HISTOCHEMICAL STUDY OF ACID MUCINS IN OSTEOARTHRITIC MENISCI OF THE HUMAN KNEE JOINT

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ABSTRACT

Knowledge regarding changes of proteoglycans (acid mucins) in human osteoarthritis (OA) meniscus may help in understanding development of meniscal degeneration. Therefore, present study was planned to know changes in acid mucins in human knee OA menisci by histochemical analysis of different parts of medial and lateral menisci of both legs. Medial and lateral OA menisci were collected from 110 human knee joints of both sexes. Normal meniscal tissue of sheep was taken as control and studied for histological stain with alcian blue pH 2.5, to find acid mucins changes in OA menisci. Data were analyzed by bivariate and one-way ANOVA using MS-Excel. Osteoarthritis is more common in females than males. OA changes were found to be more on right side in females and on left side in males, while OA was more common in both legs in number of cases in 60-69 years. Further, decreased staining intensity for acid mucins was observed in different parts of medial and lateral OA menisci of both legs than control meniscus. A significant change in level of acid mucin was observed at anterior, middle, and posterior parts of medial and lateral OA menisci of both legs (P-value=0.0306). Significant changes in acid mucins in human OA meniscus provide information on scientific evidence of OA progression, which could help health professionals in development of structure-modifying drugs for OA therapy.

KEYWORDS

Histochemical, proteoglycans (acid mucins), osteoarthritic, menisci, human knee joint

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INTRODUCTION

The knee meniscus is a specialized tissue that plays an important role in power transmission, shock absorption, and joint stability, and contributes to joint lubrication.[1, 2] osteoarthritis (OA) is a progressively disabling disease caused by a pathological imbalance between degenerative and repair processes. Patients with knee meniscus injuries are at high risk of developing the disease,[3] up to 91% of patients with symptomatic knee OA have concurrent meniscal tears,[4] and it is one of the strongest risk factors for the development and progression of knee OA.[5] The probability of horizontal meniscus tear was 63% in patients with imaging evidence of OA, but only 23% in patients without imaging evidence of OA.[6] Multiple MRI (magnetic resonance imaging) studies have shown that meniscal degeneration is a common feature of OA, and meniscal degeneration is an important risk factor for the development of OA.[7-9] Consistent with the role of the meniscus in knee function, meniscal injuries are common in athletes and the general population. The complex role of meniscal tissue components in the etiology of the subsequent development of knee OA is not fully understood, and it is increasingly clear that the meniscus plays a critical role in the long-term health of the knee. To study meniscal degeneration during the development of knee OA and to prevent its progression, changes in meniscal tissue composition must be detected before gross morphological changes occur.[10,11]

Proteoglycans and other non-collagen proteins play an important role in stabilizing the extracellular network and are therefore important in maintaining the structural integrity and mechanical properties of the meniscus. Cartilage oligomeric matrix protein (COMP) is a component of the cartilage matrix and plays an important role in the construction of the extracellular matrix.[12-16] Procollagen I/II and chondrocytes, bound to collagen types I, II, and IX, play a role in the storage and release of hydrophobic hormones, and calcium-binding proteins.[17-19] The chemical composition of the meniscus also varies by different region, with predominantly type I collagen in the outer, more fibrous, area and a mixture of type I and type II collagens in the inner, more cartilaginous area.[20] The greater part of the remaining extracellular matrix (ECM) is composed of negatively charged glycosaminoglycans (GAGs),[21] which hydrate the tissue, contribute to its compressive properties, and also enable electrical activity.[22] Later a meniscus injury, an increase in GAG levels in the synovial fluid peak early and persist for four years after injury.[23] Mucins are called mucopolysaccharides, glycosaminoglycans, and mucosubstance. More recently the term glycoconjugates have been divided into proteoglycans and glycoproteins.[24]

Proteoglycans (mucoproteins) are formed from glycosaminoglycans (GAGs) covalently linked to core proteins. Mucopolysaccharides or glycosaminoglycans are long, unbranched polysaccharides composed of repeating disaccharide units. The repeating unit (except keratan) consists of amino sugars (N-acetyl glucosamine or N- acetylgalactosamine) with a uronic sugar (glucuronic acid or iduronic acid) or galactose. Mucopolysaccharides are highly polar and absorb water. Therefore they can be useful as lubricants or shock absorbers for the body. The highly negatively charged GAG chains allow the proteoglycans to seize water and divalent cations and confer space-filling and lubricating functions.[25,26]

Meniscal degeneration in OA has been extensively studied. It has been shown that there was a much less organized network of collagen in OA menisci compared to early OA, and that collagen content was reduced in advanced OA.[27,28] However, the literature review indicates that previous studies on the OA menisci were performed using animal models, and there were few previous studies on proteoglycans changes in human OA menisci. Therefore, the chemical changes in the OA menisci are likely to be localized to different regions.[29] So the present study was undertaken to know the acid mucins (proteoglycan) changes in human OA menisci in different parts with a large sample size to have an understanding of the degenerative disease process.

