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Proteoglycan expression in the rat temporomandibular joint in response to a bite-raising appliance.

BY

Jian Mao 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Proteoglycan expression in the rat temporomandibular joint in response to a bite-raising appliance submitted by Jian Mao in partial fulfilment of the requirements for the degree of Master of Science in Clinical Sciences.

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TO

My father, who is now fighting liver cancer, for his extreme understanding and dedication to science.

ABSTRACT

It was hypothesized that a unilateral bite-raising appliance would increase the load applied to the rat temporomandibular joint (TMJ) and thereby induce increased expression of proteoglycans in TMJ articular tissues. To test this hypothesis, six- and nine-week-old, Sprague-Dawley rats received the appliances bonded to their right molars for four weeks. Some nine-week-old rats were housed for an additional four weeks after removal of the appliances they had worn for four weeks. Parasagittal sections of the TMJ disc and mandibular condyle were stained with hematoxylin-eosin. Proliferation of chondroblast-like cells was apparent in the treated specimens. Aggrecan-like proteoglycans were detected by safranin O (SO) staining in the condylar cartilage where cell proliferation was evident. A monoclonal antibody (mAb) against versican reacted strongly in the disc and the fibrous layer of the mandibular condyle in the treated groups. Computer quantification of intensities for SO and anti-versican mAb staining revealed that the average intensities of the treated specimens were significantly higher than their corresponding sham-operated controls and the average intensities of the appliance-removal specimens showed no significant differences from their corresponding sham-operated controls. Thus, the unilateral bite-raising appliance induced an increase in the expression of aggrecan in condylar cartilage and versican in the TMJ disc and the surface fibrous layer of the condyle. The elevated proteoglycan expression may be used as indirect evidence to suggest that the appliance induces an increase in the magnitude of the compressive forces in the rat temporomandibular joint.

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CHAPTER 1

INTRODUCTION

The human temporomandibular joint (TMJ) consists of the temporal bone and the mandibular condyle with an interposing disc. The TMJ articular components, namely the disc, the articular surfaces of the mandibular condyle and the articular eminence of the temporal bone, are composed of dense fibrous tissue or fibrocartilage (Provenza, 1986). The mandibular condyle can be arbitrarily divided into layers according to histology as shown in Fig. 1 (redrawn from Osborn, 1981 and Ten Cate, 1985). A growing condyle is characterized by the presence of a chondrogenic (proliferative) layer, a chondroblast layer, a hypertrophic layer and a cartilage-breakdown layer (Fig. 1A). The adult human condyle (Fig. 1B), on the other hand, is composed entirely of bone covered by a thick layer of dense (articular) fibrous tissue, a fibrocartilage mineralized to different extent underneath, and subchondral bone (Blackwood, 1966).

Both the fibrous and cartilaginous components of TMJ articular tissues are composed of cells, mostly fibroblasts and chondroblasts, and their intercellular matrix. Besides water, the intercellular matrix is largely composed of collagens (Thilander, 1964; Appleton, 1975) and proteoglycans (Mills *et al.*, 1988; Nakano and Scott, 1989). Connective tissue cells and the molecules they synthesize are by no means inert, as once thought. Instead, both cells and molecules respond to environmental stimuli. A wide variety of responses of a single connective tissue cell to the environment are brought about by a limited number of stimuli, one of which is mechanical in nature. A mechanical stimulus or force has been shown to affect the behavior of connective tissue cells and the function and turnover of the molecules they synthesize (Shimshoni *et al.*, 1984; Copray *et al.*, 1985; Koob and Vogel, 1987; Kim *et al.*, 1994; Carvalho *et al.*,

1995). In articular tissues, collagen resists tensile and shear forces and proteoglycans resist compressive forces (Maroudas, 1976; Koob and Vogel, 1987; Hukins, 1992; Bruckner and van der Rest, 1994). The experiments reported below provide one of the increasingly illustrated examples whereby cells and molecules of connective tissue adapt to a change in the mechanical stress imposed on them. In this case, the mechanical stimulus is a dental bite-raising appliance, a resin overlay placed between the upper and lower teeth in the rat.

1.1 Collagens.

Collagens of various types comprise about 60%-80% of the dry weight or 20% of the wet weight in a given articular tissue (Buckwalter *et al.*, 1990; Mow *et al.*, 1990) including the TMJ discs of the cow, mouse, rabbit and guinea pig (Nakano and Scott, 1989; Silbermann and von der Mark, 1990; Berkovitz *et al.*, 1992). In general, type I collagen predominates in the TMJ disc and the dense fibrous tissue or fibrocartilage on the articular surface of the mandibular condyle, whereas type II collagen is abundant in the condylar growth cartilage (Hirschmann and Shuttleworth, 1976).

A type I collagen molecule, consisting of two α1(I) chains and one α2(I) chain, is a product of two genes located on human chromosomes 17 and 7 (Olsen and Ninomiya, 1993). Many type I collagen molecules together form thick, interwoven fibrils and act as the mechanical frame of fibrous connective tissue including the TMJ articular components (Hirschmann and Shuttleworth, 1976; Milam *et al.*, 1991). By so doing, type I collagen fibrils are able to resist tensile and shear forces such as those

imposed on muscle tendon and knee meniscus (Grodzinsky, 1983; Koob and Vogel, 1987; Evanko and Vogel, 1990). In different articulations (Berthet *et al.*, 1978) or different regions of the TMJ disc (Berkovitz and Robertshaw, 1993; Kuc and Scott, 1994), the diameter of type I collagen fibrils varies, suggestive of differential responses to forces. Small collagen fibrils seen in the surface fibrous layer of the rat mandibular condyle before weaning are replaced by large, compact bundles of collagen fibrils after tooth eruption and occlusal function (Copray and Liem, 1989). The diameter of collagen fibrils in an area subjected to tensile forces is greater than that in an area subjected to compressive forces (Evanko and Vogel, 1990; Robbins and Vogel, 1994).

Type II collagens, each consisting of three α1(II) chains, form fibrils of small diameter (Mayne and Irwin, 1986; Bruckner and van der Rest, 1994) and account for about 90% of all collagens found in the matrix of hyaline cartilage (Nimni and Deshmukh, 1973; Mizoguchi *et al.*, 1990; Srinivas *et al.*, 1994). In the TMJ, type II collagen has been identified in the condylar growth cartilage in the rat and rabbit (Mizoguchi *et al.*, 1990; Salo and Kantomaa, 1993; Ali and Sharawy, 1995) and in chondroid bone on the articular eminence of the monkey (Milam *et al.*, 1991). Type II collagen is usually found in regions subjected to compressive forces (Benjamin and Evans, 1990; Robbins and Vogel, 1994). For example, it is abundant in the inner portion but absent from the periphery of the knee meniscus of the rabbit (Benjamin and Evans, 1990). Messenger RNA for type II collagen is abundant in cells from the compressed region of the adult bovine deep flexor tendon (Robbins and Vogel, 1994). In addition to the predominant type II collagen, hyaline cartilage also contains small

quantities of type III collagen (Gage *et al.*, 1990; Silbermann and von der Mark, 1990; Carvalho *et al.*, 1993; Wotton and Duance, 1994), VI collagen (Yasue *et al.*, 1994), IX and XI collagens (Bruckner and van der Rest, 1994).

1.2 Proteoglycans.

In contrast to collagen, glycosaminoglycans comprise only about 5% of the dry weight of the bovine TMJ disc (Nakano and Scott, 1989), but their presence is critical to the function of articular tissues (Maroudas, 1976). All glycosaminoglycans except hyaluronic acid may be covalently bound to a protein core in molecules called proteoglycans. Proteoglycan monomers may be attached noncovalently to hyaluronic acid in a process known as aggregation (Maroudas, 1976; Oegema, 1980). These large, aggregating proteoglycans are very hydrophilic with an ability to retain 50 times their weight of water (Mow *et al.*, 1990). In addition, proteoglycans in articular tissues are compressed to about 20% of their natural volume and exist in an environment where numerous negatively charged molecules produce electrostatic repulsive forces. These characteristics of proteoglycans enable articular tissues, especially the cartilage, to withstand large compressive forces (Mow *et al.*, 1990). The distribution of a given type of glycosaminoglycan in articular tissue is not uniform in terms of depth or topography (Bayliss *et al.*, 1983; Nakano and Scott, 1989; Mow *et al.*, 1990). For example, in the bovine TMJ disc, chondroitin sulfate is thoroughly distributed, whereas dermatan sulfate is found largely in the periphery (Nakano and Scott, 1989). Keratan sulfate associated with large chondroitin sulfate proteoglycans is concentrated inside and away from the

periphery but close to the inferior and superior surfaces of the bovine TMJ disc (Nakano *et al.*, 1993). Dermatan sulfate chains from the inner region of the disc are longer than those from the outer region (Scott *et al.*, 1995). These differences in the distribution and structure of proteoglycans are thought to reflect differential loading patterns in various regions of the bovine TMJ disc.

Versican was originally named in 1989 as one of the aggregating proteoglycans expressed by human fibroblasts (Zimmermann and Ruoslahti, 1989) and its gene sequence has been characterized (Naso *et al.*, 1994). A versican monomer consists of about a dozen chondroitin sulfate side chains attached to the middle of its protein core and thus is a member of the chondroitin sulfate proteoglycan family. Versican monomers, each with a molecular mass of over 1000 kDa, aggregate through the N-terminal portions of their protein core onto a hyaluronic acid backbone (LeBaron *et al.*, 1992) and thus form gigantic polymers. Its carboxyl-terminal portion includes two epidermal growth factor (EGF)-like repeats, a lectin-like sequence and a complement regulatory protein-like domain (Grover and Roughley, 1993). Despite the close resemblance between versican and aggrecan (see below) in both N- and C-terminal portions, the two are different in the central portions of their core proteins and the number of glycosaminoglycan side chains (Zimmermann, 1993). The spatial distribution of versican has been documented in a variety of tissues including two reports in the temporomandibular joints of the rat (Mao *et al.*, 1995) and juvenile pig (Roth *et al.*, 1995).

Aggrecan, a product of a single gene on human chromosome 15 (Korenberg *et al.*, 1993), is the most abundant and largest proteoglycan in hyaline cartilage with a molecular mass of about 2500 kDa (Doege, 1993). Each aggrecan molecule carries about 100-150 chondroitin sulfate side chains and keratan sulfate side chains, both of which are attached to the middle two-thirds of its protein core (Roughley and Lee, 1994). Aggrecan enables hyaline cartilage to withstand large compressive forces (Hascall, 1988; Roughley and Lee, 1994). In addition to hyaline cartilage, aggrecan is found in the compressed region of the bovine deep flexor tendon, more than in the tensile region (Vogel *et al.*, 1994). Aggrecan mRNA levels increase up to five folds in response to *in vitro* compressive loading applied to the bovine deep flexor tendon (Evanko and Vogel, 1993). In the condylar cartilage of the mandible, aggrecan-like proteoglycans have been identified by reaction of their glycosaminoglycan sides chains to safranin O (Glineburg *et al.*, 1982).

Decorin is a small proteoglycan with a single chondroitin sulfate or dermatan sulfate side chain attached to the fourth amino acid of its 38 kDa protein core (Chopra *et al.*, 1985). Dermatan sulfate side chains are frequently found in decorin molecules in articular cartilage and tendon (Fisher, 1993a). Its gene is located on human chromosome 12 (McBride *et al.*, 1990). Decorin is the only proteoglycan whose mRNA is detected in cells from the tensile region of the bovine deep flexor tendon, whereas mRNA for other types of proteoglycans, including that of decorin, is detected in the compressive region (Robbins and Vogel, 1994). The most documented function of decorin is its involvement in modulating collagen fibrillogenesis (Brown and Vogel,

1989; Scott *et al.*, 1989 and 1995; Karvonen *et al.*, 1992; Pogany *et al.*, 1994), possibly controlling the rate of collagen fibril formation. For this purpose, decorin interacts with both type II collagen fibrils of hyaline cartilage and type I collagen fibrils of other types of connective tissue (Scott and Haigh, 1985).

Biglycan is also a small proteoglycan but with two chondroitin sulfate or dermatan sulfate side chains attached to a 38 kDa core protein (Fisher, 1993b). It is of minor quantity in articular cartilage (Roughley *et al.*, 1993). Its function is unclear but it does not seem to interact with fibrillar collagens or influence collagen fibrillogenesis (Brown and Vogel, 1989). The only available antisera for biglycan are against synthesized biglycan peptides, such as LF-11 and LF-14 (Fisher, 1993b) and PS-318 (Scott *et al.*, 1995).

1.3 TMJ loading.

The human temporomandibular joint was once thought not to be loaded during function (Wilson, 1920; Robinson, 1946; Frankel and Burstein, 1970; Gingerich, 1971). This concept was echoed by misinterpretation of the histologic presence of fibrocartilage in the mandibular condyle instead of the commonly seen hyaline cartilage in most other load-bearing joints (Scott, 1955). In fact, the ability of fibrocartilage to resist compressive forces is essentially the same as that of hyaline cartilage (Benjamin and Evans, 1990): the ultimate compressive strength is 1.9 $\pm$ 0.06 kg/mm^2 for wet fibrocartilage and 2.1 $\pm$ 0.09 kg/mm^2 for wet hyaline cartilage in cattle (Yamada and Evans, 1970). The principal premise upon which the non-loading concept is based, as

pointed out by Hylander (1975), is that the resultant muscle force always passes through the bite point and is counteracted by a reaction force so that neither joint needs to be loaded. In a review article largely based on mechanical analysis in the sagittal plane, Hylander (1975) concluded that the articular surfaces of the temporomandibular joint must be loaded in function. This conclusion was further supported by *in vivo* bone-strain recordings in monkeys during both incisal and molar biting (Hylander and Bays, 1979). In humans, the condyle and disc are shown to be loaded by more recent three dimensional computer modelling studies (Osborn and Baragar, 1985; Koostra *et al.*, 1988) and jaw-muscle recruitment studies using three dimensional bite-force transducers (Van Eijden *et al.*, 1990; Osborn and Mao, 1993; Mao and Osborn, 1994).

The direction of TMJ reaction forces was ignored in the loading *vs* non-loading debate which focused entirely on the magnitude of the force (the load). With computer modelling, it is shown that different regions of the mandibular condyle are subjected to forces of different directions (Osborn and Baragar, 1992). The condyle will slide unless the direction of TMJ reaction forces is perpendicular to the articular surface.

