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Apoptosis in Temporomandibular Joint Disc with Internal Derangement Involves Mitochondrial-dependent Pathways. An *in vivo* Study

Rosario Caltabiano
University of Catania

Rosalia Leonardi
University of Catania

Giuseppe Musumeci
University of Catania

Giovani Bartoloni
University of Catania

Mugurel Constantin Rusu
'Carol Davila' University of Medicine and Pharmacy

See next page for additional authors

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Luis Eduardo Almeida was affiliated with Center for Health and Biological Sciences, Pontifical Catholic, University of Panarà, Curitibe, Brazil at the time of publication.

Authors
Rosario Caltabiano, Rosalia Leonardi, Giuseppe Musumeci, Giovani Bartoloni, Mugurel Constantin Rusu, Luis Eduardo Almeida, and Carla Loreto

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Apoptosis in temporomandibular joint disc with internal derangement involves mitochondrial-dependent pathways. An *in vivo* study

Rosario Caltabiano
Department G.F. Ingrassia, Section of Anatomic Pathology, University of Catania, Italy
Rosalia Leonardi
Department of Dentistry, University of Catania, Policlinico Universitario, Italy
Giuseppe Musumeci
Department of Bio-Medical Sciences, University of Catania, Italy
Giovanni Bartoloni
Fetal and Perinatal Pathology Unit, ARNAS Garibaldi, University of Catania, Italy
Mugurel Constantin Rusu
Department of Anatomy and Embryology, Faculty of Dental Medicine, 'Carol Davila' University of Medicine and Pharmacology, Bucharest, Romania

Luis Eduardo Almeida
Center for Health and Biological Sciences, Pontifical Catholic, University of Panará, Brazil
Carla Loreto
Department of Bio-Medical Sciences, University of Catania, Italy

Abstract

Objective. Two main apoptosis pathways have been identified: an extrinsic (or death receptor-mediated) and an intrinsic (or mitochondrial) pathway. Apoptotic cell death through the extrinsic pathway has just been described in temporomandibular joint disc (TMJ) with internal derangement (ID); in contrast, no data are available on the involvement of the intrinsic pathway in this tissue. The aim of this work was to investigate whether the intrinsic pathway participates in apoptosis activation in patients with TMJ ID and anterior disc displacement without reduction. ***Materials and methods.*** Apoptosis activation was studied in TMJ discs from 15 patients with ID and in six unaffected discs using bcl-2–associated X protein (bax), B-cell lymphoma 2 (bcl-2), cytochrome c and caspase 9 immunohistochemistry. A correlation was sought between immunohistochemical findings and degree of disc damage. ***Results.*** None of the pathological TMJ disc sections were immunopositive for bcl-2; negative bcl-2 immunostaining was detected in affected discs; cytochrome c and caspase 9 immunoreactivity was greater in pathological compared to unaffected discs; the difference was significant and correlated with histopathological degeneration score data (Spearman's rho = 0.617). ***Conclusion.*** The present findings suggest that in-human TMJ with ID and anterior disc displacement without reduction of cell apoptosis occurs, at least partly, via the mitochondrial pathway, which contributes to the subsequent disc degeneration. These data may have clinical implications and could help devise improved treatment strategies.

Key Words:: displaced TMJ discs, immunohistochemistry, programmed cell death

Introduction

Apoptosis, or programmed cell death, is a tightly regulated process aimed to remove harmful, damaged or unwanted cells. It is involved in embryogenesis, metamorphosis and normal tissue turnover; its dysregulation gives rise to a variety of pathological conditions [1]. This homeostatic mechanism works via two main alternative pathways: the extrinsic (or death receptor-mediated) and the intrinsic (or mitochondrial) pathway. In the former activation of death signaling ligands like tumor necrosis factor (TNF)-α, FasL, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and members of the TNF-receptor superfamily (e.g. TNF receptor I, DR4, DR5), results in activation of caspase 8, which in turn cleaves and activates the pro-enzyme form of caspase 3, an executioner caspase. The latter destroys the cell's cytoskeletal and reparative proteins [2–5].

