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AÇÃO ANTI-NOCICEPTIVA DO RENALETO DE ESTRÔNCIO NA HIPERNOCICEPÇÃO INFLAMATÓRIA INDUZIDO POR ZYMOSAN NA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATOS ENVOLVE A INIBIÇÃO DE TNFα

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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.

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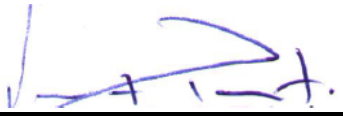

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SUMÁRIO

RESUMO

Os distúrbios na articulação temporomandibular (ATM) estão associados com dor inflamatória. O ranelato de estrôncio é utilizado no tratamento da osteoporose. Embora o mecanismo de ação de ranelato não esteja elucidado, há provas de seu efeito analgésico. Investigamos a eficácia do Ranelato na hipernocicepção inflamatória induzido pelo zymosan na ATM de ratos, avaliando o envolvimento TNF-α, IL-1β, e hemeoxygenase-1. Ratos Wistar foram tratados previamente com Ranelato (0,5, 5 ou 50 mg / kg) antes da injeção de zymosan na ATM. O Teste de Von Frey foi utilizado para avaliar hipernocicepção. Após a injeção de zymosan o lavado sinovial foi recolhido para a contagem de leucócitos e dosagem de mieloperoxidase; tecido periarticular e no gânglio trigeminal foram retirados para análise histopatológica (H & E), e dosagem dos níveis de TNF-α e IL-1β (ELISA). Para imuno-histoquímica, secções da ATM foram submetidas ao anticorpo de TNF-α e IL-1β. Além disso, os ratos foram tratados com ZnPP-IX (3 mg / kg), um inibidor específico da enzima hemeoxigenase-1 (HO-1), antes do Ranelato (0,5 mg / kg). Além disso, Azul de Evans (5 mg / kg) foi administrado para avaliar o extravasamento plasmático. Ranelato aumentou o limiar nociceptivo. Embora Ranelato não foi capaz de reduzir a contagem de leucócitos, a atividade da mieloperoxidase, o extravasamento de azul de Evans, os níveis de IL-1β, imunomarcação de IL-1β, foi eficaz na redução dos níveis de TNF-α. Além disso, ZnPP-IX não alterou a eficácia do Ranelato. Ranelato parece exercer seus efeitos de reduzir nociceptivos por meio da redução de TNF-α no gânglio trigeminal. Além disso, o efeito anti-nociceptivo do ranelato independe de IL-1β e HO-1.

Palavras-chave: articulação temporomandibular; reumatóide; zymosan; ranelato de estrôncio.

ABSTRACT

Temporomandibular joint (TMJ) disorders are associated with inflammatory pain. Strontium ranelate is used in osteoporosis. Though the mechanism of action of Ranelate is unclear, there is evidence of its analgesic effect. We investigate Ranelate efficacy in zymosan-induced TMJ hypernociception in rats evaluating TNF-a, IL-1β, and hemeoxygenase-1 involvement. Wistar rats were pretreated with Ranelate (0.5, 5 or 50mg/kg) before zymosan injection in TMJ. Von Frey test was used to evaluate hypernociception. After zymosan injection synovial lavage was collected for leukocyte counting and myeloperoxidase measurement; joint tissue and trigeminal ganglion for histopathological analysis (H&E), and TNF-a/IL-1β levels dosage (ELISA). To immunohistochemistry, TMJ sections were subjected to both TNF-a/IL-1β antibody. Also, rats were treated with ZnPP-IX (3 mg/kg), a specific HO-1 inhibitor, before Ranelate (0.5 mg/kg). Further, Evans Blue (5 mg/kg) was administered to assess plasma extravasation. Ranelate increased the nociceptive threshold. Although Ranelate was not able to reduce leukocyte counting, myeloperoxidase activity, Evans Blue extravasation, IL-1β levels, and TNF-a/IL-1b immunolabeling, it was effective in reducing TNF-α levels. Further, ZnPP-IX did not changed Ranelate efficacy. Ranelate may achieve its nociceptive-alleviating effects through reducing TNF-α levels in trigeminal ganglion. Further, the Ranelate anti-nociceptive effect is IL-1b and HO-1-independent.

Key words: temporomandibular joint; arthritis; zymosan; Strontium ranelate.

1. INTRODUÇÃO

A inflamação é uma reação de defesa do organismo, porém pode ser lesiva aos tecidos, havendo inclusive doenças em que a resposta inflamatória exacerbada representa a base do processo lesivo, sendo responsável pela elevada prevalência de morbi-mortalidade. A rigor, representa uma reação protetora, essencial para a sobrevivência do indivíduo. Entretanto, quando essa reação acontece de maneira inapropriada, comportando-se excessivamente ou de forma insuficiente, pode tornar-se prejudicial, passando a fazer parte integrante do processo patológico de algumas doenças. Ainda, a região acometida por um processo inflamatório torna-se uma área de dor notável devido, dentre outros eventos, à liberação de uma série de mediadores químicos (VANE *et al*., 1998).

A dor, definida pela International Association for the Study of Pain (IASP) como uma experiência sensorial e emocional desagradável associada a um dano tecidual real ou potencial ou descrita em termos deste dano, está presente no processo inflamatório e exerce também papel de defesa e alerta do organismo. Sua cronificação, porém, pode comprometer a qualidade de vida dos pacientes, limitando-os às suas atividades profissionais, afetando suas relações emocionais, sociais e familiares e implicando ainda aumento dos gastos financeiros para os serviços públicos de saúde (CHAVES et al., 2011).

Considerando-se os processos inflamatórios dolorosos que afetam o aparelho estomatognático, a artrite na articulação temporomandibular (ATM) representa um evento que compromete sobremaneira a qualidade de vida de suas desafortunadas vítimas. A artrite na ATM se apresenta como um dos diagnósticos diferenciais nas disfunções temporomandibulares (DTM) que, por sua vez, engloba um grupo de condições musculoesqueléticas e neuromusculares envolvendo as ATMs, os músculos mastigatórios e todos os tecidos associados. Os sinais e sintomas associados com essas disfunções são diversos, e podem incluir dificuldade em mastigar, falar, e/ou em outras funções orofaciais, estando frequentemente associadas com dor aguda ou persistente. As formas crônicas das DTMs são extremamente incapacitantes, podendo acarretar dificuldades nas atividades laborais, exclusão social, com

grandes prejuízos para os sistemas de saúde e para a vida desses doentes (GREENE, 2010).

Estima-se que 16-59% da população mundial apresenta sintomas e que 33-86% apresenta algum sinal de DTM (CARLSON e LERESCHE, 1995), sendo mais comum em mulheres do que em homens, numa proporção de 9:1. Em 2003, estudos comprovaram que são gastos cerca de 10 bilhões de reais anualmente no tratamento dessas desordens nos EUA, comprovando a necessidade de pesquisas na área para que alternativas de tratamento eficazes sejam propostas (LERESCHE, 2003).

