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ORIGINAL ARTICLE
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Aquaporin-1 and Aquaporin-3 Density Decreases in Meniscal Tissue During Aging: An Experimental Animal Study

Yaşlanma Sırasında Menisküs Dokusunda Aquaporin-1 ve Aquaporin-3 Yoğunluğu Azalır: Deneysel Bir Hayvan Çalışması

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ABSTRACT

Aim: The menisci plays a crucial role in absorbing shock and providing stability thanks to their particular anatomy and physiology. Menisci exhibit histological, biochemical, and morphological degenerative changes during the process of aging. Aquaporins (AQPs) are membrane water channels that regulate the water contents of cells. We compared expression of aquaporin1 (AQP1), aquaporin3 (AQP3), and type I collagen in the meniscal tissues of young and aged rats using immunohistochemistry.

Material and Method: In this study, 14 Wistar albino rats (180-400 g) were used. Animals were divided into two equal groups; Group I: two-month old animals (n=7), Group II: 18-month-old animals (n=7). Meniscal tissues were dissected and examined histopathological and immunohistochemical. After routine histological procedure, sections of 4-5 µm thickness were obtained and embedded in paraffin. Sections were stained immunohistochemically for AQP1, AQP3, and type I collagen with hematoxylin-eosin.

Results: Immunohistochemically, expression of AQP1, AQP3, and type I collagen were demonstrated in young and old rats. In aged rats, the number of fibrochondrocytes was too few and cracks were too many compared to young rats. It was found that the immunoreactivity of AQP1, AQP3, and type I collagen in meniscal tissues were significantly reduced by aging.

Conclusion: Our results suggest that expression of AQP1, AQP3 and type I collagen in meniscus tissue may be related to age.

Keywords: Menisci, aging, aquaporin 1, aquaporin 3, type I collagen, immunohistochemistry

ÖZ

Amaç: Menisküsler, özel anatomileri ve fizyolojileri sayesinde şok emiliminde ve stabilitenin sağlanmasında önemli rol oynarlar. Menisküsler yaşlanma sürecinde histolojik, biyokimyasal ve morfolojik dejeneratif değişiklikler gösterir. Aquaporinler (AQP'ler) hücrelerin su içeriğini düzenleyen membran su kanallarıdır. Genç ve yaşlı sıçanların menisküs dokularında aquaporin1 (AQP1), aquaporin3 (AQP3) ve tip I kollajen ekspresyonunu immünohistokimya kullanarak karşılaştırdık.

Gereç ve Yöntem: Bu çalışmada 14 adet Wistar albino sıçan (180-400 g) kullanıldı. Hayvanlar iki eşit gruba ayrıldı; Grup I: iki aylık hayvanlar (n=7), Grup II: 18 aylık hayvanlar (n=7). Menisküs dokuları diseke edildi ve histopatolojik ve immünhistokimyasal olarak incelendi. Rutin histolojik prosedürün ardından 4-5 µm kalınlığında kesitler alındı ve parafine gömüldü. Kesitler immünhistokimyasal olarak AQP1, AQP3 ve tip I kollajen için hematoksilen-eozin ile boyandı.

Bulgular: İmmünohistokimyasal olarak, genç ve yaşlı sıçanlarda AQP1, AQP3 ve tip I kollajen ekspresyonu gösterildi. Yaşlı sıçanlarda, genç sıçanlara kıyasla fibrokondrosit sayısı çok az ve çatlaklar çok fazlaydı. Menisküs dokularında AQP1, AQP3 ve tip I kollajenin immünoreaktivitesinin yaşlanma ile önemli ölçüde azaldığı bulundu.

Sonuç: Menisküs dokusunda AQP1, AQP3 ve tip I kollajen ekspresyonunun yaşla ilişkili olabileceğini düşündürmektedir.