Loading has limited analytic value when used to describe the mechanical stress in the temporomandibular joint. Instead, forces should be analyzed. Load is a scalar that has only a magnitude but not direction. Force is a vector that must be defined by a magnitude and direction. When the response of tissues to a force is measured, its point of application, frequency and duration should also be described. There are four types of force: tensile, compressive, shear and torsional. In a complex mechanical system such as the temporomandibular joint, a resultant force can be resolved into two or more

components. The force on the mandibular condyle, in reaction to a bite force, contains a shear component at all times when its direction is not perpendicular to the articular surface (Osborn and Baragar, 1992). An example of combined compressive and shear forces can be readily illustrated by the observation that the mandibular condyle and the TMJ disc are anatomically equipped with the ability to be moved while they are heavily loaded (Osborn, 1985): load being the compressive force and movement causing shear and tensile forces. This unusual biomechanical feature of the TMJ's ability to withstand forces while being moved has been attributed to the presence of dense fibrous tissue that comprises the articular surfaces of the mandibular condyle, the articular eminence of the temporal bone and the TMJ disc in humans (Osborn, 1985). Movement is enabled by the low friction of dense fibrous tissue while the joint is loaded (Mow *et al.*, 1990).

1.4 Occlusal splints.

Occlusal splints are prescribed for muscular and/or skeletal pathology associated with temporomandibular disorders (TMD) (Clark, 1984). Muscle pathology commonly manifests as pain and tenderness to palpation, both of which are subjective. Skeletal pathologies, which may be detected by radiographs or magnetic resonance imaging, include aberrant relationship of joint components such as displacement of the disc and breakdown of structural integrity such as disc perforation and osteoarthritis. Occlusal splints provide pain relief in about 70% of all TMD patients (Clark, 1984).

The design of occlusal splints has been limited only by the imagination of dental practitioners. To date, many types of splints have been used, such as the full-coverage,

flat-plane occlusal splint, the mandibular repositioning splint (to direct the mandibular condyle into a different position), anterior bite plate, posterior bite plate, pivot splint (with contact of a single molar on each side), *etc* (Boero, 1989). Some consider that the (soft) mouth guards worn by both professional and amateur athletes are also occlusal splints. Among TMD patients, the most commonly used is a full-coverage, flat-plane occlusal splint providing even occlusal contact as advocated by Ramfjord and Ash (1971).

Like many other clinical procedures, occlusal splints have been conventionally used before their range of effect and mechanisms of action are fully understood (Boero, 1989; Messing, 1991). Theories have been proposed to explain how occlusal splints achieve therapeutic effects. These are occlusal replacement (Ramfjord and Ash, 1971), mandibular repositioning (Farrar, 1972; Gausch and Kilner, 1977; Weinberg, 1979; Zamburlini and Austin, 1991), muscle relaxation (reviewed by Dahlström, 1989), psychological placebo (Greene and Laskin, 1972; Rugh and Robbins, 1981; Dao *et al.*, 1994), altered vertical dimension (Block, 1947; Ramfjord and Blankenship, 1981) and reduced joint loading (Dos Santos *et al.*, 1988; Pertes and Attanasio, 1991). The theory of interest in the present study is that a full-arch, stabilization occlusal splint decreases the load on the temporomandibular joint, as largely based on the conclusion from the mathematical analysis of Dos Santos *et al.* (1988). The two dimensional trigonometric model of Dos Santos *et al.* (1985) manipulates two forces each acting on its own inclined plane, one at the TMJ (N_1) and the other on an incisor tooth (N_2), in static equilibrium with a vertical muscle resultant force (F), which is not physiological. The point of application of F was allowed to vary in the sagittal plane. Three equations were used

for different angles of the inclined planes and values of F. None of the calculations were shown and it is difficult to understand how the results were derived because the three forces do not intersect (see N_1, N_2 and F in their Figs. 1 to 3), a condition that must exist if the system is to be in equilibrium. Anecdotal propositions of the "unloading" theory include that an occlusal splint, especially a splint with only posterior tooth contact, distracts the condyle and the disc away from the articular eminence and therefore relieves the load on these structures (Pertes and Attanasio, 1991). It has also been proposed that occlusal splints "unload" the joint so that pain is relieved and TMJ articular tissues may have a chance to recover from pre-existing damage (Dos Santos *et al.*, 1988).

Animal experiments have revealed that occlusal splints induce cellular changes in the mandibular condyle and the TMJ disc. In rats and rabbits wearing unilateral bite-raising appliances, three studies have identified proliferation of fibroblast-like and chondroblast-like cells in the TMJ disc and the mandibular condyle on the treated side (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). The longer the appliance has been worn (up to a maximum of four weeks), the more marked the cell proliferation. Incremental opening of the bite by means of full-arch occlusal splints with different thicknesses led to an increase in the volume of the mandibular condyle in monkeys (McNamara, 1977). The volume of the condylar cartilage was also found to increase after bilateral occlusal splints were used in rabbits (Rashed and Sharawy, 1993). Cellular changes have also been found in *in vivo* studies under comparable conditions. Continuous protrusion of the mandible by means of a fixed appliance induced an increasing amount of condylar growth in *Macaca mulatta* (McNamara and Bryan, 1987).

Increases in compressive forces applied to the disc and condyle after posterior relocation of the glenoid fossa induced proliferation of chondroblast-like cells in the condylar growth cartilage (Pirttiniemi *et al.*, 1993 and 1994; Kantomaa *et al.*, 1994a).

1.5 The hypothesis.

The present study was designed to test a hypothesis that a unilateral bite-raising appliance in the rat induces an increase in the magnitude of TMJ compressive forces as might be revealed by an increase in the expression of proteoglycans in TMJ articular tissues. This hypothesis was proposed on the basis of the following three lines of previous investigations.

First, the articular surfaces of the temporomandibular joint withstand forces in function (Barbenel, 1972; Hylander, 1975; Hylander and Bays, 1979). The mandibular condyle and the articular disc are moved downward and forward over the articular eminence during jaw opening (Rees, 1954), possibly including when an occlusal splint is inserted (Fig. 2B). As a result of jaw opening, different regions of the disc may be loaded (Fig. 2B'). An occlusal splint with only posterior tooth contact does not distract the disc and condyle from the articular eminence (Ito *et al.*, 1986). With an occlusal splint (Fig. 3A), the mandibular condyle may be loaded in a similar way to biting with the point of force application in front of the resultant muscle force (Fig. 3B). The condyle may only be distracted or unloaded when the point of application of bite force is posterior to the resultant muscle force (Fig. 3C), for example on M3 as in Hylander (1979) and constitutes a rare physiological situation in man (Dr. Jeffrey Osborn, personal

communication). These macroscopic studies indicate the likelihood that the disc and condyle may continue to be loaded when functioning with an occlusal splint *in situ*, just as they are without a splint.

Second, the increase in condylar volume found in two *in vivo* studies in response to bilateral occlusal splints (McNamara, 1977; Rashed and Sharawy, 1993) indicates that splints have induced condylar growth by proliferation of cells and by an increase in the volume of the intercellular matrix. Proliferation of fibroblasts and chondroblasts has been shown in three *in vivo* studies in rats and rabbits wearing unilateral bite-raising appliances (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). However, whether an increasing amount of proteoglycans is expressed in the intercellular matrix of TMJ articular tissues in response to bite-raising appliances has not been investigated.

Third, the following associations have been established between increasing compressive forces and increase in proteoglycan synthesis in both the temporomandibular joint and other connective tissues across different species. In the temporomandibular joint, increasing compressive forces have been shown to induce enhancement in glycosaminoglycan synthesis and/or proteoglycan expression. Glycosaminoglycan synthesis is found to increase in response to intermittent compressive forces applied to the rat condylar growth cartilage in organ culture (Copray *et al.*, 1985) and chondroblasts from the rabbit condylar growth cartilage in cell culture (Takano-Yamamoto *et al.*, 1991). After the TMJ disc and the mandibular condyle are subjected to larger compressive forces as a result of surgically posterior relocation of the glenoid fossa in the rabbit, there are both marked cell proliferation and an increase in the amount of

glycosaminoglycans (Kantomaa *et al.*, 1994b). Compressive forces of different duration applied to the rat TMJ disc and mandibular condyle in organ culture lead to significantly enhanced synthesis of chondroitin sulfate and dermatan sulfate (Carvalho *et al.*, 1995). Reverse situations of decreased TMJ loading have been shown to reduce proteoglycan expression and incorporation of radio-labelled sulfate into glycosaminoglycans as seen in immobilization of the temporomandibular joint in *Macaca cynomolgus* (Glineburg *et al.*, 1982) and a switch from hard to soft diet in the rat (Hinton, 1988 and 1993). In the result of Glineburg *et al.* (1982), the intensity of safranin O staining, which detects chondroitin sulfate and keratan sulfate, was lower in the condylar growth cartilage of the mandible after TMJ immobilization and rebounded two weeks after remobilization.

In connectives tissues in other regions of the body, many studies have shown that proteoglycan synthesis is enhanced as a result of applying compressive forces. In the sheep ankle articular cartilage, increased compressive loading is accompanied by an increase in uronic acid content and incorporation of labelled acetate into chondroitin sulfate fractions (Caterson and Lowther, 1978). Bovine tendon fibrocartilage subjected to compressive forces synthesizes an increasing amount of large proteoglycans, whereas that without loading synthesizes only small proteoglycans (Koob *et al.*, 1992). By dry weight of the flexor digitorum profundus tendon, regions subjected to tensile forces have a glycosaminoglycan content of 0.2% but the small sesamoid region subjected to compression has a glycosaminoglycan content of 4% (Gillard *et al.*, 1979). When the sesamoid region is relieved from compression, its glycosaminoglycan content decreases by 60% (Gillard *et al.*, 1979).

From both lines of studies in the TMJ and connective tissues in other regions of the body, it is clear that regardless of the type of tissue (cartilage or dense fibrous tissue) or the type of animal models, increased compressive forces are associated with increased proteoglycan expression and/or glycosaminoglycan synthesis (review: Mow *et al.*, 1990). Although the direction of compressive force may be different in different biological systems (the TMJ vs. the knee joint or the TMJ in the monkey vs. the TMJ in the rat), the magnitude of a force is likely to induce similar responses in cells and molecules of different types of connective tissue. If an occlusal splint in man induces an increase in the load on the TMJ, then a bite-raising appliance in a rat, for example, should have a similar effect. The increased load, in common with other loaded connective tissues, would result in an increase in the expression of proteoglycans.

CHAPTER 2

Proteoglycan expression in the rat temporomandibular joint in response to a unilateral bite-raising appliance.

Introduction

The articular surfaces of the human temporomandibular joint (TMJ) consist of dense fibrous tissue or fibrocartilage (Provenza, 1986). Besides water and sparse cells, TMJ articular tissues are largely composed of collagens (Hirschmann and Shuttleworth, 1976; Berkovitz *et al.*, 1992; Kuc and Scott, 1994) and proteoglycans (Mills *et al.*, 1988; Nakano and Scott, 1989). These intercellular matrix molecules and their parent cells respond to mechanical stimuli (Copray *et al.*, 1985; Koob and Vogel, 1987; Kim *et al.*, 1994; Carvalho *et al.*, 1995). In articular tissues, collagen resists tensile and shear forces and proteoglycans resist compressive forces (Maroudas, 1976; Koob and Vogel, 1987; Mow *et al.*, 1990; Hukins, 1992; Bruckner and van der Rest, 1994).

Glycosaminoglycans comprise about 5% of the dry weight of the bovine TMJ disc (Nakano and Scott, 1989), but their presence is essential for articular tissues to withstand compressive forces (Maroudas, 1976). The distribution of a given glycosaminoglycan type in articular tissues is not uniform (Bayliss *et al.*, 1983; Nakano and Scott, 1989; Mow *et al.*, 1990). For example, in the bovine TMJ disc, chondroitin sulfate is thoroughly distributed, whereas dermatan sulfate is largely found in the periphery (Nakano and Scott, 1989). Keratan sulfate associated with large chondroitin sulfate proteoglycans is concentrated in the middle and close to the inferior and superior surfaces (Nakano *et al.*, 1993). Dermatan sulfate chains from the inner portion of the bovine TMJ disc are longer than those from the outer portion (Scott *et al.*, 1995). These differences in the distribution and structure of proteoglycans are thought to reflect differential loading patterns in various regions of the TMJ disc.

Occlusal splints are prescribed for muscular and/or skeletal pathology associated with temporomandibular disorders (TMD). Despite their clinical effectiveness (Clark, 1984), the mechanisms of action of occlusal splints are unclear and theories have been proposed to explain how splints achieve therapeutic effects (Boero, 1989). The theory of interest here is that a full-arch, stabilization occlusal splint decreases TMJ loading (Dos Santos *et al.*, 1988; Pertes and Attanasio, 1991). This "unloading" theory states that splints decrease joint load so that joint tissues may have a chance to recover from pre-existing damage (Dos Santos *et al.*, 1988). Anecdotal propositions also include that pain relief is achieved as a result of decreased joint loading.

A wide range of effects of occlusal splints has been illustrated in animal models. Condylar volume increases after bilateral, full-coverage occlusal splints have been used in monkeys and rabbits (McNamara, 1977; Rashed and Sharawy, 1993). Proliferation of fibroblast-like and chondroblast-like cells has been found, after unilateral bite-raising appliances have been used in rats and rabbits, in the TMJ disc and the mandibular condyle on the treated side (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). Increasing compressive forces *in vitro* and *in vivo* have been shown to enhance glycosaminoglycan synthesis in TMJ articular tissues (Copray *et al.*, 1985; Takano-Yamamoto *et al.*, 1991; Kantomaa *et al.*, 1994b; Carvalho *et al.*, 1995) and other types of connective tissue (Caterson and Lowther, 1978; Gillard *et al.*, 1979; Koob and Vogel, 1987; Koob *et al.*, 1992). The present study was designed to test whether a unilateral bite-raising appliance in the rat induces increasing expression of proteoglycans in TMJ articular tissues. If so, the elevated proteoglycan expression may be interpreted as

indirect evidence to suggest that a unilateral bite-raising appliance induces an increase in the magnitude of TMJ compressive forces in the rat.