Vários modelos experimentais têm sido desenvolvidos para o estudo das algias e alterações inflamatórias da ATM. Alguns autores propõem a indução da osteoartrite através de procedimentos cirúrgicos (HELMY et al*.,* 1988; ISHIMARU et al.., 1994; LEKKAS, 1994) ou mecânicos (IMAI *et al.,* 2001), outros, entretanto, induzem alterações inflamatórias através da injeção intra-articular de estímulos químicos. Entretanto, Puzas (2003) acredita ainda não haver um modelo experimental que esclareça a fisiopatologia das DTMs em todos os estágios, da fase aguda à fase crônica.

O nosso grupo vem trabalhando nos últimos anos com modelo de artrite experimental aguda através da injeção de zymosan na ATM de ratos (CHAVES et al., 2011). O modelo consiste na injeção do zymosan, um derivado de parede fúngica, permitindo analisar parâmetros de nocicepção e inflamação. Para a avaliação da nocicepção utiliza-se o analgesímetro digital Von Frey, onda a força aplicada em gramas se faz com um sensor acoplado a um aparelho eletrônico. Para os parâmetros inflamatórios utiliza-se análise histopatológica, ensaio da atividade de mieloperoxidase, contagem total de células no lavado articular e aumento de permeabilidade vascular por Azul de Evans.

O tratamento inicial das DTMs deve ser baseado no uso de modalidades terapêuticas conservadoras e reversíveis. Apesar de nenhuma terapia específica ser uniformemente efetiva, muitas das terapias conservadoras provaram ser no mínimo tão efetivas em proporcionar alívio sintomatológico quanto às formas de tratamento invasivas. Pelo fato de essas modalidades

terapêuticas não produzirem modificações irreversíveis, elas apresentam muito menos risco de causar malefício. As modalidades de tratamento conservadoras incluem terapias com placas oclusais, terapias físicas (termoterapia, terapia de resfriamento, laser, ultra-som, estimulação elétrica neural transcutânea-TENS), fisioterapia, terapia com exercícios e terapia farmacológica (OKESON, 2008; GREENE, 2010). Com relação à terapia farmacológica, destaca-se o uso de drogas anti-inflamatórias não-esteroidais (AINEs), além de alguns pacientes também se beneficiarem do uso de relaxantes musculares. Entretanto, em virtude dos efeitos colaterais comumente associados com o uso dos AINEs, particularmente no trato gastro-intestinal, muitas são as buscas por opções terapêuticas que apresentem melhor relação custo-benefício. Neste sentido, nosso grupo demonstrou em um modelo de artrite em joelhos de ratos que o pré-tratamento dos animais com risedronato, uma medicação antireabsosrtiva utilizado para tratamento da osteoporose, possui atividade anti-inflamatória e anti-nociceptiva (CARVALHO et al., 2006).

Destarte, esse projeto de pesquisa busca avaliar a eficácia do Ranelato de Estrôncio, uma droga registrada e licenciada para o tratamento da osteoporose, que é capaz de aumentar a formação óssea e reduzir a reabsorção óssea, deslocando o *turnover* ósseo em favor da formação óssea, em um modelo de artrite na ATM induzida por zymosan em ratos (RINGE, 2010). Portanto, a análise da eficácia do Ranelato de Estrôncio em processos inflamatórios dolorosos como a artrite na ATM permitirá uma nova indicação para um fármaco já estabelecido no mercado, reduzindo o tempo necessário para que se cumpram as boas práticas no fluxo da pesquisa de novos fármacos, representando uma nova abordagem para a terapêutica das DTMs, contribuindo para uma melhor qualidade de vida dos pacientes que padecem destes processos crônicos dolorosos.

2. JUSTIFICATIVA

A despeito dos achados clínicos das Disfunções Temporomandibulares (DTM), a caracterização dos mecanismos celulares e moleculares que formam a base dessa disfunção, assim como seu diagnóstico e tratamento, ainda

carece de maior estudo. Neste sentido, abordagens que busquem a elucidação da fisiopatologia das DTMs, através do desenvolvimento de modelos animais e ferramentas farmacológicas que possam afetar seu curso evolutivo, contribuirão sobremaneira para a melhoria da qualidade de vida dos pacientes, uma vez que a terapia medicamentosa crônica com anti-inflamatórios não esteroidais (AINES) está associada com graves efeitos colaterais, principalmente no trato intestinal, estimulando, desta forma, a busca de novas terapias farmacológicas. Nesse sentido, nosso grupo se empenhou em pesquisar o Ranelato de Estrôncio, que é uma droga já utilizada no tratamento da osteoporose.

3. OBJETIVO GERAL

Investigar a eficácia do Ranelato de Estrôncio no modelo hipernocicepção inflamatória induzido por zymosan na articulação temporomandibular (ATM) de ratos.

3.1 Objetivos Específicos

Verificar a possível participação de IL-1β, TNF-α e HO-1 no modelo hipernocicepção inflamatória induzido por zymosan na articulação temporomandibular (ATM) de ratos.

4. ARTIGO

Anti-nociceptive action of strontium ranelate in zymosan-induced temporomandibular joint inflammatory hypernociception in rats involves TNF-a inhibition but not IL-1β inhibition neither HO-1 pathway activation

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Abstract: Temporomandibular joint (TMJ) disorders are associated with inflammatory pain. Strontium ranelate is used in osteoporosis. Though the mechanism of action of Ranelate is unclear, there is evidence of its analgesic effect. We investigate Ranelate efficacy in zymosan-induced TMJ hypernociception in rats evaluating TNF-α, IL-1β, and hemeoxygenase-1 involvement. Wistar rats were pretreated with Ranelate (0.5, 5 or 50mg/kg) before zymosan injection in TMJ. Von Frey test was used to evaluate hypernociception. After zymosan injection synovial lavage was collected for leukocyte counting and myeloperoxidase measurement; joint tissue and trigeminal ganglion for histopathological analysis (H&E), and TNF-α/IL-1β levels dosage (ELISA). To immunohistochemistry, TMJ sections were subjected to

both TNF-α/IL-1β antibody. Also, rats were treated with ZnPP-IX (3 mg/kg), a specific HO-1 inhibitor, before Ranelate (0.5 mg/kg). Further, Evans Blue (5 mg/kg) was administered to assess plasma extravasation. Ranelate increased the nociceptive threshold. Although Ranelate was not able to reduce leukocyte counting, myeloperoxidase activity, Evans Blue extravasation, IL-1β levels, and TNF-α/IL-1β immunolabeling, it was effective in reducing TNF-α levels. Further, ZnPP-IX did not changed Ranelate efficacy. Ranelate may achieve its nociceptive-alleviating effects through reducing TNF-α levels in trigeminal ganglion. Further, the Ranelate anti-nociceptive effect is IL-1β and HO-1-independent.

