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ABSTRACT

Temporomandibular joint (TMJ) discs frequently undergo degenerative changes in arthritis. However, the biomechanical properties of pathogenic discs remain to be explored. In this study, we evaluated the effects of chronic inflammation on the biomechanical properties of TMJ discs in rats. Chronic inflammation of TMJs was induced by double intra-articular injections of complete Freund's adjuvant for 5 weeks, and biomechanical properties and ultrastructure of the discs were examined by mechanical testing, scanning electron microscopy, and transmission electron microscopy. The instantaneous compressive moduli of the anterior and posterior bands of discs in inflamed TMJs were decreased significantly compared with those in the control group. The instantaneous tensile moduli of the discs of inflamed TMJs also showed significant decreases in both the anterior-posterior and mesial-lateral directions. The relaxation moduli of the discs of inflamed TMJs showed nearly the same tendency as the instantaneous moduli. The surfaces of the discs of inflamed TMJs became rough and porous due to the loss of the superficial gel-like stratum, with many collagen fibers exposed and degradation of the sub-superficial collagen fibrils. Our results suggested that chronic inflammation of TMJ could lead to deterioration of mechanical properties and alteration of disc ultrastructure, which might contribute to TMJ disc displacement.

KEY WORDS: temporomandibular joint, temporomandibular disorders, disc displacement, arthritis, biomechanics, collagen.

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Deterioration of Mechanical Properties of Discs in Chronically Inflamed TMJ

INTRODUCTION

Internal derangement or articular disc displacement in the temporomandibular joint (TMJ) is characterized by different displacements of the discs in the joint, such as disc anterior displacement with or without reduction, and occupies a large sub-group in the classification of temporomandibular disorders (TMD) (Emshoff and Rudisch, 2001; Stegenga, 2001). The TMJ disc has a biconcave shape and functions in absorbing and redistributing stress, which plays an important role in protecting the joint (Tanaka and van Eijden, 2003).

Patients with severe anterior disc displacement in the TMJ show strong correlations with disc deformation, including thickening, lengthening, and folding (Westesson and Rohlin, 1984; Westesson *et al.*, 1985; Orsini *et al.*, 1996; Taskaya-Yilmaz and Ogutcen-Toller, 2001). Moreover, inflammation and degeneration were found in nearly one-third of TMJs with advanced internal derangement (Dimitroulis, 2005). However, the causes of disc displacement in the TMJ remain unknown. It has been noted that some TMJs had inflammation and disc degeneration but had no disc displacement (de Bont *et al.*, 1986; Kondoh *et al.*, 1998), although inflammation of the TMJ and deformation of discs are generally believed to be the consequence of disc displacement. However, in our recent study, deformity and thickening and changes in the disc contents of collagen and proteoglycan were observed in chronically inflamed TMJs in rats (Wang *et al.*, 2012b). Therefore, we hypothesized that inflammation might cause deterioration of the biomechanical properties of discs.

The mechanical properties of the disc are indispensable to TMJ function. Several mechanical properties of normal TMJ discs from various species have been evaluated (Tanaka and van Eijden, 2003; Allen and Athanasiou, 2006; Koolstra and Tanaka, 2009; Kuo *et al.*, 2011; Juran *et al.*, 2013). Anisotropic and heterogeneous properties among diverse region of discs have been highlighted (Kuo *et al.*, 2010; Yuya *et al.*, 2010). Although the physiological functions and mechanical properties of TMJ discs have been documented to some extent, there is a lack of understanding regarding the mechanical properties of pathogenic discs. Moreover, little information is available on the relationship between biomechanical properties and ultrastructure changes of discs in inflamed TMJs.

In this study, we examined the biomechanical properties (tensile and compressive) and the ultrastructure of discs from chronically inflamed TMJs in rats.

