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Juliano Henrique Perotto

ANÁLISE IMUNO-HISTOQUÍMICA DA METALOPROTEASE 13 NA DISFUNÇÃO DA ARTICULAÇÃO TEMPOROMANDIBULAR

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Juliano Henrique Perotto

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para a obtenção do titulo de Mestre em Ciências da Saúde.

Orientadora: Prof. Dra. Paula Cristina Trevilatto

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DEDICO

Aos meus familiares, e em especial aos meus pais e namorada, por me apoiarem e participarem ativamente de todas as fases da minha vida.

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“A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original”.

Albert Einstein

SUMÁRIO

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RESUMO

A articulação temporomandibular (ATM) humana está em constante remodelação. Fatores genéticos e influências do meio externo, como hábitos parafuncionais, participam nessa remodelação e adaptação dos tecidos articulares. As metaloproteases (MMPs) são enzimas que participam nesse processo e fazem parte da ampla família das metaloproteases que assumem um papel importante na cicatrização e remodelação tecidual. Das MMPs, as colagenases (MMP-1,8 e 13) são as que clivam o colágeno e, nesse caso, a MMP-13 com maior rapidez que as demais cliva o colágeno tipo II. Este trabalho tem por objetivo identificar essa enzima nas diferentes fases fisiopatológicas das disfunções temporomandibulares e analisar sua associação com a osteoartrose. Foram analisados 39 espécimes de disco articular em diferentes estágios de degradação do disco, segundo a escala de Wilkes (n=31), e espécimes do grupo controle (n=8), compostos por fratura e hiperplasia de côndilo. Em outro corte, foram analisados n=10 com e n=29 sem osteoartrose. A marcação para MMP-13 foi realizada por meio da análise imuno-histoquímica. Diferenças significativas não foram encontradas entre os grupos controle e com deslocamento de disco com e sem redução, no que diz respeito a variável área de expressão da MMP-13 no exame de imuno-histoquímico e entre os grupos com e sem osteoartrite. Nos grupos Porém, houve um aumento, não estatisticamente significante, da MMP-13 no grupo com osteoartrose. Estudos futuros devem ser realizados com uma amostra maior, o que poderia confirmar a participação da MMP-13 nas fases mais agudas de degeneração, remodelação e osteoartrose da ATM.

ABSTRACT

Human temporomandibular joint (TMJ) is constantly being remodeled. Genetic factors and environmental influences such as parafunctional habits play a role on the remodeling/adaptation of these tissues. Metaloproteases (MMPs) are enzymes that participate on this process and belong to the metalloproteinases family, which have an important task in the healing process. Among the MMPs, the colegenase (MMP-1,8 and 13) are the ones that cleave the colagen, in this case the MMP-13 is the fastest acting enzyme on collagen type II. This study aim to identify this enzyme in different pathologic TMJ stages, comparing to control groups. 39 articular disc samples in different Wilkes stages were analyzed with a MMP-13 marker. No statistically difference was observed between the groups. Future studies are needed with a bigger sample, which may confirm the role of MMP-13 in the acute fases of TMJ degeneration or remodelation.

1. INTRODUÇÃO

1.1. Articulação Temporomandibular

A articulação temporomandibular (ATM) é uma das mais complexas articulações do corpo humano. Por sua complexidade, está sujeita a várias interferências. Está localizada bilateralmente na região pré-auricular, entre os ossos temporal e mandibular. É uma articulação gínglimo-artroidal, sendo a única articulação móvel do crânio, a qual realiza variados movimentos durante o desempenho de suas funções (FIGUN & GARINO, 1989).

A ATM, semelhante a outras articulações, pode adaptar-se às demandas funcionais, possuindo capacidade de remodelação. A cartilagem articular, que reveste a cabeça da mandíbula, e a eminência articular apresentam maior adaptação às forças funcionais, ao contrário do disco articular, que não possui tal capacidade (Fig. 1).

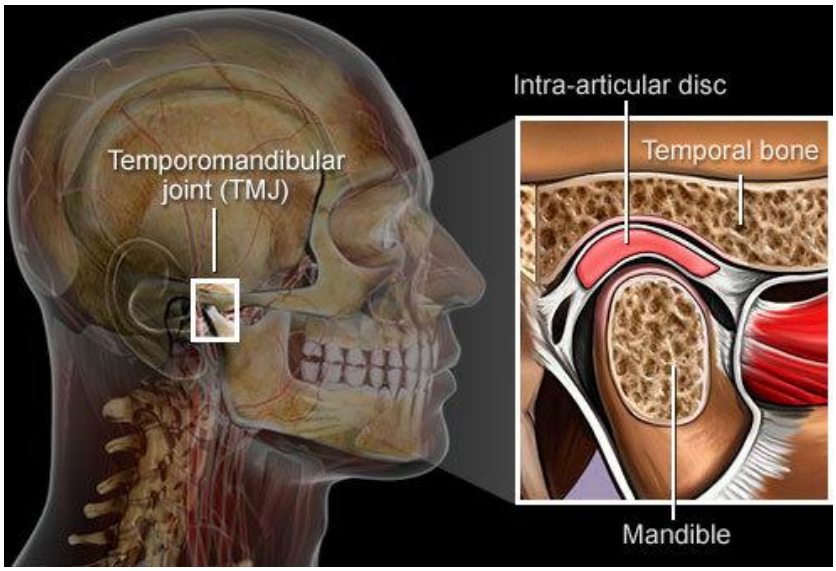

Fig. 1 - Anatomia da articulação temporomandibular (Fonte: www.dtmeoclusao.com.br).