Anahtar Kelimeler: Menisküs, yaşlanma, aquaporin 1, aquaporin 3, tip I kollajen, immünohistokimya

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INTRODUCTION

The menisci are two oval (crescent shaped) fibrocartilages that rest medially and laterally between the tibial plateau and femoral condyles (1). These structures play a crucial role in load-bearing, shock absorption, stability and joint lubrication within the knee joint. Human meniscal tissue contains 72% water, 22% collagen, 0.8% glycosaminoglycans, and 0.12% DNA (2). Menisci modulate the complex biomechanics of the knee joint. This task becomes apparent when several conditions such as meniscal tears, partial and total meniscectomy, and meniscal degeneration lead to development or progression of knee osteoarthritis (OA) (3). Underlying mechanisms of pathophysiology in aging cartilaginous tissues have been recently studied with animal models.

Aquaporins (AQPs), now numbering 13 as known human AQPs, are members of a family of water channel proteins expressed throughout the human body. AQPs play a role in transporting water and small solutes in several kinds of tissues, they also act as water reserves depending on the type of the tissue (4). AQP1 is expressed all around the human body and can be selectively permeated by water. In addition to water, AQP3 transports glycerol and urea as well, therefore it is also named aquaglyceroporin. Mobasheri et al. demonstrated expression of AQP1 and 3 in equine articular chondrocytes using immunohistochemistry, western blotting and quantitative flow cytometry (5). It was shown that AQP1 has major roles in degeneration of intervertebral discs with aging (6). Li et al. showed that AQP3 expression decreased with increasing age in both skin and normal human epidermal keratinocytes (7). Osteoarthritis (OA) is a complicated disease which involves the entire synovial joint. OA of the knee can be seen in older adults, the menisci exhibit degenerative changes too, and these alterations can be presented as tears on imaging tests.

About 70% of the wet weight of normal articular cartilage is water. Water transfer in articular cartilage is essential for a healthy cartilage environment (8). It has been well known that the amount of water decreases in the body by the aging process. However, the expression pattern of AQP1 and AQP3 of meniscal tissue in aged rats is unclear. Therefore, we aimed to investigate whether there were changes in expression of AQP1, AQP3, and type I collagen in meniscal tissue by aging.

MATERIAL AND METHOD

Animal Selection

In this study, 14 female Wistar albino rats (180-400 g) were used. Animals were divided into two equal groups according to their ages. Two-month old rats created Group 1 (n=7), and 18-month old rats formed Group 2 (n=7). Before the procedure, rats were kept at room temperature (22±1°C) and 40-50% humidity. The light pattern was set as 12 hour day and 12 hour night. Eating and drinking was ad libitum. Physical examinations of rats were performed daily during the observation period, which lasted one week. This study was performed in an experimental research unit after the approval of University Local Ethics Committee (2014-HADYEK-49).

Sample Collection

Following anesthesia with ketamine/Xylazine (50/10 mg/kg), the animals were sacrificed via exsanguination. For immunohistochemical and histopathological examinations, the menisci were removed and fixed in 10% formalin solution. Following routine histological procedures, tissues were then embedded in paraffin.

Histological examination

After routine histologic follow-ups, meniscus tissues were embedded into paraffin blocks. 4-5 µm thick sections were taken from paraffin-embedded tissues and stained with hematoxylin-eosin (H&E) method. The stained sections were examined under a light microscope (Zeiss Axio Lab A1).

Immunohistochemistry

Immunohistochemical staining was performed according to previously described protocol with minor modification (5). 4-5 µm thick-serial sections taken from paraffin blocks were placed on polylysine coated microscope slides (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 56°C. Tissue sections were deparaffinized in xylene and rehydrated by passing through graded alcohol series. Then they were taken into distilled water and boiled in citrate buffer solution pH:6 in a microwave oven (600W) for 5 minutes for antigen retrieval. It was treated with H_2O_2 to prevent endogenous peroxidase activity. In order to prevent base staining, after treating with Ultra V Block (Ultra V Block, TA-125-UB, Thermo Fisher Scientific Inc., USA) solution, it was incubated with primary antibody (Aquaporin 1 rabbit polyclonal IgG, Abcam, ab–15080, California, USA; Aquaporin 3 mouse monoclonal IgG, Abcam, ab–125219, Camridge, UK; Anti-collagen I antibody, Abcam, ab6308, California, USA) for 60 minutes. After primary antibody application, secondary antibody (biotinylated anti-mouse IgG, Diagnostic BioSystems, KP 50A, Pleasanton, USA) was applied for 30 minutes, streptavidin horseradish peroxidase was performed for 30 minutes and 3-Amino–9-ethyl carbazole (AEC) chromogen was applied and then counterstaining was performed with Mayer's hematoxylin. For the tissues prepared for negative control, phosphate buffered saline (PBS) was used instead of primer antibody and other steps were performed similarly. The tissues that were passed through PBS and distilled water were closed with an appropriate solution. The preparations were examined, evaluated and photographed under a research microscope (Zeiss Axio Lab A1).

