Flavio de Alcântara Camejo

EXPRESSÃO IMUNOHISTOQUÍMICA DE FAS-LIGAND EM DISCOS ARTICULARES HUMANOS DA ARTICULAÇÃO TEMPOROMANDIBULAR (ATM) COM DESARRANJOS INTERNOS

CURITIBA

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Dissertação apresentada ao Programa de Pós-graduação em Medicina da Pontifícia Universidade Católica do Paraná, como parte dos requesitos para obtenção do título de Mestre em Ciências da Saúde.

Orientadora: Profa. Dra. Paula Cristina Trevilatto

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2012

DEDICO

Aos meus familiares, em especial meus pais, **Fernando e Maria Zélia**, por me apoiarem e estarem sempre ao meu lado, tornando possível ter tudo que tenho e sou hoje.

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RESUMO

Apoptose é um programa de morte celular sem indução de resposta inflamatória. Estudos recentes sugerem uma correlação entre os desarranjos internos temporomandibulares e a apoptose. Este estudo tem por objetivo analisar, por imunohistoquímica, a expressão do ligante Fas (Fas-L), um fator indutor de apoptose, conhecido por desencadear a apoptose através de vias de sinalização distintas, em discos da articulação temporomandibular (ATM) de pacientes com deslocamento disco anterior com (ADDwR) e sem redução (ADDwoR), e sua associação com osteoartrose (OA). Quarenta e dois (n = 42) discos articulares da ATM foram divididos em dois cortes: 1) oito amostras controle, 17 com ADDwR, 17 ADDwoR e 2) sem OA (n=25) e com OA (n=17). A área da imunocoloração foi comparada estatisticamente entre os grupos (p<0,05). Diferenças estatisticamente significativas foram encontradas na expressão de Fas-L nos discos da ATM entre os três grupos (p=0,001). ADDwR apresentou expressão significativamente maior de Fas-L, quando comparado com ADDwoR (p<0,001). Expressão significativamente maior de Fas-L foi observada no grupo sem OA (p=0,001). Todos os pacientes sem OA apresentavam ADDwR, enquanto todos os pacientes com OA apresentavam ADDwoR. A maior área de imunomarcação *in situ* de Fas-L foi encontrada em discos articulares com redução, que é a condição menos severa. Por outro lado, uma redução da expressão de Fas-L nos discos de pacientes com osteoartrose foi encontrado, o que sugere que alguns aspectos da apoptose podem ocorrer subjacentes à progressão de desordens da ATM.

ABSTRACT

Apoptosis is a program of cell death which does not induce an inflammatory response. Recent previous research has suggested a correlation between temporomandibular internal derangement and apoptosis. This study aims to examine, by immunohistochemistry, the expression of Fas ligand (Fas-L), an apoptosis-inducing factor, known to trigger apoptosis through distinct signal pathways, in temporomandibular joint (TMJ) articular discs of patients with anterior disc deslocament with (ADDwR) and without reduction (ADDwoR) and its association with osteoarthrosis (OA). Forty-two (n=42) TMJ articular discs were divided into two cutoffs: 1) 8 control, 17 ADDwR, 17 ADDwoR, and 2) without OA (n=25) and with OA (n=17). The area of immunostaining was compared statistically between groups (p<0.05). Statistically significant differences were found in the expression of Fas-L in TMJ discs between the three groups (p=0.001). ADDwR presented significant higher Fas-L expression when compared to ADDwoR (p<0.001). Significant higher Fas-L expression was observed in the group without OA (p=0.001). All patients without OA presented ADDwR while all the patients with OA presented ADDwoR. A higher area of in situ immunostaining of Fas-L was found in temporomandibular discs with reduction, which is the less severe condition. Moreover, a reduced expression of Fas-L in the discs of patients with osteoarthrosis was found, suggesting that some aspects of apoptosis might underlie the progression of TMJ disorders.

INTRODUÇÃO

1. INTRODUÇÃO

1.1 Articulação Temporomandibular (ATM)

A articulação temporomandibular (ATM) representa um conjunto de estruturas anatômicas que, com a participação de grupos de músculos especiais, possibilita à mandíbula executar variados movimentos durante o desempenho de suas funções (Figun & Garino, 1989). Essa articulação é a unidade funcional responsável pela mastigação, deglutição, fonação e respiração. A ATM, semelhante a outras articulações, pode adaptar-se às demandas funcionais, possuindo enorme capacidade de remodelação. A cartilagem articular, que reveste a cabeça do côndilo mandibular, e a eminência articular apresentam maior adaptação às forças funcionais; já o disco articular não possui esta capacidade.

O agente etiológico das doenças da ATM, excetuando-se as doenças sistêmicas, é, na grande maioria das vezes, o trauma. Este trauma, que pode ser micro ou macro, ocasionará modificações capazes de resultar em uma disfunção dessa articulação (Dimitroulis, 2005).

Vale ressaltar que a ATM apresenta-se como uma articulação duplo-dependente, possuindo um ponto fixo e rígido de início e final de movimento, que apresenta correlação com as estruturas dentárias. Ou seja, qualquer alteração em uma ATM, com o tempo, acarretará disfunção na articulação contralateral (Brossard, 2005).

Alterações teciduais na ATM podem afetar a cartilagem articular e 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, finalmente, de forma irreversível,

reduzindo sua capacidade de suportar forças de pressão e compressão (Israel et al., 1991).

Clinicamente, a primeira alteração leve da ATM 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 (Wilkes, 1978). Sinais clínicos e sintomas têm mostrado íntima correlação com imagens radiográficas em pacientes com inflamação e disfunção da ATM (Leeuw et al., 1999), no qual os casos de deslocamento anterior de disco com redução são associados à maior clique articular e episódios de travamentos leves, enquanto que nos casos de deslocamento anterior de disco sem redução, verifica-se ausência ou diminuição no clique articular, além da ocorrência de travamento e diminuição em abertura bucal.

1.1.1 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).

Interpondo-se entre a cabeça da mandíbula e a fossa mandibular, o disco permite uma movimentação suave entre essas estruturas, pois ameniza a

discrepância em termos de forma existente entre elas. Também, absorve os choques entre estas estruturas.

Sendo coberto por uma fina camada de células sinoviais, o disco articular secreta o líquido sinovial, o qual é de suma importância, pois lubrifica o próprio 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 ocasionarão, em maior ou menor intensidade, alterações na função da ATM.

1.2 Desarranjos internos da Articulação Temporomandibular

São as desordens mais frequentes nas articulações temporomandibulares e caracterizam-se por uma relação posicional e funcional anormal entre o disco articular e superfícies articulares da ATM.