MATERIALS & METHODS

Induction of Inflammation

Female Sprague–Dawley rats (7 wk old, 180–200 g) were divided randomly into inflammation and control groups. TMJ inflammation was induced by intra-articular injections of complete Freund’s adjuvant (CFA; Sigma, St. Louis, MO, USA) twice, as described previously (Wang *et al.*, 2012b). Briefly, a 25- μ L quantity of CFA was emulsified in 25 μ L saline and then injected directly into the upper compartments of the TMJs on days 0 and 14. The control group received similar intra-articular injections of saline. All rats were sacrificed by pentobarbital overdose on day 35. Rats were housed under controlled temperatures with a 12-/12-hour light/dark cycle and free access to food and water.

Animal procedures were approved by the Peking University Animal Ethics Committee prior to the initiation of the study (approval number: LA2012-59). This investigation conformed to the ARRIVE guidelines for preclinical studies.

Macroscopic Mechanical Testing

We carefully dissected 16 discs from the TMJs of eight rats by cutting the disc-condylar ligaments and stored them in ice-cold 0.15 M phosphate-buffered saline (PBS, pH 7.4) until use (Juran *et al.*, 2013). All samples were tested within 4 hr of dissection. Right and left discs were examined for compressive and tensile properties, respectively. Discs were kept moist by being soaked in PBS buffer at room temperature throughout the entire testing process (Tanaka *et al.*, 2003).

Compressive and tensile properties, including instantaneous reaction and stress relaxation of the discs, were tested with the ElectroForce 5200 biodynamic test instrument (Bose Corporation, Bloomington, MN, USA). We modified the indenter for both compression and tension tests. The indenter in compression tests was designed to have a rounded cross-section ($r = 0.37$ mm), which was small enough for the rugged surface of the disc to be regarded as a regular sample (Appendix Fig., C). Analogously, the clamping apparatus for tensile testing was modified to have a square contact section ($a = 0.3$ mm), which is sufficiently small to allow us to ignore the deviation in internal thickness, and to consider the sample as a long bar-shape between the clamping apparatus (Appendix Fig., D). The cross-sectional areas of the compression and tensile apparatus were 0.43 mm² and 0.3 mm², respectively. Graphs of compressive and tensile tests are shown in Figs. 1A and 1B.

To maintain the integrity of discs, the tested regions were not isolated from the disc. For compressive testing, five equal regions (anterior, posterior, intermediate, medial, and lateral) were marked on each disc (Fig. 1C). For tensile testing, only anterior-posterior and medial-lateral directions were marked on the discs (Fig. 1D).

Following the application of a 0.05-N tare load, the initial thickness and length were calculated according to the exact location. During testing, the largest strain was defined (compressive strain, 40%; tensile strain, 30%). Stress and strain magnitudes were recorded at 0.02-second intervals, and

