UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

**THOMAS BARBIN**

# INFLUÊNCIA DO ÔMEGA 3 NO PROCESSO INFLAMATÓRIO INDUZIDO POR CFA NA CAUDA DE RATOS: ESTUDO PILOTO

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Trabalho de Conclusão de Curso apresentado à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Cirurgião Dentista.

Udergraduate final work presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Dental Surgeon

Orientador: Dr. Sidney Figueroba Raimundo

Coorientador: Prof. Dr. Francisco Carlos Groppo

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DO TRABALHO DE CONCLUSÃO DE CURSO APRESENTADO PELO ALUNO THOMAS BARBIN E ORIENTADO PELO DR. SIDNEY FIGUEROBA RAIMUNDO

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**RESUMO**

Disfunções temporomandibulares (DTM) abrangem alterações craniofaciais, a qual participa a ATM, os músculos da mastigação ou estruturas associadas. Muitas DTM têm características de inflamação crônica. Estudos demonstraram efeitos benéficos dos ácidos graxos poli-insaturados eicosapentaenoico do tipo 3 (ômega-3), no processo inflamatório incluindo danos neurológicos, doenças cardiovasculares e artrite reumatoide. O objetivo foi avaliar “in vivo” aspectos morfológicos/ histoquímicos e nível de citocinas inflamatórias nos tecidos da ATM em ratos com artrite induzida (AI) por Adjuvante Completo de Freund (CFA) associado ao colágeno tipo II (CII). Foram usados 32 ratos machos com 3 meses de idade (Rato heterogênico HanUnib: WH (Wistar) separados em grupos: G1 – injeção 100µL / NaCl 0,89% na base da cauda; G2 – injeção 100µL/ CFA+CII, base da cauda; G3 — injeção / 100µL / CFA+CII, base da cauda administração intragastrica única, diária, 0,3mg/kg / dexametasona / 7 dias; G4 – administração intragastrica única, diária, 300 mg/kg / ômega-3 / 7 dias, foi observado o efeito dos tratamentos com ômega-3 ou dexametasona na ATM de ratos submetidos a injeção com CFA, 15 dias após a indução. Mortos após 7 dias dos tratamentos para ambos os grupos, as ATMs foram retiradas em bloco. Uma ATM foi descalcificada para realização do processamento histológico (HE, Azul de Toluidina e Picrossirius Vermelho-Hematoxilina). A outra ATM sofreu o processo de maceração e centrifugação em (tampão tris-HCL 50mM, pH7,5). O sobrenadante submetido à Imunoensaio (ELISA) para mensurar as citocinas (IL1-β, TNF-α e IL-10). Leitura feita por espectrofotometro. Os resultados foram avaliados por ANOVA e Kruskal-Wallis, na dependência da distribuição dos dados e da homocedasticidade das variâncias. E foi possível concluir que os níveis de citocinas inflamatórias aumentaram e os níveis de citocinas anti-inflamatórias diminuíram na ATM, com a instalação da artrite induzida pela combinação de CFA e colágeno tipo II, constatarmos a diminuição da espessura da cartilagem condilar. Portanto, o tratamento com ômega-3 produziu efeito anti-inflamatório similar aquele da dexametasona.

**Palavras-chave:** Disfunção temporomandibular. Ômega 3. Citocinas

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**ABSTRACT**

Temporomandibular disorders (TMD) include craniofacial alterations, which involve the TMJ, chewing muscles or associated structures. Many TMD have characteristics of chronic inflammation. Studies have shown beneficial effects of type 3 (omega-3) eicosapentaenoic polyunsaturated fatty acids on the inflammatory process including neurological damage, cardiovascular disease and rheumatoid arthritis. The objective was to evaluate “in vivo” morphological / histochemical aspects and level of inflammatory cytokines in TMJ tissues in rats with Freund's Complete Adjuvant-induced arthritis (CFA) associated with type II collagen (CII). Thirty-two 3-month-old male rats (HanUnib heterogeneous rat: WH (Wistar) separated into groups were used: G1 - 100μL / NaCl 0.89% injection at tail base; G2 - 100μL / CFA + CII injection, tail base) ; G3 — injection / 100μL / CFA + IIC, tail basis single intragastric administration daily 0.3 mg / kg / dexamethasone / 7 days; G4 – single intragastric administration daily 300 mg / kg / omega-3/7 The effect of omega-3 or dexamethasone treatments on the TMJ of rats submitted to CFA injection 15 days after induction was observed, and after 7 days of treatment for both groups, the TMJs were removed en bloc. It was decalcified for histological processing (HE, Toluidine Blue and Picrossirius Red-Hematoxylin.) The other TMJ was macerated and centrifuged in (50mM tris-HCL buffer, pH7.5).) to measure cytokines (IL1-β, TNF -α and IL-10) Spectrophotometer reading. Results were evaluated by ANOVA and Kruskal-Wallis, depending on data distribution and homoscedasticity of variances. It was concluded that the levels of inflammatory cytokines increased and the levels of anti-inflammatory cytokines decreased in the TMJ, with the installation of arthritis induced by the combination of CFA and type II collagen, as we observed the decrease in the thickness of the condylar cartilage. Therefore, omega-3 treatment produced an anti-inflammatory effect similar to that of dexamethasone.

**Key words:** Temporomandibular dysfunction. Omega 3. Cytokines.

**SUMÁRIO**

## 1 INTRODUÇÃO

A artrite reumatoide (AR) é uma doença autoimune inflamatória crônica que afeta os articulações e ossos (Firestein, 2003). É progressiva e desencadeada por fatores imunológicos, genéticos e ambientais (Lee & Weinblatt, 2001). A causa da AR não é totalmente conhecida, no entanto, ambos os processos autoimunes, adaptativos e inatos, desempenham papéis importantes na patogênese da doença (Holmdahl et al., 2014; Wang et al., 2017).

Erosão óssea geralmente ocorre nas articulações das mãos e dos pés (Miles & Calder, 2012). A ATM tem diferentes características morfológicas, funcionais e biológicas em comparação com as demais articulações sinoviais (Embree et al., 2010). Revestida por uma fibrocartilagem é constituída por colágeno tipo I e as camadas mais profundas colágeno tipo II (Ghassemi et al., 2016). Isto diferencia a ATM de outras articulações com cartilagem hialina, constituídas de colágeno tipo II (Embree et al., 2011).

