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Meniscal Degeneration following Anterior Cruciate Ligament Tear and the Role of Intra-Articular Injection of Sodium Hyaluronate. Histochemical and Electron Microscopic Study in the Rabbit Knee

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Abstract

Objective: The purpose of this study was to demonstrate the histological changes occurring in the synovium and meniscus after transection of the anterior cruciate ligament in rabbits, and to evaluate these changes after intra-articular injection of sodium hyaluronate.

Methods: Fifteen rabbits were divided into three groups. The surgery was performed in the left knees only and the right knees served as controls. Group (I) served as sham-operated controls, Group (II) underwent unilateral anterior cruciate ligament transection of the left knees and received no treatment, and Group (III) received intra-articular injections of 0.3 ml sodium hyaluronate into the left knee beginning 1 week after surgery, once a week for 5 weeks. All rabbits were killed 8 weeks following surgery for assessment of knee meniscus by histological, histochemical and ultrastructural analyses.

Results: The histological examination of group II demonstrated the synovium with multilayered synovioblasts, and extensive cellular and matrix deterioration of meniscus in the form of altered cell distribution, decreased cell density, and abnormalities in the collagen arrangement. In groups III, the synovium showed many blood vessels and the cells of menisci apparently increased. Histochemically, safranin-O staining revealed the increased presence of proteoglycan in the sodium hyaluronate treated menisci relative to non-treated one.

Ultrastructurally, the chondrocytes of group II showed obvious decrease in their organelles associated with the synthesis and secretions of the matrix with an increase in the number of lysosomes and cytoplasmic vacuoles. In group III, some active chondrocytes containing RER and ribosomes were observed.

Conclusions: The results in the present study documented that the treatment with sodium hyaluronate after anterior cruciate ligament transection, induced an improvement of several structural features of both synovial membrane and meniscus.

Keywords: Meniscal degeneration; Anterior cruciate ligament tear; Intra-articular injection; Histochemical and electron microscopicstudy; Rabbit knee

Introduction

The meniscus of the knee joint is an intra-articular fibrocartilaginous disk of the type found in only a few human synovial joints: the temporomandibular, inferior radioulnar, and sternoclavicular joints [1]. The menisci are wedge-shaped semilunar structures that correct the incongruence of the femoral and tibial articular surfaces [2]. They provide important biomechanical functions to the knee joint such as load bearing, load distribution, shock absorption and joint stability and exhibit viscoelastic properties, which may serve to attenuate impacts sustained through the knee on loading [3]. It has been determined that approximately 50% of the body weight is transmitted through the menisci in extension and up to 90% of flexion [4].

The meniscal tissue is composed mainly of collagen type I [5]. The collagen bundles are arranged mainly in a circumferential orientation with a relatively small number of tie-bands passing in a radial direction and another small number of fibers passing from the superior to the inferior borders of the meniscus [6]. In addition, the matrix of menisci contains considerably less proteoglycan (<1%) than does hyaline cartilage (7%) [7]. Both large chondroitin sulfate and small dermatan sulfate proteoglcans have been shown to be present in meniscal tissue [8,9].

Hyaluronic Acid (HA) is a natural molecule playing a pivotal role in all the regions of the joint. It is actively synthesized by the synoviocytes and it determines the viscoelastic properties of the synovial fluid. In the cartilage, HA which permanently complexed with proteoglycans is essential in maintaining the structural and functional characteristics of this tissue? In addition, the free HA contributes to the formation of an amorphous layer about 0.6 ¼ m thick, covering the articular surface of the cartilage, and also contributes to the boundary lubrication mechanism in conditions of extreme loading [10].

Deteriorating knee function following rupture of the anterior cruciate ligament is frequently observed particularly in patients who continue with a high level of sport activity. The changes in various tissues of the knee joint, in particular articular cartilage following anterior cruciate ligament transection, have been extensively studied [11,12]. However, considerably less is known regarding the effect of anterior cruciate ligament transection on the knee joint menisci and synovium. Some authors have suggested that the outcome of a ruptured anterior cruciate ligament in the majority of active young patients, who attempt to remain active, leads to high incidence of secondary meniscus injury and early development of osteoarthritis [13,14].

A number of clinical studies have documented the therapeutic

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value of intraarticular application of hyaluronic acid but these studies relied on pain relief, knee function as well as range of motion of the affected joint as parameters for the effectiveness of such treatment [15,16].

Therefore, the purpose of the present study was to demonstrate the histological changes in the synovium and meniscus in cases of the anterior cruciate ligament insufficiency; and also after intraarticular injections of sodium hyaluronate.