Key words: temporomandibular joint; arthritis; zymosan; strontium ranelate.

1. Introduction

- Although noteworthy progress has been made in past three decades, the physiopathology of temporomandibular joint (TMJ) disorders still remains uncertain, which bring difficulty to the diagnostic and pharmacotherapy. Therefore, experimental models that allow studying the underlying pathogenesis of temporomandibular disorder-related inflammatory pain are of great clinical relevance. We have developed a rat model of TMJ inflammation using intra-articular injections of zymosan (1). Zymosan is a polysaccharide from yeast cell walls that produces a severe and erosive synovitis (2) associated with inflammatory pain in animal models of knee arthritis (3).

- Inflammatory stimuli cause mechanical hypernociception by a well-defined sequential release of cytokines (4). Within inflamed joints, a plethora of cells (synoviocytes, chondrocytes, mast cells, as well as infiltrating neutrophils and monocytes) are sources of mediators of inflammation, including cytokines. In this regard, tumor necrosis factor-a (TNF-α) is a proinflammatory cytokine, with an elevated expression in the joint s with TMJ disorders (5). Similar to TNF-α, interleukin-1β (IL-1β) is a potent multifunctional cytokine involved in the host immune and inflammatory responses. As matter of fact, some authors have also

shown considerable levels of IL-1β and TNF-α in the synovial fluid of patients suffering from painful TMJ disorders (6).

- Over the last few years numerous studies have led to the appreciation that heme oxygenase 1 (HO-1) plays an important role in the antioxidant defense system, and its induction would provide a negative feedback for cell activation and the production of inflammatory mediators, which could modulate the inflammatory pain (7,8,9).

- Strontium ranelate (Sran) {~5-[bis(carboxy-methyl)amino]-2-carboxy-4-cyano-3-thiophen-acetic acid distrontium salt} is a compound with two stable strontium atoms and ranelic acid and represents a new paradigm in the treatment of osteoporosis (10). It is considered a unique drug that has a combined effect with stimulation of bone formation and inhibition of bone resorption (11). Sran is an orally active treatment able to decrease the risk of vertebral and hip fractures in osteoporotic postmenopausal women (11). Although the mechanism of action of Sran is unclear at this time, there is some evidence of an analgesic effect of Sran (12).

- Thus, the present study was aimed at investigating the unexplored anti-nociceptive and anti-inflammatory efficacy of Sran in the model of zymosan-induced TMJ inflammatory hypernociception in the rat. Since IL-1β and TNF-α are key mediators of TMJ disorders, we also evaluate the levels of these cytokines after Sran treatment. Additional, considering our previous findings showing that the inhibition of HO-1 pathway is associated with worsening of inflammatory response (8), we also investigated whether Sran efficacy depends on HO-1 pathway.

2. Materials and Methods

2.3. Animals

- Male *Wistar* rats (160–220 g) (n=60) were housed in standard plastic cages with food and water available *ad libitum*. They were maintained in a temperature-controlled room (23 ± 2^{o} C) with a 12/12-hour light-dark cycle. All experiments were designed to minimize animal suffering and to use the minimum number of animals required to achieve a valid statistical evaluation. This study was conducted in accordance with the Institutional Animal Care and with the approval of the Ethics Committee of the Federal University of Ceara, Fortaleza, Brazil (CEPA no. 54/12).

2.4. Induction of TMJ inflammatory hypernociception

- Rats were briefly anesthetized with inhaled isoflurane and received an intra-articular (i.art.) injection of 2 mg zymosan (40 µL total volume) dissolved in sterile saline into the left TMJ using a 30-gauge needle and 0.5 mL syringe. Sham animals received only saline i.art. Before zymosan or saline injections, the TMJ skin region was carefully shaved, the postero-inferior border of the zygomatic arch was palpated, and the needle was inserted inferior to this point and advanced in a medial and anterior direction until the needle made contact with the condyle. This contact was verified by the moving of the mandible, and the puncture of the needle into the joint space was confirmed by the loss of resistance. Gentle aspiration ruled out intravascular placement, after which the specified volume of zymosan or saline was injected.

- As shown by our group previously (1) the zymosan TMJ inflammatory hypernociception is maximal at 4 h of arthritis whereas cell influx peaks at 6 h. Based on these results we used these time points to assess both nociceptive (head withdrawal threshold) and inflammatory parameters (total cell counting and myeloperoxidase assay).

2.5. Evaluation of hypernociception

- Hypernociception in the TMJ was evaluated by measuring the threshold of force intensity that needed to be applied to the TMJ region until the occurrence of a reflex response of the animal (e.g., headwithdrawal). The measurements were performed by an examiner unaware of the treatments and used a digital device (Insight, Ribeirão Preto, SP, Brazil) that consisted of a rigid filament linked in an electronic device that measured the response threshold in grams (g) when the filament was applied to the surface of the tested region (13). The facial areas to be tested around the TMJ were carefully shaved, and the animals were put into individual plastic cages 45 min before the beginning of the tests. The animals were submitted to a conditioning session of head withdrawal threshold measurements in the testing room for 4 consecutive days under controlled temperatures (23±2º C) and low illumination. On the fifth day, the basal force threshold value was recorded (in triplicate) before the i.art. injections of either zymosan or vehicle and after 4 h.

2.6. Synovial Lavage Collection and Cell Counting

- Six hours after zymosan-injections the rats were sacrificed under anesthesia and exsanguinated. The superficial tissues were dissected, and the TMJ cavity was washed to collect the synovial fluid (SF) by a pumping and aspiration technique using 0.05 mL of EDTA in neutral buffered PBS. This procedure was repeated twice. The total number of white cells in the synovial lavage was counted using a Neubauer chamber.

2.7. Myeloperoxidase Activity Analysis

- Myeloperoxidase (MPO) is an enzyme found primarily in the azurophilic granules of the neutrophils and has been used extensively as a biochemical marker of granulocyte infiltration into various tissues. The MPO activity assay measurement has been described previously by (14). In our study, the MPO

assay was conducted on the collected synovial lavage at 6h after zymosan injection. Briefly, the synovial lavage was centrifuged at 4,500 rpm for 12 min at 4º C. MPO activity was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1 % hydrogen peroxide. The results are reported as the MPO units/joint fluid. A unit of MPO activity was defined as the conversion of 1 µmol of hydrogen peroxide to water in 1 min at 22º C.

2.8. Evans Blue Extravasation Measurement

- At another time Sran (0.5 mg/kg) was administered *per os* 1 h prior to Zy, and 30 min before euthanasia, Evans Blue (5mg/kg, iv) was administered to assess plasma extravasation. Immediately after the extraction, the periarticular tissue was weighed and placed in 2mL of formaldehyde overnight. The supernatant (100 µL) was extracted, and the absorbance at 440nm was determined in spectrophotometer. The concentration was determined by comparison to a standard curve of known amounts of Evans blue dye in the extraction solution, which was assessed within the same assay. The amount of Evans blue dye (µg) was then calculated per mL of exudate (15).

