THE

SYNTHESIS OF COLLAGEN AND GLYCOSAMINOGLYCANS *IN VITRO* IN RAT AND MOUSE TEMPOROMANDIBULAR DISC

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Submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the

degree of MASTER of SCIENCE

DEPARTMENT OF PREVENTIVE DENTAL SCIENCE

WINNIPEG, MANITOBA

by

ROBERTO SILVA CARVALHO

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BY

ROBERTO SILVA CARVALHO

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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To know that we know what we know, and

that we do not know what we do not know,

that is true knowledge...

- THOUREAU, Walden (quoting Confucius)

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ABSTRACT

Glycosaminoglycans (GAGs) and collagen synthesis are predominant in articular disc tissues. The effect of growth on the synthesis of both molecular species and the effect of functional demands are poorly understood. Therefore, the main purpose of this investigation is to correlate GAG and collagen synthesis in the articular disc due to growth and to mechanical stress.

Rat articular discs were studied histologically and biochemically. Ratios of newly-synthesized type III collagen have been regarded as important parameters of collagen metabolism during rapid tissue remodeling. A correlation was suggested between the decrease in the ratio of type III collagen from the articular disc labelled in organ culture and the decrease in growth of the mandible. Distribution in the disc of $[^3H]$-proline revealed the anterior and medial bands to have an intense collagen synthesis as evidenced by the silver grains as compared to the posterior band. This pattern appears unchanged with age.

The effect of age in GAG synthesis in the rat articular disc indicated that hyaluronic acid (HA), chondroitin$_6$sulfate (C_6S) and keratan sulfate/chondroitin$_4$sulfate (KS/C_4S) were the major GAG components of the disc. The variance of such fractions was correlated to the pattern of growth as shown for collagen synthesis. HA and C_6S peaked at 6 to 8 weeks of age confirming this age period to be highly metabolic active. Preliminary *in vitro* stimulation of the loaded articular disc suggeted that under intermittent stress the fibrocartilaginous properties of the disc may switch phenotype to a more cartilaginous type as evidenced by the increase in C_6S synthesis. Ultimately, this relationship between force and molecular synthesis has a strong potential to explain the change in tissue behaviour in the pathology of the articular disc and during normal TMJ growth and development.

DEDICATION

To My Parents

For always believing and encouraging everything that I have chosen to do, they have given me not only their example, but also their love and friendship.

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I would like to thank Dr. Edwin Yen, my research supervisor, for having given me the opportunity to begin and the support to finish this project. He has been an example that I will carry in my career.

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And finally, I thank for all the courage and determination passed to me from the warm and happy environment in which I was brought up. This I owe to my family.

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Chapter ONE

Literature Review

LITERATURE REVIEW

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1. Introduction

The temporomandibular joint is a bilateral structure joining the mandibular condyle with the articular facets of the temporal bones (Bell, 1990). This is a diarthroidal or synovial joint, in which both surfaces are non-inervated and non-vascularized (Moffet, 1984; Bell, 1986).

Synovial joints are always encapsulated structures with the presence of an articulating disc or meniscus and of synovial fluid which confers a great degree of articulating motion (Barnett *et al.*, 1961; Wright *et. al.*, 1973). This feature is divided basically into two movements, rotation and sliding. Rotation is confined to the mandibular condyle-articular disc portion (disc-condyle complex), while the sliding movement is restricted to the articulation of the disc-condyle complex with the temporal bone (Bell, 1986; Bell, 1990). In addition to these basic movements are produced in many combinations, since an ideal joint has to provide a wide range of possible modes of action coordinated with a certain degree of stability (Wright *et. al.*, 1973).

The effect of specific stimuli may be an essential factor in the pathogenesis of diseases of the temporomandibular joint. Yet the complex interdependence of some of the factors involved prohibit complete understanding of this phenomena. Such factors could be determined by the structural and functional study of the major anatomical component as well as their contribution to the model of the TM joint function and the deviations responsible for TM dysfunctions. Although the disorders and interferences due to an altered function in the normal articular disc structure are responsible for a high proportion of the all TMJ complaints, second only to muscle disorders (Bell, 1990), the factors influencing the pathologic transformations that take place in the articular disc are not clear.

Therefore, the objectives of this investigation are to clarify some of the above mentioned aspects and other possible interferences in the articular disc, with organ culture

experiments and thereby to study the ultrastructural and functional behaviors of this component, focusing in how it is affected by the age-related changes and mechanical stimulations.

1.1. Anatomical Considerations of the Articular Disc

Articular Disc or meniscus is an essential component of some specialized synovial joints or articulations capable of a wide range of movements. These are: the articulation of the knee, wrist articulation, sternoclavicular joint, sometimes the acromioclavicular joint and the temporomandibular joint (Wrigth *et al.*, 1973). Menisci and discs are not morphologically synonymous, however both present similar structural and physiological activities (Barnett *et al.*, 1961). The complexity of such anatomical structure facilitates articulating motion while supports a considerable load involved. This is partly due to the cartilaginous properties of this connective tissue and the arrangement of its internal components.

The articular disc is composed of a dense fibrous tissue which is non-innervated, largely avascular (with the exception of the peripheral areas), and also elastic tissue (Rees, 1954; Miles, 1962; Frommer, 1966; Silva, 1969; Wright *et al.*, 1973) with a scattering of mature fibrocytic cells (Wright *et al.*, 1971). Also a few chondrocytes can be seen (Bell, 1986; Mills *et al.*, 1988).

The overall contour of the disc is of extreme importance in the equilibrium of the temporomandibular articulation. The forces are distributed to form a self-centering effect on the disc, resulting in its rotatory movement to bring the central thinner portion between the areas under stress. Once the pressure is released, the disc space widens allowing the disc to rotate to its thicker portion to fill the space. When the structural conformation is lost, the disc changes its shape and loses this self-centering capability (Moffet, 1984). On the other

hand, the shape of the disc not only prevents sliding movement between the disc and the condyle without an excessive inhibition of this movement but also contributes to reduce disc displacement (Bell, 1986).

Passively, the disc divides the joint cavity into two separate portions. Anteriorly, the disc is attached to the superior head of the lateral-pterygoid muscle and posteriorly it is complemented by a loose connective tissue that attaches to the posterior edge of the articular disc and to the posterior portion of the TM joint capsule, called retrodiscal tissue (Oberg *et al.*, 1971). Due to its so-called elasticity (Griffin *et al.*, 1962; DuBrul, 1981), it is common to assume that this tissue holds the disc on the top of the condyle during the resting position of the joint (Travell *et al.*, 1983). However, in the resting joint the main force in the disc is applied anteriorly by the muscle tonus in the superior head of the lateral pterygoid muscle. Therefore, the shape of the disc contour is actually responsible for the prevention of anterior displacement (Bell, 1986). The disc shape was described to be highly genetically determined, as it shows the same basic structure (gross and microscopic observations) in the newborn as in the adult (Strauss *et al.*, 1960; Wong *et al.*, 1985), even though, the disc sagittal contour in the newborn appears to be much less biconcave than in the adult (Wright, 1976; Strauss *et al.*, 1960). On the other hand, according to Osborn (1985), it is apparent that the shape of the disc adapts to the compressional forces to which it is subjected (Rees, 1954). The compression of the central portion of the disc was assumed to be responsible for the biconcave shape of the anterior collagenous part, as bone growth occurs at the articular eminence (Wright *et al.*, 1976). Laterally the disc is connected to collateral ligaments that are attached to the medial and lateral poles of the condyle (Krog-Poulsen *et al.*, 1957). These collagenous fibers are responsible for the passive restraint of discal movement (Moffet, 1984). Consequently, the disc can rotate forward on the condyle, but it is stopped from anterior or posterior displacement, as long as these ligaments are intact and functional.

1.1.1. Articular Disc Development in Rodents

The first appearance of the articular disc in rodents occurs at eighteen-days insemination age and the first clear evidence of the fibrous ligament appears around the nineteenth to twentieth-day insemination age with the form of granules or beads (Furstman, 1966). Posteriorly, these granules get thicker separating into upper and lower laminae of fibrous tissue. In the upper laminae many elastic fibers appear and extend to the post glenoid area anterior to the tympanic bulla. The lower portion merges with the connective tissue of the lateral pterygoid muscle before it inserts posteriorly to the neck of the mandible. Around the twenty-first-day insemination age, it can be seen to be continuous laterally with the anlage of the "capsular ligament" and medially with the connective tissue around the fibers of the lateral pterygoid muscle (Cunat *et al.*, 1956).

At birth the disc is formed by loose connective tissue and becomes more dense and acellular with age (Levy, 1949). At the age of two days, the disc is formed by few collagenous fiber bundles increasing in number about the fourth day. Around the fifth day there is a great amount of width of the anterior and posterior margins, but the disc still remains narrow centrally (Cunat *et al.*, 1956; Weijs, 1975). The disc structure is well formed by the sixth day of age (Levy, 1949), with a considerable number of collagenous and elastic fibers and cells which resemble chondrocytes (Cabrini and Eurasquin, 1941), however, it is considered complete around seven days after birth (Furstman, 1966). In the lateral portion, the disc is five to six cell layers thick, narrowing to become indistinct medially. The number of fiber bundles increases progressively from the fifth to the thirtieth day of life and the disc becomes a highly fibrous structure (Cunat *et al.*, 1956) consisting mainly of longitudinally oriented collagenous fibers (Cunat *et al.*, 1956; Weijs, 1975).

Similarly to the human articular disc, there aren't any nerve fibers in the rodent disc, although the surrounding tissue contain these receptors (Frommer *et al.*, 1968). According to Daniel (non published material, 1989), the rodent joint differs from that of

human in two points: (1) the rodent joint does not have a glenoid fossa and the portion of the posterior attachment is diffusely spread and attached broadly to the temporal bone, and (2) the posterior end of the zygomatic arch also forms the lateral wall of the same bone.

1.2. Articular Disc and Temporomandibular Joint

The articular disc can be classified into a shock absorption structure. This is possibly results from the resilient nature of the connective tissue that forms the disc which helps to protect the articular surfaces and increase the congruity between them, thereby improving joint stability and facilitates the motion of the entire articulation (Wright *et al.*, 1971). However, the function of the temporomandibular disc is not known. As it was stated (Jagger, 1980), is likely that it may include: (a) shock absorption, (b) component to increase the congruity between articular surfaces, (c) component to allow the joint to describe a combination of different movements, (d) ball-bearing action, (e) weight distribution by increasing the contact area between articular surfaces and (f) component to assist joint lubrication by forming thin synovial fluid films. Biochemical studies indicated that the articular disc works as a destabilizer for the mandibular condyle during mastication (Osborn, 1985; Nakano and Scott, 1989). All these features may be further present at the same time and all are related with joint function. If the disc is not adapted successfully within the TMJ, a degenerative response of the joint may occur resulting from the functional and other forces imposed upon it (Moffet, 1984).

Alterations in disc configuration are not considered "degenerations" (Moffet, 1984), they are classified in: (a) deformation, the passive change in structure that occurs in response to compressive loading; (b) deterioration, the change that occurs passively in response to frictional movement; (c) perforation or fracture, resulting either from extensive deformation or deterioration; (d) displacement, loss of structural contour resulting in the

alteration of disc-condyle relationship and (e) dislocation, gross displacement of the disc from its original site with no apparent cellular changes (Bell, 1986; Bell 1990).

1.2.1. Importance of the Articular Disc in TMD

The non-inflammatory disorders of the TMJ are designated by the articular disc-interference disorders in which the articular disc is chiefly responsible for the symptoms (Bell, 1990). This type of interference also predispose the joint to degenerative change, in other words, failure of nonpathologic adaptation of the disc tissues (Bell, 1990) This is the result of a high number of the cases which are expressed with the presence or absence of noises and pain (Solberg, 1986). Most interferences in the temporomandibular joint that originate from the articular disc are due to: adhesions between the disc and the condyle, change in the normal disc anatomy and disc displacement/dislocation.

Adhesions between the disc and the condyle can occur as a result of trauma, surgery, infection or rheumatoid arthritis, preventing normal rotatory movement. This causes an irregular cycle with noises, skidding back and forth predisposing to degenerative arthritis. The term "deformation" is used to indicate a change in configuration that occurs passively in response to physical compression (Moffet, 1984). Discs that continue to be compressed in one area, central or elsewhere, may become so thinned in the area that they will have a perforation due to over compression, not to degenerative changes of the disc tissue (Moffet, 1984). As a result of disc form alteration, the main damages consist of roughening, thinning, perforation and fracture. According to Bell (1986, 1990) disc related interferences are divided into: Class I, class II, class III, class IV and class V groups.

The first interference occurs in the closed position of the joint as a result of maximum intercuspation of the teeth, caused usually by a chronic occlusal disharmony of a kind and magnitude that displaces the disc-condyle complex. The resultant depend on the degree of the disharmony, and may predispose to a class II interference. Following the

intercuspation relationship, the second interference takes place immediately after the mouth opening. The symptoms that characterize it are an initial sensation of sticking, followed by a discrete click and some discomfort. At this stage the articular disc is considerably affected by the strain derived by this interference. If the articular disc loses its contour, it is an indication of class III interference (Bell 1986), however, this particularity is not well explained. The symptoms associated with a class III interference disorder appear during the translatory cycle. The result of such interference may be momentary locking occurring from catching of the articular disc between the condyle and the eminence. Excessive passive interarticular pressure, lack of compatibility in the shape of articular surfaces and some derangement or impairment of function involving the disc-condyle complex are the main causes of class III interference.

The interarticular pressure is usually increased in situations of emotional tension and can be an activating factor for the other situations of discal interference. Structural incompatibility is a result of many factors, especially trauma in the TMJ area; and impairment of disc-condyle complex is related to the deviations of disc form as well as disc dislocation/displacement and to the change in the proper mobility of this complex. Noises and irregular movements are typical symptoms (or signs) of this abnormality (Solberg, 1986).

The dislocation/displacement are subdivided into functional displacement and functional dislocations. The consequences that arise from the former are dependent on the location of the destructive change in the disc (thinning of the posterior margin permits functional displacement in an anterior direction and vice-versa) , extent of the discal ligaments and the type of movement executed. The latter is a further consequence from the former.

Class IV interference disorder or joint hypermobility is a subluxation characterized by a partial or incomplete dislocation of the articular surfaces of the joint, reached when the

disc can no longer rotate posteriorly on the condyle being limited by the condylar articular facet (excessive opening of the mouth, usually on a habitual level). The last interference, namely, class V or spontaneous dislocation is a deficiency of a sharp contact of the articulating surfaces during translatory cycle. Attributed to a sudden extra contraction of the upper head of the lateral pterygoid muscle (Bell, 1986), this anterior disc displacement frequently happens during yawning and when the muscles are fatigued by keeping the mouth open too long (Travell *et al.*, 1983).

2. Glycosaminoglycans and Proteoglycans in Connective Tissue

Extracellular matrix consists mostly of water and a variety of polysaccharides and proteins (Hukins, 1984; Alberts *et al.*, 1989). These molecules are secreted locally and assemble into an organized meshwork of fibers and ground substance that is largely responsible for the biochemical and physical properties of connective tissue matrix, making the tissue an ideal cushioning material that distributes the load to the bony surfaces. Among extracellular molecular complexes, soluble proteoglycans (PGs) together with insoluble fibrous protein collagen form the major structural elements of connective tissue (McDevitt, 1973). PGs are large ubiquitous molecules that are composed of a mixture of proteoglycan subunits that together with a glycoprotein-link are usually found in the form of large macromolecular aggregates (Tsiganos *et al.*, 1971). The individual polysaccharide chains that form these complexes are called glycosaminoglycans (GAGs) (Hardingham, 1981).

Due to the relative facility to extract PG molecules and the high GAG tissue content in cartilage (Stockwell, 1979; Hardingham, 1981; Bayliss 1984), these complexes were thought to be specific to that tissue (Wight and Mechan 1987) and most of the pioneering studies on the structure and properties of PGs have been done in cartilage (Frazen, 1984). The concentration of these molecules in cartilage is large as compared to other tissues, some 10% of the wet weight (Heinegard *et al.*, 1986), however the extraction of pure compounds is often an obstacle to PG study.

2.1. Structure and Morphology of GAGs and PGs

PGs play an important role as part of the supporting matrix of connective tissue. These substances are compounds of high molecular weight composed of a protein core with glycosaminoglycans (GAGs) covalently bonded as a sequence of side chains.

The structure of the GAG side chains consists of a hexosamine or amino sugar (either N-acetylglucosamine or N-acetylgalactosamine), alternating with another sugar which is uronic acid (either glucuronic acid or iduronic acid). Despite the fact that an individual chain may have a mixture of uronic acids, the hexosamine will always be a glucosamine or a galactosamine which are linked by alpha or beta glycosidic bonds (Hay, 1981; Silbert, 1982; Alberts *et al.*, 1989). Because the hexosamine occurs as every other sugar, GAGs are more easily defined as long polysaccharides chains made up of disaccharide repeating units (two sugar residues), which usually are very similar within a single chain (Silbert, 1982). GAGs are negatively charged under physiological conditions. The fixed, highly polianionic nature (due to negative charges of SO^- and COO^- mainly (Stockwell, 1979)) provides the ground substance with a great affinity to water which in turn, provides the articular cartilage with physiochemical stiffness (Kempson *et al.*, 1970). This anionic nature is associated with a cation, which in native state is predominantly Na^+. Cations such as Na^+ are osmotically active and cause large quantities of water to be trapped within the matrix creating a swelling pressure that enables the tissue to withstand compressive forces (Evered and Whelan, 1986; Hascall and Hascall, 1981).

Under physiological conditions, the conformation of GAGs is of long unbranched chains, the so-called random coil conformations. This is due to their negative charge density that creates a repulsion among them, making the molecule too inflexible to fold up into compact globular structures that polypeptide chains typically form (Evered and Whelan, 1986; Hascall and Hascall, 1981). Consequently, this arrangement reduces the number of probable conformations that a PG molecule could assume which increase considerably the space that the molecule occupies in relation to its mass (Wight and Mecham, 1987) resulting in the formation of gels even at very low concentrations. The nature of the three-dimensional network of these gels also enables the rapid diffusion of water-soluble molecules and the migration of cells through the cartilage matrix (Maroudas,

1973) exerting a sieve effect on the metabolites transported to and from the cells (Granstrom and Linde, 1973). GAGs chains are anchored to the protein core of PGs via a neutral trisaccharide (Gal-Gal-Xyl) at the reducing end of the GAG chain (Hardingham, 1981).

Compounds of GAGs are related to one another and they were classified according on the basis of different disaccharide units (Hardingham, 1981), on the number and location of sulfate groups and on the type of bonds between the sugars (Alberts *et al*, 1989). This classification formed four major groups (Alberts *et al*, 1989). The first group is composed by hyaluronic acid (HA) which is the only unsulphated GAG and is present in large amounts in most tissues. The second group comprises chondroitin$_4$sulphate (C_4S), chondroitin$_6$sulphate (C_6S) and dermatan sulphate (DS) followed by the third group with heparan sulphate (HS) and heparin (H) and the last group with keratan sulphate (KS) which is divided into KS I and KS II. For a review of GAG classes see Saamanen (1989).

The molecular weights of GAG chains varies within a range of 1-2 x 10^4, with the exception of HA which despite being the simplest of all GAGs, ranges from 10^4 to 10^7 (Toole, 1982; Bartold, 1987). HA has been related to water content, and the levels of HA may indicate the degree of hydration of the particular tissue (Toole, 1982).

Chondroitin sulfate can be classified into either C_4S or C_6S, according to the ester group in the N-acetylgalactosamine unit (Roden, 1980; Carney and Muir, 1988). Widespread throughout connective tissue, they may exist in many hybrid forms where both types (C_4S and C_6S) may appear in the same or different molecule (Hoffman *et al.*, 1956; Bartold, 1987). Classified under the same group as CS, DS differs by the presence of a L-Iduronic rather than D-glucuronic acid, however, the molecule may contain both types of uronic acids (Habuchi *et al.*, 1973; Bartold, 1987). HS and H have been the least studied GAGs. There are markedly structural heterogeneity in this group, such that they may be

more well defined as a family of similar polysaccharides (Bartold, 1987). Structurally, HS and H are the most complex group of GAGs (Lindahl and Kjellen, 1987).

KS was defined (Bartold, 1987) in two different types: KS I, typical of cornea and KS II, typical of connective tissues. Both forms have the same disaccharide sequence, however, they differ in their linkage to protein (peptide fragments) and in the degree of sulphation and galactosamine content (Carney and Muir, 1988). KS linkage to protein has no neutral trisaccharide (Gal-Gal-Xyl), but is linked via an O-glycosidic bond between N-acetylgalactosamine to serine or threonine (KS I) or via an N-glycosylamine linkage from N-acetylgalactosamine to aspargine (KS II) (Hardingham, 1981; Bayliss, 1984).

The structure of PGs may be comprised of either one type of GAGs or may be formed by several types of GAGs on the same protein core (Tsiganos and Muir, 1969; Stockwell, 1979; Silbert, 1982; Bartold, 1987). This variation also happens in the proportion of carbohydrate to protein over all the chains per core molecule (Bartold, 1987). PG molecules isolated from cartilage have shown that a single PG subunit consists of one core protein to which are attached approximately 250 carbohydrate chains (Hunziker and Schenk, 1987). From studies with enzyme and chemical degradation, a model of the PG subunit has been proposed (Heinegard and Axelsson, 1977) in which there are three regions of the molecule: one constant region rich in KS chains; a more extensive variable CS-rich region containing other types of GAGs; and the HA binding region containing a few short KS chains, which is also constant.

The common class of cartilage PGs forms very large aggregates in the extracellular matrix, which may have 30 or more PGs that are linked to a chain of HA, itself a GAG (Hardingham and Muir, 1972a). The size of the PG aggregate depends on the chain length of the HA molecule, which can bind up to 100 PG molecules, creating very large aggregates (MW of 2 X 10^8) (Hardingham, 1986). PG-HA interaction can be further stabilized by the link protein (Hardingham and Muir, 1972) which joins both PG and HA

together (Muir, 1981), and in physiological conditions it appears to be an irreversible process (Hardingham, 1979).

Another common family of PGs is the small interstitial PG, which frequently contain DS (Hascall, 1986). These molecules usually contain only small chains and are found in connective tissues with fibrillar collagen matrices which are probably associated with specific sites along the collagen fibrils (Scott, 1986). Small PGs do not have aggregation properties (Heinegard *et al.*, 1981a). In addition, there are other PG compounds, such as proteochondroitin sulfate and protodermatan sulfate (PDS) (Kresse, *et al.*, 1986) that have not received too much attention. These are members of the class of small PGs which are widely distributed in connective tissues (Poole, 1986; Scott *et al.*, 1989). PDS has been shown to be associated with the surfaces of collagen fibers (Scott, 1988; Nakano and Scott, 1989).

Aggregate PGs are also called high bouyant density PGs and non aggregating PGs are called low bouyant density PGs (Thonar and Kuettner, 1987). From the family of high bouyant density, there are two different types of PGs, one having a high content of C_6S and the other one with a relatively increased amount of KS (Heinegard *et al.*, 1981a; Frazen, 1984). Although most of the high weight PGs are aggregates, there are complexes that do not form aggregates (Tsiganos and Muir, 1969; Brandt and Muir, 1971; Stockwell, 1979; Frazen, 1984), and demonstrate and increased protein content (Heinegard and Hascall, 1979). Generally, the protein core accounts for one tenth of the total molecular weight (Hascall and Riolo, 1972).

PG aggregation is suggested to have other functions besides of their once thought, immobilization in the matrix. The destruction of aggregates can alter the physical properties of the cartilage allowing the PG to diffuse in the tissue, which enables endogenous collagenase to remain activated in the matrix (Muir, 1981), once separate components of an

aggregate are not inhibitory to mammalian collagenase as opposed to the stable link-aggregates (Harper and Muir, 1981).

PG complexes contain other components, other than GAG chains, that have been not as well characterized such as small chains of oligosaccharides (N- and O- linked) and link proteins. The characterization of oligosaccharides is due to the amino acid residue to which they bind in the protein core. N-linked is when the sugar moiety is bound to aspargine and O-linked is when the sugar moiety binds to serine or threonine residues. The latter has been suggested as primer molecules of KS II in various stages of completion (Carney and Muir, 1988). The oligosaccharide chains are most likely too short to be identifiable and are present in a rate of around 100 per core protein (Hunziker and Schenk, 1987). Their function is yet to be identifiable (Bartold, 1987), however, their abundance accounts them as having an important role in the biosynthesis or structure of PGs (Carney and Muir, 1988).

PG are found spread out in all tissues and not only participate as supporting structures but also as mediators of events that characterize development and various diseases (Wight and Mechan, 1987). PG aggregation is an important feature of cartilage, however, how this mechanism is important to cartilage function is not known. It has been suggested that aggregation would immobilize PGs within the network of collagen fibers creating a large osmotic swelling pressure rendering cartilagenous tissues stiff and resilient (Hardingham, 1986), therefore, enhancing their ability to resist compressive deformation (Hardingham, 1979).

Due to the complexity, extensive variation, and location of most GAG complexes, it is unlikely that single functions can be attributed to them (Gallagher *et al.*, 1986). Moreover, from different GAG molecules, few functions are known or even suspected (Poole, 1986). GAG chains play a central role in the regulation of the extracellular matrix

production and organization and have been reported as stabilizers to the differentiated state of cells (Toole, 1982; Bronsson *et al.*, 1988).

The most widespread GAG, HA has been involved in several biological processes, including a critical role in the aggregation of cartilage PGs (Hascall, 1986). The enzymatic degradation of HA has been postulated to control cell migration and cell differentiation in developing and remodeling tissues (Toole, 1982). When this migration ends there seems to be an increased proportion of hyaluronidase (enzyme that degrades HA, see Table II). This could be correlated to the formation of a swelling gel to facilitate cell migration during periods of development and cell repair rendering HA with a role in tissue morphogenesis (Toole, 1982; Laurent and Fraser, 1986). It has also been correlated with increased activity in macrophages and granulocytes and with cooperation in extracellular matrix in cell detachment (Laurent and Fraser, 1986).

CS-PG do not have an established role, but may perform an important function in dissipating load applied to the tissue, since it is present in regions subjected to large amounts of stress, such as cartilage and tendon (Vogel and Heinegard, 1985; Poole, 1986). The large amounts of CS in cartilage possibly indicates this GAG chain as an important parameter in cartilage formation (Toole, 1982).

DS is mostly distributed in the form of a small, interstitial PG (DS-PG) present in the extracellular matrices of connective tissue (Heinegard *et al.*, 1985). Among the important biological properties that DS-PG have, they bind non-covalently to collagen fibrils and fibronectin and inhibit fibrilogenesis *in vitro*, as well as the adhesion of fibroblasts to fibronectin. They also inhibit collagen fibril growth *in vitro* (Vogel *et al.*, 1984; Vogel and Trotter, 1987). Through the enhancement of gap-junctions they enhance cell-cell communication (Rosemberg *et al.*, 1986).

Heparan sulfate proteoglycans (HS-PGs) may be firmly anchored to the plasma membrane of all mammalian cells (Poole, 1986). This interaction is held by a protein core

that is inserted into the plasma membrane (Fransson *et al.*, 1986). When present at the cell surface it may regulate the cell-substrate adhesion and cell proliferation; it may also participate in the binding and uptake of extracellular components and in extracellular matrix formation (Hook *et al.*, 1984; Fransson *et al.*, 1986; Poole, 1986). In addition, HS-PGs have been reported to have a role in the control of cell growth (Hook *et al.*, 1984; Fransson *et al.*, 1986; Fransson, 1987).

Heparin as well as HS have been attributed a variety of biological functions, most of which involving interactions with other macromolecules. The best known example is the blood anticoagulant activity, which depends on the H binding to antithrombin, a strong protease inhibitor (Lindahl and Kjellen, 1987). It may also suppress the proliferation of smooth muscle cells *in vivo,* inhibit DNA and RNA synthesis, and produce qualitative changes in the biosynthesis of both collagenous and non-collagenous proteins (Poole, 1986). Subgroups of PGs such as protochondroitin sulfate and PDS are involved in the control of the orientation and growth of collagen fibers (Kresse *et al.*, 1986; Scott and Nakano, 1989), and the latter may have a role in inhibiting mineral deposition within the collagen fibrils on the normally uncalcified tissues (Scott *et al.*, 1989).

2.2. Synthesis and Degradation of GAGs

In view of the complexity of these molecules, there is a vast literature about GAG synthesis and degradation. Nevertheless, it is not the purpose of this review to expose details of very specific aspects of GAG synthesis that would have little relevance to this project. For a more detailed review see Roden (1980), Silbert (1982) and Hardingham *et al.* (1984).

All the GAGs are synthesized on a core protein primer, with the exception of HA (Spooner and Thompson-Pletscher, 1986). The site of biosynthesis for HA has been

determined to be the plasma membrane and the addition of sugars takes place at the reducing ends of the molecule (Laurent and Fraser, 1986). GAG chains are initiated from sugar nucleotide precursors (Silbert, 1982) with the synthesis of a sugar bridge which is carried out by the addition of N-acetylgalactosamine and glucuronic acid to the chain via their UDP derivatives (Silbert, 1964). The uridine sugar nucleotide precursors form a pyrophosphorylase type of reaction (Silbert, 1982) with UDP-glucose and UDP-N-acetylglucosamine, which in turn form the 'puddling blocks' for polymer synthesis (both can be formed by a reaction of UTP with a sugar-1-P in the presence of homogenates from proteoglycan-producing tissue). The two forms of nucleotide sugars are the products of the two metabolic pathways which are converted where necessary to forms suitable for inclusion in the various GAGs synthesized (Stockwell, 1979). UDP-glucose can be oxidized to form UDP-glucuronic acid which can be decarboxylated to form UDP-xylose. Both UDP-glucose and UDP-N-acetylglycosaminoglycan undergo epimerization at the C-4 position to form UDP-galactose and UDP-N-acetylgalactosamine respectively (Silbert, 1982). GAG sulphation occurs by the transfer of a sulphate group from PAPS to the sites on the GAG after the incorporation of the sugars (Silbert, 1973).

Synthesis of the O-linked GAGs (all except HA and KS), starts by the xylosilation of serine residues in the core protein. The subsequent addition of xylotransferase, galactotransferases I and II are going to form the linkage region, that serves as primer for the addition of the particular hexosamine and uronic acid in each GAG chain (Spooner and Thompson-Pletscher, 1986). Post-translation modifications of the core protein and the processing of O- and N-linked oligosaccharides happens further in the process when the molecules undergo extensive modifications (Spooner and Thompson-Pletscher, 1986).

PG molecules are secreted into the pericellular environment as soon as they are completed in the Golgi apparatus (Carney and Muir, 1988). Although PG are able to aggregate as soon as they are synthesized, there is some evidence that a maturation step is

also required, to convert PG from an inactive to a fully active aggregating form (Hardingham *et al.*,1984). However, not all PGs are secreted into the matrix, since some complexes remain attached to the lipid bilayer. These types of PGs usually contain a small number of GAG chains (Hook and Kjellen, 1984).

As summarized by Hardingham *et al.* (1984), the synthesis, secretion and assembly of PG aggregates for cartilage is as follows: (1) synthesis and processing of mRNA for PG protein core, (2) translocation of mRNA cisternal space of the RER (likely site for N-linked oligosaccharide transfer), (3) translocation from cisternal RER to the Golgi apparatus, (4) biosynthesis of CS, probably KS and O-linked oligosaccharides, (5) transport of completed PG to secretory vesicles and to the plasma membrane, (6) secretion of PG into the extracellular space (probably already associated with link protein), (7) secretion of HA through a different pathway and (8) assembly of PG-link protein with HA to form stable PG aggregates.

For PG and GAG degradation, the normal catabolic pathway of carbohydrate moieties of PG occurs by a set of reactions that has been regarded to be the opposite of the synthetic process (Roden, 1980), the mechanism of PG turnover is not clear. It has been suggested that there is a probable degradation of the GAG moiety, but the degree that this occurs is not known. Moreover, as studies indicate that some intact GAG can be found in the urine, both mechanisms may exist concomitantly (Silbert, 1982). The enzymatic degradation of GAGs or PGs was first noted as a spreading factor (Silbert, 1982). Experimental works were correlated with the *in vivo* situation suggesting that enzymes similar to the reported spreading factor were also present in GAG breakdown *in vivo*. Due to the structure of PG aggregates, two major groups of enzymes are needed for degradation. These are proteinases and glycosidases (Stockwell, 1979). In a stepwise manner, the enzyme groups remove the glycosyl as well as sulfate groups starting from the

non-reducing end of the molecule (Roden, 1980). The best defined GAG degrading enzyme is hyaluronidase (Silbert, 1978; Roden, 1980).

Although endoenzymes are capable of extensive fragmentation, they are not capable of adequate degradation alone due to the accumulation of the partially degraded material in the mucopolysaccharidoses (Roden, 1980).

2.3. Normal Distribution of GAGs in Connective Tissue

According to Hunziker and Schenk (1987) the extracellular matrix in connective tissue is divided in two phases. The former is composed of a variety of insoluble fibers and the latter is often called of amorphous ground substance, a homogeneous and transparent layer. Comprising almost 95% of the content of nonfibrous dry-weight material, PG complexes are the major components of the ground substance (Hunziker and Schenk, 1987). This concentration is virtually achieved in consequence of the existence of PGs in an underhydrated state (around 20 to 40 mg/ml). Such characteristics pose major problems for aqueous chemical fixation of connective tissue which reduces the scope for study of these macromolecules (Hunziker and Schenk, 1987).

Collagen and PGs are the major components responsible for the unique characteristics of the articular cartilage regarding reversible deformity, essential for its function (McDevitt, 1973). In cartilage, PGs consist of a series of subpopulations which differ in size and composition. Most of the PG content exists in the form of large aggregates in the presence of a link component (McDevitt, 1973), forming an amorphous gel (Sweet *et al.*, 1977), and nonaggregating PGs make a small percentage of the total PG content (Muir, 1980; Myers and Mow, 1983). In cartilage, these compounds are always bound to protein (McDevitt, 1973). PG distribution in cartilage has been found to be located mainly in the middle region, where about 90% of GAGs aggregate with HA

(Maroudas, 1979). The GAGs of cartilage are C_4S, C_6S, KS and HA (McDevitt, 1973; Stockwell, 1979, Carney and Muir, 1988), although there is a considerable variation between different areas of cartilage (Silbert, 1978). In hyaline cartilage the most abundant PG is a high aggregating species containing both CS and KS chains. The KS content is more variable and usually increase in proportion with age (Hardingham *et al.*, 1986).

As reported by Scott *et al.* (1989), the type, concentration and distribution of PG complexes in connective tissue reflect the load regimen that the tissue is exposed *in vivo*. The marked heterogeneity of the ground substance in relation to the cell and the staining techniques show areas of basophilia and/or alternating zones of basophilia and acidophilia around the cell. Stockwell (1979), based on this finding, noted that the matrix was divided into zones more or less concentric to the individual cell or cell groups.

However, in normal cartilage the GAG content varies in distribution according with the depth of the surface (Stockwell, 1967; Lemperg *et al.*, 1974; Bayliss *et al.*, 1983) and topographically in different areas that are essential or non-essential for articulation (Bjelle, 1974). The total amount of GAGs range from 5 to 15% of the dry weight of the cartilage. CS is the main component of cartilage (50%) and KS varies from 4 to 20% of the total, while HA is less than 1%, which reflects the aggregation of CS-PG (Silbert, 1978). In the articulating areas of maximum contact there is more HA and much more KS than in areas of minimum contact (Sweet *et al.*, 1977). CS is predominantly territorial while KS is interterritorial, except in the deeper zones of older tissues when KS is present close to the chondrocyte. Normally, there is an increase in the amount of KS in the deeper layers of cartilage (Hardingham *et al.*, 1986) and PG complexes are increased in the middle zone as compared to the articular surface (Ratcliffe *et al.*, 1984).

There is a special group of PG molecules that can be present on the cell surface, and the most predominant is HS as reported by Spooner and Thompson-Pletscher (1986).

Extracellular matrix from basement membranes is also rich in PGs, varying from CS, DS and HA (Spooner and Thompson-Pletscher, 1986).

In the articular discs, the cellular matrix consists of sheets of collagen fibrils and fibers dispersed in a sparse interfibrillary matrix, except in the immediate vicinity of the cartilage cells (Granstrom and Linde, 1973) where PG complexes and associated filaments are found (Ghadially, 1978). In the central zone of the disc, the interterritorial matrix consists essentially of dense fibrous tissue (Silva, 1969). This composite structure confers the disc with a clearly fibrochondrocytic appearance and a considerable degree of flexibility (Silva, 1969).

Fibrocartilage tissues differ from those of hyaline cartilage. For instance, approximately 50% of the total amount of GAGs of the nucleus pulposus is KS and the rest is mainly CS (Hallen, 1958). Habuchi *et al.* (1973) indicated that meniscal GAGs represent a separate family, that may be specifically adapted to the function of fibrocartilages. Interarticular disc PGs have a smaller size and a larger amount of KS chains than the articular cartilage, however, they have the same capability of aggregation (Muir, 1981). Generally, PGs are rather minor components of fibrous connective tissues comprising less than 1% of the dry weight of the tissue (Vogel and Heinegard, 1985).

Topographical distribution of GAGs is marked in the central part of the disc as revealed by a marked uptake of [^{35}S] in both guinea pigs (Oberg, 1964) and in human subjects (Kopp, 1976), however the main localization of the sulphated GAGs correspond to the infero-posterior part of the TMj tubercle with the presence of molecules with characteristics to CS/DS and KS (Kopp, 1976). By contrast, Mills *et al.* (1988) found that the distribution of GAGs is restricted mainly to the anterior and posterior bands of the rabbit articular disc, with distinct populations of C_6S and KS. This is in accordance with quantitative analysis of the GAG component from bovine articular disc (Nakano and Scott , 1989). However, the major GAG components in the latter turned to be CS and DS with

smaller proportions of HA and KS. On the other hand, Granstrom and Linde (1973) found that DS and HA are the two major GAGs of the articular disc. DS was found to be the largest fraction in the rabbit, dog and monkey articular discs, while HA was found to be the major fraction in the rat disc. The analysis of the PG content of the rabbit menisci indicated that the sulphated GAGs consisted of C_6S (72%), C_4S (19%) and DS (5%) (Webber *et al.*, 1984). Similarly, Adams and Muir (1981) characterized the PGs of the canine meniscus, finding C_6S as the major GAG, followed by C_4S and DS. KS was not detected in either study.

The variation of PGs content in different tissues of different animals, may be due to either differences in the weight-bearing role of the articulations or simple species differences (Webber *et al.*, 1984). Although, the variability of GAG concentration in different connective tissues subjected to different degrees of stress is not very distinct, the proportion of small and large PGs in each fibrous tissue may be an important indicator of their roles in producing and maintaining a functional matrix (Vogel and Heinegard, 1985).

3. Collagen Physiology

Collagen is the major protein found in all eukaryotic organisms (Miller, 1978) estimated to account for 25% of the body protein content (Parry and Craig, 1988), that is vital for the maintenance of the extracellular matrix (Berg, 1986). These characteristics *per se* render the understanding of collagen physiology as a very important step for the overall knowledge of connective tissue behavior (Parry and Craig, 1988).