São encontrados mais frequentemente nas mulheres, sendo que os fatores responsáveis por esta prevalência ainda não estão claros; porém, estudos recentes vêm demonstrando a influência de hormônios sexuais femininos, como o estradiol, progesterona e estrogênio (Ribeiro DaSilva El al., 2010; Kou et al., 2011; Torres-Chaves et al., 2011).

Sinais e sintomas associados a distúrbios intra-articulares da ATM são comuns, ocorrendo em 4% a 28% da população adulta (Katzberg, 1989), porém, destes, apenas de 3 a 4% necessitam algum tipo de tratamento cirúrgico e 70% apresentam deslocamento do disco articular (Farrar & Mccarty, 1979; Gray et al, 1995).

Frequentemente o disco articular apresenta-se deslocado anteriormente, mas também existe grande incidência de deslocamento medial ou lateral, ou

uma combinação de ambos. Existem dois tipos de deslocamento de disco: com e sem redução, sendo este último de repercussão mais severa.

1.2.1 Deslocamento Anterior de Disco com Redução

Nesta condição o disco articular encontra-se deslocado anteriormente em repouso e, com a abertura de boca, este disco retorna à posição anatômica ideal em relação ao côndilo, gerando, durante este movimento, o clique articular (Fig. 1). Clinicamente, pode-se observar o travamento de abertura da boca, seguida de estalo ou clique, podendo ser tanto na abertura como no fechamento da boca (clique recíproco), geralmente associado à dor por compressão do tecido retrodiscal pelo côndilo (côndilo articulando contra a zona bilaminar).

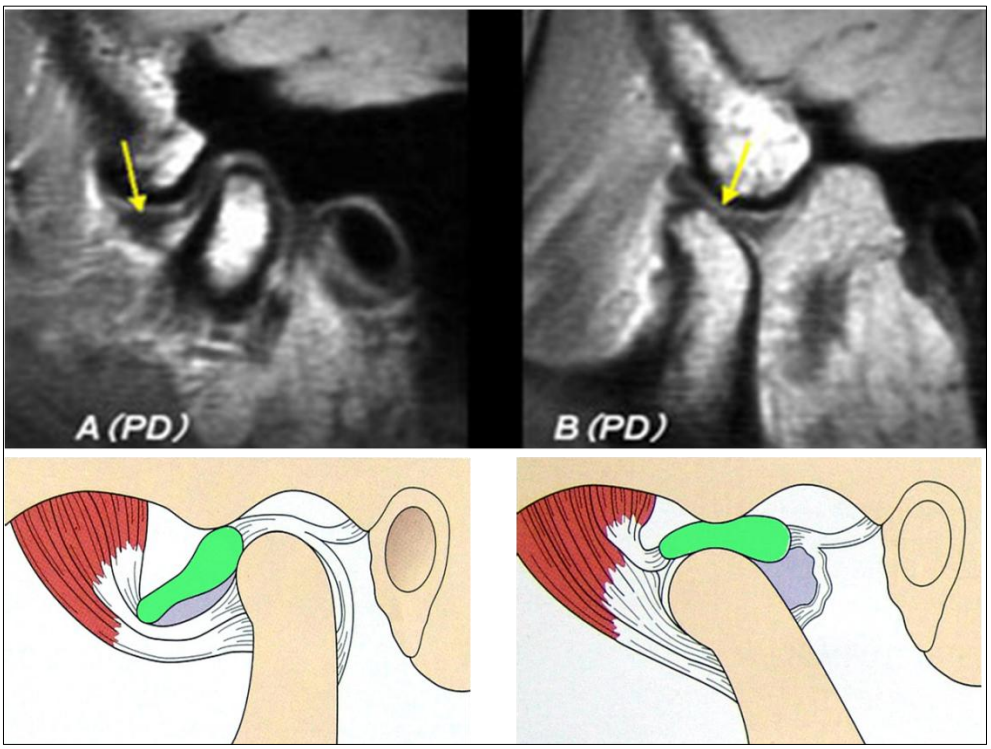

Fig.1. Deslocamento de Disco com Redução. **A** – Seta demonstrando disco articular localizado anteriormente em posição de boca fechada. **B** – Seta demonstrando disco articular retornando para sua posição correta durante movimento de abertura bucal.

Fonte: Color Atlas of Temporomandibular Joint Surgery. Peter D. Quinn; Editora Mosby,1998.

1.2.2 Deslocamento Anterior de Disco sem Redução

No deslocamento anterior do disco sem redução, o disco permanece deslocado anteriormente em todos os movimentos mandibulares, sem ocorrer o retorno deste à posição anatômica ideal em relação ao côndilo, não ocorrendo, portanto, o clique articular (Fig. 2). Clinicamente, este processo é caracterizado por redução da abertura de boca de, no máximo, 30 mm ou, até mesmo, impedindo totalmente a abertura de boca, normalmente associado com dor aguda.