Materials and Methods

Experimental design: Fifteen male mature white rabbits were used in the present study. All rabbits were divided into three groups (5 rabbits/group). The surgery was performed in the left knees and the nonoperated right knees served as controls. Group (I) served as sham-operated controls, the ligament was exposed but not transected. Group (II) underwent unilateral Anterior Cruciate Ligament Transection (ACLT) of the left knees and received no treatment. Group (III) received 0.3 ml intra-articular injections of sodium hyaluronate (Healon, Pharmacia and Upjohn Com.) into the left knee beginning 1 week after ACLT, once a week for 5 weeks. This schedule was the same as the current clinical application schedule of hyaluronic acid [10].

Surgery and treatment of animals: All rabbits were anesthetized by intramuscular injection of ketamine (l00 mg/kg). Operative knees were shaved and disinfected with betadine solution. A medial parapatellar skin incision was made and a medial arthrotomy was performed. The patella was dislocated laterally and the anterior cruciate ligament was transected. The capsule was closed with a running suture of 4-0 prolene and the skin incision was closed with interrupted 4-0 nylon sutures.

Postoperatively the animals were closely monitored for infections and other complications and were allowed unrestricted activity in cages until sacrifice. One week after surgery, anterior cruciate ligament transected knees of group III were shaved and disinfected with betadine solution, and then they were injected intra-articularly with sodium hyaluronate. The syringe was inserted beneath the patella and 0.3 ml sodium hyaluronate was injected. All animals of three groups were killed 8 weeks following surgery by overdose of anesthesia.

Histological preparation: The medial menisci with its surrounding synovium from each group were dissected carefully. Each meniscus was divided into two halves, Anterior half was immediately fixed in 10% neutral buffered formalin and was routinely processed and paraffin embedded. The sections were cut with a microtome and stained with hematoxylin and eosin for general morphology and safranin O/fast green for proteoglycan staining. The other half of samples were immediately dipped in 1% glutaraldehyde and then cut into small pieces transversely. Samples were then fixed by 2.5% glutaraldehyde, for 24 h and then for 1 h in 1% osmium tetroxide. After dehydration in ethanol and propylene oxide, specimens were embedded in resin. Semi thin sections were cut and stained with toluidine blue and examined by light microscopy to choose the selected areas. Ultra thin sections were stained 30 min in a saturated solution of uranyl acetate, 5 min in lead citrate, and examined and photographed with transmission electron microscope.

The synovium was subjected for cellular assessment by histological scoring system [17] this score assessed (1) synovial lining layer: hyperplasia of synovial lining cells (0-3 points), hypertrophy of synoival lining layer (0-3 points), and infiltration of inflammatory cells (0-3 points); (2) subsynovial tissue: proliferation of granulation tissue (0-3 points), vascularization (0-3 points), and infiltration of inflammatory cells (0-3 points), with a maximum of 18 points. The synvoitis scores were divided into three stages: 0-6 points (mild synovitis), 7-12 points (moderate synovitis), and 13 or more points (severe synovitis).The higher the score, the more the damage.

Microscopic examination was used to grade the meniscus according to Mankin's histologic grading with modification [18] this score assesses structure (0-6 points), cellularity (0-3 points), matrix staining (0-4 points), and tidemark integrity (0-1 points), and has a maximum of 14 points. Scoring was based on the most severe histological changes seen in each meniscus section. The Mankin's score was divided into three stages of 0-6 points (stage I; mild degenerative change), 7-9 points (stage II; moderate degenerative change), and 10 or more points (stage III; severe degenerative change), a higher score indicating more severe damage.

Statistical analysis: All data are presented as the mean standard error of the mean (SEM). Data was analyzed using one-way ANOVA with Fisher's post hoc tests for multiple comparisons. A p-value<0.05 was considered significant.

Results

In the sham group, the meniscus was macroscopically normal, with a glistening, smooth surface. The grade of cartilage damage of the meniscus in the HA group was not statistically different from that in the sham group. The synovium from the sham group had a white luster and transparent appearance and showed no hyperemia or evidence of synovitis. Synovium in the HA group were thinner and the discoloration less intense.

Light microscopic results: By hematoxylin and eosin stain, the synovial membrane of control and sham-operated groups was lined by one to two layers of flat cells. Underneath these cells there was a layer of loose connective tissue with areas of adipose tissue (Figure 1). In the same groups, the fibrocartilage of meniscus showed chondrocytes surrounded by a small amount of cartilage matrix and were aligned in rows between parallel bundles of collagen fibers (Figure 2). The chondrocytes were located in lacunae with very thin capsules that look basophilic but the tissue as a whole was acidophilic owing to the predominance of collagen (Figure 3). The peripheral part of meniscus showed elongated cells resembling fibroblasts and was penetrated by small blood vessels from the synovium (Figure 4).

The ACL transected knees of group II revealed stratification of the synovial lining cell layer which appeared as multilayered synovioblasts (Figure 5). In the meniscus of this group the cell density and its surrounding matrix apparently decreased with respect to control sample (Figure 6). Extreme alteration in cell distribution with the presence of some empty lacunae was observed. Some cells showed karyolysis of their nuclei which evidenced by a lessening of nuclear basophilia (Figure 7). Other cells displayed irregularity in the shape of their nuclei with the appearance of some pyknotic nuclei. Abnormalities in the collagen arrangement were also noticed that displayed the greatest changes from normal arrangement (Figure 8).