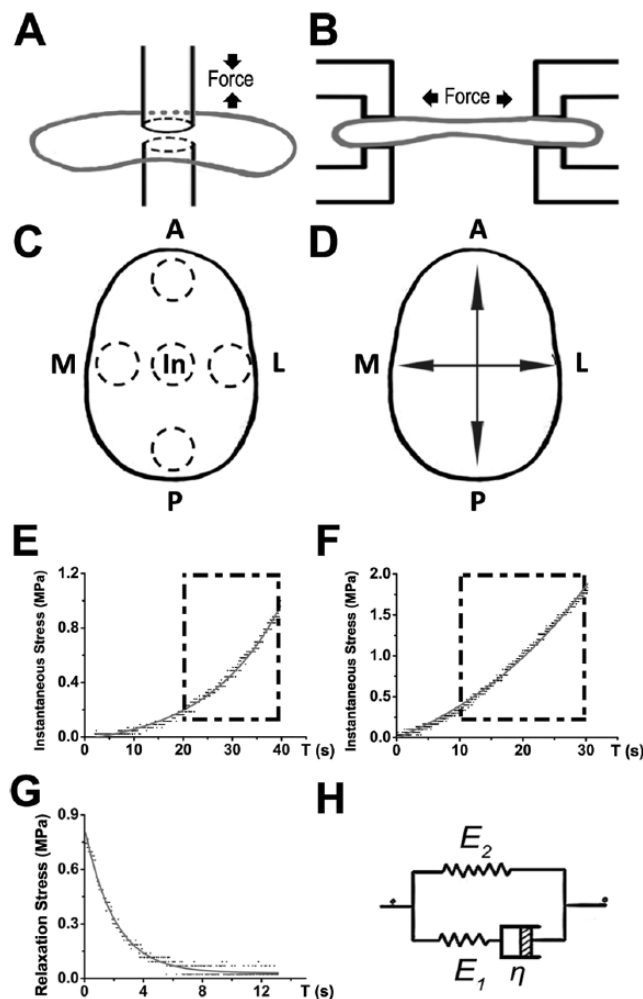


Figure 1. Description and representative stress-strain curves of biomechanical tests of TMJ disc. (A) Graph of the process of compressive tests. (B) Graph of the process of tensile tests. (C) Region division of compressive mechanical tests of TMJ disc. Dotted circle represents relevant compressive regions, including anterior, intermediate, posterior, mesial, and lateral regions. (D) Direction division of tensile mechanical tests of TMJ disc. The arrow represents the tensile direction, including anterior-posterior and mesial-lateral directions. (E) Linear fit of instantaneous compressive modulus. (F) Linear fit of instantaneous tensile modulus. (G) Nonlinear fit of relaxation modulus. (H) Kelvin model used to build viscoelastic model in relaxed tests. A, anterior; In, intermediate; P, posterior; M, mesial; L, lateral.

stress-strain and strain-time curves were generated. Disc viscoelastic properties were evaluated in terms of the instantaneous and relaxation moduli. For the instantaneous modulus, stress-strain curves were intercepted at the transition zone (Tanaka and van Eijden, 2003), and fitted to a linear model. For the test, 20% to 40% compressive strain (Fig. 1E) and 10% to 30% tensile strain were used (Fig. 1F). In the stress-relaxation test, the Kelvin model was used to fit stress-time curves (Allen and Athanasiou, 2006; Kalpakci *et al.*, 2011) (Figs. 1G, 1H). Using these original data, we calculated stress, strain, and modulus. The expression of the stress-time curve was as follows:

Table. Values of Compressive and Tensile Moduli of TMJ Discs Represented by the Mean \pm Standard Deviation

Location	Instantaneous Modulus (MPa)		Relaxation Modulus (MPa)	
	Control	Inflammation	Control	Inflammation
	A	1.74 \pm 0.23	1.34 \pm 0.43	0.34 \pm 0.08
P	2.26 \pm 0.62	1.20 \pm 0.30	0.49 \pm 0.35	0.20 \pm 0.11
In	1.51 \pm 0.45	1.51 \pm 0.56	0.21 \pm 0.18	0.23 \pm 0.17
M	1.65 \pm 0.35	1.88 \pm 0.78	0.26 \pm 0.14	0.41 \pm 0.46
L	1.62 \pm 0.36	1.71 \pm 0.69	0.22 \pm 0.09	0.33 \pm 0.27

Direction	Instantaneous Modulus (MPa)		Relaxation Modulus (MPa)	
	Control	Inflammation	Control	Inflammation
	A-P	2.69 \pm 1.01	5.03 \pm 2.08	1.39 \pm 0.88
M-L	1.98 \pm 0.85	3.74 \pm 1.09	0.19 \pm 0.21	0.07 \pm 0.05

(A) Compressive instantaneous and relaxation moduli of control and inflamed discs (n = 8).

(B) Tensile instantaneous and relaxation moduli of control and inflamed discs (n = 6).

A, anterior; In, intermediate; P, posterior; M, mesial; L, lateral.

$$\sigma = (E_2 + E_1 e^{-\frac{E_1 t}{\tau}}) \epsilon_0$$

The relaxation modulus was determined according to this formula. All experimental data showed high accuracy ($R^2 = 0.94$).