2.8. Histopathological Analysis

- After sacrifice at 6h after zymosan-induced TMJ inflammatory hypernociception, the TMJ was excised. The specimens were fixed in 10 % neutral buffered formalin for 24 h, demineralized in 10 % EDTA, embedded in paraffin, and sectioned along the long axis of the TMJ. Sections of 5 µm, which included the condyle, articular cartilage, articular disc, synovial membrane, periarticular tissue, and the skeletal muscle periarticular tissue, were evaluated under light microscopy.

- For the specimens processed for routine hematoxylin-eosin (H&E) staining, histological analysis considered a 0–4 score grade based on the following parameters: cell influx in the synovial membrane, cell influx in the connective tissue and in the skeletal muscle periarticular tissue, and the thickness of synovial membrane.

2.9. Joint tissue and trigeminal ganglion TNF-α and IL-1β ELISA assays

- TMJ tissue and the trigeminal ganglion were colected at 6h after zymosan injection in rats that received 0.5 mg/kg strontium ranelate *per os* zymosan group or sham group, and the tissues were stored at -80^{o} C. The material was 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 TNF-α and IL-1β in the TMJ tissue and the trigeminal ganglion. 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.

2.10. Immunohistochemistry for TNF-α and IL-1β

- Immunohistochemistry for TNF-α and lL-1β was performed using the streptavidin-biotin (*Labeled Streptavidin Biotin* – LSAB) method in formalin-fixed, paraffin-embedded tissue sections (5 μm thick), mounted on glass slides previously prepared with an organosilane-based adhesive (3-aminopropyltriethoxysilane, Sigma Chemical Co®, St Louis, MO, USA). Briefly, it consisted of the following steps: the sections went through 2 baths in xylol, each

lasting ten minutes. After this, they were immersed in three passages of absolute alcohol, and then washed in running water, and immediately after this, a passage in distilled water.

- Antigen recovery was performed with citrate at pH 6.0, for 30 minutes at 99^{o} C. After returning to ambient temperature, the sections were immersed in a 3 % hydrogen peroxide blocking solution for 10 minutes. After returning to ambient temperature, the sections were incubated overnight (4º C) with a primary rabbit anti-TNF-α and anti- IL-1β antibody (ABCAM®, England, UK), at the dilution of 1:200, and afterwards washed with a phosphate buffered saline solution, PBS (*phosphate buffered saline*).

- The samples were incubated with the secondary antibody LSAB Kit for 10 minutes at ambient temperature. Next, incubation was performed in a chromogen solution prepared with 3,3´ diaminobenzidine (DAB) (DAKO®, Carpentaria, CA, USA), for 10 minutes in a dark chamber. Afterwards, the specimens were washed in running water and then in distilled water. Counter-staining was performed with hematoxylin, and afterwards the specimens were dehydrated in alcohol and diaphanized in xylol. Lastly, they were mounted on glass slides, which were examined under optical microscope. The negative control sections were performed, excluding the application of the primary antibody. The parameter of positivity for the immunohistochemical marking of the antigen in all the specimens included in the sample consisted of the cells that exhibited brown staining in their cytoplasm, irrespective of the intensity of the immunomarking.

2.11. Treatment

- Strontium ranelate (Sran) (PROTOS® 2g, Les Laboratoires Servier Industrie, 45 520 Gidy, France) (0.5, 5 or 50 mg/kg) was injected (*per os*) 1 h prior to zymosan i.art. In order to avoid any changes in pharmacokinetic profile of strontium ranelate, food was removed 1 h before treatment. Zymosan group consisted of rats that received (*per os*) strontium ranelate vehicle (0.9 % sterile

saline) 1h prior to zymosan i.art. To validate the data, a positive control group of rats was pretreated (s.c.) with indomethacin (5 mg/kg) 1 h before zymosan injection. Sham group received (*per os* and i.art.) 0.9 % sterile saline.

- To analyze the possible effect of HO-1 pathway on anti-nociceptive and anti-inflammatory efficacy of strontium ranelate in the model of zymosan-induced TMJ inflammatory hypernociception in the rat, another series of experiments was performed. Animals were pretreated (s.c.) with ZnPP IX (3 mg/kg), a specific HO-1 inhibitor, followed by an injection (*per os*) of Sran (0.5 mg/kg) 30 min later. After 1 h, zymosan (2 mg) was injected (i.art.), and on the 4^{th} h inflammatory hypernociception in the TMJ was evaluated as described above.

2.12. Statistical Analysis

- The data are presented as the mean±S.E.M. or medians, where appropriate. Differences between means were compared using a one-way ANOVA followed by the Bonferroni test. The Kruskal-Wallis test followed by Dunn's test was used to compare medians. A probability value of $P < 0.05$ indicated significant differences.

3. Results

3.1. Efficacy of strontium ranelate on the zymosan-induced TMJ inflammatory hypernociception

- The intra-articular (i.art.) 2 mg injection of zymosan resulted in inflammatory hypernociception as measured by a clear decrease in the mechanical threshold for head withdrawal at 4 h (Figure 1A). Further, a 2 mg injection of zymosan resulted in a significant increase in the number of polymorphonuclear cells (Figure 1B). This increase in neutrophils was certified by the increase of MPO activity in the TMJ synovial lavage following zymosan injection (Figure 1C).

These changes were accompanied by plasma extravasation that occurred in the TMJ during the 6h, which was certified by of Evans blue dye extravasation (Figure 1D). In the intra-articular saline-injected animals (Sham) no significant changes in withdrawal thresholds, polymorphonuclear cells counts or MPO activity were observed (Figure 1 A, B, C). Sran (0.5, 5 or 50 mg/kg) injected (*per os*) 1 h prior to zymosan i.art. injection significantly ($P < 0.05$) increased the nociceptive thresholds (Figure 1A). Nevertheless, Sran failed in decreasing the number of polymorphonuclear cells (Figure 1B), MPO activity (Figure 1C), and Evans blue dye extravasation in the synovial lavage (Figure 1D).