Evaluation immunohistochemistry

Evaluation of the AQP1 and AQP3 immunohistochemical labeling was performed using H-SCORE analyses as previously described protocol (9). Immunohistochemical labeling of the menisci tissues were semi-quantitatively evaluated using the following categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining). For each tissue, an H-SCORE value was derived as follows. First, the sum of the percentages of cells that stained at each intensity category was calculated, and then, that value was multiplied by the weighted intensity of the staining using the following formula: H-SCORE = ∑Pi (i+ l). In this formula, 'i' represents the intensity scores, and 'Pi' is the corresponding percentage of the cells. Five randomly selected areas were evaluated under a light microscope on each slide (40x objectives). Two investigators, who were not informed about the type and source of the tissues, determined the percentage of cells for each intensity within these areas at different times. The combined average score of both observers was used.

Type I collagen expression was evaluated immunohistochemically according to the method previously described by Kanter et al. (10). In this method, density of immuno-reactivity was evaluated at two sections for each animal, at 400x magnification, and using eight fields at each section. The evaluation was made according to the following classifications: absence (-), a few (+), medium (++), high (+++), and very high (++++).

Statistical analysis

Statistical analyzes was performed using IBM-SPSS 20 program. Data are presented as mean±standard deviation (SD). Independent-sample t test was used for comparing groups in terms of H-score values. $p<0.05$ was considered statistically significant.

RESULTS

Histological findings

The meniscal tissue from young and aged rats were stained with hematoxylin-eosin (HE) and examined by light microscopy (**Figure 1A** and **Figure 1B**). Meniscal tissue of young rats didn't show any pathological findings in meniscal tissue, although meniscal tissue of aged rats represented mild decrease in number of fibrochondrocytes and high number of cracks.

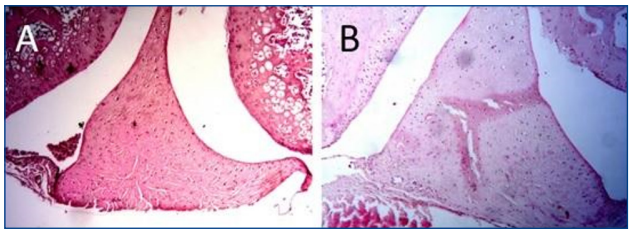

Figure 1. A) Hematoxylin and eosin (H&E) staining x10 of meniscal tissue of young rats. B) Hematoxylin and eosin (H&E) staining x10 of meniscal tissue of aged rats.

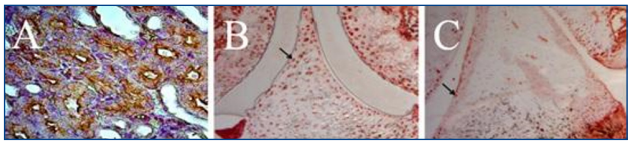

Figure 2. A) AQP1 positive control tissue staining section in the rat menisci. B) AQP1x10 meniscal tissue section in young rats. C) AQP1x10 meniscal tissue section in elderly rats. The tip of the arrows indicates the positive fibrochondrocytes stained with AQP1 antibody.

Immunohistochemical findings

Immunohistochemistry was used to show the localization of AQP1 and AQP3 in young and aged rats shown in **Figure 3** and **Figure 4**.