The functional integrity of tissues such as bone, cartilage and skin and the structure of others such as blood vessels and most organs is dependent on the pattern of their collagenous framework (Nimni and Harkness, 1988), since collagen is not only a family of highly fibrous proteins (Alberts *et al.*, 1989) that contributes for the structure of connective tissue, but also those structures that are subjected to mechanical stresses. Collagen molecules are very diverse, which may be account for the variation of functional demands in different types of connective tissues.

3.1. Collagen Structure

Collagen molecules have been divided in many different types, and so far 11 have been identified. Each collagen type is identified by an array of the 20 different α-chains isolated. Each of these α-chains is encoded by a different gene, which when combined express different collagen types in different tissues (Martin *et al.*, 1985; Miller, 1988).

The monomeric unit of collagen is a triple-stranded helix made of three α-chains composed of about 1050 amino acids each (Miller, 1978). The collagen chains are wound around each other forming a regular superhelix to generate a ropelike collagen molecule. Collagen α-chains are also called polypeptides due to the name of the linkage between amino acids that is called a peptide bond (Alberts *et al.*, 1989). The triple-helix structure generates a symmetrical pattern of three left-handed helical chains, that can be slightly

displaced to the right (Nimni and Harkness, 1988). The collagen types differ from each other in the primary sequence of their α-chains, in the extent of their posttranslational modifications, and their distribution in different tissues (Berg, 1986).

The distribution and function of most of the collagen types is not very clear. However, from all the known types, the types I, II, III and IV are the most widespread (Alberts *et al.*, 1989). The types I, II and III are connective tissue collagens and are also called fibrilar or interstitial collagens, since they are apparent always located between the cellular component of a given tissue or organ (Miller, 1978). Type IV collagen is present at the basement membranes, forming a network structure (Berg, 1986). The other types of collagen will not be included in this review.

Type I collagen is composed of two α_1(I) and one α_2(I) chain and is found widespread in connective tissue. Type II collagen is typical of cartilage and has three identical α_1(II) chains (Miller, 1973). Type III collagen contains three identical α_1(III) and is found in skin and in arterial walls. Initially called foetal collagen, due to its abundance in fetal tissue, type III collagen is often associated with tissues or conditions that require fast collagen turnover, such as during development and tissue repair (Uitto *et al.*, 1989). The last of the common collagens or type IV collagen has three identical α_1(IV) chains and is typical of basement membranes. The primary structure has been revised by Berg (1986) and Uitto *et al.* (1989).

The most common collagen types, or fibrilar collagens are secreted by connective cells, mainly fibroblasts into the extracellular matrix where they will form organized cable-like structures, that are called collagen fibrils (Alberts *et al.*, 1989). Among cell types that synthesize collagen, chondroblasts and osteoblasts are specific for cartilage and bone, respectively. The organization of the extracellular matrix in connective tissue will be, therefore, dependent on the interaction of the collagen fibrils that usually form fibers which are disposed according to the functional requirements of the tissue.

The most important and evident function of the collagen molecule is to provide a tensile mechanical resistance (Parry and Craig, 1988). The maintenance of the shape of the tissue is the most obvious mechanical function, since the special arrangement of collagen is responsible for the resistance of stretching forces that will tend to alter the tissues original shape (Nimni and Harkness, 1988).

3.2. Biosynthesis and Degradation of Collagen

Biosynthesis of collagen is a complex process that involves several specific intracellular steps (Berg, 1986). It is important to mention that the majority of information of synthesis and degradation is available only for the well known fibrilar collagens,types I, II and III. As they constitute by far the most widespread collagen molecules, most of this review will concentrate on these types.

Transcription from the collagen genes is followed by gene splicing to yield a functional mRNA with approximately 3000 bases, that are translated to the RER on the membrane bound ribosomes (Nimni and Harkness, 1988) that synthesize the larger precursors that are called proαchains (Olsen, 1981; Berg, 1986). These molecules are high molecular weight and can be also known as procollagen proαchain and they are characterized by the presence of additional polypeptide extensions located at the amino and carboxyl terminal of the α-chains (Berg, 1986). These are the signal peptide and the propeptide sequences. The main function of the signal peptides is the recognition and insertion of the molecule in the lumen of the ER.(Alberts *et al.*, 1989), and has been shown to be proteolytically removed coincidently with the transport into the lumen of the ER (Palmiter *et al.*, 1979).

Collagen is also subjected to various posttranslational modifications that are numerous potential sites for regulating the amount of final product that becomes

incorporated in the extracellular matrix (Berg, 1986). Following the modification of procollagen in the lumen of the RER, these molecules are secreted via the secretory vesicles of the Golgi apparatus (Berg, 1986). Secretion of collagen differs from the other secretory proteins, since collagen is continuously secreted by the cell (Tartakoff and Vassali, 1977) as compared to other proteins that are secreted upon signal transduction mechanisms (Berg, 1986). Collagen molecules must be in triple-helical form to permit efficient secretion, and therefore, any condition that prevents the attainment of this helicity reduces the rate of secretion (Miller, 1978). After secretion, the procollagen molecules are proteolytically separated from the amino- and carboxyl-terminal ends, so the chains can then assemble to form the fibril arrangement (Uitto *et al.*, 1989).

In the extracellular space, procollagen is transformed in tropocollagen molecules, which requires the activity of at least two types of proteases (Miller, 1978). The subsequent extracellular aggregation of collagen molecules does not occur as a result of a spontaneous process, but by a regulated cellular mechanism (Trelstad, 1982) and only occurs after the modifications of procollagen into tropocollagen have been completed. Tropocollagen or only collagen molecules (ranging about 1.5 nm in diameter) will associate to form collagen fibrils (Alberts *et al.*, 1989).

After formation, collagen molecules display a remarkable resistance to proteolysis (Jeffrey, 1986; Stricklin and Hibbs, 1988). The incorporation of molecules into fibrils and the subsequent cross-linking further stabilizes the molecules, raising the denaturation temperature of a single molecule from 40°C to more than 60°C (Kuhn, 1987). To deal with this problem, a class of special enzymes called collagenases have been identified. Collagenase types form a heterogeneous group of proteinases that participate in collagen degradation and they were classified according to their substrate specificity (Stricklin and Hibbs, 1988). They are very widespread in mammalian tissues, and some tissues such as skin contain natural collagenase in the extracellular matrix (Bornstein and Sage, 1980;

Stricklin and Hibbs, 1988). Collagenase attacks collagen at specific sites in the molecule, splicing it into two fragments that will denature in physiologic conditions (Harris, 1978; Jeffrey, 1986). Collagen cross-linking appears to reduce the degree of degradation, which suggests that cross-linking plays a role in the mechanical stability of collagen and in the regulation of collagen turnover *in vivo* (Nimni and Harkness, 1988).

3.3. Collagen Distribution in Connective Tissue

From the common collagen types, type I collagen is by far the most abundant and it can be found in skin, bone, tendon, periodontal ligament, dentin, fascia, placenta, blood vessels, interarticular discs and meniscus (Bornstein and Sage, 1980; Yen *et al.*, 1989). Collagenous tissues are more likely to be organized in specific arrays disposed in order to accommodate the physiological stresses that are placed upon them (Baer *et al.*, 1988). The disposition of such stresses will determine the orientation and organization of the collagen network (Baer *et al.*, 1988).

The shear stiffness of tissues like cartilage and articular disc is given by the tensile stiffness of collagen fibrils, since the arrangement of the fibers is such that they are stretched when the tissue is sheared (Myers *et al.*, 1988). Collagen content in cartilage is highest in the superficial layer and declines with depth. This represents an inverse correlation of collagen and cartilage (McDevitt, 1973). In hyaline cartilage, the collagen fibers are relatively small in size and thickness. They are often masked by an amorphous ground substance in which they are embedded (Yasui and Nimni, 1988). Electron microscopy shows a branching meshwork of fine collagen fibers without a cross-striation pattern as compared with adult articular cartilage that contains large fibers.

In the the articular disc it has been reported that the collagen content, as in other fibrocartilages, may be exclusively type I or a combination of Type I and II collagens

(Miller, 1978). The major constitution of the articular disc is of a dense, packed fibrocartilagenous network of type I collagen fibres that surrounds chondrocyte-like cells and their matrices (Mills *et al.*, 1988; Yasui and Nimni, 1988). Hirschmann and Shuttleworth (1976) also described the pattern of collagen distribution in the articular disc as represented by its densely packed fibrils in a well defined band, which is characteristic of that of type I collagen. Collagen comprises about 83% of the dry weight of the articular disc extracted from bovine subjects (Nakano and Scott, 1989). Similar tissue such as the intervertebral disc has been defined as a composite structure in which a gel-like nucleus pulposus of type II collagen is contained within the concentric fibrous lamellae of the annulus fibrosus (Baer *et al.*, 1988). However, the wall of the annulus is also composed of collagen type I fibers whose morphology is similar to that of the tendon (Baer *et al.*, 1988). The fibrocartilage of the annulus fibrosus was shown to contain both type I and type II collagens (for review see Miller, 1978).

In cartilage, collagen comprises 50% to 80% of the dry weight or 20% of the wet weight as compared with the meniscus, where collagen comprises up to 90% of the dry weight or 20% to 25% of the wet weight (Myers *et al.*, 1988).

4. Normal Development and Mechanical Stress in Connective Tissue

The differences in functional requirements of different stages in life could account for major compositional changes closely related to maturity. Compositional changes could be a more appropriate criteria of development or aging. As Thonar and Kuettner (1987) pointed out, not all age-related changes would play causative roles in the development of a functional impairment.

Some of the age-related changes in cartilage have been related to the regimen of load and stress applied to the articular cartilage surface of a joint (Thonar *et al.*, 1978). Even though, the changes may reflect alterations of the level of synthesis of macromolecules that constitute the extracellular matrix, it has been suggested that some of the changes may result in modifications of these macromolecules (Thonar and Kuettner, 1987). The alterations in the net quantity of PG and their structure may either be the result of an increased catabolism or of a decrease in synthesis (Frazen, 1984).

4.1. Normal Development of GAGs and Collagen

The distribution of GAGs depends on age or degree of maturation (Lemperg *et al.*, 1974). There is some controversy regarding the concentrations of individual GAGs. Kaplan and Meyer (1959) reported an increase in KS associated with the decrease in concentration of CS. On the other hand, Bollet and Nance (1966) noted no change with age. According to Roughley *et al.* (1981), PG monomer subunit and its CS decrease, while the KS, oligosaccharide and protein contents increase. There seems to be a trend towards an increase in the ratio of KS to glycoproteins and a decrease in CS (Kaplan and Meyer, 1959; Rosemberg *et al.*, 1965; Bjelle *et al.*, 1972; Hjertquist and Lemperg, 1972), an increase in KS and/or HA (Bayliss and Ali, 1978; Bayliss and Ali, 1981) in the aging

process. After development the changes in GAGs are much slower and most of GAG molecules seem to stabilize (Carney and Muir, 1988), but it seems that the normal aged cartilage lacks the ability to bind to HA. PGs may not lose their ability to aggregate with HA, which causes a decrease in extractability of PG with age (Bayliss and Ali, 1978, Saamanen, 1984). Also, the age-related change parameters are not supposed to be influenced by the degree of weight bearing in cartilage (Roughley *et al.*, 1981).

Collagen fibril diameter has been shown to increase with age (Parry and Craig, 1988). Although, there is a relative variability of changes from collagen of different human tissues and donor animals, the type I collagen fibers remain relatively constant as the larger fibers, while the smaller fibers or collagen type III fibers tend to increase slightly (Luder *et al.*, 1988). In fetal or immature connective tissues, collagen is present in small and very widespread bundles with relatively constant diameter collagen fibrils (Parry and Craig, 1988). With advancing age, the pattern of collagen shows a more strictly organized structure with an increased amount of inter- and intra-molecular cross-links (Viidik, 1980).

In cartilage, the collagen content progressively increases in the developmental phase undergoing considerable organization to become in later stages a relatively inert fibrilar network (McDevitt, 1973). The ratio of PG/collagen decreases with maturation and age, due mainly to an increase in collagen content (Saamanen *et al.*, 1987). Collagen chemical properties change with age. While soluble collagen tends to decrease, the amount of insoluble collagen becomes increased (Nimni and Harkness, 1988). The process of normal degradation is by itself a non-dependent age phenomena (Nimni and Harkness, 1988).

4.2. Normal Mechanical Stress in Connective Tissue

Connective tissue which is subjected to pressure and shear forces tend to develop into a fibrocartilagenous tissue (Remagen and Morscher, 1984; Daniel and Mills, 1988).

Numerous stress analyses indicated that the stress history plays an important role in controlling connective tissue biology (Carter, 1987).

In connective tissue, the ability to absorb the high loads takes place through the retention and diffusion of water and is particularly related to PG and GAG molecules (Myers and Mow, 1980). The amount of stress in which the chondrocytes are subjected to regulates the synthesis and degradation of these components (Lohmander, 1988). As reported by Scott *et al.* (1989), the type, concentration and distribution of PG complexes reflect the load regimen that the tissue is exposed *in vivo*.

There have been a number of studies of response of cartilage to mechanical load and other aspects of its metabolism in tissue culture (De Witt *et al.*, 1984). Most of the works used isolated cultured chondrocytes from epiphyseal cartilage, focusing on the changes of Ca^{++} uptake and cyclic AMP levels (Rodan *et al.*, 1975; Veldhuijzen *et al.*, 1979) and in the increase of [^{3}H]-thymidine, [^{35}S]-sulphate and [^{3}H]-glucosamine incorporation (Van Kampen and Veldhuijzen, 1982; De Witt *et al.*, 1984, Copray *et al.*, 1985; Veldhuijzen *et al.*, 1985; Van Kampen *et al.*, 1985; Saamanen *et al.*, 1987). Chondrocytes cultured *in vitro* for up to 24 hours under continuous load showed an increase in the incorporation of [^{35}S]-sulphate and [^{3}H]-glucosamine into GAGs and the size of the PGs synthesized appeared to be slightly larger (De Witt *et al.*, 1984). According to De Witt *et al.* (1984), under the influence of the compression of the collagen network, the chondrocytes would respond the same way as if compressed directly. Intermittent compressive force applied for the same period of time described above (24 hours) *in vitro* similarly resulted in increased PG synthesis and increased non-extractable PG component which are firmly anchored in the matrix (Van Kampen *et al.*, 1985; Saamanen *et al.*, 1987). This means that intermittent force improves the aggregating capacity of PGs and its coherence capacity with other matrix components. The entrapment of PGs in the collagen network certainly plays a role, once more PGs were extracted when the tissue was minced

before the extraction. Matrix production is enhanced in the initial response of the repetitive articular trauma which describes intermittent type of force (Muir, 1977). Studies of mechanical stress in condylar cartilage *in vitro* show that small loads may reflect alterations that are not seen in cartilages subjected to higher forces (Thomas *et al.*, 1984). *In vivo* studies of cartilage show that there seems to be only a modest loss of GAGs and PGs as compared with degenerated tissue (Sin *et al.*, 1984).

The synthesis of GAGs and PGs also increased in tendons cultured *in vitro* with mechanical load. Longer periods of load increase the synthesis of GAGs while shorter periods stimulate DNA synthesis (Slack and Flint, 1984). Although, the synthesis of GAGs seems to increase in specimens with the application of mechanical stress, it will return to its control level when the absence of pressure on the matrix is restored (Schneiderman *et al.*, 1985). The distribution of PG in tendon varies according to the distribution of forces in the tissue. In parts of the bovine flexor tendon subjected to tensional forces, PG molecules are formed mostly by small DS chains (Vogel and Heinegard, 1985), while in zones subjected to both compressive and tensional forces there is a considerable increase in large CS that have the ability to bind to HA (Vogel and Heinegard, 1985; Vogel *et al.*, 1986). In the fibrous proximal portion of the bovine tendon, the predominant PG was a small DS containing molecule, that comprised more than 88% of the total PGs extracted, while the minor reported was a CS-PG (Vogel and Heinegard, 1985).

4.2.1. Health and Disease in Connective Tissue

PGs are found in most of the matrices of all tissues providing not only support for cells and tissue turgor but also participating as mediators of many events that characterize a variety of diseases (Wight and Mechan, 1987). Degeneration particularly in cartilage,

results initially in an increased water content without any significant increase in the PG content (Mankin and Lipiello, 1970). In general, collagen stability is not as markedly affected by pathological conditions as compared with PG complexes (McDevitt, 1973). Maroudas (1981) reported that a damaged collagen network is often related to the basis of cartilage degeneration and PG breakdown.

Abnormal mechanical stress in connective tissue may be responsible for degenerations such as osteoarthritis in joint tissues (McDevitt, 1973). Osteoarthritis is a very common joint disease that is produced from various conditions, including injuries and diverse pathogenic mechanisms. This alteration is often related to the aging process, where the amount of physical stresses, generated by both weight bearing and muscle pull tends to be transformed by other factors or pathological conditions (McDevitt, 1973). In osteoarthritis, the balance between destruction and repair determines whether or not the changes are progressive (Thonar and Kuettner, 1987). Osteoarthritic cartilage shows a less stiff and weaker structure than the normal cartilage (Kempson *et al.*, 1972) which is due to the loss of GAGs, especially CS and KS, which has a greater influence on the stiffness component of cartilage (Kempson *et al.*, 1970). The meniscus of the knee in studies of experimental osteoarthrosis in dogs, which were subjected to increased joint loadings, showed an increased proportion of C_6S in the central portion, and HA in the lateral portion. The changes observed differ from those of cartilage, suggesting that some degree of meniscus repair may occur (Adams *et al.*, 1981). The osteoarthritic cartilage also has a reduced capacity to resist water loss under a given applied pressure than normal cartilage. As contradictory as it may seem, this is not due to a change in the quality of the PGs but to a decreased fixed charge (Maroudas *et al.*, 1985). There have been suggestions that senescence chondrocytes synthesize a PG that is less able to structure water that results in tissue fragmentation such as in osteoarthritis (Thonar and Kuettner, 1987).

Cartilage loading has also been studied in the joint tissues. The understanding of the nature of the phatological processes in the joint is partialy dependent on the assessment of the regulatory factors that maintain or restore a steady state metabolism of PGs in cartilage (Morales and Roberts, 1988). Generally, the proportion of GAGs and PGs tends to increase in joints subjected to heavy weight bearing experiments (see Saamanen, 1989). The aggregation of PGs in cartilage may also be influenced by the mechanical stress. It appears that PG aggregation is an irreversible process, and the turnover will either occur by protetolytic attack (Hardingham, 1979) or may be a result of an external mechanical stress. Saamanen (1989) reviwed the effects of running exercise on the proportion of PGs and GAGs in the load bearing joints. Intensive exercise increase the percentage of PGs, as compared to strenuous exercise that leads to injuries with an increase in cartilage fibrilation and reduction of PG content, PG aggregation and PG size.

Pathologic mechanical stress may act similarly to joint immobilization. Joint motion is fundamental for the integrity of the articular cartilage, however, as Palmoski *et al.* (1980) observed in the knee articulation, the absence of loading will lead to similar changes than those of created in immobilized joint. Systematic joint immobilization is associated with signs of osteoarthrosis, cartilage surface defects (Saamanen, 1989) and degenerative disease, such as massive intra-articular adhesions, cystis degeneration and possibly joint fusion (Behrens *et al.*, 1989). Among the alterations in the articular cartilage, there is a general decrease in the amount of uronic acid and an accumulation of water (Saamanen, 1989). Studies in joint fixation have been divided due to the time the joint is immobilized. Short term fixation does not have significant alterations for the most time (Enneking and Horowitz, 1972; Palmoski *et al.*, 1979) as compared to long term fixation that generally creates an irreversible alteration (Palmoski *et al.*, 1979; Tammi *et al.*, 1983; Behrens *et al.*, 1989). There is a variation according to the type of fixation used and the species and age of the animals used in the experimentations (Langenskiold, 1979; Tammi *et al.*, 1987). In

partially immobilized joints, a decrease in the dry weight has been reported (Behrens *et al.*, 1989), mostly due to a decrease in the amount of PGs counteracted by an incease in water content. However, this type of fixation allowed a complete recovery of the normal joint metabolism as compared to externally fixed joints that continued to deteriorate. Partially immobilized joints also showed a slightly reduced than normal PG-HA binding (Saamanen *et al.*, 1987). Other investigators have observed that the PG synthesis is reduced in immobilized joints and fails to aggregate with HA (Palmoski *et al.*, 1979; Palmoski *et al.*, 1980; Tammi *et al.*, 1983; Saamanen *et al.*, 1987). Comparatively, the contralateral joints that were not subjected to fixation, were exposed to an increased load regimen and the consequences were, for the most time, an increase in the hexuronate content and PG synthesis (De witt *et al.*, 1984; Saamanen *et al.*, 1987; Behrens *et al.*, 1989). Joint fixation supports the hypothesis that few PG remain in the matrix if they are not able to form stable aggregate complexes (Sandy *et al.*, 1978; Ratcliff *et al.*, 1986; Behrens *et al.*, 1989). These results demonstrate that the constant stress applied in this experiment may be the initial stage of molecular breakdown in the articular disc.

The correlation between mechanical force, organization and distribution of PGs and collagen fibers is very important for normal equilibrium of the articular disc function. This relationship is characteristically altered in cases of human disc displacement (Scapino, 1983; Blaustein and Scapino, 1986). Displacement of the disc resulted in extensive collagen fiber remodelling and decreased GAG staining. The artificially created environment of abnormal mechanical pressure causes the articular disc to respond with an internal reorganization and biochemical changes to adapt to the new function (Mills *et al.*, 1988). Similarly, the posterior attachment of the disc revealed fibrosis as a remodeling effect caused by the abnormal compressive loading of the condyle (Scapino, 1983). The same areas also had some evidence of hyalinization with the presence of some cartilage

cells (Hall *et al.*, 1984; Isberg and Isacsson, 1986) which were also scattered throughout the tissue (Carlsson *et al.*, 1968).

There seems to be a normal function of repair in long term organ culture, however, the loss of PGs that fails to aggregate is not recovered (Verbruggen *et al.*, 1985). This was demonstrated in the increased synthesis of GAGs in knee cartilage *in vitro*, which can then be interpreted as a repair mechanism (Luyten *et al.*, 1987). Although, the effect of repair in cartilage can be enhanced by the application of passive motion (Shimizu *et al.*, 1987), studies *in vivo* showed that repair tissue is of poor mechanical quality that does not contribute appreciably to weight bearing (Nelson *et al.*, 1988). In cartilage from osteoarthritic joints may not accumulate PG synthesis as evidenced by radioisotope studies, which can be considered as a failure to repair (Luyten *et al.*, 1987).

Mechanical stress forces have been proven to enhance the strength of wounds (eg. ligaments and tendons) (Urschel *et al.*, 1988). This enhancement may also be the result of an increased motion-induced enhancement of cellular nutrition (Gelberman *et al.*, 1981). This effect is also beneficial in wound healing of bone fractures (Urschel *et al.*, 1988) as has also been described by Goodship and Kenwright (1985), who looked at the stimulation of osteogenesis in bones subjected to intermittent forces. Although stresses are known to influence the wound tissue, the cells from these tissues show distinct phenotypic characteristics which do not seem to be the result of the environmental influences on the cells (Bronson *et al.*, 1989).

5. *In Vitro* Organ Culture for the Study of the Articular Disc

Connective tissue matrix has been extensively studied. The early approaches to explain the various components of this biological phenomena, independent of the animal studied, presented the inherited variability of results that could not be "individually" observed without the multifactorial relationship in the *in vivo* experiment. Such limitation of controlling all the *in vivo* factors, gave raise to more developed techniques, that were able to isolate parts of the tissue (or explants) to be analyzed independent of the interference of external factors that otherwise could account for the variability observed. These methods were denominated: *In vitro.*

With *in vitro* techniques or also called organ culture techniques, it is possible to develop a more consistent experimental model for the testing of *in vivo* theories. Although, there is a vast applicability for *in vitro* experiments, it is fundamental to determine wether or not the changes observed *in vitro* reflect what is observed *in vivo*, and not only an artifact caused by the procedures involved. From the original studies (for review see Yen, 1978), there has been a considerable amount of work in this field to establish variables such as the medium necessary to maintain the viability of the tissue and the oxygen concentration to which the culture is exposed.

To date, one of the best culture systems is that described by Trowell (1959), due to its economy and efficiency. The use of the Trowell type organ culture dishes and the establishment of a balanced continuous flow of oxygen (95%) and carbon dioxide (5%) mixture by Yen and Melcher (1978) has created a consistent and reliable organ culture technique for the study of different types of connective tissues, including periodontium (Yen, 1978), interparietal sutures (Yen *et al.*, 1984) and temporomandibular disc (Yen *et al.*, 1989; Carvalho *et al.*, 1990).

5.1. Molecular Turnover in Organ Culture

Organ culture can be used to study the various components of a particular tissue under analysis. It is not intention of this review to deal with all the variables that normally would account for the connective tissue explant turnover, in this case. Therefore, only collagen and PGs will be briefly described.

In organ culture, steady states of PG turnover have been reported after 5 days (Hascall *et al.*, 1983; Campbell *et al.*, 1989). However, the information about the turnover of PGs in organ culture is not very clear. The initial event in normal PG turnover is the cleavage of the protein core between the binding region and the CS/KS chains of the molecule. This constitutes a main step, once the survival of the PG in the extracellular matrix is limited, as it separates form the part that maintains the molecule attached in an aggregate form (Hardingham, 1986). PG turnover was estimated for cartilage as a fast mechanism in young animals, varying from 5 to 10 days, and as a slow mechanism for mature animals, varying from 100 to 300 days (Maroudas, 1979; Hardingham *et al.*,1984).

Collagen normal turnover in adult tissues is thought to be normally very slow (Alberts *et al.*, 1989). Collagen turnover is assessed by the analysis of hyp synthesis and by the measurement of the activity of specific enzymes, such as proline and lysil hydroxylases (Nimni and Harkness, 1988). The time required for the synthesis of a complete α-chain was reported to be around 6.7 minutes (Nimni and Harkness, 1988).

5.2. Interaction between Collagen and Proteoglycans *In Vitro*

PG complexes do not exist *in vivo* as separate entities (Bartold, 1987). There is a complex series of interactions that involve not only carbohydrate chain-chain interaction but also PG and cell surface, PG with HA and PG with collagen, among others. Although, the relationships among all PG interactions must be correlated, a more detailed description of

all factors will not be included in the present review. However, since PG and collagen are the major components of connective tissue (McDevitt, 1973), their interaction seems to be essential for a detailed understanding of both structures.

The stability of the matrix and of the physico-chemical and mechanical properties of cartilage depend of the maintenance of the integrity of the collagen network and the immobilization of the PG-complex aggregates (Stockwell, 1979). The specific mechanisms of the PG-collagen interactions may involve electrostatic forces (Muir and Hardingham, 1975, Obrink *et al.*, 1975) and forces that are originated from the binding of the GAG's acidic groups and the basic amino groups of collagen (Montes and Junqueira, 1988). According to Obrink (1974), sulphated GAGs formed electrostatic complexes with collagen under physiologic conditions, while HA and collagen interacted by steric exclusion. Possibly, PGs may also be associated with collagen indirectly via non-collagenous proteins or glycoproteins (Stockwell, 1979).

In vitro experiments have greatly clarified collagen-PG interaction. However, aqueous solutions *in vitro* show fundamental differences between those of functioning tissues (Scott, 1986). The orientation of the tropocollagen molecules *in vivo* align to produce fibers in a way that excludes PGs once they utilize an increased number of binding sites which might otherwise associate with PGs demonstrating *in vitro* a fictitious pattern Studies have shown that the small DS-PG has the unique capacity to inhibit the development of collagen fibrils *in vitro* (Vogel *et al.*, 1984), and that collagen fibrils formed in the presence of PGs are thinner (Vogel and Trotter, 1987) and at reduced rate (Oegama *et al.*, 1975) than those of formed in the absence of PGs.

Chapter TWO

Statement of the Problem

STATEMENT OF THE PROBLEM

In the incidence of non-inflamatory derangement in temporomandibular joints the articular disc is mainly responsible for the signs and symptoms in the joint (Solberg, 1986; Bell, 1990). Although the temporomanibular disc is probably affected directly by mechanical articulating forces, the basic disc mechanical properties and the biological activities that determine these properties are poorly understood.

Due to the fast growth and remodeling of the TMJ area, the assessment of the collagen synthesis may elucidate the metabolic state and change in cellular activity in remodeling tissues (Bornstein and Sage, 1980; Gage *et al.*, 1990) while the analysis of GAG complexes may clarify the role of the mechanical stimulations to the integrity and function of the articular disc.

Therefore, the purposes of this study are:

1. To study the synthesis of collagen *in vitro* from the normal development of articular disc of both mouse and rat. Collagen synthesis and distribution was determined in animals of different ages in order to establish the profile of maturation of articular disc development by monitoring type III collagen (Chapter III).

2. To determine the synthesis of newly-synthesized GAG molecules *in vitro* in the normal development of the rat articular disc (Chapter IV). These experiments were used to determine the pattern of GAG changes and ultimately to estimate the most suitable age periods for mechanical stimulation.

3. To compare the synthesis of GAGs in the rat articular disc between animals of selected ages using different regimens of mechanical stress *in vitro* (Chapter V). The articular disc behaviour in such experiments may indicate a pattern of alteration

that will help to determine the modulation of cellular activity during the stages of disc development.

Chapter THREE

Effect of Age in Collagen Synthesis *In Vitro* : In Rat and Mouse

SUMMARY

Four Sprague Dawley rats were divided into two different age groups of 3, 4, 5, 6, 7, 8 and 9 and 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8 weeks, and received the application of [^{3}H]-proline to determine the radioautographical distribution of silver grains of collagen synthesis and [^{14}C]-glycine respectively to determine the pattern of proportion of newly-synthesized type III collagen. The synthesis and distribution of collagen was also characterized for the Swiss-Webster mouse articular disc *in vitro*. The explants from animals aged 3, 4, 5, 6, 7, 8 and 9 weeks were cultured for a period of 4 hours using [^{3}H]-proline and animals aged 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 weeks were cultured with [^{14}C]-glycine.

The purpose of this investigation was to compare the behaviour of the rat articular disc with the mouse articular disc that was subjected to the same procedures. The pattern of synthetic activity as demonstrated by the disposition of the silver grains showed to be very similar in both rat and mouse, in which the articular disc seemed to have an increased percentage of labeling in the anterior and medial bands as compared with the posterior band, however, this must be confirmed by grain counting and statistical analysis. The mouse articular disc showed more labeling in the ventral region of the disc, but the same feature could not be demonstrated in the rat disc.

The SDS-PAGE polyacrylamide gel electrophoresis and fluorographic visualization of separated radio-labeled collagen bands determined that the highest proportion of type III collagen was at 4 weeks of age for the mouse articular disc and around 6 weeks of age for the rat disc. Thus, the analysis of the articular disc from these two different species demonstrated to be very similar, which is likely to be a reflection of the pattern of mastication and overall functional muscle behaviour that is comparable for both rat and mouse.

INTRODUCTION

The derangement of the articular disc may be cause of many TMJ signs and symptoms (Solberg, 1986; Bell, 1990). It has been suggested that predisposition of derangement may be related to developmental problems of the articular disc (Yen *et al.*, 1989).

Among possible mechanisms the biochemical content of the articular disc may provide information of the ability of discal tissues to resist derangement of perforation. The latter is closely related to the regenerative capacity of the disc.as suggested by the variation in fibril size of the collagen network, which is usually observable in either developing or recently formed tissue (Peach *et al.*, 1961; Ross *et al.*, 1961).

Despite that the articular disc has been extensively regarded as a very important component of TMDs (Solberg, 1986; Bell, 1990), to date little is known about the intrinsic changes in the major structural components of the disc. Collagen, among other connective tissue components, is an important structural protein of the disc, and the monitoring of collagen formation due to normal development may clarify the behaviour of the articular disc during maturation (Cage *et al.*, 1990). Currently, there is no data available on the effect of age on the biochemical content of the articular disc.

In order to investigate TMJ normal structure, function and derangements, several studies have been performed using a series of different species of animals such as rat (Cabrini *et al.*, 1941; Cunat *et al.*, 1956; Frommer, 1966; Furstman, 1966; Koski, 1969; Apleton, 1975; Petrovic *et al*, 1975; Katz *et al.*, 1979; Buchner, 1982; Koski, 1982; Petrovic *et al.*, 1983; Gazit *et al.*, 1987; Kantomaa *et al.*, 1988; Lekkas *et al.*, 1988), mouse (Levy, 1949; Frommer, 1964; Glasstone, 1971), guinea pigs (Silva, 1969), rabbits (Barnett *et al.*, 1963; Kantomaa, 1984a; Kantomaa, 1984b), monkeys (Cutler, 1968; Zimmerman, 1971; McNamara, 1973; McNamara *et al.*, 1974; McNamara *et al.*, 1975), chick embryo (Murray, 1969), human fetus (Furstman, 1963; Van Dongen, 1977; Wong *et*

al., 1985; Morimoto *et al.*, 1987) and post mortem human material (Coleman *et al.*, 1955; Oberg, 1971; Oberg, 1973; Carlsson *et al.*, 1974; Hansson *et al.*, 1976; Hellsing *et al.*, 1985). However, the big concern in animal research is to extrapolate these results to the human subjects. McNamara (1975) has suggested the relevance of the relationship between experimental studies in rats and monkeys compared to human in clinical orthodontics. In addition, the induction of structural adaptation in the temporomandibular joint has been reported in experimental studies of young rats (Petrovic *et al.*, 1975) and monkeys (Stockli *et al.*, 1971; McNamara *et al.*, 1975), as a response to altered function. This suggests the logic of considering that the same response may occur in man, since there is the evidence of similar responses in both different laboratory animals (McNamara *et al.*, 1975) with marked differences in craniofacial anatomy (Angel, 1948; Zimmerman, 1971; McNamara *et al.*, 1974). It has been suggested that the histologic findings of such experimental animals in both *in vivo* and *in vitro* studies may be applicable to the human subjects. This is mainly true for biological studies in which different experimental animals are used.

The present study has focused on the histological evaluation of collagen distribution in the articular disc from rats and mouse and its changes due to normal development. The articular disc was also studied biochemically by monitoring the changes in type III collagen, to determine the pattern of disc maturation in order to establish baseline data for further study the effect of mechanical stress on the articular disc.

MATERIALS & METHODS

1. *In Vitro* Organ Cultures

The first part of this study selected Sprague-Dawley rats, inbred in our facility, that were randomly divided in number of four for each of the following age groups: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 weeks of age for biochemical analysis and in age groups of 3, 4, 5, 6, 7, 8 and 9 weeks of age for histological analysis. The second part of this study selected Swiss-Webster white mice, inbread in our facility, that were randomly divided in the number of four for each of the following age groups: 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9 weeks of age for biochemical analysis and in age groups of 3, 4, 5, 6, 7, 8 and 9 weeks of age for histological analysis. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The articular disc and attached ligaments from both joints of each animal were carefully dissected through a lateral incision to expose the disc/condyle complex following the removal of the masseter muscle. The removal of the articular disc from the head of the condyle was precisely done so as to remove the disc and small portions of both retrodiscal tissue and superior head of lateral-pterygoid muscle, which were important for the orientation of the different bands of the tissue *in vitro*.

Each articular disc was washed in Waymouth's medium (Waymouth's 752/1, Grand Island Biological Co., Grand Island, New York) and placed on special stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California) at the gas:medium interface in Trowell type (Trowell, 1959) organ culture dishes (60 x 16 mm, Falcon #3037, Becton, Dickinson and Co., Cockeysville, Maryland) with a gas mixture of 95% oxygen and 5% carbon dioxide humidified by bubbling through distilled water. Each organ culture dish contained 1 ml of Waymouth's medium (Waymouth, 1959) supplemented with 300 μgm/ml of ascorbic acid, 330 U/ml of penicillin, 0.75 μg/ml

amphotericin B and 330 μg/ml streptomycin and surrounded by sterile distiled water. The dishes were cultured at 37°C in a humidified incubator.

For biochemical analysis, following 1 hour incubation, 20 μCi/ml of [^{14}C]-glycine (Amersham Corporation, Oakville, Ontario) with specific activity of 52.2 mCi/mmol was added to the culture dishes. After 3 hours of culture in the presence of the isotope, the explants were removed and placed in 1.5 ml plastic tubes (Eppendorf tubes, Baxter/Canlab, Winnipeg, Manitoba) following the carefully wash with 0.5 ml of fresh culture medium to remove free isotopes and frozen at - 20°C.

For histological analysis, immediately after dissection the explants were placed in culture with 10 μCi/ml of [^{3}H]-proline (Amersham Corporation, Oakville, Ontario) with specific activity of 60 mCi/mmol in each dish. After 4 hours in culture the explants were removed and placed in 10% phosphate-buffered formalin containing 0.5% of cetylpyridinium chloride or CPC (Sigma Chemical Co., St. Louis, Missouri) for fixation of the tissue.

2. Histological Procedures

The explants were fixed in 10% phosphate-buffered formalin containing 0.5% of cetylpyridinium chloride or CPC (Sigma Chemical Co., St. Louis, Missouri) for 24 hours. Following fixation each articular disc and attached ligaments were oriented longitudinally in the anteroposterior direction in order to embed in paraffin blocs (Fisher Scientific, Winnipeg, Manitoba). The paraffin blocs were serially sectioned at 5 μm of thickness and mounted on glass slides (Corning Glass Works, Corning, New York) using albumin (Fisher Scientific, Winnipeg, Manitoba) as fixative. Prepared slides were incubated in a 60°C oven for a minimum of 6 hours (to permanently fix the sections onto the glass slides with the removal of the excess of paraffin) and allowed to cool at room temperature. Every

fourth slide was stained with Alcian Blue 8GX OR (Mallinckrodt Inc., Paris, Kentucky) at pH of 2.5 according to the method of Lev and Spicer (1964) and examined under the photomicroscope (Zeiss, West Germany). Randomly selected adjacent unstained sections were processed for radioautography by the technique of Kopriwa and Leblond (1962).

The selected slides were dewaxed, dipped in Kodak NTB2 emulsion (Kodak Canada Inc., Brampton, Ontario) and exposed in the dark at 4°C for 28 days. The radioautographs were developed with Dektol (Kodak Canada Inc., Brampton, Ontario), fixed with rapid fixer (Kodak Canada Inc., Brampton, Ontario) and stained through the emulsion with Haematoxylin and Eosin (Fisher Scientific, Winnipeg, Manitoba). Stained radioautographs were assembled with cover slips (Corning, Corning Glass Works, Corning, New York).

Photomicrographs were taken of selected autoradiographs by using a 35 mm Panatomic-X film (Kodak Canada Inc., Brampton, Ontario) which were subsequently developed using Microdol (Kodak Canada Inc., Brampton, Ontario). Black and white prints were made on Kodak Polycontrast Rapid II RC paper with an enlargement of 6.5 X of the original magnification. Prints were developed with Kodak Dektol and fixed with Kodak Fixer.