Mesmo com essas diferenças, a ATM pode ter as mesmas intercorrências que afetam outras articulações, incluindo AR (Aliko et al., 2011), pode ser grave e comprometer todo o sistema mastigatório (Dimitroulis, 2005). As lesões articulares apresentam infiltração de células imunes que produzem altos níveis de mediadores químicos (Miles & Calder, 2012). Aumento do nível das citocinas IL-1β, IL-6, TNF-alfa e prostaglandina (PGE2) no líquido sinovial têm sido encontrados no desarranjo interno do ATM e na osteoartrite (Figueroba et al., 2018).

Vários métodos têm sido utilizados para induzir inflamação aguda ou crônica na ATM, aumento de dimensão vertical, rompimento da zona bilaminar e hipermobilidade condilar (Embree et al., 2011; Figueroba et al., 2014). injeção de agentes irritantes químicos podem desencadear a inflamação aguda entre eles a carragenina, a ovalbumina (Habu et al., 2002) e o adjuvante completo de Freund ou CFA (Denadai-Souza et al., 2009; Wu et al., 2010; Kou et al, 2011; Wang et al., 2012; Holwegner et al., 2015).

O CFA é constituído de Mycobacterium tuberculosis mortos e secos em óleo. A indução patológica da AR é semelhante ou igual a que ocorre em humanos (Bendele, 2001). Com ênfase na fase aguda da inflamação a injeção do CFA é simples e reprodutível, (Flake et al., 2006; Wu et al., 2010; Kou et al., 2011). A inflamação dos tecidos da ATM pode ser um fator predisponente para as anormalidades estruturais (Wang et al., 2012).

A IL-1β é um importante fator no desenvolvimento da patologia da AR, que pode ser detectada na cavidade articular (Morin et al., 2015), baixos níveis da IL-10 anti-inflamatória foram detectados em pacientes com AR e animais induzido por CFA (Arab et al., 2017). Células imunológicas envolvidas na AR geralmente contêm uma alta proporção do ácido araquidônico n-6 (AA) e baixa proporção de outros ácidos graxos poli-insaturados de 20 carbonos (PUFAs), sendo que o AA é considerado principal substrato para síntese de eicosanoides (Calder et al., 2009).

Anti-inflamatórios hormonais e não-hormonais são utilizados para aliviar a dor, em processos inflamatórios e em doenças autoimunes, sem reduzir a cartilagem e a destruição óssea articular (Cessak et al., 2014). O uso sistêmico de anti-inflamatórios em ratos saudáveis mostrou uma diminuição da espessura da cartilagem articular da ATM (Figueroba et al., 2018).

No entanto, a administração em longo prazo destes fármacos podem causar efeitos colaterais graves, tais como risco cardiovascular e distúrbios gastrointestinais (Cessak et al., 2014), osteoporose, diabetes mellitus, ganho de peso, aumento da pressão sanguínea, do risco de insuficiência cardíaca e cardiovascular (Calder et al., 2009). O tratamento para a artrite reumatoide é tratar a remissão dos sintomas para um estado em que a inflamação desapareça, ou seja, muito menor (Flurey et al.,2014). Os corticosteroides, injetados intra-articular ou extra-articular, tornaram-se referência no tratamento da AR (Caporali et al., 2013). O uso prolongado ou repetitivo via intra-articular pode causar um colapso na articulação (Sidebottom & Salha, 2013).

Outros fármacos são descritos no controle da reabsorção condilar como ômega-3, tetraciclinas, estatinas e inibidores do receptor de IL-6 (denosumab, tocilizumab) (Gunson et al.,2012) e bloqueadores de TNF-α, como o infliximab (Bevaart et al.,2010).

Omega-3 de ácidos graxos poli-insaturados de cadeia longa (PUFA) têm propriedades anti-inflamatórias, benéficas nas doenças cardiovasculares, artrite reumatoide (Olson et al., 2013), constituintes importantes dos fosfolipídios das membranas celulares, onde desempenham um papel na permeabilidade iónica da membrana (Calder, 2013). Pesquisa em animais relatam que os ácidos graxos reduziram a incidência e gravidade da AR (Knott et al.,2011). Redução no nível de IL-1 em um modelo in-vitro de inflamação de cartilagem mostrou que o ômega 3 tem potencial terapêutico no tratamento de doenças inflamatórias articulares (Wann et al., 2010). Sugerindo que suplementos de ácidos graxos melhoram a resposta inflamatória e diminuem a dose diária de NSAID em pacientes com artrite idiopática juvenil (Gheita et al., 2012).

Outra hipótese de ação do ω-3 está na competição com o estoque de ácido araquidônico ocasionando à inibição da produção de eicosanoides pró-inflamatórios. Podem servir como substratos alternativos para a cicloxigenase (COX1 e COX2) resultando na formação de produtos menos potentes que os pró-inflamatórios (Serhan et al., 2009). Dessa forma, o ômega 3 poderia desempenhar um papel modulador das reações inflamatórias, sejam elas agudas ou crônicas.

# 2 ARTIGO: THE EFFECT OF OMEGA-3 IN TMJ SYNOVIAL TISSUES OF RATS WITH INDUCED ARTHRITIS: PILOT STUDY

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**Key words:** Temporomandibular Joint; Cartilage; Omega-3; Anti-inflammatory; Cytokine

**Short Title:** “The Effect of Omega-3 in TMJ”

**ABSTRACT**

The aim of this study was to evaluate the effect of systemic administration of omega-3 in the expression of IL-1β, IL-10 and TNF-α and in the cartilage thickness of temporomandibular joint (TMJ) inflammatory model induced by complete Freund's Adjuvant (CFA). Thirty-two adult rats were equally divided into four groups: 1) control and induced arthritis (IR) rats who received administration during seven 7 days of 2) 0.89% NaCl (CFA), 3) dexamethasone and 4) omega-3. After that, the TMJs were removed and assigned for histomorphometric analysis and immunoassay. Kruskal–Wallis followed by Dunn was applied to the data and the significance level was set at 5%. The highest levels of IL-1β and TNF-α were found in the CFA group ($P<0.050$) and there were no differences amongst the control, omega-3 and dexamethasone groups ($P>0.050$). IL-10 levels were the lowest in the CFA group ($P<0.050$), and, likewise, no differences were found amongst the control, omega-3 and dexamethasone groups ($P>0.050$). In addition, omega-3 group presented the largest fibrous and hypertrophic layer thickness ($P<0.050$). In conclusion, omega-3 can successfully reduce the damage in the TMJ of IR rats and, therefore, can be considered a promising alternative to the use of glucocorticoids.