The intrinsic mitochondrial pathway is partly influenced by members of the bcl family bound to the mitochondrial membrane, including bax and bcl-2, which act as pro- or anti-apoptotic regulatory proteins, respectively [2]. The anti-apoptotic proteins bcl-2 and bcl-XL inhibit release of cytochrome c, whereas pro-apoptotic Bcl-2-associated X protein (bax), Bcl-2 homologous antagonist/killer (bak) and BH3 interacting-domain death agonist (bid) promote its release from mitochondria. Cytochrome c and deoxyadenosine triphosphate (dATP) bind to apoptotic protease activating factor (Apaf-1), forming a

multimeric complex that recruits and activates pro-caspase 9, an executioner protease mediating apoptosis, which in turn activates caspase 3, resulting in cell apoptosis [6,7].

Chondrocyte apoptosis plays a significant role in the pathogenesis of temporomandibular joint (TMJ) disorders. The commonest TMJ arthropathy is internal derangement (ID), characterized by anatomical disturbance in disc–condyle complex relationships. ID is likely a precursor of histopathological changes in TMJ disc tissue. Disc displacement is associated to degenerative tissue changes and to cell phenotype modification, possibly as a response to abnormal loading [8–10], although the underlying mechanism has not yet been completely elucidated. These degenerative joint changes are known to be influenced by the type and degree of disc displacement [11].

In a recent study of human displaced TMJ discs with ID we described increased chondrocyte-like apoptosis via the extrinsic pathway, which acted as a repair-limiting mechanism [10]; in particular we found over-expression of DR5 receptor, its ligand (TRAIL) and chondrocyte-like cells positive for terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL). These findings correlated with severity of the disc displacement, suggesting a possible pivotal role for the TRAIL system in the pathogenesis of disc degeneration. In the present study we investigated the intrinsic apoptosis pathway in displaced TMJ discs with ID using bax, bcl-2, cytochrome c and caspase 9 immunohistochemistry. We also attempted to correlate the immunohistochemical findings to the degree of TMJ disc damage using a recently devised histopathological degeneration scoring (HDS) system [12].

Materials and methods

Patients and tissues

Surgical TMJ disc specimens (nine right, six left) from 12 female and three male patients with a mean age of 33.1 years (range 25–43) were obtained from the archives of the University of the Pontifical Catholic University of Paranà, Brazil. ID was confirmed by clinical history, physical examination and magnetic resonance imaging. The study was approved by the ethics committee of the Pontifical Catholic University of Paraná (Resolution 196/96 of the National Health Council, registration number 104). The informed consent of each patient was obtained after tissue collection. Patients' age distribution and symptom severity are detailed in Table I. Unassisted maximum mouth opening (MMO) was measured with a millimeter ruler as the inter-incisal distance on maximum mouth opening as a measure of disease severity. Pain intensity in the preceding week was measured on a 100 visual analog scale (VAS) with anchor points 0 for ‘no pain' and 100 for the ‘worst imaginable pain'.

Table I. Visual analog scale (VAS); anchor points 0 ‘no pain' and 100 ‘worst imaginable pain'.

	Age		**MMO**		**VAS**	
	MV	**SD**	**MV**	**SD**	**MV**	**SD**
ADDwoR	35.4	4.2	27.2	4.4	81	6

The diagnosis that led to disc excision was painful disc derangement with impaired function. All patients had previously undergone unsuccessful conservative treatment including bite splinting, physical therapy, chiropractic manipulation, drug therapy and vitamin supplements, for at least 6 months. Discectomy has