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

In total, six TMJ discs of three rats were fixed with 2.5% glutaraldehyde solution (Sigma) at 4°C for 12 hr, with half (right discs) for SEM and the other half (left discs) for TEM. SEM and TEM were performed as described previously (Wang *et al.*, 2012a). For SEM, the superior surface of the anterior band, intermediate zone, and posterior band of the disc was scanned. For TEM, the superior surface of the anterior band, intermediate zone, and posterior band of the disc was examined. Samples were double-fixed with 1% osmium tetroxide (Sigma), stained with lead citrate and uranyl acetate, embedded in epoxy resin, and sectioned at 100 nm.

Statistical Analysis

Statistical analyses were performed with SPSS software (ver. 11.0 for Windows). Data for macroscopic mechanical testing are presented as means \pm standard deviations (SDs). Two of the samples were damaged during tensile properties testing, and the corresponding data were excluded in statistical analysis. Thus, the sample sizes for compressive and tensile tests were eight and six, respectively. Statistical comparisons of the two groups were performed an independent *t* test. *P* values < .05 were considered to indicate statistical significance.

RESULTS

Decreased Instantaneous and Relaxation Moduli of Discs in Chronically Inflamed TMJ

Biomechanical properties of discs from TMJs with and without chronic inflammation were evaluated by macroscopic mechanical testing. The instantaneous and relaxation moduli of discs in compression and tension tests are shown in the Table. The instantaneous compressive moduli of the anterior and posterior bands of the discs of inflamed TMJs were decreased significantly vs. those of control discs (1.34 \pm 0.43 MPa and 1.20 \pm 0.30 MPa vs. 1.74 \pm 0.23 MPa and 2.26 \pm 0.62 MPa, respectively; n = 8; *p* < .05; Fig. 2A). However, the compressive moduli of the intermediate, medial, and lateral regions of discs from inflamed TMJs did not differ from those of control discs. The instantaneous tensile moduli in the anterior-posterior and mesial-lateral directions of discs from inflamed TMJs showed significant decreases compared with those of the control group (2.69 \pm 1.01 MPa and 1.98 \pm 0.85 MPa vs. 5.03 \pm 2.08 MPa and 3.74 \pm 1.09 MPa, respectively, n = 6; *p* < .05; Fig. 2B). Two samples were broken during tension testing, and their data were excluded from the analyses. The relaxation compressive moduli of the anterior and posterior bands of discs from inflamed TMJs were also decreased compared with those of the control group (0.24 \pm 0.08 MPa and 0.20 \pm 0.11 MPa vs. 0.34 \pm 0.08 MPa and 0.49 \pm 0.35 MPa, respectively; n = 8; *p* < .05; Fig. 2C). The relaxation tensile modulus in the anterior-posterior direction of discs from inflamed TMJs showed a significant decrease compared with that of the control group (0.19 \pm 0.18 MPa vs. 1.39 \pm 0.88 MPa), whereas the relaxation tensile modulus in the mesial-lateral direction showed no difference between the groups (Fig. 2D).

Loss of Gel-like Structure of Superior Surfaces of Discs from Inflamed TMJ

To evaluate the effects of inflammation on ultrastructural morphology in TMJ discs, we examined ultra-morphological changes of the superior surfaces of discs by SEM. At lower magnification (2,000 \times), the superficial strata of the anterior and posterior bands of the control disc appeared even and intact, covered with a gel-like structure; the superficial stratum of the intermediate zone of the control disc showed obvious folded features or a corrugated surface. Despite variation among bands, the surfaces of the control discs showed regularity at higher magnification (10,000 \times). All three control discs showed similar ultrastructural surfaces. However, at lower magnification, the superficial strata of discs from inflamed TMJs for all three discs examined were observed to lack the gel-like structure of the anterior and posterior bands and the corrugated surface of the intermediate zone; at higher magnification, superficial fractional damage and exposure of sub-superficial collagen fiber-like tissue were evident for all three discs examined (Fig. 3).

