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Changes in Composition of ADAMTS-5 and CD-68 in the Knee Joint Synovial Fluid Cells of Meniscal Tears Patients an Immunohistochemical Study

Cambios en la Composición de ADAMTS-5 y CD-68 en las Células del Líquido Sinovial de la Articulación de la Rodilla en Pacientes con Desgarro de Menisco. Un Estudio Inmunohistoquímico

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SUMMARY: Meniscus tear is an important injury affecting the quality of life. This work is aimed to investigate the activity of CD68 and ADAMTS-5 in cells in synovial fluid in male and female patients with meniscal tear. In this study ,18 male and 22 female patients with meniscal tears were included. Local pain sensation during patients' physical examination, swelling, performing daily activities and difficulty in running-walking complaints were determined. 5 cc synovial fluids were aspirated from the lateral suprapatellar pouch part of the knees with meniscal pain. After routine histological follow-up of the samples, they were embedded in paraffin and sectioned with microtome and 5 micrometer thickness. CD68 and ADAMTS-5 primary antibodies were used for immunohistochemical analysis. Sections were taken and evaluated with a stylish microscope. The distribution of blood cells after meniscus tear was higher in female patients than in male patients. CD68 distribution in female patients appeared higher than in male patients. CD68 expression was high in macrophage cell cytoplasm. ADAMTS-5 expression was higher in female patients in degenerative cells and apoptotic cells. ADAMTS-5 is an important metallo-protein involved in the development of apoptotic signal and extracellular matrix synthesis in patients with ADAMTS-5 meniscus tear, and it may be an important criterion for the treatment developed after injury. CD68 and ADAMTS-5 activity was thought to be one of the important signal pathways that can be identified in the treatment of meniscus tear.

KEY WORDS: Meniscus tears; Sinovial cells; CD68,ADAMTS-5.

INTRODUCTION

Meniscus tears are among the most common conditions that can affect all age groups (Salata *et al*., 2010). Meniscal tears may interrupt meniscal functions to be performed with resulting changes in structure. These alterations in joint function can lead to degenerative changes in the knee and facilitate the development of osteoarthritis (Liu *et al*., 2017).

Biomarkers are useful for the measurement of biological, and pathological processes and also pharmacologic reaction of tissue to a therapeutic application (Biomarkers Definitions Working Group, 2001; Kraus *et al*., 2015). There are studies presenting changes in biomarker composition of synovial fluid that differs in normal knees compared with those with traumatic or degenerative meniscal tears (Cook *et al*., 2017; Brophy *et al*., 2017). After the formation of meniscal tear, the change in synovial fluid composition persists months after injury, suggesting a chronic inflammatory state resulting in meniscal degeneration and the initiation of osteoarthritis (Larsson *et al*., 2015; Bigoni *et al*., 2017). For instance, degradative enzymes, including metalloproteinases (MMPs) and aggrecanases (ADAMTs), contribute to meniscal degeneration through proteoglycan and collagen degradation (Goldring & Otero, 2011). The cytokines IL-1b and TNF-a take part in joint inflammation because they contribute to the process of tissue degeneration with apoptosis and degradative enzyme production, studied in various researches (Catterall *et al*., 2010; Monibi *et al*., 2016). Inflammatory cytokines; IFN-d, IL-6, MCP-1, and MIP-1 are involved in the formation of degeneration in meniscal tears of the knee (Cuellar *et al*., 2009). ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) are a group of secreted proteases one of which is ADAMTS-5, also

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known as aggrecanase-2. The group including ADAMTS-5 is known to cleave the aggrecan, Aggrecan is the major proteoglycan of cartilage and is responsible for the ability of this tissue to resist compression by hydrating and swelling against the type II collagen (Porter *et al*., 2005).

CD68 (Cluster of Differentiation 68) is a protein that is highly expressed by cells in the phagocytes by circulating macrophages, and by tissue macrophages such as monocytes, Kupffer cells and microglia (Holness & Simmons, 1993). It presents the proliferation or abnormality of macrophage cells.

Nonoperative therapy with anti-inflammatory drugs is preferred for pain relief and improving mechanical function of the knee joint for meniscal tears (Katz *et al*., 2013; Howell *et al*., 2014; Woodmass *et al*., 2017). Hence, we aimed to observe the changes in these biomarker compositions to find out if anti ADAMs-5 and anti-CD 68 therapy can be used for potential clinical applications in the treatment of meniscal tears.