MATERIALS AND METHODS

Medial and lateral osteoarthritic menisci were collected from 110 human knee joints of both sexes. The design of the study was hospital based cross-sectional study, and the sample size was calculated by the below-mentioned formula;

$$n = \frac{Z^2_{1-\alpha/2} \times SD^2}{(0.2 \times SD)^2} \times 1.1 = 110$$

Where,

$Z_{1-\alpha}$ = 1.96 with 95% C.I

SD = Standard Deviation

d = Tolerable error

Sample size at 95% CI, 20% error, and 10% lost to data entry/data collection or outliers.

Menisci were collected from 65 females and 45 males, aged 50-84 years. These menisci specimens were collected from consecutive osteoarthritis (OA) patients who had undergone total knee joint replacement surgery, and lower limb amputation surgery from the Orthopedics department unit of KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi, Karnataka, India. Patients were excluded in case of any malignancy in the menisci or torn menisci and injuries to the menisci. Meniscal specimens were collected intraoperatively during total knee replacement (TKR) surgery. Normal meniscal tissue from 8 months old (n=1) male domesticated (cud-chewing) ruminant sheep (ovine) average weight 13 kg was used in this study. Sheep meniscal tissue was used as a control and showed no signs of knee-related musculoskeletal disease. It was obtained from a commercial source at Nehru Nagar in Belagavi and dissected after 4 hours of defeat.[30] Meniscal samples were stored in 10% formalin for 3 to 5 days. After that menisci were cut in a standardized way. For each meniscus, three separate parts (anterior, middle, and posterior) were processed. The menisci were sectioned in two places vertically at 45º and 135º angles relative to the sagittal plane. After that, each part was sectioned along the horizontal plane from the inner border to the outer border. Then tissue of medial and lateral meniscal samples was fixed in 10% buffered formalin for 24 hours. Tissue samples were brought in for routine tissue processing and studied for histological stain with a color intensity of Alcian blue pH 2.5.

Ethical consideration: Prior approval was taken from the institutional ethical committee (ref. no. KLEU/EC/17-18/D-97; Dated 16/5/2017). Informed written consent was obtained from the participants before initiating the data collection process. Privacy and confidentiality of information were maintained and informed about their freedom of choice.

Histological processing and staining: Tissue samples of the medial and lateral menisci were taken and routinely processed in the histology laboratory. After fixation, dehydrated with graded alcohol, then cleared with xylene and infiltrated with paraffin. Tissue was embedded with paraffin and blocks were prepared. After that using the rotatory microtome, 5 µm sections were cut from each different parts of the medial and lateral menisci and stained with alcian blue pH 2.5, and evaluated for proteoglycan (acid mucins).

Development of histological scoring/grading system: The scoring system reported in this study was developed after reviewing slides from patients of different ages with OA. For microscopic evaluation of meniscus, the staining color intensity of Alcian blue pH 2.5 for acid mucins was graded as a negative stain (- : 0%), weak or variable stain (± : <25%), slight or mild stain (+ : 26-50%), moderate stain (+ + : 51-75%), and strong stain (+ + +: 76-100%).[31,32] All images were captured with an Olympus BX-41 microscope equipped with Graphix software elements. (U-TV1X-2) T7 Tokyo, Japan. The histochemical grading of the meniscal OA was done by a Pathologist of KAHER's, JN Medical College, Belagavi.

Statistical analysis: Descriptive statistics were used to generate histochemical scores and distributions to summarize negative, weak, mild, moderate, and severe acid mucins in meniscal osteoarthritis. Further chi-square test was used to see the association between AB-2.5 Staining Intensity of Acid Mucins and sides of osteoarthritis menisci. One-way ANOVA (F-test) was applied to test various parts of Alcian Blue 2.5 Staining Intensity of Acid mucins in medial and lateral OA menisci of both left and right legs. Also, significance was seen at 5% level. Analyses were performed using MS Excel.

