1-1-2014

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Accepted version. *Journal of Oral Pathology & Medicine*, Vol. 43, No. 1 (January 2014): 69-75. DOI.

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Journal of Oral Pathology & Medicine, Vol. 43, No. 1 (2014): 69-75. DOI.

FasL Expression in Articular Discs of Human Temporomandibular Joint and Association with Osteoarthrosis

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Abstract

Background

Apoptosis is a programme of cell death which does not induce an inflammatory response. Recent previous research has suggested a correlation between temporomandibular internal derangement and apoptosis. Fas ligand (FasL) is an apoptosis-inducing factor, known to trigger apoptosis through distinct signal pathways. This study aims to examine, by immunohistochemistry, the expression of FasL in temporomandibular joint (TMJ) articular discs of patients with anterior disc displacement with reduction (ADDwR) and without reduction (ADDwoR) in patients with and without osteoarthrosis (OA).

Methods

Forty-two (*n* = 42) TMJ articular discs were divided into two cut-offs: (i) 8 control, 17 ADDwR, 17 ADDwoR, and (ii) 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 with 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.

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].

Anterior disc displacement with reduction (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 with 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].

Clinically, TMJ with mild ID is characterized by disc displacement with or without osseous remodelling, while severe derangement includes disc or attachment perforations, osseous remodelling and osteoarthritic changes [7].

Temporomandibular joint ID results from an imbalance between anabolic and catabolic processes, predominantly controlled by fibrochondrocytes, and is characterized by progressive degradation of the extracellular matrix of the articular disc [8-10].

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

Under normal physiological conditions, a balance exists in synovial joints between tissue breakdown and repair. When the balance is disturbed by a biomechanical or inflammatory insult, the discal fibrocartilaginous remodelling 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].

Previous research has demonstrated a correlation between TMJ ID and apoptosis [22-28]. Apoptosis is a physiopathological process implicated in various aspects of mammalian development, including embryogenesis, normal tissue turnover and homoeostasis, and also as a protection mechanism in immune reactions and diseases [22, 29]. It can be triggered either by a mitochondrion-dependent intrinsic pathway or via a cell surface death receptor–mediated extrinsic pathway. Fas ligand (FasL, also called CD95L), tumour necrosis factor (TNF)-α and TNF-related apoptosis–inducing ligand (TRAIL) are common apoptosis-inducing factors, known to trigger apoptosis through distinct signal pathways [30, 29, 31-35].

Fas ligand 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 [36, 37]. FasL has a pathogenic involvement in a variety of inflammatory diseases, including hepatitis, graft-versus-host diseases and pulmonary fibrosis [38-43].

In this study, we tested the hypothesis that apoptosis may be involved in the progression of TMJ ID, as shown by previous research [22-28]. Therefore, the present investigation was designed to evaluate, by immunohistochemistry, the expression of FasL in temporomandibular joint (TMJ) articular discs of patients with anterior disc displacement with (ADDwR) and without reduction (ADDwoR) in patients with and without osteoarthrosis (OA), focusing on the role, apoptosis may have in the association between severity of disc displacement and osteoarthrosis.

Materials and methods

Sample selection

A sample of 42 temporomandibular discs from 29 patients (mean age 32.7 years old, range from 18 to 56 years) was recruited for study from the patient pool at the Evangelico School Hospital, Curitiba, 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. 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 anti-inflammatory 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.

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

Affected side								
Patient	**Ethnical group**	**Gender**	**Age (yrs)**	**Diagnosis**	**Right**	**Left**	**Wilkes stage**	**Osteoarthritic 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,* condyle hyperplasia; *CFx,* condyle fracture; *OA*, osteoarthrosis.

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 non-surgical 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 four subjects displayed active condyle hyperplasia (CH), diagnosed by radiographs, CT scan and scintigraphy, as follows:

- Subjects without any signs of disc displacement (control group; *n* = 8; 8 specimens);
- Patients presenting anterior disc displacement with reduction (ADDwR; *n* = 10; 17 specimens);
- 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) [44].