MATERIAL AND METHOD

Study design. Patients with meniscal tears treated between 2019-2020, in the Dicle University Orthopaedic Clinic were involved in the study who consulted with knee pain and difficulty in performing their daily activities. Informed consent form was obtained from all of the patients (18 male and 22 female patients). Synovial fluid (5ml) from each patient was taken from the knee lateral suprapatellar pouch with an injector. Blood samples were taken from the patients for analysis for cell count. The synovial fluid was centrifuged and the supernatant was discarded, fixed in 10 % neutral formalin for 2 h. Supernatant was again discarded and the pellet was taken on a filter paper, and eosin stain solution was added. After this procedure was completed samples were placed in a cassette, routine histological tissue processing was performed. Paraffin blocks were cut with a microtome and sections were examined under the light microscope (Carl Zeiss, Germany) after Harris haematoxylin and eosin (H&E) staining.

Immunohistochemical staining. Sections for immunohistochemical analysis were placed in distilled water and washed three times for 5 min with phosphate-buffered saline (PBS) (catalogue no. 10010023; Thermo Fisher Scientific, Fremont, CA, USA). Antigen retrieval was performed in a microwave oven (Bosch, 700 W) for 3 min at 90 °C in citrate buffer (pH 6). The sections were washed three times for 5 min with PBS and incubated with hydrogen peroxide (catalogue no. K-40677109, 64271; Merck, Dortmund, Germany) (3 mL 30 % [v/v] H2O2 + 27 mL methanol) for 20 min. The sections were washed 3 times for 5 min with PBS and blocked with Ultra V Block (lot PHL150128; Thermo Fisher Scientific) for 8 min. After draining, primary antibodies were directly added to the sections. The antibodies were CD-68 (1:100, lot#MA5-13324, Thermo Fischer, Fremont, CA, USA) and ADAMTS5 (catalogue no. ab41037 Abcam, UK) followed by incubation overnight at 4 °C. The sections were washed 3 times for 5 min with PBS and incubated with biotinylated secondary antibody (lot PHL150128; Thermo Fisher Scientific) for 14 min. After washing with PBS, streptavidin peroxidase (lot PH L150128; Thermo Fisher Scientific) was added for 15 min followed by washing 3 times for 5 min with PBS and the addition of DAB (lot HD36221; Thermo Fisher Scientific) for up to 10 min. As the reaction developed, the slides were placed in PBS, counterstained with Harris haematoxylin (haematoxylin and eosin, H&E) for 45 s, dehydrated through baths of ascending alcohol proportions; cleared in xylene (catalogue no. HHS32; Sigma-Aldrich, St. Louis, MO, USA); mounted with Entellan (lot 107961; Sigma-Aldrich), and examined under the light microscope (Carl Zeiss, Germany).

Statistical analysis. The data obtained were subjected to normal distribution test before statistical analysis. Shapiro-Wilk values were taken into consideration in the normal distribution evaluation.Samples showing normal distribution were analyzed statistically with independent T test. Parameters not showing normal distribution were evaluated by nonparametric Mann-Whitney U test. All parameters were shown as mean ± SD (Table I).

Table I. Statistical analysis results of the groups. Values are shown as mean + SD and p <0.05 was considered statistically significant.

	Male	Female	*p Value*
Lymphocyte (%)	30.41±3.52	39.85±1.60	***p<0.05***
Monocyte cells(%)	6.78±0.62	9.03±0.58	***p<0.05***
Basophil cells(%)	0.67±0.25	0.86±0.25	***p>0.05***
Neutrophil cells(%)	52.58±3.76	59.14±4.62	***p<0.05***
Eosinophils(%)	2.65±0.36	3.20±0.29	***p<0.05***
ADAMTS-5	2.90±0.74	3.50±0.53	***p>0.05***
CD68 expression	2.20±0.63	3.10±0.57	***p<0.05***

RESULTS

Synovial fluid samples from male and female patients were compared (Fig. 1). It was determined that lymphocyte, monocyte, neutrophil and eosinophil percentages were normally distributed and basophil cell percentage, ADAMTS-5 and CD68 expressions did not show normal distribution.