In group III which injected with sodium hyaluronate after ACL transection, the synovium was lined by a single layer of squamous cells, and many blood vessels with thick wall were also noticed (Figure 9). The cells of menisci apparently increased in comparison with the pervious group (Figure 10). Focal hyper-cellular areas with increase in its surrounding matrix were noticed in some specimens (Figure 11).

The Mankin grading has shown significant differences between the sham group (0.75 ± 0.15) and both the control (3.42 ± 0.16, p=0.0004) and HA groups (2.62 ± 0.24, p=0.006), and between the control and

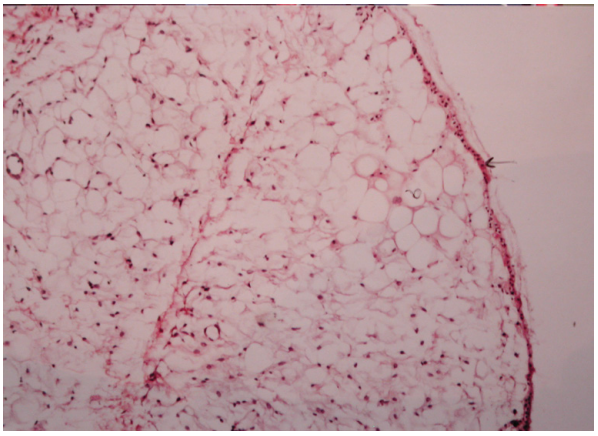

Figure 1: Photomicrograph of the synovial membrane of control group showing synovial lining cell layer. Underneath these cells is a layer of loose connective tissue with areas of adipose tissue (Hx&E, X100).

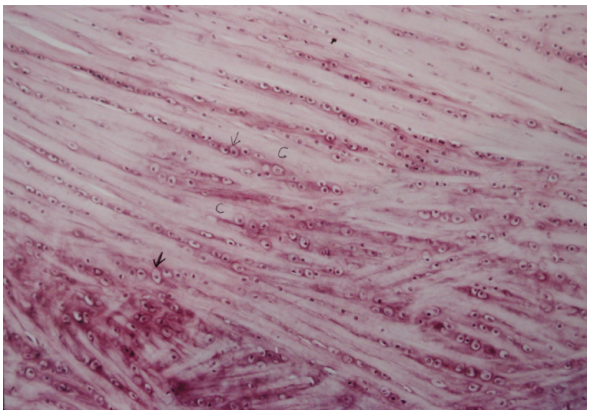

Figure 2: Photomicrograph of the meniscus of control group showing chondrocytes in lacunae and surrounded by a small amount of cartilage matrix (Hx&E, X100).

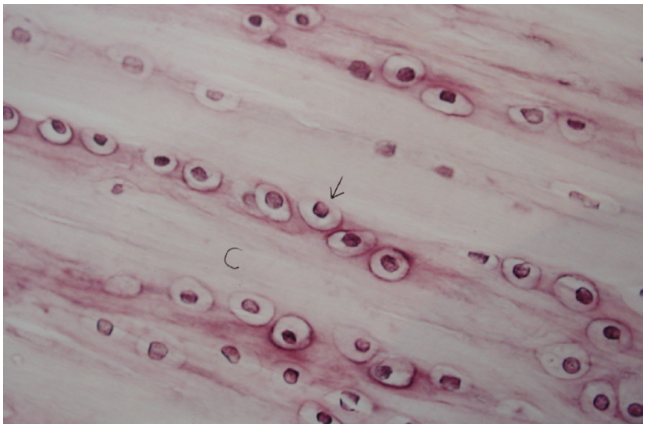

Figure 3: Photomicrograph of the meniscus of control group showing chondrocytes in lacunae arranged in rows. The cells are surrounded by a rim of condensed matrix between bundles of collagen fibers (Hx&E, X400).

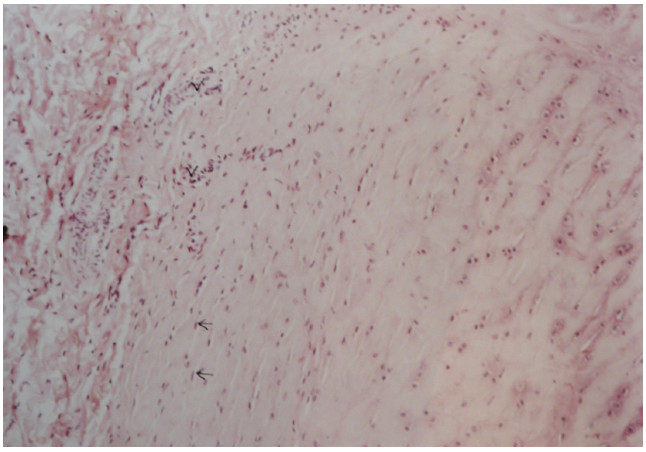

Figure 4: Photomicrograph of the meniscus of control group showing the peripheral part of meniscus with elongated fibroblasts and small blood vessels (Hx&E, X100).