## INTRODUCTION

Rheumatoid arthritis (RA) is a progressive, chronic, inflammatory and autoimmune disease that affects the joints and bones [1] and it is triggered by immune, genetic, and environmental factors [2]. The causative factors of RA are not fully established yet, but both autoimmune processes (innate and adaptive) seem to play an important role in the disease pathogenesis [3, 4].

One of the pathological characteristics of RA is bone erosion, which generally occurs in the joints of the hands and feet [5]. The temporomandibular joint (TMJ) presents different characteristics (morphological, biomechanics, functional, and biological) from other joints, such as hands and feet [6]. In particular, the most superficial cellular layer in TMJ is a fibrocartilage composed of collagen type I, and deeper cellular areas contain collagen type II [7], which is a unique feature of the TMJ [8]. In spite of these differences, the TMJ is subject to disorders that can affect other synovial joints, including RA [9], which may be severe and debilitating and compromise the masticatory function [10].

Increased levels of inflammatory cytokines (IL-1β, IL-6, TNF-α, and PGE2) in synovial fluid have been found in the internal derangements of TMJ, e.g., anterior disc displacement and osteoarthritis [11]. IL-1β is an important factor in the development of RA and seems to guide cell migration and stimulating endothelial cells [12]. Low levels of IL-10 were detected in RA patients and animals models of induced arthritis (IR) by the complete Freund adjuvant (CFA) injection [13]. A high proportion of arachidonic acid (AA) and a low proportion of other 20 carbons n-6 polyunsaturated fatty acids (PUFAs) are present in immune cells involved in RA [14]. In addition, COX-2 expression is increased in synovial fluid of RA patients and in articular tissues of rats with IR [12].

There is still no cure for RA and the main goal is to decrease and keep the inflammation to a doable level [15]. The gold-standard treatment for RA is the intra- or extra-articular injection of corticoids [16]. However, prolonged or repetitive use of corticoids leads to the joint collapse and it can also decrease the TMJ articular cartilage thickness in healthy rats [17]. The long-term administration of corticoids can cause serious side effects and toxicity,

such as increased cardiovascular risk and gastrointestinal disorders, osteoporosis, diabetes, weight-gain and increased blood pressure [14, 18]. Therefore, other drugs, such as tetracycline, statins and IL-6-receptor inhibitors have been applied to delay the condylar resorption [19]. In particular, omega-3 polyunsaturated fatty acid (PUFA) has anti-inflammatory properties, and it can be therapeutic beneficial for cardiovascular disease, arthritis, and inflammatory bowel disease [20]. Omega-3 reduces the incidence and severity of IR in rats [21] and it induces a significant reduction in IL-1 level in a cartilage in-vitro model [22]. In addition, it improved the anti-inflammatory effect and reduced the daily dose of NSAIDs, in patients with juvenile idiopathic arthritis [23].

Based on that, the aim of this study was to evaluate the effect of systemic administration of omega-3 in the expression of IL-1β, IL-10 and TNF-α and in the cartilage thickness of a TMJ inflammatory model induced by complete Freund's Adjuvant (CFA) injection.

## MATERIALS AND METHODS

### Animals

The study was approved by the Ethics Committee of Animal Experimentation (CEEA) of the Biology Institute-UNICAMP under the protocol #4182-1. Thirty-two adult rats, aging 10 to 12 weeks and weighing 350 to 400 g, were used. They were kept under *ad libitum* water and food, at 22 (± 1) ºC. The following formulations were used sterile saline (0.89% NaCl); dexamethasone (Decadron® 4 mg/mL - Aché pharmaceutical labs, São Paulo, Brazil); and omega-3 (OmegaPure 90%®, omega-3 fatty acids, eicosapentaenoic acid (EPA) 235mg and docosahexaenoic acid (DHA) 165 mg, Biobalance Nutraceuticals, Porto Feliz-São Paulo, Brazil).

### Induction of arthritis and drug administration

The arthritis model used in the present study was modified from a previously described method [12]. In brief, type II bovine collagen (CII) obtained from Becton Sigma-Aldrich (St. Louis, MO, USA) was dissolved in 0.05 M acetic acid to 2.0 mg/mL (suspension 1). The arthritis-induction formulation (CFA+CII) was obtained by emulsifying (1:1) the suspension 1 in CFA (Sigma-Aldrich, St. Louis, MO, USA). In order to immunize the animals, 100 µL of CFA+CII was injected intradermically at the tail base of 24 animals. After five days, a new injection was applied at the same site. Eight animals of the control group received only an injection of 100 µL of 0.89% NaCl intradermically at the tail base of the animals. After 15 days of the first injection, the animals of the control group received a single administration of 1 mL of 0.89% NaCl, p.o., daily, for 7 days. The animals with arthritis were divided into the following 3 groups (n=8):

CFA group – administration of 1 mL of 0.89% NaCl, p.o., daily, during 7 days;

Dexa group – administration of 0.1 mg/kg dexamethasone (Decadron® 4 mg/mL - Aché pharmaceutical labs, São Paulo, Brazil), via I.M., daily, during 7 days;

Omega group – administration of 300 mg/kg omega-3 (OmegaPure 90%®, omega-3 fatty acids, EPA + DHA 235mg 165mg, Biobalance Nutraceuticals, Porto Feliz - São Paulo,

Brazil), p.o., daily, during 7 days. The dose (300 mg/kg) was chosen according to the Health Canada Guidelines in order to obtain a dose equivalent to omega-3 used in humans (3 g/day) [12].

**Obtaining the tissues or tissue processing**

After 7 days of the treatments, all animals were anaesthetized by 2% xylazine (10 mg/kg IM) and 10% ketamine (90 mg/kg IM) and killed by asphyxiation ($CO_2$). The TMJs on both sides were then removed and designed for histological analysis or immunoassay.