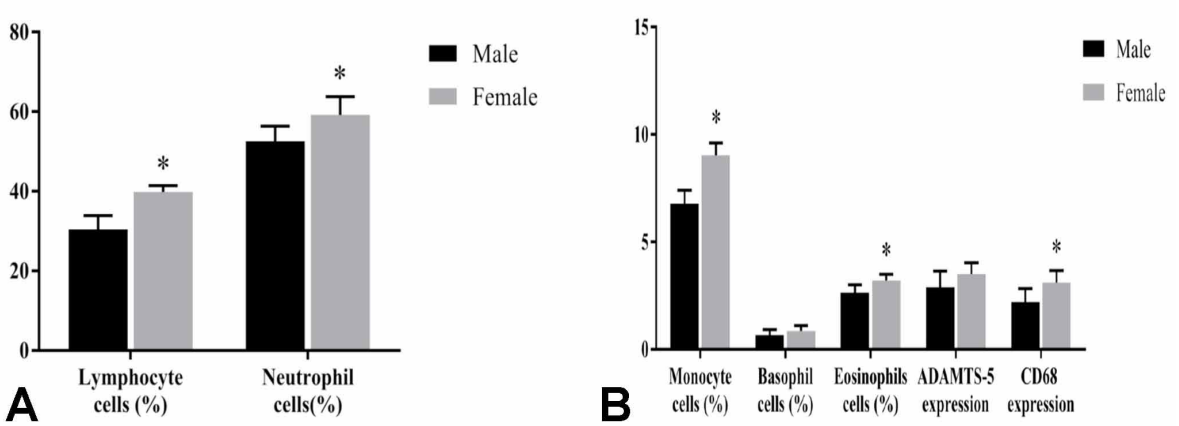

Fig. 1. A. Graphical view of the statistical result of the percentages of lymphocyte and Neutrophil cells in male and female. The symbol on the bar shows the significant difference between the groups (*; $p < 0.05$). B. Graphical view of the statistical result of the ratio of monocyte, basophil, eosinophil cells and ADAMTS-5 and CD68 expression scores in men and women. The symbol on the bar shows the significant difference between the groups (*; $p < 0.05$).

In the synovial fluid sample from male patients with meniscal tear, CD68 expression was positively observed in peripheral stoplasms of some synovial cells and macrophage cells (Fig. 2A). In the synovial fluid sample taken from female patients with meniscal tear, CD68 expression was increased in synovial cells and in the stoplasms of macrophage cells (Fig. 2B). Macrophage activity became evident due to increased inflammation reaction.