3. Biochemical Procedures

3.1. Pepsin Extraction of Newly-Synthesized Soluble Collagen

The frozen samples were allowed to thaw and therefore proceed to limited pepsin digestion of each explant in order to solubilize the radio-labeled newly-synthesized collagen. In first place, individual samples were digested with 1 ml of crystallized pepsin (2 X crystallized, Sigma Chemical Co., St. Louis, Missouri) solution of 0.1mg/ml of pepsin in 0.5 N acetic acid (Sigma Chemical Co., St. Louis, Missouri) at 16°C for 12 hours. After

the first digestion, the samples were centrifuged (Fisher Scientific Microcentrifuge Model 235A, Winnipeg, Manitoba), the supernatants were removed and the pellets were re-digested with 0.5 mls of the same pepsin solution. After 4 hours the samples were centrifuged again and the supernatants were pooled together with the initial extracts. The pellets were washed with 0.5 mls of 0.5 N acetic acid and re-centrifuged. The final extract was added to the previous digestions up to a volume of 2 mls and mixed thoroughly.

The mixture corresponding to each sample was dialyzed (Spectra/Por Membranes, Spectrum Medical Institutes Inc., Los Angeles, California) against 0.1 N acetic acid for 2 days at 4°C. Following dialysis from each sample 100 µl was removed and placed in mini-scintilation vials with 5 mls of scintillation cocktail (Aquasol, New England Nuclear, Boston, Massachussets) and counted for [^{14}C] in a liquid scintillation counter (Nuclear Chicago Isocap 300, Searle Instrumentation, Toronto, Ontario). The remaining of the samples were frozen at - 80°C and lyophilized for 24 hrs (Labconco Model Freeze-dryer 3) at - 50°C in preparation for gel electrophoresis.

3.2. Separation and Quantitation of Collagen α-chains

Collagen and procollagen α-chains were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 20 cm slab gels to allow the analysis of type III collagen. The method used was a modification of the original method by Laemmli (1970) in which a delayed reduction step was introduced using 7.5% cross-linked separating gel, a 2.5% stacking gel and tris/glycine buffers (Bio-Rad Laboratories, Richmond, Virginia). Type I and III collagen α-chains were separated using the interrupted electrophoresis method of Sykes *et al.* (1976).

Freeze-dried samples were dissolved in 70 µl of reservoir buffer containing 2 M urea, 2% SDS and 0.1% bromophenol blue (tracking dye) and were heated at 60°C for 30 minutes to denature the collagen. Samples were introduced to the sample wells of the

electrophoresis apparatus (Bio-Rad Laboratories Model Mini Protean II, Mississauga, Ontario) and electrophoresis was performed at 130 volts under non-reducing conditions. After 1 hour, the samples were reduced by the addition of 20% β-mercaptoethanol (Sigma Chemical Co., St. Louis, Missouri) to the sample wells to allow type III α-chains to penetrate the gel. After 60 minutes of reduction, electrophoresis was resumed at 24 mA/gel until the tracking dye reached the base of the gel.

Fluorographic visualization of separated radio-labeled collagen bands in each gel was performed by the dehydration with two washes of dimethyl sulfoxide (Fisher Scientific, Fair Lawn, New Jersey) and impregnated with 2,5-diphenyloxazole/dimethyl sulfoxide (Dupont-New England Nuclear, Boston, Massachussets) as described by Bonner and Laskey (1974). The gels were then placed on filter paper (Bio-Rad Laboratories, Richmond, Virginia), dried on a slab drier (Bio-Rad Laboratories, Richmond, California) and exposed to Kodak XRP-1 x-ray films (Kodak Canada Inc., Toronto, Ontario) at - 80°C for varying periods of time as indicated by the scintillation counts of the samples.

Individual tracks corresponding to one sample from the developed fluorographs were scanned at 550 nm with a spectrophotometer (Bio-Rad Laboratories Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd, Japan). The proportions of type III α-chains relative to the total of type I and type III α-chains were calculated from integrator (Bio-Rad Laboratories Model 3392A Integrator, Richmond, California) readings. Finally, the values obtained for the collagen ratios across age were subjected to statistical analysis using a one-way analysis of variance with Tukey's multiple multiple comparison test.

4. Study of Mandibular Growth

Thirty Sprague-Dawley male rats, inbred in our facility, were randomly divided in number of three for each of the following age groups: 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 weeks of age. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The mandible was removed and measured with precision calipers (Staedtler, West Germany) to record the normal growth for the following dimensions: 1) Mandibular Lenght (ML), taken from the posterior border of the condyle to the dento-alveolar-bone junction of the incisor; 2) Condylar Length (CL), taken from the most anterior to the most posterior point in the mandibular condyle and 3) Condylar Width (CW), taken from the most lateral points in the mandibular condyle, as shown in Appendix K, Figure K-1 (Bouvier and Zimny, 1987; Bouvier, 1988).

RESULTS

The articular disc was well developed by 3 weeks of age. The silver grain distribution of [^{3}H]-proline labelling showed that the articular disc of both mouse and rat have similar patterns of protein synthesis, as evidenced by the increased concentration of labeling in the anterior and medial bands respectively (Figures III-1 to III-8), however, this information has to be confirmed by grain counting and statistical evaluation. The amount of collagen synthesis was demonstrated in the mouse articular disc as well as the rat articular disc (Figures III-1 to III-8) as evidenced by the labeling of [^{3}H]-proline.

The percentage of type III collagen that incorporated [^{14}C]-glycine in the mouse disc showed to increase significantly at 4 weeks of age and persisted at a high ratio after that age (Figure III-9), as shown by ANOVA analysis which compared the percentage of type III collagen to the different age groups for both rat and mouse. The proportion of newly-synthesized type III collagen in the rat articular disc increased from 3 to 6 weeks of age and peaked at 7 and 8 weeks of age, decreasing after that, however, the mouse disc had an increased percentage of type III as compared with the rat articular disc (Figure III-10). On the other hand the rat disc did not show any significant age variation using the same statistical analysis. The percentage of type III collagen from the mouse and rat articular disc was summarized in table III-I.

Type III collagen in the rat articular disc was compared with the velocity curve of the growth of the mandible and there seems to be a correlation between mandibular growth and collagen synthesis, since both yielded a similar pattern of growth up to the 8th week of life, when collagen decreases steadily (Figure III-11).

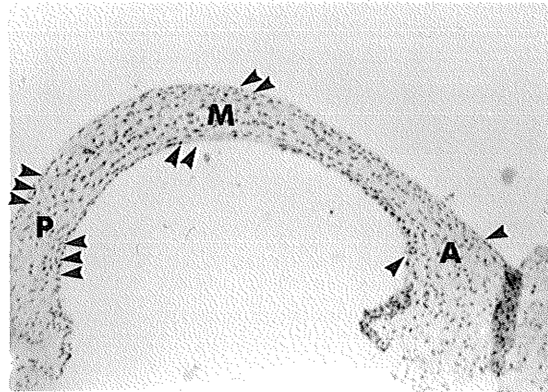


Figure III-1: Radioautograph of cross-section of the rat temporomandibular disc. Donor age is 5 weeks. Explant cultured *in vitro*. Legends are: A = anterior band, M = medial band and P = posterior band. Haematoxylin and Eosin. X 12

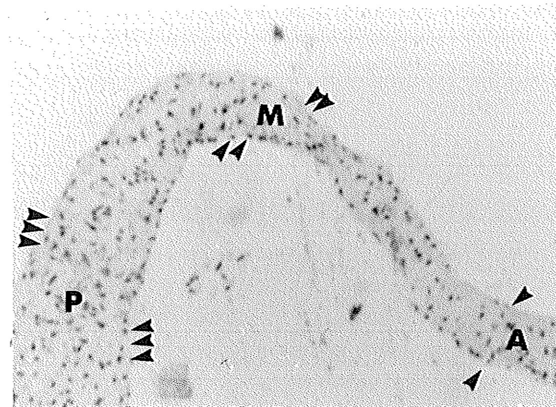


Figure III-2: Radioautograph of cross-section of the mouse temporomandibular disc. Donor age is 5 weeks. Explant cultured *in vitro*. The legends are the same as Figure III-1. Haematoxylin and Eosin. X 12

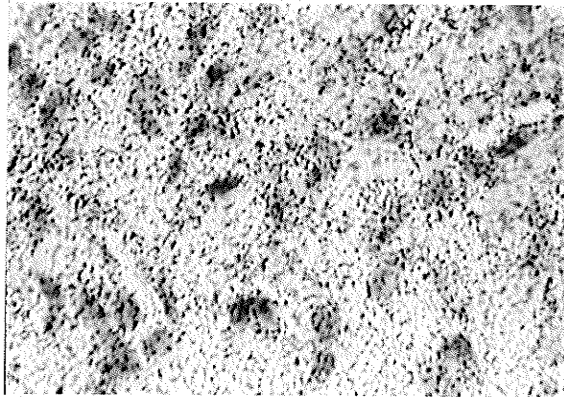


Figure III-3: Magnification of the anterior band of the rat disc delineated by the single arrows in Figure III-1. X 205

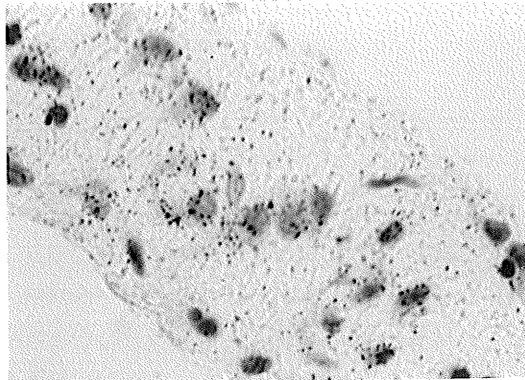


Figure III-4: Magnification of the medial band of the rat disc delineated by double arrows in Figure III-1. X 205

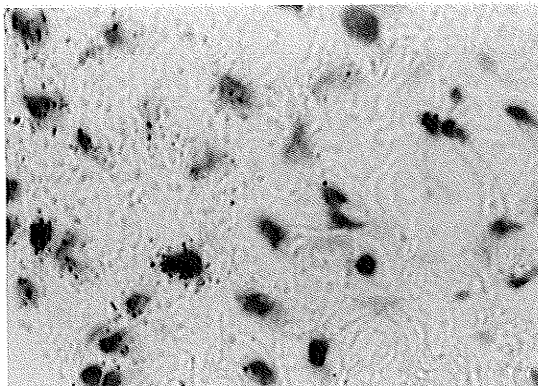


Figure III-5: Magnification of the posterior band of the rat disc delineated by the triple arrows in Figure III-1. X 205

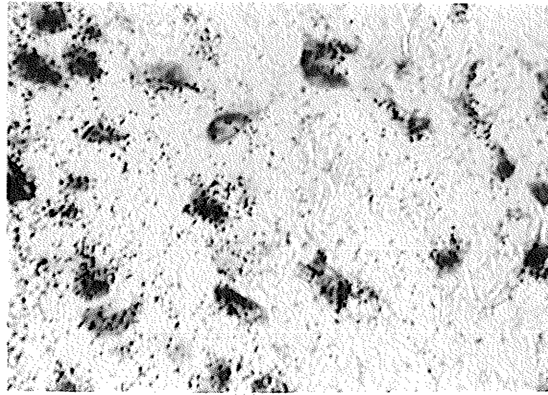


Figure III-6: Magnification of the anterior band of the mouse disc delineated by the single arrows in Figure III-2. Note that the proportion in labeling was similar to the anterior band of the rat disc. X 205

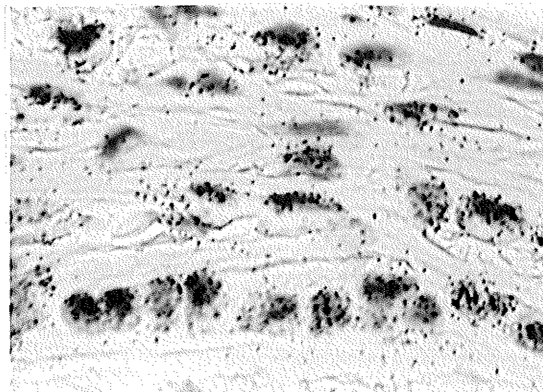


Figure III-7: Magnification of the medial band of the mouse disc delineated by double arrows in Figure III-2. Similarly, the medial band of the mouse disc appeared proportionally correspondent to that of the rat disc. X 205

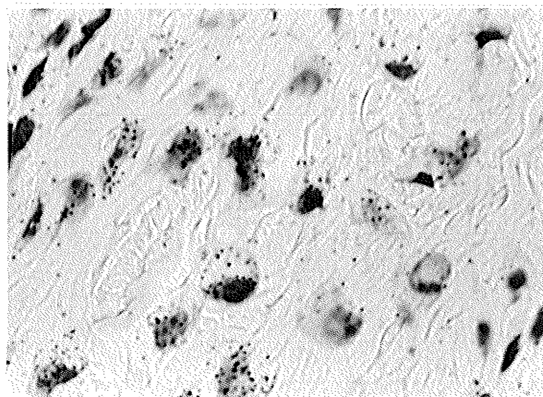


Figure III-8: Magnification of the posterior band of the mouse disc delineated by the triple arrows in Figure III-2. Note that there is a proportionally reduced amount of silver grains in the posterior band of the mouse disc as well as that of the rat. X 205

Figure III-9: Graph showing the percentage of type III collagen distribution in the rat articular disc as a result of normal development.

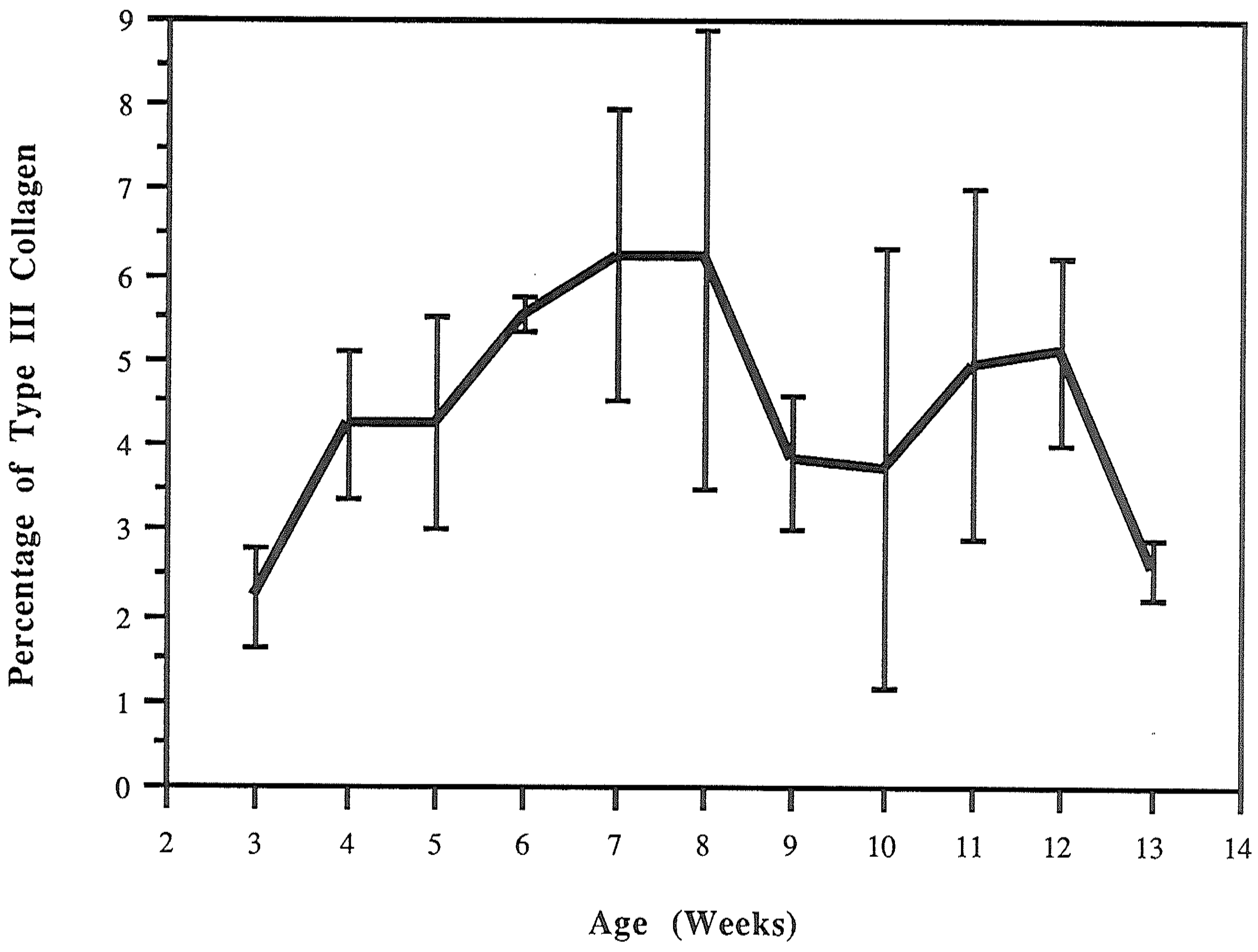
Figure III-10: Graph showing the percentage of type III collagen distribution in the rat articular disc compared to the mouse articular disc as a result of normal development.

Figure III-11: Graph showing the percentage of type III collagen distribution in the rat articular disc as a result of normal development and the velocity curve of the rat mandibular growth. Note that the collagen synthesis seems to follow the apparent peak of mandibular growth spurt at 7 weeks of age.

Table III-I: Table of the percentage of type III collagen distribution in the rat articular disc and in the mouse articular disc as a result of normal development.

Figure III-9

Effect of Age on the Percentage of Type III Collagen in the Rat Articular Disc



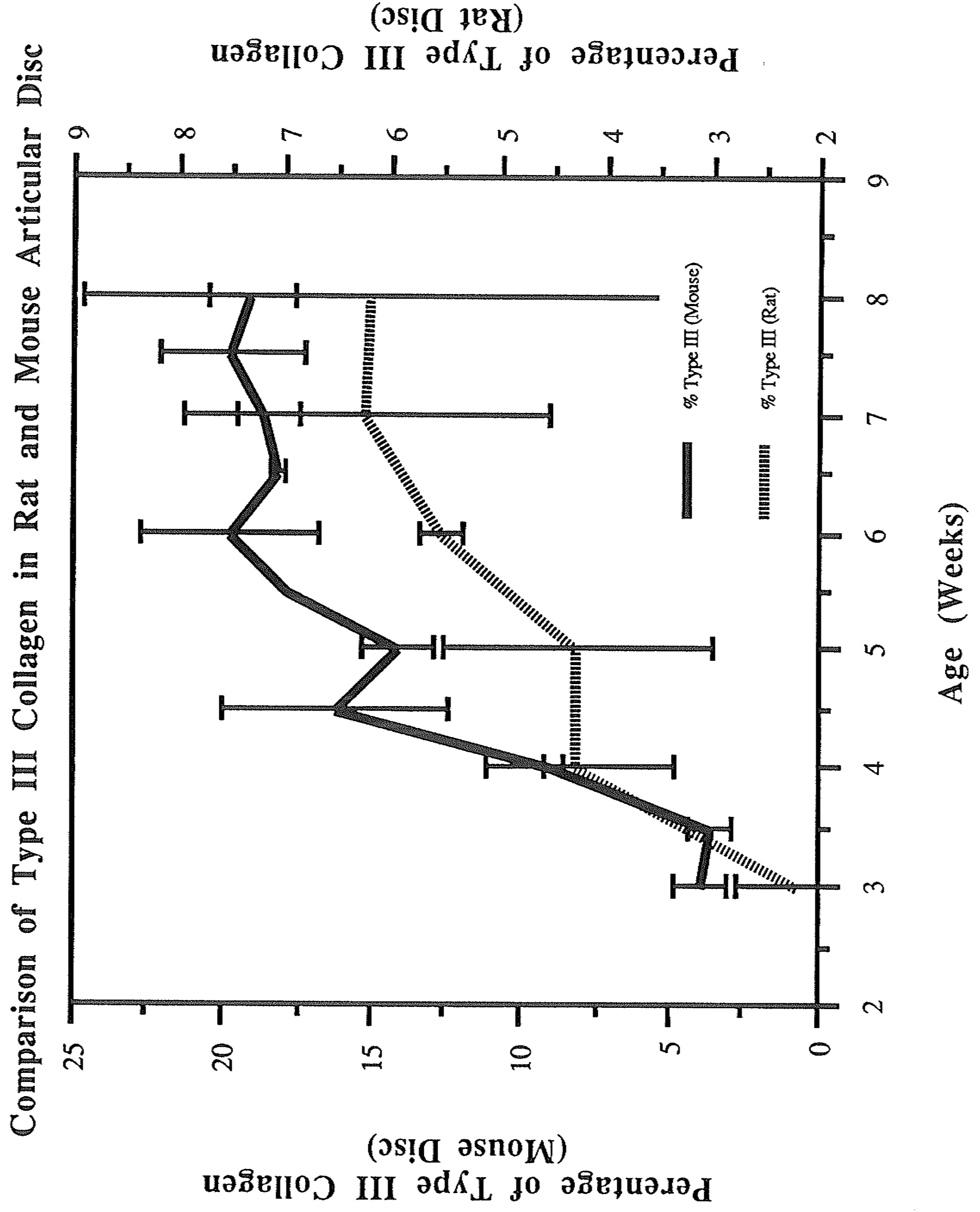


Figure III-10
Comparison of Type III Collagen in Rat and Mouse Articular Disc
Perentage of Type III Collagen
(Mouse Disc)
Percentage of Type III Collagen
(Rat Disc)
25
20
15
10
5
0
9
8
7
6
5
4
3
2
2
3
4
5
6
7
8
9
% Type III (Mouse)
% Type III (Rat)
Age (Weeks)

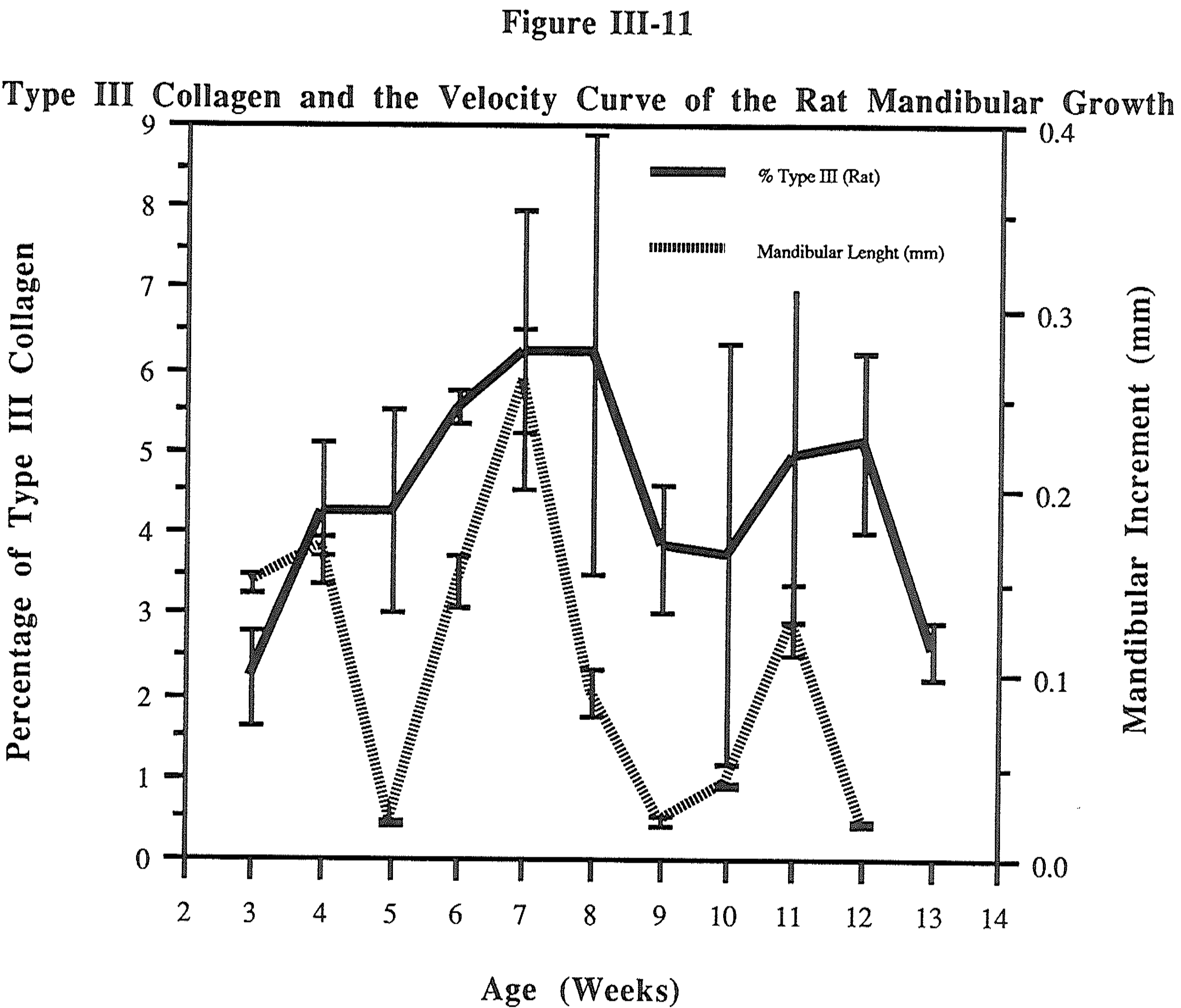


Figure III-11
Type III Collagen and the Velocity Curve of the Rat Mandibular Growth
% Type III (Rat)
Mandibular Lenght (mm)
Percentage of Type III Collagen
Mandibular Increment (mm)
Age (Weeks)
0
1
2
3
4
5
6
7
8
9
0.0
0.1
0.2
0.3
0.4
2
3
4
5
6
7
8
9
10
11
12
13
14

Table III-I

Percentage of Newly-synthesized Type III Collagen in the Rat and Mouse Articular Discs

Collagen Type (Rat Disc)	α_3(III)		Collagen Type (Mouse Disc)	α_3(III)	
Age (Weeks)	Mean	St. Dv.	Age (weeks)	Mean	St. Dv.
3	2.20	0.57	3	3.91	1.92
4	4.24	0.88	3.5	3.62	0.69
5	4.26	1.25	4	8.89	0.29
6	5.54	0.21	4.5	16.19	3.80
7	6.23	1.71	5	14.05	1.20
8	6.20	2.69	5.5	17.75	0.07
9	3.81	0.80	6	19.75	3.04
10	3.74	2.60	6.5	18.13	0.25
11	4.95	2.06	7	18.47	1.11
12	5.11	1.12	7.5	19.69	2.42
13	2.53	0.35	8	19.02	1.46

DISCUSSION

The limited understanding of the pathological changes that take place in the temporomandibular joint has yielded a great deal of work to characterize the factors that play a role in such functional alterations. To date, the articular disc or meniscus articularis has been regarded as one of the main factors that contribute to the establishment of dysfunctions in abnormal temporomandibular joints (Bell, 1990). The articular disc is a biconcave structure that covers the head of the mandibular condyle and that is mainly formed by bundles of type I collagen fibers (Gage *et al.*, 1990), with a scattered distribution of chondrocytes (Silva, 1969), which have been defined as fibrochondrocytes (Webber *et al.*, 1985), and are embeded in a PG matrix (Hardingham, 1979; Gage *et al.*, 1990). Due to the characteristics of the tissue, articular disc as a fybrochondrocytic structure has a potential capacity for adaptation and remodelling when subjected to altered functional demands.

The fibrous structure of the disc resembles that of a elongated structure with a compact distribution of fibers along the longitudional axis that are mainly composed of type I collagen. This inherited characteristic gives the disc a high degree of resistance to tensional forces, however, the presence of type III collagen, which is typically found in connective tissues that are more distensible (Bornstein and Sage, 1980; Gage *et al.*, 1990), may account for the stretching and degeneration of the articular disc structure. Type III collagen is present in high proportions in newly-formed tissue or in areas with increased cellular activity, and therefore establishes a pattern of remodelling and degree of maturation of the tissue studied (Bornstein and Sage, 1980). The characteristics of the articular disc clearly demonstrate that type III collagen forms an interaction with type I collagen, that can vary through age and skeletal development.

Due to the limited data of the effect of age on the collagen synthesis from the articular disc, a series of experiments were undertaken to evaluate the pattern of adaptation,

that the articular disc may undergo due to normal development as evidenced by the assessment of the protein synthetic activity, both histologically and biochemically. In this chapter, the articular disc from the albino rat was analyzed for the distribution of collagen synthesis and the proportion of type III collagen synthesized in different ages and compared with the articular disc in the swiss mouse using the same experiments to evaluate the degree of comparison that the protein synthesis may be subjected to and if both animal models can be related.

The monitoring of collagen formation due to normal development may clarify the behaviour of the articular disc. The observations in the mouse articular disc seemed to be very comparable with the same results in the rat. The assessment of the silver grains distribution revealed that the anterior and medial areas disposed longitudinally in the articular disc presented the most relevant amount of labeling as compared to the posterior area, for both the rat and the mouse, however, this information must be confirmed by silver grain-counting and statistical evaluation. It seems that the functional demands for both species are very simmilar and despite the proportional increased concentration of silver grains in the rat disc, both models can be used interchangeably.

Type III α-chains appeared more concentrated in the mouse disc as compared to the rat disc. The fast increase of type III collagen synthesis in the rat seemed to be slightly earlier than that of the mouse (Figure III-11), however, the curve of synthesis for both animals remained high up to 8 weeks of age.

The normal development of soft tissue structures does not seem to follow the same pattern than that of calcified structures. The rate of bone growth appears highly increased during the second and third weeks of life, decreasing rapidly after that (Massler and Schour, 1951). Although, it is speculative to affirm such differences since the proportion of type III collagen did not show significant correlation with age, the pattern of changes seems to reflect the remodeling capacity of the TMJ in rats (Cunat *et al.*, 1956). The factors

and control mechanisms that are responsible for the biomechanical and histological changes during this period are unknown.

The analysis of the percentage of rat type III collagen showed a fast increase in synthesis at the age of 7 weeks old (Figure III-9), period that coincided with an appearant mandibular fast growth spurt (Figure-12). According to Petrovic *et al.* (1975), the normal growth of the albino rat never ceases, but it seems negligible after 110 days of life (or 15.71 weeks). At this stage, the synthesis of type III collagen is to be expected to drop to 0, since the capacity of remodeling would have reached its maximum. The steady drop of percentage of type III collagen at 8 weeks of age (Figure III-9), which was also showed to be just after the period of an apparant mandibular growth spurt, and the lowest drop at week 13 may represent this observation. However, the large standard deviations in the normal growth of the rat show a high variability among the ages studied. One explanation may be the variation in the recovery of disc material during extraction procedures, which can be minimized by calculating and correcting the loss of material throughout the procedure.

The type III collagen synthesis from mouse disc was not studied at ages higher than 8 weeks, therefore, it is speculative to affirm that a drop in synthesis similar to the rat disc would also occur in the mouse. Although, the analysis of both tissues has revealed a great deal of similarities and therefore it would be assumed that the same drop above mentioned would occur, the lack of information on the mouse mandibular growth is a limiting factor, since the overall craniofacial development in the mouse may not take place at the same time than that of the rat. Independently, the analysis of the articular disc from both animals suggests that they are likely to have compatible mandibular movements and to be exposed to the same pattern of muscle functional activity.

CONCLUSIONS

1. Anterior and medial bands of articular disc appear to have the most active sites of protein incorporation at all ages for the rat as well as the mouse, however, this information may be confirmed by grain-counting and statistical evaluation. Older ages showed a generalized reduction in protein synthetic activity. Such distribution may be the result of the functional demand to specific stress and strain mechanisms.

2. The significant increase of proportion of newly-synthesized type III collagen at 4 weeks of age for the mouse is very similar than at 3.5 weeks of age for the rat. The tissue of both models demonstrated a high level of synthesis after that, until the 8th week. Collagen type III in the rat increases steadily up to 7 and 8 weeks of age when it starts to decrease, showing the lowest level in animals of 13 weeks of age.

3. Type III collagen in the rat seemed to follow the same pattern as mandibular growth until just after an apparant period of the mandibular growth spurt, which took place in the rat at 8th weeks of age, when the percentage of collagen decreased steadily. This observation may suggest that the articular disc follows the same pattern of maturation that that of the overall craniofacial development.

4. The similar pattern of collagen synthesis and distribution suggests that the albino rat and the swiss mouse are subjected to the same mastication pattern and overall muscle behaviour.

5. The model for organ culture *in vitro* that maintains the synthetic activity of the articular disc can be used to further investigate the metabolism of disc behaviour when subjected to mechanical or hormonal stimulation.

Chapter FOUR

Effect of Age in GAG Synthesis *In Vitro*

SUMMARY

The basic mechanical properties and the contributing biological activities that determine pathologies such as articular disc derangement cause poorly understood structural changes in the disc. However, it is well known that the function of the tissues under mechanical stress, most often compressive load, is only maintained through the balance of extracellular complexes of PGs and GAGs (Hardingham , 1981; Saamanen, 1989). In this investigation, an organ culture model was used to correlate the synthesis of the various GAGs synthesized *in vitro* with the normal growth and development of the articular disc of young rats to provide baseline data for future comparison with mechanically stressed and pathological disc tissues. The articular disc was dissected from male Sprague-Dawley rats of 3 to 12 weeks of age and labeled in culture with [^{3}H]-glucosamine for 24 hours. The radiolabeled samples were digested with pronase-E and different GAG types were separated following ethanol and CPC precipitation. The qualitative assessment of GAG synthetic activity demonstrated that HA, C_6S and KS/C_4S are the major GAG molecules of the rat articular disc. DPM counts/mg wet tissue suggested a steady increase in HA and C_6S at 6 and 7 weeks in the rat articular disc respectively, decreasing after that and KS/C_4S appeared to be increased at 3 weeks of age showing an inverse relationship as the animals aged. The overall percentage of each GAG type over the total amount of isolated GAGs showed KS/C_4S and C_6S to vary significantly with age although the patterns of change were similar to the DPM counts per mg of tissue.

All GAG types showed a significant interaction with the pattern of changes due to aging. The changes within groups seemed to be restricted to ages 3 and 5 to 7 weeks. From these observations it appears that the articular disc shows more characteristics of cartilage as evidenced by the amounts of C_6S and KS/C_4S, during the mandibular growth spurt within the ages 6 and 7 weeks (Figure K-2 and Table K-I in Appendix K). This rise in cellular activity is also determined by the increased overall amounts of HA that is typically

correlated with areas of intense cellular migration, suggesting that during ages 5 to 7 weeks the rat articular disc is subjected to a more drastic degree of remodeling. This data now provides baseline data for further study on mechanical loading and trauma and articular disc response.

INTRODUCTION

The main function of the articular disc is to serve as a cushioning material that absorbs the high loads that are applied by the bony surfaces (Wrigth *et al.*, 1971; Saamanen *et al.*, 1987). This is accomplished by the structural composition of the disc which resembles that of cartilage (Silva, 1969; Kopp, 1976; Isberg and Isacsson, 1986; Mills *et al.*, 1988), although its composition is mostly formed by a fibrous tissue (Miles and Dawson, 1962; Frommer and Monroe, 1966; Silva, 1968; Wright *et al.*, 1971) of collagen fibers (Strauss *et al.*, 1960; Dixon, 1962; Wong *et al.*, 1985; Mills *et al.*, 1988). Despite being not as abundant as collagen, the sparse extracellular matrix of the articular disc is rich in PG, that are responsible for the compressional behaviour of articular disc function (Hardingham, 1971; Adams and Muir, 1981).

The composition of connective tissue often represents its functional requirements (Daniel and Mills, 1988). The differents types of loads that are applied to the tissues will establish the orientation and disposition of fibers and extracellular matrix. Tissues that are subjected to compressional loads display a structure composed of bundles of collagen fibers disposed in a parallel arrangement and a scant matrix while tissues subjected to tensional stresses show an irregular arrangement of collagen fibers and an abundant extracellular matrix rich in PGs, ions and water (Daniel and Mills, 1988).

Although, the extracellular matrix is not the major component of the articular disc structure, the relationship of the loads and stresses applied to the disc are highly dependent on the proper arrangement of the matrix and fibers. Therefore, the monitoring of the normal alteration of PGs and GAGs, the predominant complexes of the matrix, may suggest the mechanisms of disc behaviour.

The structure of the extracellular matrix in connective tissue endure considerable change and remodeling during normal development due to the aging process. Even if aging is not necessarily an imposed process of impairment (Thonar and Kuettner, 1987), it

constitutes a biological process that progressively modifies the functioning and the interrelation of the various components of a tissue (Bouissou and Pieraggi, 1988).

Age or degree of maturation are important to the distribution of GAGs in connective tissue (Lemperg *et al.*, 1974). Although, it is often difficult to draw the line between age and maturation, some investigations reported a marked increased change in GAGs in the first two decades of life (Hjertquist and Lemperg, 1972; Carney and Muir, 1988). Among the different GAG types, the ratio of HA, KS and glycoproteins have been reported to increase with age while CS have been reported to decrease (Kaplan and Meyer, 1959; Rosemberg *et al.*, 1965; Bjelle *et al.*, 1972; Hjertquist and Lemperg, 1972; Bayliss and Ali, 1978; Bayliss and Ali, 1981). In general, any alteration in GAG content is more marked during development, due to the rapid changes that take place in the tissues (Carney and Muir, 1988).

Maturation of the articular disc not only affects the distribution of the GAG types but also other factors such as ability to aggregate with HA (Bayliss and Ali, 1978, Saamanen, 1984) and the change in hydrodynamic size of the aggregate molecules (Frazen, 1984; Thonar and Kuettner, 1987).

Although, the mechanism of extracellular aging is not completely understood, a logical question to investigate was the pattern of changes in the extracellular composition that can be due to the maturation and the possible correlations of such changes. To reduce the variability of the *in vivo* study this investigation focused on the metabolic changes that regulate the formation of the extracellular matrix complexes. The change in molecular synthesis was studied due to normal development by using an organ culture system that showed to maintain the viability of the tissue (Yen, 1978; Yen *et al.*, 1984; Yen *et al.*, 1989; Carvalho *et al.*, 1990), allowing the measurement of the newly-synthesized molecules. Therefore, the objectives were two fold: first to analyze the proportion of GAG

synthesized at different ages in the articular disc of the rat and second to test the organ culture model as a viable method for GAG synthesis *in vitro*.

MATERIALS & METHODS

1. *In Vitro* Organ Cultures

This study selected Sprague-Dawley male rats, inbred in our facility, that were randomly divided in number of six for each of the following age groups: 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 weeks of age for the extraction and separation of PG complexes in different GAG types and consequently biochemical analysis. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The articular disc from both joints of each animal was removed, its weight carefully recorded and 4 discs of each age were pooled together to constitute one individual sample.

Each articular disc was washed in Waymouth's medium (Waymouth's 752/1, Grand Island Biological Co., Grand Island, New York) and placed on special stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California) at the gas:medium interface in Trowell type (Trowell, 1959) organ culture dishes (60 x 16 mm, Falcon #3037, Becton, Dickinson and Co., Cockeysville, Maryland) with a gas mixture of 95% oxygen and 5% carbon dioxide humidified by bubbling through distiled water. Each organ culture dish contained 1 ml of Waymouth's medium (Waymouth, 1959) supplemented with 300 μgm/ml of ascorbic acid, 330 U/ml of penicillin, 0.75 μg/ml amphotericin B and 330 μg/ml streptomycin and surrounded by sterile distiled water. The dishes were cultured at 37°C in a humidified incubator.