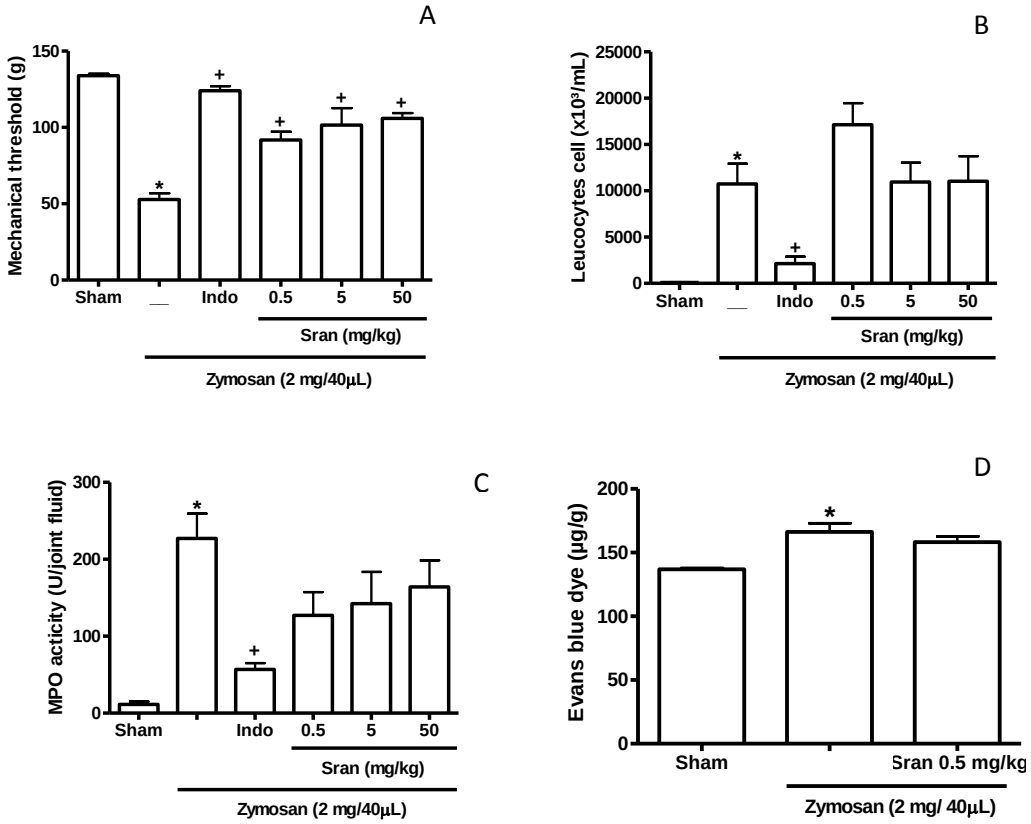

Figure 1: Efficacy of strontium ranelate on the zymosan-induced TMJ inflammatory hypernociception. Zymosan (2 mg; 40 *µL)* or saline was injected i.art. into the left TMJ of the rat. Strontium ranelate (0.5, 5 or 50 mg/kg) was injected (*per os*) 1 h prior to zymosan i.art. injection. (**A**) **Head withdrawal threshold in strontium ranelate-treated animals**: The mechanical nociceptive threshold was measured in strontium ranelate treated animals before and 4 h after an i.art. injection of zymosan. (**B**) **Leukocyte counting in strontium ranelate-treated animals**: 6 h after zymosan injection (i.art.) leukocyte migration was evaluated in strontium ranelate-treated animals

by cell counting in TMJ synovial lavage. **(C) MPO activity from TMJ synovial lavage in strontium ranelate-treated animals.** The MPO activity was measured in TMJ synovial lavage at 6 h after zymosan injection (i.art.). **(D) Plasma extravasation in strontium ranelate-treated animals.** The Evans blue (5 mg/kg) was injected i.v. 30 minutes prior to euthanasia. Data are expressed as the mean±SEM of 6 mice for each group. $^{*}P < 0.05$ indicates a significant difference from the sham group, $^{+}P < 0.05$ indicates a significant difference from the zymosan group (ANOVA, Bonferroni).

3.2. Effect of zinc protoporphyrin IX (ZnPP IX), a specific HO-1 inhibitor, on the strontium ranelate efficacy on the zymosan-induced TMJ inflammatory hypernociception

- To investigate the role of HO-1 activity in the antinociceptive effect of Sran, the animals were pretreated (s.c.) with ZnPP IX (3 mg/kg), a specific HO-1 inhibitor. The effects of strontium ranelate (0.5 mg/kg) on the zymosan-induced TMJ hypernociception (Figure 2) were not changed in the presence of ZnPP-IX (3 mg/kg).

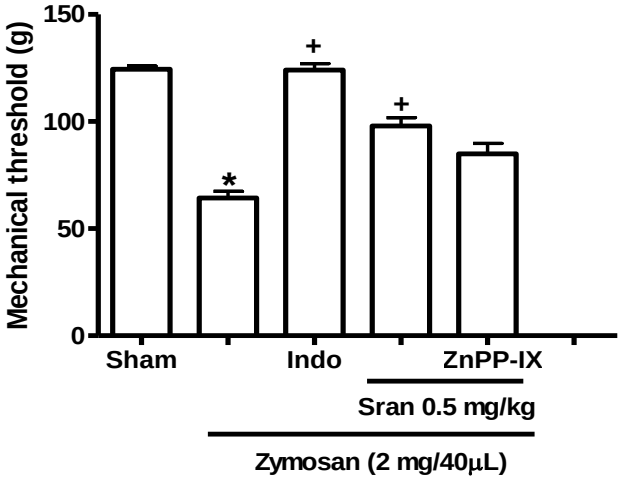

Figure 2: Effect of zinc protoporphyrin IX (ZnPP IX), a specific HO-1 inhibitor, on the Strontium ranelate efficacy on zymosan-induced TMJ inflammatory hypernociception. Animals were pretreated (s.c.) with ZnPP IX (3 mg/kg), a specific HO-1 inhibitor, followed by an injection (*per os*) of Strontium ranelate Pers (0.5 mg/kg) 30 min later. After 1 h, zymosan (2 mg) was injected (i.art.). The mechanical nociceptive threshold was measured before and 4 h after an i.art. injection of zymosan.

Data are expressed as the mean±SEM of 6 mice for each group. $^{*}P < 0.05$ indicates a significant difference from the sham group; $^{+}P < 0.05$ indicates a significant difference from the zymosan group (ANOVA, Bonferroni).

3.3. Joint tissue and trigeminal ganglion TNF-α and IL-1β ELISA assays

- The intra-articular (i.art.) injection of zymosan (2 mg) resulted in a significant increase in TNF-α (Figures 3A e 3B) and IL-1β (Figures 3C e 3D) levels in both joint tissue and trigeminal ganglion after. Although Sran treatment was not able to significantly reduce IL-1β levels, when compared with the zymosan group (Figures 3C e 3D), Sran did reduce TNF-α levels in both joint tissue and trigeminal ganglion (Figures 3A e 3B).