**Histological analysis**

Histomorphometric analyses were performed in the TMJs stored in 10% paraformaldehyde (maintained during 24 hours at 4 $^{o}$C). TMJs were decalcified in 6% ethylenediaminetetraacetic acid (EDTA) and 0.1 M phosphate buffer (pH 7.4) during 3 months. The TMJs were then submitted to nine serial coronal sections (nine sections per animal 6 mm thick) after inclusion in paraffin blocks. The sections were then stained with hematoxylin-eosin (HE), toluidine blue, and Picrossirius hematoxylin-red. Thickness of fibrous, proliferative, mature, and hypertrophic cartilage layers were measured at the apical region of TMJ condyle with the aid of the Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Rockville, MD, EUA) in three different areas [24]. Finally, an Olympus BX51 microscope (Melville, NY, USA), equipped with polarized light filters, was used to observe the organization of collagen fibers at ×5 and ×20 magnification.

**ELISA assays**

The TMJs assigned for immunoassay were macerated and centrifuged in 50 mM Tris HCl (pH 7.5). The levels of IL-1β, IL-10, and TNF-α were analyzed in the supernatants by using ELISA kits (PeproTech Inc., Rocky Hill, NJ, USA). The plates were coated with individual cytokine-specific capture anti-bodies, being the samples, detection antibodies, and enzyme-linked secondary antibodies later added. The color change following the addition of

2,20-azino-bis (3-ethylben-zothiazoline-6-sulfonic acid) (ABTS) was determined by measuring the optical density at 405 nm with the wavelength correction set to 650 nm.

**Statistical analysis**

Sample size calculation indicated that seven animals in each group would provide 95% power for a minimal mean difference of total thickness cartilage of 10 μm ± 4 μm among groups considering 5% of significance level. Thus, eight animals were included in order to prevent any animal loss during the experiment.

The Levene's and Shapiro–Wilk tests were applied to assess, respectively, homogeneity of variances and the data distribution. The cytokine levels and the thickness of the articular cartilage layers were analyzed by Kruskal–Wallis followed by Dunn test (*post hoc*) and they were expressed as median and interquartile range (IQR). Statistical analyses were performed using Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA), and the significance level was set at 5%.

## RESULTS

### Cytokines levels

The levels of IL-1β (Fig. 1A) were higher (Kruskal–Wallis, H = 23.2; df = 3, P < 0.0001) in the CFA group (median 46.4 (IQR 39.4-53.3) ng/ml) than in the control group (median 1.81 (IQR 1.47-5.41) ng/ml). In addition, there were no differences (P > 0.050) amongst control, omega-3 and dexamethasone groups for IL-1b.

IL-10 levels (Fig. 1B) were the lowest (Kruskal–Wallis, H = 23.63; df = 3, P < 0.0001) in the CFA group (median 73.5 (IQR 52.8-90.5) ng/ml). Likewise, no differences were found amongst the control, omega-3 and dexamethasone groups (P > 0.050).

TNF-α levels (Fig. 1C) were also higher (Kruskal–Wallis, H = 49.2; df = 3, P < 0.0001) in the CFA group (median 122.7 (IQR 92.9-284.7) ng/ml) than in control (median 29.1 (IQR 23.7-31.3) ng/ml), omega-3 (median 57.9 (IQR 48.8-62.4) ng/ml) and dexamethasone (median 57.4 (IQR 50.6-68.9) ng/ml) groups. Both omega-3 (P = 0.0313) and dexamethasone (P = 0.0081) induced higher TNF-α levels than in control group.

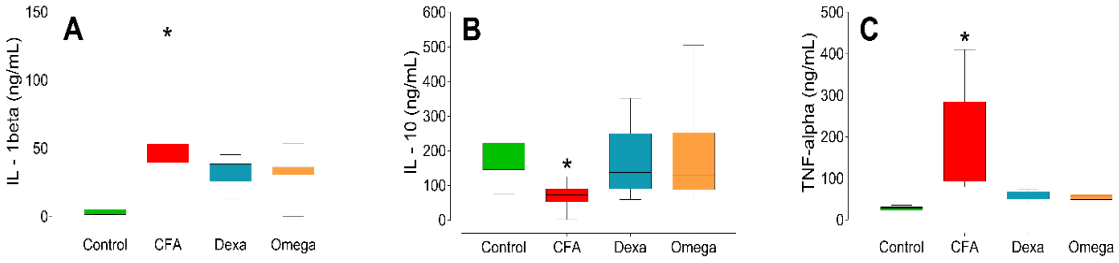


Figure 1. Levels (central bar = median value; boxes = first and third quartiles; whiskers = maximum and minimum values) of IL-1β (A), IL-10 (B), and TNF-α (C) in the synovial fluid. * significant differences between groups (p<0.050).

**Cartilage layer thickness**

The histomorphometric outcomes are presented in Table 1 and Fig. 2. The smallest (Kruskal–Wallis, H = 23.2; df = 3, P < 0.0001) total thickness (Table 1) was found in the CFA group, whereas the dexamethasone group presented the largest total thickness.

**TABLES**

Table 1. Thickness (median and interquartile range) of the total cartilage layer (in µm).

| | **CII in CFA** | **Control** | **Dexamethasone** | **Omega-3** |
|---|---|---|---|---|
| 1st quartile | 145.4 | 193.8 | 178.6 | 162.9 |
| **Median** | **160.1 a** | **202.7 b** | **265.9 c** | **172.9 d** |
| 3rd quartile | 203.8 | 213.9 | 287.5 | 211.9 |

The thickness of fibrous layer (Fig. 2A) was smaller (Kruskal–Wallis, H = 70.52; df = 3, P < 0.0001) in CFA (median 19.0 (IQR 16.7-21.2) µm) when compared to control (median 23.4 (IQR 20.9-27.2) µm), omega-3 (median 31.4 (IQR 27.2-34.6) µm) and dexamethasone (median 26.5 (IQR 22.7-30.0) µm). No significant differences (P = 0.30) were observed between control and dexamethasone, but omega-3 induced larger fibrous layer thickness than both control (P < 0.0001) and dexamethasone (P = 0.0409).