come under increasing attack as the standard therapy for disc displacement, as a number of surgeons now favor a conservative approach; however, other researchers still believe in the value of discectomy [13–15]. Open surgery for patients with ID therefore has an ethical justification. This small sample of whole TMJ discs was used because they provide exhaustive information on degree of degeneration. Inclusion criteria were unsuccessful non-surgical treatment; a diagnosis of TMJ ID; and tenderness to TMJ palpation or interference with mandibular movements. Exclusion criteria were other TMJ disorders, dentofacial deformity; major jaw trauma; previous TMJ surgery; and previous steroid injections in the TMJ. All discs had anterior disc displacement without reduction (ADDwoR). After surgical excision, all discs were found to be well conserved; they were macroscopically deformed and none had a normal biconcave shape. The anterior and intermediate band and the posterior disc attachment were preserved in all specimens.

Six macroscopically normal human TMJ discs from cadavers of the collection of the Department of Anatomy of Catania University, Catania, Italy, were studied along with the diseased discs. These paraffin-embedded autopsy specimens, obtained from two male and four female subjects aged 40.3 years (range 22–60), were selected because the donors' clinical histories were negative for generalized joint disease or TMJ arthropathy; none had macroscopic signs of degenerative or inflammatory joint disease on dissection and none were displaced. They stained with Harry's hematoxylin on light microscopy and exhibited no detectable pathological changes.

Immunohistochemistry

For routine immunohistochemical staining, the specimens were fixed overnight in 10% neutral buffered formalin (Bio-Optica, Milan, Italy). After fixation and overnight washing, each disc was sectioned through its center along a parasagittal plane, perpendicular to its long axis. Each tissue block was dehydrated in graded ethanol, paraffin-embedded with preservation of the longitudinal anatomical orientation, cut into 5-µm-thick sections and placed on silanized glass slides with the anterior band on the right. Slides were dewaxed in xylene, hydrated using graded ethanol (100°, 95°, 80°, 70°, 50°) and washed in distilled water. Endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 for 10 min. Non-specific antibody binding was blocked by normal horse/goat serum (diluted 1:20 in phosphate buffered saline (PBS), 0.1% bovine serum albumin (BSA)). Sections were irradiated (5 min × 3) in capped polypropylene slide-holders with citrate buffer (pH 6), using a microwave oven (750 W) to unmask antigenic sites.

A rabbit anti-human bax polyclonal antibody diluted 1:100, a ready to use mouse anti-human bcl-2 monoclonal antibody (both from Dako Corporation, Glostrup, Denmark), a rabbit anti-cytochrome c antibody diluted 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA), which detects only cytosolic cytochrome c, and a rabbit anti-human caspase 9 polyclonal antibody (diluted 1:100) (Abcam, Cambridge, MA, USA) were employed. They were applied directly onto sections and slides were incubated overnight (4°C) in a humid chamber. Sections were de-waxed 3-times in PBS. Immune complexes were then treated with a biotinylated link antibody, de-waxed again 3-times in PBS and then detected with peroxidase-labeled streptavidin, both incubated for 10 min at room temperature (LSAB + System-HRP, Dako). After more PBS washings, sections were incubated for 4 min in 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Sections were lightly counterstained with Mayer's hematoxylin (Histolab Products

AB, Goteborg, Sweden) and finally mounted on GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA).

The immunoreaction was visualized with an Axioplan light microscope (Zeiss, Oberkochen, Germany).

Immunohistochemical evaluation

Immunostained slides were evaluated on a consensus basis by three anatomists, who were blinded to patient identity, clinical status and group identification, using a light microscope.

We applied a semi-quantitative scoring system already used in previous studies that considers staining intensity (SI) and area extent [16]. SI and the proportion of immunopositive cells were assessed and recorded. SI was graded on a 0–4 scale: 0: no detectable staining; +: weak staining; ++: moderate staining; +++: strong staining; ++++: very strong staining. Immunopositive cells, observed at 200× magnification, were scored as a proportion of 100 cells as 0: <5%; 1: 6–30%; 2: 31–50%; 3: >50%; and 4: >75% (extent score, ES). The final staining score (FSS) was the sum of SI and ES.