Disordered Arrangement of Collagen Fibrils in Discs from Inflamed TMJ

For further examination of the ultra-morphological changes in discs from inflamed TMJs, TEM was performed. The superior

surfaces of the anterior band, intermediate zone, and posterior band of the control disc were covered by a 150- to 400-nm-thick superficial stratum, which had a collagen-free, electron-dense, and amorphous structure. A deeper stratum of less-electron-dense proteoglycan lamina with well-arranged collagen fibrils was also evident. However, in the inflamed group, the collagen-free superficial stratum was partially diminished; the collagen fibrils of the deeper stratum were disorganized and degraded, resulting in diffused and scattered individual collagen fibrils (Fig. 4).

DISCUSSION

In this study, we demonstrated the deterioration of biomechanical properties and ultrastructural changes of discs from chronically inflamed TMJs. Both the instantaneous and relaxation moduli of discs from the inflamed TMJs were significantly lower than those of control discs. SEM and TEM showed disturbance of the superficial stratum, and exposure and degradation of the underlying collagen fibers in the discs of inflamed TMJs, suggesting a relationship between the biomechanical alterations and ultrastructural changes in the discs. To the best of our knowledge, this is the first report of a decrease in mechanical properties in discs from chronically inflamed TMJ discs.

The compressive and tensile moduli of discs were significantly decreased in chronically inflamed TMJs. This decrease in instantaneous moduli in different locations and directions of the discs from the inflamed TMJs indicated deterioration in the capacity to absorb and distribute stress from the inflamed TMJ discs. This deterioration of biomechanical properties of discs could be an indication of early degeneration, and is consistent with changes in articular cartilage due to inflammation (Silver *et al.*, 2002). During mandibular movements, discs often endure and absorb peak loads by deformation in a certain area (Tanaka *et al.*, 2004). In the present study, to observe biomechanical properties under significant deformation, we set 40% strain of compression and 30% strain of tension as loading limits. A diminished instantaneous modulus of the disc indicated that the disc was relatively softer or underwent greater strain under the same stress, which may subsequently cause a decrease in adaptive capacity of the disc and result in overloading of the TMJ, ultimately giving rise to tissue breakdown.

The decreasing instantaneous moduli of discs from the chronically inflamed TMJs were the opposite of the changes in aging discs, which showed an increase in instantaneous modulus (Tanaka *et al.*, 2006; Ahn *et al.*, 2007). These differing changes in mechanical properties may result from the different characteristics of the degeneration process. Age-related biomechanical