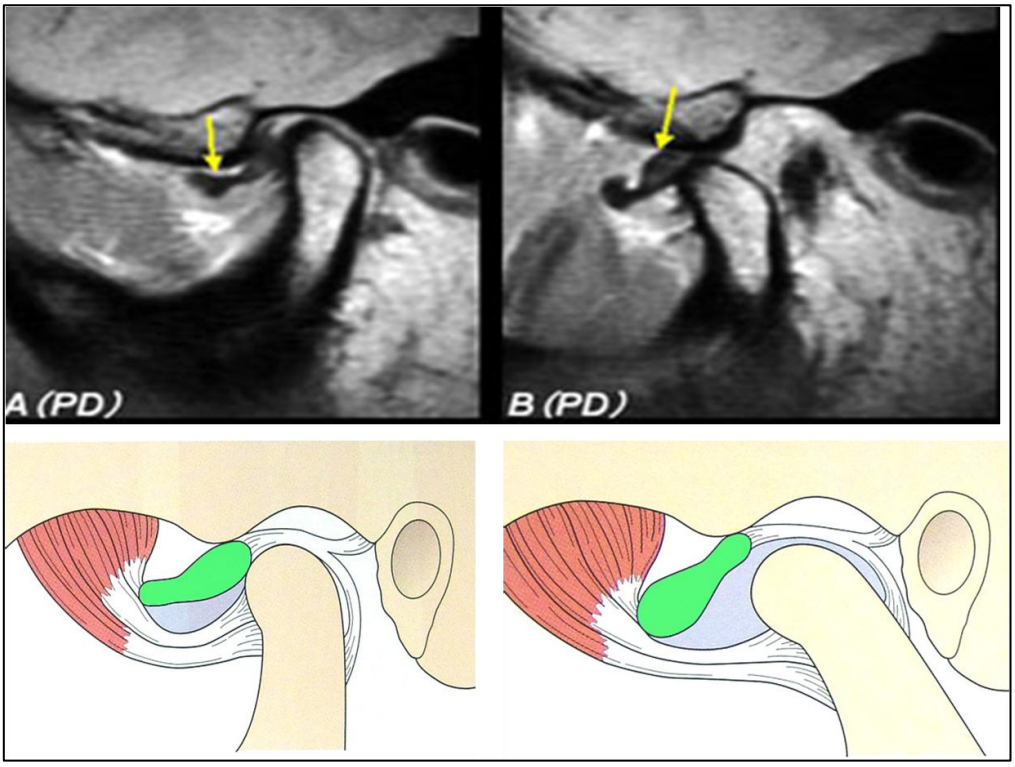

Fig.2. Deslocamento de Disco sem Redução. **A** – Seta demonstrando disco articular localizado anteriormente em posição de boca fechada. **B** – Seta demonstrando disco articular permanecendo em mesma posição de boca fechada durante movimento de abertura bucal, sem ocorrer portanto redução à sua posição correta.

Fonte: Color Atlas of Temporomandibular Joint Surgery. Peter D. Quinn; Editora Mosby,1998.

1.3 Osteoartrose

A osteoartrose é uma desordem degenerativa focal que afeta primariamente a cartilagem articular e o osso subcondral de articulações sinoviais, como a ATM (Stegenga, De Bont, Boering & Van Willigen, 1991).

Neste processo, os condrócitos vão morrendo e produzem menor quantidade de proteoglicanas e de colágeno. Em consequência disto, a cartilagem articular ulcera e o osso que está embaixo da cartilagem, chamado osso subcondral, reage espessando-se e dando origem a excrescências ósseas chamadas osteófitos, levando a alterações osteoartríticas na articulação. Sendo assim, o colapso e erosão da superfície articular do côndilo mandibular são sinais indicativos de osteoartrose.

Já está bem documentado na literatura que a osteoartrose frequentemente ocorre em conjunto com os desarranjos internos da ATM (De Bont et al., 1986). Entretanto, um estudo de Dimitroulis, em 2005, descreve que em apenas 6 articulações de 18 (33%) diagnosticadas com desarranjos em disco articular foram encontrados sinais de osteoartrose e sugere que o fato de a osteoartrose não ter sido encontrada em todos os casos poderia ser justificado pelos desarranjos internos precederem a osteoartrose (Dimitroulis, 1995).

1.4 Apoptose

Apoptose é fisiologicamente envolvida em vários aspectos do desenvolvimento dos mamíferos, incluindo embriogênese e homeostase. É um programa de morte celular extremamente regulado e de grande eficiência, que requer a interação de inúmeros fatores, e cuja função é o de remover células prejudiciais, danificadas ou indesejáveis, sem induzir uma resposta inflamatória, por liberação do conteúdo celular, como é observado durante a morte celular por necrose (White, 1996; Alenzi, 2005). As alterações morfológicas observadas são consequência de uma cascata de eventos

moleculares e bioquímicos específicos e geneticamente regulados (Saraste & Pulkki, 2000).

Diversos são os fatores que podem desencadear a apoptose, entre eles: ligação de moléculas a receptores de membrana, agentes quimioterápicos, radiação ionizante, danos no DNA, choque térmico, deprivação de fatores de crescimento, baixa quantidade de nutrientes e níveis aumentados de espécies reativas do oxigênio (Hengartner, 2000).

A ativação da apoptose pode ser iniciada de duas diferentes maneiras: pela via intrínseca (mitocondrial), ou pela via extrínseca (citoplasmática), sendo esta última induzida por ligantes sinalizadores de morte celular. A ativação dessa cascata irá culminar com a ativação das caspases (cysteine-containing aspartate-specific proteases), que, por sua vez, sinalizam para a apoptose, levando à condensação e fragmentação nuclear, e externalização de fosfolipídios de membrana, que irão sinalizar para estas células serem fagocitadas por macrófagos (Nicholson & Thornberry, 1997; Boatright & Salvesen, 2003) (Fig. 3).