RESULTS

Meniscal samples were taken from 110 patients of different ages with OA in this study. In that 65 (59%) females and 45 (41%), males participated (Fig. 2). Among them 56 (51 %) have left leg OA and 54 (49 %) a right leg OA (Fig. 1). Further, 43% of females have left leg OA and 57 % have a right leg. On the other hand, 62 % and 38 % of males got left and right leg OA respectively (Fig. 3). In this study, the patient's sample was taken from the age group 50-84 years and minimum number of cases of osteoarthritis were found in

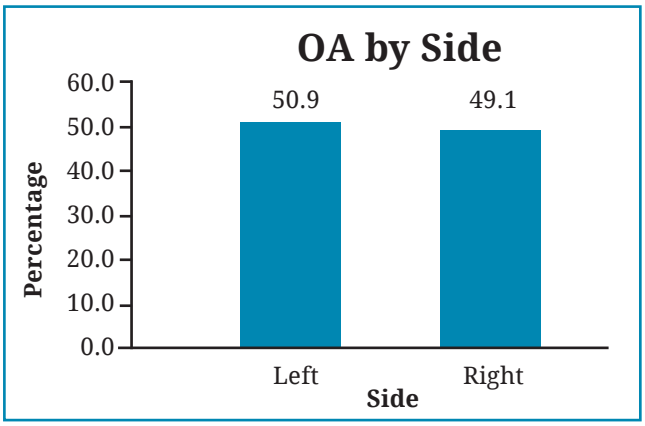


Fig. 1: Osteoarthritis (OA) menisci by side of legs

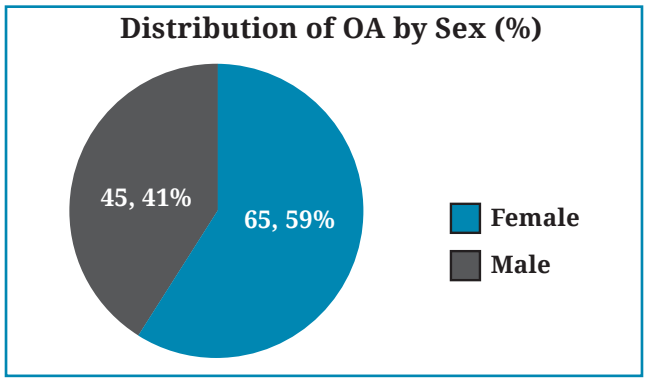


Fig. 2: Distribution of osteoarthritis menisci by sex

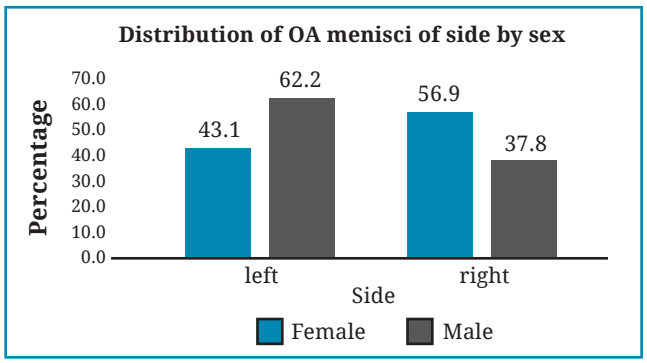


Fig. 3: Distribution of OA menisci of side by sex

50 – 59 and 70+ years, whereas OA cases were higher in the age group 60 - 69 years in both left and right legs of knee joint (Fig. 4).

Fig. 1 shows the distribution of Osteoarthritis (OA) menisci percentage on the side of the legs. Approximately 51 % of people have left leg OA and 49 % in the right legs. Among 110 OA patients, about 59% and 41% of OA problems are in females and males respectively (Fig. 2).

Fig. 3 shows the distribution of OA menisci of side by sex. 43% of females have left leg OA whereas 57% have a right leg OA. On the other hand, 62% and 38% of males got left and right leg OA respectively.

Osteoarthritis menisci are varying by age and side, as shown in Fig. 4. The minimum number of cases of osteoarthritis was found in 50–59 and 70+ years, whereas OA cases were higher in the age group 60-69 years in both left and right legs of knee joint.