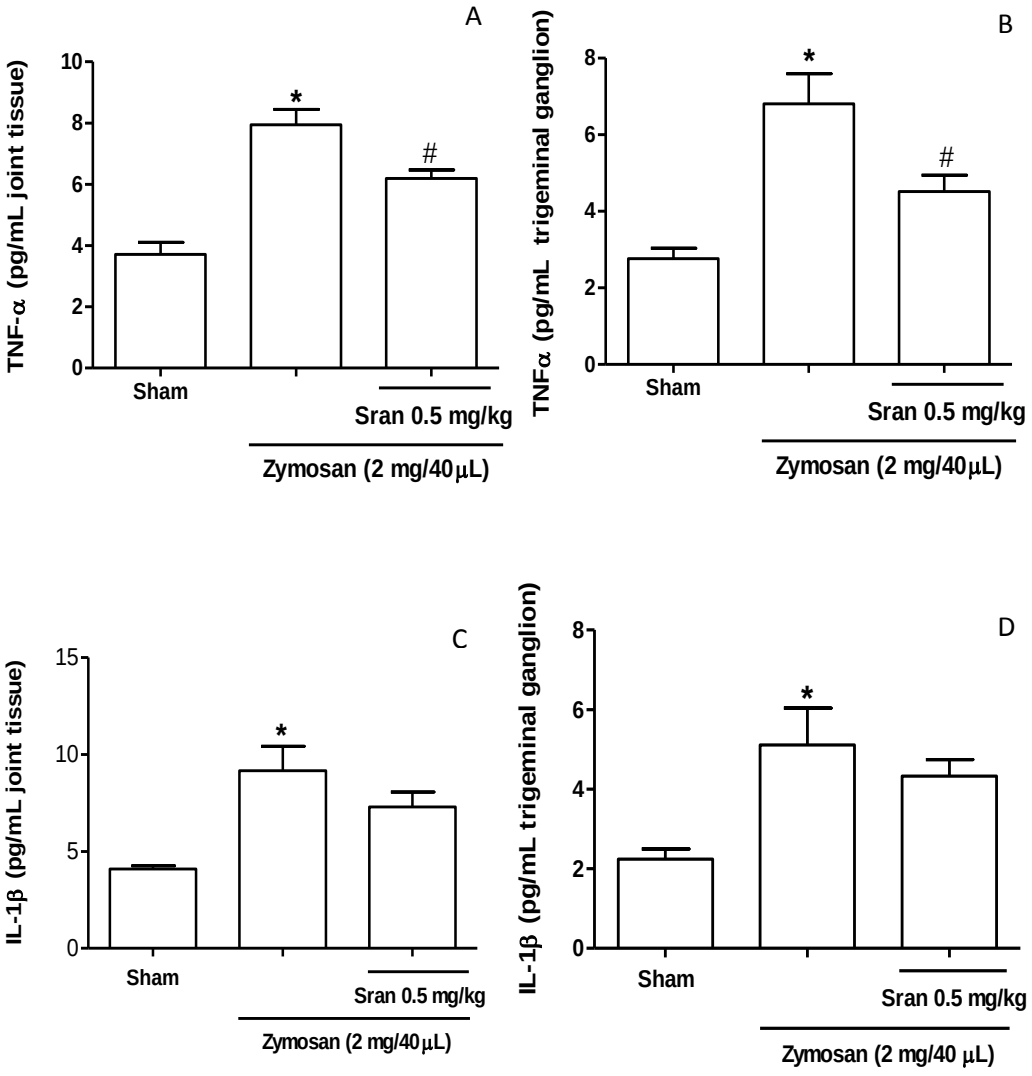

Figure 3: Joint tissue and trigeminal ganglion TNF-α (A/B) and IL-1β (C/D) levels from study rats either subjected or not to on zymosan-induced TMJ inflammatory hypernociception and assayed on the 6 hour post challenge. Zymosan (2 mg/kg) or saline was injected i.art. into the left TMJ of the rat. Strontium ranelate (0.5 mg/kg) was injected (*per os*) 1 h prior to zymosan i.art. injection. Data are expressed as the mean ± SEM of 6 mice for each group. *P < 0.05 indicates a significant difference from the sham group (ANOVA, Bonferroni).

3.4. Histopathological analysis

- In the 6th h after zymosan-induced TMJ inflammatory hypernociception, an inflammatory cell influx was observed in the synovial membrane (Figure 4B) compared to the Sham group (Figure 4A). The cell types were predominantly neutrophils, which characterized acute inflammation. Edema was also observed in the synovium (Figure 4B). Table 1 shows the scores attributed to TMJ's histopathological analysis and compares the values between the TMJs of sham and zymosan groups. A significant (P<0.05) increase in the inflammatory parameters was observed in the zymosan group. Table 1 also shows the scores attributed to TMJ's histopathological analysis and compares the values between the TMJ of zymosan and Sran (0.5, 5 or 50 mg/kg) groups. Sran (0.5, 5 or 50 mg/kg) did not reduce the inflammatory parameters. In figure 4C and 4D is depicted TMJ of rats pretreated (*per os*) with Sran.