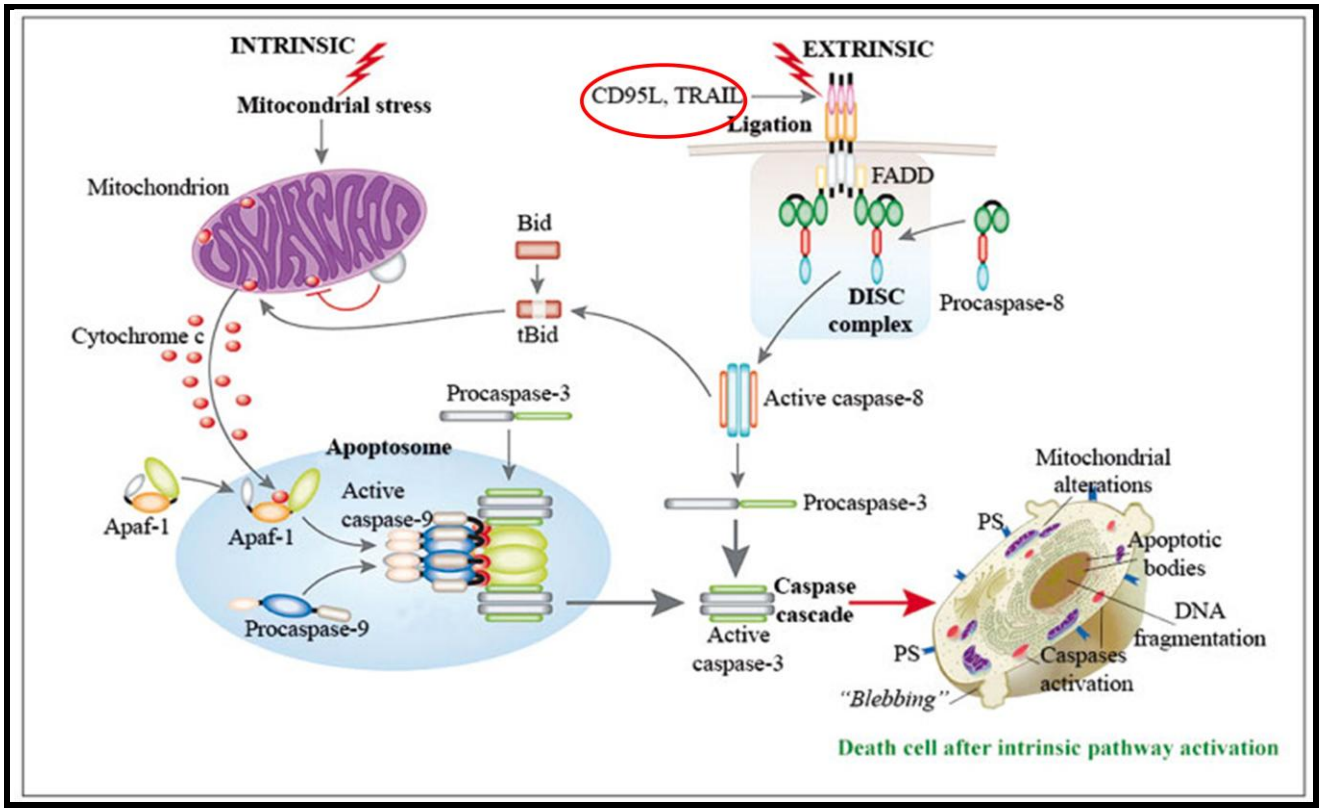

Fig. 3. Vias do mecanismo de apoptose celular, evidenciando a participação da molécula de FasL (CD95L) na ativação do mecanismo extrínseco.

Fonte: Calvino Fernández M, Parra Cid T. H. pylori and mitochondrial changes in epithelial cells. The role of oxidative stress. Rev Esp Enferm Dig. 2010 Jan;102(1):41-50.

A via intrínseca é ativada por estresse intracelular ou extracelular, como a deprivação de fatores de crescimento, danos no DNA, hipóxia ou ativação de oncogenes. A mitocôndria integra os estímulos de morte celular, induzindo a sua permeabilização e consequente liberação de moléculas pró-apoptóticas nela presentes (Desagher et al., 2000).

A via extrínseca é desencadeada pela ligação de ligantes específicos a um grupo de receptores de membrana da superfamília dos receptores de fatores de necrose tumoral (rTNF), sendo os principais: Fas, TNF e TRAIL (Liu & Pope, 2003). Essa ligação é capaz de ativar a cascata das caspases (Budihardjo et al., 1999). Quando os receptores de morte celular reconhecem um ligante específico, os seus domínios de morte interagem com moléculas

conhecidas como FADD/MORT-1. Essas moléculas têm a capacidade de recrutar a caspase-8 que irá ativar a caspase-3, executando a morte por apoptose (Daniel et al., 2001).

1.4.1 Fas-Ligand (CD95L)

Fas ligante (FasL, também chamado de CD95L) é um fator que induz a morte celular por apoptose, pertencente à família do fator de necrose tumoral (TNF), que faz parte da via extrínseca da apoptose.

Uma vez que FasL se liga a uma molécula de Fas na superfície da célula-alvo, um sinal é transmitido para o citoplasma e uma cascata de apoptose é iniciada, através da ativação de proteases. A cascata iniciada com a ligação do FasL ao receptor Fas da célula-alvo induz a apoptose através de um domínio de morte citoplasmático, que interage com as proteínas adaptadoras de sinalização, como FADD (Fas *Associated Death Domain*). FADD, então, dá inicio a uma cascata de ativação das caspases 8, 3, 6 e 7, culminando com a liberação da molécula CAD (Caspase-activated DNase) ao interior do núcleo, a qual irá causar a apoptose na célula-alvo (Fig. 4).

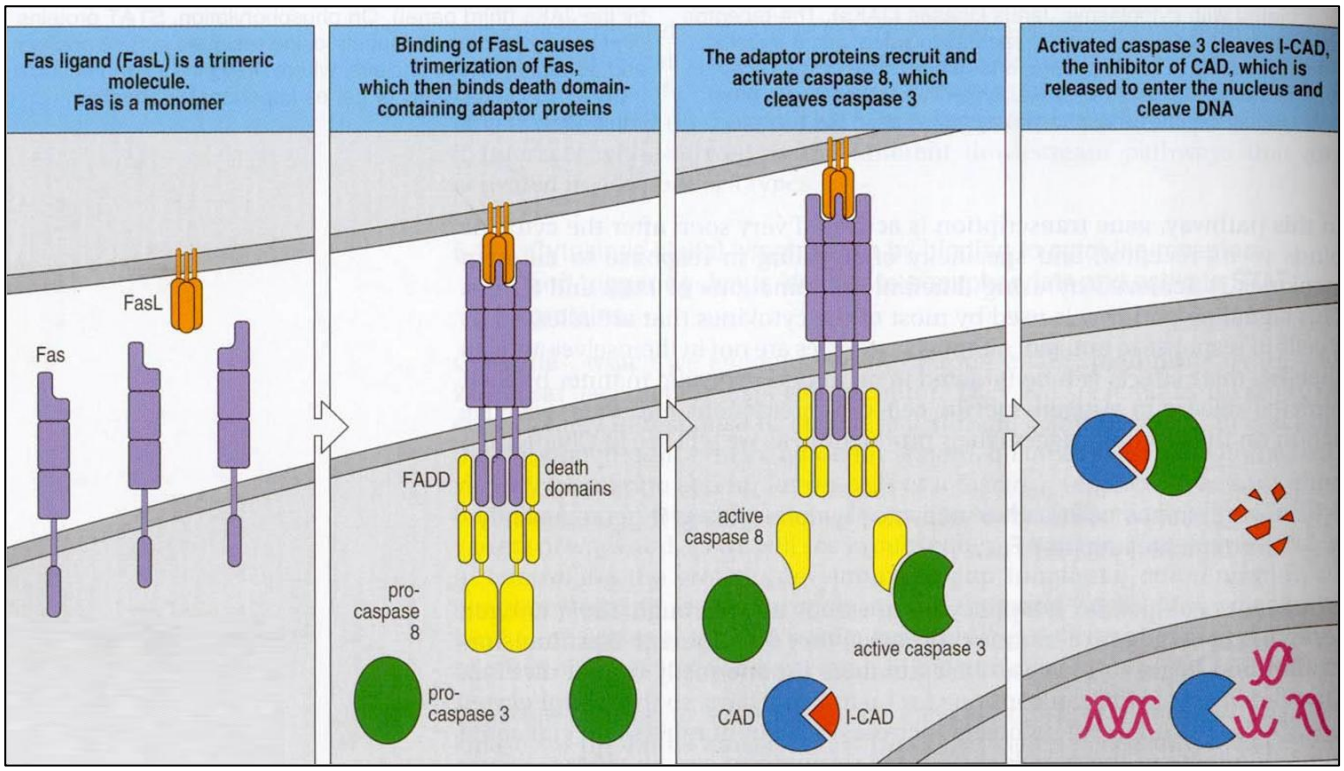

Fig. 4. Via extrínseca da apoptose .