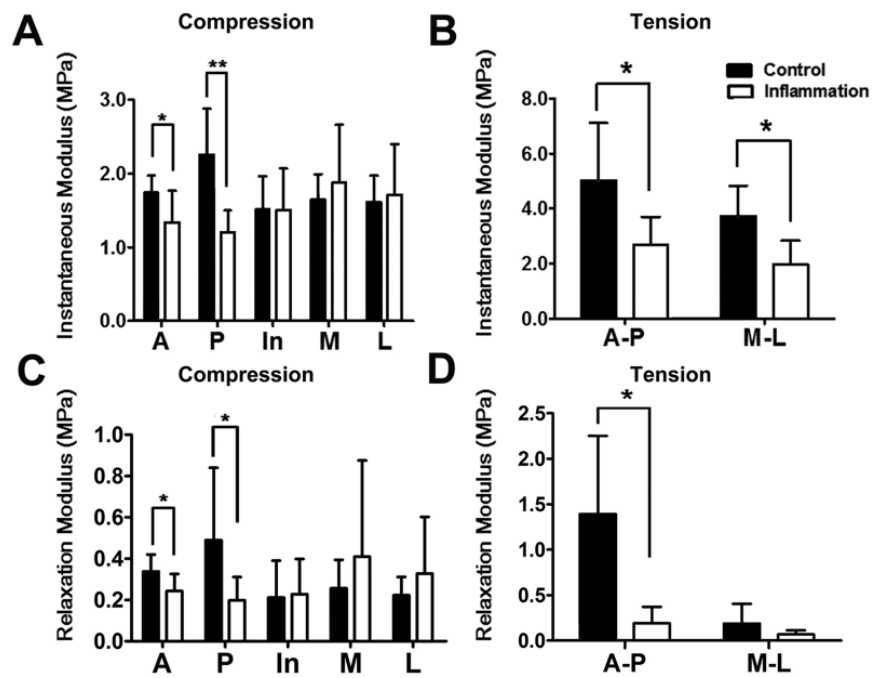


Figure 2. Biomechanical properties of TMJ discs with or without inflammation. (A) Instantaneous moduli under compression from 20% to 40% strain. Instantaneous compressive moduli of the anterior and posterior bands of discs of inflamed TMJs were significantly lower than those of the control group, whereas there was no statistically significant difference among the other three regions ($n = 8$; $*p < .05$, $**p < .01$). (B) Instantaneous moduli under tension from 10% to 30% strain. Instantaneous tensile moduli of anterior-posterior and mesial-lateral directions of discs of the inflamed TMJ were significantly lower than those of the control group ($n = 6$; $*p < .05$). (C) Relaxation moduli under compression. (D) Relaxation moduli under tension. Relaxation moduli of discs of the inflamed TMJs showed nearly the same trend as the instantaneous moduli. A, anterior zone; In, intermediate zone; P, posterior zone; M, medial zone; L, lateral zone.

changes of TMJ discs imply stiffening of discs (Tanaka *et al.*, 2006) and may be related to an increase in calcium content in the discs (Takano *et al.*, 1999). In contrast, the decreasing instantaneous moduli of the discs of chronically inflamed TMJs in the present model imply softening of the discs, which are more similar to the early degenerative changes of condylar cartilage, including swelling and softening (de Bont and Stegenga, 1993). Moreover, glycosaminoglycan (GAG) and collagen contents are considered to be responsible for the elastic properties of discs (Scapino *et al.*, 1996; Tanaka *et al.*, 2003; Tanaka and van Eijden, 2003). It is reported that the concentration of GAG showed a decrease with age in rats (Okazaki *et al.*, 1996). However, in our previous study, both GAG and collagen, the composition of the disc matrix, were increased in the inflamed discs (Wang *et al.*, 2012b). We evaluated the change of matrix of the disc as a whole, but did not examine the regional content of GAG and collagen in each band of the disc. Therefore, we could not exclude the possibility that changes of regional matrix components might also contribute to the regional alteration in biomechanical properties.

The relaxation modulus in discs from inflamed TMJs were also decreased. The stress relaxation of discs reflects their capacity to dissipate stress (Tanaka and van Eijden, 2003). Changes in the relaxation modulus are related to the moisture level of the disc and involve their viscoelastic properties, which