Micro e macro trauma são os principais responsáveis pelas alterações patológicas (excetuando doenças sistêmicas) que levam a uma disfunção das articulações temporomandibulares (DIMITROULIS, 2005; WANG, 2008).

Devido ao trabalho concomitante das articulações, essas alterações na ATM, com o tempo, darão origem à disfunção na articulação contralateral (BROSSARD, 2005) e podem afetar todas as estruturas anatômicas adjacentes, incluindo a cápsula e ligamentos articulares, a membrana sinovial e estruturas ósseas da articulação (DIMITROULIS, 2005).

Alterações degenerativas na ATM modificam propriedades físicas e funcionais, primeiramente de forma reversível e, posteriormente, de forma irreversível, reduzindo sua capacidade de suportar forças de pressão e compressão (ISRAEL et al., 1991).

A disfunção na ATM é uma coleção de problemas clínicos que envolvem os músculos da mastigação e a articulação temporomandibular e estruturas adjacentes. (SILVA et al. 2014) O sintoma mais comum é a dor, mas outros sinais como ruídos e estalos podem estar associados. A evolução do quadro pode levar à restrição de função da articulação e até mesmo à degeneração. A prevalência dos sintomas pode chegar a 75% na população adulta (LEITE et al. 2009).

Clinicamente, a primeira alteração leve da articulação temporomandibular é caracterizada pelo deslocamento do disco articular, com ou sem remodelação óssea. Já em uma fase mais severa, podem-se observar desordens do tipo perfuração de disco, remodelação óssea e mudanças

osteoartríticas, conforme tabela I (WILKES, 1978). Sinais clínicos e sintomas têm mostrado mínima correlação com imagens radiográficas em pacientes com inflamação e disfunção da ATM em estágios iniciais (LEEUW et al., 1999).

Tabela I. Estágios de Wilkes para disfunções temporomandibulares.

ESTÁGIO	ASPECTO CLÍNICO	ASPECTO DAS IMAGENS	ASPECTO CIRÚRGICO
0 - NORMAL	Sem clique e sem dor	Disco bem posicionado	Não indicado
I - INICIAL	Clique sem dor; nenhuma restrição de movimentos	Disco levemente anteriorizado com redução*; contornos ósseos normais	Disco com formato normal; pequeno deslocamento anterior; descoordenação passiva (clique)
II - INICIAL/INTERMEDIÁRIO	Clique com dor ocasional; travamento intermitente; cefaléias	Disco levemente anteriorizado com redução; deformidade inicial do disco; contornos ósseos normais	Deslocamento anterior do disco; disco espessado
III - INTERMEDIÁRIO	Dor freqüente; sensibilidade articular; cefaléias; travamento; mobilidade restrita; mastigação dolorosa	Deslocamento anterior do disco com redução no início, progredindo para não redução* tardiamente; espessamento moderado a acentuado do disco	Deslocamento e deformidade do disco; adesões variáveis; nenhuma mudança óssea
IV - INTERMEDIÁRIA/AVANÇADA	Dor crônica; cefaléia; mobilidade restrita	Deslocamento anterior do disco sem redução; acentuado espessamento do disco; contornos ósseos anormais	Remodelação degenerativa das superfícies ósseas; osteófitos; adesões; disco deformado sem perfuração
V - AVANÇADO	Dor variável; crepitação articular; função com dor	Deslocamento anterior do disco sem redução com perfuração e grosseira deformação; mudanças ósseas degenerativas	Mudanças degenerativas grosseiras do disco e dos tecidos duros; perfuração; adesões múltiplas

Fonte: Wilkes, 1989.

1.2. **Disco Articular**

O disco articular na ATM apresenta-se como uma placa fibrocartilaginosa que recobre totalmente a superfície superior da cabeça da mandíbula. Superiormente este disco não se prende ao osso temporal. Já inferiormente, ele encontra-se fortemente unido à cabeça da mandíbula em dois pontos: pólo medial e lateral. Isto explica porque a mandíbula pode girar abaixo do disco articular sem movê-lo, ao passo que, em movimento de translação, o disco articular obrigatoriamente acompanha os movimentos mandibulares. Um desequilíbrio entre o disco articular e a mandíbula nestes movimentos poderá provocar ruídos articulares (FIGUN & GARINO, 1989).

O disco permite uma movimentação suave das estruturas da ATM, pois compensa a discrepância de forma existente entre elas e serve também para absorver os choques entre as estruturas.