Fig. 5a shows assessment of histological staining intensity of alcian blue pH 2.5 in the extracellular matrix of sheep meniscus used as control group at 3 regions (anterior, middle, posterior) of medial and lateral menisci of right leg: RMA, RMM, RMP, RLA, RLM and RLP showed strong staining with score (+ + +).

Fig. 5b shows an assessment of histological staining intensity of alcian blue pH 2.5 in the extracellular matrix of OA human meniscus used as a test group at 3 regions (anterior, middle, posterior) of medial and lateral menisci of both legs: figure 'A' score: negative (-), figure 'B' score: weak or variable stain (±), figure 'C' score: Slight or mild stain (+) and figure 'D' score: moderate stain (+ +).

Table 1a reveals a histochemical assessment of alcian blue pH 2.5 staining intensity level in the extracellular matrix of medial menisci of left and right legs. The study showed strong staining intensity of alcian blue pH 2.5 in the control and its score of three plus (+ + +) acid mucins. It is present in both legs of the medial and lateral menisci of sheep. The observation made in the left leg medial meniscus anterior part (LMA) was 1.8% negative, 51.8% weak, and 46.4% mild staining intensity of AB-2.5 while, it was 1.8% negative, 46.4% weak, and 51.8% mild in the left leg medial meniscus middle part (LMM). Similarly, the staining intensity in the left leg medial meniscus posterior part (LMP)

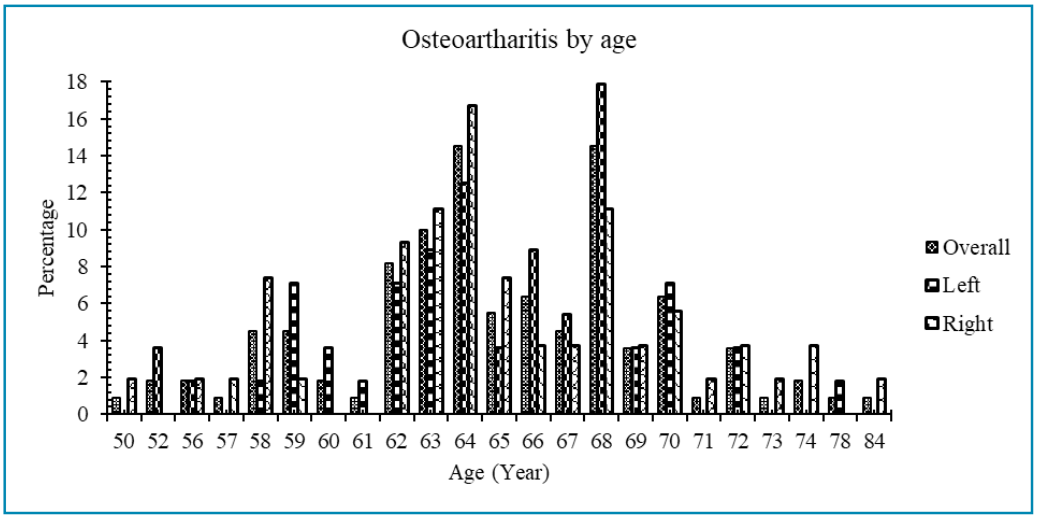
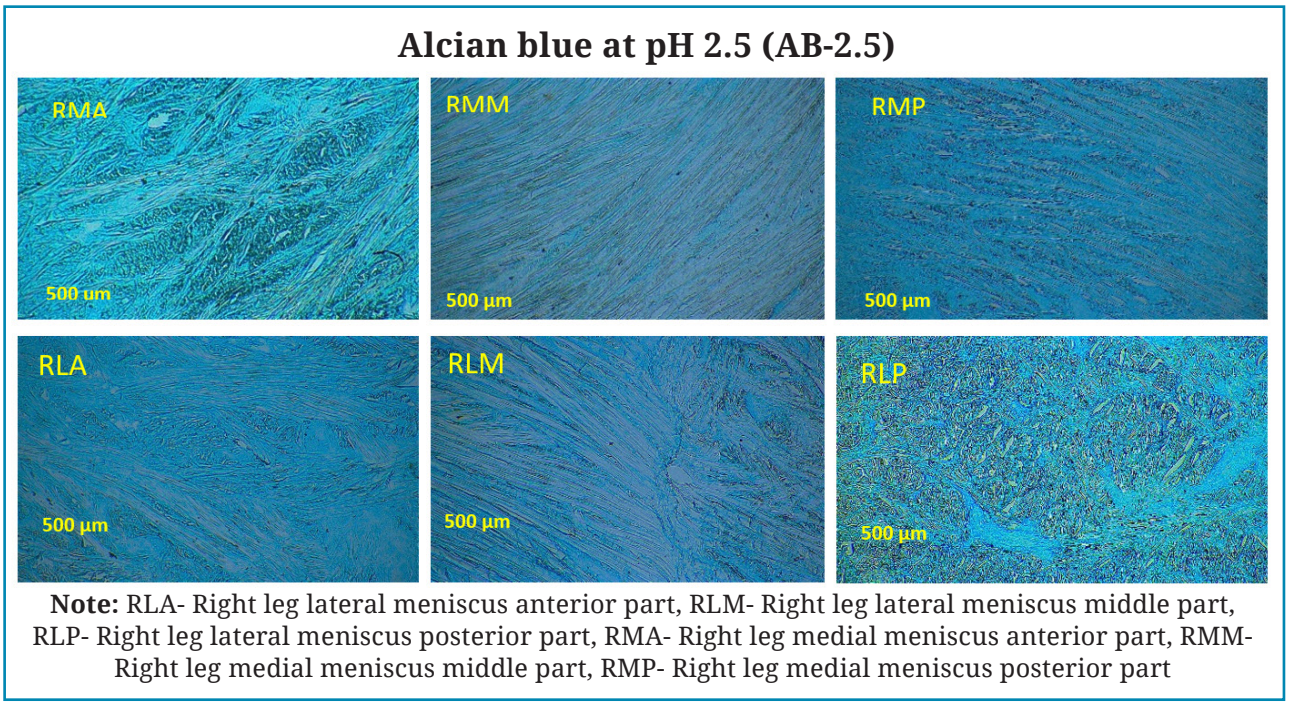