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 [44]. 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 remodelling 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) [44]. 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:

- Patients without OA (control group + Wilkes stage III; *n* = 18; 25 specimens).
- Patient with OA (Wilkes stage IV and V; *n* = 11; 17 specimens).

Table 1 shows the baseline characteristics of the sample.

Surgical technique

Temporomandibular joint surgery was performed according to the technique described by Mehra and Wolford [45].

First, the displaced disc 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 non-resorbable 2–0 or 3–0 suture is placed through the hole and through the disc at the junction of the posterior and intermediate bands, and the disc is tied down to the condylar neck. The deformity of the disc precludes repositioning it into a more normal position, and recontouring the thickened disc 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. Post-surgical physical therapy was indicated at the discretion of the surgeon.

Histological sections obtained by scalpel of disc excess were prepared for observation of the *in situ* expression of FasL by immunohistochemistry.

Immunohistochemistry

The TMJ disc sections were deparaffinized with xylol (2 × 10 min) and rehydrated with absolute ethylic alcohol (3 × 1 min) and 80% ethylic alcohol (1 × 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 FasL antibody (Novocastra, New Castle, UK), diluted 1:50 in phosphate-buffered saline (PBS), 0.1% bovine serum albumin (BSA). This monoclonal antibody immunostains only the cellular fraction of FasL. The soluble fraction (sFasL), which is an inhibitor of the ligation between FasL and Fas in the target cell, is not immunostained. For negative controls, the primary antibody was not added. PBS was used instead. The secondary antibody, Advance™ (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 FasL because immunoreactivity was not observed in the negative controls.

The colour morphometry method was used to analyse the anti-FasL-immunostained area in the TMJ disc tissue. For this purpose, images of consecutive fields were captured by the 40× 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.

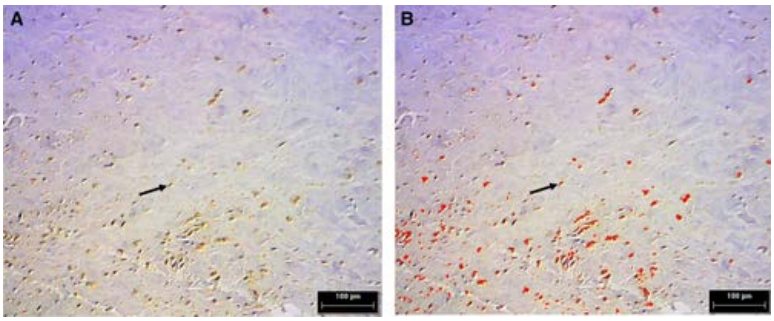

Figure 1. (A) Microphotography showing the positive immunohistochemical reaction for Fas ligand in brown, used as the *mask*. (B) Image Pro Plus™ analysis of the positive immunohistochemical reaction identified by the software through a sample of brown coloration considered appropriated by the observer, pattern followed for all fields analysed. The mean of all positive reaction represented the immunohistochemical expression of FasL measured in μm^2.

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 Student's *t*-test for independent samples or nonparametric Mann–Whitney test was employed. To meet symmetrical condition of the variable, data of area are previously submitted to a logarithmic transformation. *P* value <0.05 was considered statistically significant. Data were analysed with the software Statistica V. 8.0 (StatSoft Inc., Tulsa, OK, USA).

Results

Fas ligand 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

Significant differences were found in the expression of FasL 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 ($P < 0.001$) (Table 3, Figs 2 and 3), with higher area of expression in the ADDwR (Table 2).

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	Standard deviation	P-*value*[a]
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	

- [a] One-factor anova, $P < 0.05$.

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

Groups	P-value[a]
Control × ADDwR	0.062
Control × ADDwoR	0.187
ADDwR × ADDwoR	<0.001

- [a] Student's *t*-test for independent samples, $P < 0.05$.