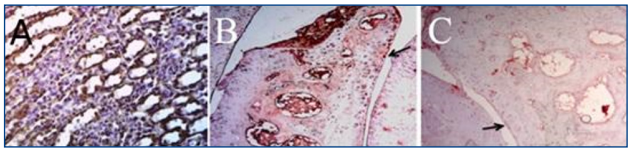

Figure 3. A) AQP3 positive control tissue staining section in the rat menisci. B) AQP3 x10 meniscal tissue section in young rats. C) AQP3 x10 meniscal tissue section in elderly rats. The tip of the arrows indicates the positive fibrochondrocytes stained with AQP3 antibody.

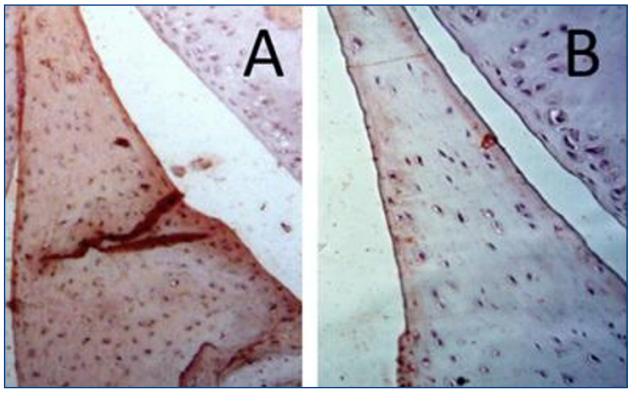

Figure 4. Immunohistochemical staining for type I collagen in meniscus tissues of rats x40. A) Type I collagen immunoreactivity in young rats. Arrow points to immunopositive staining. B) Type I collagen immunoreactivity in elderly rats. Arrow points to immunopositive staining.

H-SCORE analysis revealed that the staining intensity and the number of cells positively stained for AQP1 and AQP3 in menisci significantly decreased in aged rats. H score values presented in **Figure 5**.

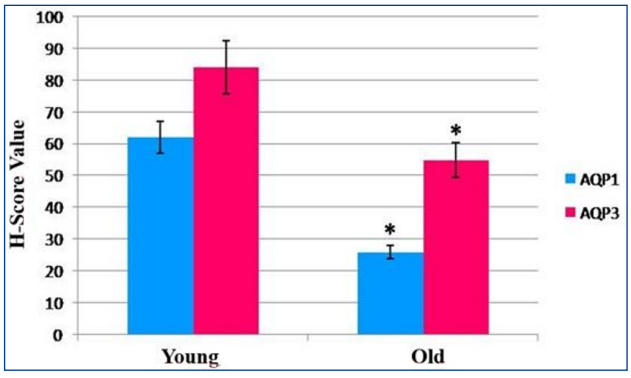

Figure 5. H-score values of AQP1 and AQP3 in young and elderly rats. The data are represented as mean±SD. *p< 0.0001, #p=0.001 old rats versus young rats.

Positive control staining for AQP1 and AQP3 have been made (**Figure 2A – 3A**). When young and aged rats were compared, the AQP1 and AQP3 expression in the meniscal tissue were significantly lower in aged rats ($p < 0.001$) (**Figure 2B-C**, **3B-C**, **5**). In addition, the meniscal tissue of aged rats showed significantly decreased type I collagen immunoreactivity (+) ($p < 0.001$), whereas increased type I collagen immunoreactivity in young rats (+++) was observed (**Figure 4**).

DISCUSSION

Main functions of the menisci include buffering axial, rotational and shearing forces and protecting the cartilage (11). Meniscal cells are usually regarded as a cross between chondrocytes and fibroblasts, known as fibrochondrocytes. Therefore they display features of both fibroblasts and chondrocytes. These cells are essential for tissue homeostasis because they produce, maintain and/or degrade the extracellular matrix, a primarily composed type I collagen. Proteoglycans, elastin, water and other collagen types constitute the other matrix components (12). The high hydration rate and strong permeability of the meniscal tissue enables the transportation of nutritions and metabolic substances.