Fig. 4: Osteoarthritis (OA) menisci by age and side of legs



Note: RLA- Right leg lateral meniscus anterior part, RLM- Right leg lateral meniscus middle part, RLP- Right leg lateral meniscus posterior part, RMA- Right leg medial meniscus anterior part, RMM- Right leg medial meniscus middle part, RMP- Right leg medial meniscus posterior part

Fig. 5a. The intensity of acid mucins in control group: magnification 10x

Table 1a: Alcian blue pH 2.5 Staining Intensity of Acid Mucins in Medial Meniscus of left and right legs.

Alcian blue 2.5 staining intensity of acid mucins		Left leg		Right leg		Overall	
		n1	%	n2	%	n	%
Control (normal menisci)	Strong (+ + +)	1	100	1	100		100
Test (OA menisci)							
Medial anterior	Negative (-)	1	1.8	2	3.7	3	2.7
	*Weak (±)	29	51.8	27	50	56	50.9
	#Mild (+)	26	46.4	25	46.3	51	46.4
Medial middle	Negative (-)	1	1.8	0	0	1	0.9
	*Weak (±)	26	46.4	25	46.3	51	46.4
	#Mild (+)	29	51.8	29	53.7	58	52.7
Medial posterior	Negative (-)	1	1.8	1	1.9	2	1.8
	*Weak (±)	27	48.2	33	61.1	60	54.5
	#Mild (+)	25	44.6	20	37	45	40.9
	Moderate (++)	3	5.4	0	0	3	2.7

Note: - : - : 0 %, ±: < 25 %, +: 26 -50 %, ++: 51-75 %, +++: 76 -100 % acid Mucins, *,F= 20.35 (Weak), p=0.037 #; F=37.44 (Mild), p= 0.030, n1+n2=n

was 1.8% negative, 48.2% weak, 44.6% mild, and 5.4% moderate. Right leg medial meniscus anterior part (RMA) was 3.7% negative, 50% weak, and 46.3% mild staining intensity while, it was 46.3% weak, and 53.7% mild staining intensity in RMM. Similarly, the staining intensity in RMP was 1.9% negative, 61.1% weak, and 37% mild. Staining intensity in the medial OA menisci is significantly varying by parts ($p < 0.05$).

Table 1b. the result made in left leg lateral meniscus anterior part (LLA) had 64.3% weak, and 35.7% mild staining intensity of AB-2.5 while, it was 5.4% negative, 53.6% weak, and 41.1% mild in the left leg lateral meniscus middle part (LLM). Similarly, staining intensity in the left leg lateral meniscus posterior part (LLP) was 1.8% negative, 66.1% weak, and 32.1% mild. The right leg lateral meniscus anterior part (RLA) showed 3.7% negative, 51.9% weak, and 44.4% mild staining intensity.