Materials and Methods

Fifty Sprague-Dawley rats were divided into the following groups (Appendix 1). The first group of five six-week-old rats wore the unilateral bite-raising appliances (Fig. 4 and described below) for four weeks. Three age-matched rats served as sham-operated controls. The second group of 14 nine-week-old rats wore the unilateral bite-raising appliances for four weeks. Ten age-matched rats served as sham-operated controls. The third group of eight nine-week-old rats wore the unilateral bite-raising appliances for four weeks and were then housed for an additional four weeks after appliance removal. Ten age-matched rats served as sham-operated controls. Rats were assigned to different groups without strict randomization. All the rats were conventionally housed and fed with regular hard pellet diet. The project was approved by a local Animal Welfare Committee.

Unilateral bite-raising appliances were placed in rats sedated under general anesthesia with intraperitoneal injection of 45 mg/kg sodium pentobarbital along with 0.1 mg/kg atropine sulfate to reduce oral and tracheal secretions. The maxillary molars on the right side were dried with compressed air, etched with a liquid containing 40% phosphoric acid (SCI Pharmaceuticals, Duarte, CA) for about 15 seconds and then washed with water while suction was applied to prevent water inhalation. After air-drying, light-cure resin (Dentsply Lab Products, York, PA) was placed on all the three

maxillary molars on the right side (Fig. 4). The mandible was manually closed onto the maxilla with light force to obtain an even occlusal surface on the resin. The resin was cured with a halogen light source (Demetron, Danbury, CT). After these treatments, the resin formed a unilateral bite-raising appliance with a approximate thickness of 1 mm between the distal molars (M3) as estimated from measuring the separation between the upper and lower incisors by taking into consideration the difference between interincisal and intermolar clearance.

Inspection of each rat was conducted on the following three consecutive days and then once every three to four days for two purposes: first, to observe feeding behavior and measure weight gain and second, to ensure retention of the appliance. The rats wearing the unilateral bite-raising appliances lost weight after appliance insertion (Appendix 2). After this initial weight loss, the treated rats regained weight and by the time of termination, there were no statistically significant differences (see p values of the Student's *t*-test in the caption for Appendix 2) in body weights between the treated rats and their corresponding age-matched, sham-operated control rats: 362.6±14.2 g (mean±S.D.) and 379.7±15.0 g for the six-week-old treated and control rats respectively, 437.6±29.1 g and 452.8±24.2 g for the nine-week-old treated and control rats respectively, and 488.3±22.5 g and 497.4±17.9 g for the nine-week-old appliance-removal and control rats respectively. During each inspection, rats were also checked under brief halothane inhalation anesthesia (Halocarbon Lab, North Augusta, SC) to ensure retention of the unilateral bite-raising appliances. The maximum time period during which an appliance exfoliated from a rat was two or three days. A new appliance

was installed, following the above procedures, whenever a rat in a treated group was found without an appliance. Unilateral bite-raising appliances in the appliance-removal group were removed with a pair of crown and bridge scissors. All sham-operated control rats were subjected to all of the above procedures except for those related to insertion of the unilateral bite-raising appliances.

The disc and condyle of the temporomandibular joint were dissected as one piece and immediately placed in 3% paraformaldehyde after the rats were sacrificed by sodium pentobarbital overdose. Following demineralization with formic acid-sodium citrate, a midsagittal section split each specimen into two halves. It was not always possible to cut a perfect midsagittal section largely due to the small size of the specimen. In a few cases, specimens were ruined (Appendix 3). Sections, each 5 μm thick, were cut in the parasagittal plane using a microtome. Each section was stained with hematoxylin-eosin and additional sections were cut and sequentially utilized for the following stainings.

Safranin O (National Auline Div., New York, NY) and fast-green (SO-FG) staining was performed after sections were deparaffinized. Safranin O, a cationic dye, binds to polyanions such as chondroitin sulfate and keratan sulfate (Rosenberg, 1971; Kiviranta *et al.*, 1985; Lammi and Tammi, 1988). All specimens (from both six- and nine-week-old rats) were stained with safranin O and fast green on the same day under the same conditions with frequent changes of the staining solutions prepared in the same jars. Sections were stained in 0.002% fast green for four minutes. After being quickly rinsed once in 1% glacial acetic acid, sections were stained in 0.1% safranin O for five minutes. Detailed SO-FG staining protocol is included in Appendix 4.

Immunohistochemical staining was carried out with the following antibodies. 1) An anti-versican monoclonal antibody (mAb), 5D5, was a generous gift from Dr. Firoz Rahemtullah, Department of Oral Biology, University of Alabama. It was raised in mice against versican from bovine sclera. Cross-reaction with human versican and lack of cross-reaction with aggrecan have been documented (Larjava *et al.*, 1992). 2) Anti-decorin mAbs (3B3) and (6D6), developed in the Department of Oral Biology, University of Alberta (Pringle *et al.*, 1985; Scott *et al.*, 1993), were both raised in mice against different segments of bovine skin decorin. 3) A polyclonal antibody against biglycan (PS-318), also developed in the Department of Oral Biology, University of Alberta (Scott *et al.*, 1995), was raised in rabbits against a synthetic core peptide of human biglycan.

Protocols for immunohistochemical staining followed essentially as described in Sternberger (1986). Deparaffinized sections were treated with 2% (v/v) H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase. After washing with TBS (0.05 M Tris-HCl/0.15 M NaCl, pH 7.6), sections were treated with testicular hyaluronidase (1 mg/ml in TBS) at 37°C for 30 min to digest glycosaminoglycans that may otherwise mask protein epitopes. After washing in TBS, sections were soaked in normal goat serum (for 5D5, 3B3 and 6D6) or normal rabbit serum (for PS-318), both diluted 1 in 20 in TBS, for 60 min. Primary antibodies against versican, decorin and biglycan were added and incubated at 4°C overnight. The following antibody dilutions in TBS were used: 1:500 for 5D5, 1:10 for 6D6 and 3B3, and 1:30 for PS-318. Positive control sections included bovine articular cartilage, hypertrophic scar and fetal rat skin, all of which were treated identically to the above sections. Control sections (without primary

antibodies) were incubated with cell culture supernatant for 5D5, 3B3 and 6D6 or normal rabbit serum for PS-318 under the same conditions. The next day, antibody and control sections were washed separately in TBS and incubated for 45 min with goat anti-mouse IgG(Fab)$_2$ (Organon Teknika, Durham, NC) diluted 1:50 for 5D5, 3B3 and 6D6 and goat anti-rabbit IgG,A,M diluted 1:50 for PS-318. After washing, sections were incubated with mouse peroxidase-antiperoxidase complex diluted 1:300 for 30 min. Sections were washed again and incubated for 4 min in 0.05% diaminobenzidine tetrahydrochloride/0.01%/H_2O_2 to develop the color. Detailed immunohistochemical staining protocol is included in Appendix 5. The protocol (*e.g.* time, antibody dilution) for the same antibody staining on different occasions was consistent for comparison of staining intensity. All stained sections were mounted with Permount after dehydration with graded concentrations of ethanol and treatment with xylene.

Quantification of the intensities of both SO and anti-versican monoclonal antibody stainings was performed by means of a computer-assisted image analysis system. The number of tissue sections for either SO staining or anti-versican antibody (5D5) staining was as follows (Appendix 3): 1) ten for the nine-week-old, treated specimens, five for their contralateral controls and five for their age-matched, sham-operated controls; 2) six for the nine-week-old, appliance-removal specimens, four for their contralateral controls and four for their age-matched, sham-operated controls; 3) four for the six-week-old, treated specimens, four for their contralateral controls and three for their age-matched, sham-operated controls. All specimens (N=45 for either SO or 5D5 staining in Appendix 3) were "blinded" from the operator for quantification of staining intensities.

Each tissue slide was placed under the microscope under low power. The light intensity of the microscope was kept constant throughout. For SO staining, the middle one-third of the condylar growth cartilage of the mandible was selected. For 5D5 staining, the middle one-third of the TMJ disc and the surface articular fibrous layer of the condyle was selected. The computer program (Northern Exposure, Scarborough, Ont.) assigned the staining intensity on a scale from 0 to 255 in a reversed pattern: 0 being the darkest staining and 255 being complete lack of staining. In the following data presentation in Fig. 5, staining intensity was converted, for the ease of comprehension, from the above reversed pattern: 0 being complete lack of staining and 255 being the darkest staining. The Kruskal-Willis test was used to compare whether there were differences in the average staining intensities within each group, but not among different groups, between the treated or appliance-removal specimens, their contralateral control specimens and the corresponding age-matched, sham-operated control specimens. A p value less than 0.05 was considered to be statistically significant.

Results

Slides chosen for photomicrographic presentation are typical, representing 1) rats (both the six- and nine-week-old) wearing unilateral bite-raising appliances for four weeks (treated), 2) a nine-week-old rat wearing the unilateral bite-raising appliance for four weeks followed by four weeks without the appliance (appliance-removal) and 3) a nine-week-old rat that served as the sham-operated control. Fig. 5 contains four photomicrographs with hematoxylin-eosin staining of the mandibular condyles and TMJ

discs of the treated (both the six- and nine-week-old), appliance-removal and control rats. There was marked proliferation of chondroblast-like cells in the condylar growth cartilage of the six-week-old specimen (Fig. 5A), consistent with previously reported hyperplasia in rats and rabbits wearing unilateral bite-raising appliances (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). Hyperplasia was also marked in the nine-week-old treated rat (Fig. 5B), less marked in the appliance-removal rat (Fig. 5C) and the control rat (Fig. 5D).

Aggrecan-like proteoglycans were abundant in the growth cartilage portion of the mandibular condyle as detected by safranin O staining (Fig. 6). The fibrous articular components of the TMJ, namely the disc and the surface fibrous layer of the mandibular condyle, were scarcely stained with SO in any case. Differences in safranin O staining intensities were found in the condylar cartilage between the treated (both the six- and nine-week-old), appliance-removal and control specimens (Fig. 6). The intense staining in the pericellular matrix of the condylar cartilage in both the six- and nine-week-old treated specimens (Fig. 6A and 6B) indicated the presence of abundant chondroitin sulfate and/or keratan sulfate (Rosenberg, 1971). In both sections (Fig. 6A,B), the regions of strong safranin O staining corresponded with the regions of hyperplasia of chondroblast-like cells shown in H-E stained sections in Fig. 5A,B. In contrast to the strong SO staining in the treated specimens (Fig. 6A,B), weaker staining intensities were found in the appliance-removal specimen (Fig. 6C) and the sham-operated control specimen (Fig. 6D). The overall staining intensities for safranin O as measured by the computer image analysis system are illustrated in Fig. 7. The average intensity of the nine-week-old,

treated specimens was significantly greater than the average intensities of their age-matched, sham-operated controls ($p < 0.01$) and their corresponding contralateral control ($p = 0.037$). The average staining intensity of the contralateral control specimens was not significantly different from that of the age-matched, sham-operated specimens. For the six-week-old rats, the average SO staining intensity of the treated specimens was greater ($p < 0.05$) than both the contralateral control and the sham-operated control; no statistically significant difference was found between these two types of controls. No statistically significant difference was found among the three types of specimens of the appliance-removal group.

Versican was identified in the fibrous articular components, namely the TMJ disc and the surface fibrous layer of the mandibular condyle but not in the cartilaginous portion of the condyle (Fig. 8). Different staining intensities for versican were identified between the treated (both the six- and nine-week-old) specimens, the appliance-removal specimen and the sham-operated control specimen (Fig. 8). The fibrous layer of the condyle of the nine-week-old treated specimen (Fig. 8B) reacted strongly to the anti-versican mAb. Moderate staining intensity was found in the entire disc of the six-week-old treated specimen (Fig. 8A) and the anterior and posterior portions of the disc of the nine-week-old treated specimen (Fig. 8B). In contrast, the posterior portion of the TMJ disc of the appliance-removal specimen was only weakly stained (Fig. 8C). Weak staining with the anti-versican antibody was found in the sham-operated control specimen (Fig. 8D). Computer image analysis revealed that the average staining intensities for the anti-versican antibody (5D5) (Fig. 9) were of somewhat similar patterns to those for the

above-described SO staining (Fig. 7). The average intensity of the nine-week-old treated specimens was significantly greater than that of the corresponding sham-operated controls ($p < 0.01$) but not significantly different from that of the contralateral control specimens. For the six-week-old rats, the average anti-versican mAb staining intensity of the treated specimens was greater ($p < 0.05$) than the sham-operated control but not different from the contralateral control; no statistically significant difference was detected between the two types of controls. Also, no statistically significant difference was detected among the three types of specimens of the appliance-removal group.

Decorin was detected by monoclonal antibodies against different segments of its protein core (3B3 and 6D6) in some, but not all, treated and appliance-removal sections (Fig. 10). In any case, the reaction was not nearly as strong as in the fetal rat skin used as the positive control (not shown). For example, moderate staining was identified in the posterior portion of the disc of the nine-week-old treated specimen (Fig. 10A). There was also faint staining in the entire disc and condylar fibrous layer in both the treated (Fig. 10A) and appliance-removal specimen (Fig. 10B). No staining was observed in the control section (Fig. 10C).

Biglycan could not be identified with the polyclonal antibody (PS-318) used in the present study (Fig. 11). This staining was repeated many times and the absence of staining was confirmed by the very positive reaction to the same antibody in the fetal rat skin used as the positive control (not shown).

Discussion

Strong safranin O staining in the condylar cartilage of the treated rats (Figs. 6A and 6B, 7) indicates the presence of a proteoglycan or proteoglycans that carry abundant chondroitin sulfate and/or keratan sulfate side chains (Rosenberg, 1971; Kiviranta *et al.*, 1985; Lammi and Tammi, 1988). Judged from their location in the condylar cartilage, these strongly stained glycosaminoglycans are very likely side chains of aggrecan that is the most abundant proteoglycan in hyaline cartilage and is known to carry up to 150 chondroitin sulfate and keratan sulfate side chains per molecule (Roughley and Lee, 1994). One molecule of safranin O, a cationic dye, binds stoichiometrically to a negatively charged group of chondroitin sulfate or keratan sulfate *in vitro* (Rosenberg, 1971).