Fonte: www.bio.davidson.edu/courses/immunology/students/spring2003/holmberg/protein.htm

Assim, Fas e FasL são indutores de apoptose, por meio da ligação do FasL ao Fas na superfície da membrana (Hasunuma et al., 1997; Nozawa et al., 1997; Imirzalioğlu et al., 2009), desempenhando um papel importante em vários aspectos do sistema imunológico, incluindo a citotoxicidade mediada por células e autotolerância. FasL parece desempenhar um papel imunossupressor não só contra antígenos endógenos, mas também contra antígenos exógenos (Nagata & Suda, 1995).

FasL é principalmente expresso pelos linfócitos T citotóxicos e células *natural killer* (NK) (Houston & O'Connell, 2004), porém, alguns estudos demonstram expressões anômalas de FasL em outras células, tais como neutrófilos (Montes-Berrueta et al., 2012), células do timo (Bai, et al., 2012), células cancerígenas (Taliouri et al., 2012) e condrócitos (Tu et al., 2012).

O aumento da apoptose de condrócitos contribui para as alterações degenerativas e perda tecidual nas articulações. Estudos em líquido sinovial demonstraram maior expressão de FasL em pacientes com artrite reumatoide grave que em pacientes com artrite reumatoide leve ou osteoartrose (Hashimoto, et al., 1998; Bremer, et al., 2011). Já em outro estudo, FasL demonstrou suprimir artrite experimental em ratos, após injeção local, sugerindo, assim, apresentar um potencial terapêutico (Li, et al., 2004).

FasL (CD95L) parece desempenhar papel importante em vários processos fisiológicos e patológicos, como por exemplo, na reabsorção óssea (Kovacic, 2010), na osteoartrite (Pennock et al., 2007), na doença periodontal (Gamonal et al., 2001), na doença de Alzheimer (Ethell & Buhler, 2003; Douraghi-zadeh et al., 2009), na diabetes (Mollah et al., 2011; Xial et al., 2011), na tuberculose (Abebe et al., 2010; Wu et al., 2010), em doenças coronarianas (Purevjav et al., 2007; Ristic et al., 2009), nos variados tipos de câncer (Huang et al., 2011; Kaufmann et al., 2011; Liang et al., 2011; Shao et al., 2011), na artrite reumatoide (Lundy et al., 2009; Pundt et al., 2009; Taar, 2010), no lúpus eritematoso sistêmico (Habib et al., 2009), na síndrome de Sjögren (Manganelli & Fietta., 2003; Herrera-Sparza et al., 2008) e na disfunção da articulação temporomandibular (Guz et al., 2002 & Nagai et al., 2003; Imirzalioglu et al., 2009).

1.5 Apoptose em Desarranjos Internos da Articulação Temporomandibular

Em doenças na articulação temporomandibular, a sinóvia e a cartilagem articular produzem vários mediadores, como interleucina-1 (IL-1) e FasL (CD95L), que possuem o potencial de induzir a morte de condrócitos

através da apoptose ou necrose (Carson & Ribeiro, 1993; Gu et al., 2002; Nagai, et al., 2003). Dentro da cartilagem articular, condrócitos são as únicas células que produzem e mantêm a matriz cartilaginosa. Assim, mudanças na sobrevivência de condrócitos (devido à proliferação, apoptose e outras formas de morte celular), induzidas por mediadores, podem ter importância patogênica no desenvolvimento da degradação da cartilagem. Apoptose em tecidos sinoviais da ATM tem se mostrado associada com a progressão de desarranjos internos (Guz et al., 2002 & Nagai et al., 2003).

Estudos recentes vêm demonstrando a correlação entre o desenvolvimento de desarranjos internos na ATM e apoptose. Imirzalioğlu et al., 2009, em estudo investigando fluido sinovial obtido por artrocentese de 17 articulações em 17 pacientes (11 mulheres e 6 homens, média de idade de 31,5 anos (±11,9, 19-55), encontraram baixos níveis de sFas (inibidor da ligação entre FasL e o Faz na célula-alvo) e sugerem vulnerabilidade à apoptose em pacientes com desarranjos internos de ATM. Mais recentemente, um grupo de pesquisadores italianos, utilizando as amostras de discos articulares da ATM (sem alteração, e com deslocamento anterior de disco com e sem redução) coletadas por pesquisador do nosso grupo (L.E.A.), em uma série de estudos com marcadores de apoptose, encontraram maior expressão de caspase-3, ligante indutor de apoptose relacionado ao TNF (TRAIL) e células DR5-positivas em tecidos discais com deslocamento anterior de disco com e sem redução, quando comparadas às células do grupo controle, com discos sem alterações (Loreto, Musumeci, Leonardi., 2009; Leonardi et al., 2010; Loreto et al., 2010; Leonardi et al., 2011; Loreto et al., 2011)

Frente a resultados promissores, o esforço do nosso grupo continua sendo analisar a expressão de marcadores relacionados à via extrínseca da

apoptose, com o intuito de aumentar as bases moleculares para a compreensão dos mecanismos que controlam o processo de morte celular, resultante de desarranjos internos da ATM.

PROPOSIÇÃO

2. PROPOSIÇÃO

Este estudo foi projetado para examinar, através de análise imunohistoquímica, a expressão de FasL (CD95L) 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 de apoptose.

ARTIGO

Fas-L Expression in Articular Discs of Human TMJ

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ABSTRACT

Background: Apoptosis is a program of cell death which does not induce an inflammatory response. Recent previous research has suggested a correlation between temporomandibular internal derangement and apoptosis. This study aims to examine, by immunohistochemistry, the expression of Fas-ligand (FasL), an apoptosis-inducing factor, known to trigger apoptosis through distinct signal pathways, in temporomandibular joint (TMJ) articular discs of patients with anterior disc deslocament with (ADDwR) and without reduction (ADDwoR) and its association with osteoarthrosis (OA). **Methods:** Forty-two (n=42) TMJ articular discs were divided into two cutoffs: 1) 8 control, 17 ADDwR, 17 ADDwoR, and 2) without OA (n=25) and with OA (n=17). The area of immunostaining was compared statistically between groups (p<0.05). **Results:** Statistically significant differences were found in the expression of FasL in TMJ discs between the three groups (p=0.001). ADDwR presented significant higher FasL expression when compared to ADDwoR (p<0.001). Significant higher FasL expression was observed in the group without OA (p=0.001). All patients without OA presented ADDwR while all the patients with OA presented ADDwoR. **Conclusion:** A higher area of in situ immunostaining of FasL was found in temporomandibular discs with reduction, which is the less severe condition. Moreover, a reduced expression of FasL in the discs of patients with osteoarthrosis was found, suggesting that some aspects of apoptosis might underlie the progression of TMJ disorders.