Positive and negative controls

Positive and negative controls were performed to test the specific reaction of the primary antibodies used in the study. Positive controls were specimens of basal cell carcinoma. For negative controls, randomly collected sections of diseased discs were treated with normal rabbit serum instead of the specific antibodies.

Rating of histopathological degeneration

One section in every three from each disc was stained with hematoxylin & eosin (H&E) and graded using a recently devised [11] histopathological degeneration rating tool, the HDS, which rates three measures (collagen bundles, non-specific degenerative changes and presence of vessels) with a score that ranges from 0 (no pathological changes) to 8 (severe degeneration), as described in Table II.

Table II. Histopathological degeneration grading system.

Collagen bundles	
0 points	Preservation of multidirectional collagen bundles
1 point	Altered collagen bundle architecture and/or fragmentation
2 points	Tears
3 points	Splitting
Non-specific degenerative changes	
0 points-	No degeneration
1 point	One of the following: fatty degeneration, calcified areas, hyalinization (fibrosis), or chondroid metaplasia
2 points	Two of th e following: fatty degeneration, calcified areas, hyalinization (fibrosis), or chondroid metaplasia
3 points	Three or more degeneration features.
Degree of vascularization	
0 points	Absence
1 point	Capillaries
2 points	Arterioles and venules

Sum 0 or 1 = No TMJ disc degeneration; Sum 2–3 = Moderate degeneration; Sum 4–8 = Severe degeneration.

Statistical analysis

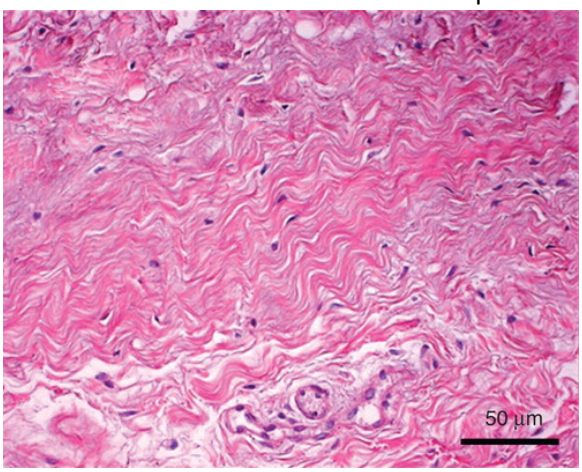
Mean and standard deviation were calculated for FSS and HDS data. Bax, bcl-2, cytochrome c and caspase 9 expression levels were compared using Spearman' test. All data were analyzed with the SPSS program (release 16.0, Chicago, IL, USA).

Cohen's kappa was applied to measure the agreement between the three observers and averaged over all three to evaluate overall agreement using the following grading: 0–0.2 (slight), 0.21–0.40 (fair), 0.41–0.60 (moderate), 0.61–0.80 (substantial), and 0.81–1.0 (almost perfect).

Results

Histological examination of H&E sections documented changes in collagen fiber arrangement, i.e. fragmentation, tears or splitting, and new vessel formation, in all specimens (Figure 1). Non-specific disc degeneration was less frequent. The mean HDS was 4.78 ± 1.30 (range 2–7). The damaged discs also displayed diminished cellularity and an altered cell population ratio with an increase in chondrocyte-like cells related to the severity of the morphological disc damage, and decreased fibroblast-like cells.

Figure 1. Histological examination of a H&E section showing alterations in collagen fiber arrangement and new vessel formation. Scale bar 50 µm.



The six control discs exhibited preserved multi-directional collagen bundles, absence of vessels and no signs of non-specific disc tissue degeneration.