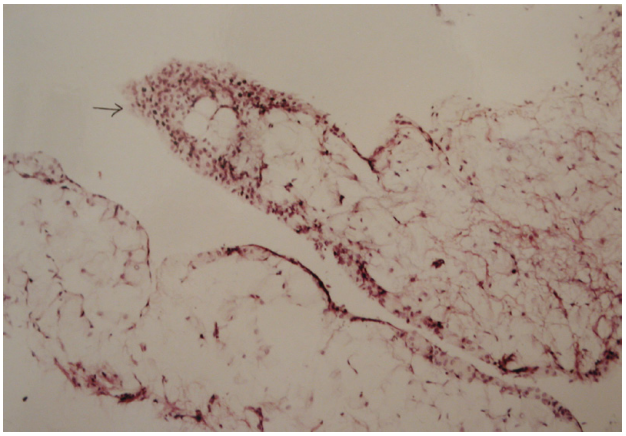

Figure 5: Photomicrograph of synovial membrane of group II showing stratification of synovial lining cell layer (Hx&E, X100).

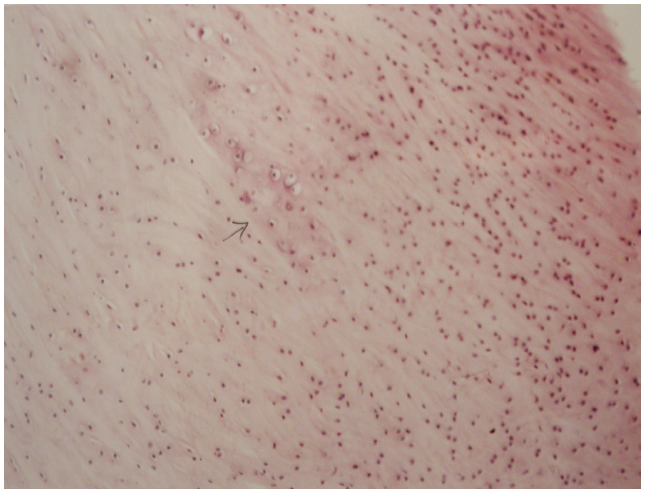

Figure 6: Photomicrograph of the meniscus of group II showing an apparent decrease in the cell density and its surrounding matrix (Hx&E, X100).

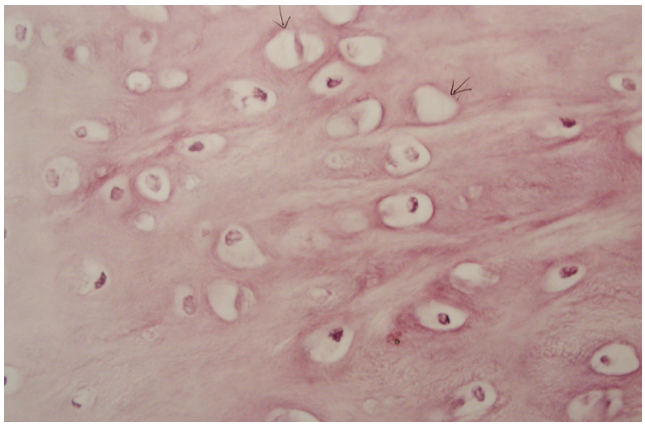

Figure 7: Photomicrograph of the meniscus of group II showing extreme alteration in cell distribution with the presence of some empty lacunae and karyolytic nuclei (Hx&E, X400).

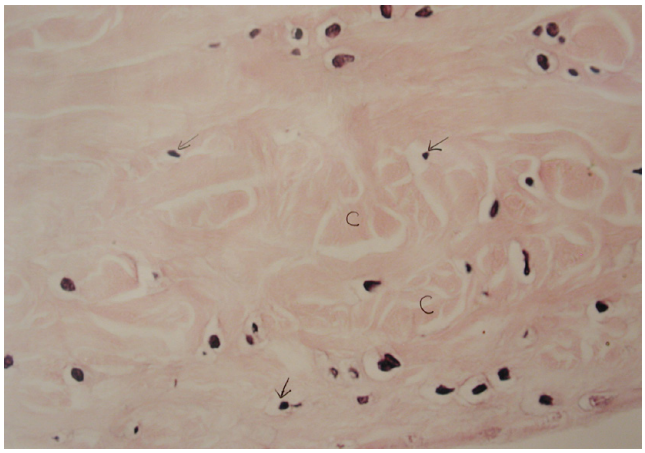

Figure 8: Photomicrograph of the meniscus of group II showing irregularity in the shape of nuclei with the appearance of some pyknotic nuclei, and abnormalities in the collagen arrangement (Hx&E, X400).