Sendo coberto por uma fina camada de células sinoviais, o disco articular é envolto pelo líquido sinovial, de importância para lubrificar o disco e as superfícies articulares em ambos os compartimentos. Além disso, essas células sinoviais mantêm íntimo contato com inúmeros vasos linfáticos e capilares, ao longo de todo o perímetro discal.

Modificações na posição, forma ou estrutura anatômica do disco articular poderão ocasionar, em maior ou menor intensidade, alterações na função da ATM (ABRAMOWICZ & DOLWICK, 2010).

1.3 Inflamação

A inflamação tecidual é uma resposta benéfica, pois repara e protege após uma infecção ou trauma. Envolve várias reações moleculares e a efetividade dessa resposta é a habilidade do hospedeiro em interromper este processo, assim que o agente causador da inflamação seja removido, prevenindo a autodestruição tecidual. Esta auto-regulação ocorre através de um balanço entre mediadores pró- e antiinflamatórios.

Estudos têm mostrado que o processo inflamatório está intimamente ligado ao processo de destruição da ATM (MALEMUND & GOLDBERG, 1999). Frequentemente, as primeiras células a responder a um estímulo inflamatório são os mastócitos e macrófagos (NATHAN, 2002). Essas células liberam vários fatores pró-inflamatórios, incluindo citocinas, as quais vão desencadear múltiplas cadeias reacionais, culminando com a destruição da matriz tecidual, por ação das metaloproteases da matriz (MMPs) (KURODA, 2009). Células constitutivas (ex. células ósseas, fibrobrocondrócitos) liberam MMPs, com a estimulação inflamatória (Fig. 2).

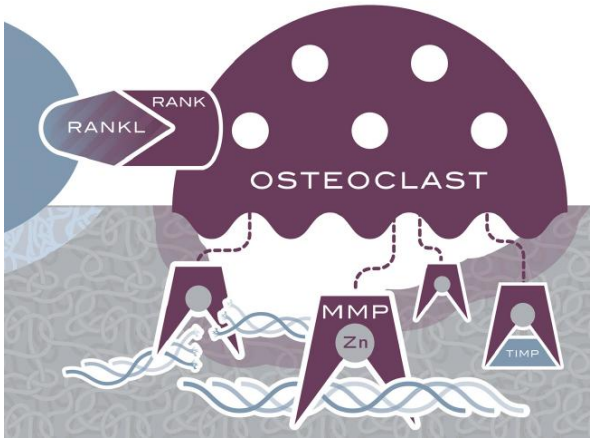

Fig. 2 - Osteoclasto produzindo MMPs, que atuam na degradação do colágeno da matriz. Fonte: Gunson et al. (2012).

1.4 Metaloproteases da Matriz Extracelular (MMPs)

As matrizes metaloproteases (MMPs) fazem parte da família das enzimas metaloproteases e assumem um papel importante na remodelação das lesões.

A matriz extracelular participa de muitos fenômenos celulares, como diferenciação e crescimento. Esta matriz está constantemente sendo sintetizada e degradada. Membros da família das metaloproteases são os principais reguladores da degradação da matriz extracelular (BIRKEDAL-HANSEN, 1993).

As MMPs são proteases conhecidas como reguladoras de proteólise da matriz extracelular e classicamente reconhecidas como enzimas de remodelação tecidual na presença de resposta inflamatória (VU, 2000) (Fig. 3). As MMPs compreendem uma família de enzimas que apresenta especificidade por macromoléculas que compõem a matriz extracelular. A família das MMPs é formada por pelo menos vinte membros que exibem similaridades funcionais e estruturais. Essas enzimas são secretadas na forma inativa, como zimógeno

e/ou como complexo enzima/inibidor (STRICKLIN et al., 1983; EMONAR/GRIMAUD, 1990). A ativação ocorre em duas etapas. Inicialmente o zimógeno sofre uma clivagem proteolítica por várias enzimas, como a tripsina, a plasmina, a catepsina B e a elastase (VAN WART & BIRKEDAL-HANSEN, 1990), o que resulta na remoção da porção aminoterminal. Em uma segunda etapa a enzima sofre autodigestão, resultando na sua forma ativa. Acredita-se que a ativação seja causada pela ruptura da ponte existente entre o aminoácido cisteína e o íon zinco, que bloqueia o sítio ativo da molécula. Outra característica comum entre as metaloproteases é a dependência dos íons zinco e cálcio. A interação do zinco com resíduos de histidina, presentes no domínio catalítico da molécula, tem importância crucial para o funcionamento adequado das metaloproteases. E dois átomos de cálcio conferem estabilidade para a estrutura terciária da proteína (DIOSZEGI, CANNON & VAN WART, 1995) (SOUZA et al., 2000).