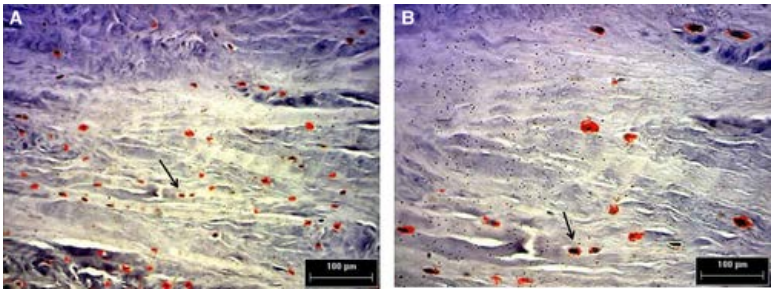

Figure 2. Immunostaining for Fas ligand in the temporomandibular joint disc of patients affected by disc displacement with reduction (ADDwR). (A) Magnification of 10×. Arrow = fibrochondrocyte. (B) Magnification of 20×. Arrow = fibrochondrocyte.

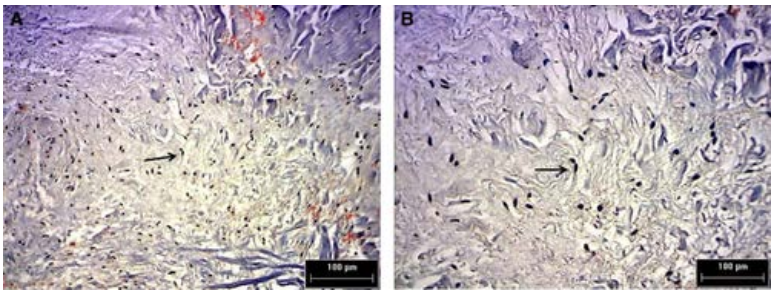

Figure 3. Immunostaining for Fas ligand in the temporomandibular joint disc of patients affected by disc displacement without reduction (ADDwoR). (A) Magnification of 10x. Arrow = fibrochondrocyte. (B) Magnification of 20×. Arrow = fibrochondrocyte.

Expression of FasL in TMJ discs of patients with and without osteoarthrosis

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 ($P = 0.001$) (Table 4).

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

Variable	Group	n	Mean	Median	Minimum	Maximum	Standard deviation	P-value[a]
FasL area (μm^2)	Without osteoarthrosis	25	20.43	16.26	7.64	45.68	10.33	0.001a
	With osteoarthrosis	17	12.26	10.85	5.97	20.65	4.43	

- [a] Student's *t*-test for independent samples, $P < 0.05$.

Discussion

Increasingly, studies have shown that mechanism of apoptosis plays an important role in the progression of ID of the TMJ 22-28.

In diseases of the TMJ, the synovium and articular cartilage produce various mediators, such as IL-1 and FasL, that have the potential to induce chondrocyte death through necrosis or apoptosis 46-48. Some studies showed correlation between TMJ ID and apoptosis mechanism. Imirzalioğlu et al. 28, in a study using synovial fluid through arthrocentesis from 17 joints in 17 patients 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 28. 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 22-27.

In this study, the analysis of FasL in TMJ discs of patients with and without disc displacement showed an increased area of expression in fibrochondrocytes of the ADDwR group. Indeed, although physiologically FasL is expressed by some cells as T lymphocytes or natural killer (NK) cells 49, our study shows FasL expression by fibrochondrocytes. Biomechanical stress activates multiple parallel and converging signals for hypertrophy and apoptosis 50. 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 50. Thus, the higher 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, may

induce the apoptosis process. This phenomenon can set up an endogenous reaction, which aims to restore homoeostasis and/or tissue remodelling. Therefore, the apoptotic event might work as a protective mechanism to overcome progression of disease.

Osteoarthrosis is a focal degenerative disorder that primarily affects the articular cartilage and subchondral bone of synovial joints such as TMJ 51-53. 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, 50, 52-60. With physiological 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 FasL expression in TMJ discs with osteoarthrosis process as the mechanism of apoptosis may influence the progression of ID. It was found an increase in the expression of FasL 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 61. Moreover, considering that mechanical damage of articular cartilage is often associated with OA pathogenesis 61 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 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 was obtained from patients, and not from cadavers, as most study samples, which allows clinical examination and anamnesis.

In conclusion, a higher expression of FasL 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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