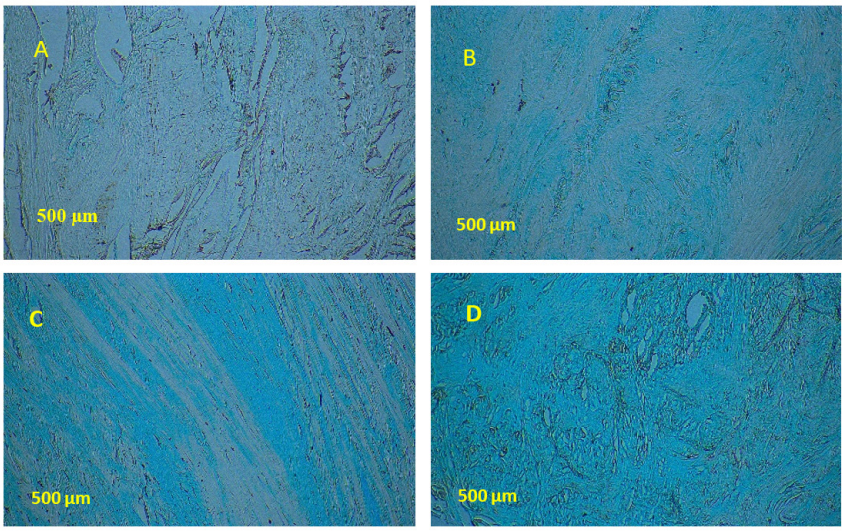


Fig. 5b: The intensity of Acid Mucins in the test group: magnification 10x

Table 1b: Alcian blue pH 2.5 Staining Intensity of Acid mucins in Lateral Meniscus of left and right legs.

Alcian Blue 2.5 Staining Intensity of Acid mucins		Left leg		Right leg		Overall	
		n1	**%**	**n2**	**%**	**n**	**%**
Control (Normal menisci)	**Strong (+ + +)**	**1**	**100**	**1**	**100**		**100**
Test (OA menisci)							
Lateral Anterior	Negative (-)	0	0	2	3.7	2	1.8
	*Weak (±)	36	64.3	28	51.9	64	58.2
	#Mild (+)	20	35.7	24	44.4	44	40
Lateral Middle	Negative (-)	3	5.4	2	3.7	5	4.5
	*Weak (±)	30	53.6	36	66.7	66	60
	#Mild (+)	23	41.1	16	29.6	39	35.5
Lateral Posterior	Negative (-)	1	1.8	1	1.9	2	1.8
	*Weak (±)	37	66.1	30	55.6	67	60.9
	#Mild (+)	18	32.1	23	42.6	41	37.3

Note: - : - ; 0 %, ±: <25 %, +: 26-50 %, ++: 51-75 %, +++: 76 -100 % acid Mucins, *F= 3.38 (Weak),p=0.048 # F= 3.80 (Mild); p = 0.0436, n1+n2=n

Table 2: Association of AB-2.5 intensity of Acid Mucins – medial and lateral menisci with side of the legs

AB-2.5 Staining Intensity of Acid Mucins		**Side of legs**				**Chi-square (df), P-value**
		Left		**Right**		
		n	**%**	**n**	**%**	
Medial menisci	Weak	27	48.2	26	48.1	3.058 (2), 0.217
	Mild	26	46.4	28	51.9	
	Moderate	3	5.4	0	0	
Lateral menisci	Weak	36	64.3	33	61.1	0.119 (1), 0.731
	Mild	20	35.7	21	38.9	

While, it was negative 3.7%, weak 66.7%, and mild 29.6% staining intensity in RLM. Similarly, the staining intensity in RLP was 1.9% negative, 55.6% weak, and 42.6% mild. Staining Intensity in lateral OA menisci significantly ($p < 0.05$) varied by parts.

Table 2 stated the association in the medial and lateral OA meniscus of AB-2.5 intensity of acid mucins on both sides of the legs. Medial menisci of left and right leg have 46.4 and 51.9% respectively mild intensity of acid mucins. However, lateral menisci of the left and right leg have respectively 35.7 and 38.9% mild intensity of acid mucins. Moreover, the sides of the legs do not show a significant association between the medial and lateral meniscus.