Keywords: temporomandibular disorder, disc derangement, osteoarthrosis, apoptosis

Introduction

Disc derangement is defined as a malpositioning of the articular disc in relation to the condyle and eminence. The two most common types of internal derangement (ID) are anterior disc displacement with (ADDwR) and without (ADDwoR) reduction (1-4).

ADDwR is typically defined as a condition in which the articular disc of the temporomandibular joint (TMJ) is displaced while the mouth is closed and the teeth are in contact in maximal occlusion and slide into its normal functional position as the jaw open.(5, 6).

In ADDwoR, the condyle is unable to slide or snap back underneath the disc. The displaced disc thus does not reduce to its position on top of the condyle during the opening movement (5, 6).

TMJ ID results from an imbalance between the anabolic and catabolic processes, predominantly controlled by fibrochondrocytes, and is characterized by progressive degradation of the extracellular matrix of the articular disc (7-9). Clinically, TMJ with mild ID is characterized by disc displacement with or without osseous remodeling, while severe derangement includes disc or attachment perforations, osseous remodeling, and osteoarthritic changes (10).

Disc displacement is associated with degenerative tissue changes and it is considered to be a risk factor for osteoarthrosis (OA) development, with abnormal remodeling of the condyle and mandibular fossa, however, the underlying mechanisms remain unclear (11-20).

Under normal physiologic conditions, a balance exists in synovial joints between tissue breakdown and repair. When the balance is disturbed by a mechanical, biomechanical or inflammatory insult the discal fibrocartilaginous remodeling system may fail, resulting in accelerated tissue breakdown (17). In

TMJ diseases, the synovium and articular cartilage produce several mediators that have the potential to induce apoptosis (21). Discal fibrochondrocytes are the only cells that produce and maintain the disc extracellular matrix. Thus, changes in fibrochondrocyte survival (due to cell proliferation, apoptosis, and other forms of cell death) induced by endogenous mediators may be of pathogenic significance in the development of articular disc degradation (17, 18).

Recent previous research has demonstrated a correlation between TMJ ID and apoptosis (20-25). Apoptosis is a physiologic process implicated in various aspects of mammalian development, including embryogenesis, normal tissue turnover and homeostasis. It can be triggered either by a mitochondria-dependent intrinsic pathway or via a cell surface death receptor-mediated extrinsic pathway. Fas ligand (FasL, also called CD95L), tumor necrosis factor (TNF)-α, and TNF-related apoptosis-inducing ligand (TRAIL) are common apoptosis-inducing factors, known to trigger apoptosis through distinct signal pathways (26-31).

FasL is a cell membrane associated factor that induces apoptotic cell death and is related to various aspects of immune system, including cell-mediated cytotoxicity and self-tolerance. FasL seems to play an immunosuppressive role against not only itself but also exogenous antigens (32, 33). FasL has a pathogenic involvement in a variety of inflammatory diseases, including hepatitis, graft-versus-host diseases and pulmonary fibrosis (34-38).

In this study we tested the hypothesis that the apoptosis may be involved in the progression of TMJ ID, as shown by recent previous research (20-25). Therefore, the present investigation was designed to evaluate, through

immunohistochemistry, the expression of FasL in TMJ articular discs of ADDwR and ADDwoR patients. Moreover, the presence of TMJ OA was also investigated to better understand the relationship between TMJ disc displacement and apoptosis.

Materials and methods

Sample selection

A sample of 42 temporomandibular discs from 29 patients (mean age 32.7 years, range from 18 to 56 years) were recruited for study from the patient pool at the Evangelico School Hospital, Curitiba, PR, Brazil (Table I), 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. The patients were from the southern region of Brazil. Subjects did not present any of the following criteria: 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 by an experienced operating oral and maxillofacial surgeon. The clinical examination consisted of palpation of the TMJ region, the

occurrence of painful opening/closing mouth, and crepitation. 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. Regarding complementary exams, all patients had a panorex. These patients were from the Brazilian public health system, therefore, a few of them had financial conditions to afford other exams such as computerized tomography (CT) scan or a TMJ magnetic resonance imaging. Accordingly, the diagnoses were primarily clinical.

Patients presenting disc displacement with and without reduction were grouped together for analysis. Out of the control patients, 4 individuals presented condyle fracture (CFx), confirmed by radiographs and CT scan, which needed to be operated for the fracture reduction and 4 subjects displayed active condyle hyperplasia (CH), diagnosed by radiographs, CT scan, and scintillography, as follows:

1) Subjects without any signs of disc displacement (control group; n=8; 8 specimens);
2) Patients presenting anterior disc displacement with reduction (ADDwR; n=10; 17 specimens);
3) Patients presenting anterior disc displacement without reduction (ADDwoR; n=11; 17 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) (39).

Patients' selection for OA analysis was based on the primary diagnosis of advanced 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 (39). 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) (40). Patients of 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=18; 25 specimens);
2) Patient with OA (Wilkes stage IV and V; n=11; 17 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 (40).

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 FasL by immunohistochemistry.

Immunohistochemistry

For immunostaining, the TMJ disc sections were deparaffinized with xylol (2 x 10 min), absolute ethilic alcohol (3 x 1 min) and 80% etilic alcohol (1 x 1 min). Endogenous peroxidase activity was quenched by treatment with H_2O_2 (3%) for 10 min. Non-specific binding site blocking was achieved by treating the specimens with imuno retriever (Dako™) at 99ºC for 40 min. The sections were incubated with dilutes monoclonal FasL antibody (Novo Castra, New Castle, UK), diluted 1:50 in PBS, 0.1% BSA. This monoclonal antibody mark only the cellular fraction of FasL, so the soluvel fraction (sFasL) which is an inhibitor of the ligation between FasL and Fas in the target cell is not mark. For negative controls, the primary antibody was not added. The secondary antibody, advanced link / advanced enzyme (Dako™) were applied for 30 min.

The immunoreactions were visualized by incubating the sections using DAB cromogenon (1:1). The sections were lightly counterstained with Harris haematoxylin for 5 min and finally mounted. Immunostain was considered to be

specific to Fas-L because immunoreactivity was not observed in the negative controls.

The positive areas were marked using the color morphometry method, which consisted of an analysis of the anti-FasL reaction area with the TMJ disc tissue. For this purpose, images of consecutive fields were captured by the 40x objective lens coupled to 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 made the mask for the staining other slides. This procedure was performed by a single observer with a blind study. Moreover, it automatically calculates the area of the positive reaction. The data was entered into a spread sheet and Microsoft Excel (Redmond, WA) 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 regarding the area the model of analysis of variance with one factor (ANOVA) was considered. To compare control and affected group the t'-student test for independent samples or 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 Statistica V. 8.0.