For biochemical analysis, 5 μCi/ml of [^{3}H]-glucosamine (Amersham Corporation, Oakville, Ontario) with specific activity of 27 Ci/mmol was added to the culture dishes. After 24 hours of culture in the presence of the isotope, the explants were removed, pooled together in the number of four and placed in 1.5 ml plastic tubes (Eppendorf tubes,

Baxter/Canlab, Winnipeg, Manitoba) following a careful wash with 0.5 ml of fresh culture medium to remove free isotopes and frozen at - 20°C.

2. Biochemical Analysis

2.1. Isolation of GAG Components

Samples of all the ages were incubated with 1 ml from solution of 10 mg of Pronase-E (Sigma Chemical Co., St. Louis, Missouri) in 50 mls of distiled water for 48 hrs in a humidified incubator at 37°C. This procedure is a modification of the method of Bronson *et al.* (1989). Following incubation the samples were boiled for two minutes to inactivate the enzyme, and the samples were centrifuged (Fisher Scientific Microcentrifuge Model 235A, Winnipeg, Manitoba) for the removal of the insoluble material which was discarded. The different GAG types were separated from the supernatant by precipitation with the addition of 2 mls of cetylpyridinium chloride or CPC (Sigma Chemical Co., St. Louis, Missouri) at concentration of 1% for 12 hours and placed in a humidified incubator at 37°C.

Following centrifugation (Jouan, Model CR 3000, Ontario) at 5°C and 3,000 rpm. for 30 minutes the pellets (original pellet material) were separated and frozen at - 20°C; the supernatants were dyalized (Spectra/Por Membranes, Spectrum Medical Institutes Inc., Los Angeles, California) against distilled water for 2 days at 4°C, when they were removed and frozen at - 80°C and lyophilized (Labconco Model Freeze-dryer 3) for 24 hours at - 50°C. Lyophylized samples were re-suspended in 2 mls of 1% CPC in 0.02 M/L of NaCl and incubated overnight at room temperature with the addition of 20 µl of GAG carrier (Sigma Chemical Co., St. Louis, Missouri) mixture (4 mg/ml). Once more centrifuged (0-5°C and 3,000 rpm. for 30 minutes) the supernatants were discarded and the pellets were re-suspended in 1 ml of 2 M/L NaCl-Ethanol (100:15)(v/v) and the polysaccharides were

precipitated with the addition of 3 vls of cold Ethanol (Commercial Alcohool Ltd., Toronto, Ontario) for 2 nights at 20°C or room temperature.

Once again centrifuged (0-5°C and 3,000 rpm. for 30 minutes) the supernatants were discarded and the pellets were re-suspended in 1 ml of distilled water with the addition of 20 μl of GAG carrier mixture (4 mg/ml) for 12 hrs at room temperature and the polysaccharides were precipitated by the addition of 3 vls of cold ethanol for 2 nights at 20°C or room temperature. Following centrifugation (0-5°C and 3,000 rpm. for 30 minutes), the supernatants were discarded and the pellets were combined with the original pelleted material to prepare the samples for acetate plate electrophoresis.

2.2. Analysis and Quantitation of GAG Components

Combined samples and original pelleted material were collected by centrifugation (Fisher Scientific Microcentrifuge Model 235A, Winnipeg, Manitoba), re-suspended in 10 μl of distilled water and labeled with a trace of Phenol Red (Sigma Chemical Co., St. Louis, Missouri) as a marker for the acetate plate electrophoresis gel according to a modification of the original articles by Cappelletti *et al.* (1979a and 1979b).

Aliquots of 2.5 μl of each sample Cellulose Acetate Plates were separated for electrophoresis on cellulose acetate plates (Helena Laboratories, Beaumont, Texas). Each plate (dimensions of 6 x 7.5 cm) was soaked 2-3 sec. (dist. water) to 1.5 cm of one of the borders along the smaller dimension. The opposite end was immersed in 0.1 M barium acetate (Sigma Chemical Co., St. Louis, Missouri) buffer with pH of 5.0 (by acetic acid, Fisher Scientific, Winnipeg, Manitoba) leaving a 2-4 mm band before reaching the water soak. Plates were removed and blotted before the application of the samples. The samples were then applied in the water zone of the plate and loaded on the electrophoresis apparatus (Bio Rad Laboratories, Model 1405 Horizontal Electrophoresis Cell, Mississauga, Ontario).

The procedure is divided in three different stages. The first stage is the electrophoresis run for about 2 to 3 minutes at 250 volts, until the samples align at the interface water/unsoaked plate as a thin yellow line. The plate is then blotted, soaked in 0.1 M barium acetate with pH of 5.0 for 1 to 2 minutes and blotted again. The second electrophoretic run is a longer stage for 9 to 12 minutes at 250 volts. Removed from the apparatus, the plate is once more blotted and immersed in barium acetate buffer containing 25% ethanol (v/v) for 2 minutes and blotted. The last stage is the electrophoretic run for 25 minutes at 250 volts. Following electrophoresis the samples were stained with 0.1% alcian blue 8GX OR (Mallinckrodt Inc., Paris, Kentucky) at pH of 2.5 in 0.1% of Acetic Acid, and destained with several changes of 5% acetic acid. Some of the plates were immersed in OptiClear solution (Gelman Science Inc., Rexdale, Ontario), blotted and dried by heated air at 60°C.

GAG standards were co-electrophoresed and the GAG bands were identified by comparison of the migration distances with the reference standards and by selective enzyme digestions of the polysaccharides (See appendix L). Radioactive bands were localized by the slicing of each lane into 3 mm strips and placed into mini-scintillation vials with 5 mls of scintillation cocktail (Aquasol, New England Nuclear, Boston, Massachussets) and counted for [^{3}H] in a liquid scintillation counter (Nuclear Chicago Isocap 300, Searle Instrumentation, Toronto, Ontario).

3. Study of Mandibular Growth

Thirty Sprague-Dawley male rats, inbred in our facility, were randomly divided in number of three for each of the following age groups: 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 weeks of age. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The mandible was removed and measured

with precision calipers (Staedtler, West Germany) to record the normal growth for the following dimensions: 1) Mandibular Length (ML), taken from the posterior border of the condyle to the dento alveolar-bone junction of the incisor; 2) Condylar Length (CL), taken from the most anterior to the most posterior point in the mandibular condyle and 3) Condylar Width (CW), taken from the most lateral points in the mandibular condyle, as shown in Appendix K, Figure K-1 (Bouvier and Zimny, 1987; Bouvier, 1988).

RESULTS

The effect of age in the newly-synthesized GAG molecules was evaluated by monitoring the ratio between the percentage concentration of each GAG molecule synthesized and the total GAG molecules produced throughout the organ culture period. In addition, the rate of synthesis of each molecule in each age group was determined by evaluation of DPM counts per mg of wet tissue weight for each age group. Therefore, both percentage and content of GAGs according to age were evaluated from rat articular disc cultured *in vitro* and were illustrated in the Figures IV-1, IV-2, IV-3 and IV-4. Among the types of GAG studied, the qualitative assessment of GAG synthetic activity as shown by the labeling pattern of $[^3H]$-glucosamine, suggested that HA, C_6S and KS/C_4S were the major fraction components of the articular disc.

A one-way analysis of variance model was used to test whether or not there were any significant differences between the synthesis of each GAG molecule as a function of age during the normal development of the rat articular disc. The results of the statistical tests for percentage distribution showed that HA, KS/C_4S and C_6S each appeared to vary significantly with age (HA, KS/C_4S, $p<0.05$), especially C_6S (C_6S, $p<0.0001$). In these GAG groups that showed to vary with age, Tukey's multiple comparison tests were used to identify which ages were significantly different from one another. KS/C_4S appeared to be similar in all the extremes of the age groups studied. At both the ages 3 and 11 weeks KS seemed to represent the highest fraction, which contrasted to 7 weeks of age when this molecule showed the lowest concentration. C_6S appeared to increase at 6 weeks of age and to drop to its lowest level at 8 weeks of age. These concentrations were significantly different from the other ages which did not show a big variation. Tukey's multiple comparison tests showed that the age groups with the largest significant variability of GAG types were 3, 5, 6 and 7 weeks. At age 3 weeks, HS molecules showed significant difference from KS/C_4S, C_6S and DS. At age 5 weeks KS/C_4S was different from C_6S. In

articular discs aged 6 weeks, KS/C_4S appeared to be distinct from C_6S, at the same time that C_6S and DS were significantly different than all the other types as evidenced in Figure IV-3. This situation was also evident at 7 weeks of age.

The one-way analysis of variance test for content of GAG per mg of wet tissue also suggested that KS/C_4S, C_6S and HA concentrations varied with age ($p<0.0001$). KS/C_4S appeared to be increased at 3 weeks of age and decreased after that. C_6S and HA increased significantly at 6 and 7 weeks respectively where they showed the highest peak and dropped significantly after that. The concentration of newly-synthesized molecules of DS, HS and H did not show any significant changes with age. Comparison of GAG synthesis and the rate of mandibular growth suggested a correlation between the synthesis of KS/C_4S and C_6S to the velocity of the apparent mandibular growth spurt, which in rats takes place from ages 5 to 7 weeks (Appendix K). However, tests of correlation were not attempted to determine the significance of that correlation. The synthesis of KS/C_4S decreased during the mandibular growth spurt while C_6S peaked during the period of intense growth (Figures IV-5 and IV-6). HA also demonstrate the same relationship as C_6S as shown in Figure IV-7.

The was a significant interaction between specific GAG synthesis and age ($p<0.005$). However, when the samples were tested with a two-way analysis of variance model to determine if the differences over age were consistent for all the GAG groups, it was evident that the heterogeneous molecules did not follow the same pattern of changes. The mean values for the percent concentration and DPM/mg with respective standard deviations for each GAG molecule for all age groups studied are summarized in Table IV-I and in Table IV-II respectively.

Modified acetate plate electrophoresis showed a clear and distinct separation of all different known classes of GAGs, with the exception of KS and C_4S which migrated together (Figure N-1 in Appendix N) and were therefore classified as one group. However,

enzymatic treatment of GAGs and GAG separation (Figure J-1 in Appendix J) showed that the actual content of C_4S was very low; therefore, the changes described above can be accounted mostly for KS alone. This was due to the percentage of ethanol selected in one of the buffers during the actual procedure (for more detail see Appendix N).

Figure IV-1: Graph showing the percentage of GAG distribution in the rat articular disc as a result of normal development. Legends are: C_4S/KS - chondroitin$_4$ sulfate/keratan sulfate, C_6S - chondroitin$_6$sulfate, DS - dermatan sulfate, HS - heparan sulfate and H - heparin.

Figure IV-2: Graph showing the percentage of hyaluronic acid distribution in the rat articular disc as a result of normal development. Legends is: HA - hyaluronic acid.

Figure IV-3: Graph showing the DPM/mg GAG content in the rat articular disc as a result of normal development. Legends are: C_4S/KS - chondroitin$_4$ sulfate/keratan sulfate, C_6S - chondroitin$_6$sulfate, DS - dermatan sulfate, HS - heparan sulfate and H - heparin. Note the increase in GAG content, mainly C_6S and DS at 7 week old animals.

Figure IV-4: Graph showing the DPM/mg hyaluronic acid content in the rat articular disc as a result of normal development. Legends is: HA - hyaluronic acid.

Figure IV-5: Graph showing the DPM/mg C_4S/KS content in the rat articular disc as a result of normal development compared to the velocity curve of the rat mandibular growth. Legends are: C_4S/KS Synt. - chondroitin$_4$sulfate/ keratan sulfate synthesis and Mandibular Inc. (mm) - mandibular growth increment. Note that the synthesis of C_4S/KS decreases during the mandibular growth spurt.

Figure IV-6: Graph showing the DPM/mg C_6S content in the rat articular disc as a result of normal development compared to the velocity curve of the rat mandibular growth. Legends are: C_6S Synt. - chondroitin$_6$sulfate synthesis and Mandibular Inc. (mm) - mandibular growth increment. Note that although the correlation of mandibular growth spurt and C_6S synthesis at age 7 and 8 weeks appears significant, C_6S synthesis did not folllow the second peak in mandibualr increment at 11 weeks old animals.

Figure IV-7: Graph showing the DPM/mg HA content in the rat articular disc as a result of normal development compared to the velocity curve of the rat mandibular growth. Legends are: HA Synt. - hyaluronic acid synthesis and Mandibular Inc. (mm) - mandibular growth increment. Note the correlation of mandibular growth spurt and HA synthesis at age 7 and 8 weeks. Similarly to C_6S synthesis, HA did not follow the second peak in mandibular increment at 11 weeks old animals.

Table IV-I: Table of the percentage of GAG distribution in the rat articular disc as a result of normal development.

Table IV-II: Table of the DPM/mg GAG content in the rat articular disc as a result of normal development.

Figure IV-1

Percentage of GAG Distribution in the Rat Articular Disc

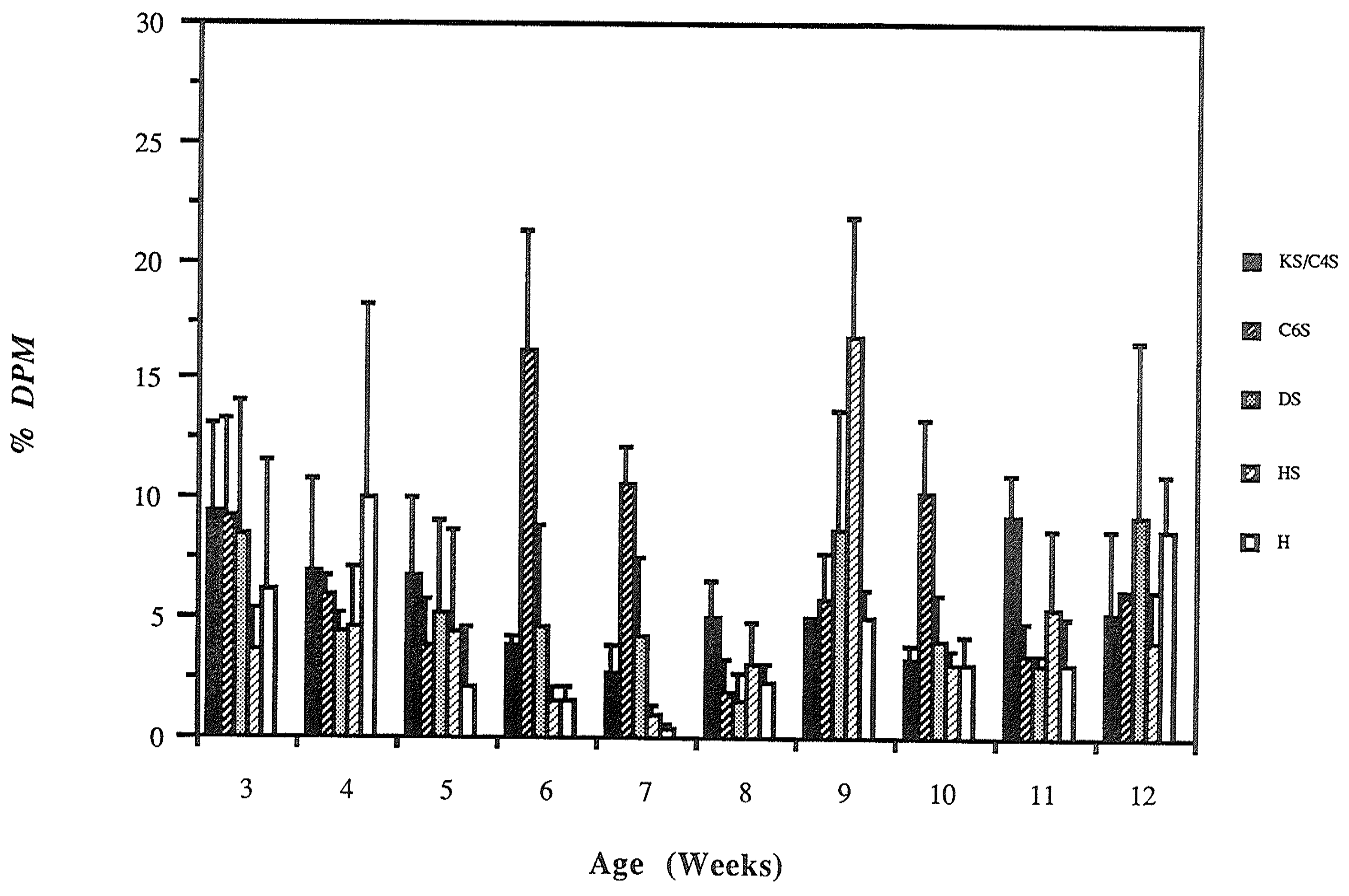


Figure IV-2

Percentage of Hyaluronic Acid Distribution in the Rat Articular Disc

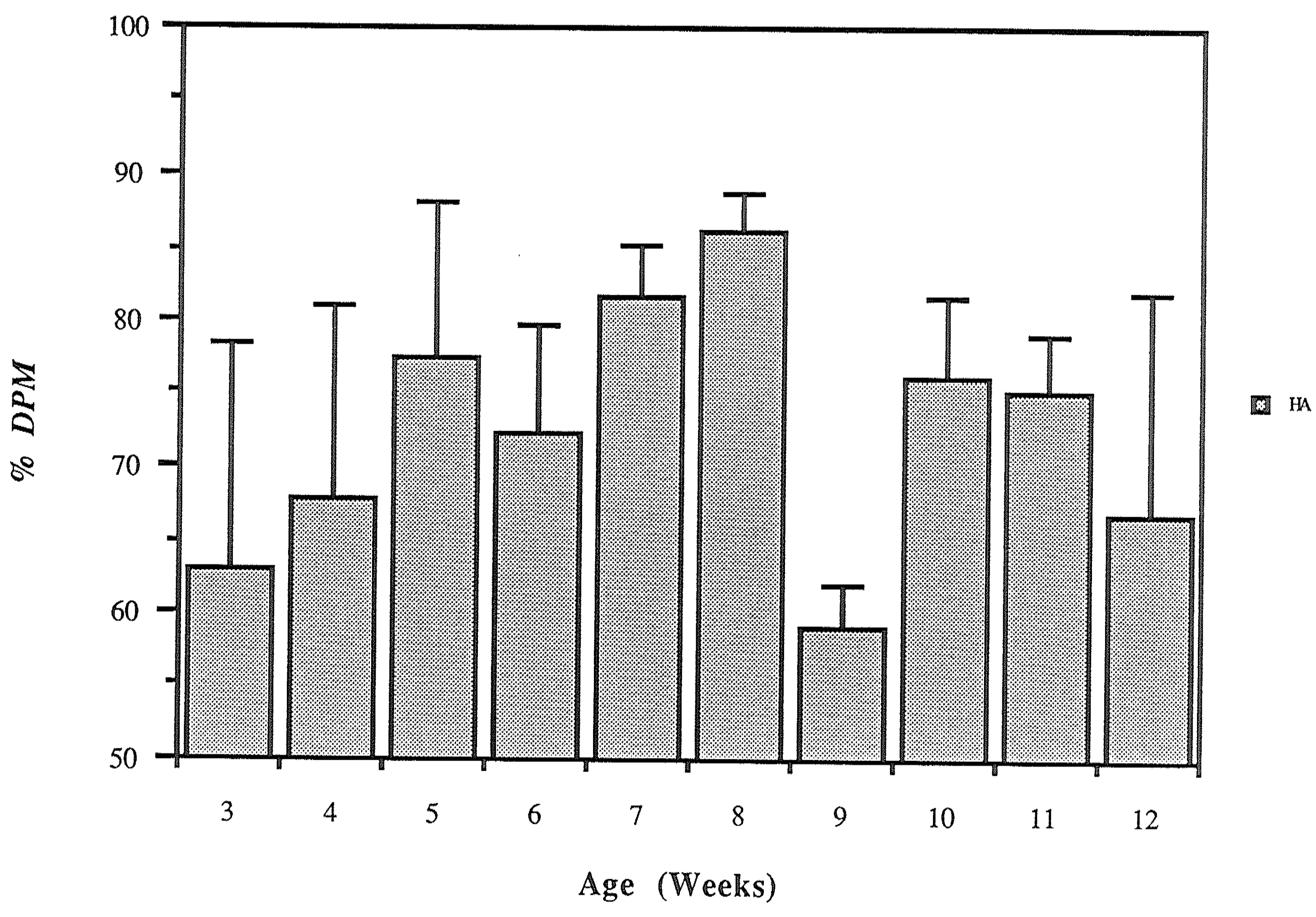


Figure IV-3

DPM/mg GAG Content in the Rat Articular Disc

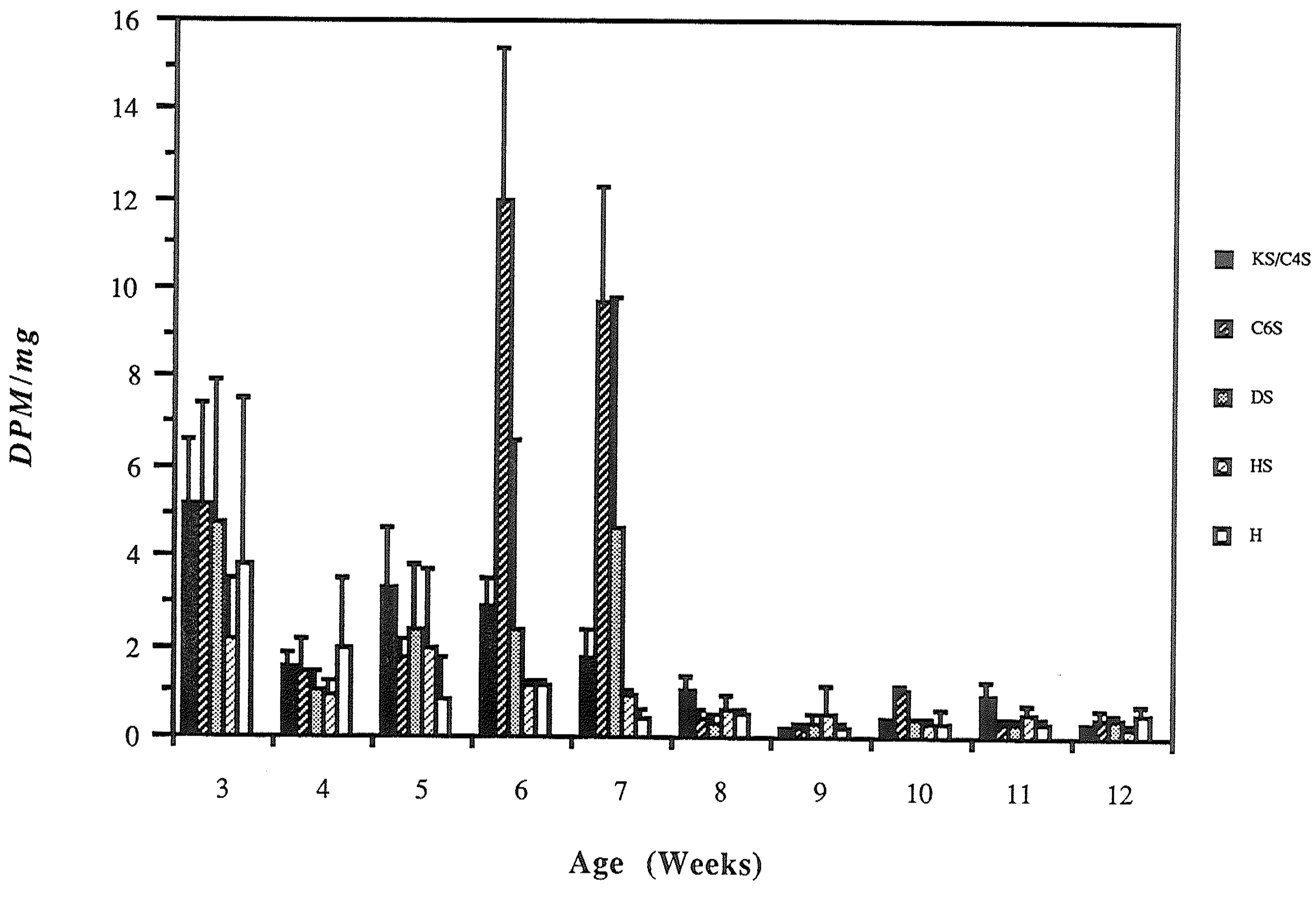


Figure IV-4

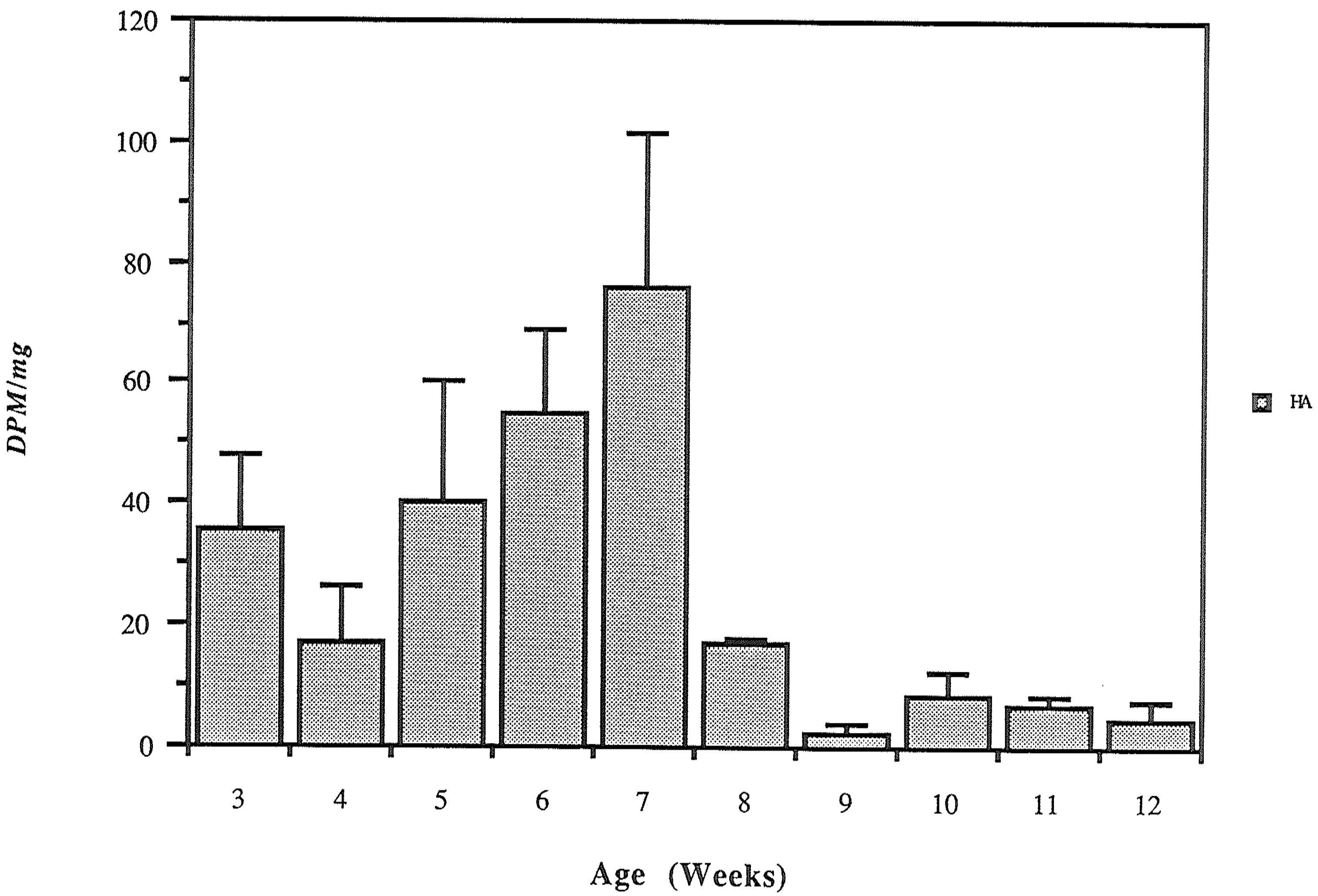


Figure IV-5

C4S/KS Synthesis and the Velocity Curve of the Rat Mandibular Growth

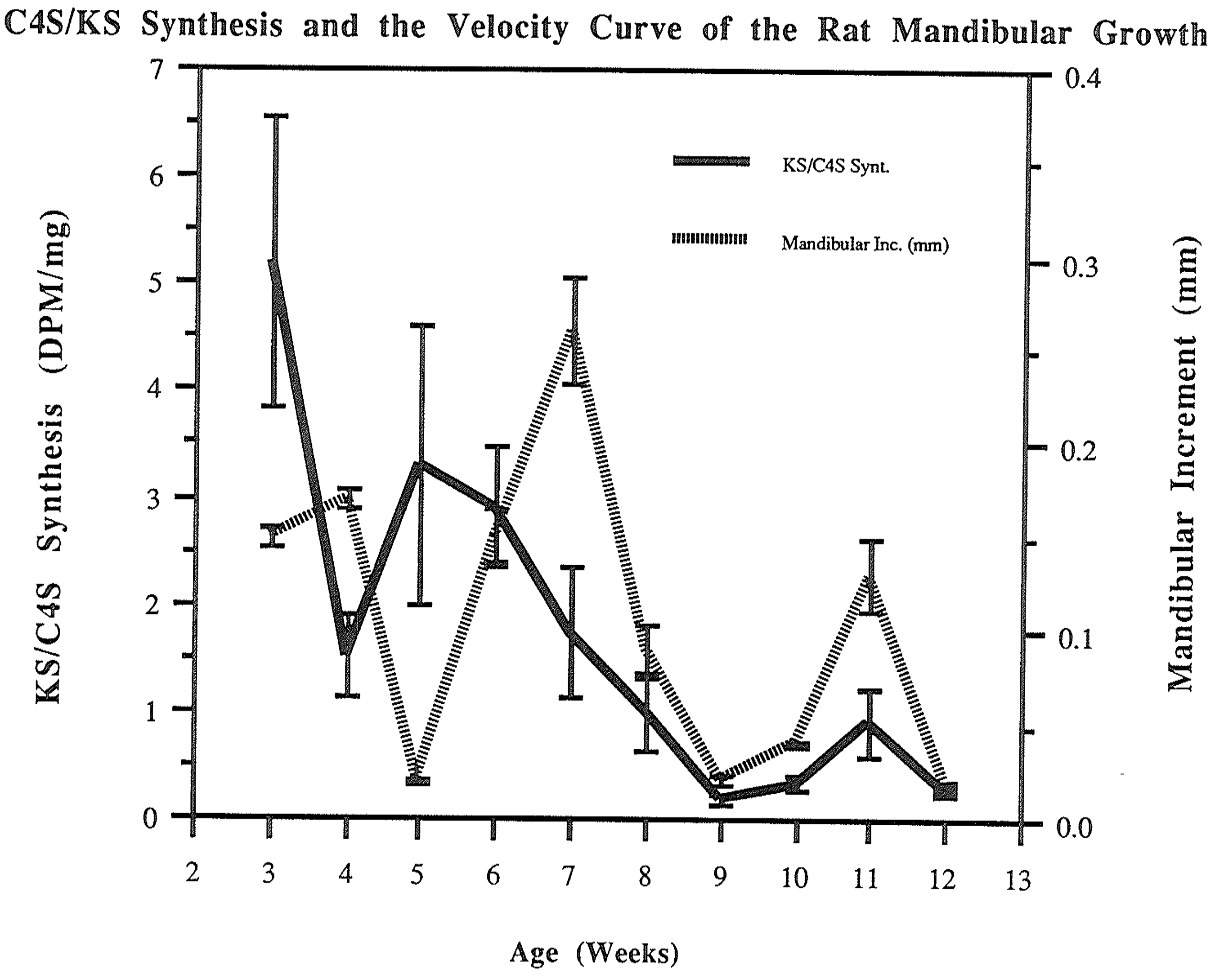


Figure IV-6

C6S Synthesis and the Velocity Curve of the Rat Mandibular Condyle

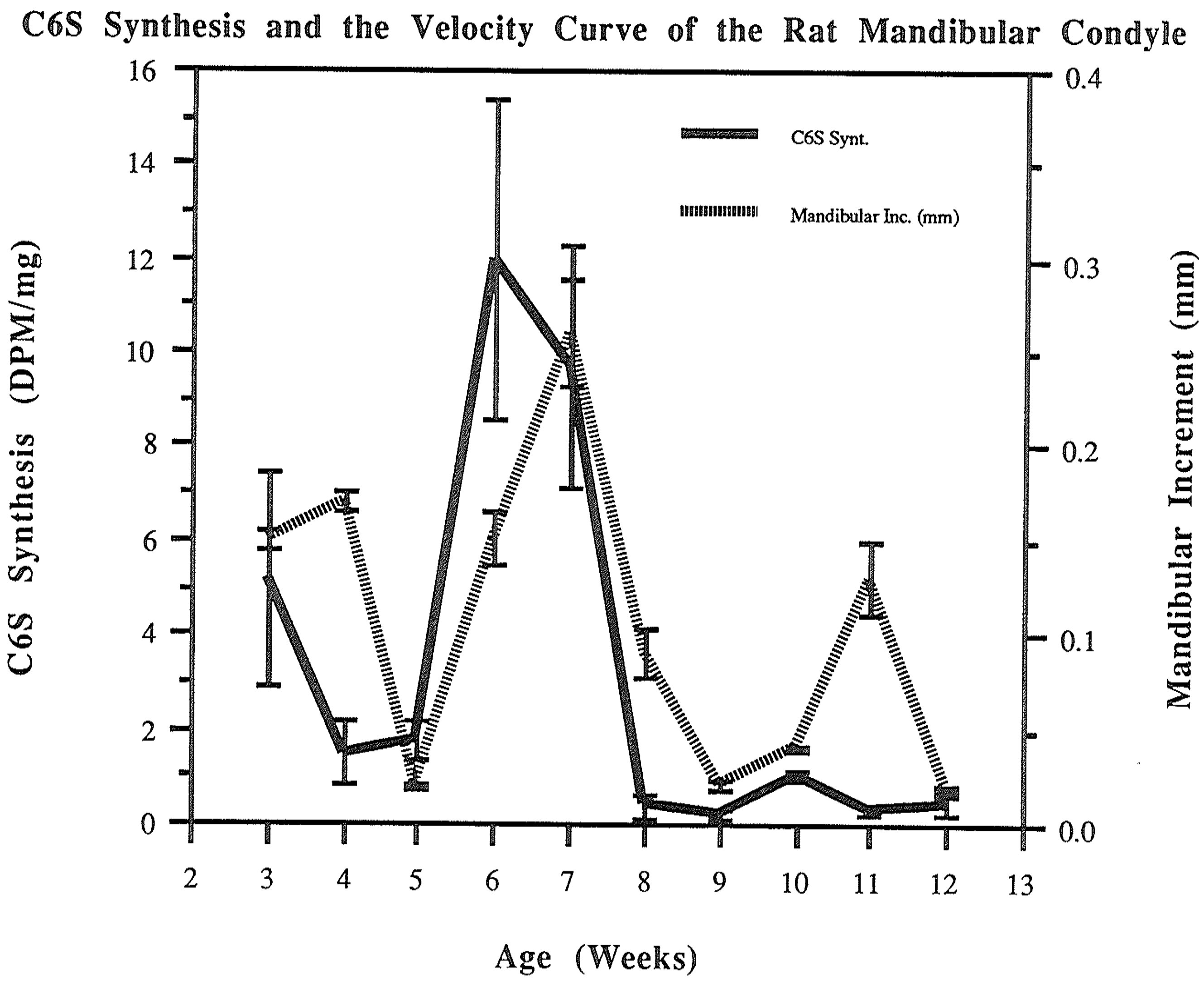


Figure IV-7

HA Synthesis and the Velocity Curve of the Rat Mandibular Growth

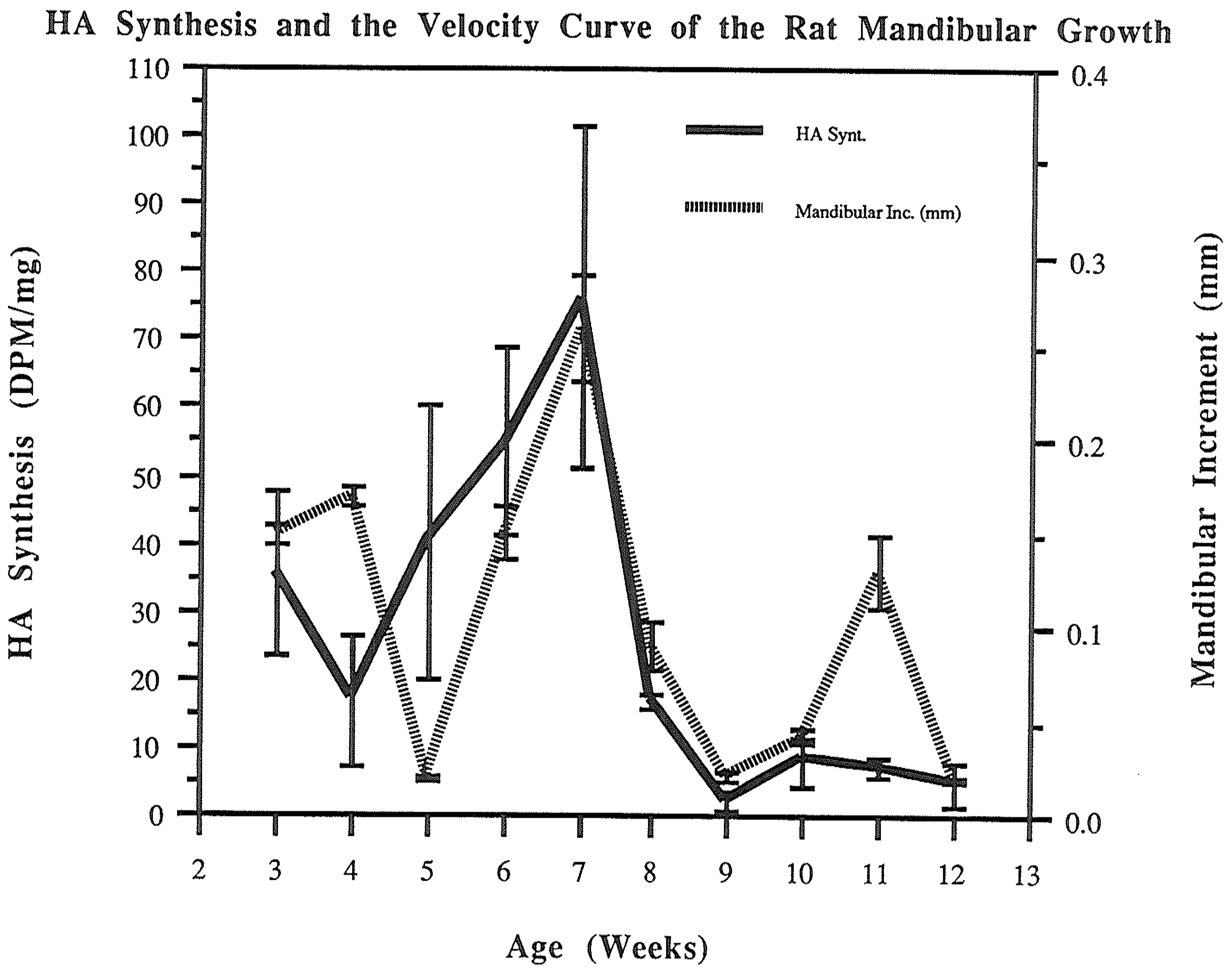


Table IV-I

Values for the Percentage of Different Glycosaminoglycans from the Rat Articular Disc

Type of GAGs	KS/C_4S		HA		C_6S		DS		HS		H	
Age (Weeks)	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.
3	9.51	3.58	62.86	15.63	9.32	4.06	8.55	5.63	3.61	1.74	6.12	5.55
4	7.06	3.72	67.62	13.18	6.08	0.62	4.51	0.90	4.63	2.53	10.02	8.25
5	6.87	3.23	77.31	10.85	3.87	1.97	5.29	3.85	4.49	4.22	2.15	2.48
6	3.87	0.34	72.17	7.42	16.17	5.11	4.70	4.16	1.55	0.60	1.53	0.51
7	2.72	1.15	81.63	3.54	10.62	1.64	4.24	3.40	1.03	0.37	0.39	0.25
8	5.03	1.54	86.00	2.67	2.03	1.26	1.46	1.17	3.10	1.68	2.37	0.68
9	4.77	0.34	58.99	3.06	5.77	2.01	8.65	5.13	16.76	5.08	5.04	1.18
10	3.20	0.65	76.27	5.25	10.24	3.06	4.07	2.01	3.16	0.56	3.05	1.18
11	9.29	1.76	75.02	4.17	3.40	1.35	3.07	0.43	5.40	3.27	3.83	1.89
12	5.19	3.44	66.68	15.18	5.91	0.23	9.37	7.18	4.16	2.07	8.68	2.33

Table IV-II

Values of DPM/mg Content of Different Glycosaminoglycans from the Rat Articular Disc

Type of GAGs	KS/C_4S		HA		C_6S		DS		HS		H	
Age (Weeks)	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.
3	5.19	1.37	35.72	11.98	5.17	2.31	4.71	3.28	2.15	1.35	3.83	3.75
4	1.50	0.39	16.89	9.68	1.49	0.71	1.07	0.34	0.92	0.37	1.96	1.56
5	3.30	1.30	40.18	20.08	1.72	0.42	2.33	1.54	1.95	1.79	0.84	0.88
6	2.91	0.55	54.96	14.07	11.97	3.40	2.39	4.21	1.11	0.15	1.11	0.16
7	1.73	0.60	76.20	25.11	9.74	2.58	4.67	5.12	0.89	0.15	0.37	0.24
8	0.99	0.34	16.87	1.00	0.39	0.25	0.28	0.21	0.59	0.29	0.47	0.15
9	0.18	0.05	2.32	1.28	0.22	0.07	0.33	0.21	0.47	0.65	0.19	0.08
10	0.33	0.08	8.46	4.26	1.02	0.14	0.38	0.03	0.33	0.08	0.36	0.23
11	0.90	0.31	7.11	1.54	0.31	0.08	0.29	0.09	0.48	0.24	0.34	0.12
12	0.25	0.03	4.69	3.40	0.39	0.21	0.44	0.04	0.23	0.07	0.51	0.20

DISCUSSION

Maturation of connective tissue has demonstrated to be a complex process. The effects of time dependent mechanisms such as aging in development and growth is not *per se* the establishment of an impairment process (Thonar and Kuettner, 1987), in spite of its suggestion in the literature through studies of arthritis and induced osteoarthritis (Mankin and Lippielo, 1971; Lust *et al.*, 1981; Adams *et al.*, 1981; McDevitt *et al.*, 1981). However, it is well known that connective tissue's structural molecules vary with age (Stockwell and Scott, 1965; Stockwell and Scott, 1967; Stockwell, 1970). The present study focused in this process through the assessment of the amount of GAGs, the widespread group of molecules that participate actively in the functional behavior of weight bearing structures such as bone and cartilage, and in this case the articulating disc from the temporomandibular joint. The proportion and amount of different types of GAGs were demonstrated in the articular disc by extraction with protease, precipitation with CPC and ethanol (Bronson *et al.*, 1989) and separation of different GAG types in cellulose acetate plates (Cappelletti *et al.*, 1979a and 1979b).