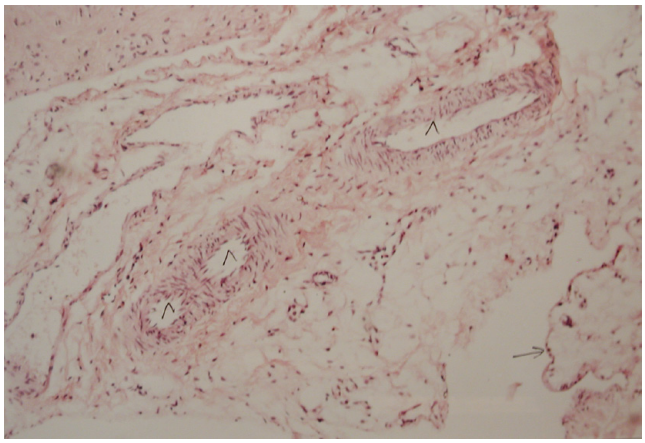

Figure 9: Photomicrograph of synovial membrane of group III showing single layer of synovial lining cells and many blood vessels (Hx&E, X100).

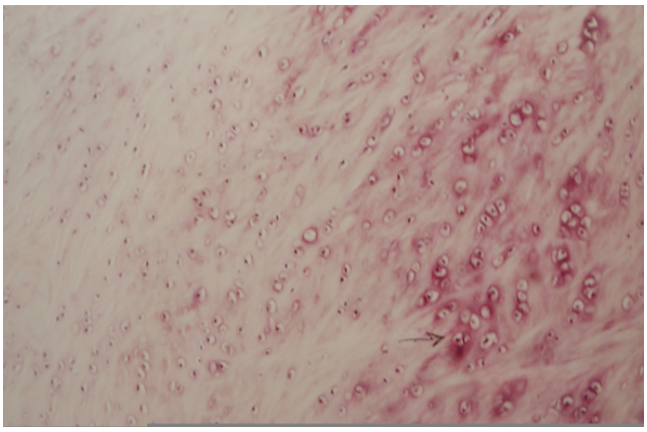

Figure 10: Photomicrograph of the meniscus of group III showing an apparent increase in the chondrocytes and their surrounding matrix (Hx&E, X100).

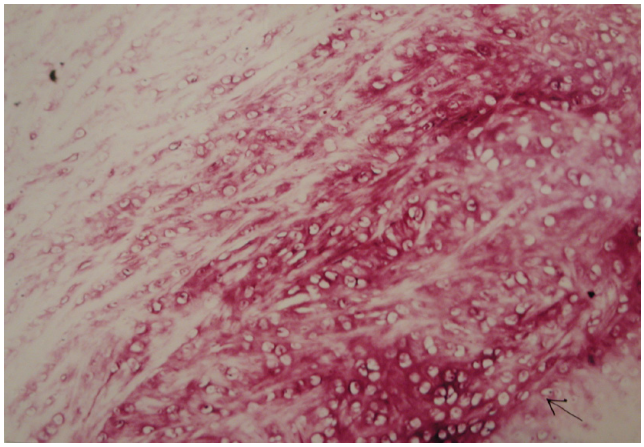

Figure 11: Photomicrograph of the meniscus of group III showing focal hypercellular areas and condensed matrix (Hx&E, X100).

HA groups (p=0.005) (Table 1).

The synovitis scores by microscopic evaluation are shown in Table 1; significant differences were found between the control group (2.25 ± 0.37) and both the sham (6.42 ± 0.28, p=0.0004) and HA groups (3.25 ± 0.31, p=0.006), and between the HA and sham groups (p=0.005).

Histochemically, the extracellular matrix of the control meniscus homomgenusly and moderately stained with safranin-O stain and appeared reddish in color (Figure 12). Compared to control meniscus, the meniscus of group II stained less intensely (Figure 13) and areas of diffuse loss of safranin-O staining were observed (Figure 14). In group III, the extracellular matrix of the meniscus showed extensive alteration in the pattern of staining with areas of high intensity and areas of weak staining (Figure 15).

Electron microscopic results: At the electron microscopic level, specimens of the control meniscus revealed the normal structure of chondrocytes. Their outlines varied from fusiform to rounded (Figures 16 and 17) and their cytoplasm contained RER which is distended with secretory material, lysosomes, and lipid droplets (Figure 16). The parallel bundles of collagen fibers separated from the cells by a zone which may correspond to extracellular matrix (Figure 16). In group II, various types of changes with varying degrees of severity were

observed, some cells showed obvious decrease in their organelles with irregularities in their outlines (Figures 18 and 19).

Some nuclei demonstrated irregularity and folding of the nuclear membranes (Figure 19), and others became shrunken with condensed chromatin (Figure 20).

In group III, some cells showed an increase in the number of lysosomes and cytoplasmic vacuoles (Figure 20). Some chondrocytes containing RER and ribosomes were observed and surrounded with bundles of collagen fibers (Figure 21).