Patients' discs showed moderate–strong bax, cytochrome c and caspase 9 immunoreactivity (SI: ++/+++; ES: 2–3) in the cytoplasm of fibroblast- and chondrocyte-like cells, albeit with different reaction patterns (Figures 2,3,4), whereas in control discs it was nearly undetectable (SI: 0; ES: 0). The difference in bax, cytochrome c and caspase 9 immunostaining between the two sets of samples was significant ($p < 0.01$) and correlated with HDS data (Spearman's rho = 0.617). The mean sample FSS of pathological discs was 4.70 ± 0.64 (range 3.6–5.6). As regards bcl-2, a small number of scattered cells in control discs were immunopositive (SI: 0/+; ES: 0/+) (Figure 5) as opposed to none of the pathological disc sections (0).

Figure 2. Bax immunoreactivity in degenerated TMJ disc with ADDowR (×400). Spindle fibroblast-like cells (black arrow) showed strong cytoplasmic immunopositivity. Red arrow indicates a round, strongly immunopositive chondrocyte-like cell. Scale bar 50 µm (28-year-old woman with TMJ disc with internal derangement and ADDowR).

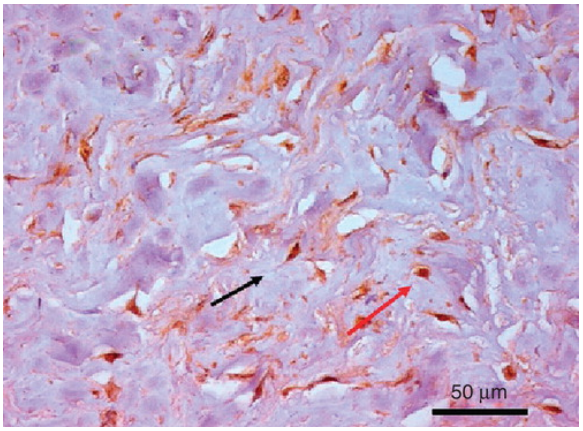


Figure 3. Cytochrome c immunoreactivity in degenerated TMJ disc with ADDowR (400×). Red arrow indicates a strongly immunopositive chondrocyte-like cell. Scale bar 50 µm (37-year-old man with TMJ disc with internal derangement and ADDowR).

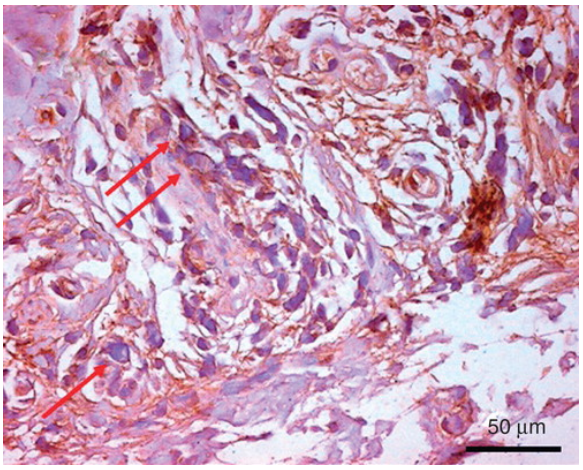


Figure 4. Caspase 9 immunoreactivity in degenerated TMJ disc with ADDowR (×400). The black arrow indicates a spindle fibroblast-like cell showing strong cytoplasmic immunopositivity. Scale bar 50 µm (34-year-old woman with TMJ disc with ADDowR).