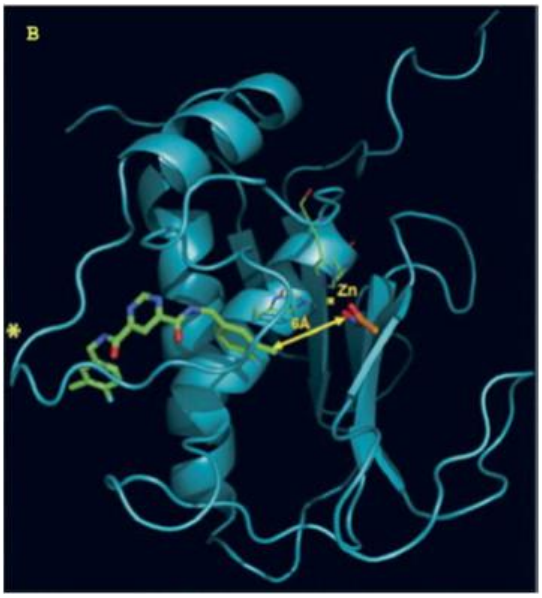

Fig. 3 - Figura representando a MMP-13. **Fonte:** Fields (2015).

As MMPs estão divididas em três importantes classes: as colagenases intersticiais, as gelatinases e as estromelisinas. Esta classificação baseia-se na

especificidade do substrato. As colagenases intersticiais são as mais específicas. Foram as primeiras a ser descritas (GROSS & NAGAI, 1962) e durante as últimas décadas têm sido objeto de diversos estudos que versam sobre sua expressão, distribuição, estrutura química e molecular, em processos normais e patológicos (BIRKEDAL-HANSEN, 1993). Estas enzimas são as únicas com capacidade de clivar a tríplice hélice dos colágenos I, II, III em condições fisiológicas, tornando estas moléculas suscetíveis à ação de outras enzimas (SOUZA et al., 1993). Existem três tipos de colagenases intersticiais que, apesar de exibirem semelhanças estruturais, são codificadas por genes distintos: a colagenase 1 (MMP-1), a colagenase 3 (MMP-13) e a colagenase de neutrófilo (MMP-8). As colagenases 1 e 3 são encontradas na cartilagem artrósica, em níveis proporcionais à severidade da doença (TAKAISHI et al., 2008). Atuam na quebra da tríplice hélice do colágeno e são sintetizadas nos condrócitos, estimulados por IL-1 (YAMAGUSHI et al., 2005). A colagenase 3 (MMP-13) foi descrita recentemente e tem uma função tão importante quanto à colagenase 1 (MMP-1), sendo 10 vezes mais rápida que esta (OTTERNESS et al., 2000). Experiências sugerem que ambas agem sobre a mesma ponte de cadeia do colágeno, sendo: MMP-13 de ação mais rápida, além de possibilitar uma segunda clivagem (ISHIGURO et al., 1999). A MMP-8 é produzida exclusivamente por leucócitos polimorfonucleares. Ao contrário da colagenase de fibroblasto, que é rapidamente secretada após sua síntese, a colagenase de polimorfonucleares é armazenada em grânulos intracelulares, somente liberados após a ativação dessas células (HIBBS & BAINTON, 1989).

Os genes que codificam as MMPs respondem a diferentes fatores de crescimento e citocinas inflamatórias. O TGF-β1 (Fator Transformador do Crescimento-β1) é aparentemente um dos mais importantes fatores

reguladores da síntese de MMPs em fibroblastos (TORRE-AMIONE et al., 1990). Este fator atua como estimulante da atividade reparadora nos tecidos, inibindo a expressão, síntese e liberação de MMPs pelos fibroblastos, além de estimular a produção de TIMP (inibidor tecidual de metaloproteinases), que neutraliza a atividade da enzima na matriz extracelular. Classicamente o TGF-β1 é considerado um fator promotor de fibrose. Entretanto, alguns autores classificam o TGF-β1 como um fator pró-inflamatório, uma vez que ele atua como agente quimiotático para neutrófilos, monócitos e linfócitos do sangue (TORRE-AMIONE et al., 1990).

A regulação da atividade proteolítica das metaloproteases ocorre em nível extracelular, através de proteínas inibidoras específicas de tecido, os TIMPs. Alterações nos níveis de síntese de TIMPs e MMPs podem levar a um desequilíbrio na taxa de degradação da matriz extracelular, podendo causar destruição anormal da matriz (MACNAUL et al., 1990). MMPs e seus inibidores (TIMPs) participam da remodelação tecidual fisiológica, mas em condições inflamatórias a sobre-expressão e superatividade das MMPs pode resultar em destruição tecidual (BROUSSARD, 2005; WANG, 2008).

1.5 Metaloproteases da Matriz em Doenças e Processos Degenerativos

As metaloproteases da matriz desempenham papel importante em vários processos fisiológicos e patológicos, como na involução pós-parto (WEEKS et al., 1976), na reabsorção óssea (OKADA et al., 1995), na inflamação (KNAUPER et al., 1993), na tuberculose (NINOMIYA et al., 2004) (BIRKEDAL-HANSEN, 1993), no infarto agudo do miocárdio (KAI et al., 1998), no

aneurisma aórtico abdominal (MCMILLAN & PEARCE, 1999), no crescimento e expansão de tumores benignos (AUTIO-HARMAINEN et al., 1993), na invasão e metástase de tumores malignos (DECLERCK et al, 1992; JUNG et al., 1997; TORII et al., 1997; LEIN et al., 2000), na artrite reumatóide (LIU et al., 2005; KANEKO et al., 2001; PAP et al., 2000; LINDY et al., 1997), artrite reumatóide juvenil (GATTORNO et al, 2002), na osteoartrite (WOESSNER, 1991; NAITO et al., 1999), na doença periodontal (de Souza et al., 2003) e na disfunção da articulação temporomandibular (YOSHIDA et al., 1999; GEPSTEIN, 2003; XU et al., 2003).