The temporomandibular disc is composed mainly of fibrous tissue of collagen fibers with some characteristics resembling cartilage (Silva, 1969). The amount of GAG molecules accounts for most of the fibrocartilagenous properties of the disc, and they have shown to vary consistently in distribution (Kopp, 1976; Mills *et al.*, 1988). Despite the composition of the articular disc in relation to its GAG content has been reported in the rabbit disc (Grandstrom and Linde, 1972; Mills *et al.*, 1988), rat disc, monkey disc, dog disc (Granstrom and Linde, 1972) and bovine disc (Nakano and Scott, 1989) the variation of these molecules as a result of age it is still not clear.

Nakano and Scott (1989) reported that the major GAG fractions of the bovine disc were C_6S and DS with smaller proportions of HA and KS. Similarly, Mills *et al.* (1988) reported that the disc is composed mainly of C_6S and KS. On the other hand, the analysis

of the GAG content of the dog, rabbit and monkey discs by Granstrom and Linde (1972) described DS as the major portion and in rat disc the major GAG was reportedly HA. In this study, the amount of HA has proved to be the major fraction in the rat articular disc in all the ages followed by C_6S. This variability may be explained by the intrinsic species differences (Webber *et al.*, 1986) in load-bearing TMJ function (Daniel *et al.*, 1988), and the methods for GAG separation. The variation in composition and the high polydispersity of GAG molecules may also play important roles in the analysis of GAGs in the different tissues studied. According to Granstrom and Linde (1972), hybridization between C_6S, C_4S and DS, the variation in sulphation and the polydispersity of molecular weights can not be determined by the isolate differences in GAG migration.

Molecules may be present in a reduced number or be masked by the larger aggregate complexes. Soluble GAG molecules can be extracted at a rate of 85% of the total tissue hexuronate for cartilage (Sajdera and Hascall, 1969), which also depends on the methods of isolation and purification. Due to the difficulty in purifying and isolating GAG from the collagen meshwork and PG aggregates, most of the studies have been qualititave. PG aggregation not only immobilizes the PG complexes in the matrix, but the dissociation of these large macromolecules enables endogenous collagenase to remain activated in the matrix (Muir, 1981). The presence and concentration of small oligosaccharide chains and link protein may also contribute to the selectivity of GAG extraction. The changes in the molecular structure of GAGs and PGs in studies of aging and maturation of cartilage have shown that in older cartilage the PGs are smaller, have shorter CS chains and have more KS (Inerot *et al.*, 1978; Bayliss and Ali, 1978; Roughley and White, 1980; Glant *et al.*, 1986). These studies also suggested that in adult cartilage the heterogeneous and polydisperse PG population represents more than one type of population with more than one core protein.

In the present investigation, only the newly-synthesized GAGs were measured by the incorporation of radioisotope labelling and separated using acetate plates electrophoresis. The different GAGs were evidenced by co-migration of standards and selective enzyme digestion (see Appendix J and N). The percentage of each fraction per age group relative to the total amount of radiolabel incorporated into GAGs was recorded as well as the GAG content in relation to the wet weight of each specimen. It was demonstrated in all the ages studied that at 7 weeks there was a relative increase in the total amount of radioactivity in comparison to the other ages. HA appears to peak at 7 weeks, decreasing after that. C_6S also rises at 6 and 7 weeks of age as well as DS. These results are in agreement with studies of human articular cartilage in which as C_6S decreased with age with a concomittant increase in KS and sialic acid (Roughley *et al.*, 1981). The present data failed to detect a progressive increase in the percentage of KS which seemed to be increased at 11 weeks of age, decreasing after that. Such differences may be partly due to the variability of the *in vitro* results that artificially represent a more complex *in vivo* environment.

According to Carney and Muir (1988) the most pronounced changes in the content of GAG molecules in human cartilage takes occur during the first two decades of life. After the developmental phase of the tissue, the age-related changes in GAG content are much slower as evidenced by the constant proportion of C_6S (Anderson *et al.*, 1964; Bollet and Nance, 1966). In this study, by the comparison of C_6S content in rats to the rat mandibular growth rate, C_6S seems to be highly correlated with the period of fast growth of ages 6 and 7 weeks (see Figure IV-6). After that, there is hardly any changes in C_6S molecular content.

GAG chains are very important in the maintanance of the resistance and resilence of connective tissue (Hardingham, 1981). However, from the known GAG types, few of them have been clearly related to specific functions that could be correlated to the overall

tissue behaviour. HA has been involved in several biological processes, including a critical role in the aggregation of cartilage PGs (Hascall, 1986). HA is also known to play an important role in temporal control of cell migration and cell differentiation in developing and remodeling tissues (Tomida *et al.*, 1974; Toole, 1982; Bartold and Page, 1985), which can be evidenced by the increase in hyaluronidase in non developing tissues (Alberts *et al.*, 1989). This is an important evidence of the role of HA in tissue morphogenesis (Toole, 1982; Laurent and Fraser, 1986). In this investigation, the role of HA in development can be correlated with a period of fast growth of the rat mandible (Figure IV-7). However, it can not be explained why the mandible growth seemed to peak at 8 weeks of age, when HA synthesis peaked at 7 weeks decreasing after that. This may reflect a more active role of HA in the initial stages of development, where relatively immature GAGs are being synthesized. According to Toole (1982), the sequences of HA were divided into a morphogenic phase during which the cells accumulate by proliferation and migration (HA production), and a differentiation phase, with more HA activity. In addition, Glant *et al.* (1986) have found the presence of two populations of PGs as age related molecules, divided in foetal type and adult type due to their chemical characteristics. However, in this case it seems more like a substitution of molecular synthesis, in which both types can not be found concomitantly.

The second most abundant GAG here or C_6S has been related mostly with the GAG chain that is directly involved in tissue resistant to mechanical forces (Hardingham, 1979; Daniel and Mills, 1988). C_6S is abundantly present in cartilage. Sweet *et al.* (1977) reported an increased percentage of C_6S in the growing zone of immature cartilage, particularly in the area of minimum contact. This suggests that C_6S may not change with the increase in stress on the cartilage as evidenced by the areas of maximum contact. Roughley *et al.* (1981) reported that the parameters responsible for producing the age-related changes in cartilage are not influenced by the degree of weight-bearing on the

cartilage. Although, the type of cartilage studied and the species variability may account for such differences, the mechanical stress such as mechanical compression induce C_6S to increase (Daniel and Mills, 1988). C_6S has been shown to decrease in cases of osteoarthritis (Matthews, 1953; Bollet and Nace, 1966), that seems to be related with the severity of the process (Mankin and Lipiello, 1971). However, this finding may depend on the region of the cartilage studied. In rib cartilage, C_6S seems to be unaltered (Bollet and Nance, 1966; Mankin and Lippiello, 1971) while in hips cartilage it appearently increases (Mankin and Lippiello, 1971).

DS is usually present in connective tissue mostly in the form of a small, interstitial PG (DS-PG) that is present in the extracellular matrices (Heinegard *et al.*, 1985). This type of GAG has been shown to bind non-covalently to collagen fibrils and fibronectin and inhibit fibrillogenesis *in vitro*, as well as the adhesion of fibroblasts to fibronectin. DS also inhibit collagen fibril growth *in vitro* (Vogel *et al.*, 1984; Vogel and Trotter, 1987; Scott, 1988; Nakano and Scott, 1989). It seems that the change of the amount of newly-synthesized DS in the disc can not be fully understood based on these functions alone. However, it seems logical that the variability of the weight bearing capacity of the disc in different ages may reflect a stronger interaction of collagen fibers and PGs and that possibly DS may exert a favorable effect in this type of mechanism. DS may not be correlated to the withstanding of mechanical compressive forces as CS, but to a means of stabilizing the collagen network, since according to Nakano and Scott (1989), the percentage of DS in the aticular disc is similar to skin and tendon, which are tissues that are more subjected to tensional forces.

The total amount of the uronic acid as well as hexosamine and glucosamine content were not evaluated here. Post-translational modifications, such as N-and O-linked oligosaccharides and the structure of link proteins were not accounted either. It is important to keep in mind that other age-related changes in PG structure, GAG molecular length, and

degree of sulfation of GAG chains will complement the understanding of GAG modification as a result of tissue remodeling. There seems to be more than one mechanism by which age affects PG structure (Carney and Muir, 1988), like a continuous variation in the core protein, synthesis of PG isoforms, altered peri- or extracellular modifications and altered synthesis of carbohydrate components.

The observations of this study have demonstrated that the GAG content in the articular disc varies with age in a relative linear pattern. Although very little is known about GAG functions, it is possible to speculate that functional demands cause the articular disc to yield a cartilagenous phenotype at ages of intense cellular activity as compared to a fibrocartilagenous behaviour in the adult tissue, however, this information needs to be confirmed with a histological analysis of the disc specific for cartilage tissue. The structural components of the articular disc that are demanded by the forces in which they are subjected suggests a good model for studies of mechanical stimulation and disc remodeling, which according to Nakano and Scott (1989) may advance the understanding of the relationship between chemical structure and biochemical function; this was investigated in Chapter V.

CONCLUSIONS

1. HA, C_6S and KS/C_4S were shown to be the major GAG fractions in the rat articular disc.

2. The percentage of HA peaked at 8 weeks of age while the content of DPM/mg of wet tissue peaked at 7 weeks of age. After the age of 7 weeks there was a generally reduced GAG synthetic activity as evidenced by the incorporation of $[^3H]$-glucosamine. Similarly, both the percentage and the DPM/mg content of C_6S appeared to peak at 6 and 7 weeks of age whereas KS/C_4S showed a progressive decrease in concentration in all the ages studied.

3. Our findings are agree with similar results in the dog, rabbit, monkey and rat articular disc (Grandstrom and Linde, 1972) and rabbit (Mills *et al.*, 1988) and bovine articular disc (Nakano and Scott, 1989). However, these studies were of only specific age groups.

4. The increased proportion of GAG content at 6 and 7 weeks of age of the rat articular disc may be correlated with the period of fast growth in the rat mandible. This indicated that the articular disc undergoes a greater degree of remodeling and functional demand during the same ages as a result of growth and possibly stress/strain applied to the disc and disc ligaments.

5. Analysis of variance suggested a significant interaction between GAG concentration and aging ($p<0.005$). From all the age groups studied ages 6 and 7 weeks demonstrated the largest concentration of GAGs, particularly HA and C_6S.

6. Although the functions of different GAGs have been poorly characterized, there is a suggestion that variable degrees of sulfation and differences in ratios of GAG types

play important roles in the overall tissue behaviour of PG aggregates. Further investigations are, therefore, also required of other PGs components (e.g. protein cores, link protein, N- and O-linked oligosaccharides) during growth and development of the human TMJ tissues.

7. This method of organ culture of articular disc tissues that maintain synthetic patterns *in vitro* will permit future studies on control mechanisms using mechanical stress for disc remodeling.

Chapter FIVE

Synthesis of GAGs *In Vitro* Mechanically
Stressed Rat Articular Disc

SUMMARY

Mechanical stress is important for the maintenance of connective tissue with cartilagenous properties. The variation of mechanical pressure or tension affects the underlying molecular structure that distributes itself according to the stimulus applied. Fibrocartilage is closely related to hyaline cartilage functionally, in which there is a marked change in polysaccharide content given by GAGs synthesis and aggregation in PGs.

The mechanism by which compressive mechanical stress affect polysaccharide complexes was investigated in the articular disc with a modified organ culture technique. Forty eight Sprague-Dawley rats were divided into 4 different groups comprising the ages of 7 and 9 weeks. Each group had 12 animals divided equally for both ages. In each group, three animals were experimental and one control. Each of the experimental groups followed a different regimen of stress; 25%, 75% and 100% of stress out of the total stress period of 24 hours. The stress was applied in an intermittent pattern for the correspondent duration of each group within the total span of the organ culture. The mandible of each animal was extracted and each hemi-mandible was dissected and carefully cleaned in organ culture medium of all soft tissue, except the articular disc that remained attached to the condylar head. The explants were then trimmed and mounted in special organ culture dishes with flexible bottoms and incubated with [^{3}H]-glucosamine for the period of 24 hours.

The application of stress was possible by using an apparatus that has the ability to apply different vacuum pressure regimens in the organ culture dishes with flexible bottoms. Under normal conditions, the vacuum pressure causes the bottom of the dishes to deform in the direction of the applied pressure and the more vacuum applied, the more deformation will result. Therefore, each hemi-mandible was placed in such a way that the articular disc was compressed by the condylar head and the bottom of the dish. During a period of vacuum, the bottom of the dishes would deform releasing the pressure applied on the discs.

By varying the time and frequency that the vaccum pressure was applied, it was possible to represent different regimens of stress similarly to the normal joint *in vivo*.

Following culture, the samples were collected and digested with Pronase-E, and following precipitation with CPC and ethanol, the different GAGs were separated using cellulose acetate plate gel electrophoresis. The percentage of each of the GAG types showed a directly proportional increase with the amount of pressure applied. C_6S was the most abundant GAG and seemed to increase steadily with stress. On the other hand HA and the other GAG types showed a progressive decrease with the increase in stress. There was no significant relationship between age and regimen of stress applied. Both ages demonstrated similar results and 75% and 100% showed significantly higher results for C_6S. As C_6S is the major component of hyaline cartilage, the results of this study suggest that compressive forces in the articular disc may select for a more cartilagenous-like properties with respect to GAG content.

INTRODUCTION

Articular disc behavior under pathologic situations is regulated by the degree of adaptation of its molecular components and by the biomechanical properties of its fibrocartilage constitution, which in turn is due to the complex regimen of stress and strain constantly present in the temporomandibular joint.

The properties of all types of cartilage are relative to their composition. Biomechanically, the most important component of cartilage is water which makes 60 to 80% of its volume allowing a large portion of the interstitial fluid to move freely under a load or pressure gradient (Myers and Mow, 1983). The response to mechanical stress applied to cartilage is divided into two components (Kempson, 1973). The first is an instantaneous phase, which is independent of fluid flow, followed by a long phase, which is due to fluid flow and accounts for the most change in shape (Stockwell, 1979). The rate of fluid expression relates with the magnitude of deformation and the stiffness of the cartilage, and is dependent of the hydraulic permeability of the matrix, which is inversely proportional to the PG concentration. This is in accordance with the concept of Newtonian mechanics where the result of the deformation of a given material when subjected to external mechanical stimuli depends on the intrinsic properties of the material as well as on its shape (Myers and Mow, 1983).

Water diffusion plays an important role in connective tissue mechanics (Fessler, 1960; Sokoloff, 1963; Myers and Mow, 1983). A model correlating equations of stress (force per unit of original area) and strain (deformation per unit of original length) with the fluid and the solid component of cartilage has been created to explain time dependent processes such as creep and stress relaxation (Myers and Mow, 1983). The liquid diffuses through the porous and permeable solid part of the tissue while the movement of the fluid is restricted by frictional drag between water and the pore walls. This is the theory of a biphasic material in which the stress changes with time when the material is externally

deformed, until fluid motion ceases. On the other hand, when the material is externally loaded the strain varies with time (Mow *et al.*, 1980)

Cartilage matrix is responsible for resistance to compressive, tensile and shearing forces. Stockwell (1971b) in studies of interrelationships of cell density, cartilage thickness and species size suggested that the amount of matrix per cell varies according to the stresses applied by the block of cartilage tissue. The large loads applied to the cartilage surfaces are not static, in other words, during the motion of the opposite sliding of articular surfaces the magnitude and direction of the applied force is being altered.

PGs are an important constituent of cartilage and are extremely compressed, exerting a considerable swelling pressure which is resisted by the collagen network (Maroudas, 1980; Muir, 1981). Applied load increase the internal swelling pressure, through the increase of PG concentration offering a resistance against fluid flow until an equilibrium with the external applied force is achieved, when fluid movement ceases (Myers *et al.*, 1983). The relationship between resistance to fluid flow and PG content is thought to be the result of frictional interaction with the fine structure of GAG molecules within PG aggregates (Maroudas, 1979). The loss of these complexes results in a tenfold decrease in the compressive modulus (Stahurski *et al.*, 1981), which affects the hydraulic permeability and therefore the load carrying characteristics of cartilage (Myers and Mow, 1983).

Although, often correlated with pathology, mechanical influence is necessary to stimulate the normal cartilage metabolism (Meickle, 1973; Stutzman and Petrovic, 1974; Simon, 1977). Articular cartilage subjected to immobilization or alteration of normal mechanical stress degenerates and becomes thin which is probably due to loss of matrix, since numerous chondrocytes are found (Collins *et al.*, 1960). When compression is present, together with immobilization, there is loss of metachromasia and cartilage thickness followed by chondrocyte degeneration and cartilage fibrillation and erosion

(Salter *et al.*, 1960; Thompson and Bassett, 1970). Degeneration in cartilage is often accompanied by fibrilation (Freeman and Meachim, 1979). Armstrong and Mow (1982) related the degree of degeneration with the compressive equilibrium modulus of human articular cartilage, but no correlation was found. In addition, the diseased state of cartilage, mainly in its osteoarthritic form, has many effects on mechanical behavior with a progressive weakness, lack of stiffness and increased permeability (Myers and Mow, 1983).

In general, mechanical wear and biological degradation will influence the mechanical properties of the tissue and breakdown will occur (Kempson, 1973; Armstrong and Mow, 1982). Therefore, the correlation of the connective tissue matrices to their particular properties and mechanical stress is an important step for the characterization of abnormal function (Hukins 1984). The present investigation attempted to determine the changes in the pattern of newly-synthesized GAG molecules in two different ages of rat articular disc that were subjected to mechanical forces of different durations.

MATERIALS & METHODS

1. *In Vitro* Organ Cultures

This study selected Sprague-Dawley male rats, inbred in our facility, that were randomly divided into four different groups, each group consisting of twelve animals of the ages 7 and 9 weeks. From the four groups, three were experimental, namely: (1) compressive force of short duration (25%), (2) compressive force of long duration (75%) and (3) continuous compressive force. The remaining group (4) served as the control group.

The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. Both hemi-mandibles of each animal were removed and immediately transported to a laminar flow hood (Nuaire, Minnesota), where they were placed on a petri dish (100 X 20 mm/Corning, Corning Glass Works, New York) containing growth medium which consisted of Dubelco's minimun essential medium (Gibco/BRL, Burlington, Ontario), 400 U/ml of penicillin G (Gibco Laboratories, Grand Island, New York), 0.56 mg/ml of streptomycin sulphate (Gibco Laboratories, Grand Island, New York), 0.2 mg/ml of ascorbic acid (Sigma Chemical Co., St. Louis, Missouri) and 2.2 mg/ml of sodium bicarbonate (Fisher Laboratory Chemical, Fair Lawn, New Jersey). All the soft tissue was removed with the exception of the articular disc which remained attached to the condyle through the lateral ligaments. The medium was sterilized using a Millipore 0.22 μm filter (Millipore Corp., Mississauga, Ontario). Each hemi-mandible was then carefully measured and sectioned to fit in the organ culture apparatus for the application of artificial mechanical stress (See Appendix O, figure O-1). The sectioned hemi-mandibles were fixed to the lid of a specially modified organ culture dish with flexible bottoms (Flex I - Generic Type I collagen, McKeesport, Pennsylvania) with the use of

Cyanoacrylate cement (Instabond, Le Page, Boucherville, Quebec) as shown in Figure V-1.

Each modified organ culture dish contained 6 articular discs properly attached to the hemi-mandibles in six separate wells (see figure V-2), each containing 1.5 mls of growth medium. The placement of the explants was done in such a way that the articular disc had a constant load applied in between the condylar surface and the flexible bottom of the well from the organ culture dish. In order to keep an effectively active load in the disc, a standardized weight (mean weight of 257.6 grams) was placed on top of the culture dish. The mechanism of stress used in this study can be seen in Figure V-3 and the activated stress samples are shown in Figure V-2.

All dishes from all groups were cultured for 24 hours in a humidified incubator (National Incubator Model 4200, Portland, Oregon) with an atmosphere of 5% carbon dioxide at 37°C. The experimental groups (1) and (2) were cultured under specified regimens for the organ culture apparatus (Flexercell Strain Unit, Flex Cell Corp., McKeesport, Pensylvania). Group (1) regimen consisted of stress 75% of the time and relaxation 25% of the time in 24 hours as compared to the second group that consisted of stress 25% of the time and relaxation 75% of the time in the same period. The group (3) had stress 100% of the time, while the group (4) had no stress at all. All the stress described from the apparatus ranged from 10 to 15 KPa. The regimen for 25% stress of the total time of culture was of a cycle of 10 seconds of stress and 10 seconds of relaxation, in a period of 30 minutes in every 2 hours of culture. Therefore, the total cycle was of a total of 6 hours proportionally spaced in the total culture time. Comparatively, the 75% regimen of stress had the same cycle for a period of 90 minutes in every 2 hours of culture, with a total amount of 18 hours proportionally spaced in the total culture time of 24 hours. In the 100% stress group there was no relaxation periods, and the control group was maintained without any stress. All the procedures were identical for both age groups studied (7 and 9

weeks). For a detailed description of the method used and the application of the mechanical stress *in vitro* refer to Appendix O. The biochemical procedures for GAG labeling, extraction and quantitation were described in the previous chapter.

RESULTS

Mechanical stress applied to the articular disc *in vitro* has been observed to affect the newly-synthesized GAG molecules. Similarly to Chapter IV, GAG molecules were evaluated by monitoring the ratio of the percentage and by the inverse relationship of total DPM counts per mg of wet tissue weight of each GAG synthesized over the total GAG molecules produced throughout the organ culture period. Therefore, both percentage and content of GAGs according to age were evaluated from rat articular disc cultured *in vitro* as a result of the mechanical stress and are illustrated in the Figures V-4, V-5, V-6 and V-7.

From the aging study that demonstrated the changes in GAG pattern (see Chapter IV), the selected ages with the highest (7 week old samples) and the lowest amount of synthesis (9 week old samples) were chosen to study a new model of organ culture, which is able to test different mechanical stresses of different magnitudes and duration between different age groups. This experiment used stress time periods of 0% (or control group), 25%, 75% and 100% (or constant stress) from the total period of 24 hours. The mechanism used is schematically depicted in Figure V-3. For a more detailed description of the apparatus used refer to Appendix O.

A two-way analysis of variance model was used to determine the difference in stress levels would influence GAG synthesis in both age groups studied. The results of these statistical tests showed that the different regimens of mechanical stress had a significant influence in the type of GAG that was synthesized. The assessment of the [^{3}H]-glucosamine synthetic activity showed that the main GAG produced as a result of stress was C_6S and HA for the more frequent and less frequent stress regimens respectively. The increase in stress level was directly proportional to the increase in C_6S; therefore the more stress applied, the more C_6S molecules were synthesized. KS/C_4S and clearly HA showed that the increase in mechanical stress caused a decrease in the amount of molecules

synthesized. These results were similar for percentage of concentration of GAGs and DPM/mg content as illustrated in Figures V-4 to V-7.

At the end of the experimental period of 24 hours, the two-way analysis of variance model indicated that both age groups did not appeared to have significant differences in the mean values of GAG concentration as a factor of the stress regimens used. Although, newly-synthesized GAG types seemed to be unaltered with age correlated with stress, the percentage concentration of DS and HS appeared to be significantly changed in respect to age, where they appeared increased in animals of 7 weeks of age. The GAG synthetic rates also revealed that DS and HS were significantly altered in the age groups studied ($p<0.005$). There was a non-significant increase in C_6S in the 7 week old specimens as compared with the 9 week old ones in the constant stress group. This relationship was somehow inverted in the 75% group as shown in Figures V-4 and V-6. The percentage of the total GAG was observed to be significant at the level of $p<0.005$ for KS/C_4S, C_6S, HA, DS and HS and the DPM/mg counts were significant only for KS/C_4S and C_6S.

Tukey's multiple comparison tests were used to determine if the the levels of stress used determined the type of the GAG molecules that were synthesized. For each GAG type, there seemed to be groups of stress levels that affected the molecular synthesis. The percentage of KS/C_4S, C_6S and DS showed that the control group and 25% stress formed a similar group that contrasted with 75% and 100% stress levels, suggesting a variance as 5 of GAG types as a factor of light and heavy forces. At the same time the percentage of HA showed that control group was different from the constant stress group and both were different from 25% and 75% stress groups. The DPM/mg for C_6S was similar than that of the percentage in which there seemed to be a distinct separation between frequency of force applied. The mean values of percentage and DPM/mg with respective standard deviations for each GAG molecule for both ages through all the stress regimens are summarized in Table V-I and in Table V-II respectively.

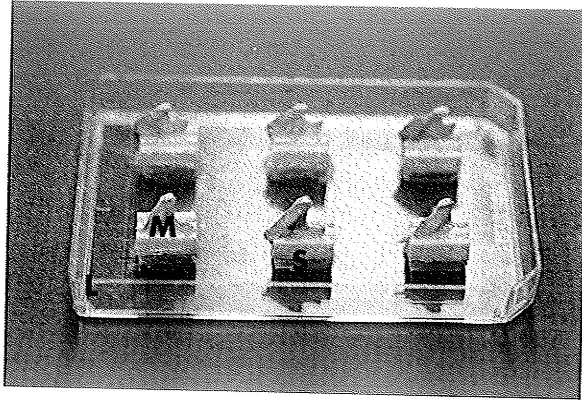


Figure V-1: Photograph of the lid (L) of the organ culture dishes (Flex I - Generic type I collagen, McKeesport, Pensylvania) used in the stress experiment. The sectioned hemi-mandibles (M) were attached to a acrylic support (S) using cyanoacrylate cement.

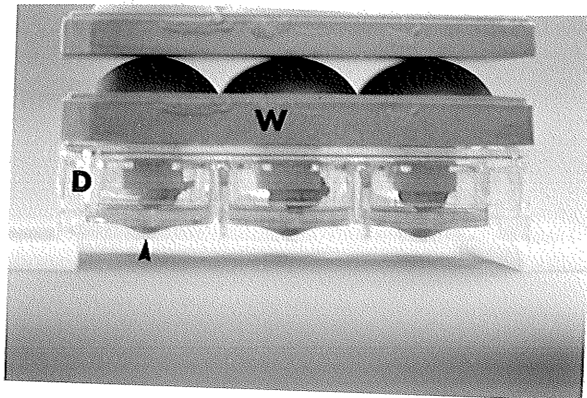
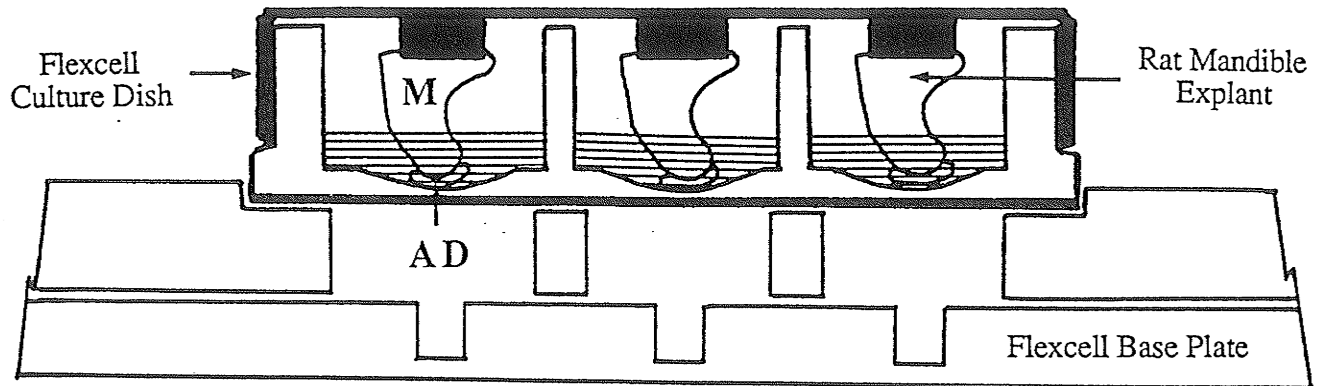
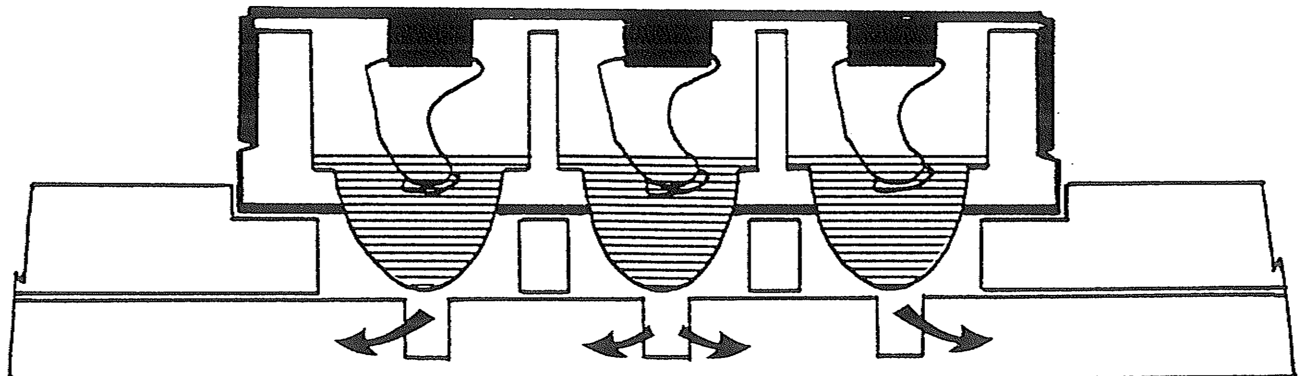


Figure V-2: Photograph of the organ culture dish (D) under the application of the mechanical stress. On top of the dish a standard weight (W) is placed to obtain the desired stress. Note the deformation of the bottom of the dish indicated by the arrow.

Rat Temporomandibular Disc *In Vitro* Stress Model



Partial View Without Vacuum - Stress



Partial View With Vacuum - Non-stress

Figure V-3: Schematic representation of the organ culture system for the study of the response of the temporomandibular disc to the application of mechanical stress. In diagram A the articular discs (AD) are stressed by compression in between the bottoms of each well of the dish and the condyle from the sectioned hemi-mandibles (M). In diagram B the elastic bottoms of the wells are deformed by the application of vacuum pressure (arrows) in the system, which in turn eliminates the stress applied in the articular discs.

Figure V-4: Graph showing the percentage of GAG distribution in the rat articular disc as a result of stress. The legends are: 7w/C- 7 week old samples of the non-stress control group; 9w/C- 9 week old samples of the non-stress control group; 7w/25%- 7 week old samples of the group with stress 25% of the total time in culture; 9w/25%- 9 week old samples of the group with stress 25% of the total time in culture; 7w/75%- 7 week old samples of the group with stress 75% of the total time in culture; 9w/75%- 9 week old samples of the group with stress 75% of the total time in culture; 7w/100%- 7 week old samples of the group with stress 100% of the total time in culture (constant stress) and 9w/100%- 9 week old samples of the group with stress 100% of the total time in culture (constant stress).

Figure V-5: Graph showing the percentage of HA distribution in the rat articular disc as a result of stress. The legends are: 7w/C- 7 week old samples of the non-stress control group; 9w/C- 9 week old samples of the non-stress control group; 7w/25%- 7 week old samples of the group with stress 25% of the total time in culture; 9w/25%- 9 week old samples of the group with stress 25% of the total time in culture; 7w/75%- 7 week old samples of the group with stress 75% of the total time in culture; 9w/75%- 9 week old samples of the group with stress 75% of the total time in culture; 7w/100%- 7 week old samples of the group with stress 100% of the total time in culture (constant stress) and 9w/100%- 9 week old samples of the group with stress 100% of the total time in culture (constant stress).

Figure V-6: Graph showing the DPM/mg GAG content of the rat articular disc as a result of stress. The legends are: 7w/C- 7 week old samples of the non-stress control group; 9w/C- 9 week old samples of the non-stress control group; 7w/25%- 7 week old samples of the group with stress 25% of the total time in culture; 9w/25%- 9 week old samples of the group with stress 25% of the total time in culture; 7w/75%- 7 week old samples of the group with stress 75% of the total time in culture; 9w/75%- 9 week old samples of the group with stress 75% of the total time in culture; 7w/100%- 7 week old samples of the group with stress 100% of the total time in culture (constant stress) and 9w/100%- 9 week old samples of the group with stress 100% of the total time in culture (constant stress).

Figure V-7: Graph showing the DPM/mg HA content of the rat articular disc as a result of stress. The legends are: 7w/C- 7 week old samples of the non-stress control group; 9w/C- 9 week old samples of the non-stress control group; 7w/25%- 7 week old samples of the group with stress 25% of the total time in culture; 9w/25%- 9 week old samples of the group with stress 25% of the total time in culture; 7w/75%- 7 week old samples of the group with stress 75% of the total time in culture; 9w/75%- 9 week old samples of the group with stress 75% of the total time in culture; 7w/100%- 7 week old samples of the group with stress 100% of the total time in culture (constant stress) and 9w/100%- 9 week old samples of the group with stress 100% of the total time in culture (constant stress).

Table V-I: Table of the percentage of GAG distribution in the rat articular disc as a result of stress.

Table V-II: Table of the DPM/mg GAG content in the rat articular disc as a result of stress.

