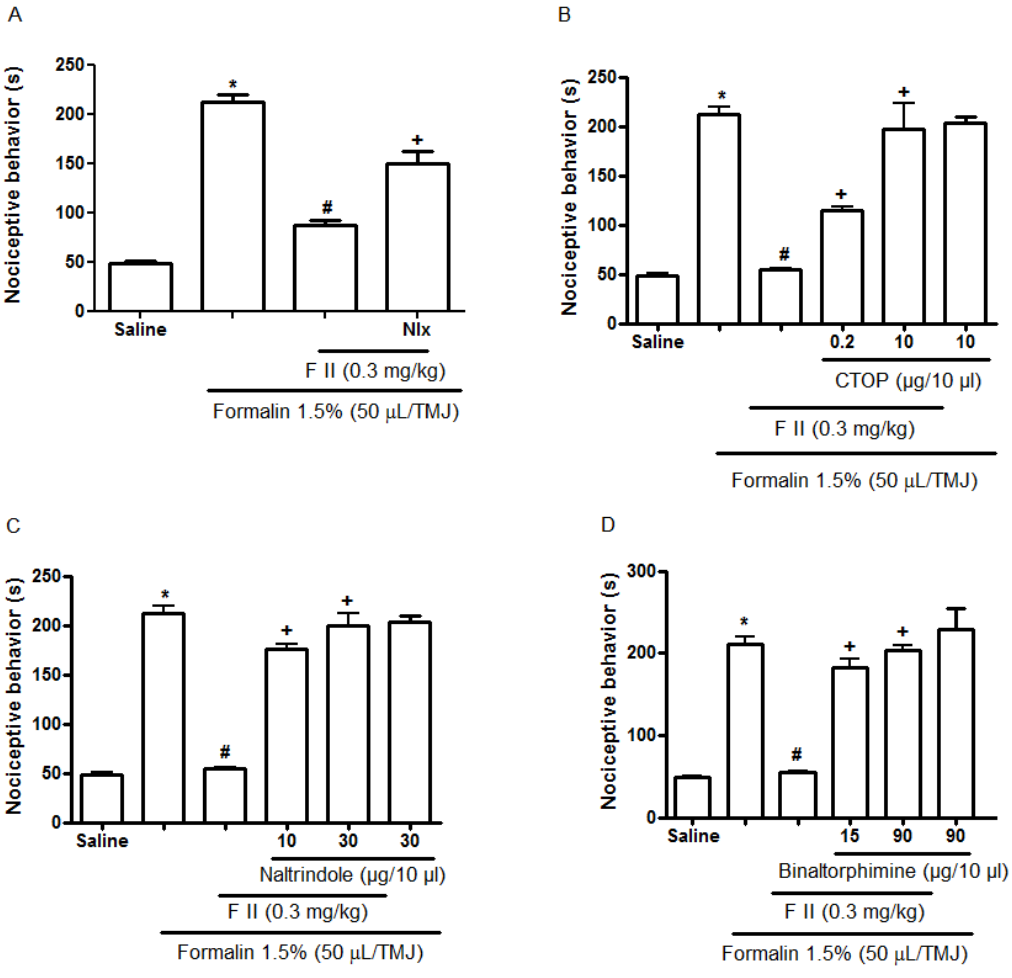


Fig. 3

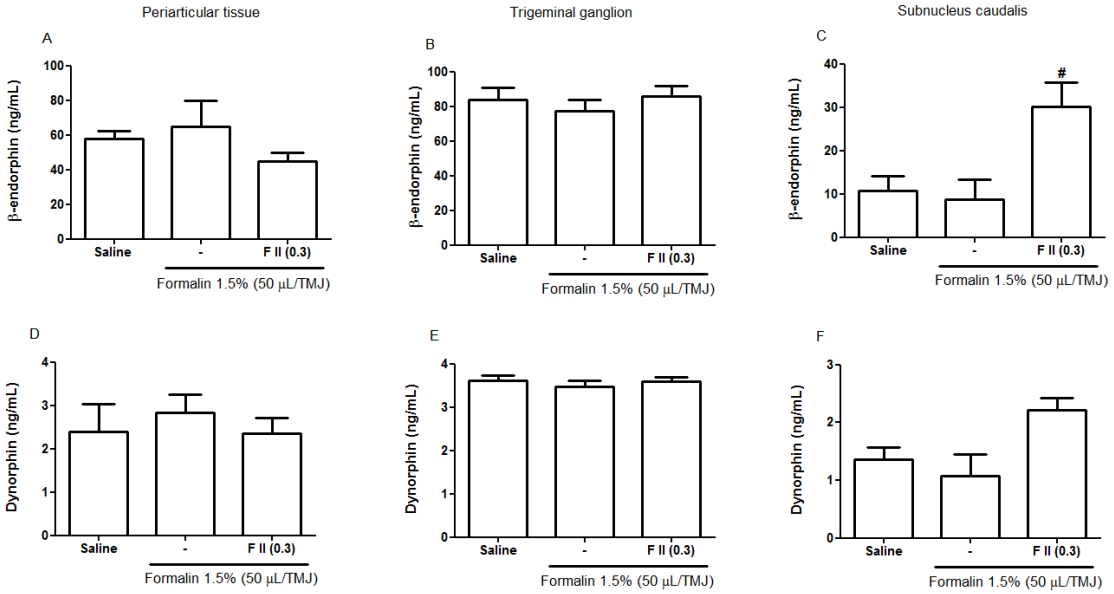


Fig. 4

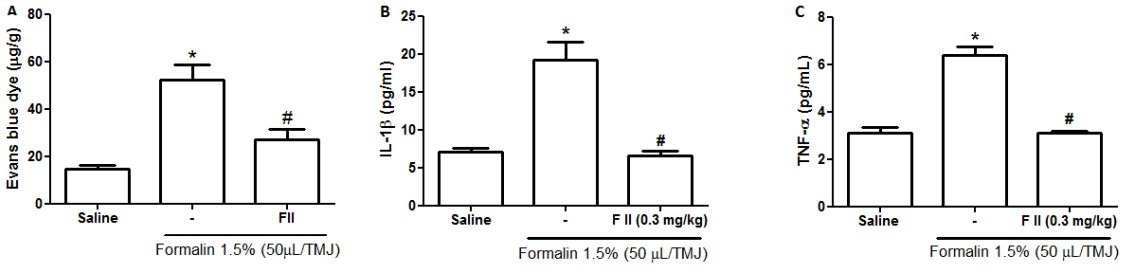
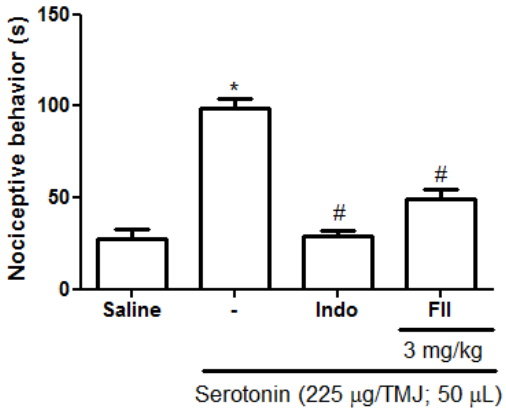


Fig. 5



4. CONCLUSÃO

Os resultados sugerem que FII tem um potente efeito antinociceptivo e anti-inflamatório na ATM mediada pela ativação de receptores opióides no subnucleo caudal e inibição da libertação de mediadores inflamatórios nos tecidos periarticulares. Assim, a presente investigação aponta que o polissacarídeo sulfatado da alga *Solieria filiformis* são ferramentas importantes no estudo das DTMs.

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ANEXO

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DECLARAÇÃO

Declaramos que o protocolo para uso de animais em experimentação n° 71/2012, sobre o projeto intitulado: **"POTENCIAL ANTI-INFLAMATÓRIO DE POLISSACARÍDEOS SULFATADOS DE ALGAS MARINHAS DO LITORAL CEARENSE: MECANISMO MOLECULAR E MEDIADORES ENVOLVIDOS"**, de responsabilidade de Ianna Wivianne Fernandes de Araújo está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA).

Declaramos ainda que o referido projeto foi aprovado pela Comissão de Ética em Pesquisa Animal – CEPA – em reunião realizada em 28 de novembro de 2012.

Fortaleza, 25 de janeiro de 2013

Profa. Dra. Nylane Maria Nunes de Alencar
Coordenadora da Comissão de Ética em Pesquisa Animal – CEPA