Alguns estudos mostram RNAm de MMP-13 em níveis elevados em carcinomas de mama e osteoartrite articular, tornando a MMP-13 um alvo de estudo no tratamento dessas alterações.

As MMPs são vistas como as maiores responsáveis pela destruição tecidual em doenças articulares degenerativas, com diferenças nas expressões de MMP-1, MMP-8, e MMP-13 em articulações sadias e doentes (WOESSNER Jr & GUNJA-SMITH, 1991).

Em um artigo de Leonardi et al. (2007) com discos humanos, foi identificado que quanto mais severamente danificado o disco, maior é o número de células imunomarcadas pela MMP-13. Vasos recém-formados no disco também foram imunomarcados com a MMP-13 e a proporção de células imunopositivas foi significativamente maior que no grupo controle.

OBJETIVOS

2. OBJETIVOS

Este estudo tem por objetivo examinar, por meio de análise imuno-histoquímica, a expressão de MMP-13 em discos da articulação temporomandibular (ATM) de humanos com deslocamento anterior de disco com e sem redução. Além disso, uma associação com osteoartrose da ATM também foi investigada, para melhor compreender a relação entre o deslocamento de disco da ATM e o processo degenerativo.

ARTIGO

Expression of MMP-13 in Disc Derangement of Human Temporomandibular Joint and Association with Osteoarthrosis

Juliano Henrique Perotto[1], Flavio de Alcântara Camejo, MS, PhD[1], Andrea Duarte Doetzer, PhD[1], Luis Eduardo Almeida, MS, PhD[2], Marina Azevedo[1], Marcia Olandoski[3], PhD, Lucia Noronha PhD[3], Paula Cristina Trevilatto, PhD[3]

[1] Graduate student of the School of Health and Biosciences at Pontifícia Universidade Católica do Paraná (Curitiba, BRA)

[2] Assistant professor of the School of Dentistry at Marquette University (Milwalkee, USA)

[3] Professor of the School of Health and Biosciences at Pontifícia Universidade Católica do Paraná (Curitiba, BRA)

Correspondence to:
Paula Cristina Trevilatto
School of Health and Biosciences
Pontifícia Universidade Católica do Paraná (PUCPR)
Rua Imaculada Conceição, 1155
Curitiba-PR 80215-901 BRAZIL
Phone: +55 (41) 3271-2582
Fax: +55 (41) 3271-1657
Email: **paula.trevilatto@pucpr.br**

ABSTRACT

Background: Collagenase 3 (MMP-13) is one of the most important enzyme, member of collagenases family, which perform preferential digestion of type II collagen over type I and III collagens. Since the temporomandibular joint (TMJ) articular disk is formed by these types of collagen, the present investigation was designed to evaluate, through immunohistochemistry, the expression of MMP-13 in patients with anterior disc displacement with (ADDwR) and without reduction (ADDwoR). Moreover, the presence of TMJ osteoarthrosis (OA) was also investigated to better understand the relationship between TMD advanced stages and MMP-13 expression.

Methods: 39 human temporomandibular joint samples were collected. In first analysis the samples were divided in patients with anterior disk displacement with reduction (n = 21) and without reduction (n = 10), and 8 samples in the control group; and in a second analysis: without osteoarthrosis (29 samples, n=22) and with osteoarthrosis (10 samples, n=6). The immunostaining of the TMJ disks was statistically compared between the groups (p<0.05). **Results:** There wasn't a statistically significant difference for the area of MMP-13 immunostaining between the control group, displacement disks with reduction group (ADDwR), and displacement disk without reduction group (ADDwoR) (p = 0.288), and between groups with and without osteorthrosis (p=0.185). **Conclusion:** No statistically significant difference was found between the variable area of MMP-13 expression in the disk with and without disk displacement, and between groups with and without osteoarthrosis.

Running Title: MMP-13 and temporomandibular disorders.

Keywords: temporomandibular disorder, disc derangement, osteoarthrosis, MMP-13

Introduction

Temporomandibular joint derangement (TMD) is a common disease that affects many people around the world, with major prevalence in females (1-4). The most commonly found disorder in temporomandibular joint (TMJ) is the abnormal positioning of the articular disc (1, 5-8)

The two most frequent types of disc displacement are anterior disc displacement with (ADDwR) and without reduction (ADDwoR). In ADDwR, the disc returns to its normal position during mouth opening, and it goes back to its displaced position when the mouth close completed, which is clinically represented by an articular click. Hence in ADDwoR the disc is unable to return to the normal position, which leads to a decrease in mouth opening and is usually painfull (9,10).