Results

FasL expression was observed at cytoplasmic membrane, especially in fibrochondrocytes and statistically significant differences were found between TMJ samples of ADDwR and ADDwoR, and between TMJ discs of patients with and without osteoarthrosis.

Expression of FasL in TMJ sample ADDwR and ADDwoR

Statistically significant differences were found in the expression of Fas-L in TMJ discs between the three groups for the variable area (p=0.001) (Table 2) However, it was observed significant difference only between ADDwR and ADDwoR groups (p<0.001), with higher area of expression in the ADDwR (Table 3).

Expression of FasL 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 found in the expression of FasL in TMJ discs between the groups with and without OA for the variable area (p=0.001), being the higher area of expression in the ADDwR (Table 4).

Discussion

Increasingly, studies have shown that mechanism of apoptosis plays an important role in the progression of ID of the TMJ (20-25).

In diseases of the TMJ, the synovium and articular cartilage produce various mediators, such as IL-1 and Fas-L (CD95L) that have the potential to induce chondrocyte death through necrosis or apoptosis (41-43). Some studies showed correlation between TMJ ID and apoptosis mechanism. Imirzalioğlu et al. (2009), in a study using synovial fluid through arthrocentesis from 17 joints in 17 patients (11 female, 6 male; mean age, 31.5 +/- 11.9 years; range, 19 to 55) found lower levels of sFas suggesting vulnerability to apoptosis in patients with internal derangement. Increased levels of sFas blocked apoptosis by inhibiting binding of FasL to Fas on the cell membrane (25). Immunohistochemical studies with TMJ discs using some apoptosis markers found higher expression of caspase 3, TRAIL and DR5-positive cells in disc tissues in patients with ADDwR and ADDwoR than in control discs (20-24).

In this study, the analysis of FasL in TMJ discs expression of individuals with and without disc displacement showed an increased area of expression in fibrochondrocytes of the ADDwR group. Although normally FasL is expressed by T lymphocytes or NK cells, our study its expression was verified by abnormally fibrochondrocytes as shown in Figure 1. Biomechanical stress activates multiple parallel and converging signals for hypertrophy and apoptosis (44). Considering the physiopathology of disc displacement, the mechanical stress generated in jaw movement during the opening and closing of the mouth, which can be stimulated by sliding the disc into and out of its normal position, prompts the activation of apoptosis in these areas. Additionally, cartilage trauma may induce accelerated fibrochondrocyte apoptosis (44). Thus, the apoptosis process may induce the expression of the marker FasL (CD95L) found in cases of ADDwR compared with ADDwoR, in which a less intense inflammatory process and more mechanical stress may coexist. This phenomenon can set up

an endogenous reaction, which aims to restore homeostasis and/or tissue remodeling. Therefore, the apoptotic event might work as a protective mechanism to overcome progression of disease.

OA is a focal degenerative disorder that primarily affects the articular cartilage and subchondral bone of synovial joints such as TMJ (45-47). It is a consensus that TMJ disc displacement and OA often occur concomitantly. The most frequently reported relationship is that disc displacement causes OA (17, 39, 46-57). With physiologic loading, there is a balance between synthesis and breakdown within the tissue. When this adaptive capacity is exceeded, an inflammatory response may become clinically evident and may result in damage to the cells, leading to cell destruction. Thus, in OA an inflammatory reaction reflects increased degenerative activity.

In this study, we investigated a possible association of Fas-L expression in TMJ discs with ostheoarthrosis process since the mechanism of apoptosis may influence the progression of ID. It was found an increase in the expression of Fas-L in TMJ discs of individuals without OA. It worth mentioning that in our study we found a strong association between OA and ADDwoR, the most extreme phenotype, in which a greater inflammatory process and minor mechanical stress coexist.

Our findings suggest that apoptosis process is a protective mechanism against TMJ disorder progression and reinforce that necrosis should be the main way of cell death in OA fibrochondrocytes (58). Moreover, considering that mechanical damage of articular cartilage is often associated with OA pathogenesis (58) and that fibrochondrocyte necrosis occurs in damage of articular cartilage, we can suggest that apoptosis mechanism may precede necrosis on the TMJ ID. This might explain why there are some cases in which

the progression from ADDwR to ADDwoR might not occur, and cases of patients with OA who do not present TMJ problems.

Future studies should be performed with a larger sample size, which may make it clear the association of a marker of apoptosis FasL (CD95L) with temporomandibular joint dysfunction. Although the sample number is small, to the authors' knowledge it is the largest sample reported in literature, Besides, the sample is obtained from patients, and not cadavers, as most study samples, which allows clinical examination and anamnesis.

In conclusion, a higher expression of FasL (CD98L) was found in temporomandibular discs with reduction when compared with discs without reduction. Moreover, a lower expression of FasL in the discs of patients with osteoarthrosis was found, which suggests that apoptosis may protect against TMJ disorders progression.

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Table 1. Baseline clinical characteristics of the study group with and without TMJ dysfunction, associated with Wilkes Stage.

					Affected Side			
Patient	Etinical Group	Gender	Age (yrs)	Diagnosis	Right	Left	Wilkes Stage	ostoarthritic group
1	Caucasian	F	39	ADDwoR	X		V	with OA
2	Caucasian	M	27	CFx	X			without OA
3	Caucasian	F	25	ADDwoR	X		V	with OA
4	Caucasian	F	46	ADDwoR		X	V	with OA
4	Caucasian	F	46	ADDwoR	X		V	with OA
5	Caucasian	F	20	ADDwoR		X	V	with OA
5	Caucasian	F	20	ADDwoR	X		V	with OA
6	Caucasian	F	41	ADDwR		X	III	without OA
6	Caucasian	F	41	ADDwR	X		III	without OA
7	Caucasian	F	35	CH		X		without OA
8	Caucasian	F	32	ADDwR		X	III	without OA
8	Caucasian	F	32	ADDwR	X		III	without OA
9	Caucasian	F	41	ADDwoR		X	V	with OA
9	Caucasian	F	41	ADDwoR	X		V	with OA
10	Caucasian	F	26	ADDwR		X	III	without OA
10	Caucasian	F	26	ADDwR	X		III	without OA
11	Caucasian	F	28	ADDwR		X	III	without OA
11	Caucasian	F	28	ADDwR	X		III	without OA
12	Caucasian	F	33	CH		X		without OA
13	Caucasian	F	36	ADDwR		X	III	without OA
13	Caucasian	F	36	ADDwR	X		III	without OA
14	Caucasian	F	18	ADDwR		X	III	without OA
14	Caucasian	F	18	ADDwR	X		III	without OA
15	Caucasian	F	38	ADDwoR		X	IV	with OA
15	Caucasian	F	38	ADDwoR	X		IV	with OA
16	Caucasian	F	45	ADDwoR	X		IV	with OA
17	Caucasian	F	23	CH		X		without OA
18	Caucasian	F	51	ADDwoR		X	V	with OA
19	Caucasian	F	33	ADDwoR	X		V	with OA
19	Caucasian	F	33	ADDwoR		X	V	with OA
20	Caucasian	M	22	CFx	X			without OA
21	Caucasian	F	35	ADDwoR	X		IV	with OA
22	Caucasian	F	22	ADDwR	X		III	without OA
22	Caucasian	F	22	ADDwR		X	III	without OA
23	Caucasian	F	24	ADDwR		X	III	without OA
24	Caucasian	M	18	CFx	X			without OA
25	Caucasian	F	32	CH		X		without OA
26	Caucasian	M	37	CFx	X			without OA
27	Caucasian	F	23	ADDwoR	X		IV	with OA
27	Caucasian	F	23	ADDwoR		X	IV	with OA
28	Caucasian	F	42	ADDwR	X		III	without OA
29	Caucasian	F	56	ADDwR	X		III	without OA