An increase in the expression of versican and aggrecan was accompanied by hyperplasia of chondroblast-like cells (Fig. 5A and 5B), consistent with proliferation of these cells reported before in response to unilateral bite-raising appliances (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). Cell proliferation starts as soon as 12 h after insertion of a unilateral bite-raising appliance in the rat and reaches its peak in about two weeks (Lindsay, 1977). It is conceivable that expression of aggrecan (Figs. 6 and 7) and versican (Figs. 8 and 9) identified four weeks after appliance insertion in the present study was enhanced after the two-week cell division period reported in Lindsay (1977). Marked cell proliferation has been shown to accompany increasing mechanical stimuli by other means such as protrusion of the mandible (McNamara *et al.*, 1982; McNamara and Carlson, 1979; McNamara and Bryan, 1987) and posterior

relocation of the glenoid fossa (Kantomaa *et al.*, 1994a; Pirttiniemi *et al.*, 1993 and 1994). A logical outcome of cell proliferation in TMJ articular tissues appears to be an increase in proteoglycan expression, shown as enhanced expression of aggrecan and versican (Figs. 6-9).

The present findings are consistent with the identification of different types of glycosaminoglycans in TMJ articular tissues by previous biochemical and immunohistochemical analyses. For example, the chondroitin sulfate detected by an antibody in the rabbit TMJ disc by Mills *et al.* (1988) seems to be equivalent to the same glycosaminoglycan that is likely carried as side chains in versican molecules in the rat TMJ fibrous tissue in the present study. Distribution of chondroitin sulfate throughout the bovine TMJ disc found by Nakano and Scott (1989) matches the distribution of versican, which carries chondroitin sulfate side chains, in the fibrous layer of the condyle and the TMJ disc in the present study. Somewhat surprisingly, decorin was not consistently detected in the present study. Dermatan sulfate, accounting for 14% of all the glycosaminoglycans in the bovine TMJ disc, is mostly located in the periphery (Nakano and Scott, 1989; Nakano *et al.*, 1993). Some or all of this dermatan sulfate is expected to be side chains of decorin and biglycan. Nevertheless, the location of decorin identified mostly in the periphery of the disc (Fig. 10A and 10B) is consistent with the location of decorin in the bovine TMJ disc (Nakano and Scott, 1989). Variation in the consistency of decorin staining may be due to positioning of the mandible in the presence of the unilateral bite-raising appliance. Biglycan, which is a minor proteoglycan in the

bovine TMJ disc (Nakano *et al.*, 1993; Scott *et al.*, 1995), was not identified with the polyclonal antibody (PS318) used in the present study.

The increase in the expression of versican observed in dense fibrous tissue (Figs. 8A and 8B, 9) and aggrecan in the condylar growth cartilage (Figs. 6A and 6B, 7) was the responses of these proteoglycans' parent cells, fibroblasts and chondroblasts respectively, to a unilateral bite-raising appliance which, in this case, induced mechanical stimuli. Reversal of the enhanced expression of versican and aggrecan four weeks after appliance removal (Figs. 6C, 7, 8C and 9) confirms their elevated expression with the appliance *in situ*. The present study, however, does not provide direct evidence whether the rat temporomandibular joint is more or less loaded in response to a unilateral bite-raising appliance. Nevertheless, it is interesting to speculate whether TMJ reaction forces are increased or decreased. Findings from previous studies all show that proteoglycan expression and/or glycosaminoglycan synthesis are increased in response to increasing compressive forces applied to TMJ articular tissues (Copray *et al.*, 1985; Takano-Yamamoto *et al.*, 1991; Kantomaa *et al.*, 1994b; Carvalho *et al.*, 1995) and decreased as a result of a reduction in TMJ reaction forces (Glineburg *et al.*, 1982; Hinton, 1988 and 1993). Specifically, the increase in chondroitin sulfate found by Carvalho *et al.* (1995) in the rat TMJ disc and in [^{35}S]-sulfate incorporation found in Copray *et al.* (1985) in the rat condylar growth cartilage when these structures are subjected to increasing compressive forces in organ culture provides complementary evidence in support for the enhanced expression of versican and aggrecan observed in the present study. Thus, the enhanced expression of aggrecan and versican observed in the

present study suggests an increase in the magnitude of compressive forces projected onto the mandibular condyle in response to a unilateral bite-raising appliance in the rat. It also appears from the above studies that the present results of increasing expression of versican and aggrecan are likely a universal effect of an increase in compressive forces rather than the isolated effect of a unilateral bite-raising appliance *per se*.

The inference that a unilateral bite-raising appliance in the rat induces an increase in TMJ loading is at variance with a mathematical prediction of a decrease in TMJ loading in response to full-arch occlusal splints in humans (Dos Santos *et al.*, 1988) and anecdotal propositions of TMJ unloading with occlusal splints (Pertes and Attanasio, 1991). Extreme caution, however, must be taken before any clinical significance can be inferred from the present results. First, both the direction and point of application of TMJ compressive forces are probably very different between rats and humans due to numerous anatomical differences. Second, the duration of TMJ compressive forces is very likely longer in rats wearing unilateral bite-raising appliances than in humans because rats spend much time grinding their teeth. Third, the treated rats were observed to feed usually on the side of the appliance, similar to the observation in rabbits and pigs wearing unilateral bite-raising appliances (Shaw and Molyneux, 1993; Zhang *et al.*, 1995). Unilateral biting may induce an increase in the magnitude of compressive forces on the treated side, a situation that is different from the most commonly used (full-arch) occlusal splints in humans. Fourth, the thickness of the unilateral bite-raising appliance in the rat (1 mm over 10 mm jaw separation) is proportionally greater than the thickness of a commonly used full-arch occlusal splint in humans (1 or 2 mm over an average of

40 mm jaw separation). The response of TMJ articular tissues to the unilateral bite raising appliance in the present study of the rat is expected to be much stronger than equivalent tissues to full-arch occlusal splints in humans. Nevertheless, the present inference of increasing TMJ loading in response to a unilateral bite-raising appliance in the rat provides a hypothesis, alternative to decreasing TMJ loading, that can be further studied using computer models or experimental approaches in animal models. The ultimate proof of whether occlusal splints increase or decrease TMJ loading may require *in vivo* bone-strain recordings.

CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1 Clinical significance.

The vertical facial dimension is frequently altered in clinical practices such as orthodontic intrusion/extrusion of molars, prosthodontic restoration with crowns and full dentures, surgical increase or reduction of maxillary height and the use of occlusal splints. Despite the widespread practice of altering vertical facial dimensions, including that by means of occlusal splints, adaptation of TMJ articular tissues under various conditions is yet to be fully understood. The present study may be considered as the first *in vivo* attempt to investigate the expression of proteoglycans in TMJ articular tissues in response to a unilateral bite-raising appliance. It complements the macroscopic and cellular studies that have explored the effects of bite-raising appliances in various animal models (McNamara, 1974; Lindsay, 1977; Ehrlich *et al.*, 1980; Rashed and Sharawy, 1993; Shaw and Molyneux, 1993).

Lack of animal models has impeded experimental research in TMD and related areas. Recent efforts have been made to overcome this shortcoming. For example, the following conditions have been created in the following *in vivo* studies: anterior disc displacement by surgically severing the posterior attachment of the disc and suturing it to the zygomatic arch in the rabbit (Ali and Sharawy, 1995), posterior relocation of the glenoid fossa in the rabbit (Pirttiniemi *et al.*, 1993 and 1994; Kantomaa *et al.*, 1994a,b), prolonged protrusion of the mandible in monkeys (McNamara and Carlson 1979, McNamara *et al.*, 1982) and both unilateral and bilateral bite-raising appliances in monkeys, rats and rabbits (McNamara, 1974; Lindsay, 1977; Ehrlich *et al.*, 1980; Rashed and Sharawy, 1993; Shaw and Molyneux, 1993; Mao *et al.*, 1995). A general

conclusion from these studies seems to be that manipulations aimed at increasing the magnitude of the compressive forces on TMJ articular tissues lead to enhanced expression of proteoglycans and/or proliferation of their parent cells, fibroblasts and chondroblasts. In comparison, procedures aimed at decreasing TMJ loading are associated with a reduction in proteoglycan expression or reduced incorporation of radio-labelled sulfate into glycosaminoglycans in TMJ articular tissues as seen in immobilization of the temporomandibular joint in *Macaca cynomolgus* (Glineburg *et al.*, 1982) and a switch from a hard to a soft diet in the rat (Hinton, 1988 and 1993). These *in vivo* studies, which inevitably suffer from a lack of precise knowledge of the magnitude and duration of the forces applied to TMJ articular tissues, are complemented by *in vitro* studies in which known magnitude and duration of compressive forces are applied to TMJ articular tissue components in cell or organ culture (Copray *et al.*, 1985; Takano-Yamamoto *et al.*, 1991; Carvalho *et al.*, 1995). Conclusions from both the above *in vivo* and *in vitro* studies suggest that cell proliferation and elevated proteoglycan synthesis are induced by increasing compressive forces in TMJ articular tissues.

The clinical significance of the present study by itself is limited for the following reasons. First, both the direction and point of application of TMJ compressive forces are probably very different between rats and humans due to numerous anatomical differences. Second, the duration of TMJ compressive forces is very likely longer in rats wearing unilateral bite-raising appliances than in humans because rats spend much time grinding their teeth. Third, the treated rats were observed to feed usually on the side of the appliance, similar to the observation in rabbits wearing unilateral bite-raising appliances

(Shaw and Molyneux, 1993; Zhang *et al.*, 1994). Unilateral biting may induce an increase in the magnitude of compressive force on the treated side, a situation that is different from the most commonly used (full-arch) occlusal splints in humans. Fourth, the thickness of the unilateral bite-raising appliance in the rat (1 mm over 10 mm jaw separation) is proportionally greater than the thickness of a commonly used full-arch occlusal splint in humans (1 or 2 mm over an average of 40 mm jaw separation). The response of TMJ articular tissues to the unilateral bite-raising appliance in the present study of the rat is expected to be much stronger than equivalent tissues to full-arch occlusal splints in humans. Nevertheless, the present inference of increasing TMJ loading in the presence of a unilateral bite-raising appliance in the rat provides a hypothesis, alternative to decreasing TMJ loading, that might be tested in future studies using computer models or experimental approaches using animal models. The ultimate proof of whether occlusal splints increase or decrease TMJ loading may require *in vivo* bone-strain recordings.

The present study was not designed to explain how symptoms, for example pain, are alleviated by appliance use. TMD pain results more frequently from craniofacial musculature than from the tissues in the temporomandibular joint in man (Clark, 1988). Even if the pain originates from the joint, both the disc and the articular surfaces of the mandibular condyle and articular eminence are devoid of nociceptors (Greenfield and Wyke, 1966; Klineberg, 1971). Therefore, "unloading" the joint, even if it is the case, may not provide pain relief from these structures.

3.2 Growth and ageing.

The present study was not designed to test the influence of a bite-raising appliance on condylar growth as many previous studies (*e.g.* McNamara, 1974; Carlson *et al.*, 1978; McNamara and Carlson 1979; McNamara *et al.*, 1982). However, growth and ageing should be considered in any study involving animals that age during the course of an experiment. Proliferation of chondroblast-like cells was found in both the six- and nine-week-old treated rats (Fig. 5A and 5B). At the sixth week, the rat temporomandibular joint is near its adult form (Furstman, 1966). The articular surface is covered by a thin layer of connective tissue but its thickness is greater than that in younger rats. The articular disc is increasingly fibrous. The growth cartilage in the condyle is much thinner than in younger rats. By the ninth week, the TMJ is largely, if not entirely, of its adult form (Furstman, 1966). The articular disc is composed of dense fibrous connective tissue and has assumed its characteristic shape. The condyle has flattened mediolaterally and has assumed its mature morphology. Articular surfaces are lined with dense fibrous connective tissue. The bone matrix of the mandibular condyle is well mineralized. In consideration of all the above, the choice of using the nine-week-old rats as the principal sample in the present study appears justified. Ageing should not have been a principal factor that might have otherwise contributed to the outcome of the present study because age-matched control rats were used for each treatment group.

Ageing has been shown to have effects on the distribution and structure of proteoglycans (Roughley and White, 1980). For example, ageing is associated with a

general decrease in proteoglycan content in cartilage, an increase in protein relative to glycosaminoglycan content in aggrecan molecules and increased ratios of both keratan sulfate to chondroitin sulfate and 6-sulfate to 4-sulfate (Roughley and White, 1980). However, irrespective of age, the majority of proteoglycans maintain their ability to interact with hyaluronic acid (Roughley and White, 1980). In the present study, the potential influence of ageing on proteoglycans should be comparable between the treated rats and their corresponding, age-matched control rats.

The four-week duration for treatment with unilateral bite-raising appliances was chosen largely due to previous reports of cell proliferation in the disc and condyle two to three weeks after rats and rabbits wore unilateral bite-raising appliances (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). Four weeks were thought to be an appropriate period to detect whether these cells had changed their metabolism of proteoglycans. Use of bite-raising appliances for various times may be adopted in future investigations for studying the correlation between cell proliferation and enhanced proteoglycan expression.

Growth and maturation of TMJ articular tissues should also be considered when the present results are related to patients wearing occlusal splints. The majority of TMD patients wearing occlusal splints are 30-40 years of age and have fully matured TMJ articular tissues. Growth of TMJ articular tissues is presumably present in a small proportion of TMD patients who are adolescents and wear occlusal splints (Dr. Paul Major, personal communication). It is not clear in humans at what age the chondrogenic layer of the mandibular condyle stops reacting to mechanical stimuli although in young

adult monkeys (*Macaca mulatta*) subjected to a protruded jaw position, condylar hyperplasia similar to that in juvenile monkeys has been found (McNamara *et al.*, 1982). There is also evidence that unilateral bite-raising appliances induce fibroblasts and chondroblasts to proliferate in the mandibular condyles of fully mature rats older than 15 months (Lindsay, 1977). Blackwood (1967) showed that cells in the proliferative zone of the mandibular condyle in man are capable of proliferation and differentiation during adult life. In addition, it is not clear whether the remaining fibroblasts and chondroblasts in adult human or animals, without addition of newly proliferated ones as a result of marked hyperplasia, are able to synthesize more proteoglycans when these cells are subjected to greater *in vivo* mechanical stimuli. In parallel with addressing the above questions in human individuals, the next logical experiment seems to be investigation of glycosaminoglycan synthesis in various animal models of different ages using bite-raising appliances.