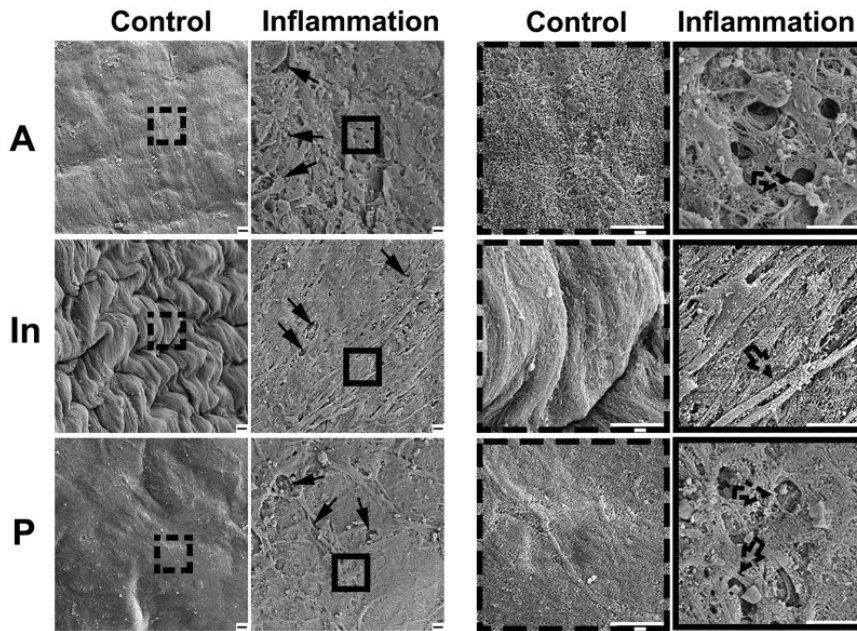


Figure 3. SEM analysis of surface topography of discs of TMJs with or without inflammation. **Left panels** were lower magnification (magnified 2,000 \times), and **right panels** were higher magnification (magnified 10,000 \times) from the dashed and solid boxes on the left panels. Control group showed relative flat and smooth surfaces in anterior and posterior bands and folded integrated surface in the intermediate zone; the superficial gel-like layer was distinct at the lower magnification. Inflammation group showed rough and porous surfaces in anterior and posterior bands of discs of inflamed TMJs; the intermediate zone lost the folded structure and became flattened, and the gel-like layer was discontinued (arrow), with collagen fibers exposed (dotted arrow). Bar = 2 μ m. Representative images of three samples.

play important roles in TMJ disc function (Kuboki *et al.*, 1997; Yamashita *et al.*, 2001). Previously, we showed that moisture content is increased in discs from inflamed TMJs (Wang *et al.*, 2012b); thus, the decrease in viscoelasticity in the anterior and posterior bands may also be due to the increased moisture content of the disc following chronic inflammation of the TMJ.

The changes in morphological features may also be associated with changes in the mechanical and functional properties of discs from inflamed TMJs. The proteoglycans and arrangements of collagen fibers are considered to influence the compressive and tensile properties of discs (Stankovic *et al.*, 2013). We observed by SEM and TEM that the superficial stratum was disordered, with a rough and porous structure in all three bands of discs from inflamed TMJs, and the underlying collagen fibers exhibited marked degradation. The superficial gel-like stratum of the three bands likely was comprised of chondroitin-sulfate-rich proteoglycans, similar to the surface layers of articular cartilage, providing lubrication and compression resistance to the articular surface (Orford and Gardner, 1985). Thus, the ultrastructural alterations observed may also account, in part, for the decrease in the mechanical properties of discs from inflamed TMJs.

Regional variations in compressive modulus were found in discs from inflamed TMJs. Studies have reported various magnitudes of tensile modulus or Young's modulus and relaxation modulus. Analysis of data from animals showed marked differences in tension compared with humans, and regional variations

are seen consistently among species (Kalpakci *et al.*, 2011). We observed that the compressive modulus was higher in the anterior and posterior bands than in the intermediate zone, and the apparent Young's modulus and ultimate tensile strength of the disc were higher for anterior-posterior loading than for mesial-lateral loading in both the control and inflammation groups. These results are consistent with those of other mechanical tests (Beatty *et al.*, 2001; Kalpakci *et al.*, 2011). Although the morphological changes were clear in all three zones of discs from inflamed TMJs, the intermediate zone showed no significant difference in mechanical tests compared with the control discs. This might be due to the thickness of the intermediate zone, which was the thinnest part of the TMJ disc. Thus, obtaining accurate data for the intermediate zone may not be feasible, resulting in the large variation. However, the compressive modulus of the mesial and lateral bands of the discs also showed no significant difference between the groups; this could be due to the fact that rats do not have lateral translator movement of the mandible (Hiimäe and Ardran, 1968; Weijjs, 1975), resulting in significant changes in compressive

modulus only in the anterior and posterior bands. Moreover, various contents and types of proteoglycans have been found in different regions of discs in several mammals (Tanaka and van Eijden, 2003); this may also explain the differences in mechanical properties among regions. Additionally, regional variations were identified only in the viscoelasticity test under instantaneous loading; mechanical tests with a range of frequencies and cyclic loading, as well as shear stress testing, are necessary for a full assessment.

In this study, we demonstrated deterioration in the superficial ultrastructural morphology and mechanical properties of TMJ discs following the induction of chronic inflammation. Based on our previous and the current results, we suggest that TMJ inflammation-induced deformities and alterations in the contents and mechanical properties of discs might be a predisposing factor for disc displacement in the TMJ. Although there was a lack of direct evidence to support this suggestion, whether the distribution of stress of inflamed TMJ discs and surrounding tissues was also altered and how changes in disc mechanical properties contribute to functional changes in TMJ discs require further investigation.