The influence of the disc displacement in the progression of TMJ derangement, may lead to osteoarthrosis in TMJ (which is characterized by presenting abnormal remodeling of osseous structures in the joint) (11-14).

Previous studies have shown that alteration of the disc structure may lead to the expression of some mediators, related to apoptosis and inflammatory mediators, and maybe associated with TMD progression (15-26). The articular disc of TMJ is formed basically by proteoglycan aggregates and collagen fibers that are composed mainly of type I and II collagen; and its physiologic maintenance is through a balance between degradation of collagen fibers performed by MMPs and their inhibitors, TIMPs (26-28).

In this study the hypothesis that MMP-13 may be involved in the progression of TMD was analyzed. Therefore, the present investigation was designed to evaluate, through immunohistochemistry, the expression of MMP-13 in TMJ articular discs of ADDwR and ADDwoR patients. Moreover, the

presence of TMJ osteoarthiritis (OA) was also investigated to better understand the relationship between TMD advanced stages and MMP-13 expression.

Materials and methods

Sample selection

A convenient sample of temporomandibular discs from 27 patients, mean age 33.59 years old, (17 to 57), was recruited for study from the patient pool at the Evangelico School Hospital, Curitiba, Southern region of Brazil (Table 1), as approved by the Ethical Committee on Research at Pontifical Catholic University of Paraná, according to Resolution 196/96 of the National Health Council and approved under registration number 104. Subjects were not include if presenting: use of orthodontic appliances, chronic usage of antiinflammatory drugs, history of diabetes, hepatitis, HIV infection, immunosuppressive chemotherapy, history of any disease known to severely compromise immune function, current pregnancy or lactation, dentofacial deformity major jaw trauma, previous TMJ surgery and previous steroid injection in the TMJ.

Subjects completed personal medical history questionnaires and, within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. All patients were asked to complete a pain questionnaire, and a clinical examination was performed according to Clinical Practice Guidelines for TMJ surgery of the American Association of Oral Maxillofacial Surgeons. The patients were considered to be affected and treated surgically when presenting painful clinical signs of disc displacement after unsuccessful nonsurgical treatment for at least 6 months.

Out of the control patients, 5 individuals presented condyle fracture (CFx), confirmed by radiographs and CT scan, which needed to be operated for the

fracture reduction and 3 subjects displayed active condyle hyperplasia (CH), diagnosed by radiographs.

CT scan, and scintigraphy, as follows:

1) Subjects without any signs of disc displacement (control group; n=7; 8 specimens);
2) Patients presenting anterior disc displacement with reduction (ADDwR; n=14; 21 specimens);
3) Patients presenting anterior disc displacement without reduction (ADDwoR; n=6; 10 specimens).

Subjects were included in clinical categories according to the presence or absence of disc displacement and, at a second moment, according to the presence or absence of osteoarthrosis (using Wilkes classification) (29).

Patients' selection for OA analysis was based on the primary diagnosis of severe TMJ ID. The stages of TMJ ID were classified into mild, intermediate and severe according to Wilkes classification based on clinical, surgical and pathological stages (29). Mild internal derangement (Wilkes stage III) is characterized by simple disc displacement without any morphological alteration of the disc and with or without osseous shift. The intermediate stage (Wilkes stage IV) is characterized by disc displacement and morphological deformity and/or osseous remodeling changes. Severe derangement (Wilkes stage V) is characterized by perforations of the disc attachments and osseous shift and/or osteoarthritic changes (sclerosis, osteophyte formation, articular surface flattening, depression and/or cystic alterations) (29). Patients from the control group and those classified as Wilkes III were considered not presenting OA and patients classified as Wilkes IV or V were included in the OA group, as follows:

1) Patients without OA (control group + Wilkes stage III; n=22; 29 specimens);
2) Patient with OA (Wilkes stage IV and V; n= 6; 10 specimens).

Table I shows the baseline characteristics of the sample.

Surgical technique

TMJ surgery was performed according to the technique described by Mehra and Wolford (30).

First the displaced disk is freed by the surgeon entering the upper and lower joint spaces and lysing adhesions. At this point a small hole is placed through the lateral pole of the condyle from posterior to anterior direction. The Mitek bone-cleat introducer is inserted and pushed into the bone, where two small coils unlock and attach the cleat to the inner surface of the condyle cortical bone. A nonresorbable 2-0 or 3-0 suture is placed through the hole and through the disk at the junction of the posterior and intermediate bands, and the disk is tied down to the condylar neck. The deformity of the disk precludes repositioning it into a more normal position, and recontouring the thickened disk with a scalpel is necessary (this scalpelled material constitutes the sample).

This procedure was conducted for all patients with disc displacement and the control group. In the CFx patients, the disc displaced by fracture was repositioned and in the CH patients the disc was sutured to prevent disc displacement caused by the gap that was created after the high condylectomy. Postsurgical physical therapy was indicated at the discretion of the surgeon.