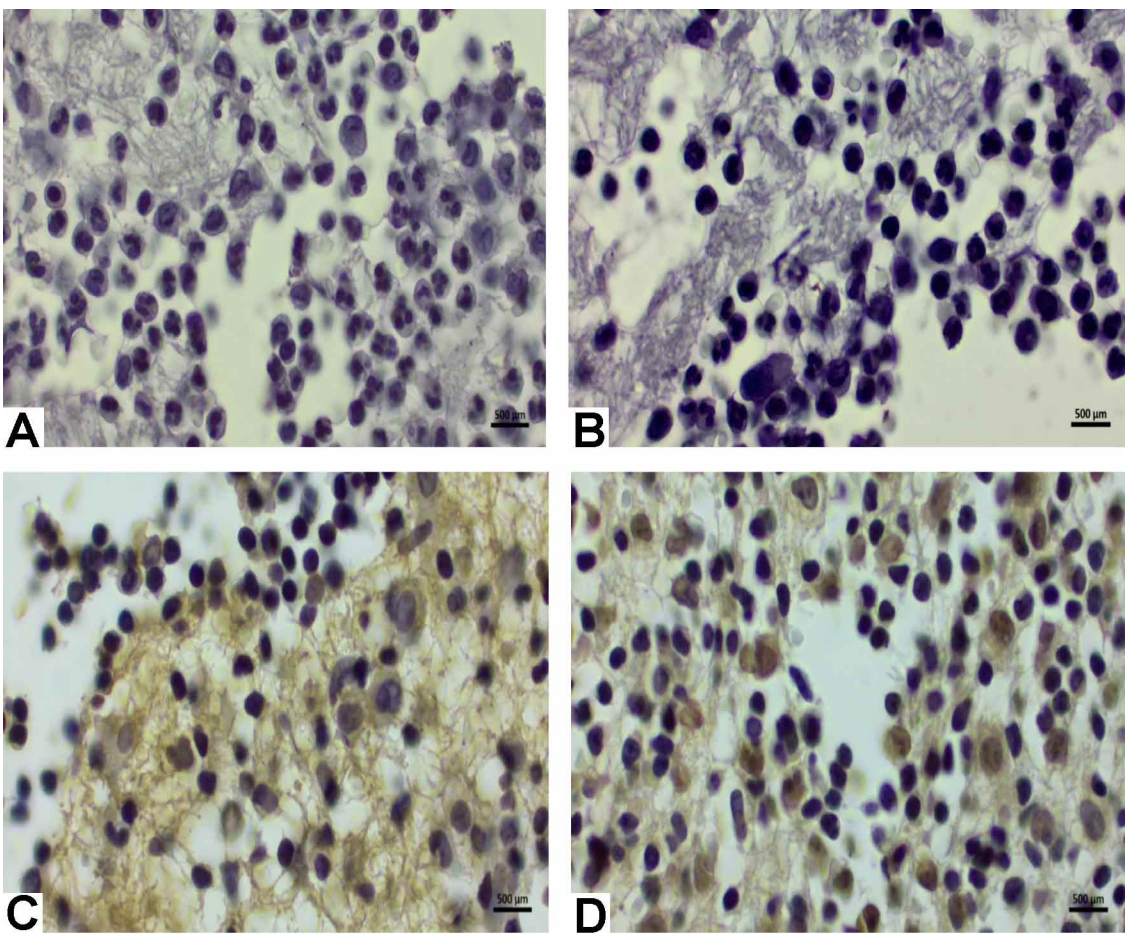

Fig. 2. CD68 and ADAMTS-5 expression distribution in synovial fluid from male and female patients.

In the synovial fluid sample from a male patient with a meniscal tear, ADAMTS-5 expression was observed in some areas of synovial cells with ADAMTS-5 expression, and ADAMTS-5 activity was negative in some of the synovial cells with nucleus degeneration and picnosis (Fig. 2C). In the synovial fluid sample from a female patient with a meniscal tear, ADAMTS-5 expression activity was observed to be positive especially in hypertrophic cells in the extracellular matrix areas. In polymorph nucleus cells, apoptosis was observed, while in fibroblast cells, the cell nucleus was observed as peripherally located degenerative (Fig. 2D). An increase in ADAMTS-5 expression was seen in both structures.

DISCUSSION

Meniscal tears are the most frequently seen and treated injuries of the knee joint from every age of patients (Baker *et al*., 1985). Meniscal tear surgery is an option but, non-operative treatment is useful for acute knee trauma and degenerative meniscal tears For this purpose anti-inflammatory and analgesic drugs, muscle strengthening, and intra-articular injections are being applied. Thus, alternative biological healing and pain relieving solutions are being investigated for meniscus tears management to interrupt the degradative cascade in the tissue (Yim *et al*., 2013; Mordecai *et al*., 2014; Beaufils *et al*., 2017; Doral *et al*., 2018).

Various biomarkers have been examined and still being investigated to determine whether there are differences between healthy and meniscus with tears. The cytokines and chemokines in synovial fluid of meniscal injury from animal models (Garner *et al*.,2011) and patients were studied such as; IL-1b, TNF-a, IL-6, IL-8, IL-15, MCP-1, MIP-1b, Gro, and IFN-g (Attur *et al*., 2013; Mabey & Honsawek, 2015; Monibi *et al*.). IFN-d, IL-6, MCP-1, and MIP-1b, were found to be associated with pain in meniscal tears patients that here inflammatory biomarker levels ; IL-6, IL-8, and TNFa in the synovial fluid were observed to be higher (Cuellar *et al*.). Cytokines MCP-1, IL-6, and IL-8 were observed in synovial fluid several months after injury, meaning that pro-inflammatory cytokines are present after meniscal trauma or in the begining of osteoartritis (Catteral *et al*., 2010). In our study, male patients with a meniscus tear were observed less frequently than female patients in macrophage activity, where inflammation rate was lower than female patients.CD68 expression rate was higher in female harvests. It was thought that cytokine activity increased more prominently in female patients and treatment would be required according to macrophage activity.