Figure V-4

Percentage of GAG Distribution in the Rat Articular Disc as a Result of Stress

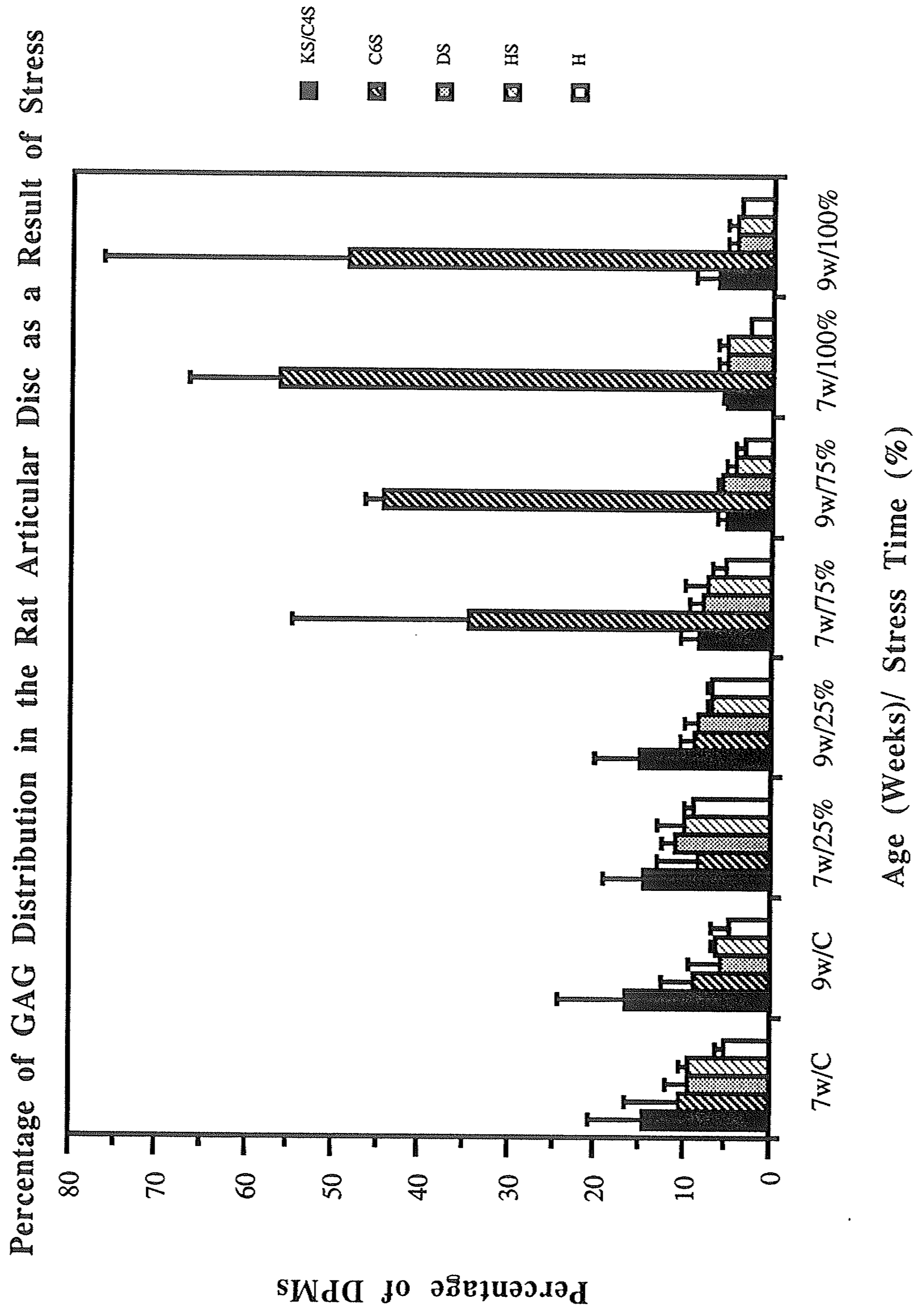
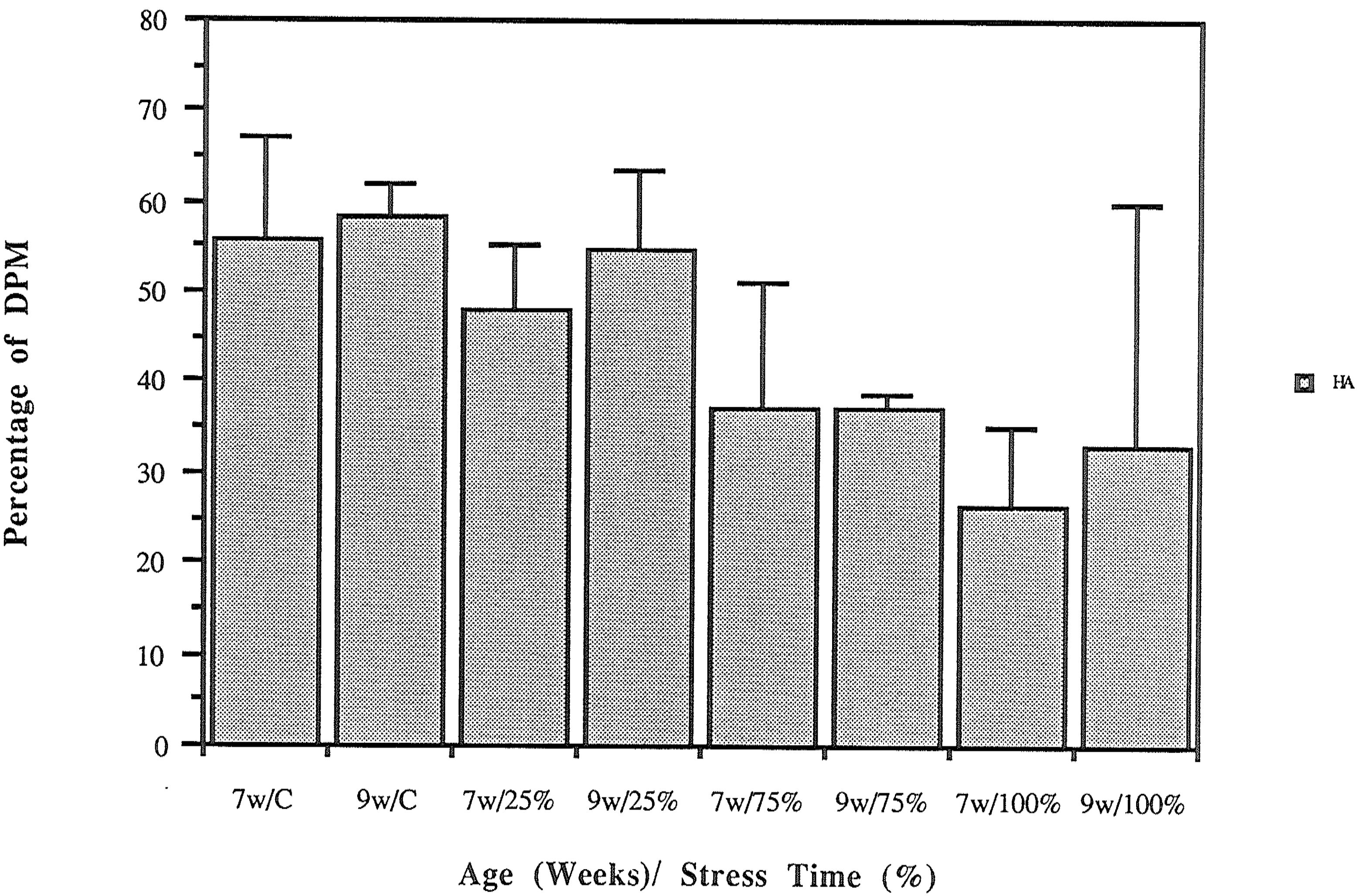


Figure V-5

Percentage of Hyaluronic Acid Distribution in the Rat Articular Disc as a Result of Stress



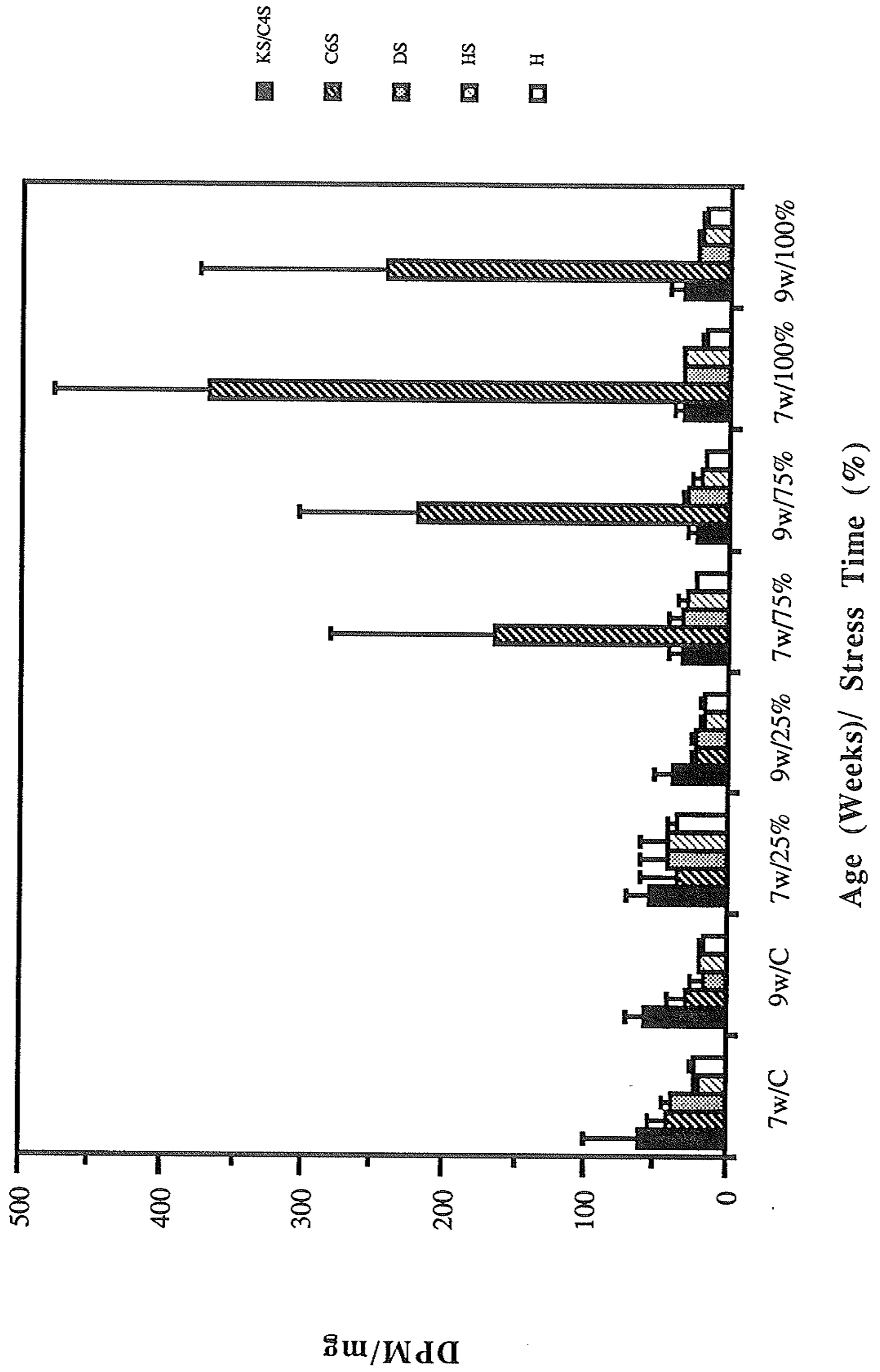


Figure V-6

DPM/mg GAG Content of the Rat Articular Disc as a Result of Stress

Figure V-7

DPM/mg GAG Content of Hyaluronic Acid of the Rat Articular Disc as a Result of Stress

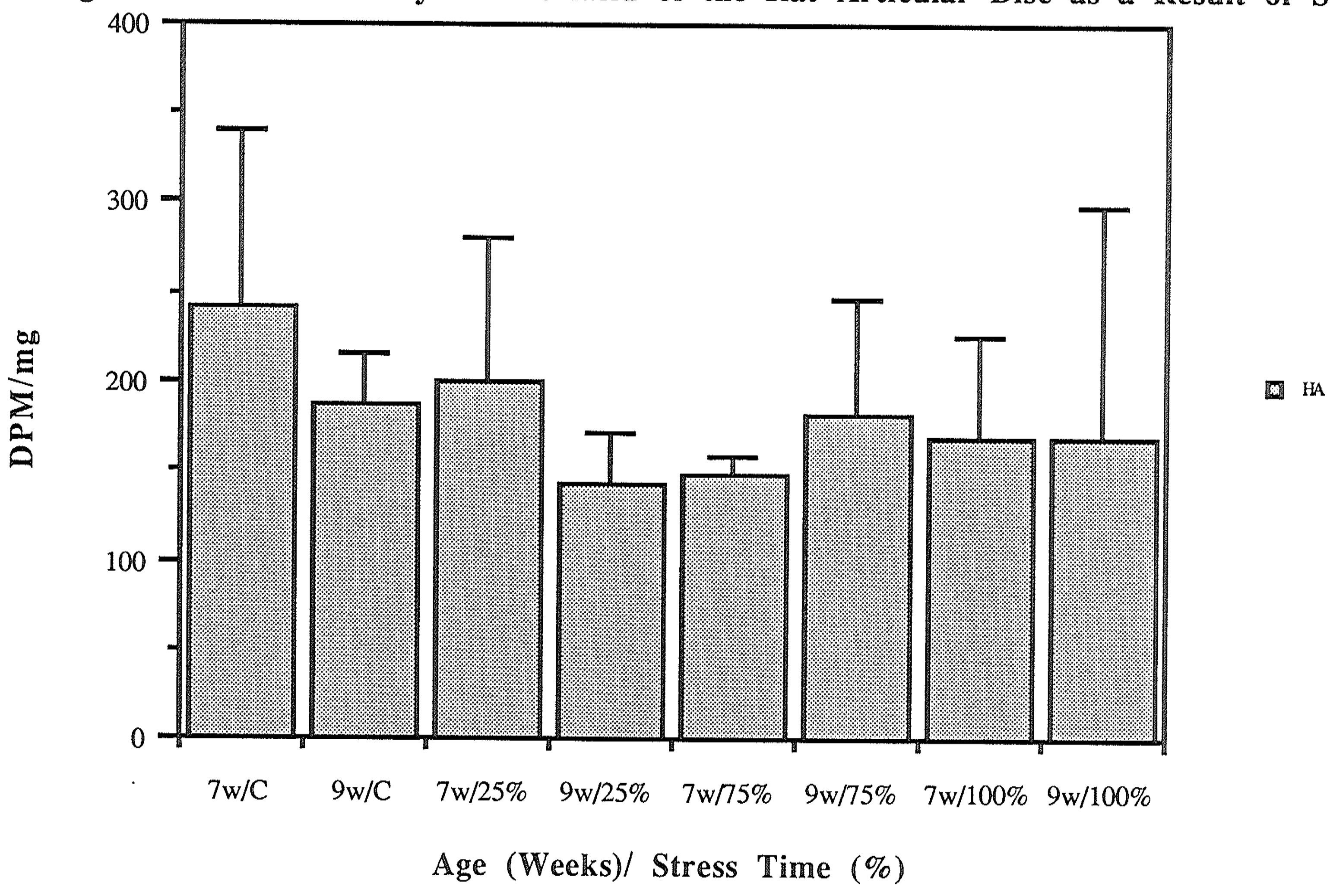


Table V-I

Values for the Percentage of Different Glycosaminoglycans from the Rat Articular Disc Due to Mechanical Stress

Type of GAGs	KS/C_4S		HA		C_6S		DS		HS		H	
Age (Weeks) /Stress Time (%)	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.
7/0%	14.65	6.17	55.79	11.56	10.28	6.10	9.40	2.28	9.40	0.72	5.03	1.11
9/0%	16.45	7.77	58.31	3.76	8.77	3.45	5.93	3.12	5.94	0.53	4.86	1.73
7/25%	14.62	4.32	47.78	7.21	8.44	4.26	10.66	1.89	9.88	2.94	8.61	1.45
9/25%	14.74	5.64	54.72	8.86	8.96	1.39	8.39	1.49	6.70	0.38	6.48	0.91
7/75%	8.15	2.24	37.08	14.26	34.51	20.04	7.89	1.20	7.06	2.63	5.30	1.37
9/75%	5.14	0.87	37.07	1.43	44.63	1.99	5.80	0.64	4.19	1.18	3.16	0.73
7/100%	5.16	0.53	26.42	8.66	56.15	10.26	4.93	1.27	4.93	1.06	2.39	0.31
9/100%	6.34	2.59	33.08	26.73	48.71	27.87	4.35	1.04	4.35	0.76	3.41	0.13

Table V-II

Values of DPM/mg Content of Different Glycosaminoglycans from the Rat Articular Disc Due to Mechanical Stress

Type of GAGs	KS/C_4S		HA		C_6S		DS		HS		H	
Age (Weeks) /Stress Time (%)	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.
7/0%	61.52	39.66	242.30	97.44	40.34	16.00	39.01	7.48	20.12	1.08	21.35	5.83
9/0%	56.46	13.70	185.80	30.30	28.31	12.05	17.69	7.07	18.00	2.95	14.84	2.90
7/25%	56.40	13.07	198.80	80.49	35.38	26.19	43.51	18.06	40.35	19.91	33.91	6.95
9/25%	37.73	12.84	142.10	28.58	23.10	2.86	21.58	2.88	17.32	1.09	16.72	2.14
7/75%	33.76	6.85	148.90	10.01	166.00	116.00	33.64	8.84	28.08	8.44	21.82	1.63
9/75%	24.05	3.98	181.40	65.49	219.40	84.73	27.55	6.26	19.62	5.87	14.57	1.49
7/100%	33.12	4.70	168.40	58.06	366.40	109.50	30.97	2.45	31.08	2.34	15.50	3.51
9/100%	30.79	9.84	170.00	129.06	243.50	132.20	21.14	1.27	20.04	1.96	17.08	3.34

DISCUSSION

There is no evidence about the mechanism by which compressive and tensile forces *in vivo* induce internal changes in the articular disc structure and the extent this may be responsible for the breakdown of the underlying tissue.

Most of the biochemical studies using mechanical forces have been done in cartilage. This seems very reasonable since most of the structures directly under mechanical stress have some degree of cartilagenous composition. The ability of cartilage to absorb the high loads that take place through the retention and diffusion of water is particularly related to PG and GAG molecules. As reported by Scott *et al.* (1989), the type, concentration and distribution of PG complexes, not only in cartilage but also in connective tissue, reflect the load regimen that the tissue is exposed *in vivo*.

PG molecules and PG aggregates possess a high viscosity and a large molecular size that at the same time reduce their capacity to diffuse through the collagenous fibrillar network of cartilage retaining large amounts of water. The energy of the stresses in cartilage is stored as potential energy, which is released upon removal of the stress. In normal conditions, the cartilage regains its original shape in a process that the elastic capability of the PG complexes play a major role (McDevitt, 1973). In other words, PGs indirectly modulate the stiffness of the collagen network through the difference in the osmotic pressure (Muir and Hardingham, 1975; Muir, 1981). On the other hand, there seems to be some evidence that the water content of cartilage from joints that are maximally loaded is lower than that of cartilage from other regions of the body that do not have strong load patterns (Thonar *et al.*, 1978). Reduction of the water content due to high loads is caused by a reduction of PG and GAG synthesis which tends to normalize when the external pressure was eliminated (Schneiderman *et al.*, 1986). From the known GAG types, C_6S is the main component of hyaline cartilage and has been highly correlated with the application of mechanical stress (Hardingham, 1979, Daniel and Mills, 1988). HA and

KS may also act as a biological absorbant to mechanical stress (Bartold, 1987; Saamanen *et al.*, 1987). In fibrochondrocytic cells cultured *in vitro* PG synthesis also represented 87% of C_6S (Webber and Hough, 1988).

However, quoting Mechan (1986): "To view regulation of matrix accumulation by cells in isolation is almost certainly to see the process too narrowly", the analysis of tissue culture by itself may not be sufficiently descriptive of the entire process. As compared with tissue culture studies, the study of the intact structure in organ culture allows the study of PGs and GAGs interacting with collagen fibers. Studies on mechanical stress of the knee joint (Saamanen *et al.*, 1987), demonstrated that the collagen content relative to the PG portion, did not show any change with manipulation of forces applied to the articular cartilage of the joint. This demonstrated that quantities of collagen and PGs are regulated separately. While the content of non-extractable PGs decreases in the unloaded joint, it increases in the heavily loaded contralateral side (Tammi *et al.*, 1983). This accumulation of PGs was reported to be associated with an increase in GAGs that contain glucosamine, particularly KS (Saamanen *et al.*, 1987). This observation is in agreement with the incorporation of radioisotope into newly-synthesized GAGs in this investigation which suggested that the increase in mechanical pressure resulted in an increase in selected groups of GAGs, such as C_6S and KS. However, this relationship may indicate that the results found in the group with 100% stress is more pathological and may not be a condition likely to occur *in vivo*. The results from the 75% stress group seemed to represent a more physiologic *in vivo* condition, as it could appear in cartilage of knee joints as a result of running exercise. According to Saamanen (1989), intensive exercise increases the percentage of PGs, as compared to strenuous exercise (similar to the 100% stress group of this study) that leads to injuries with an increase in cartilage fibrilation and reduction of PG content, PG aggregation and PG size. The use in future studies of different times of stress

regimens may allow a more accurate comparison between the degree of physiological and/or pathological response *in vitro*.

Biomechanical studies suggest that the differences in intermittent and constant forces play an important role in normal growth of the craniofacial structures and in particular of the articular surfaces of the temporomandibular joint (Petrovic *et al.*, 1975; Simon, 1977; Copray *et al.*, 1985a; Hinton *et al.*, 1986). The four different regimens of time and stress applied to the rat articular disc in this study suggested that the synthesis of GAG molecules was dependent on the time that the force was applied in the culture. This demonstrated a significant influence in the type of GAG that was synthesized. The time of stress could then be divided in no force varying in different degrees of intermittent to pure constant forces.

Synthesis of C_6S appeared to be the result of the more constant forces while HA resulted from more intermittent to no force at all. The composition of fibrocartilagenous tissue in the articular disc seems to render the disc with both cartilagenous and fibrous behaviours that must be correlated to the long term stimulus that would generate tissue breakdown, since long periods of constant forces inhibit matrix formation to stimulate cellular proliferation. According to Vogel *et al.* (1986) the tendon that was subjected to mechanical forces showed the GAG content to increase 3 to 5 times higher with 50% of a large form CS, therefore, demonstrating cartilage-like characteristics.

Autoradiographic studies of condylar cartilage under constant forces showed considerable cellular proliferation with a fall in matrix production, whereas intermittent pressure resulted in a fall in cellular proliferation and an increase in matrix formation (Copray *et al.*, 1985), as evidenced by the increased rate of [^{35}S]-sulphate incorporation into GAGs, due to an increased deposition of PGs in the pericellular matrix (Veldhuijzen *et al.*, 1985). The synthesis of C_6S in both ages studied as a result of constant stress demonstrate that the disc has the capacity to adapt and remodel to a more cartilagenous

composition. However, this must be confirmed with future experiments using histological evaluation of the stressed samples.

The total duration of 24 hours of organ culture may not represent enough stress to inhibit generalized matrix formation, as reported by Copray *et al.* (1985). Although, this study can not explain the inconsistency that the increased stress appears to decrease the synthesis of HA, which have been related to increase in the synovial fluid of patients with TM disorders (Bjelle, 1975; Sweet *et al.*, 1977; Kiviranta, 1987b; Saamanen, 1989), constant stress may inhibit HA synthesis through an independent mechanism. Analysis of variance indicated that both age groups did not appeared to have major significant differences in the mean values of GAG content among the stress regimens used. This is an interesting observation since the ages of 7 and 9 weeks were chosen to contrast the highest and the lowest amount of GAGs synthesized without the application of stress respectively (see Chapter IV, Figure IV-1, IV-2, IV-3 and IV-4).

According to these observations, the articular disc seems to undergo a remodeling process when subjected to extensive mechanical stress. Although, the adaptability of the articular disc is assumed to be very limited (Miller, 52; Ermshar, 82), Osborn (1985) suggested that the shape of the disc adapts to the compression forces to which is subjected (Rees, 1954).

The mode of action of the forces is not known. However, the strain energy stored within the collagen fibers of the loaded tissue can be transfered into a potential field formed by the charged GAG components and their electrostatic interaction with collagen fibrils (Egan, 1987). Intermittent compression improves the exchange of nutrients and catabolites (Maroudas, 1974) and the increase in mechanical stresses causes a correspondent increase in cellular activity (Urschel *et al.*, 1988), however, this type of force will not increase the rate of transport of nutrients considerably above that which can be achieved by diffusion alone, according to Maroudas (1974). It has been hypothesized that stresses may affect the

cells through mechanisms that are translated to the cellular level, but there is still a great deal of speculation on how the mechanical stimulus is sensed by the cell to develop a particular response (Urschel *et al.*, 1988). The analysis of studies of second messengers such as cyclic AMP showed it to increase after the application of the intermittent mechanical hydrostatic pressure (Veldhuijzen *et al.*, 1979). Bourret and Rodan (1976) using continuous compressive forces with an attempt to elucidate the mechanism of the translation of the mechanical stimulus into cellular signal, found a reduction in cAMP levels which they attributed to an increased Ca^{++} uptake.

CONCLUSIONS

1. The rat articular disc responded to the artificial mechanical stress by a change in newly-synthesized GAG profile, which was demonstrated to be specific to the regimen of stress applied.

2. The major GAG molecules in the disc following stress were C_6S and HA. C_6S appeared to increase in regimens of constant stress, which may indicate that the disc remodels to a more cartilaginous phenotype to resist compressive forces. However, this evidence must be confirmed in future experiments using histological evaluation. On the other hand, HA seemed to decrease with the progressive increase of the time of stress application. This suggests that under short periods of constant stress, HA synthesis is inhibited.

3. There seemed to be no differences in response to mechanical stress between the age groups studied. Analysis of variance showed that independent of the synthesis of GAGs under no stress, both age groups responded to stress with the same pattern of GAG synthesis.

4. The different stress levels under different time periods showed significant variability ($p<0.005$) in the type of GAG synthesized. For the synthesis of KS/C_4S, C_6S and DS, stress could be divided into low, intermittent to no stress at all, and constant stress. HA synthesis was significantly ($p<0.006$) divided into control, intermittent and constant stress response groups.

5. The variance in GAG synthesis to form different groups as a result of stress indicates that each GAG molecule has its own pattern of synthesis. This reveals that the articular disc controls its composition by the regulation of matrix deposition in accordance to the environment to which it is subjected.

Chapter SIX

Summary and Future Research

SUMMARY AND FUTURE STUDIES

Synthesis of GAG molecules and collagen chains from the rat articular disc has been shown *in vitro*. The observations resulted from the molecular synthesis of these structures rended the articular disc a dynamic pattern for remodeling:

1. The results of the distribution of collagen in the anterior and medial bands in the disc possibly resulted from the increased relationship of these areas to functional activity as compared to the less prominent posterior band. The preliminary investigation of the percentage of type III collagen confirmed the period of 7 to 8 weeks as remodeling active in comparison to early age periods showing a correlation with the mandibular growth spurt. Later periods of development showed a drop in collagen synthesis.

2. HA and C_6S were the main groups of newly-synthesized GAG molecules *in vitro* in the rat articular disc. Synthesis of GAGs seemed to peak in animals of 7 and 8 weeks of age, demonstrating a good correlation with the apparent mandibular period of fast growth, more likely a growth spurt, that could explain the reason for a high GAG synthesis. Ages 7 and 9 weeks were selected for the application of mechanical stress.

3. The synthesis of GAGs with the application of mechanical stress *in vitro* showed to be significant for the regimen of stress applied. The synthesis of C_6S was highly increased with stress in comparison with the other GAG types. It has been speculated that this pattern of synthesis may indicate an increase in cartilaginous properties in the disc, but it needs histological confirmation. Both age groups studied did not show any singnificant difference in GAG synthesis.

This was a preliminary study in the analysis of the articular disc normal development and metabolical response to mechanical stress *in vitro*. Possible future studies may include:

1. Study of the response of the normal development and mechanical stress on the articular disc by assessing the collagen and GAG distribution on the disc by grain counting of histological specimens with the use of more specific techniques, such as monoclonal antibodies, to identify collagen α-chains and GAG types within the disc.

2. Study of the effect of various levels and duration of mechanical stress on the articular disc by assessing collagen and GAG synthesis. The comparison of an increased range of different levels of stress of different magnitudes may give important insights in the relationship between physiological and pathological articular disc response.

3. Study of the effect of mechanical stress in normal development of the articular disc from a larger range of age groups that those of used in this study.

Chapter SEVEN

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Chapter EIGHT

Appendices

APPENDIX A

Articular Disc Early Development in the Albino Rat

The study of the normal growth and development of the temporomandibular joint was undertaken in the albino rat to clarify the early changes that take place in the same joint with focus to the articular disc early development. Both the TMJ formation in rats and humans have been described in Chapter I.

MATERIALS & METHODS

Sprague-Dawley rats, inbred in our facility, were randomly divided in number of four for each of the following age groups: 11, 13, 15, 17, 19, 21, 25 insemination days of age and newborn animals for histological analysis. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The entire head of each animal was carefully washed in Waymouth's medium (Waymouth's 752/1, Grand Island Biological Co., Grand Island, New York) and fixed in 10% phosphate-buffered formalin containing 0.5% of cetylpyridinium chloride or CPC (Sigma Chemical Co., St. Louis, Missouri) for 24 hours. The samples were decalcified with EDTA (pH 7.2) for 4 weeks and dehydrated in alcohools and chloroform. Following fixation each explant was oriented anteroposteriorly in order to embed in paraffin blocs (Fisher Scientific, Winnipeg, Manitoba). Histological procedures follow the ones indicated in Chapter III.

RESULTS & DISCUSSION

The assessment of the staining pattern in the temporomandibular area revealed that the first appearance of the articular disc took place at 20 days insemination age (Figure A-1, A-2 and A-3). This is in accordance with the work of Furstman (1966), in which the articular disc appeared as a fibrous ligament at the age of 20 to 21 days insemination age. The antero-posterior orientation of this fibrous ligament was clearly defined in the ages older than 20 days. In the early ages it was possible to demarcate any differences in articular disc bands which did not appear nearly until birth. After that, most of the structural composition of this ligament formed over the mandibular condyle showed little change with development.

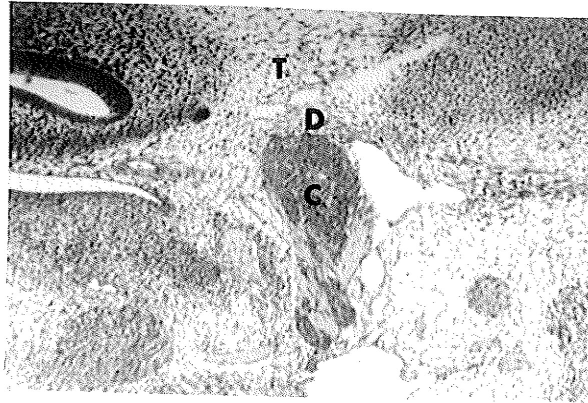


Figure A-1: Radioautograph of cross-section of the rat temporomandibular joint. Donor age is 20 days of insemination. Explant cultured *in vitro*. Legends are: C = condyle, T = temporal bone and D = articular disc. Haematoxylin and Eosin. X 12

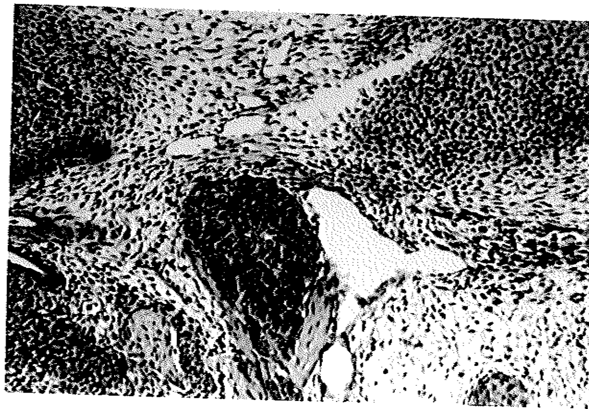


Figure A-2: Magnification of Figure A-1. Haematoxylin and Eosin. X 82

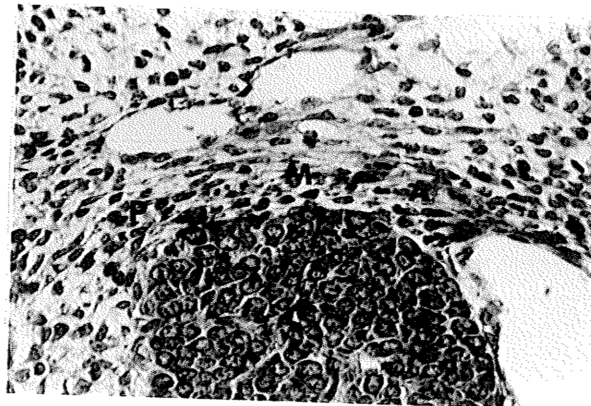


Figure A-3: Magnification of Figure A-2. Note the antero-posterior disposition of the fibres in the early articular disc. Legends are: A = anterior band, M = medial band, P = posterior band and C = condyle. Haematoxylin and Eosin. X 205

APPENDIX B

Collagen Synthesis and Distribution from Rat Articular Disc *In Vitro* and *In Vivo*

The synthesis and distribution of collagen was characterized in the rat articular disc *in vitro* and *in vivo*. Although, *in vitro* studies are widely used, it is important to compare the consistency of the results with similar experiments *in vivo* that will more likely characterize the normal changes and synthesis that take place in the anatomical structure studied.

Age-changes may be considered the result of the biological process that progressively modifies the functioning and the interrelation of the various components of a tissue (Bouissou and Pieraggi, 1988). Although not all age-related changes can be counted as causative roles of the development of an impairment (Thonar and Kuettner, 1987), aging consists of an eventual degenerative process that accounts for an increased occurrence of diseases which are frequently typical of advanced ages. The causes for this phenomena are varied, but the regimen of load and stress applied to the articular cartilage surface of a joint have been related to age changes in cartilage (Thonar *et al.*, 1978).

The objectives of this investigation were to clarify some of the cellular changes and other possible interferences in the articular disc, by isolating this structure *in vitro* and *in vivo*. Among the mechanisms of cellular adaptation, the synthesis of well known molecules help to determine the behaviour of the tissue as a result of the distribution and function of the same molecule. It is well known, that collagen is the main extracellular component in connective tissue (Bornstein and Sage, 1980) and consequently, its histological and biochemical analysis may help to elucidate disc behaviour. For visualization purposes the articular disc has been divided anteroposteriorly in different areas or bands (Thilander, 1964; Mills *et al.*, 1988).

MATERIALS & METHODS

Sprague-Dawley rats, inbred in our facility, were randomly divided in number of four for each of the following age groups of the *in vitro* study: 3, 4, 5, 6, 7, 8 and 9 weeks of age for histological analysis. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The articular disc and attached ligaments from both joints of each animal were carefully dissected through a lateral incision to expose the disc/condyle complex following the removal of the masseter muscle. The removal of the articular disc from the head of the condyle was precisely done so as to remove the disc and small portions of both retrodiscal tissue and superior head of lateral-pterygoid muscle which were important for the orientation of the different bands of the tissue *in vitro*.

In the *in vivo* study, Sprague-Dawley rats were randomly divided in number of four for each of the following age groups: 3, 5 and 7 weeks of age for histological analysis. The animals were also anaesthetized with ether and injected with 10 μCi/g (body weight) of [^{3}H]-proline (Amersham Corporation, Oakville, Ontario) with specific activity of 60 mCi/mmol. After 4 hours in culture the animals were reanaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The temporomandibular joint from both sides was removed in bloc. The following organ culture and histological procedures were described in Chapter III.

RESULTS

The qualitative assessment of protein synthetic activity as shown by labelling pattern of [^{3}H]-proline, suggested that there is a more active collagenous protein synthesis *in vitro* in the anterior band and medial regions of the articular disc as compared with the posterior band (Figures B-2 and Figures B-6 to B-8), however, these patterns will have to be confirmed by grain-counting and statistical evaluation. The *in vivo* results seemed to show similar results for the correspondent tissue explants that were labeled *in vitro*.

DISCUSSION

The concept of normal development and remodeling of the articular disc has been little studied. Although the articular disc is a homogeneous structure of fibrocartilage (Bell, 1986; Rees, 1954; Osborn, 1985; Gage *et al.*, 1990), for visialization purposes the synthesis of molecules was classified antero-posteriorly into three bands or areas, namely anterior, medial and posterior bands. Collagen distribution in the articular disc of the rat by the analysis of [^{3}H]-proline labeling, was located at the anterior and medial bands primarily. At the same time, the same areas appear to contain increased synthesis of GAG molecules in the mouse articular disc (refer to Appendix C). This observation suggests that the synthesis of both collagen and GAG molecules are affected by stimuli taking place concomitantly.

Concluding, the description and correlation of events happening during growth and development do not clearly explain the regulation and control mechanisms responsible for the histodifferentiation and morphogenesis of the craniofacial structures. The monitoring of collagen metabolism that is regulated following a specific pattern of synthesis can, therefore, contribute for the understanding of the overall mechanism of cellular interaction and protein synthesis through normal development and maturation. The findings from this study prove that collagen synthesis can be maintained in the rat articular disc *in vitro* in a system which is able to reproduce the developmental changes that take place in the living tissue *in vivo*. This approach may have a strong potential for the study of disc pathology.