The thickness (Kruskal–Wallis, H = 88.8; df = 3) of mature layer (Fig. 2B) was larger in dexamethasone (median 65.9 (IQR 59.6-72.1) µm) than in CFA (median 27.3 (IQR 22.1-39.0) µm, P < 0.0001), control (median 35.8 (IQR 31.1-38.7) µm, P < 0.0001), and omega-3 (median 40.4 (IQR 34.8-45.2) µm, P < 0.0001) groups. No significant differences were observed between control and CFA (P = 0.33) or omega-3 (P = 0.40) groups. However, omega-3 induced larger thickness when compared to CFA group (P = 0.0010).

Proliferative layer thickness (Fig. 2C) was larger (Kruskal–Wallis, H = 47.1; df = 3, P <0.0001) in omega-3 (median 55.1 (IQR 50.1-65.2) µm) and dexamethasone (median 59.5 (IQR 51.1-65.9) µm) than in both control (median 46.5 (IQR 30.2-50.5) µm) and CFA (median 35.0 (IQR 26.2-52.9) µm) groups.

The thickness (Kruskal–Wallis, H = 57.4; df = 3) of hypertrophic layer (Fig. 2D) was larger in omega-3 (median 109.6 (IQR 93.3-120.5) µm) than in CFA (median 72.7 (IQR 65.8-82.8) µm, P < 0.0001), control (median 86.4 (IQR 72.5-99.5) µm, P < 0.0001), and dexamethasone (median 88.2 (IQR 84.7-96.4) µm, P = 0.0057) groups. CFA induced the smallest thickness for the hypertrophic layer. No significant differences were observed between control and dexamethasone (P = 0.99) groups.

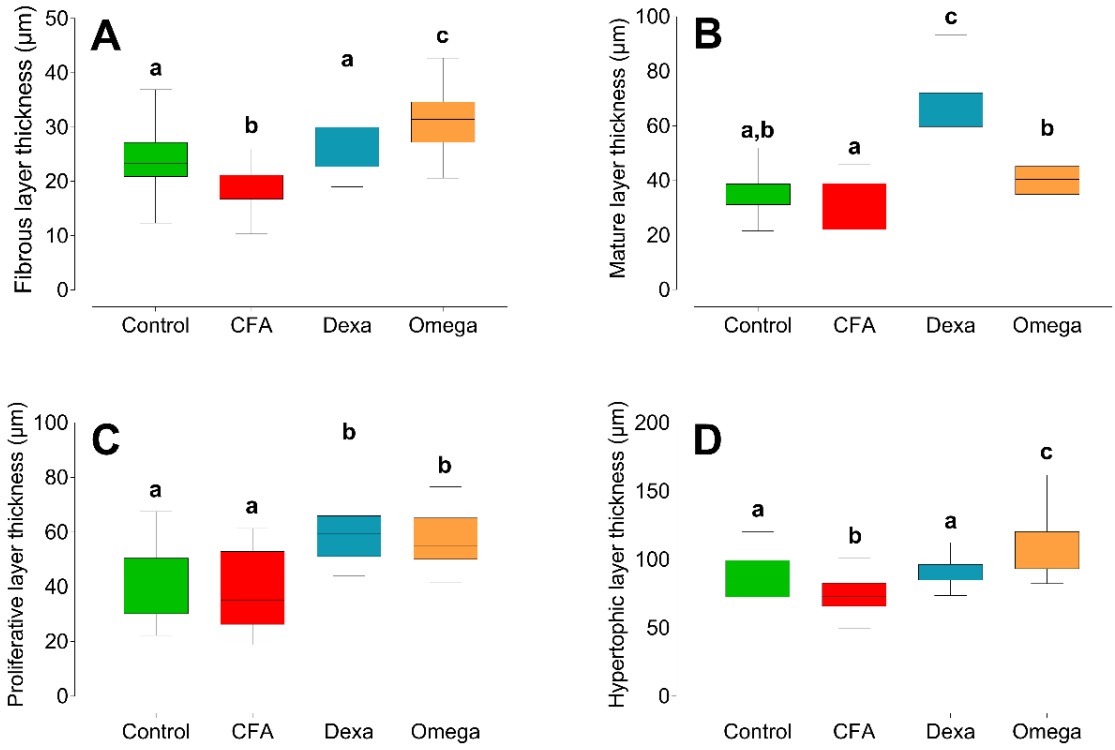


Figure 2. Thickness (central bar = median value; boxes = first and third quartiles; whiskers = maximum and minimum values) of the fibrous (A), mature (B), proliferative (C), and hypertrophic (D) layers. Different lowercase letters indicate significant between-group differences (p<0.050).

**Histomorphometric analysis**

Cellular disorder, flat-amorphous chondrocytes and decreased density of proteoglycans were observed in the animals of CFA group when compared to the control

group (Figs. 3F and 3J). Animals treated with dexamethasone and omega-3 showed cell morphology reorganization close to the normal aspect and a decreased number of chondrocytes in the mature layer (Fig. 3K). Omega-3 also increased the density of proteoglycans, which was similar to the control group (Fig. 3L).

The polarization microscopy of Sirius-red staining shows the organization of collagen fibers (Figs. 3M, 3N, 3O and 3P). All the animals in the CFA group showed an organizational deterioration in the collagen fibers of condylar cartilage and subchondral bone with decreased amount of fibers (Fig. 3N). On the other hand, the organization and amount of fibers in the animals treated with dexamethasone and omega-3 were similar to the control group. (Fig. 3O and 3P).