Histological sections obtained by scapel of disk excess were prepared for observation of the in situ expression of MMP-13 by immunohistochemistry.

Immunohistochemistry

The TMJ disc sections were deparaffinized with xylol (2 x 10 min) and rehydrated with absolute ethylic alcohol (3 x 1 min) and 80% ethylic alcohol (1 x 1 min). Endogenous peroxidase activity was quenched by treatment with H_2O_2 (5% in methanol) for 10 min. Target Retrieval Solution™ (Dako, DK-2600 Glostrup, Denmark) was used prior to slide staining for heat-inducing epitope retrieval (for formalin-fixed, paraffin-embedded material), according to the manufacturer's instructions. The sections were incubated with monoclonal MMP-13 antibody (Abcam plc., Cambridge, UK), diluted 1:50 in phosphate-buffered saline (PBS), 0.1% bovine serum albumin (BSA). For negative controls, the primary antibody was not added. PBS was used instead. The secondary antibody, AdvanceTM (Dako, DK-2600 Glostrup, Denmark), was applied for 30 min, according to the manufacturer's instruction.

The immunoreactions were visualized by incubating the sections using 3,3' diaminobenzidine (DAB) chromogen (OriGene, Rockville, MD), (1 drop in 1 mL distilled water). The sections were lightly counterstained with Harris haematoxylin for 5 min and finally mounted. Immunostaining was considered to be specific to MMP-13 because immunoreactivity was not observed in the negative controls.

The colour morphometry method was used to analyze the anti-MMP-13 immunostained area in the TMJ disc tissue. For this purpose, images of consecutive fields were captured by the 20x objective lens coupled with the BX50 Olympus microscope with the Sony camera, Model DXC-107A, and image analysis was performed with specific software called Image Pro Plus software (Media Cybernetics Inc., Silver Spring, USA). This software allows an observer to select and paint the positive areas to obtain an image model and

make the mask for the other stained slides, being automatically calculated the area of the positive reaction (Fig. 1). This procedure was performed by a single examiner in a blind manner. The data were entered into a spread sheet, and Microsoft Excel (Redmond, WA, USA) was used to obtain the statistical analysis. The variable area was measured in square micrometers (μm^2) and was obtained with the mean of all positive areas.

Statistical Analysis

To compare the groups (control, ADDwR and ADDwoR) regarding area, the non-parametric Kruskal-Wallis analysis was considered. To compare groups with and without osteoarthrosis the non-parametric Mann-Whitney test was employed. To meet symmetric condition of the variable, data of area are previously submitted to a logarithmic transformation. *P* value <0.05 was considered statistically significant. Data were analyzed with the software IBM SPSS Statistics v.20.0 (IBM Corporation, Armonk, NY, USA).

Results

Expression of MMP-13 was observed at cytoplasm level.

Expression of MMP-13 in TMJ sample ADDwR, ADDwoR and Control

Significant differences were not found in the expression of MMP-13 in TMJ discs between the three groups for the variable area (p=0.288) (Table 2).

Expression of MMP-13 in TMJ discs of patients with and without osteoarhrosis

It was observed that all the patients with ADDwoR presented OA. On the other hand, all patients without OA presented ADDwR.

Statistically significant differences were not found in the expression of MMP-13 in TMJ discs between the groups with and without OA for the variable area (p=0.185) (Table 3).

Discussion

In the last few years, the expression of matrix metalloproteiases and some others markers, such as FasL and ADAM-17, were shown in the progression of TMJ ID using TMJ discs and synovial liquid (15-26).

The articular disc of the temporomandibular joint is formed by proteoglycan aggregates and collagen types I and II (5,10,13). A balance between degradation of collagen fibers performed by MMPs and their inhibitors, TIMPs, are responsible for functional remodeling of TMJ (21, 26-28)

Matrix Metalloproteiases (MMPs) are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the Fas ligand), and chemokine/cytokine inactivation MMPs. They play an important role in tissue remodeling associated with various physiological or pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis (21, 27,28).

Loreto et al. (21) showed that MMP-7 and MMP-9 are expressed in arthritic joints and provided evidence of a role for those MMPs in TMJ ID disc damage, with higher expression being detected in the posterior rather than in the anterior and intermediate bands of ADDwR and ADDwoR discs.

Almeida et al. (24) in a study using MMP-2 and MMP-9 did not find statistically significant difference between the variable area of MMP-9 expression in the disk with and without disk displacement, as determined by

immunohistochemical analysis. However, an elevation of MMP-2 expression in the disks of patients with ADDwoR (more severe alteration) was identified.

One of the most important metalloproteinase involved in degradation of collagen within the cartilage, is MMP-13 (collagenase-3), a metalloproteinase of collagenase group, which perform preferential digestion of type II collagen over type I and III collagens (26-28). Leonardi et al., (26) found correlation of an increased MMP-13 immunoreactivity in TMJ diseased disc tissue with the increased severity of the histopathological changes, however this study was performed with cadavers disc.