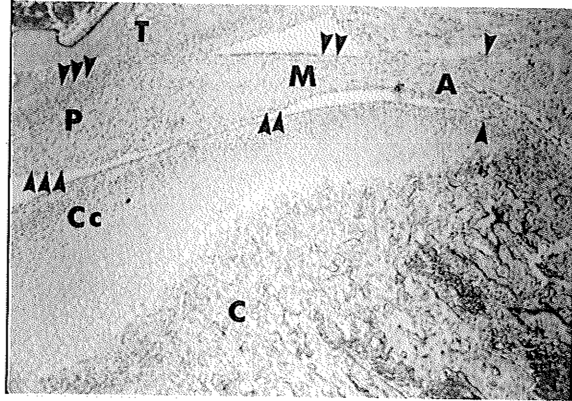


Figure B-1: Radioautograph of cross-section of the rat temporomandibular joint. Donor age is 3 weeks. Explant cultured *in vivo*. Legends are: C = condyle, T = temporal bone, A = disc anterior band, M = disc medial band, P = disc posterior band and CC = condylar cartilage. Haematoxylin and Eosin. X 12

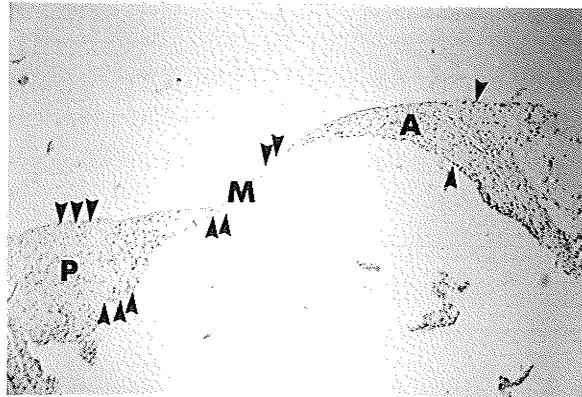


Figure B-2: Radioautograph of cross-section of the rat temporomandibular disc. Donor age is 3 weeks. Explant cultured *in vitro*. Legends are: A = anterior band, M = medial band and P = posterior band. X 12

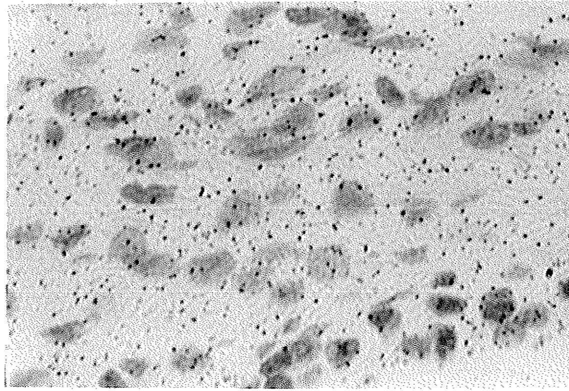


Figure B-3: Magnification of the anterior band area delineated by single arrows in Figure B-1. Haematoxylin and Eosin. X 205

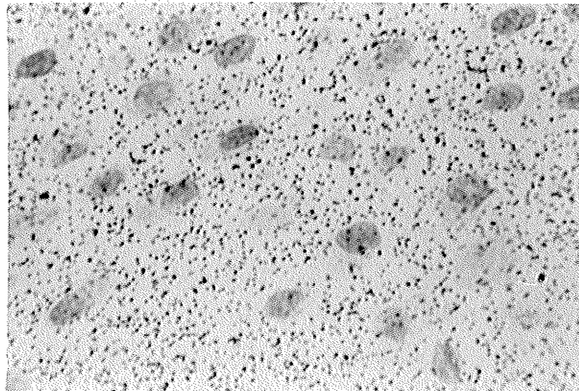


Figure B-4: Magnification of the medial band area delineated by double arrows in Figure B-1. Haematoxylin and Eosin. X 205

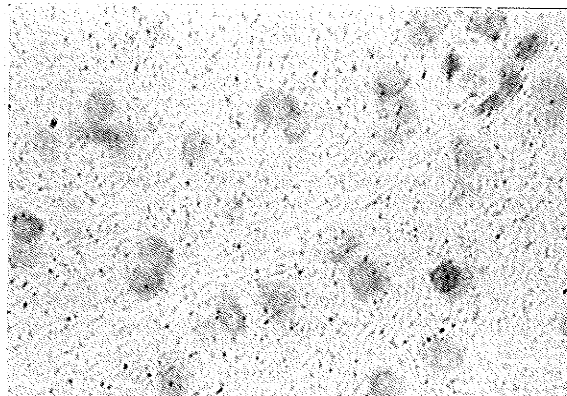


Figure B-5: Magnification of the posterior band area delineated by triple arrows in Figure B-1. Haematoxylin and Eosin. X 205

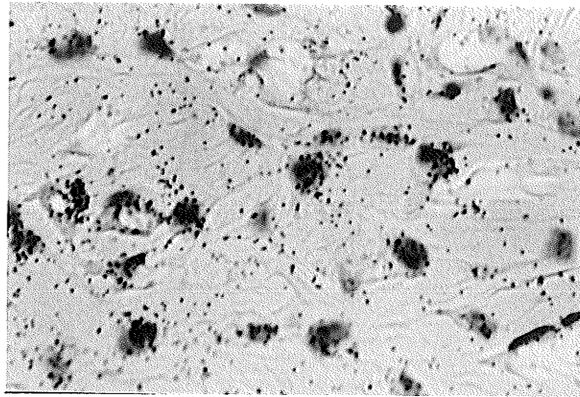


Figure B-6: Magnification of the anterior band area delineated between single arrowsin Figure B-2. Note the amount of siver grains. X 205

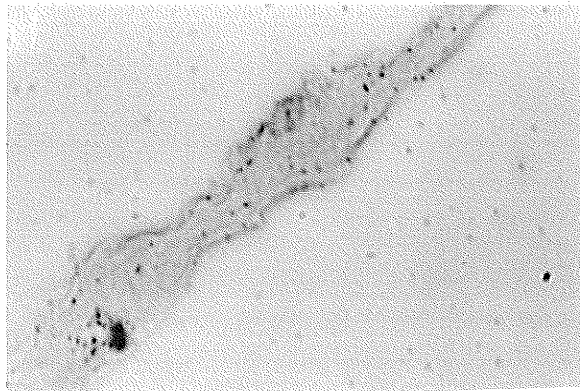


Figure B-7: Magnification of the medial band area delineated between double arrows in Figure B-2. Note the amount of siver grains. X 205

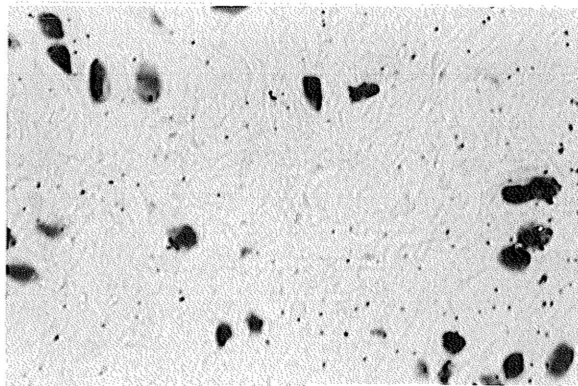


Figure B-8: Magnification of the posterior band area delineated between triple arrows Figure B-2. Note the reduced labeling as compared to the Figures B-6 and B-7. X 205

APPENDIX C

GAG Synthesis and Distribution from the Mouse Articular Disc Cultured *In Vitro* and *In Vivo*

GAG complexes were studied in the rat articular disc biochemically as a result of the normal aging process and as a result of mechanical stress. The articular disc from the Albino Rat was compared to that of the Swiss Mouse and both demonstrated to be very similar in not only the structure but also in the synthesis and distribution of collagen (See Chapter III). In this Appendix the synthesis and distribution of GAG molecules was studied in the mouse articular disc using both the organ culture *in vitro* model and the *in vivo* experimental procedure. The results of the both types of experiments were then compared.

MATERIALS & METHODS

Swiss-Webster white mice, inbred in our facility, were randomly divided in number of four for each of the following age groups: 3, 5 and 7 weeks of age for histological analysis of the *in vivo* experiment and in the age groups: 1, 2, 3, 4, 5, 6, 7 and 8 weeks of age for histological analysis of the *in vitro* experiment.

In the first group, the animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and injected with 5 μCi/g (body weight) of [^{35}S]-sulfate (Amersham Corporation, Oakville, Ontario) with specific activity of 43 Ci/mg in each dish. After 4 hours in culture the animals were reanaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The temporomandibular joint from both sides was removed in bloc, carefully washed in Waymouth's medium (Waymouth's 752/1, Grand Island Biological Co., Grand Island, New York) and placed in fixative.

The animals from the second group were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The articular disc and attached ligaments from both joints of each animal were carefully dissected through a lateral incision to expose the disc/condyle complex following the removal of the masseter muscle. The removal of the articular disc from the head of the condyle was precisely done so as to remove the disc and small portions of both retrodiscal tissue and superior head of lateral-pterygoid muscle which were important for the orientation of the different bands of the tissue *in vitro*. The following organ culture and histology procedures have been described in Chapter III.

RESULTS & DISCUSSION

The qualitative assessment of the protein synthetic activity as shown by the silver grain pattern of [^{35}S]-sulfate in the animals labeled *in vivo* showed to be very similar for the correspondent tissue explants of the animals labeled *in vitro*. The GAG distribution in the articular disc seemed to correspond with the pattern of labeling of collagen. The *in vitro* experiment was also compared with the *in vivo* explants as shown in the Figures C-1 to C-8. The appearant correlation of GAG molecules with collagen in the anterior and medial bands suggests that these areas of the articular disc may be influenced by an increased amount of stress and remodeling throughout the rodent aging and development.

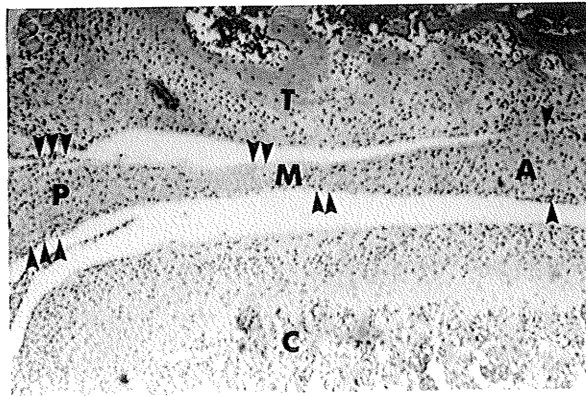


Figure C-1: Radioautograph of cross-section of the mouse temporomandibular joint. Donor age is 4 weeks. Explant cultured *in vivo*. Legends are: C = condyle, T = temporal bone, A = disc anterior band, M = disc medial band and P = disc posterior band. Haematoxylin and Eosin. X 12

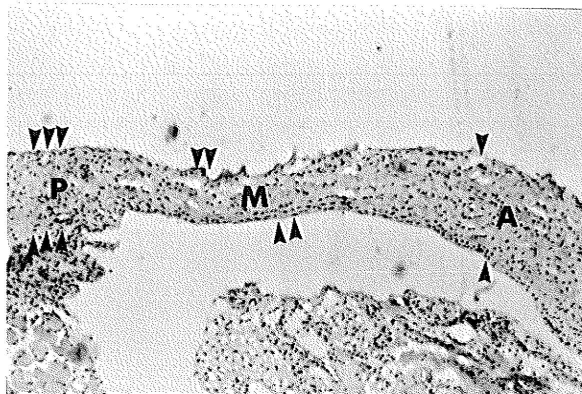


Figure C-2: Radioautograph of cross-section of the mouse articular disc. Donor age is 4 weeks. Explant cultured *in vitro*. Legends are: A = disc anterior band, M = disc medial band and P = disc posterior band. Haematoxylin and Eosin. X 12

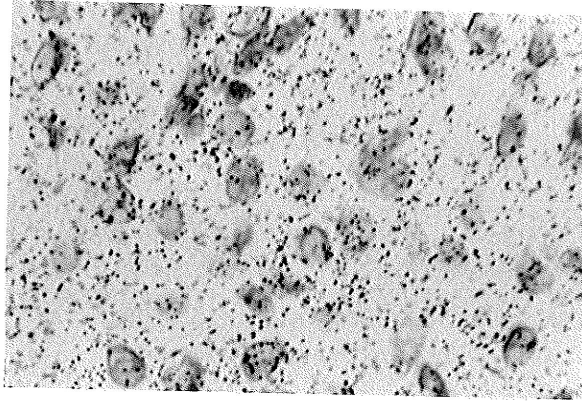


Figure C-3: Magnification of the anterior band area delineated by single arrows in Figure C-1. Haematoxylin and Eosin. X 205

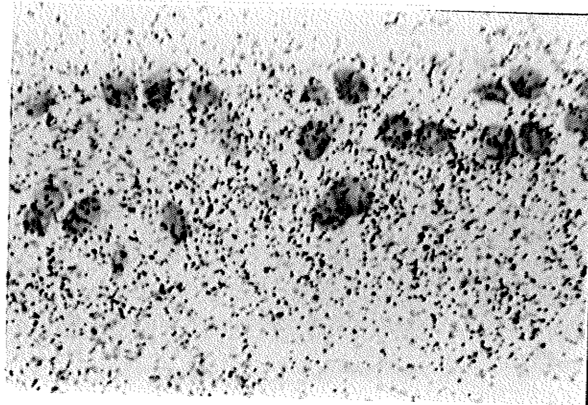


Figure C-4: Magnification of the medial band area delineated by double arrows in Figure C-1. Haematoxylin and Eosin. X 205

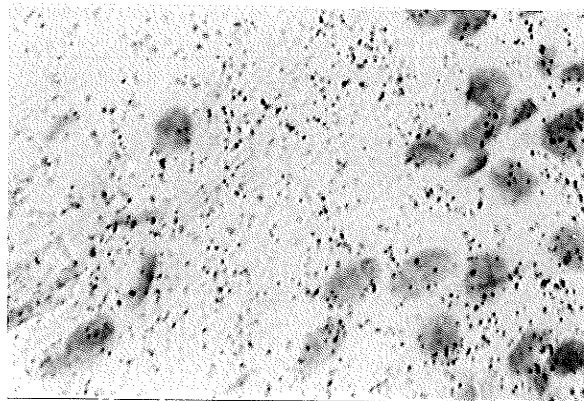


Figure C-5: Magnification of the posterior band area delineated by triple arrows in Figure C-1. Haematoxylin and Eosin. X 205

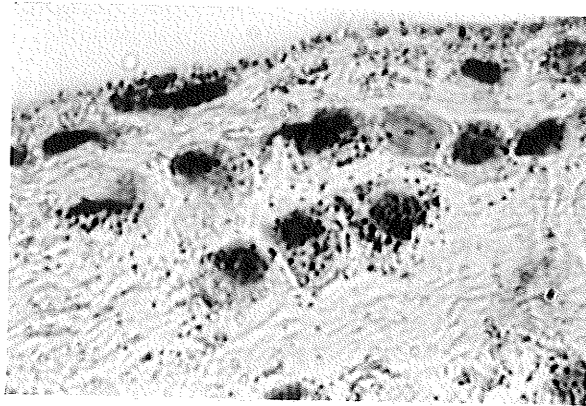


Figure C-6: Magnification of the anterior band area delineated by single arrows in Figure C-2. Note that the synthesis of collagen *in vitro* is simillar to its *in vivo* counterpart. Haematoxylin and Eosin. X 205

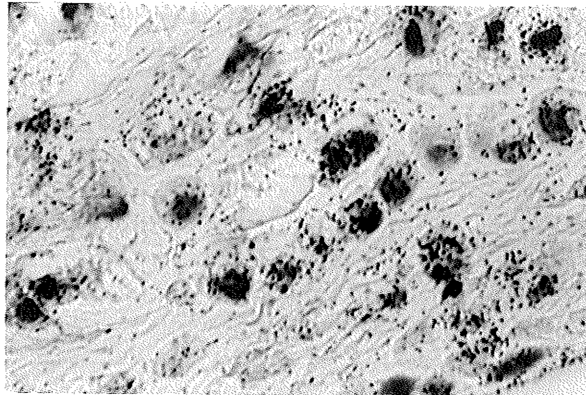


Figure C-7: Magnification of the medial band area delineated by double arrows in Figure C-2. Haematoxylin and Eosin. X 205

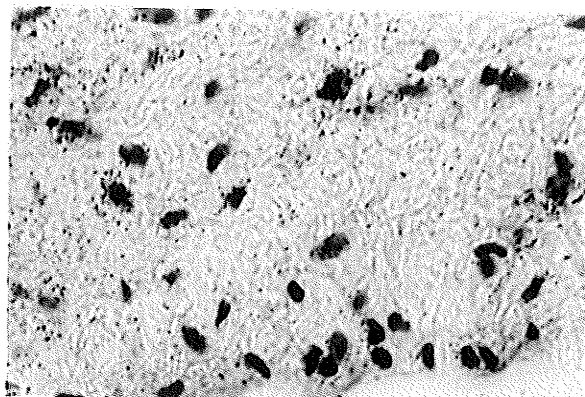


Figure C-8: Magnification of the posterior band area delineated by triple arrows in Figure C-2. Haematoxylin and Eosin. X 205

APPENDIX D

Isolation and Characterization of the Articular Disc Cells Cultured *In Vitro*

The articular disc cells were isolated and evaluated for chondrocyte characteristics. The structural composition of the articular disc that covers the head of the mandibular condyle is mainly formed by bundles of type I collagen fibers (Gage *et al.*, 1990), with a scattered distribution of chondrocytes (Silva, 1969), or fibrochondrocytes (Webber *et al.*, 1985), that are embeded in a PG matrix (Hardingham, 1979; Gage *et al.*, 1990). The presence of chondrocytes renders the fibrous structure of the articular disc an increased potential for remodeling and regeneration through the progressive synthesis of PGs and collagen. This pilot study attempted to isolate and characterize the articular disc cells as chondrocytes or fibrochondrocytes in the albino rat articular disc.

INTRODUCTION

1. Normal Chondrocyte Metabolism

The normal chondrocyte structure is of an ovoid cell that has an irregular surface with projecting cell processes, but the overall shape can vary from a spheroidal to a flattened form. The shape, as well as other characteristics, can be altered by the type of cartilage, the position of the cells in the tissue together with the cell density and the age (Stockwell, 1979). Many of the chondrocyte's features are very similar to those of the other cells and it is often difficult to identify particular characteristics precisely. However, typical features of chondrocytes are the well formed ER, an extensive Golgi complex, prominent lakes of glycogen and large lipid droplets (Barnett *et al.*, 1963; Matukas *et al.*, 1967; Weiss, 1968; Silva, 1969).

The average size of the chondrocytes was stated as ranging from 30-40 µm (Barnett *et al.*, 1961), however, most recently the mean is accepted as 10 µm in mouse and 14 µm in man (Stockwell, 1971). The diameter of chondrocyte is dependent on cartilage thickness and perhaps cell density. Chondrocyte size increases during maturation of permanent cartilage (Beneke, 1973). Stockwell (1979) correlated this finding with the inverse relationship between tissue cellularity and cell size, since cell density falls during maturation.

Respiration in normal cartilage takes place by diffusion of oxygen over long distances. The rate of glycolysis and of the respiration are in accordance with the oxygen tension and the concentration of glucose. However, the slow metabolism of cartilage is due to its low celularity, 1-10% of the tissue. It is logical to assume that oxygen utilization varies directly with cell density of the cartilage. The concept that cartilage

grows under near anaerobic conditions leads to the idea that low oxygen tension is conductive of cell differentiation (Hall, 1970). Connective tissue cells cultured under compressive stress in low oxygen atmosphere differentiate into cartilage, while high oxygen atmosphere produces bone (Bassett and Herrmann, 1961). Low concentrations of oxygen also increase the synthetic activity of cultured chondrocytes (Nevo *et al.*, 1972), but concentrations inferior to 1% inhibit the percentage of sulphate incorporation (Marcus, 1973; Brighton *et al.*, 1974). Chondrocyte oxygen consumption is greater where the oxygen tension is high, since the cells of superficial layer seem to have a higher utilization of oxygen than the deeper layers (Stockwell, 1979), and it decreases with advancing age.

In adult cartilage, the chondrocytes are localized within the matrix in cavities termed lacunae and in normal conditions is in direct contact with the pericellular surrounding matrix (Silberberg, 1968; Meachim and Stockwell, 1972). However, this condition seems to be altered histologically, once the chondrocytes appear shrank away from the matrix which according to Barnett *et al.* (1961) is due to a probable loss of the pericellular matrix (Meachim and Stockwell, 1972). From studies in rabbits, Ghadially and Roy (1969) have suggested that this artificial representation of the chondrocytes association to their lacunae is actually an area adjacent to the chondrocyte that contain few or no collagenous fibrils but does contains some fine granules. Supposedly this region would coincide with the human chondrocyte pericellular matrix (Stockwell, 1974). Several other studies with transmission electron microscope in various numbers of different animals including rats, dogs, frogs and rabbits demonstrated some electron-dense material within the region of the pericellular matrix (Shepard and Mitchell, 1976; Luft, 1971; Laros and Cooper, 1972; Green and Fergunson, 1975). This indicates proteoglycans or the presence of glycoprotein molecules (Van Sickle and Kincaid, 1976). Similarly, the area at the circumference of the chondrocyte lacunae with a concentration of collagen fibrils (Szirmai, 1969), presumably protects mechanically the enclosed chondrocyte (Meachim and Stockwell, 1972).

The chondrocyte is responsible for the matrix production and maintenance, being itself under the direct influence of the matrix that it produces (Van Sickle and Kincaid, 1976).

1.1. Articular Cartilage Cells

There is a common heterogeneity of chondrocytes among the area of articular cartilage as demonstrated by enzyme histochemical studies (Dorfman, 1970; Wilsman and Van Sickle, 1971). Their morphologic appearance in the tangential layer is also heterogeneous (Weiss *et al.*, 1968). This potential metabolic feature is a natural result of the intrinsic heterogeneous composition and the continuous physiological turnover (Van Sickle and Kincaid, 1976).

In the electron microscope the chondrocytes have a trilaminar cell membrane, with a few short cell processes which are thought to be involved in pinocytosis. The nucleus appears oval or elongated, being

more rounded in deeper zones and often shallow identations and undulations can be seen (Ghadially *et al.*, 1978). Nucleoli are not common, however they were correlated as a response to increased metabolic activity (Fuller and Ghadially, 1972). As it would be expected, the number of mitochondria is increased in metabolic more active chondrocytes, which also have best developed RER and Golgi complex. This is an indication that the major synthesis of the precursors of fibrillar and interfibrillar matrix is carried out in these structures. Presence of filaments was reported to depend on age, increasing in cases of osteoarthritis and injured cartilage (Fuller and Ghadially, 1972). Microtubules are rarely seen as well as glycogen. In the deeper articular cartilage, however, there are large deposits of glycogen which increase in size with age (Ghadially *et al.*, 1976). Lipid is also present, not in the same amount as the hyaline cartilage, but similarly, it is most prominent in the middle zone (Stockwell, 1979). The presence of intracellular lipids was reported in chondrocytes of articular cartilage (Stockwell, 1967) in man and in a series of different animals including rats (Anderson *et al.*, 1964), pigs and rabbits (Collins *et al.*, 1965). Despite of the lipid content has been showed to increase with age in animals, there is still some controversies about the same factor in man (Stockwell, 1967; Van Sickle and Kincaid, 1976).

1.2. Fibrocartilage Cells (Interarticular Disc and Menisci)

Fibrocartilage is present in intervertebral discs, some articular cartilages, in the symphysis pubis and in the insertion of some tendons in bones (Granstrom and Linde, 1973).

The chondrocytes in this particular tissue tend to be more rounded or polygonal (Ghadially *et al.*, 1978, Daniel and Mills, 1988) and show a well-developed RER and Golgi complex. However, the cells of the superficial zone of the tissue are usually oval and fusiform. Other cell processes and structures are seen in this tissue as in other sites (Ghadially *et al.*, 1978). Some fusiform cells that at light microscopy can be mistaken for fibroblasts, resembled zone I chondrocytes of articular cartilage (Silva, 1969). However, most of the cells are large and many nest in pairs like hyaline cartilage (Mills *et al.*, 1988).

MATERIALS & METHODS

Ten Sprague Dawley rats were divided into two experiments. The first experiment utilized 2 groups of 3 animals each, aged 2, 3 and 4 weeks old, whereas the second experiment involved 2 groups with 2 animals each, aged 3 and 4 weeks old respectively. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The joints were exposed in aseptic conditions. Both articular discs were surgically removed and immediately transported to a laminar flow hood (Nuaire, Minnesota), where they were placed on a petri dish (100 X 20 mm/Corning, Corning Glass Works, New York) containing growth medium which consisted of Dubelco's minimun essential medium (Gibco/BRL,

Burlington, Ontario), 400 U/ml of penicillin G (Gibco Laboratories, Grand Island, New York), 0.56 mg/ml of streptomycin sulphate (Gibco Laboratories, Grand Island, New York), 0.2 mg/ml of ascorbic acid (Sigma Chemical Co., St. Louis, Missouri) and 2.2 mg/ml of sodium bicarbonate (Fisher Laboratory Chemical, Fair Lawn, New Jersey), where all the soft tissue was removed. The medium was sterilized using a Millipore 0.22 μm filter (Millipore Corp., Mississauga, Ontario). The samples were washed three times to prepare for tissue culture.

In the first experiment the discs were minced and placed in collagenase (Sigma Chemical Co., St. Louis, Missouri) at a concentration of 0.095g/100ml of distilled water according to the procedure of Van Kampen and Veldhuijzen (1982). The articular discs were digested 4 times (the first was discarded) of 30 minutes each that were combined and centrifuged at 1500 rpm. The pellets were resuspended in 1 ml of DMEM and after cell counting, 35 mm tissue culture plates were seeded at an aggregation concentrtion of 2 X 10^5 cells in a drop of 5 μl of medium.

In the second experiment the discs were carefully divided in three bands, namely: anterior, medial and posterior bands. The bands of each age were divided in two groups, consisting of 1°) the regular procedure of cellular migration from the tissue attaching to the bottom of the dish, and 2°) the coating of the bottom of the dishes with collagen type I gel, prepared from vitrogen (Collagen Corp., Palo Alto, California, USA) according to the manufacturer's directions. The specimens were then divided in dishes with collagen and without collagen, containing one band of each age at least in one type of dish. The collagen coating provided a three-dimensional artificial matrix in which the tissue was imbedded prior to the gel solidification. The same size of dishes were used for the second experiment.

In both experiments, after being minced and placed into the dishes, the specimens were fed with 2 ml of DMEM plus 10% of Fetal Calf Serum (Flow Laboratories Inc., Missussauga, Ont.) plus 5% of fungizone (Flow Laboratories Inc., Missussauga, Ont.) and maintained at 37°C in a humidified atmosphere of 5% CO_2. After one week, the medium was replaced regularly every 2 days until the end of the time period.

The cells were monitored with a phase contrast microscope (Nikon, Japan) and photomicrographs were taken of selected cultures by using a 35 mm Panatomic-X film (Kodak Canada Inc., Brampton, Ontario) which were subsequently developed using microdol (Kodak Canada Inc., Brampton, Ontario). Black and white prints were made on kodak polycontrast rapid II RC paper with an enlargement of 6.5 X of the original magnification. Prints were developed with Kodak Dektol and fixed with Kodak Fixer.

RESULTS

The first experiment demonstrated a fast growth rate with the cells resembling chondrocytes (Figure D-1). After 14 days, the cells had formed an aggregate formation (Figure D-2) in culture and shortly

after that, a constant of growth rate was noted with the cells starting to show signs of deterioration with visible signs of cell death. This may have been due to the low amount of medium that could not be changed through the entire culture time, even though, the high density cultures were able to support the chondrocyte cell growth for a short period of time. This experiment showed typical presence of chondrocyte-like or fibrochondrocyte-like cells during the whole culture period.

The cells in the second experiment took some time to growth. After one week of culture there were not enough cells to be subcultured and no significant observation was made. This situation changed only after 3 weeks of the initial culture when the dishes showed a considerable number of chondrocyte-like cells (FigureD-5) which appeared to be attached to the bottom of the dishes, forming groups in same areas tending to confluency, similar to conluent areas in high density culture (Figure D-3). At the same time there was a clear evidence of the fibroblast-like cells population together with the former (Figure D-4). The first group (without the collagen coating) of the second experiment was finished after 40 days of initial culture without enough number of cells to be subcultured. At this time there were black dots around the medium and empty spaces among the cells which were filled previously. The collagen portion did not show much difference and chondrocyte-like cells were found in both groups. Apparently, the cell migration occurred more intensively in the collagen coated bottom dishes in the beginning of the culture (after 3 weeks) as is seen in Figure D-5. Following the end of the first group, the second group was still healthy and the cells were followed with a considerable presence of chondrocytes and fibrochondrocytes (Figures D-3 and D-6). This could indicate that the gels protected the cells for a longer period of time, once the tissue was embedded into the gels creating a more similar situation to the mother tissue. The gels were not digested with collagenase due to the reduced number of cells in all of the dishes, and as a consequence, the pieces of minced tissue were kept into the gels which probably served as an extra protection for the chondrocyte-like cells to grow.

After 7 weeks of initial culture there was a less marked presence of chondrocyte-like cells with an increase in the fibroblastic population. Now, clearly there was lack of the partial confluency and it was evident from now on that the cells would not increase the growth rate. Therefore, the remaining dishes were subcultured (same size of dishes) and the gels were discarded, once there was evidence that the cells were attaching to the bottom of the dishes and the gels were considerably ripped. This suggest that the gels also protect the cells in order for them to migrate to the bottom of the dishes. The subculturing procedure selected the number of cells and there was a reduction in chondrocyte-like cells (Figure D-7) with exception of one of the dishes. After 10 weeks from the initial culture this part of the experiment was also finished, since the cells demonstrated a slow growth rate and cell death.

DISCUSSION

The isolated populations of cells from the articular disc has been studied in culture. The primary objectives of this pilot study were to evaluate the articular disc cells as regarded to the possibility of phenotype characterization, the isolation of different cell populations with its maintenance in high density culture aggregates, the effect of cellular migration and cellular proliferation into collagen gels and the differences of cultures with collagen coating gels dishes and regular culture bottom dishes.

The ultrastructure of the articular disc cells was suggested as that of proper chondrocytes, based on the evidence from *in vivo* and *in vitro* morphological, immunohistochemical and biosyntetic observations (Silva, 1969; Isberg and Isacsson, 1986; Mills *et al.*, 1988). In addition, as evidenced by high metachromasia and alcianophilic properties, the articular disc shows rich zones of ground substance in connection with chondroid cells (Silva, 1969; Kopp, 1976). Although, a big variation was noted, there is evidence to affirm that the articular disc has at least two "different" cell populations. According to Ghadially *et al.* (1978) and Daniel and Mills (1988), the chondrocytes in this particular tissue tend to be more rounded or polygonal and the cells of the superficial zone are usually oval and fusiform. Some of the fusiform cells that at light microscopy can be mistaken for fibroblasts, resembled zone I chondrocytes of articular cartilage (Silva, 1969). As a matter of fact, many of the chondrocyte's features are very similar to those of the other cells and it is often difficult to identify particular characteristics precisely.

Nevertheless, the present work can not establish definite conclusions about the predominance of one particular population in one specific area of the disc, because the sample was small and the different ages contributed for the variation in the results. The findings included the presence of chondrocyte-like cells in all the bands as well as fibroblast-like cells, however, no attempt in correlating the observations with a specific area can be made from the data described here. Further work is necessary to determine the means to characterize the bands individually.

It is known that the connective tissue cultured cells tend to alter their characteristics in an artificial environment and frequently the cellular phenotype resulted from subsequent culture passages does not resemble the original explant cells. In addition, the tissues have the ability to switch phenotypes *in vivo* (Daniel and Mills, 1988). In other words, the flexibility to modulate the phenotype of a given cell under compression can transform the cell to allow it's adaptation under a tensional environment, for instance. After the subculturing procedures, it was not possible to observe the same chondrocytic-like growth rate, however Mills *et al.* (1988) reported that the chondrocyte-like phenotype was stable in monolayer culture through three passages with a gradual change in fibroblast-like phenotype. The aggregate formation of high density cultures showed that under a specific environment the disc cells retain the chondrocyte-like phenotype. This may reflect the natural environment of cartilagenous tissue (Veldhuijzen *et al.*, 1987), in which the cells develop and maintain their function.

It is assumed that the typical phenotype of chondrocytes from hyaline cartilage would be represented as true chondrocytes and they would act as such. According to Green (1971) the chondrocytes grown *in vitro* are of two types: one with fusiform shape resembling fibroblasts and a more polygonal ones more like hyaline cartilage chondrocytes. This finding is in agreement with the present work. Such conclusion may jeopardize the assumption that both fibroblasts and chondrocytes are present in the articular disc. Rather two different types of chondrocytes would be present and their phenotypic expression is provided by the fact that typical chondrocytes produce a methaccromatic extracellular matrix while fibroblasts do not do so. On the other hand, this could only be a problem of nomenclature, once the important issue is that one type of cell would react more like a chondrocyte and the other more like a fibroblast. The latter as well as the former could be furthermore altered to a more typical fibroblastic form as a result of lack of function.

Although, the high density culture showed signs of viability for cell function, the short term culture and the small amount of the sample make it difficult to monitor the articular disc in respect to function and molecular synthesis. In order to evaluate this effect of lack of function, the articular disc cells could be cultivated in a constant stressed environment, and not only the maintenance of cellular phenotype and behavior would be established but also the potential of cell proliferation in the stressed environment.

It is known that connective tissue cells cultured *in vitro* vary their response to different situations of mechanical stress. The form and metabolism of chondrocytes is a relative consequence of the mother tissue environment. In articular cartilage, the cells of the superficial layer are flattened and discoidal with a low rate of GAG synthesis (Maroudas and Evans, 1974) as compared to the cells in deeper zones that have a round form with a high rate of matrix production.

The size of the dishes was an important factor for cellular stability. The growth rate was considerably affected by the apparent lack of support provided by the bigger dishes in monolayer culture, which is in accordance with Bronson *et al.* (1989) that used smaller dishes with wells of 2 cm^2 each. This observation was clearly contrastant wit the high density cultures. It is evident that the small amount of tissue placed in first instance into monolayer culture was the responsible for the slow growth rate. However, by increasing the amount of tissue present in a larger area a higher degree of infections and tissue deterioration is likely to occur.

Both chondrocytic and fibroblastic phenotypes are altered by the culture medium and the serum supplement (Green, 1971; Webber *et al.*, 1985; Webber *et al.*, 1988). Dubelcco's MEM greatly increase sulphate incorporation into chondrocytes (Green, 1971; Webber *et al.*, 1985) with a slower growth rate as compared with F-12 (Webber *et al.*, 1988). It was suggested that the mediums played a selective role for either types of subpopulations of cells (Webber *et al.*, 1985).

The incorporation of isotope was not the issue of these pilot works. However, this culture was basically a monolayer culture. It has been observed that aggregation of chondrocytes in high-density

cultures, consisting of a well defined number of cells, was maintained *in vitro* with an increase in the incorporation of [^{35}S]-sulphate proving to be a more favorable system to study the factors modulating the behavior of the chondrocytes (Van Kampen and Veldhuijzen, 1982) under intermittent compressive forces (Veldhuijzen *et al.*, 1985). The conditions of the culture considerably affected the synthesis of sulphated PGs (Marsh *et al.*, 1979; Van Kampen and Veldhuijzen, 1982; Marsh *et al.*, 1982; Veldhuijzen *et al.*, 1985). Although aggregate type of culture favours cell behavior under mechanical stress, it was reported that aggregates tended to change chondrocyte surface properties (Kuroda, 1963; Nordling, 1967).

Disaggregation of cells in culture prior to the subculturing procedure may result in some damage to the cell and in many cases to a considerable decrease in cell number. However, the method of subculturing used in this study has been shown to be satisfactory without a significant alteration from the original culture (Nevo and Dorfman, 1972; Waymouth, 1974). In addition, the trypsin treatment apparently does not affect the biosynthesis of sulfated PGs, as long as there is a period of incubation in the growth medium prior to assay for synthesis (Nevo and Dorfman, 1972; Marsh *et al.*, 1982). This suggests that the same model can be applied to the description of the matrix production of articular disc cells by using mechanical stress.

It has been demonstrated in this work that the mouse articular disc and consequently the rat articular disc can be used as models for tissue culture. The amount of tissue required still has to be determined, however, aggregate high density cultures are more indicated for the study of mechanical influences on the articular disc. To this end, the density used is still dependent on the proper concentration to support chondrocyte viability for an increased amount of time, than that of this experiment.

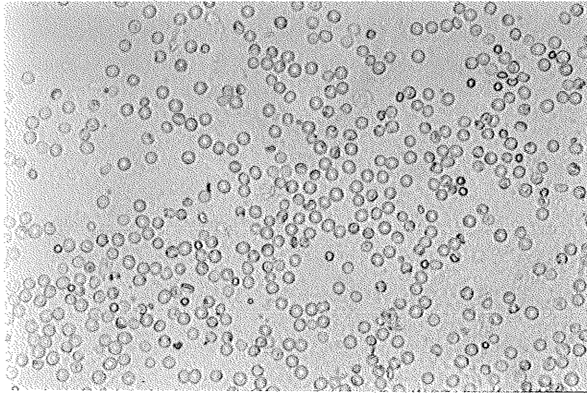


Figure D-1: Phase-contrast micrograph of a monolayer culture from rat articular disc cells resembling chondrocytes. Note the round morphology of the cells.

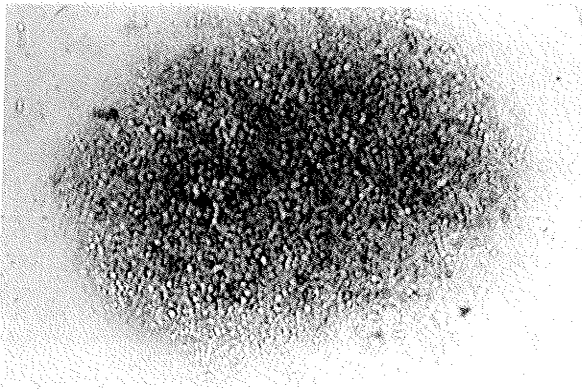


Figure D-2: Phase-contrast micrograph of a high density culture of an aggregate formation from rat articular disc cells.

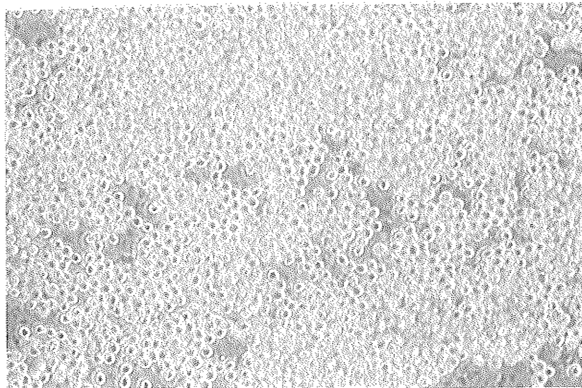


Figure D-3: Phase-contrast micrograph of a high density culture from rat articular disc cells resembling chondrocytes in confluency.

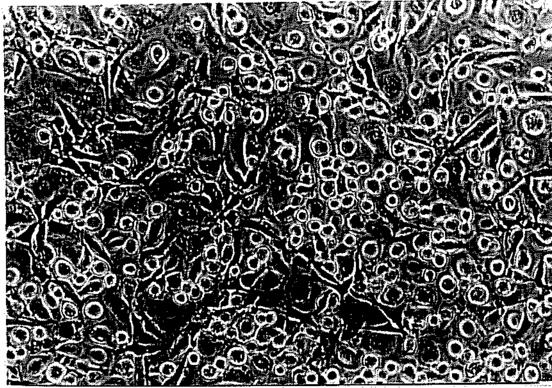


Figure D-4: Phase-contrast micrograph of a monolayer culture from rat articular disc cells showing the presence of both chondrocytes and fibroblasts.

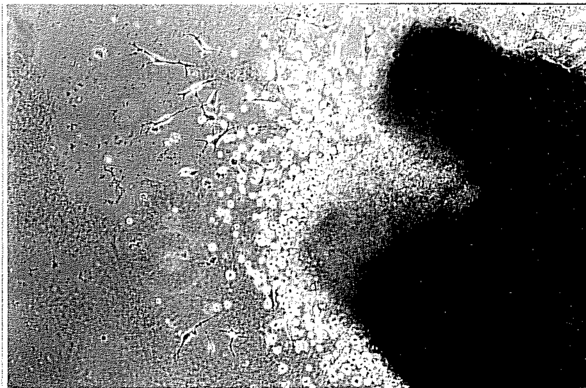


Figure D-5: Phase-contrast micrograph showing cell migration from rat articular disc explant (E) in collagen coated bottom dishes.

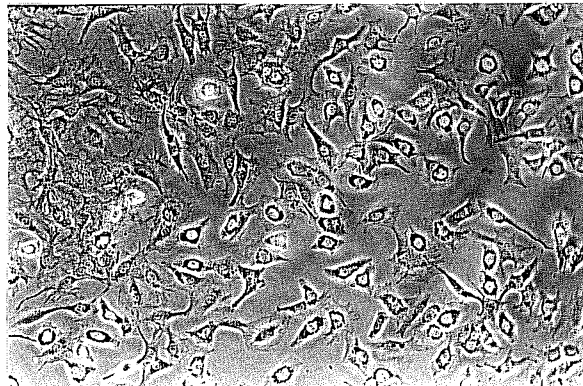


Figure D-6: Phase-contrast micrograph of a monolayer culture from rat articular disc cells resembling fibrochondrocytes. Note that the morphology of the cells is a transition from the more round shape to the fibroblastic morphology.

Figure D-7: Phase-contrast micrograph of a monolayer culture from rat articular disc cells resembling fibroblasts. Note that most of the cells resemble fibroblasts.