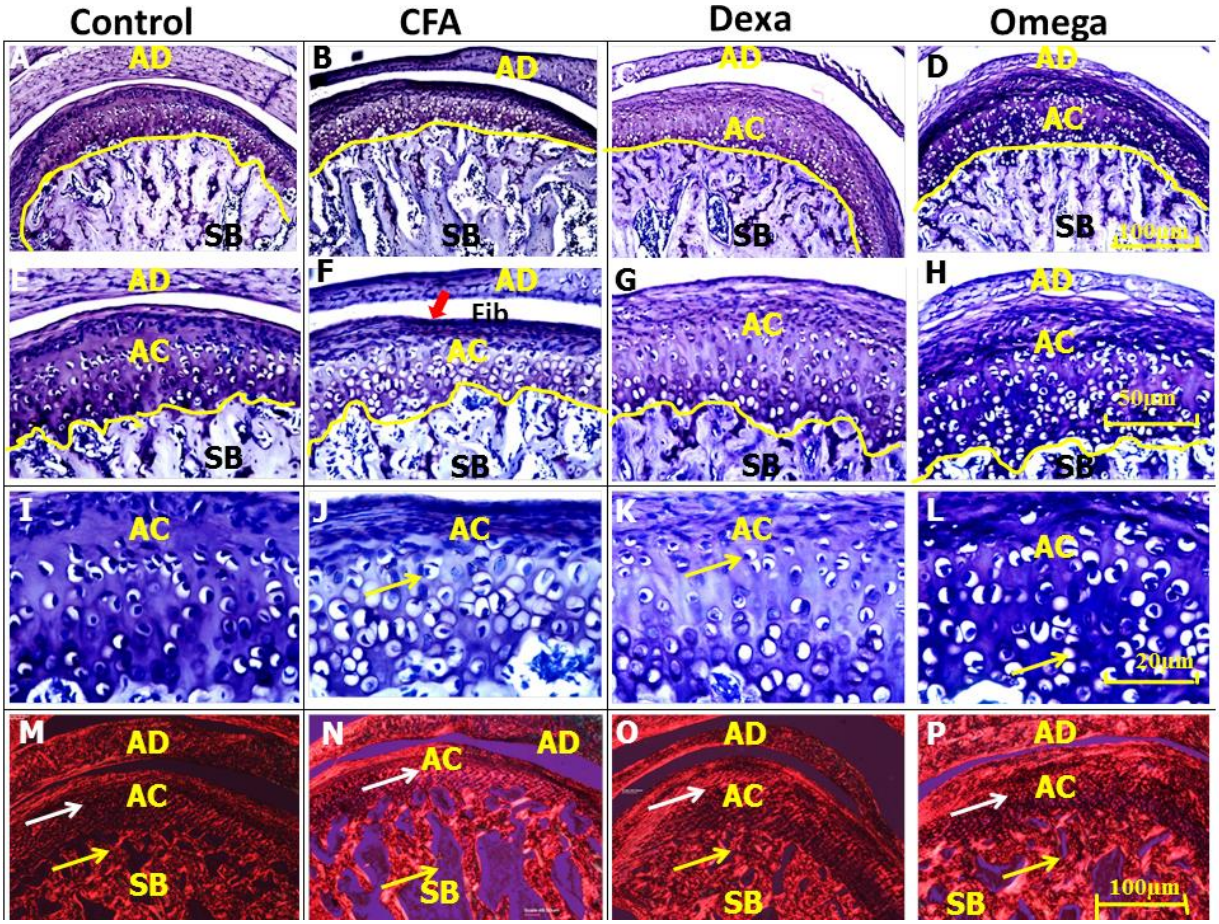


Figure 3. Coronal sections of TMJs in toluidine blue staining A–D: 10× magnification, 100 mm scale bar; E–H: 20× magnification, 50 mm scale bar; I–L: 40× magnification, 20 mm scale bar. Increased density of proteoglycans is indicated by yellow arrows. M-P indicated polarization with Sirius-red staining. The condyle regions are as follows: AD= articular disc;

AC= articular cartilage; SB= subchondral bone. Cartilage and subchondral bone are divided by the dashed yellow lines. P = proliferative layer; H = hypertrophic layer.

**DISCUSSION**

The present investigation primarily aimed to assess the anti-inflammatory effect of omega-3 in the TMJ of arthritic rats and the main findings were: 1) omega-3 stimulated significant changes in the expression of IL-1β, IL-10, and TNF-α; 2) omega-3 induced the largest thickness of the fibrous and hypertrophic condylar cartilage layers.

Dexamethasone doses described in IR rat models vary in the indexed literature (REF). The most used dexamethasone doses were similar or equal to the dose used in the present study (0.1 mg/kg/7days) (REF). However, the periods of treatment varied from 24 days (Ashraf et al., 2011), 5, 14 or 21 days (Cuzzocrea et al., 2005), 10 days (Jaffré et al., 2003), and more recently from 1 to 7 days (Wong et al., 2019). This dose has been proved effective in reducing pain behavior and synovial inflammation in IR model (REF). Similarly, omega-3 doses were based in previous studies showing effect on inflammation models (Estevão-Silva et al., 2016; Adeyemi e Olayaki, 2017). Nonetheless, further investigations systematically comparing different dosages should better clarify the effects of dexamethasone and omega-3 in the TMJ of arthritic rats.

This is the first study to associate omega-3 PUFA, EPA and DHA in order to assess the anti-inflammatory effects in the TMJ, although previous studies have reported the effects of omega-3 in the limb joints of IR rodents [20, 22]. Fish oils derivative omega-3, EPA and DHA can reduce the inflammatory response in IR through the competitive inhibition of PGE2 and LTB4 production, which, in turn can inhibit the activation of NFκB and the release of proinflammatory cytokines such as IL-1β and TNF-α [12]. Evidence from *in vitro* studies has confirmed that both EPA and DHA can inhibit the production of proinflammatory cytokines IL-1β, TNF-α and IL-6 in various cell types [5]. In addition, the analgesic and anti-inflammatory effect of omega-3 PUFAs may be associated with mediators of endogenous lipids, e.g., lipoxins and resolvins proteins derived from fatty acids [25] and cutting-edge RA therapy investigations include antibodies to TNF-α, IL-1β and IL-6 [26]. Therefore, the lower levels of IL-1β and TNF-α in the TMJ of IR rats following the systemic administration of omega-3,

which were similar to the administration of dexamethasone, could corroborate the abovementioned mechanism of action.

Our results also showed a positive effect of omega-3 treatment considering the overall thickness of the articular cartilage. Recent animal investigations have reported that omega-3 can attenuate the damage and can inhibit the degradation of the knee cartilage matrix [27, 28]. In the present study, omega-3 was able to increase the thickness of the fibrous layer, which is the responsible for resistance against compressive forces, allowing tissue deformation [35]. Fibro-cartilaginous cells, which are the main component of fibrous layer, have limited ability to self-reparation [36]. Thus, the preservation of this layer is important for the maintenance of the articulation function. In addition, omega-3 also preserved the hypertrophic layer. The hypertrophic chondrocytes are important to maintain the cartilage integrity and there is evidence that apoptosis and the autophagy of these cells are presented is RA cartilage [37]. Possible mechanisms underlying this protective response of the omega-3 are the increasing proteoglycan synthesis, chondrocyte proliferation, thickening and reorientation of collagen fibers. Furthermore, some studies have reported that EPA can inhibit the oxidative stress-induced apoptosis and loss of chondrocyte matrix [27], which might be associated with increased collagen synthesis and decreased PGE2.