In this study the hypothesis is that MMP-13 may be involved in the progression of TMD, since other studies have already demonstrated the correlation of MMPs with this progression.

Significant differences were not observed between the groups control and with and without anterior disc reduction of the articular disc with respect to the variable area for the expression of MMP-13 measured by immunohistochemical examination.

Osteoarthrosis is characterized by deterioration and abrasion of articular cartilage. Some papers have been demonstrating the association of MMPs (MMP-7, MMP-9, MMP-13) with osteoarthrosis, an inflammatory disorder of movable joints, in degenerative process of joints, included in TMJ (21,27,28).

Schlopov et al., (31) suggest a key role of MMP-13 and MMP-8, as well as MMP-1 in osteoarthritis.

This study has some limitations, such as the restrictive sample size, however this still seems to be the biggest sample size so far in TMJ studies to the authors' knowledge. This reduced number refers to the fact that only a low number of patients must be submitted to surgical treatment.

In our study, it was not observed significant differences between the groups with and without osteoarthrosis with respect to the variable area for the expression of MMP-13 measured by immunohistochemical examination.

In conclusion, the expression of MMP-13 was not associated with TMD and with osteoarthritis in the study population.

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Table I. Baseline clinical characteristics of the control and study groups with and without TMJ dysfunction, according to Wilkes stage.

					Affected Side		
Patient	Race	Gender	Age (yrs)	Diagnosis	Right	Left	Wilkes Stage
1	Caucasian	F	33	ADDwR	X		III
2	Caucasian	M	27	CFx		X	
3	Caucasian	M	33	CFx	X		
4	Caucasian	F	26	ADDwR	X		III
4	Caucasian	F	26	ADDwR		X	III
5	Caucasian	F	43	CH		X	
6	Caucasian	F	17	CH		X	
7	Caucasian	F	30	ADDwR		X	III
8	Caucasian	F	25	ADDwR		X	III
8	Caucasian	F	25	ADDwR	X		III
9	Caucasian	F	37	ADDwR	X		III
9	Caucasian	F	37	ADDwR		X	III
10	Caucasian	M	42	CFx	X		
11	Caucasian	F	20	ADDwR		X	III
12	Caucasian	F	23	ADDwoR		X	V
12	Caucasian	F	23	ADDwoR	X		V
13	Caucasian	F	36	ADDwR	X		III
13	Caucasian	F	36	ADDwR		X	III
14	Caucasian	F	38	ADDwR	X		III
14	Caucasian	F	38	ADDwR		X	III
15	Caucasian	F	22	ADDwR	X		III
15	Caucasian	F	22	ADDwR		X	III
16	Caucasian	F	26	ADDwoR	X		IV
16	Caucasian	F	26	ADDwoR		X	IV
17	Caucasian	F	32	ADDwoR		X	IV
17	Caucasian	F	32	ADDwoR	X		V
18	Caucasian	F	45	ADDwoR	X		V
19	Caucasian	F	35	ADDwoR		X	IV
19	Caucasian	F	35	ADDwoR	X		IV
20	Caucasian	F	24	ADDwoR		X	V
21	Caucasian	F	34	ADDwR		X	III
22	Caucasian	F	57	ADDwR		X	III
23	Caucasian	F	18	CFx		X	
23	Caucasian	F	18	CFx	X		
24	Caucasian	F	46	ADDwR		X	III
24	Caucasian	M	46	ADDwR	X		III
25	Caucasian	F	40	CH		X	
26	Caucasian	F	56	ADDwR	X		III
27	Caucasian	F	42	ADDwR	X		III

ADDWOR, Anterior disc displacement without reduction; *ADDWR,* anterior disc displacement with reduction; *CH,* condylar hyperplasia; *CFx,* condylar fracture.

Table II. MMP-13 area of immunostaining (μm^2) in the discs of the control and study group with and without TMJ dysfunction.

Variable	Group	n	Mean	Median	Minimun	Maximum	Standart deviation	*p*-value*
Area	Control	8	17428	7292	1432	52714	19534	
	With redution	21	11155	5947	108	54225	13058	
	Without redution	10	6364	2466	418	19862	6951	0.288

* non-parametric Kruskal-Wallis test, $p<0.05$

Table III. Differences between groups with and without osteoarhrosis with respect to area of *in situ* expression (μm^2) of MMP-13 cytokine.

Variable	Group	n	Mean	Median	Minimun	Maximum	Standart deviation	*p*-value*
Area	With osteoarthrosis	10	6364	2466	418	19862	6951	
	Without osteoarthrosis	29	12885	6933	108	54225	15011	0.185

*Teste não-paramétrico de Mann-Whitney, $p<0.05$.

CONCLUSÕES

4. CONCLUSÕES

Diferenças significativas não foram encontradas entre os grupos controle, e com e sem redução do disco articular, no que diz respeito à variável área de expressão da MMP-13 no exame de imuno-histoquímica.

Nos grupos com e sem osteoartrite, também não foram observadas diferenças significantes, no que diz respeito à variável área de expressão da MMP-13.

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