APPENDIX E

Pilot Study to Determine The Best Time Period for Organ Culture *In Vitro*

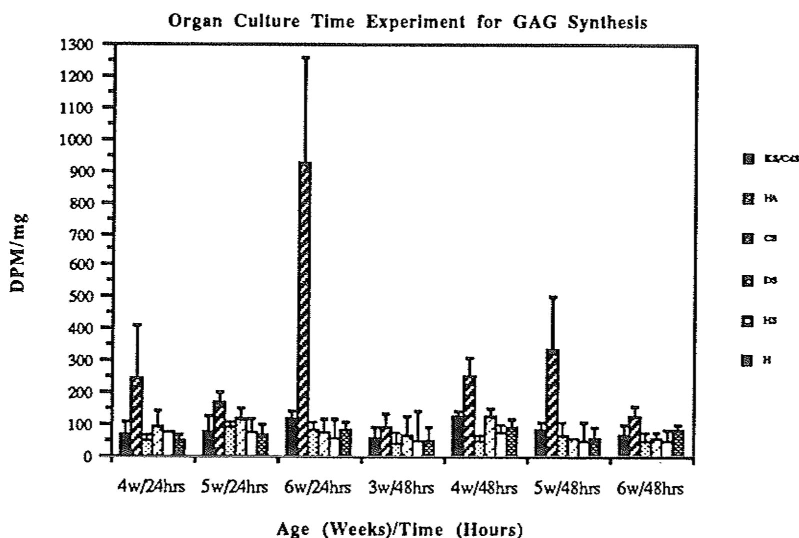
This pilot study has investigated the most appropriate time period in which the organ culture *in vitro* system was able to sustain GAG synthesis in the articular disc. The materials & methods used here has been extensively described in Chapters IV and V.

RESULTS & DISCUSSION

As shown in Figure E-1, the results have indicated that the time culture of the articular disc explants for 24 hours has been successful in maintainig a more intense GAG synthesis, given by the amount of GAG synthesis at the end of the respective time period.

Initially, the reason that this experiment has focused in two time periods only is due to the type of organ culture used as well as the turnover of GAGs in culture. The synthesis of collagen in culture has been successfully studied in the course of 4 hours to several days using the same type of organ culture model described here (non published material), however, the longer the time period the explant is left in culture the less probability the *in vitro* system will reproduce the *in vivo* situation without a significant change in phenotype. Therefore, 48 hrs has been taken as a maximum period for controlled *in vitro* culture, and has already shown signs of lack of function in the explants. Concluding, the time period of 24 hours has been determined as the time base for the study of GAG synthesis using the *in vitro* organ culture system in the experiments of Chapter IV.

Figure E-1



APPENDIX F

Pilot Study to Determine the Best Time Period for Organ Culture with Mechanical Stress *In Vitro*

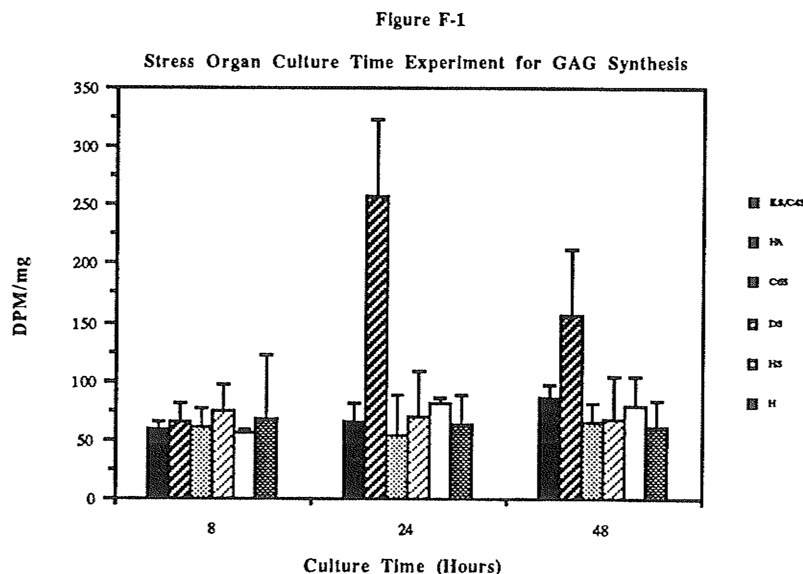
This pillot study has investigated the most appropriate time period in which the organ culture *in vitro* system was able to sustain GAG synthesis in the articular disc with the application of artificial mechanical stress. The materials & methods used here has been extensively described in Chapters IV and V.

RESULTS & DISCUSSION

The time periods used in this experiment were 8, 24 and 48 hours. The explants used were from rats aged 7 weeks old. Figure F-1 shows that 24 hours was the time period in which the articular disc was able to maintain a higher pattern of GAG synthesis as compared to the other time periods. In the previous Appendix (E), the same time period had been selected for the normal organ culture *in vitro*. This observation and the suggestion that under mechanical stress the articular disc cells did respond differently, the times of 8 and 48 hours have been incorporated and all time period samples used were proccessed together. Based on the increased proportion of GAG molecules synthesized at time period 24 hours as compared to the other ones, this variable was used as the time period standard to all the stress experiments of Chapter V.

Figure F-1

Stress Organ Culture Time Experiment for GAG Synthesis



APPENDIX G

Pilot Study to Demonstrate that the Organ Culture with Mechanical Stress *In Vitro* is Capable of Maintaining GAG Synthesis

The objective of this pilot study was to demonstrate that the organ culture modified to study the effects of mechanical stress in the articular disc was able to maintain the synthesis of GAG molecules for the period of the experiment.

MATERIALS & METHODS

Four Sprague-Dawley rats, inbred in our facility, were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The mandible was dissected and prepared for the modified organ culture as described in Chapter V. In the beginning of the experiment, 5 μCi/ml of [^{3}H]-glucosamine (Amersham Corporation, Oakville, Ontario) with specific activity of 27 Ci/mmol was carefully added to the culture dishes. After 24 hours, the articular discs were removed from the mandibular condyle (see entire procedure in Chapter V), washed in Waymouth's medium (Waymouth's 752/1, Grand Island Biological Co., Grand Island, New York) and fixed in 10% phosphate-buffered formalin containing 0.5% cetylpyridinium chloride or CPC (Sigma Chemical Co., St. Louis, Missouri) for 24 hours. The histological procedures have been described in Chapter III.

RESULTS & DISCUSSION

The distribution of silver grains in the articular disc as seen in Figure G-1 to G-3, demonstrates that the organ culture system *in vitro* that was modified to allow the application of mechanical stress is capable of maintaining GAG synthesis.

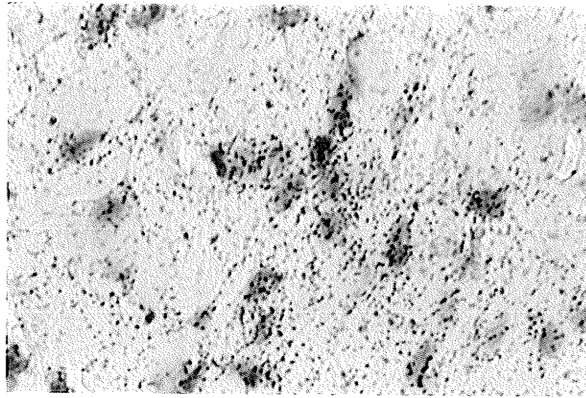


Figure G-1: Radioautograph of cross-section of the anterior band of the rat temporomandibular disc. Donor age is 5 weeks. Explant cultured *in vitro*. Note that the labeling pattern by tritiated glucosamine is similar to tritiated glycine. Haematoxylin and Eosin. X 205

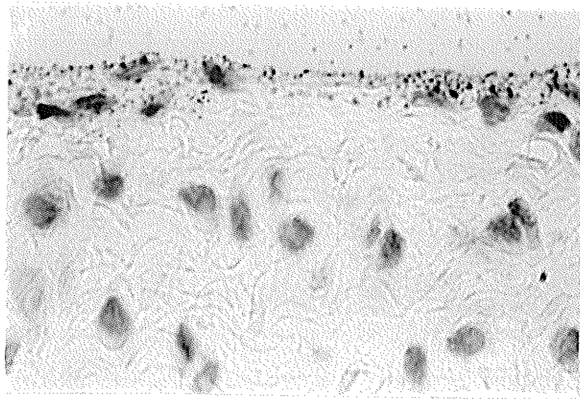


Figure G-2: Radioautograph of cross-section of the medial band of the rat temporomandibular disc. Donor age is 5 weeks. Explant cultured *in vitro*. Haematoxylin and Eosin. X 205

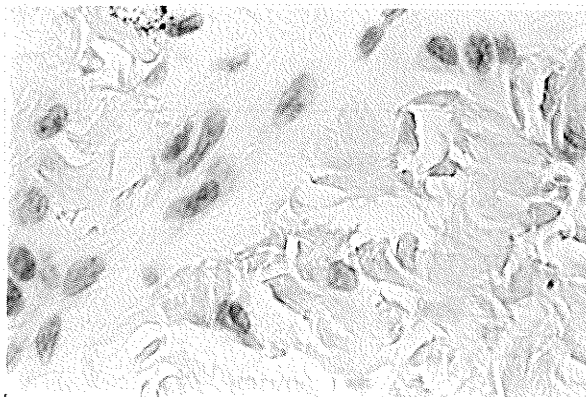


Figure G-3: Radioautograph of cross-section of the posterior band of the rat temporomandibular disc. Donor age is 5 weeks. Explant cultured *in vitro*. Note that there is very little labeling in this area. X 205

APPENDIX H

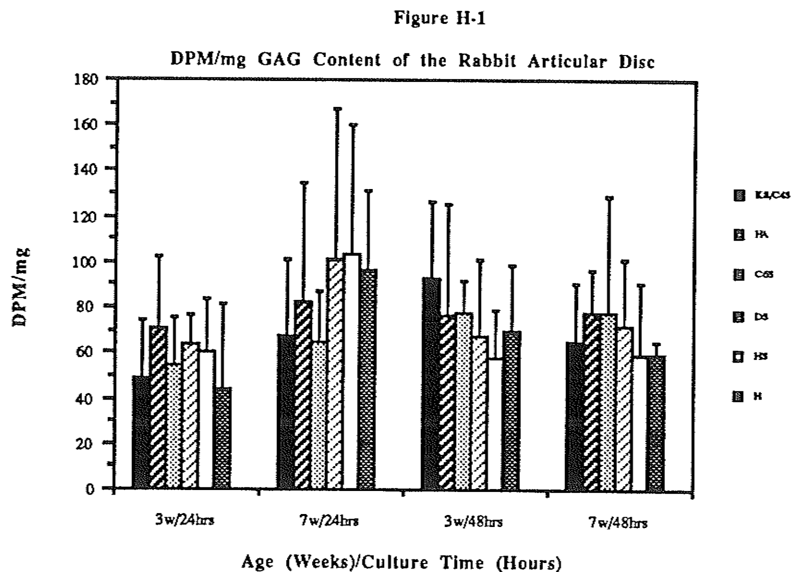
Pilot Study of GAG Synthesis and Distribution from Rabbit Articular Disc *In Vitro*

GAG synthetic activity has been studied in rat and mouse using organ culture experiments. However, due to the high similarities of the rabbit and the human temporomandibular joint (Mills *et al.*, 1988) this animal has been selected as an important model for TMJ studies. Therefore, this pillot study attempts to show the viability of this organ culture system to maintain GAG synthesis in the rabbit articular disc.

Two New Zeland white rabbits aged 3 and 7 weeks old were used in this experiment. The extraction and the proccessing of the articular disc samples have been detailed described in Chapter IV. Although, the pattern of GAG synthesis have been shown in Figure H-1, the limitted sample size does not allow any significant conclusions.

Figure H-1

DPM/mg GAG Content of the Rabbit Articular Disc



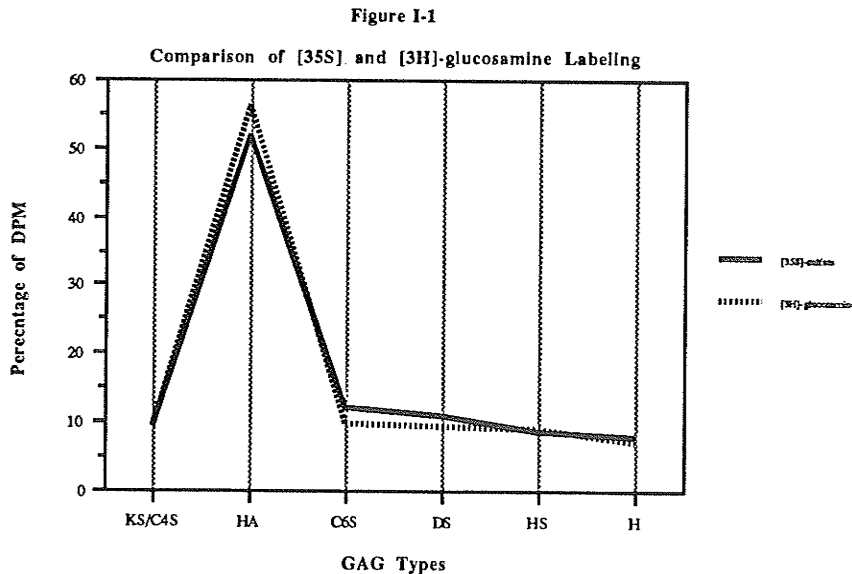
APPENDIX I

Comparison of [^{3}H]-glucosamine and [^{35}S]-sulfate labeling in the Rat Articular Disc *In Vitro*

The synthesis of GAG molecules *in vitro* has been compared using [^{3}H]-glucosamine and [^{35}S]-sulfate labeling. In this experiment, samples from animals aged 8 weeks old were used. The procedures used have been already described in Chapter IV, V and Appendices E, F and G.

RESULTS & DISCUSSION

Figure I-1 shows the comparison of both types of labeling used here. Both [^{3}H]-glucosamine and [^{35}S]-sulfate demonstrated very simmilar pattern of labeling. As it was expected, HA appeared decreased in the sample labelled with [^{35}S]-sulfate, since HA is the only GAG chain that does not have sulfate groups, however, the presence of HA in the same sample may indicate that the isotope was incorporated by labeling of other negative radicals. Similar factor happened in the case of [^{3}H]-glucosamine labeling of C_6S, since the hexosamine of this GAG chain is thought to be galactosamine and not glucosamine. Based on this results, [^{3}H]-glucosamine was picked as the choice isotope.



APPENDIX J

Enzyme Digestions of GAGs Synthesized from the Rat Articular Disc *In Vitro*

Selective enzyme digestions of GAG synthesized *in vitro* have been used to identify the different types of GAGs that were compared with the migration of purified standard GAG mixture.

MATERIALS & METHODS

Samples selected from the previous experiments were used for selective enzyme digestions. Each sample was digested with at least one different enzyme. The enzymes used were Streptomyces hyaluronidase (Sigma Chemical Co., St. Louis, Missouri), chondroitinase ABC (Sigma Chemical Co., St. Louis, Missouri) and keratanase (Sigma Chemical Co., St. Louis, Missouri). Streptomyces hyaluronidase (from *Streptomyces hyalurolyticus*) was prepared at a concentration of 3 µl/ml in 0.15 M NaCl and 1.0 M NaAc buffer at pH 5.0 (Bertolami, 1989), totalling 0.05 U/digestion. The samples were digested for 16 hours at 60°C. This enzyme removes hyaluronic acid. Chondroitinase ABC (from *Proteus vulgaris*) was prepared at a concentration of 2 µl/ml in chondroitnase buffer (Bertolami, 1989), totalling 0.05 U/digestion at pH 8.0 for 25 minutes at 37°C. This enzyme removes chondroitin$_6$sulfate,.chondroitin$_4$sulfate and dermatan sulfate. Keratanase (from *Pseudomonas species*) was prepared at a concentration of 10 µl/ml in TRIS buffer, totalling 0.05 U/digestion at pH 7.4 for 37°C. This enzyme removes keratan sulfate. Following digestion, reactions were stopped by boiling and the samples were quickly frozen and lyophilized. All digested samples were resuspended in standard mix and were separated using acetate alates gel electrophoresis (for a complete description of the procedures, refer to Chapter IV and V). Digested and non-degested standards and control samples were run concomitantly. The standard mixture used was composed of HA (from bovine tracheae), C_6S (from shark cartilage), C_4S (from bovine tracheae), DS (from bovine mucosa), HS (from bovine intestinal mucosa), KS (from bovine cornea) and H (from bovine lung).

RESULTS & DISCUSSION

Enzyme digestion of GAG organ culture samples are shown in Figures J-1 to J-4. In the Figure J-1, the digestion of the samples with chondroitinase ABC reduced the synthesis of C_6S considerably as compared with the control sample. This was also observed for DS and C_4S, although the slight difference in experiment as compared to the control sample suggests that the amount C_4S in the original sample was

very small, therefore, most of the variability from the first band has been attributed to KS alone. HA also showed reduced synthesis in the sample treated with chondroitinase ABC. This may have been due to the hig concentration of the enzyme and the variability in pH (Bertolami, 1989), however, based on previous trials the proportional decrease was non significant.

The samples digested with keratanase showed reduced incorporation of KS as compared with non-digested samples (Figure J-1 and J-3), while hyaluronidase showed reduced HA synthesis (Figures J-1 and J-4).

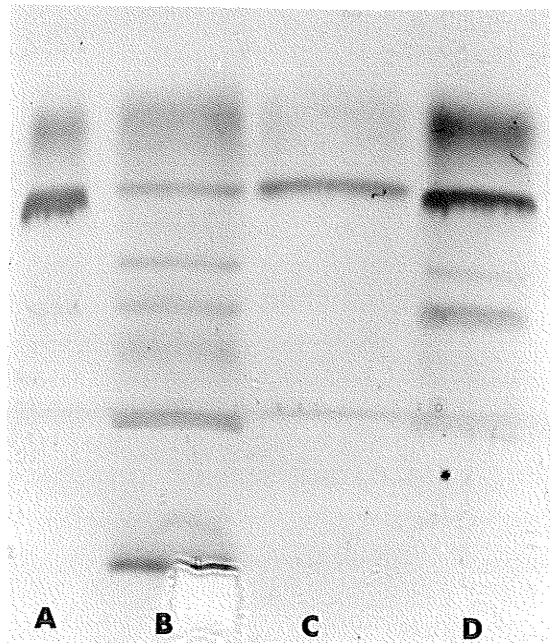


Figure J-1: Photograph illustrating the selective enzyme digestion in the GAG samples. Legends are: A = 5 week old sample treated with chondroitinase, note that CS and DS are not visible; B = 5 week old sample treated with hyaluronidase, note that HA appears less intense as compared to the other samples; C = 5 week old sample treated with keratanase, note that KS and CS have been digested and D = standard GAG mixture.

Figure J-2

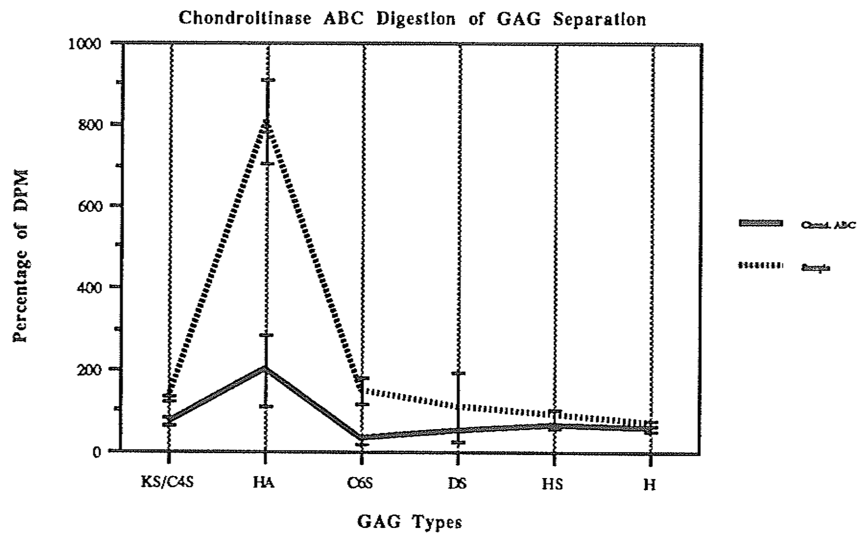


Figure J-3

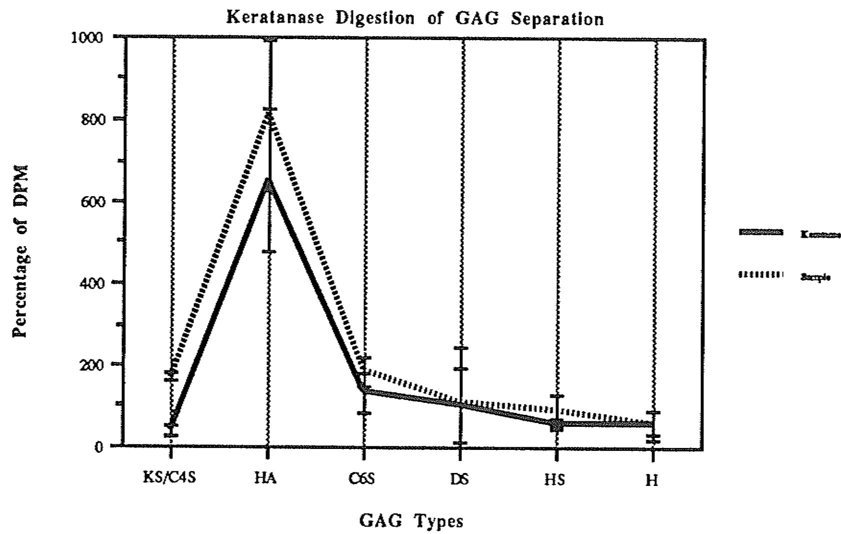
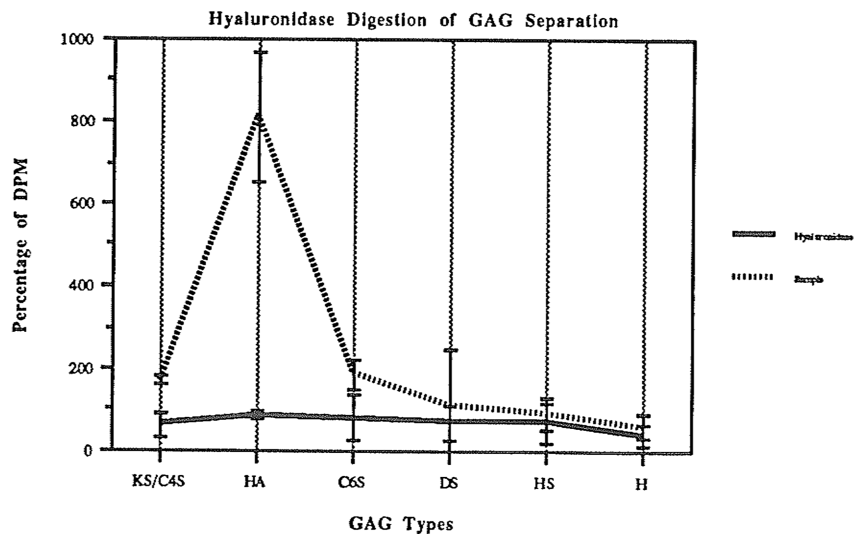


Figure J-4



APPENDIX K

Normal Growth of the Mandible and Condyle of the Albino Rat

The normal process of development and aging of the rat mandibular and condylar growth has been studied in animals from 3 to 12 weeks of age.

MATERIALS & METHODS

Thirty Sprague-Dawley male rats, inbred in our facility, were randomly divided in number of three for each of the following age groups: 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 weeks of age. The animals were anaesthetized with ether (Mallinckrodt Inc., Paris, Kentucky) and killed by cervical dislocation. The mandible was extracted and measured with precision calipers (Staedtler, West Germany) to record the normal growth for the following dimensions: 1) Mandibular Length (ML), taken from the posterior border of the condyle to the dento-alveolar-bone junctionof the incisor; 2) Condylar Length (CL), taken from the most anterior to the most posterior point in the mandibular condyle and 3) Condylar Width (CW), taken from the most lateral points in the mandibular condyle, as shown in Figure K-1 (Bouvier and Zimny, 1987; Bouvier, 1988).

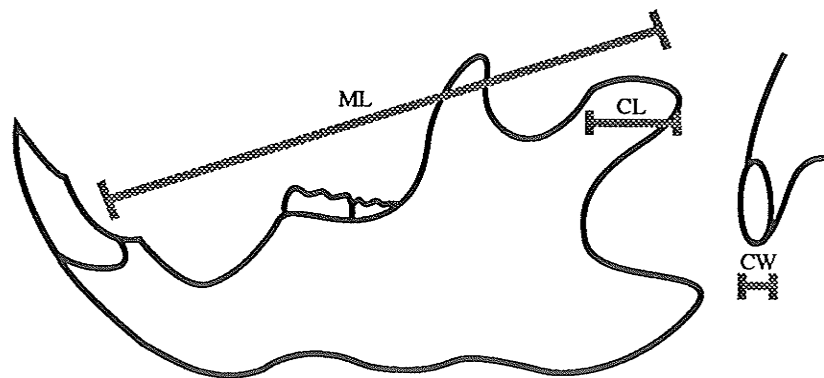
RESULTS & DISCUSSION

The mandibular growth of the albino rat is shown in Figure K-2 to K-4 and the values are resumed in Table K-I. The longitudinal growth of the mandible or the mandibular length dimension showed that there are basically two periods of fast growth, the first between ages 3 and 4 weeks and the second between ages 6 and 8 weeks (Figure K-2). Although, the growth in the first period between ages 3 and 4 weeks is significant, it is more likely to represent a fast difference in proportion from the very young to the adolescent rat. On the other hand, the ages 6 to 8 weeks represent a growth spurt at the end of adolescence to start of adulthood. According to Petrovic (1985), the growth of the albino rat takes place in three different stages; the fisrt period from birth to 20 days of age; the second period or period of fast growth, which goes from 21 to 48 days and the period in which most of the growth is completed, from 49 days to 120. Petrovic (1985) also demonstrated that in spite of the rat never stops growing, after 110 days the appositional growth is practically negligible. These results are in accordance with the observations from this study, since after 8 weeks or 56 days, the rat mandible showed little growth. On the same token, the condylar growth peaked at

8 weeks, decreasing after that (Figure K-3). The width of the condyle seemed to stabilize at 7 weeks of age, and in animals older than 10 weeks (Figure K-4), it was reduced probably due a resorptive adaptation in the TMJ. The data presented here indicates that the period from 6 to 8 weeks in the albino rat is more likely to represent a drastic change in the normal rat methabolism.

Figure K-1

Lateral View of Rat Mandible



LEGEND:

ML - Mandibular Length
CL - Condylar Length
CW - Condylar Width

Figure K-2

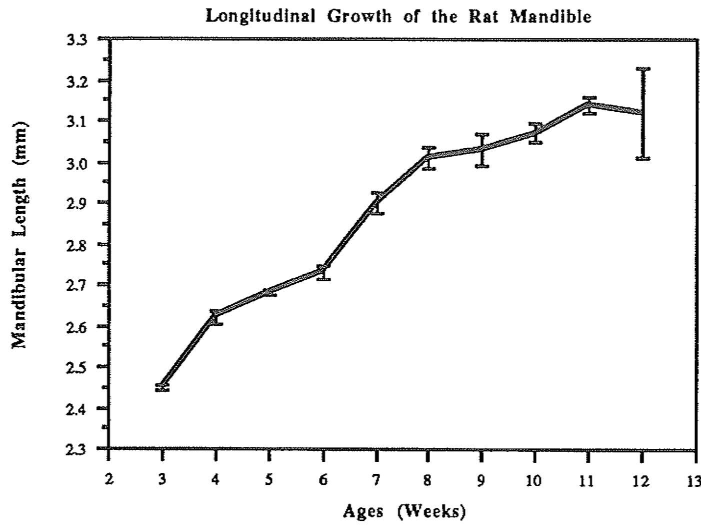


Figure K-3

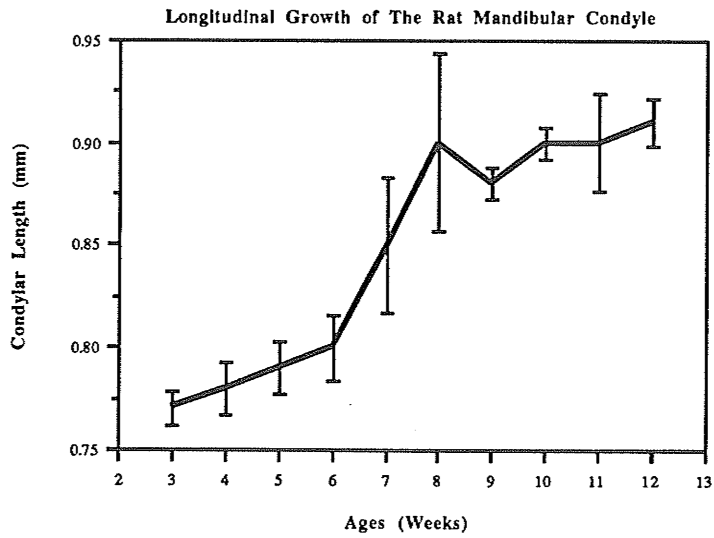


Figure K-4

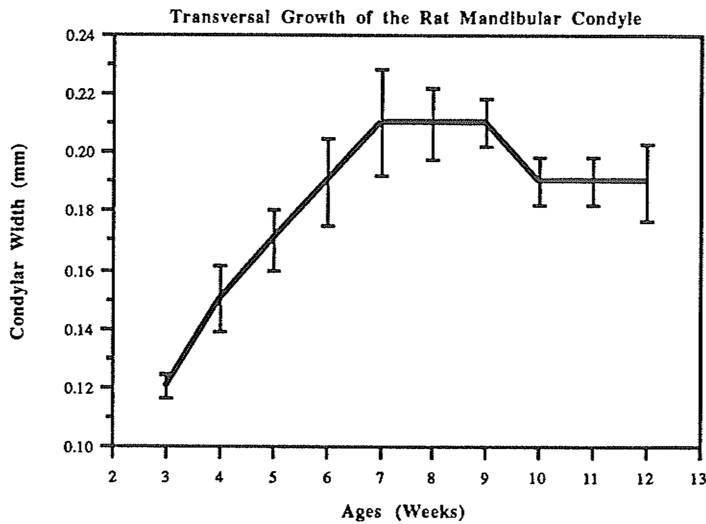


Table K-I

Rat Mandibular Growth

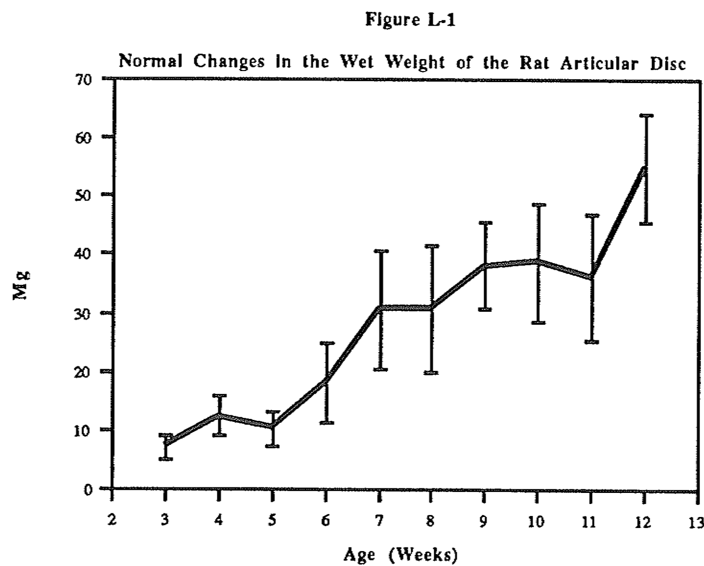
Dimensions (mm)	ML (1)		CL (2)		CW (3)	
Age (Weeks)	Mean	St. Dv.	Mean	St. Dv.	Mean	St. Dv.
3	2.45	0.005	0.77	0.008	0.121	0.004
4	2.62	0.014	0.78	0.013	0.15	0.010
5	2.68	0.005	0.79	0.013	0.17	0.010
6	2.73	0.014	0.81	0.016	0.19	0.015
7	2.90	0.028	0.85	0.032	0.21	0.018
8	3.01	0.023	0.91	0.043	0.21	0.012
9	3.03	0.038	0.88	0.007	0.22	0.008
10	3.07	0.021	0.91	0.08	0.19	0.008
11	3.14	0.020	0.90	0.024	0.19	0.008
12	3.12	0.112	0.90	0.012	0.20	0.013

APPENDIX L

Wet Weight of the Rat Articular Disc

This appendix recorded the wet weight of the articular disc from the albino rat. The same animals from the previous Appendix (J) were used. The articular disc from each joint was removed and its weight carefully recorded. Similar to the mandibular growth, the articular disc weight seems to increase rapidly until 7 to 8 weeks of age, continuing growing after that. The values are shown in Figure L-1.

Figure L-1



APPENDIX M

Photograph of Fluorogram Illustrating Collagen Bands Separated by SDS-PAGE

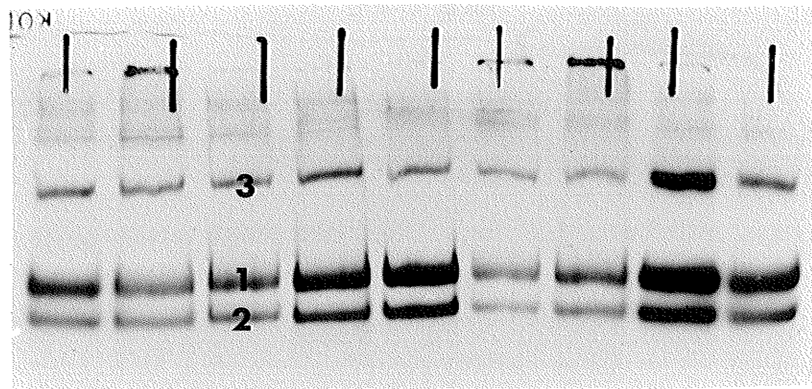


Figure M-1: Photograph of fluorogram illustrating collagen bands separated by SDS-PAGE. Legends are: (1) α_1(I)-collagen, (2) α_2(I)-collagen and (3) α_1(III)-collagen.

APPENDIX N

Photograph of Gel Illustrating GAG Bands Separated by Acetate Plate Gel Electphoresis

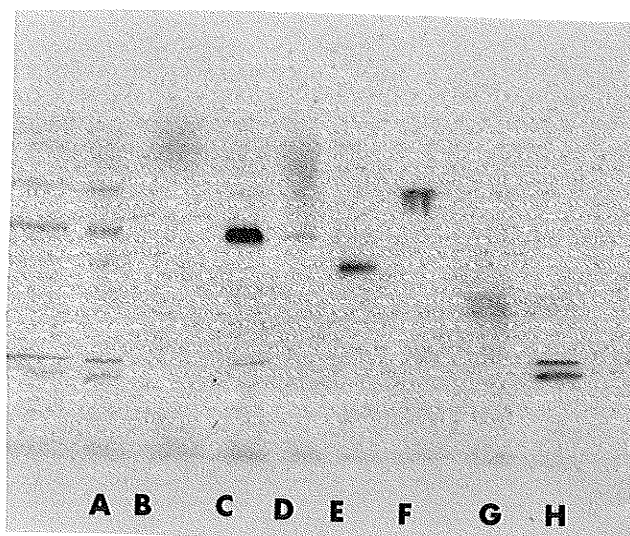


Figure N-1: Photograph of gel illustrating GAG standard bands separated by Acetate Plate Gel Electrophoresis. Legends are: A = standard GAGs, B = keratan sulfate, C = chondroitin$_6$sulfate, D = chondroitin$_4$sulfate, E = dermatan sulfate, F = hyaluronic acid, G = heparan sulfate and H = heparin.

APPENDIX O

Flexercell Strain Unit for the Study of Molecular Synthesis *In Vitro*

The cyclic strain regimen to which the organism is normally subjected *in vivo* suggests that structures like cartilage and ligament when studied without any amount of strain can not be represent *in vitro* the same environment that they are exposed *in vivo*. Although, such an assumption is a consequence of the tissue's demands, it is not always possible to explore this type of investigation. To reduce this gap between strain exposed tissues and strain non exposed ones, the use of the Flexercell Strain Unit provides a useful approach to the study of force application and deformation in cell and organ culture.

The majority of the organs *in vivo* are not always subjected to the same type or strain magnitude, however, all cell types will respond to the mechanical stimulation through similar mechanisms: a) rapid response in seconds it may involve a membrane depolarization, Ca^{++}, Na^{+} or K^{-}, or other ion flux changes, elaboration of cAMP or prostaglandin E_2; b) in minutes or hours it may involve morphologic change, change in F-actin distribution, transcription or translation and c) long term response in hours, days or weeks it may involve changes in the rate of cell division, the type of extracellular matrix formed or pathways in differentiation.

The Flexercell Strain Unit has been created and improved by Banes *et al.* (1985), and it has been under use since then. Several works by Banes *et al.* (1985) and Sumpio *et al.* (1987, 1988) have described this unit in detail. The central unit is composed of a computer terminal IBM PC compatible attached to the central vacuum unit (Figure O-1), which has inlet and outlet vacuum hoses at the rear of the instrument. The vacuum hose is inserted through a vent hole in the top of a conventional cell culture incubator, in a way to avoid disturbances in the incubator environment. Inside the incubator the vacuum hose attaches to a base plate or the unit of the apparatus which consists of a vacuum system with 8 vacuum ports and a gasket, which fits 8 flexible bottom plates (see Chapter V, for description), as shown in Figure O-2. The computer program can then control all variables related to frequency of the applied force as well as duration, amplitude and strain rates in the tissue or organ culture.

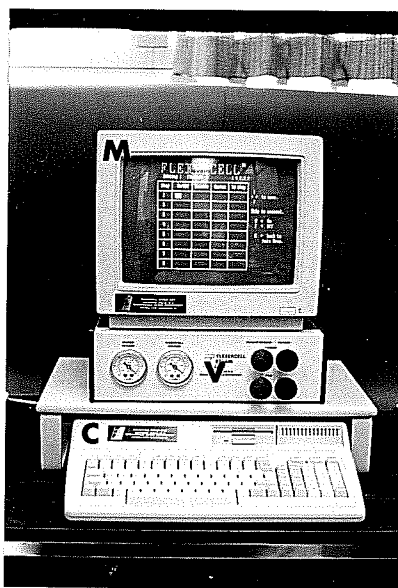


Figure O-1: Photograph of Flexercell central unit. Legends are: C = computer, M = monitor and V = vacuum central system.

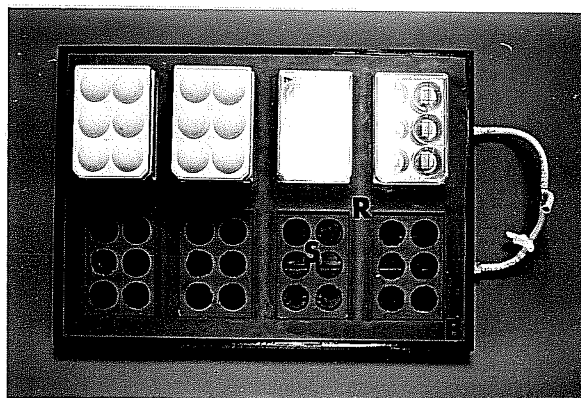


Figure O-2: Photograph of Flexercell base plate. Legends are: B = base plate, R = rubber linning and S = slot to fit the organ culture dishes.