Dexamethasone-treated rats presented lower levels of IL-1β and TNF-α and higher levels of IL10 when compared to the non-treated arthritic animals, which was a expected results considering previous evidence from the knee joint of IR rats. These positive effects are probably related to the increase transcription of anti-inflammatory cytokines, such as IL-1Ra (IL-1 receptor antagonist) and IL-10, and the inhibition of pro-inflammatory, such as IL-1β, IL-6 and TNF-α. In addition, dexamethasone also increased the overall thickness of the articular cartilage, in particular, the mature and proliferative layers. Previous evidence has already shown that dexamethasone can increase the proliferation and differentiation of chondrocytes and the synthesis of proteoglycans [29]. Besides this anabolic effect in the cartilage tissue metabolism, glucocorticoid treatment could also increase water retention between intercellular spaces.

The applied IR model (CFA + CII) effectively damaged the TMJ as it was expected. The total cartilage thickness was the smallest, in particular the proliferative and hypertrophic layers, where it could be observed reduced proteoglycans, presence of mature chondroblasts and morphological alterations when compared to the other groups. It has been reported that systemic induction of arthritis with bovine type II collagen in rats promotes enhanced synovial inflammation, bone erosion, cartilage damage and leukocytes infiltration in the knee joints and hind paws [30]. The structure of the TMJ condyle cartilage is macroscopically similar to the articular cartilage of other synovial joints, and, therefore, pathological changes induced by RA would also be equivalent [31]. Furthermore, joint damage can induce the release of pro-inflammatory cytokines and decrease the level of anti-inflammatory mediators within the joint space [32, 33]. Interestingly, it was observed lower levels of IL-10 in the CFA group, which may indicate a severe inflammatory response, considering that IL-10 can attenuate the release of pro-inflammatory cytokines from macrophages and neutrophils [34].

In conclusion, omega-3 can successfully reduce the damage in the TMJ of IR rats and, therefore, can be considered a promising alternative to the use of glucocorticoids.

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## 3 CONCLUSÃO

A atrite sistêmica induzida pela combinação de CFA e colágeno bovino tipo II provocou alterações na ATM dos animais, aumentando níveis de citocinas pró-inflamatórias (IL1-β e TNF-α) e reduzindo o anti-inflamatória (IL-10), além de diminuir a espessura da cartilagem articular. O tratamento com dexametasona e ômega-3 melhoraram significativamente o quadro inflamatório. O tratamento com ômega-3 produziu efeito anti-inflamatório similar aquele da dexametasona.

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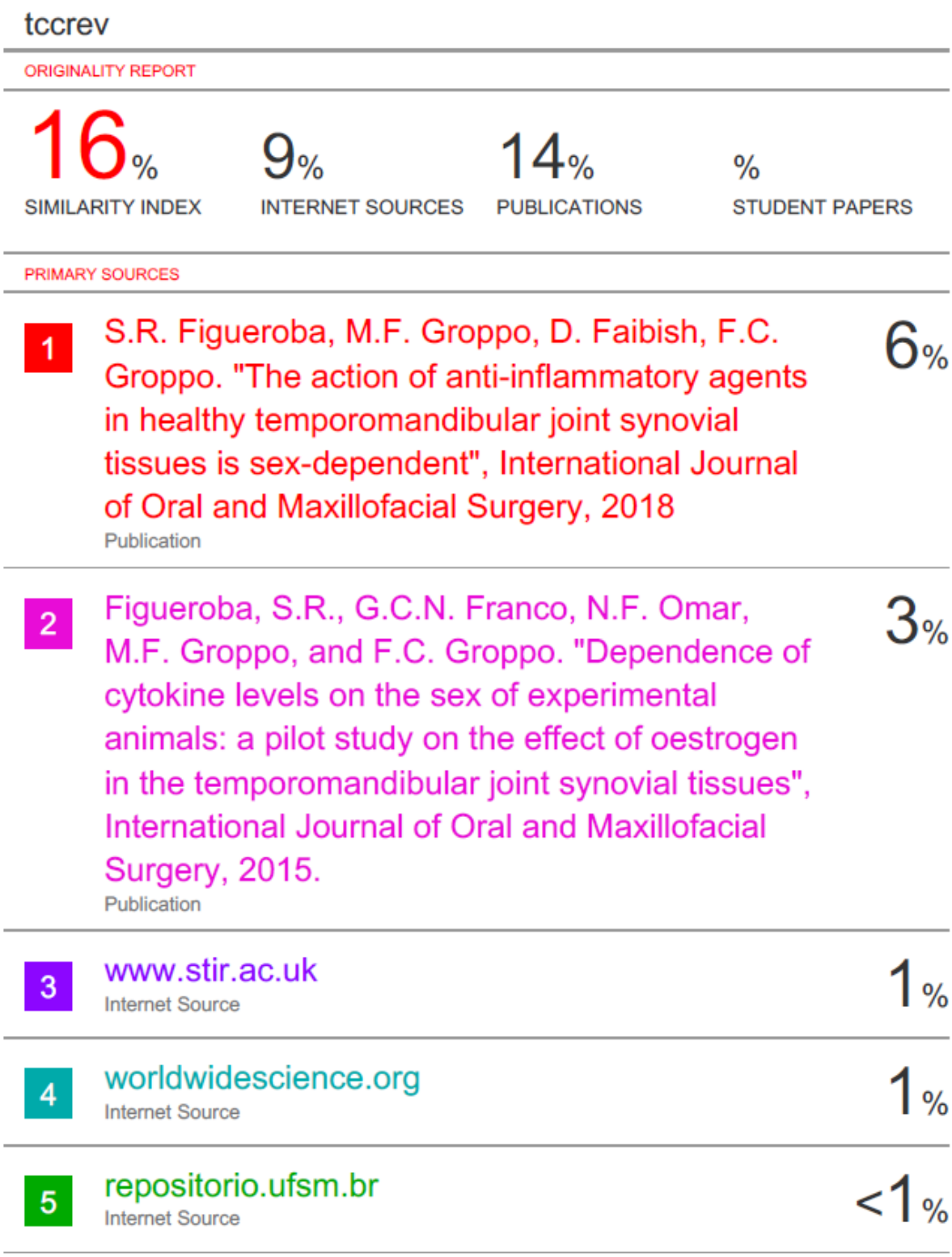
**ANEXOS**