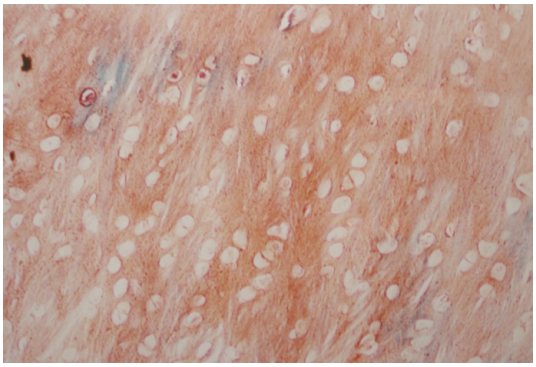

Figure12: Photomicrograph of the meniscus of control group which appeared homomgenusly and moderately stained with safranin O staining (safranin O/ fast green, X200).

Figure 13: Photomicrograph of the meniscus of group II showing less intensity of safranin-O staining (safranin O/fast green, X200).

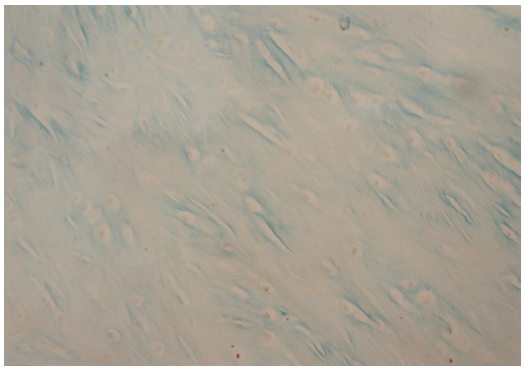

Figure 14: Photomicrograph of the meniscus of group II showing areas of diffuse loss of safranin-O staining (safranin O/fast green, X200).

Figure 15: Photomicrograph of the meniscus of group III showing areas of high intensity and areas of weak staining (safranin O/fast green, X200).

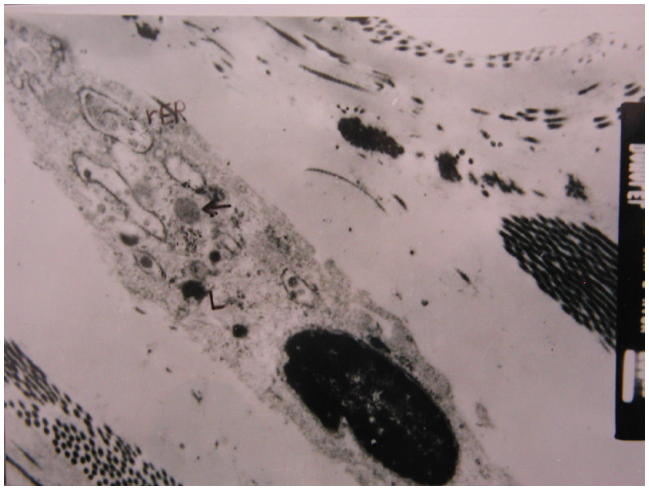

Figure 16: Electron micrograph of the meniscus of control group showing fusiform chondrocytes containing RER, nucleus, lysosomes, and lipid droplets. Notice, Collagen fibers (X10.000).

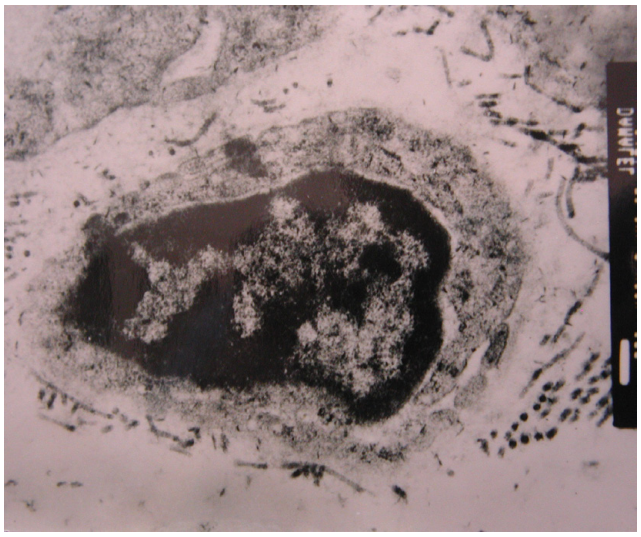

Figure 17: Electron micrograph of the meniscus of control group showing a rounded chondrocyte surrounded by collagen fibers (X20.000).