3.3 Rat model and the unilateral bite-raising appliance.

The rat model was used in the present study largely due to its convenient size and to budget limitation. Numerous anatomic differences between rat and human temporomandibular joints have functional implications that may impede extrapolation of the present results to humans wearing occlusal splints. For example, the two halves of the mandible in the rat are linked by a fibrous symphysis (Hebel and Stromberg, 1976) that transmits forces in a fashion different from the bony symphysis of the human mandible (Hylander, 1984). It is obvious from the present results that elevation in

proteoglycan expression is more significant in the ipsilateral (to the appliance side) joint than in the contralateral joint (Figs. 7 and 9), suggesting that the ipsilateral joint is more loaded than the contralateral joint. In humans, the contralateral condyle is more loaded than the ipsilateral condyle during unilateral biting (Hylander, 1975). Therefore, in terms of loading, the present results suggest that the ipsilateral condyle of the rat is equivalent to the contralateral condyle in humans. Without consideration of the available budget, monkeys are a better animal model for a study of this nature that is ultimately aimed at advancing understanding the human temporomandibular joint. However, no matter which animal model is used, anatomical differences remain a problem when results are extrapolated to explain the human situation. This is analogous to the step from animal trials to clinical trials during the development of a new drug. However, two arguments may help justify utilization of an animal model in TMJ related research: 1) force is universal irrespective of model and 2) proteoglycans enable both animal and human articular tissues to withstand compressive forces.

A unilateral bite-raising appliance, as opposed to a bilateral occlusal splint, was used in the present study largely due to the fact that a unilateral bite-raising appliance was found to be better retained in the oral cavity of the rat when inserted in the way used here. In addition, unilateral bite-raising appliances were used in three previous *in vivo* studies all of which reported proliferation of chondroblasts and fibroblasts in the disc and/or the mandibular condyle (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). By means of unilateral bite-raising appliances, the present results showed an enhancement in the expression of aggrecan (Figs. 6 and 7) and versican (Figs.

8 and 9) that are products of chondroblasts and fibroblasts whose proliferation has been reported in the three *in vivo* cellular studies (Lindsay, 1977; Ehrlich *et al.*, 1980; Shaw and Molyneux, 1993). Increasing proteoglycan expression appears to be a logical outcome of cell proliferation in TMJ articular tissues.

Bilateral occlusal splints have been used in monkeys (McNamara, 1977) and rabbits (Rashed and Sharawy, 1993). Both studies identified an increase in condylar volume, suggesting proliferation of chondroblasts and fibroblasts and increase in the volume of their extracellular matrix. Based on these results, it should not be surprising if future studies reveal an enhancement in the expression of proteoglycans in TMJ articular tissues in response to bilateral occlusal splints. Potential differences in the response of cells in TMJ articular tissues to unilateral and bilateral bite-raising appliances are likely quantitative rather than qualitative. One temporomandibular joint may be increasingly loaded in response to a unilateral bite-raising appliance, whereas both temporomandibular joints may share the forces induced by a bilateral occlusal splint.

3.4 Tissue section and staining methods.

The orientation of tissue sections was not standardized in the present study. For example, it is difficult to know whether a section was cut perpendicular to the condylar surface or somewhat obliquely. Part of this problem is intrinsic due to the small size of the specimen and the difficulty of defining to what structure a section should be perpendicular. Adoption of a larger animal model may address part of this problem but may still be susceptible to the difficulty with the curved surface of the mandibular

condyle. Tissue orientation is not critical for the purpose of the present study in which histological and immunohistochemical staining was used to identify the expression of proteoglycans. However, lack of tissue orientation does prevent effective comparison of thicknesses of different layers of structures between sections from different treatment groups.

The working dilutions of antibodies used in the present study were determined by previous experience in the laboratory (Nakano and Scott, 1989; Nakano *et al.*, 1993; Scott *et al.*, 1993) and by the color intensities of the positive control sections used for each group of staining slides. Were it not for the scarcity of some antibodies, effort would have been directed to explore optimal dilutions.

It is not entirely clear why staining with anti-versican antisera gave generally stronger positive results, as qualitatively observed, in the surface fibrous layer of the mandibular condyle than in the TMJ disc in the same specimen. The magnitude of compressive forces on the TMJ disc and the mandibular condyle should be equal in the same temporomandibular joint. The stronger versican expression in the condylar fibrous layer is probably due to more marked proliferation of fibroblasts here than in the TMJ disc perhaps because of the presence of the chondrogenic layer in the mandibular condyle. Alternatively, the articular tissue contains more proteoglycans than the disc, as in a finding between articular cartilage and the meniscus of the knee joint (Mow *et al.*, 1990). The temporomandibular disc, instead of the mandibular condyle, has often been used in previous studies for qualification and quantification of glycosaminoglycan contents (Mills *et al.*, 1988; Nakano and Scott, 1989; Carvalho *et al.*, 1995) probably

because the disc can be readily dissected as a separate anatomic entity. In the future, it may be meaningful to compare glycosaminoglycan contents of the TMJ disc with those in the surface fibrous layer of the mandibular condyle.

3.5 Major findings and limitations of the study and suggestion for future studies.

The principal findings from the present study are as follows. First, versican was identified in the TMJ disc and the fibrous layer of the mandibular condyle but not in the condylar cartilage (Figs. 8 and 9). Its expression was enhanced after the rat wore a unilateral bite-raising appliance for four weeks (Figs. 8A and 8B, 9). Four weeks after appliance removal, versican expression was reduced but still identifiable (Figs. 8C and 9). Reaction to the anti-versican antibody was weak in the control rats (Figs. 8D and 9). Second, aggrecan was identified in the condylar growth cartilage but only a trace amount was detected in TMJ fibrous tissue, by safranin O staining for the presence of chondroitin sulfate and keratan sulfate. Its expression was enhanced in the treated specimens (Figs. 6A and 6B, 7) and reduced in the appliance-removal specimens (Figs. 6C and 7). Third, marked condylar hyperplasia occurred after unilateral bite-raising appliances were used (Fig. 5). The area of condylar hyperplasia corresponded with the area with enhanced expression of aggrecan (Figs. 5 A,B and 6A, 6B). Fourth, with the antibodies (3B3 and 6D6) used in the present study, decorin was identified in some but not all sections in the periphery of the TMJ disc and condylar fibrous tissue of the treated and appliance-removal rats (Fig. 10A and 10B). Fifth, biglycan could not be identified with the antibody (PS-318) used in the present study (Fig. 11).

The following are limitations of the present study in addition to those already discussed above. 1) The duration of loading induced by the unilateral bite-raising appliance was not precisely controlled. The exact timing of appliance exfoliation was not recorded because each rat was inspected on every third or fourth day. If the bite-raising appliance was found missing, it was not possible to know exactly when it had been lost. This is attributed to an intrinsic difficulty of knowing the precise duration of force application by using a bite-raising appliance in an *in vivo* animal model. The precise magnitude and duration of compressive forces could be controlled in *in vitro* studies (Copray *et al.*, 1985; Takano-Yamamoto *et al.*, 1991; Carvalho *et al.*, 1995). However, one may argue that cells respond to forces *in vitro* differently from those *in vivo*. A solution to this problem may be that by using electrical stimulators for jaw-closing muscles, both the intensity and duration of force application can be precisely controlled. 2) Food consistency was not specified beyond an instruction that all the rats were to be fed with the same food during the entire course of experiments. A possible difference in food texture between different batches of the food even from the same supplier may have had an impact on the results, as it has been shown that foods of different texture have an effect on glycosaminoglycan synthesis in TMJ articular tissues (Hinton, 1988 and 1993). 3) "Blinding" was performed when color staining intensities were assessed but not during the experimentation itself. Other than the fact that "blinding" is not conventionally conducted in laboratory experimentation at least in other published studies along the same lines (Lindsay, 1977; McNamara, 1977; Ehrlich *et al.*, 1980; Copray *et al.*, 1985; Takano-Yamamoto *et al.*, 1991; Rashed and Sharawy, 1993;

Shaw and Molyneux, 1993; Kantomaa *et al.*, 1994a, b; Carvalho *et al.*, 1995), some of the present results are partially repeated and indirectly confirmed by results from other studies using similar and different experimental methods (Mills *et al.*, 1988; Nakano and Scott, 1989; Nakano *et al.*, 1993; Takano-Yamamoto *et al.*, 1991; Carvalho *et al.*, 1995). 4) Sample size was not rigorously determined by consultation with a statistician. Probably as a result of the expectation that far less variance exists in the control group, three or four times more animals are conventionally assigned to the experimental group than to the control group (Lindsay, 1977; McNamara, 1977; Ehrlich *et al.*, 1980; Rashed and Sharawy, 1993; Shaw and Molyneux, 1993; Kantomaa *et al.*, 1994a,b). To avoid deficiency in sample size or waste of resources, a statistician should be consulted for determination of optimal sample size. 5) Only six-week-old and nine-week-old rats were used in the present study in which unilateral bite-raising appliances were placed *in situ* for only four weeks. Use of rats of different ages and placement of bite-raising appliances for various times could help to illustrate temporal changes in the expression of proteoglycans in TMJ articular tissues. 6) Due to limitations of budget and time, glycosaminoglycan content was not quantified by biochemical means. Quantification of proteoglycans by assessment of staining intensities (Figs. 7 and 9) helps to visualize the overall staining patterns but still does not address the need for quantifying glycosaminoglycan content. A future study may use the method of incorporation of radiolabeled sulfate followed by immunoprecipitation (Goding 1983) as a means to quantify newly synthesized glycosaminoglycans.

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Figure 1. The human mandibular condyle arbitrarily divided into different layers.

Fig. 1A: The mandibular condyle of a growing mammal. Fig. 1B: The mandibular condyle of a mature human individual.

A. Growing individual.

B. Mature individual.

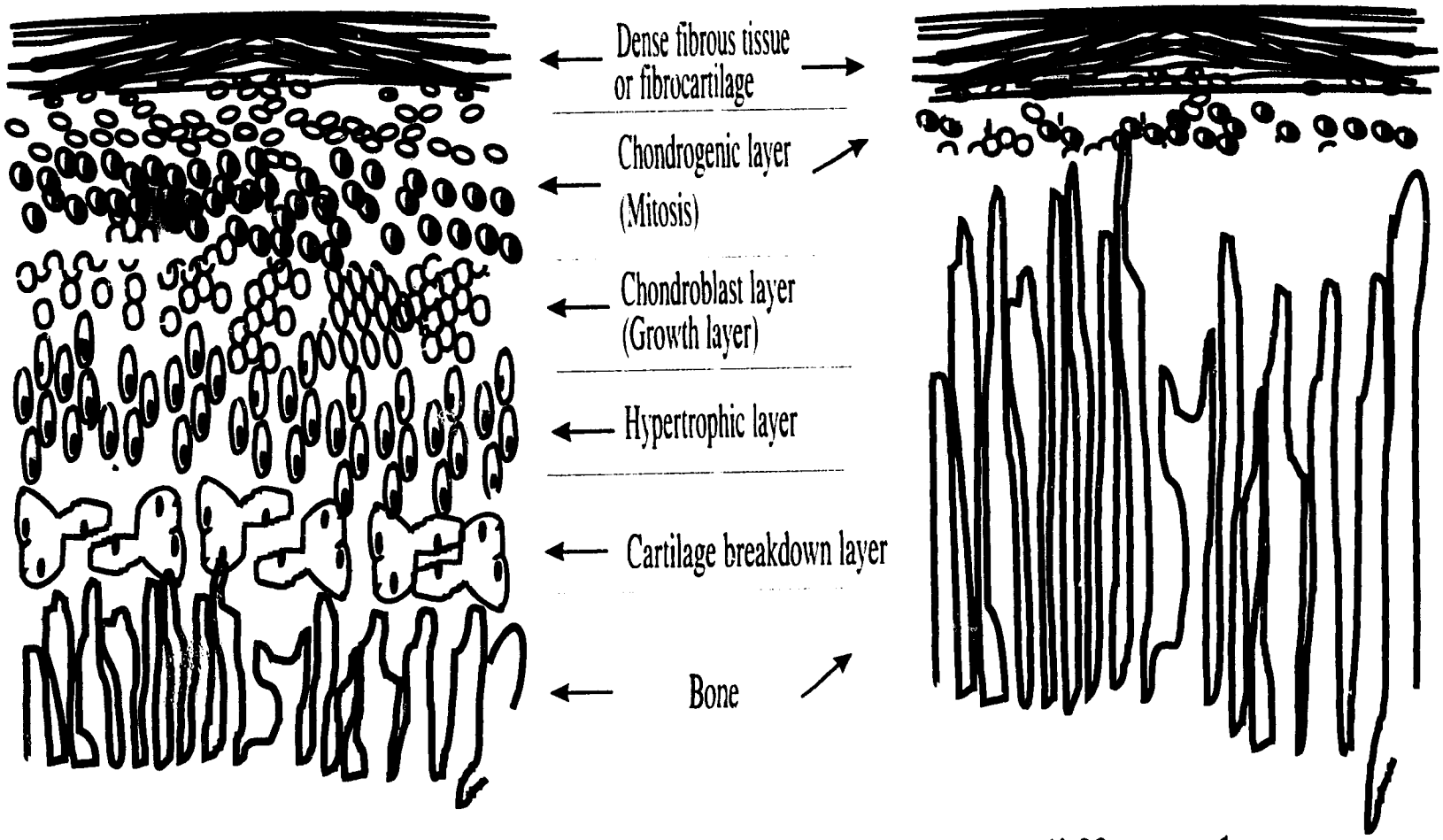


Fig. 1 The mandibular condyle divided into different layers.

Figure 2. The TMJ disc and mandibular condyle may be continuously loaded with occlusal splint *in situ*.

Fig. 2A: The articular eminence and the condyle with the disc wedged in between. Fig. 2A': A coronal view of the disc and condyle from the top. The lightened area is the center of loading. Fig. 2B: After insertion of an occlusal splint, the disc and condyle are projected downward and forward against the articular eminence. Fig. 2B': A coronal view of the disc and condyle from the top. The lightened area indicates a region of loading different from that in Fig. 2A'.