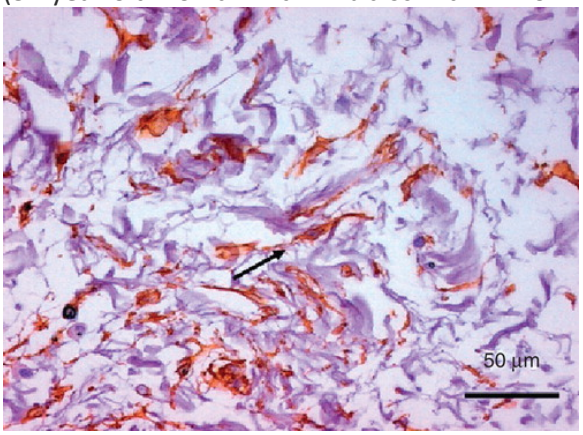
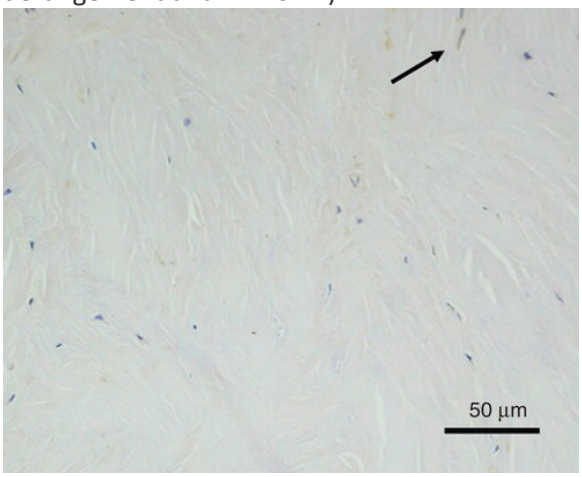


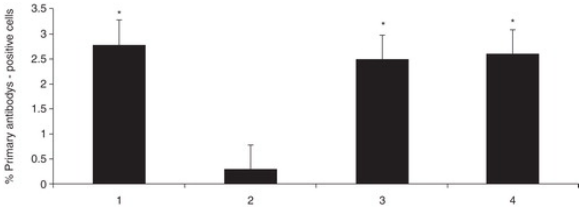
Figure 5. Bcl-2 immunostaining in normal TMJ disc (400×) demonstrating weak immunopositivity in few spindle fibroblast-like cells. Scale bar 50 µm (36-year-old woman with TMJ disc with internal derangement and ADDowR).



In positive controls, basal cell carcinoma tissue demonstrated cytoplasmic immunostaining for bax, bcl-2, cytochrome c and caspase 9.

Staining intensity and the proportion of immunopositive cells were assessed and recorded for each antibody (Figure 6).

Figure 6. Staining intensity and proportion of immunopositive cells were assessed and recorded in discs from temporomandibular joints with internal derangement and anterior disc displacement without reduction. Staining intensity (SI) was graded on a 0–4 scale: 0: no detectable staining; +: weak staining; ++: moderate staining; +++: strong staining; ++++: very strong staining. Immunopositive cells, observed at 200× magnification, were scored as a proportion of 200 cells as 0: <5%; 1: 6–30%; 2: 31–50%; 3: >50%; and 4: >75% (extent score, ES). The final staining score (FSS) was the sum of SI and ES. * $p < 0.01$. 1 = Rabbit anti-human bax polyclonal antibody, SI: ++–+++; ES: 3–4. 2 = Mouse anti-human bcl-2 monoclonal antibody, SI: 0; ES: 0/+. 3 = Rabbit anti-cytochrome c polyclonal antibody, SI: ++–+++; ES: 3–4. 4 = Rabbit anti-human caspase 9 polyclonal antibody, SI: ++–+++; ES: 3–4.



Discussion

Apoptosis-induced morphological changes play an important role in the postnatal functional adaptation of the TMJ to external stimuli, such as mechanical strain [17]. A quantitative relationship between repetitive loading and apoptosis has been demonstrated in some types of cartilage [18]. *In vitro* studies of inter-vertebral disc (IVD), very similar to TMJ disc, under increased tension and compression, suggest the existence of a tissue strain threshold that initiates cell death in the annulus fibrosus [19].