In another study on meniscal biomarkers (Vance, 2014), synovial fluid collected at surgery from patients with meniscal tear. Patients were divided into groups of short term (≤2 month) or long term (≥3 month) injury, and LAIRI (an immune inhibitory receptor), IL-10, and TMSB4X (a potential activator of metalloproteinases (MMP) expression) were found to be highly expressed in cell pellet and the supernatant of the synovial fluid. These findings demonstrate that inflammation in meniscal injury persists though, molecular biomarkers have become indicators in synovial fluid for determining of disease. Therefore, biomarkers of inflammatory pathways are valuable in determining meniscal tears (Attur *et al*.; Mabey & Honsawek). There are studies depiciting loss of proteoglycan and collagen which are signs of meniscal degradation. Nishimuta & Levenston (2015) showed that secretion of adipokines in synovial fluid may alter in diseases such as osteoarthritis (OA). They have studied degradive enzymes, metalloproteinases MMP-2 and MMP-3 activities using gelatin and casein zymography and revealed that active MMP-2 and total MMP-3 in meniscus were elevated. Atiç & Deveci (2019) have investigated the expression of matrix MMP-9 and TNF - alpha in the synovial cells of patients with meniscus tears. They have found that fluid levels and inflammation in inflamed joints with meniscal tears were increased, and that disrupted the matrix of cartilage. TNF-a and MMP-9 increment has disrupted cells such as fibroblasts, and concluded that anti-TNF-a treatment may prevent meniscal tearing. In a study of Liu *et al*. synovial fluid total MMP and PGE2 were elevated in meniscal tears patients in order to facilitate meniscal repair and prevent OA development. However, ADAMTS activity was analyzed with the increases in proteoglycan breakdown products indirectly. On the other hand, direct evaluation of ADAMTS concentrations in synovial fluid has not been clearly presented (Cook *et al*.). In this respect, our work can fill the gap in the field. In patients with meniscus tears, degenerative changes were observed especially in the nucleus in the synovial fluids with apoptosis. ADAMTS-5 expression activity was positive in the extracellular matrix areas with the cells. ADAMTS-5 expression distribution was higher in female patients than in male patients. It is an important metallo-protein involved in the development of apoptotic signal and extracellular matrix synthesis in patients with ADAMTS-5 meniscus tear, and it may be an important criterion for the treatment developed after injury. CD68 and ADAMTS-5 activity was thought to be one of the important signal pathways that can be identified in the treatment of meniscus tear.

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RESUMEN: La rotura del menisco es una lesión importante que afecta la calidad de vida. El objetivo fue investigar la activi-

dad de CD68 y ADAMTS-5 en células del líquido sinovial en pacientes masculinos y femeninos con desgarro meniscal. Se incluyeron 18 pacientes masculinos y 22 femeninos con desgarros meniscales. Se determinó la sensación de dolor local durante el examen físico de los pacientes, la hinchazón, la realización de actividades diarias y la dificultad al correr y caminar. Se aspiraron 5 cc de líquido sinoviale de la parte de la bolsa suprapatelar lateral de las rodillas de los pacientes con dolor meniscal. Después del seguimiento histológico de rutina, las muestras se incluyeron en parafina y se seccionaron con un micrótomo de grosor de 5 micrómetros. Para el análisis inmunohistoquímico se usaron los anticuerpos primarios CD68 y ADAMTS-5 . La distribución de las células sanguíneas después del desgarro del menisco fue mayor en pacientes femeninos que en pacientes masculinos. La distribución de CD68 en pacientes femeninos fue más alta que en pacientes masculinos. Además la expresión de CD68 fue alta en el citoplasma de los macrófagos. La expresión de ADAMTS-5 fue mayor en pacientes femeninos en las células degenerativas y células apoptóticas. ADAMTS-5 es una metaloproteína importante en el desarrollo de la señal apoptótica y la síntesis de matriz extracelular en pacientes con rotura de menisco ADAMTS-5, y puede ser un criterio importante para el tratamiento después de la lesión. La actividad de CD68 y ADAMTS-5 era una de las vías de señal importantes que se pueden identificar en el tratamiento de la rotura del menisco.

PALABRAS CLAVE: Desgarros del menisco; Células sinoviales; CD68, ADAMTS-5

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