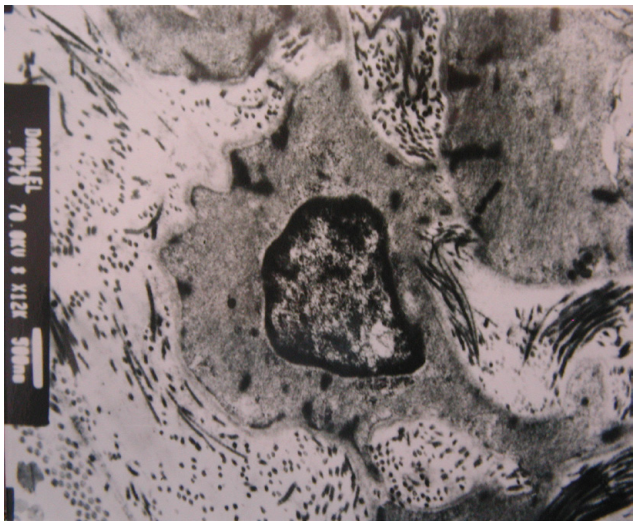

Figure 18: Electron micrograph of the meniscus of group II showing irregularly shaped chondrocytes surrounded with collagen fibers (X12.000).

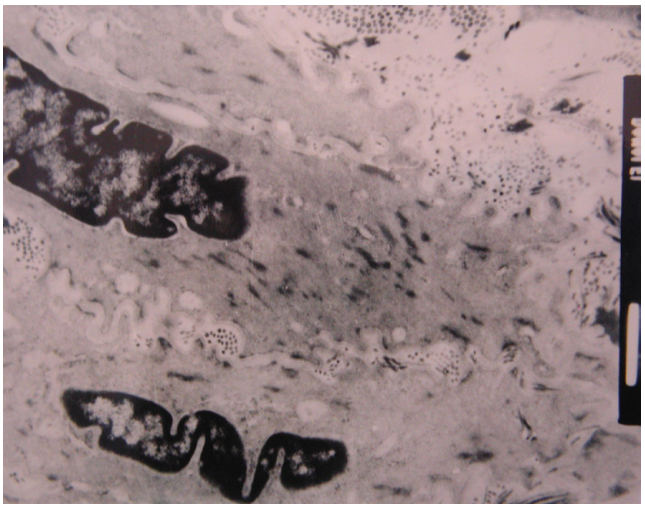

Figure 19: Electron micrograph of the meniscus of group II showing irregularly elongated chondrocytes with folded nuclei (X 7500).

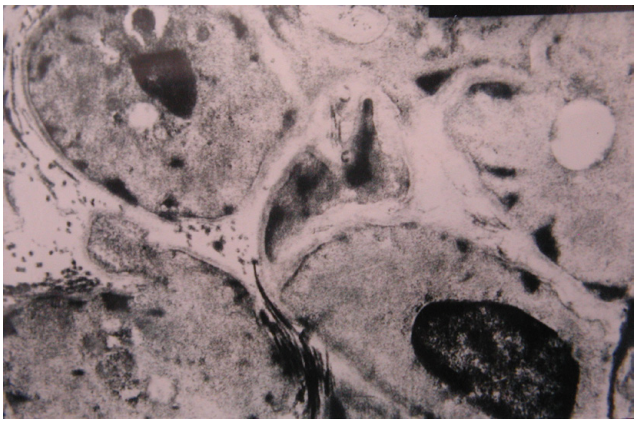

Figure 20: Electron micrograph of the meniscus of group III showing rounded chondrocytes nucleus with condensed chromatin and vacuoles (X 12000).

Figure 21: Electron micrograph of the meniscus of group III showing chondrocytes containing numerous lysosomes and vacuoles (X12.000).

Group	Meniscus Score	Synovium Score
Control group	3.42 ± 0.16 (P=0.0004)	2.25 ± 0.37
Hyaluronic acid group	2.62 ± 0.24 (P=0.006)	3.25 ± 0.31
Sham group	0.75 ± 0.15	6.42 ± 0.28

Table 1: Histological evaluation of the meniscal cartilage and synovial tissue.

Discussion

Knee instability following a complete tear of the ACL often induces OA. Animal studies are essential, as the cellular changes of osteoarthritis are almost impossible to study in the natural disease because the time of onset is not usually known; in addition, a control knee is available in the same animal, eliminating individual variation. The ACL transection model has proved a useful tool in the investigation of osteoarthritis development, because the pathological changes are identical to those seen in human osteoarthritis [14,19-20].

It is known that the presence of the intact functioning meniscus decreases the load between the femur and the tibia. Without the meniscus there would be high point loading between the femur and the tibia resulting in early failure of the articular cartilage [4]. Any injury to the meniscus may deprive the knee joint of this important functions. Consequently, the concept of injury and repair of the meniscus is extremely important.

The medial meniscus was selected in this work according to the finding of Hillo et al. [21] who found that the medial meniscus was more severely altered than the lateral meniscus after anterior cruciate ligament transection. This was explained by the fact that the medial meniscus was more restrained than the lateral meniscus particularly in the postero-medial corner [4]. The present study described dramatic histological alterations of the meniscal tissue in group II following ACL transection, including the irregularities in the chondrocytes and their nuclei. The morphology of the cells may reflect in some way the forces to which they are subjected [22]. Accordingly, these irregularities indicate that these cells were subjected to severe compressional loading after the anterior cruciate ligament transection.