Apoptosis is controlled by a variety of cell signals that may originate outside cells (extrinsic pathway) or intra-cellularly (intrinsic pathway). Studies of herniated lumbar and TMJ discs suggest that apoptosis is associated with disc degeneration [16,20,21]. In a recent paper we demonstrated a correlation between tumor necrosis factor-related apoptosis-inducing ligand, which activates the extrinsic pathway, and TMJ disc degeneration [22]. In the present study we focused on the intrinsic pathway, investigated the immunohistochemical expression of bax, bcl-2, cytochrome c and caspase 9 in degenerated discs from patients with TMJ ID and hypothesized that immunoreactivity might correlate with extent of pathological transformation.

The bax, cytochrome c and caspase 9 upregulation associated to immunonegative bcl-2 expression documented in our disc sections suggest that disc damage involves activation of fibrocartilage cell apoptosis through the mitochondrial pathway. As we demonstrated in a previous study [10], apoptosis activation through the extrinsic pathway was closely related to HDS values.

The healthy TMJ usually undergoes a variety of functional strains and continuously adapts to changing functional demands to preserve structural and functional integrity. TMJ disc displacement is associated to a general remodeling response caused by abnormal mechanical loading that generally evolves as tissue degeneration, because cells can sense and convert the overload into biological signals that result in tissue responses [23–25]. In this respect disc cells play an important role in maintaining disc matrix turnover; in fact disc degeneration seems to be associated with decreased cellularity through apoptosis-related processes which in turn lead to impaired extracellular matrix generation, organization and repair [12,20,21,26–31].

It is a common notion that pro-inflammatory mediators and mechanically-induced signaling events strongly affect the IVD cell matrix and also have the potential to promote disc cell apoptosis, which in trauma appears to involve heavily the intrinsic mitochondria-mediated signaling sequence [23,32].

Bcl-2 and bax are, respectively, anti- and pro-apoptotic oncoproteins involved in apoptosis regulation and modulate cell survival and death [33]. Huang et al. [34] were the first to describe these molecules in normal rabbit craniomandibular joint (CMJ), documenting high bcl-2 and low bax expression in fibrocartilage chondrocytes and suggesting that they are metabolically active, long-lived cells not subject to growth or cycling. Equilibrium expression of proto-oncogenes such as bcl-2 and bax ensures their prolonged survival and metabolic action in maintaining a functional fibrocartilage. Therefore, the expression pattern of these oncoproteins suggests that they play an important role in cell survival and death in normal CMJ cartilage.

Deactivation of bcl-2 results in the loss of mitochondrial wall integrity and consequent egress of cytochrome c from mitochondria. In cytosol, cytochrome c activates caspase 9, which activates the executioner caspase 3, resulting in cell apoptosis. Wu et al. [35] detected a decrease in bcl-2 and a gradual increase in bax expression on Western blots from surgically displaced rabbit disc. They also noted that, in some conditions, including a change in biomechanical force, changes in their expression were associated to little histological change [36], but they did not correlate this value with a histopathological score. These data indicate that appropriate bax and bcl-2 levels may contribute to maintaining the articular cartilage and that an altered bcl-2/bax ratio may be a key to the progression of cartilage destruction [35,36].

Our findings also provide evidence that bax, cytochrome c and caspase 9 immunohistochemical expression correlates with HDS values, whereas bcl-2 immunostaining was not expressed, in line with previous IVD data [12,20,21].

Apoptosis is initiated by extrusion of cytochrome c, an intra-mitochondrial protein that is essential for mitochondrial survival. Extruded cytochrome c activates caspase 9, which in turn activates caspase 3 [5,37]. Caspase 9 is the executioner protease acting in the mitochondrial pathway, whereas caspase 8 belongs to the extrinsic pathway; the two enzymes activate caspase 3, resulting in cell death.

In conclusion we demonstrated that in human TMJ with ID and ADDwoR cell apoptosis occurs, at least partly, via the mitochondrial pathway, and that the immunohistochemical expression of bax, cytochrome c and caspase 9 correlates with the degree of disc damage. Cell loss due to the involvement of this apoptotic pathway therefore seems to have a role in TMJ disc degeneration. These data may have clinical implications and help devise improved treatment strategies.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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