DISCUSSION

The knee menisci are specialized tissues that play a vital role in load transmission, shock absorption, and joint stability.[1] In accordance with the role of menisci play in knee joint function, meniscal injuries are common in athletes and the general population. The complex role of meniscal tissue composition in the etiology of meniscal tears and the subsequent development of knee OA is not entirely clear.[5,10] The ability to perform these mechanical functions is based on their cellular and chemical composition and, perhaps more importantly, on the organization and interactions of their constituents.[2] Mucopolysaccharides or Proteoglycans and other non-collagenous proteins play an important role in stabilizing the extracellular meshwork and therefore, are very important for the maintenance of the structural integrity and mechanical properties of the menisci.

In this study, for comparison purposes, sheep's normal meniscal tissue was taken as control of three different parts (anterior, middle, and posterior) of the medial and lateral menisci of both legs because gait analyses in this model have demonstrated a similar pattern of hind limb loading to humans, and post-surgical GRF (Ground reaction forces) changes comparable to OA patients.[33,34] Another study compared several viscoelastic properties of the bovine, ovine, and porcine menisci biomechanically with the human meniscus and reported that the ovine (sheep) model showed the greatest resemblance to the human meniscus. In addition, few studies compared ovine and rabbit menisci with human menisci using histology and scanning electron microscopy in terms of vascularization pattern, cell density, and extracellular matrix collagen and reported that sheep (ovine) menisci have greater structural similarity to the ultrastructure.[35,36]

In this study, decreased acid mucins (proteoglycan) staining intensity was observed in the various parts (anterior, middle, and posterior) of the medial and lateral menisci of both legs compared with normal control menisci. However, in other studies, normal meniscal tissue showed a predominance of acid mucins (80%) and sparse neutral mucins (20%), suggesting that the menisci are rich in acid mucins and play a major role in viscosity. About 80% of the total GAGs in the meniscus were identified as sulfated. Normal human meniscal proteoglycans contain approximately 40% chondroitin 6-sulfate, 10-20% chondroitin 4-sulfate, 20-30% dermatan sulfate, and 15% keratan sulfate.[37-39]

Severe collagen and proteoglycan loss occurred in OA cartilage, signifying that collagen and proteoglycan are more actively involved in the degenerative process and development of OA. The findings of Videman *et al*[40] on proteoglycan content (1979). reported that proteoglycan content in the menisci was increased after limb immobilization-induced OA in rabbits. Djurasovic *et al*[41] reported a decrease in proteoglycan content in the menisci of adult beagle dogs after OA was induced by limb immobilization. Adams *et al*[42] reported that proteoglycan content in the menisci decreased during the first trimester but gradually increased in the following months after induction of OA.

Peters and Smillie[43] reported elevated proteoglycan levels in the portion of the meniscus with degenerative tears in patients with meniscal injuries.[43] Herwig *et al*[39] reported that in patients with meniscal lesions, the proteoglycan content (µg/mg dry weight) in the meniscus increased with the severity of meniscal degeneration. In a study using human meniscus specimens obtained from OA patients, Ghosh *et al*[44] found increased proteoglycan content in the degenerative region of the OA meniscus compared to normal control menisci. Another study showing proteoglycan changes in human menisci by Alcian blue staining (pH 2.5) observed acidic mucin (proteoglycan) and a tendency to degenerate the meniscus due to reduced acidic mucin. Decreased matrix proteoglycans in degenerative human menisci due to decreased synthesis and development of OA.[32]

Osteoarthritis in the left leg was higher than in the right leg. Women are more likely to develop OA than men. OA changes were more

common in women on the right leg and men on the left leg, OA cases were higher in the age groups from 60-69 years in both left and right legs. The control group showed strong acid mucins staining intensity. Whereas, in the test group, the meniscus showed varying degrees of reduced (moderate to negative) staining intensity levels. Histochemical analysis of acid mucins staining intensity of the medial and lateral meniscus of the left and right legs showed a reduction compared to the control meniscus. The staining intensity of the medial and lateral menisci showed significant [$p<0.05$ (F-test)] differences in different parts of the OA meniscus in both legs. The changes of the acid mucins in human OA menisci provide information on the scientific indications of the progressive process of OA. Therefore, this study could help health professionals in the development of structure-modifying drugs for OA therapy.

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