**Anexo 1 – Verificação de originalidade e prevenção de plágio**



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| 1 | S.R. Figueroba, M.F. Groppo, D. Faibish, F.C. Groppo. "The action of anti-inflammatory agents in healthy temporomandibular joint synovial tissues is sex-dependent", International Journal of Oral and Maxillofacial Surgery, 2018<br>Publication | 6% |
| 2 | Figueroba, S.R., G.C.N. Franco, N.F. Omar, M.F. Groppo, and F.C. Groppo. "Dependence of cytokine levels on the sex of experimental animals: a pilot study on the effect of oestrogen in the temporomandibular joint synovial tissues", International Journal of Oral and Maxillofacial Surgery, 2015.<br>Publication | 3% |
| 3 | www.stir.ac.uk<br>Internet Source | 1% |
| 4 | worldwidescience.org<br>Internet Source | 1% |
| 5 | repositorio.ufsm.br<br>Internet Source | <1% |

**Anexo 2 – Comitê de Ética em Pesquisa**

UNICAMP

CEUA/UNICAMP

# CERTIFICADO

Certificamos que a proposta intitulada **INFLUÊNCIA DO ÔMEGA 3 NO PROCESSO INFLAMATÓRIO INDUZIDO POR CFA NA ATM DE RATOS**, registrada com o nº **4182-1**, sob a responsabilidade de **Prof. Dr. Francisco Carlos Groppo e Sidney Figueroba Raimundo**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **16 de junho de 2016**.

| | |
|---|---|
| **Finalidade:** | **( ) Ensino ( X ) Pesquisa Científica** |
| **Vigência do projeto:** | **16/05/2016-16/05/2018** |
| **Vigência da autorização para manipulação animal:** | **16/06/2016-16/05/2018** |
| **Espécie / linhagem/ raça:** | **Rato Heterogênico / HanUnib: WH (Wistar)** |
| **No. de animais:** | **64** |
| **Peso / Idade:** | **03 meses / 300g** |
| **Sexo:** | **machos** |
| **Origem:** | **CEMIB/UNICAMP** |

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

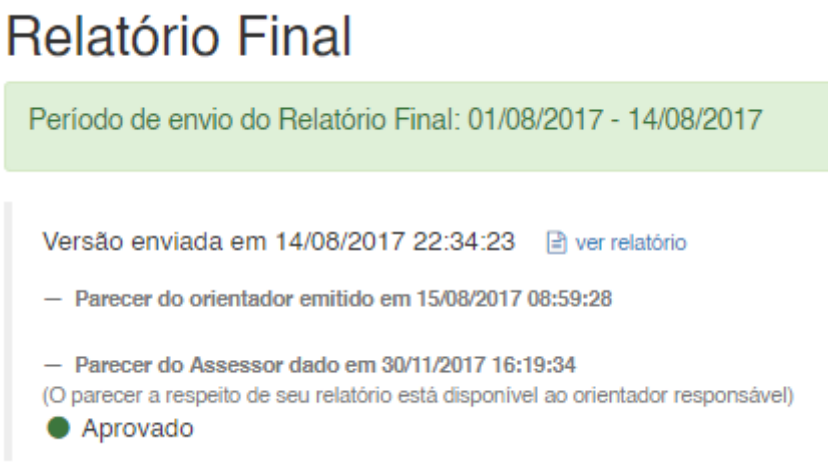
Campinas, 16 de junho de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

**IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.**

**Anexo 3 – Iniciação Científica**



**Anexo 4 – Comprovante de submissão do Artigo**

# International Journal of Oral & Maxillofacial Surgery

## The Effect of Omega-3 in TMJ Synovial Tissues of Rats with Induced Arthritis: Pilot Study.

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | IJOMS-D-19-00330R1 |
| **Article Type:** | Research Paper |
| **Keywords:** | Temporomandibular Joint; Cartilage; Omega-3; Anti-inflammatory; Cytokine |
| **Corresponding Author:** | Yuri Martins Martins Costa, DDS, PhD<br>Piracicaba Dental School - University of Campinas<br>Piracicaba, BRAZIL |
| **First Author:** | Thomas Barbin |
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| | Felipe C Toledo |
| | Yuri Martins Martins Costa, DDS, PhD |
| | Juliana T Napimoga, DDS, PhD |
| | Sidney R Figueroba, DDS, PhD |
| **Manuscript Region of Origin:** | BRAZIL |
| **Abstract:** | The aim of this study was to evaluate the effect of systemic administration of omega-3 in the expression of IL-1β, IL-10 and TNF-α and in the cartilage thickness of temporomandibular joint (TMJ) inflammatory model induced by complete Freund's Adjuvant (CFA). Thirty-two adult rats were equally divided into four groups: 1) control and induced arthritis (IR) rats who received administration during seven 7 days of 2) 0.89% NaCl (CFA), 3) dexamethasone and 4) omega-3. After that, the TMJs were removed and assigned for histomorphometric analysis and immunoassay. Kruskal–Wallis followed by Dunn was applied to the data and the significance level was set at 5%. The highest levels of IL-1b and TNF-α were found in the CFA group ($P<0.050$) and there were no differences amongst the control, omega-3 and dexamethasone groups ($P>0.050$). IL-10 levels were the lowest in the CFA group ($P<0.050$), and, likewise, no differences were found amongst the control, omega-3 and dexamethasone groups ($P>0.050$). In addition, omega-3 group presented the largest fibrous and hypertrophic layer thickness ($P<0.050$). In conclusion, omega-3 can successfully reduce the damage in the TMJ of IR rats and, therefore, can be considered a promising alternative to the use of glucocorticoids. |