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

Table 2. FasL area of immunostaining (μm^2).in the discs of the study group with and without TMJ dysfunction.

Variable	Group	n	Mean	Median	Minimum	Maximum	Standart deviation	*P*-value*
Área	Control	8	15.17	15.44	7.64	20.79	4.08	
	ADDwR	17	22.91	19.85	11.21	45.68	11.50	0.001
	ADDwoR	17	12.26	10.85	5.97	20.65	4.43	

* One-factor ANOVA, $p<0.05$

Table 3. Comparation 2x2 between groups with and without TMJ dysfunction with respect to area of expression (μm^2).of FasL.

Groups	*P*-value
Control x ADDwR	0.062
Control x ADDwoR	0.187
ADDwR x ADDwoR	<0.001

*Student`s t test for independent samples, $p<0.05$

Table 4. Diferences between groups with and without osteoarhrosis with respect to area of in situ expression (μm^2) of FasL.

Variable	Group	n	Mean	Median	Minimun	Maximum	Standart deviation	*P*-value*
Fas-L area	Without osteoarthrosis	25	20.43	16.26	7.64	45.68	10.33	
	With osteoarthrosis	17	12.26	10.85	5.97	20.65	4.43	0.001*

*Student`s t test for independent samples, $p<0.05$

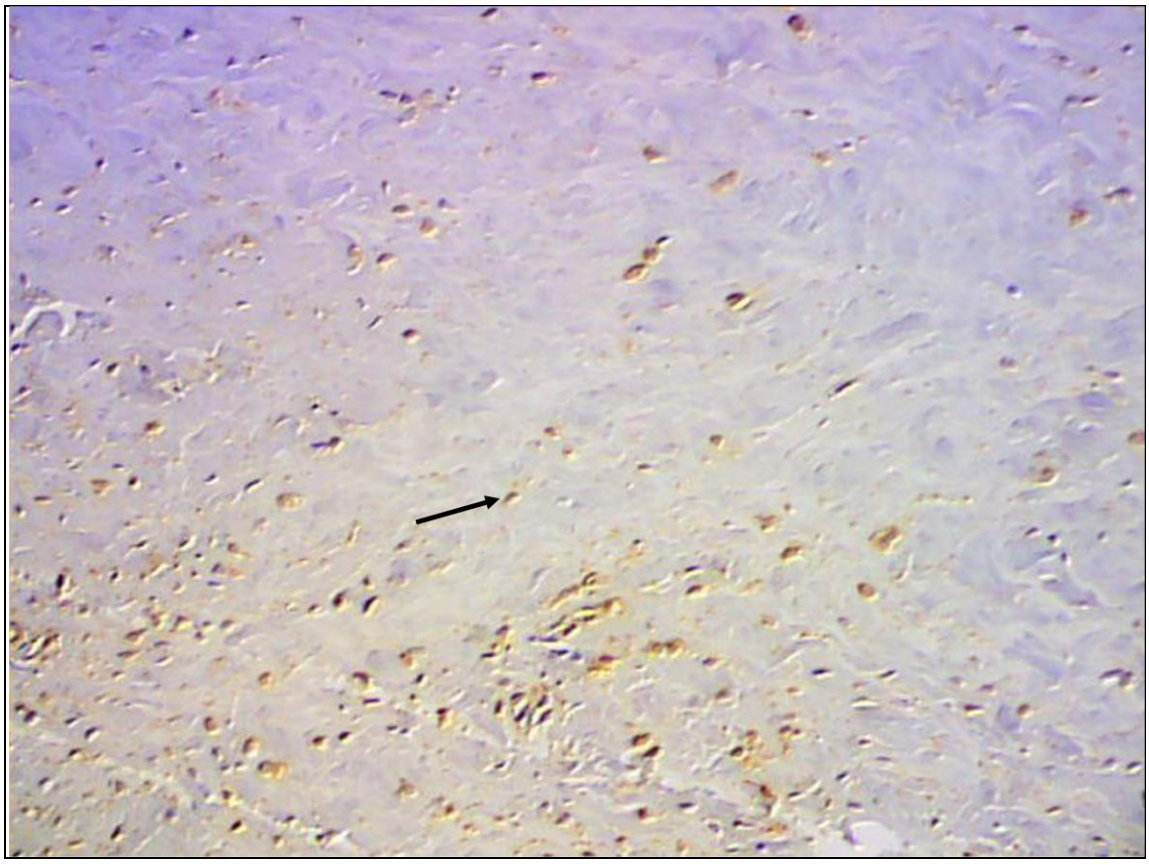

Figure 1. FasL expression evidenced by brown chromogen DAB (at arrow). 100 µm (40x)

CONCLUSÕES

4. CONCLUSÕES

Nesse estudo, verificou-se que houve:

1) Maior expressão de FasL (CD95L) no grupo de pacientes com ADDwR quando comparados com ADDwoR, demonstrando associação entre o mecanismo de apoptose e a forma mais branda de deslocamento de disco articular da articulação temporomandibular;
2) Reduzida área de expressão de FasL em discos articulares de pacientes com sinais de osteoartrose e maior expressão nos casos com ausência de sinais de osteoartrose;
3) Associação entre osteoatrose e ADDwoR, fenótipo mais extremo, onde todos os pacientes que foram diagnosticados com ADDwoR apresentavam OA, e nos quais coexistem um menor estresse mecânico e maior processo inflamatório.

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5. REFERÊNCIAS

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