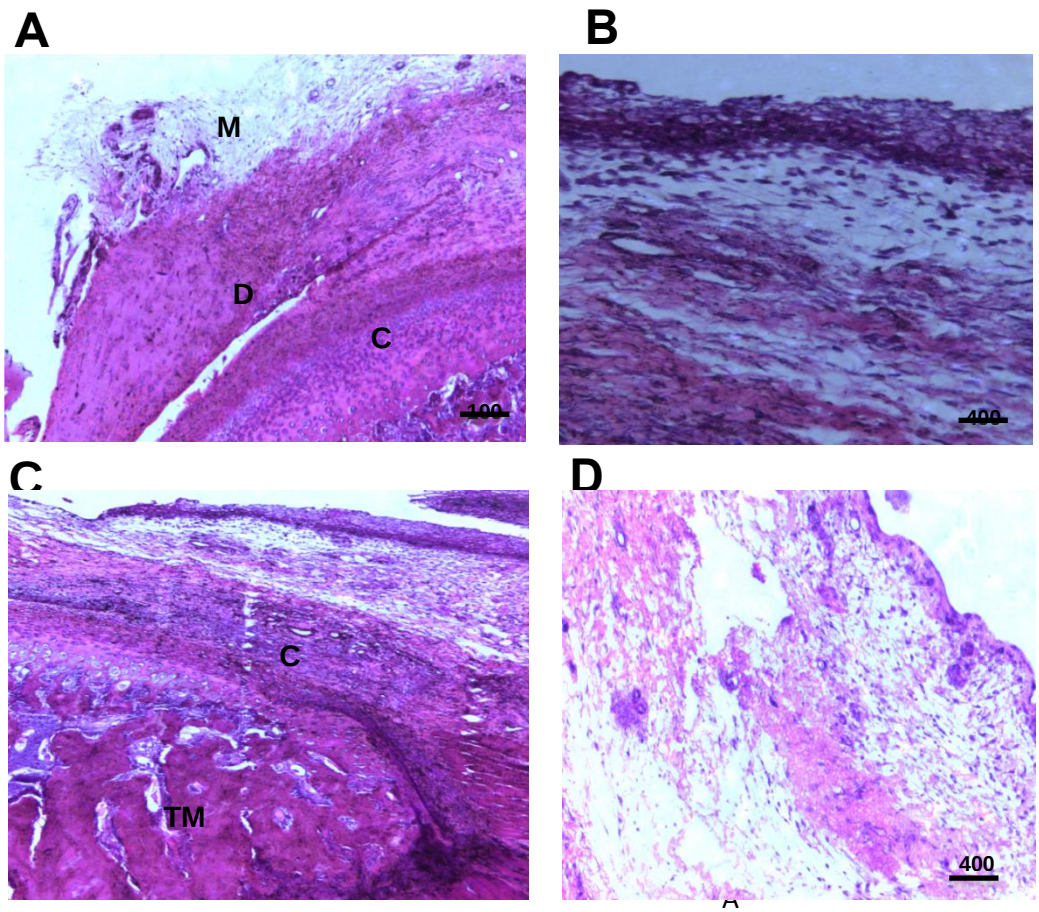

Figure 4: Photomicrographs of the histopathological analysis of temporomandibular joints (TMJ). (**A**) sham group TMJ (100x); (**B**) zymosan 2 mg group (400x) showing inflammatory cell influx in the synovial membrane; (**C**) and (**D**) TMJ of rats pretreated (*per os*) with strontium ranelate (0.5 mg/kg) and injected (i.art.) with zymosan 2 mg (100 and 400 x, respectively). Strontium ranelate did not reduce cell influx. **C**: condyle; **AC**: articular cartilage; **AD**: articular disc; **SM**: synovial membrane; **PAT**: periarticular tissue. Hematoxylin and eosin (H&E) staining.

3.5. Immunohistochemical analysis

- An immunohistochemical analysis for TNF-α and IL-1β showed increased immunolabeling for both TNF-α and IL-1β in synoviocytes and neutrophlis after zymosan-challenge that was characterized by brown-colored cells in the synovial membrane of the zymosan-induced TMJ inflammatory

hypernociception (Figure 5). The synovial cells in the synovial membrane of the zymosan-induced TMJ inflammatory hypernociception and treated with Sran (0.5 mg/kg) also showed both TNF-α and IL-1β expression (Figure 5). However, in conjunctive tissue Sran (0.5 mg/kg) treatment reduced TNF-α expression. The negative control group sections were composed of zymosan-induced TMJ inflammatory hypernociception that were not treated with anti-TNF-α or anti-IL-1β antibody. None of the negative controls showed TNF-α or IL-1β immunoreactivity.

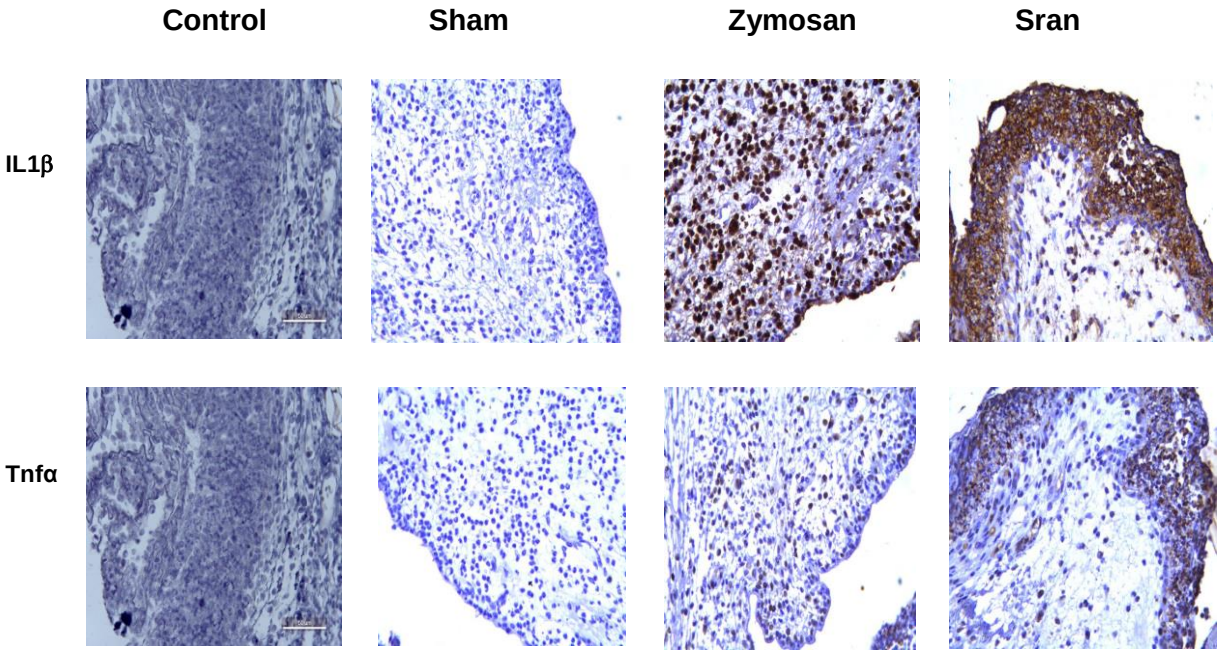

Figure 5: Representative immunohistochemistry of TMJ tissues for IL-1β (upper panel), and TNF-α (lower panel) from rats on the sixth hour after zymosan injection. The upper panel shows increased IL-1β immunolabeling of synoviocytes (black arrow) and neutrophlis (white arrow) after zymosan-challenge, an effect that was not reduced by strontium ranelate-treatment (400x). The bottom panel shows increased TNF-α immunolabeling of synoviocytes (black arrow) and neutrophlis (white arrow) after zymosan-challenge, an effect that was not reduced by strontium ranelate-treatment (400x). **Control**: negative control (sections in the absence of anti-IL-1β and anti-TNF-α antibody); **Sham**: unchallenged rats; **Zymosan**: zymosan-challenged rats receiving 0.9% saline solution; **Ran** 0.5: zymosan-challenged rats receiving strontium ranelate (0.5 mg/kg).

4. Discussion

- In this study, we demonstrated the anti-nociceptive effect of the Strontium Ranelate (Sran) in the model of zymosan-induced TMJ inflammatory hypernociception in rats. We found evidence that Sran may achieve its nociceptive-alleviating effects in zymosan-induced temporomandibular joint inflammatory hypernociception in rats through reducing TNF-α levels. We also found evidence that the anti-nociceptive effect of the Sran in zymosan-induced TMJ inflammatory hypernociception is IL-1β and HO-1-independent.

- Experimental animal models of TMJ inflammatory hypernociception have been used to study inflammation and pain conditions. We performed the first demonstration of a zymosan-induced arthritis in the TMJ, showing evidence that in TMJ, zymosan caused a time-dependent leucocyte migration, plasma extravasation, mechanical hypernociception, and neutrophil accumulation peaked at 6h (1). Thus, zymosan-induced TMJ arthritis is a reproducible model that may be used to assess both the mechanisms underlying TMJ inflammation and the potential tools for therapies.