Aging is a physiologic process that continues lifelong and can lead to progressive changes in adaptation response to stress and functions of organs and systems. Aging causes degeneration of meniscal tissue and decreases the number of fibrochondrocytes (11,13). OA is a progressive disease involving all joint structures such as bone, cartilage, meniscus and synovial fluid. Underlying pathophysiology of the disease involves imbalance between production and breakdown of matrix components in the articular cartilage. This eventually leads to ongoing destruction of the tissue. At cellular and tissue level, cartilage in OA exhibits a lack of balance in matrix biosynthesis and degeneration. Aging is the most frequent risk factor of OA. Meniscal degeneration contributes to the development or progression of knee OA. During the aging process, the menisci degenerate at micro and macro levels and this leads to pain and dysfunction in the knee joint.

AQPs are widely expressed in the human body, especially in cell types in which fluid transport occurs, including epithelial cells in many organs. However it is also expressed in some cell types that do not involve fluid transport, such as adipocytes (14). AQP1 is permeable to water and O_2. This inhibits rapid volume deformation under osmotic pressure and facilitates O_2 diffusion across the plasma membrane(6).AQP1 in chondrocytes favors a role for AQP1-mediated water transport across the synovial small vessels and the plasma membrane of chondrocytes in load-bearing joints(15). AQP3 plays a role as a water channel to facilitate glycerol permeability and water transport across cell membranes (16).AQP3 is also highly expressed in several tissues such as renal, tracheal and bronchial and epithelial tissues, as well as choroid plexus, articular chondrocytes, subchondral osteoblasts and synovial epithelium in bone/cartilage tissue, expression of three distinct AQPs has been described: AQP1 and AQP3 in equine articular chondrocytes (5). AQP1 in human articular chondrocytes (17), and AQP9 in mouse osteoclast cells (18). Liang et al. reported that AQP1 expression in mouse articular chondrocytes results in high plasma membrane water permeability and increases chondrocyte migration and adhesion (19). Using AQP1 knock-out mice, this study shows for the first time that an AQP water channel in chondrocytes has a functional role. In many other cell types, AQP1 enhances cell migration, probably through its polarized distribution in lamellipodium and its co-localization with ion channels including Na+/H+, Cl-/HCO3; and Na4/HCO3-. Fast fluid transfer through AQP water channels driven by ion transport increases lamellipodium movement, and this in turn leads to cell migration (20).

Lack of AQP3 is related with impaired corneal (21), and cutaneous wound (22) healing in mice, and with improper colonic epithelial cell proliferation in a mouse model of colitis (23). Immune cells express AQP3, and its deficiency in these cells in mice weakens the function of macrophages (24),and T cells (25).Since AQP3 has various roles, including both beneficial and deleterious, it would be very difficult to establish a feasible therapeutic window for an AQP3 modulator (26).

It has been known for a long time that water content decreases in the body with the aging process. In meniscal tissue, aging causes reduction of collagen tissue, fibrochondrocytes and water (27,28). It was reported that AQP1 expressed extensively in the body. It was shown that the expression of AQP3 decreases the degeneration tissue of the human lumbar disc (29). Tas et al. reported that AQP1 and AQP3 expression significantly decreased the nucleus pulposus and annulus fibrosus in aged rats compared to young rats (30). Kyung et al stated the relation between AQPs and extracellular matrix quality of knee hyaline cartilage (31). On the contrary, information about presence of AQP3 in meniscal tissue is limited. Besides, the effects of AQP1 and AQP3 on the pathogenesis of meniscal degeneration still remain unclear. Therefore in the present study, we aimed to evaluate whether AQP1 and 3 expression changes in the aging process. According to our knowledge, this study is the first in analyzing the AQP1 and AQP3 expression in meniscal tissue with aging.

Our data revealed that AQP3 expression exists in meniscal tissue. In addition, the expression of AQP1, AQP3, and type 1 collagen decreased significantly in aged rats compared to young rats.

CONCLUSION

Meniscal degeneration increases with aging. The decreased expression of AQP1 and AQP3 in meniscal tissue as a result of aging supported the fact that AQPs play an important role in meniscal degeneration and these may be initial factors in knee degeneration. These findings should be supported with further studies using different methods. Advances in research on therapeutic agents that regulate water permeation through AQPs have been helpful in treating the meniscal diseases.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Gaziosmanpaşa University Animal Experiments Ethics Committee (Decision No: 2014-HADYEK-49).

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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