There is a close relationship between arthritis and disc displacement, but the cause for disc displacement is still unclear. Guler *et al.* reported that 10 out of 16 discs with disc displacement without reduction presented joint effusion (Guler *et al.*, 2005); Emshoff *et al.* reported that 11 out of 23 patients with arthritis had disc displacement without reduction (Emshoff *et*

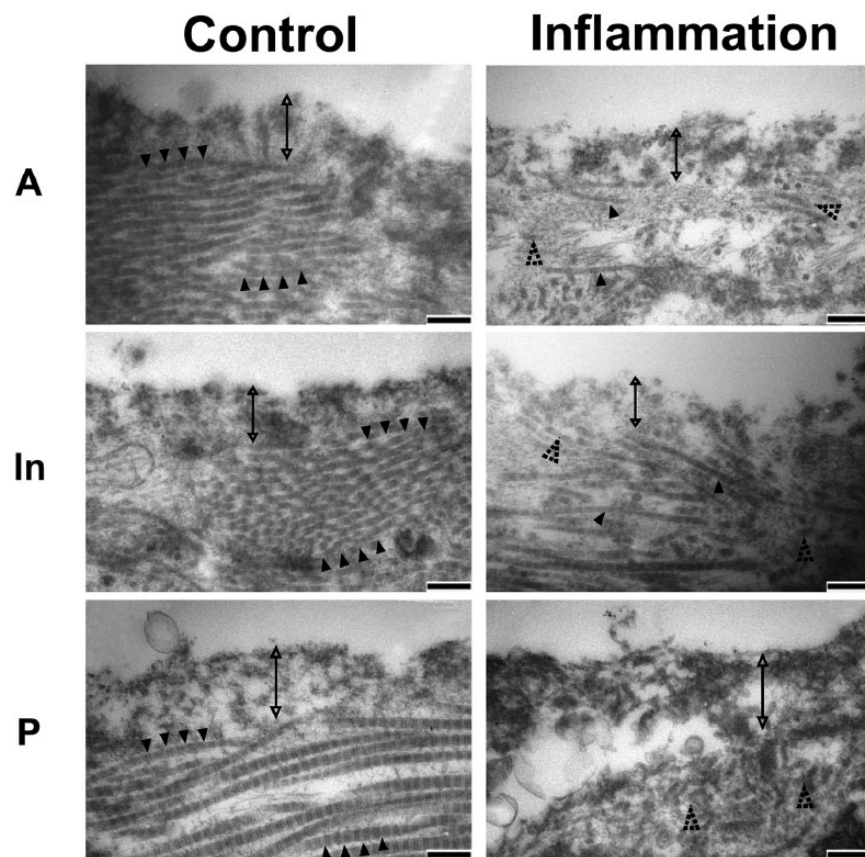


Figure 4. TEM analysis of superficial sections of discs of TMJs with or without inflammation. The surface of the control disc consisted of a collagen-free lamina (double arrow) covering the underlying collagen fibrils (black arrowhead), whereas the superficial structure became disorganized in the inflamed discs, with collagen-free lamina becoming irregular and the underlying collagen fibrils degraded and irregularly arranged (dotted arrowhead). Bar = 200 nm. Representative images of three samples.

al., 2000). These clinical observations imply that there would be a correlation between the inflammatory changes and disc displacement. Nevertheless, the deterioration of disc biomechanical properties of chronic inflammation of TMJ in rats in the present study would suggest, or provide at least one possibility, that the discs of TMD patients, who usually have chronic inflammation in the TMJs, might also have decreased instantaneous and relaxation moduli, which would lead to the decrease in adaptive capacity of the disc and gradual breakdown of the harmony of the disc-condyle complex, ultimately leading to disc displacement. Therefore, our findings might be simply a new view to explain the cause of disc displacement.

In conclusion, chronic TMJ inflammation resulted in disruption of the surface morphology and deterioration of the viscoelasticity of the disc.

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