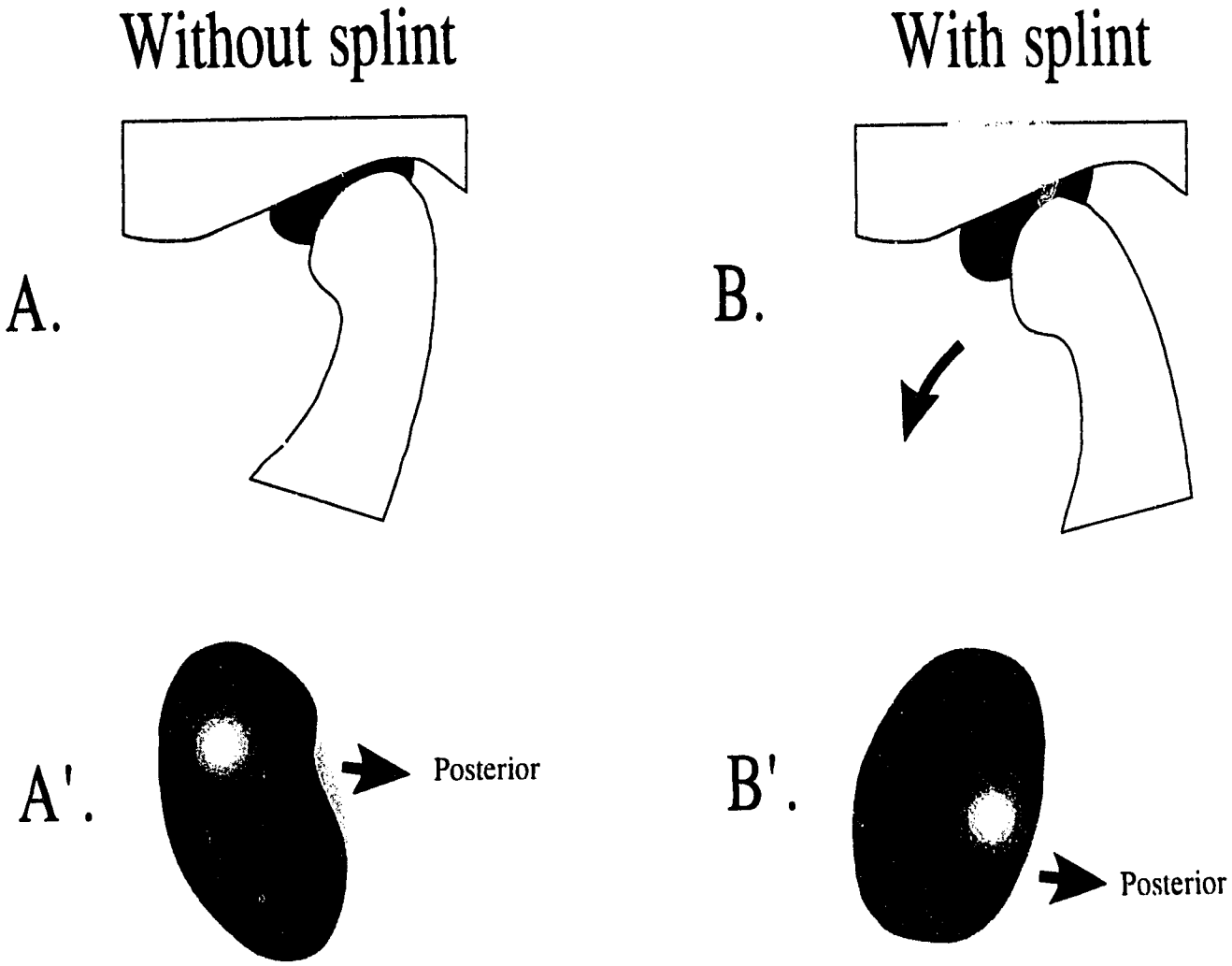


Fig. 2 Disc and condyle may be continuously loaded with occlusal splint *in situ*.

Figure 3. Mechanical analysis of TMJ loading in the sagittal plane with and without occlusal splint.

Solid line with arrow: muscle resultant force and its direction. Dashed line with arrow: bite force and its direction. Dotted line and its arrow: TMJ reaction force and its direction. Curved arrows: direction of condylar movement.

Fig. 3A: With an occlusal splint *in situ*. Fig. 3B: With the point of application of bite force on M1, in front of muscle resultant. Fig. 3C: With the point of application of bite force on M3, posterior to muscle resultant force.

The condyles are loaded in Fig. 3A and 3B. The condyle is distracted or unloaded in Fig. 3C.

A. With occlusal splint.

B. Bite point in front of muscle resultant.

C. Bite point posterior to muscle resultant.

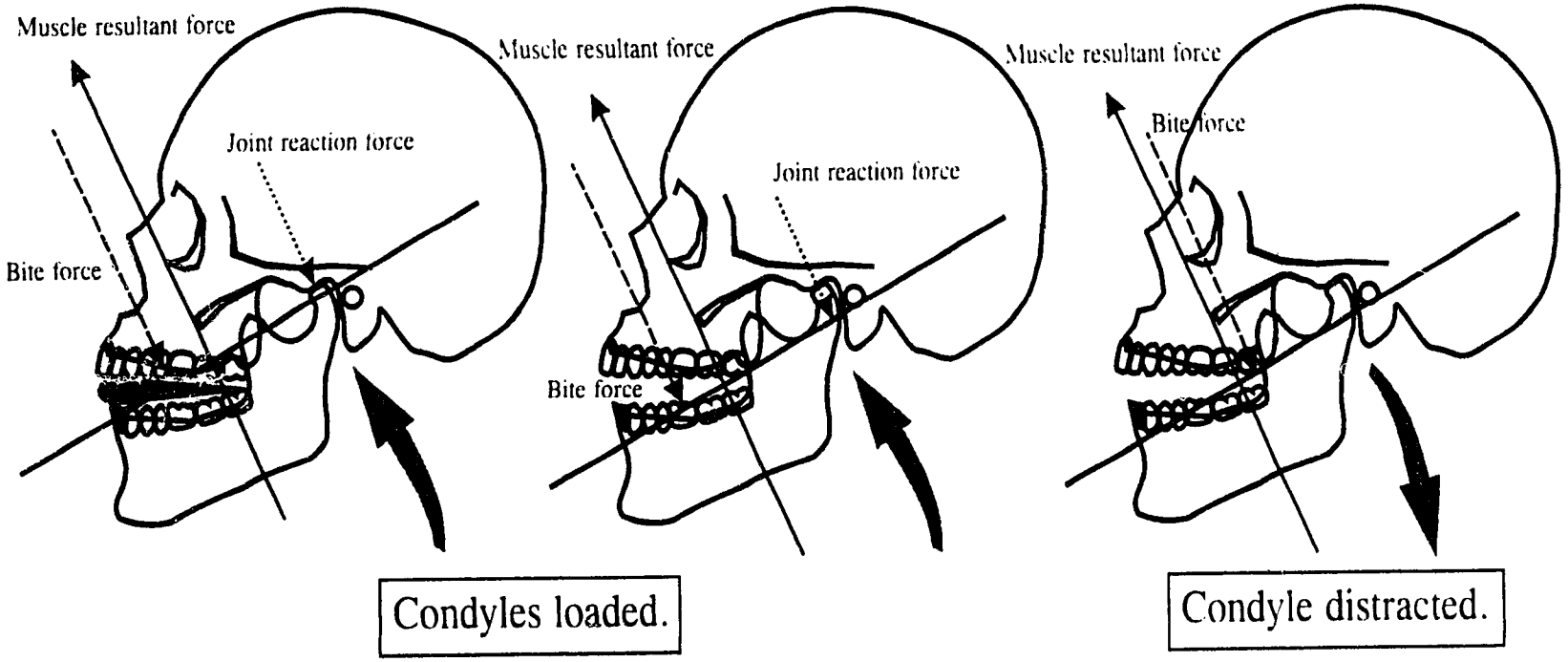


Fig. 3 Mechanical analysis of TMJ loading with and without occlusal splint.

Figure 4. Occlusal view of the rat palate with a unilateral bite-raising appliance *in situ*.

The appliance covers all three molars on the right side. For illustration, the location of the three molars on the right side is revealed.

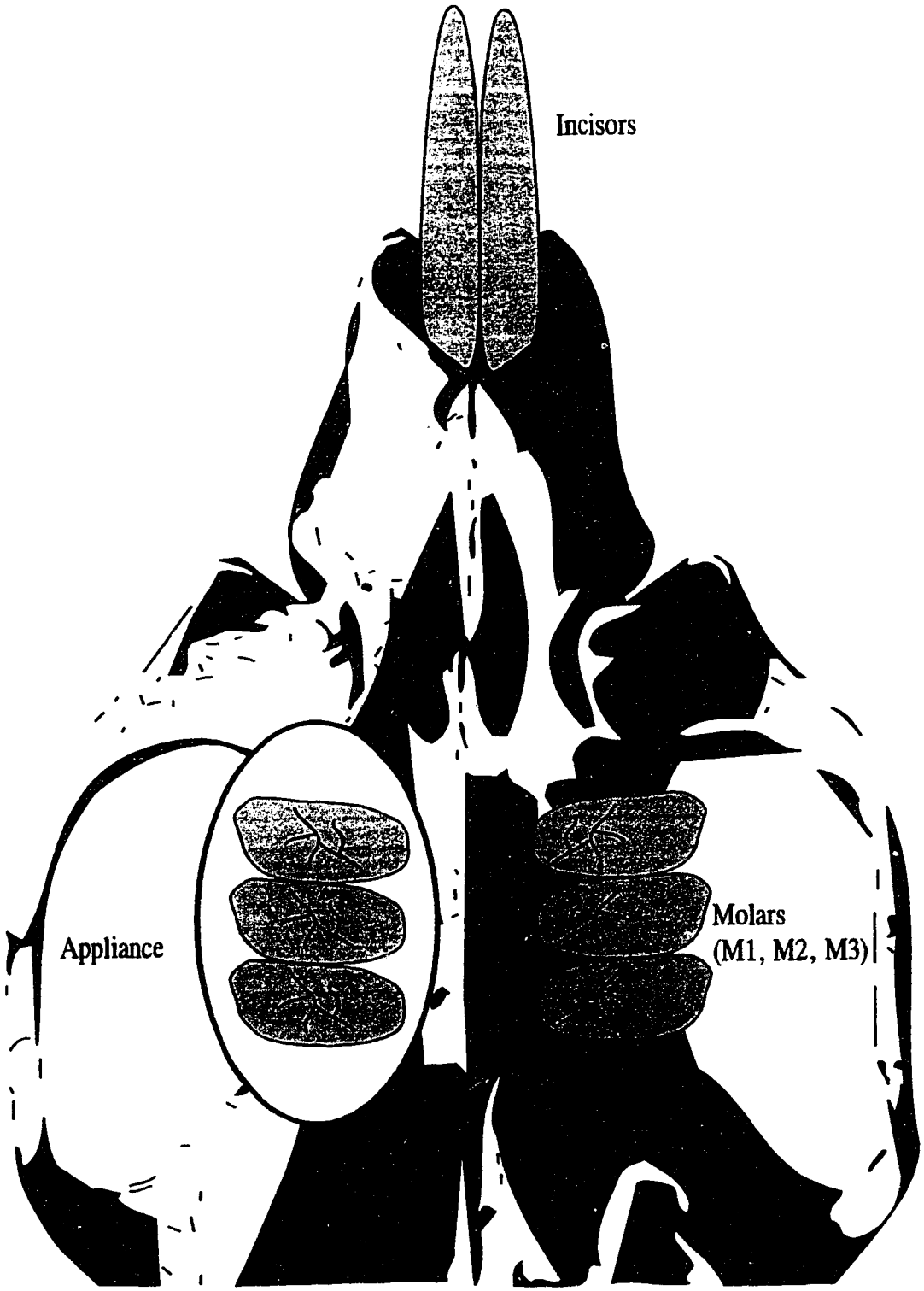


Fig. 4 Occlusal view of the rat palate with a unilateral bite-raising appliance bonded to all the three molars on the right side.

Figure 5. Hematoxylin-eosin staining of the disc and condyle.

Photomicrographs of the mandibular condyles and TMJ discs. Fig. 5A: The six-week-old treated specimen. Fig. 5B: The nine-week-old treated specimen. Fig. 5C: The nine-week-old, appliance-removal specimen. Fig. 5D: The nine-week-old control specimen. Bar = 200 μm. $\times$ 55.