- Sran is effective in reducing bone loss, increasing bone mass, and bone resistance in intact mice, rats, monkeys and human (16,17,18,19). Actually, it has been used for osteoporosis for a long time, and Sran is considered an antifracture drug able to activate osteoblasts (20). Further, a large body of evidence in preclinical, molecular, cellular, and animal models shows that Sran may have an effect in osteoarthritis (21). In fact, very recently, Sran has produced positive data in a pivotal phase III clinical study for osteoarthritis (22). Although the mechanism of Sran is not yet fully understood there is some evidence that it acts on bone surface and stimulates the differentiation of osteoblasts by stimulating the calcium sensor receptor, and inhibits osteoclast differentiation by inhibiting RANKL production and increasing osteoprotegerin (OPG) activity (23,24).

- In addition, patients treated with Sran had a greater reduction in Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) total score and pain subscore than the placebo. Moreover, the drug was previously found to reduce back pain in women with osteoporosis and osteoarthritis after a 3-year treatment (25). Since osteoporosis and osteoarthritis are associated with a variety of symptoms, including pain, it could be hypothesized that Sran may also be effective at reducing the inflammatory hypernociception in zymosan-induced temporomandibular joint inflammatory hypernociception in rats.

- In fact, we demonstrated in the present study that zymosan intra-articular (i.art.) injection decreased the mechanical nociceptive thresholds, which was increased by Sran treatment. However, regarding the inflammatory parameters, as determined by cell influx and MPO activity, showed that there was only a slight decrease following Sran treatment but this did not reach statistical significance. Also, Evans Blue Extravasation Measurement in the synovial lavage was not changed by Sran treatment.

- In current study, evaluating TMJ's histopathological analysis after zymosan, we found an inflammatory cell influx in the synovial membrane, periarticular tissue, and musculoskeletal tissue, associated with thickness in synovial membrane. The treatment with Sran did not reduce the inflammatory parameters to a normal status. Similarly, it was recently show that Sran treatment had no particular effect of reducing the histological severity of synovitis in dogs (29).

- Over the last few years, numerous studies have demonstrated that HO-1 activity results in the inhibition of oxidative damage and apoptosis, with significant reductions in production of proinflammatory cytokines (7). In this regard, it was recently demonstrated that the HO-1 pathway plays antinociceptive effects during acetic acid-evoked nociception (8). Also, heme-induced HO-1 was reported to result in a positive outcome in a zymosan-induced air pouch inflammation model (26). Considering these data, we evaluated the putative involvement of HO-1 pathway in Sran antinociceptive effect. After the pretreatment with ZnPP-IX the anti-nociceptive efficacy of Sran

was not changed, suggesting that HO-1 activity is not involved in the antinociceptive effects of Sran.

- Cytokines are produced by various cells types in response to a variety of stimuli and constitute a link between cellular injury or recognition of nonself and the development of local and systemic signs and symptoms of inflammation (27). In rats there is a cascade of release of cytokines that constitutes a link between the injuries and the release of primary hypernociceptive mediators. This concept allows us to understand why the inhibition of cytokines causes analgesia (27). In this regard, the literature has largely demonstrated the contribution of TNF-α to inflammatory hyperalgesia (27) and the clinical success of anti-TNF-α in rheumatoid arthritis also exemplifies this concept (30).

- During inflammatory response, TNF-α is the first cytokine released, and like TNF-α, IL-1β is a potent multifunctional cytokine involved in the host immune and inflammatory responses (27). In this regard, TNF-α and IL-1β and are recognized contributors to the pathogenesis of joint diseases, thus leading to synovial fibroblast hyperplasia and the destruction of the extracellular matrix (31,32).

- In the present study, zymosan injection (i.art) resulted in a significant increase in both TNF-α and IL-1β levels. Accordingly, previous studies have also shown appreciable amounts of TNF-α in the synovial fluid of patients with TMJ disorders (5). During inflammatory response it was shown in rats that TNF-α has an early and crucial role in the development of inflammatory hyperalgesia (4). Our results are in accordance with literature showing that TNF-α plays important role as a proinflammatory cytokine in TMJ disorders (5,6). In the current study we showed that Sran treatment reduced TNF-α levels in both joint tissue and trigeminal ganglion.

- TNF-α is the first cytokine released which triggers the release of IL-1β (27). IL-1β is a proinflammatory cytokine, with an elevated expression in the joints during TMJ disorders known to result in the activation of the inflammatory and degradative pathways in synovial cells (5). IL-1β has been detected in the

synovial fluid from patients with TMJ disorders (28). Nevertheless, in the present study Sran treatment was not able to reduce IL-1β levels, when compared with the zymosan group.

- Furthermore, analyses of immunohistochemical stains showed increased TNF-α and IL-1β immunolabeling in the synovial cells in the synovial membrane of the zymosan-induced TMJ inflammatory hypernociception. Although Sran treatment promoted a slight reduction in TNF-α immunolabeling in conjunctive tissue, it was not able to reduce IL-1β immunolabeling. On the other hand, it was recently showed by others that in the synovial membrane of dogs underwent sectioning of the anterior cruciate ligament, the gene expression level of IL-1β was significantly reduced by Sran treatment in doses of 50 or 75 mg/kg per day for 16 weeks (29). A possible explanation of this apparently contradictory result could be due to the different animal model used (rats *versus* dogs), and the time/dose of treatment.

- In summary, although the exact mechanisms of action of Sran remain relatively elusive, this study provides novel information about the Sran antinociceptive effect in zymosan-induced TMJ inflammatory hypernociception. As a matter of fact, the Sran efficacy in this model is through reducing the concentration levels of TNF-α. The proposed effect of Sran on TNF-α levels with the purpose of reducing TMJ inflammatory hypernociception brings new hope and it also hints Sran at a potential role interesting candidate for the treatment of TMJ disorders. Well-designed studies of longer duration are necessary to prove this additional effect of Sran and, ultimately, benefit patients with TMJ disorders.

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Author Contributions

Induction of TMJ inflammatory hypernociception: Chaves HV and Alves SM; **Evaluation of inflammatory hypernociception**: Lemos JC;
Synovial Lavage Collection and Cell Counting: Chaves HV; Alves SM; Alves SM; Abreu SC; and Freitas, RS; and de Freitas, AR;

Myeloperoxidase Activity Analysis: Abreu, SC and Alves, SM;

Evans Blue Extravasation Measurement: Alves, SM and Freitas, RS;

Histopathological Analysis: Pereira KMA; and Alves SM;

Joint tissue and trigeminal ganglion IL-1β and TNF-α ELISA assays: Chaves HV; do Val DR and Freitas, RS;

Immunohistochemistry for IL-1β and TNF-α: Chaves HV and do Val DR;

Statistical Analysis and manuscript redaction: Chaves HV; Bezerra MM; Pinto VPT; and Cristino-Filho G;

Editorial support: Benevides NMB; Brito GAC; Silva AAR.

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