The decreased intensity of safranine-O staining observed in group II; indicate decreased amounts of proteoglycans. This may be due to necrosis of chondrocytes which is responsible for proteoglycans synthesis. This was supported by what was observed by light microscopy in which there were some karyolytic and pyknotic nuclei of chondrocytes.

Moreover, by electron microscopy, the chondrocytes after ACL transaction appeared severely affected with decrease in their organelles responsible for the synthesis of matrix as RER and an increase in those organelles that have catabolic functions as lysosomes.

In the group III which was injected with sodium hyaluronate after ACLT, focal hyper-cellular areas in all specimens were observed. Mitrovic et al. [23] suggested that the focal accumulation of cells in clusters or clones will keep them from reaching the remote devitalized areas, moreover they are replacing damaged matrix. In addition, Dowd [6] explained that the observation of chondrocyte clusters in meniscus help in the production of matrix and he added that the two explanations for clustering of chondrocytes are either mitotic division of the cells producing a total greater number of cells or a redistribution of cells adjacent to the injury without a total increase in the number of cells.

The present study demonstrated significant improvement of the meniscal matrix in group III, as shown by the increased intensity of safranine-O staining. As reported, this is could be related to the increase in proteoglycan content and confirmed by the presence of chondrocytes containing numerous RER and ribososmes observed by electron microscopy. These cells are metabolically hyperactive and have increased capacity to synthesize cartilage matrix.

This result has been confirmed by similar findings observed by Guidolin et al. [10] who found that the number of viable chondrocytes in articular cartilage has significantly increased after hyaluronic acid (HA) treatment.

More interestingly, they also found by ultrastructure study that the cells had demonstrated significant improvement in their metabolism, with a shift towards more anabolic activity indicated by the increased extension of the synthetic network contradictory to the structure having catabolic or storage functions. In further supporting view, Yasui et al. [24] and Aihara et al. [25] previously reported that HA is known to inhibit the release of proteoglycan from articular cartilage tissues and delay the degeneration of cartilage.

The histological observations reported in this work showed that synovial stratification appeared more pronounced in the ACLT group and returned to almost normal structure in the sodium hyaluronate treated group. This result is in consistent with what was previously reported by Frizziero et al. [26] who found that the treatment by hyaluronate can lead to control of the synovial membrane inflammation in terms of infiltration of inflammatory cells and hyperplasia of the synovial lining. Furthermore, the increased blood vessels observed in the synovium after sodium hyaluronate injections, was explained by Dowd [6] who found that the vessels from the plexus around the periphery of the meniscus proliferated and allowed entrance to pluripotential mesenchymal cells which eventually produce a fibrous tissue.

He added also that the meniscal lesion which communicates with the peripheral blood supply has the potential to heal and has a reparative response similar to that in other areas of connective tissue.

It is well known that in pathological processes, such as osteoarthritis following ACLT, the molecular weight and the concentration of the hyaluronate in the synovial fluid can be reduced due to accumulation of liquid derived from the inflamed synovial vessels in the joint cavity. The result is a reduction in the viscoelasticity of the fluid and an increased susceptibility of cartilage to breakdown [27]. The improvement observed in meniscus and synovium after sodium hyaluronate injections was explained by many authors [28-30]. They suggested that the injection of exogenous hyaluronate stimulates the synovial cells (Type B) to synthesize endogenous hyaluronic acid of higher molecular weight [27,28]. This intrinsic hyaluronic acid is important for cell movement [31] and the regulation of extracellular matrix aggregation and synthesis [32].

The obtained results in this work are in consistent with previously reported clinical data which showed a reduction of several markers of cartilage breakdown and a decrease in extension and arthroscopic grading of the cartilage lesions following intraarticular treatment with hyaluronate [21,33].

So, it is advisable for the patients suffering from anterior cruciate ligament injury to recognize the nature of the injury, the need for modification of athletic activity, and the importance of lower extremity exercise.

As maintaining a sports activity level after anterior cruciate ligament injury leads to secondary meniscal injury, so, reconstruction of anterior cruciate ligament is widely advocated for the young active individual. From this study it appears that the non-operative management has a degree of success, and the intraarticular injection of sodium hyaluronate enhances meniscal regeneration, provides some chondroprotection, and postulates an anti-inflammatory effect on the knee joint.

Accordingly, Sodium hyaluronate may be useful in some diseases as a drug candidate for potential structure modification.

Conclusion

Anterior cruciate ligament and meniscal injuries may lead to early-onset post-traumatic osteoarthritis, regardless of whether patients had reconstruction performed. In younger patients, intra-articular injection of hyaluronic acid (HA) may be useful for improving short-term outcomes and possibly slowing or arresting the progression of OA.

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