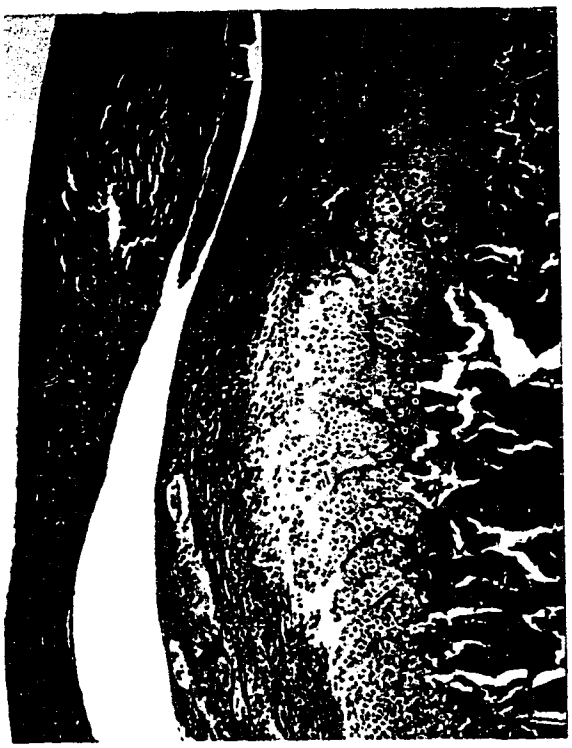


Fig. 5B

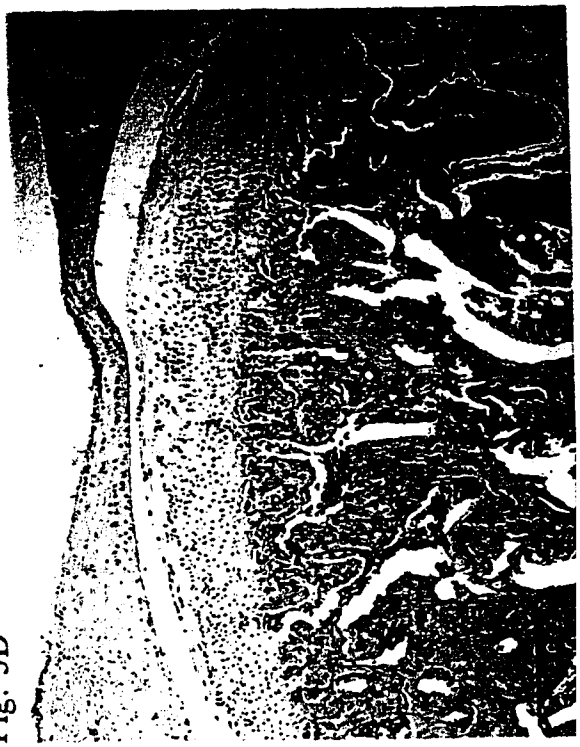


Fig. 5D

Fig. 5A

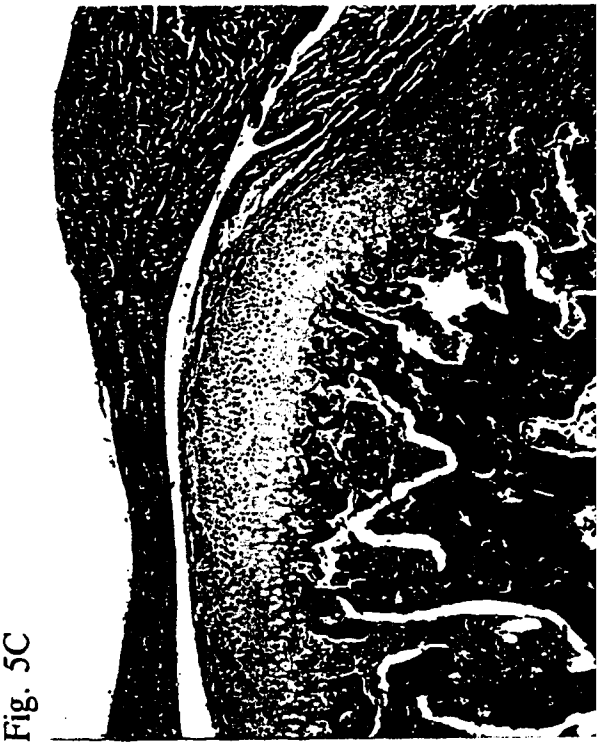


Fig. 5C

Figure 6. Safranin O and fast-green staining in TMJ articular tissues.

Photomicrographs of the mandibular condyles and TMJ discs. Fig. 6A: The six-week-old treated specimen. Fig. 6B: The nine-week-old treated specimen. Fig. 6C: The nine-week-old appliance-removal specimen. Fig. 6D: The nine-week-old sham-operated control specimen. Bar = 200 μm. $\times$ 55.

Fig. 6A

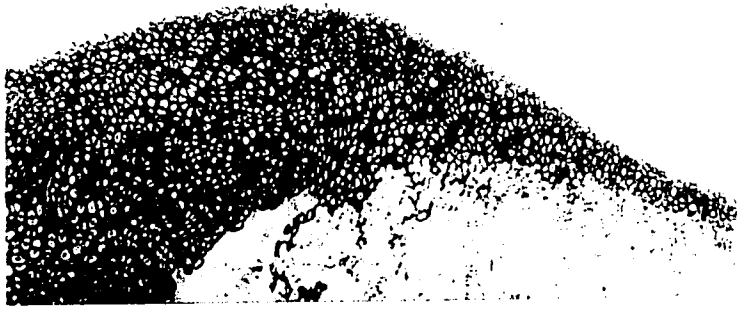


Fig. 6B

Fig. 6C

Fig. 6D

Figure 7. Color intensity measurements of safranin O staining in TMJ articular tissues quantified by a computer image analysis system.

**: $p < 0.01$. *: $p < 0.05$. Sample sizes for the contralateral control specimens (N=5,4,4) and the age-matched, sham-operated control specimens (N=5,4,3) were for the 9-week-old rats treated with the appliance, the 9-week-old appliance-removal rats and the 6-week-old rats treated with the appliance respectively.

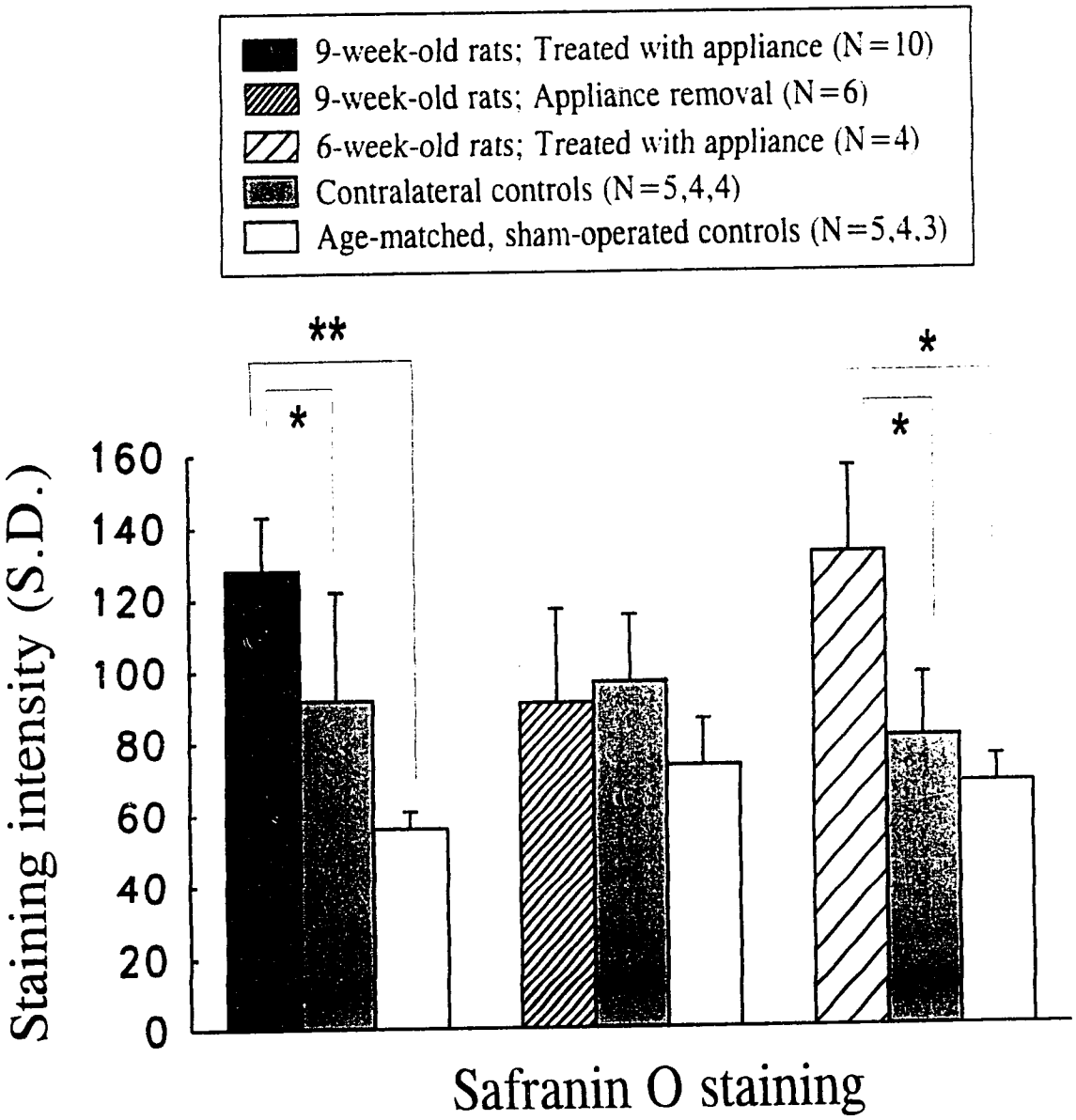

9-week-old rats; Treated with appliance (N=10)
9-week-old rats; Appliance removal (N=6)
6-week-old rats; Treated with appliance (N=4)
Contralateral controls (N=5,4,4)
Age-matched, sham-operated controls (N=5,4,3)
**
*
*
*
160
140
120
100
80
60
40
20
0
Staining intensity (S.D.)
Safranin O staining

Figure 8. Anti-versican antibody (5D5) staining in TMJ articular tissues.

Photomicrographs of the mandibular condyles and TMJ discs. Fig. 8A: The six-week-old treated specimen. Fig. 8B: The nine-week-old treated specimen. Fig. 8C: The nine-week-old, appliance-removal specimen. Fig. 8D: The nine-week-old sham-operated control specimen. Bar = 200 μm. $\times$ 55.

Fig. 8A

Fig. 8B

Fig. 8C

Fig. 8D

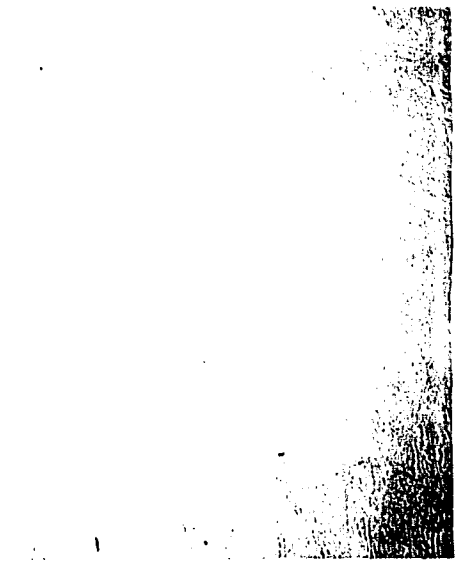


Figure 9. Color intensity measurements of anti-versican monoclonal antibody (mAb) staining in TMJ articular tissues quantified by a computer image analysis system.

**: $p < 0.01$. *: $p < 0.05$. Sample sizes for the contralateral control specimens (N=5,4,4) and the age-matched, sham-operated control specimens (N=5,4,3) were for the 9-week-old rats treated with the appliance, the 9-week-old appliance-removal rats and the 6-week-old rats treated with the appliance respectively.

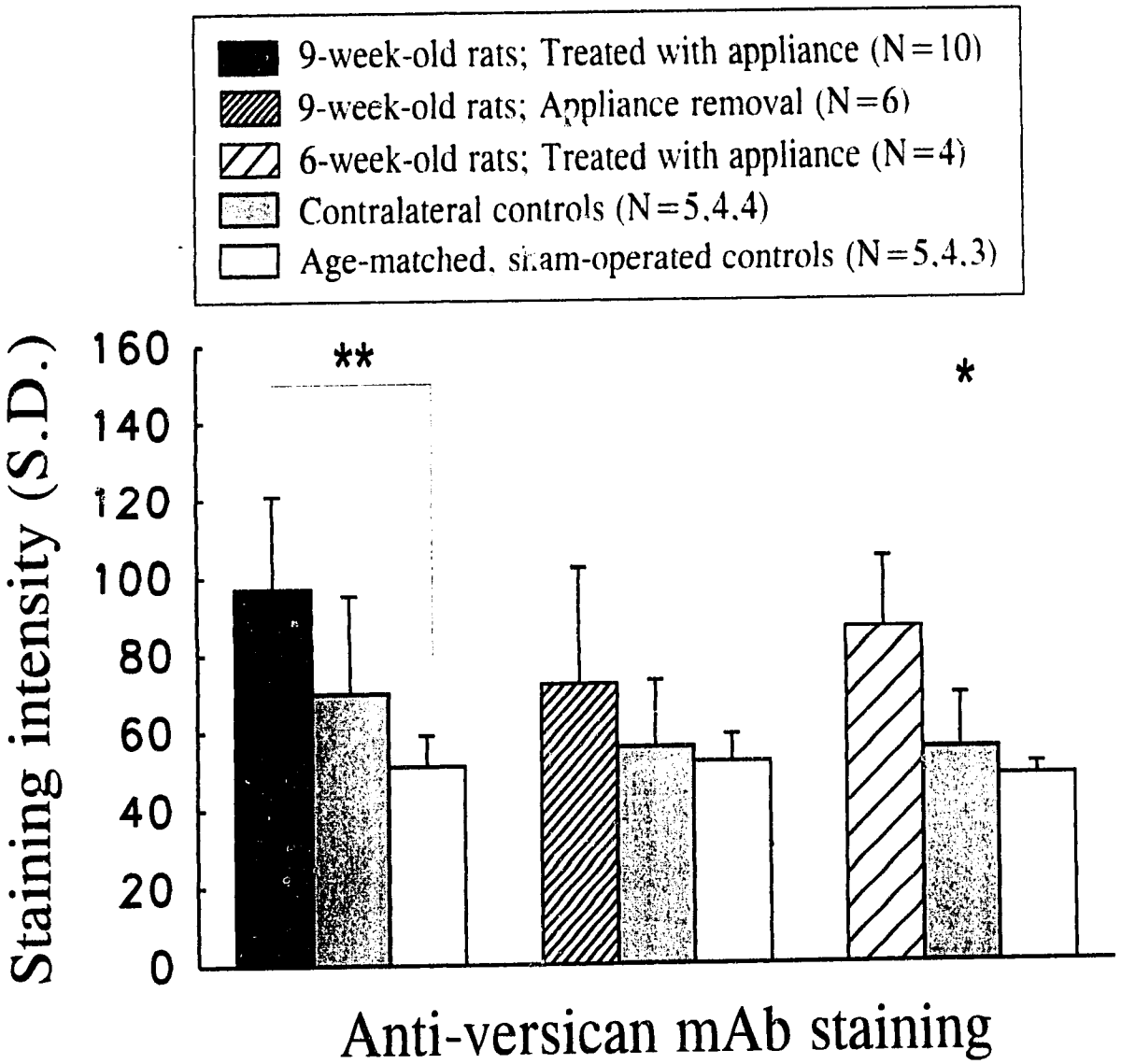

9-week-old rats; Treated with appliance (N=10)
9-week-old rats; Appliance removal (N=6)
6-week-old rats; Treated with appliance (N=4)
Contralateral controls (N=5,4,4)
Age-matched, sham-operated controls (N=5,4,3)
Staining intensity (S.D.)
160
140
120
100
80
60
40
20
0
**
*
Anti-versican mAb staining

Figure 10. Staining against anti-decorin antibodies (6D6 and 3B3) in TMJ articular tissues.

Photomicrographs of the mandibular condyles and TMJ discs. Fig. 10A: The nine-week-old treated specimen. Fig. 10B: The nine-week-old, appliance-removal specimen. Fig. 10C: The nine-week-old sham-operated control specimen. Bar = 200 μm. $\times$ 55.

Fig. 10A

Fig. 10B

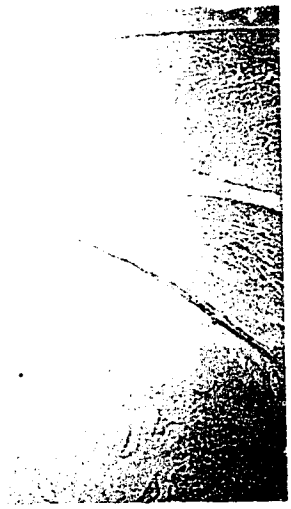


Fig. 10C

Figure 11. Anti-biglycan antibody (PS-318) staining in TMJ articular tissues.

Photomicrographs of the mandibular condyles and TMJ discs. Fig. 11A: The nine-week-old treated specimen. Fig. 11B: The nine-week-old, appliance-removal specimen. Fig. 11C: The nine-week-old sham-operated control specimen. Bar = 200 μm. × 55.

Fig. 11A

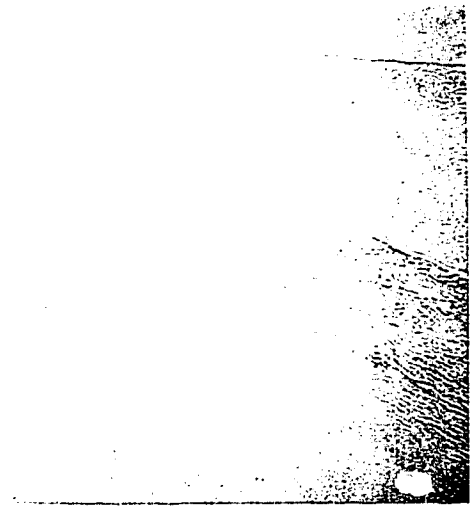


Fig. 11B

Fig. 11C

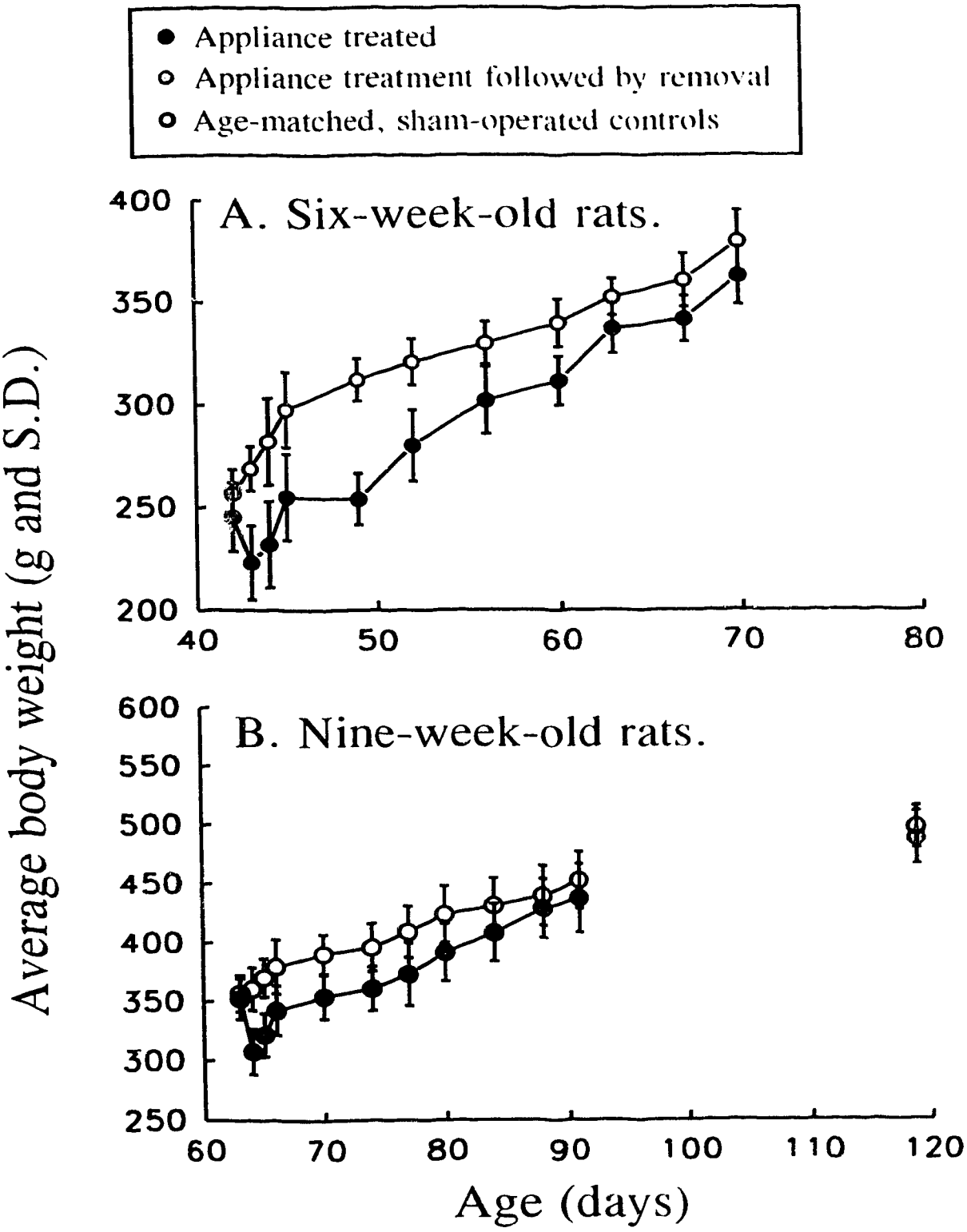
Appendix 1. A list of all rats used in the experiments.

	Age (wk)	Start date	End date	Treatment	Death	Comments
1	6	06/23/94		Uni splint	07/09/94	Somatol overdose
2	6	06/23/94	07/14/94	Uni splint		
3	6	06/23/94		Uni splint	06/23/94	Water inhalation
4	6	06/23/94	07/14/94	Uni splint		
5	6	06/23/94		Uni splint	07/05/94	Somatol overdose
6	6	06/23/94	07/14/94	Uni splint		
7	6	06/23/94	10/27/94	Bi splint		Kept to observe aging
8	6	06/23/94	07/14/94	Sham Con		
9	6	06/23/94	07/14/94	Sham Con		
10	6	06/23/94	07/14/94	Sham Con		
11	9	07/28/94	08/18/94	Uni splint		Started to use halothane
12	9	07/28/94		Uni splint	?	Water inhalation
13	9	07/28/94	08/18/94	Uni splint		
14	9	07/28/94	08/18/94	Sham Con		
15	9	07/28/94	08/18/94	Sham Con		
16	9	07/28/94	08/18/94	Sham Con		
17	6	08/25/94	09/15/94	Uni splint		
18	6	08/25/94	09/15/94	Uni splint		
19	9	02/16/95	03/16/95	Uni splint		
20	9	02/16/95	03/16/95	Uni splint		
21	9	02/16/95	03/16/95	Uni splint		
22	9	02/16/95	03/16/95	Uni splint		
23	9	02/16/95	03/16/95	Uni splint		
24	9	02/16/95	03/16/95	Sham Con		
25	9	02/16/95	03/16/95	Sham Con		
26	9	02/16/95	03/16/95	Sham Con		
27	9	02/16/95	03/16/95	Sham Con		
28	9	02/16/95	03/16/95	Sham Con		
29	9	05/27/95	07/22/95	Splint rem		Arrived 05/25/95
30	9	05/27/95	07/22/95	Splint rem		"
31	9	05/27/95		Splint rem	06/15/95	Water inhalation
32	9	05/27/95	07/22/95	Splint rem		Arrived 05/25/95
33	9	05/27/95	07/22/95	Splint rem		"
34	9	05/27/95		Splint rem	?	Water inhalation
35	9	05/27/95	07/22/95	Splint rem		Arrived 05/25/95
36	9	05/27/95	07/22/95	Splint rem		"
37	9	05/27/95	07/22/95	Splint rem		"
38	9	05/27/95	07/22/95	Splint rem		"
39	9	05/27/95	07/22/95	Sham Con		"
40	9	05/27/95	07/22/95	Sham Con		"
41	9	05/27/95	07/22/95	Sham Con		"
42	9	05/27/95	07/22/95	Sham Con		"
43	9	05/27/95	07/22/95	Sham Con		"
44	9	05/27/95	07/22/95	Sham Con		"
45	9	05/27/95	07/22/95	Sham Con		"
46	9	05/27/95	07/22/95	Sham Con		"
47	9	05/27/95	07/22/95	Sham Con		"
48	9	05/27/95	07/22/95	Sham Con		"
49	9	06/01/95	06/29/95	Uni splint		
50	9	06/01/95	06/29/95	Uni splint		
51	9	06/01/95	06/29/95	Uni splint		
52	9	06/01/95		Uni splint		Removed from study (at large) for 2 d
53	9	06/01/95	06/29/95	Uni splint		
54	9	06/01/95	06/29/95	Uni splint		
55	9	06/01/95	06/29/95	Uni splint		
56	9	06/01/95	06/29/95	Uni splint		
57	9	06/01/95	06/29/95	Sham Con		
58	9	06/01/95	06/29/95	Sham Con		

Caption for Appendix 2. Body weight measurements of all rats used in the study.

The two symbols on the right in Appendix 2B (separated from the rest) are the final average weight measurements for the appliance-removal rats (gray-filled circle) and their corresponding sham-operated controls (open circle). No statistically significant differences were found in the final weight measurements between the treated and sham-operated control groups: for the six-week-old rats ($p = 0.16$), the nine-week-old rats ($p = 0.19$) and the nine-week-old, appliance-removal rats ($p = 0.35$).

Appendix 2. Body weight measurements.



Appendix 3. Tissue sections used in the experiments.

	Total specimens	Stained SO/5D5	Lost specimens	Unstained
9S	14	10	2	2
9C	14	5	1	8
9SC	10	5	0	5
9SR	8	6	1	1
9SRC	8	4	1	3
9SRSC	10	4	1	5
6S	5	4	1	0
6C	5	4	1	0
6SC	3	3	0	0
Total		45	8	24

9S: 9-week-old rats assigned to splint group; 9C: side contralateral to the splinted side; 9SC: sham control rats; 9SR; 9-week-old-rats assigned to splint-removal group, i.e. 4-week splint wear followed by 4-week survival after splint removal; 9SRC: side contralateral to the splinted side; 9SRSC: sham-operated control rats; 6S; 6-week-old rats assigned to splint group; 6C: side contralateral to the splinted side; 6SC: sham-operated control rats; SO: safranin O staining; 5D5: anti-versican antibody staining.

Appendix 4. Protocols for safranin O and fast-green staining used in the experiments.

PREPARATION

all stain solutions to be filtered before use

0.002% fast green
- fast green....40 mg
- distilled water...200 ml
- filter before use

0.1% safranin O
- safranin O...200 mg
- distilled water...200 ml
- filter before use

1% glacial acetic acid
- 99.7% glacial acetic acid...500 µl
- distilled water...200 ml

STAINING

1. Select blank slides

2. Heat slides
   - Corning Hot Plate/Stirrer to heat slides at Level 1

3. De-wax in fumehood
   - Xylene #1...5 min
   - Xylene #2...5 min
   - 100% ethanol...3 min
   - 95% ethanol...3 min
   - 80% ethanol...3 min

4. wash in running water...10 min

5. fast green
   - 4 min

6. rinse quickly in 1% GAC

7. safranin O
   - 5 min

8. dehydrate

95% ethanol #1...2.5 min
95% ethanol #2...2.5 min
100% ethanol #1...2.5 min
100% ethanol #2...2.5 min
Xylene #1...5 min
Xylene #2...5 min

9. Coverslip with Permount

Cover tissue completely
Ensure no bubbles

Appendix 5. Protocol for immunohistochemical staining used in the experiments.

Day 1

1. Select blank slides
   one slide for each tissue must be negative control
   must always have positive control

2. Heat slides
   Corning Hot Plate/Stirrer to heat slides to soften the tissue

3. De-wax in fumehood
   Xylene: 3 × 2 min
   methanol: 3 min and discard

4. Kill endogenous peroxidase
   13 ml 2% H_2O_2 → 200 ml MeOH; seal + mix
   30 min at room temperature (R.T.)

5. Wash with TBS
   TBS (Tris buffered solution) 200 ml × 5 min × 3 times

6. Hyaluronidase Treatment
   1 mg / 1 ml buffer (0.05 M Tris-HCl/0.15 M NaCl pH 7.6)
   incubate at 37° C; 30 min

7. wash
   same as Step 5 above

8. Blocking
   blocking serum: 5% serum: 500 μl serum in 9500 μl TBS
   60 min at R.T.

9. 1° antibody
   Control slides (no 1° antibody)
      for 5D5: cell culture supernatant
      for 6D6 and 3B3: cell culture supernatant
      for PS318: normal rabbit serum
   Antibody slides
      for 5D5: 1:500
      for 3B3 and 6D6: 1:10
      for PS318: 1:30
   overnight in cold room at 4°C

Day 2

---

1. Slides out of cold room
   warm up at R.T. for 10 min

2. Wash
   wash antibody and control slides in separate jars

3. 2° antibody
   goat anti-mouse IgG (Fab_2) for 5D5, 6D6 and 3B3
   rabbit anti-mouse IgG.A.M for PS-318
   1:50
   (45 min at R.T.)

4. Wash
   same as Step 5 on Day 1

5. PAP (30 min at R.T.)
   mouse peroxidase-antiperoxidase (PAP) complex
   1:300
   5 μl PAP, 75 μl serum, 1420 μ TBS
   (30 min at R.T.)

6. Wash
   same as Step 5 on Day 1

7. Diaminobenzidine (DAB) staining
   diaminobenzidine in fumehood
   100 mg DAB → 200 ml TBS with 60 μl H_2O_2
   mix thoroughly
   ensure DAB is completely dissolved
   4 min

8. Wash with distilled H_2O
   10 min

9. Dehydrate
   70% Alcohol, 95%, 98%, MeOH
   3 min each
   Xylene 3 min

10. Mount with coverslip
    Cover tissue completely and ensure no bubbles