VIA MEDICA

ORIGINAL ARTICLE

Expression of matrix metalloproteinase-9 and tumour necrosis factor-alpha in the synovial cells of patients with meniscus tears

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Background: A meniscus tear is a serious trauma that develops during swinging motion of the fixed foot. Meniscus tears may also be accompanied by divergence of the lateral ligaments of the knee joint.

Materials and methods: We enrolled 45 males and 35 females with meniscal tears in the present study. Patients with local joint pain, swelling, difficulty climbing stairs, patellar creeping, difficulties with daily living activities, local pain on palpation, and walking and running complaints, were included. We performed preoperative magnetic resonance imaging. Synovial fluid (5 mL) was aspirated from the lateral suprapatellar pouch of each knee with meniscal pain with the patient in the supine position. Blood samples were taken and biochemical parameters were analysed. The Harris haematoxylin and eosin staining protocol was used to evaluate tissue samples, and the levels of anti-matrix metalloproteinase (MMP)-9 and anti-tumour necrosis factor alpha (TNF-α) antibodies were measured immunohistochemically.

Results: Increased numbers of lymphocytes and neutrophils, hyperplastic erythrocytes, and fibroblasts were observed in the joint fluid of females. In males, the fibroblast cells were hyperplastic and plasma cell numbers were increased. MMP-9 expression was elevated in plasma cells, fibroblasts, and neutrophils; and TNF-α expression was observed in lymphocytes and polymorphic nucleated cells. We suggest that increased fluid levels in inflamed joints with meniscal tears, and the associated inflammation, disrupt the cartilage matrix and elevate the production of cytokines such as TNF-α and MMP-9 via release from cells such as fibroblasts that synthesise these mediators.

Conclusions: Anti-TNF-α treatment may prevent meniscal tears and prevent or slow the development of osteoarthritis. (Folia Morphol 2019; 78, 3: 535–544)

Key words: meniscus tear, synovial cells, matrix metalloproteinase 9, tumour necrosis factor alpha

INTRODUCTION

The meniscus is a fibrocartilage-like tissue consisting mainly of collagen and water in which cells reside. The meniscus stabilises the knee joint, absorbing snare forces. Good shock absorption and weight transmission are necessary to maintain normal anatomical function of the knee joint. The meniscus facilitates cartilage nutrition by pushing the synovial fluid toward the articular surface and protecting the synovial film layer that forms on that surface. Meniscal tears are common. Meniscus movement during index flexion prevents injury by improving the fit between joint faces. The posteromedial

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part of the meniscus is less mobile than other parts, explaining why tears are more often observed in this region [13]. Depending on the age, the meniscus tends to become more susceptible to lesions and tears caused by degenerative disease and trauma, leading to severe musculoskeletal disorders [11].

In young subjects, intact meniscal tissue may be ruptured by trauma; meniscal tearing often induces fluid accumulation, perceived as swelling and a sense of knee fullness. This type of damage triggers local biological and mechanical responses in the meniscus and joint tissues [20, 29]. A normal meniscus may suffer acute tearing creating trauma, or degenerative tearing after abnormal meniscal loading. Traumatic tears are usually found in those 10–40 years of age and degenerative tears in older subjects, often associated with other degenerative changes in knee cartilage and bone tissue.

Meniscal tearing is a major risk factor for the development of post-traumatic arthritis and increases, by 50%, the probability of symptomatic osteoarthritis (OA) within 10–20 years of post-injury repair [26]. Such biological changes can be quantified by measuring biomarkers reflecting extracellular matrix (ECM) synthesis and degradation, and inflammation, presumably acting as surrogates of joint remodelling and disease progression [20, 27]. The balance between matrix metalloproteinase (MMP)-mediated matrix synthesis and inhibition is crucial in terms of meniscal repair [7]. Growth factors and levels of MMPs in synovial fluid have been reported to be significant criteria for the size and progression of OA and meniscus injury. The joint synovial fluid provides direct physical contact with the meniscus, thereby reducing joint friction during movement [2]. MMPs contribute to the degradation of ECM components in joint tissues, triggering post-traumatic OA [22]. Tumour necrosis factor alpha (TNF-α) is a 17-kDa protein produced principally by activated macrophages [1]. Following joint injury, the synovial fluid levels of the pro-inflammatory cytokines interleukin (IL)-1 and TNF-α become elevated, with the highest (acute) levels observed within the first 24 h after injury [4, 18, 24, 46]. Here, we used biochemical, histopathological, and immunohistochemical methods to investigate the effects of meniscal tearing on synovial fluid cells, caused by the inflammatory response and the decrease in knee movement.

MATERIALS AND METHODS

Patient

We studied patients with meniscal tears treated in the orthopaedic clinic of the medical faculty of Dicle University. The use of human tissues was approved by Dicle University Medicine Faculty Ethical Committee. All patients provided written informed consent. We enrolled patients who visited the Department of Orthopaedics, Medical School, Dicle University from June 2017 to January 2018 with local knee pain and/ or swelling, difficulty climbing stairs, patellar creeping, difficulties with daily living activities, local pain on palpation, and walking and running complaints.

MRI protocols

We performed preoperative magnetic resonance imaging (MRI) of 45 males (aged 35–53 years) and 35 females (aged 45–64 years) with meniscal tears. In total, 52 patients had one-sided meniscal tears and 28 patients two-sided tears. All knees were imaged with the same 1.5 T MR (Philips Achieva) scanner and an eight-channel knee coil on each system. All patients were evaluated using the same MRI protocol. Standard knee MRI included coronal T1 sequences, sagittal T2 sequences, and sagittal-coronal T2 fat-suppressed sequences.

Synovial fluid sampling procedure

With each patient in the supine position, a minimum of 5 mL of synovial fluid was aspirated from the lateral suprapatellar pouch of the affected knee via an injector. Blood samples were also collected. The synovial fluid was subjected to high-speed centrifugation, the supernatant was discarded, and 10% (v/v) neutral formalin was added (pellet:formalin volume ratio, 1:3). After 2 h of fixation, the clear bright supernatant was discarded and the tube was inverted on filter paper to allow excess fluid to drain. The pellet was then placed on filter paper using a spatula and eosin stain solution was added using a Pasteur pipette. After the cell aggregate developed a red colour, it was wrapped in filter paper and placed in a cassette, which was then stored in a fixation box for tissue processing. The cells were examined following Harris haematoxylin and eosin (H&E) staining. The synovial fluid was also immunostained and examined under a light microscope.

Immunohistochemical staining

Sections 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] H_2O_2 + 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 MMP-9 (monoclonal antibody; 1:100; catalogue no. PA5-13199; Thermo Fisher Scientific) and TNF-α (catalogue no. PHC3015; 1:100; Thermo Fisher Scientific) 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 PHL150128; 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 using an Olympus BH-2 light microscope.

Western blotting

The protein expression levels of TNF-α and MMP-9 were examined by Western blotting. Briefly, proteins were extracted from synovial tissues [47], separated on 10–12% SDS-PAGE gels (40 mg/lane) and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk in TBST buffer (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.05% Tween 20, pH 7.1) for 2 h and incubated with primary antibodies overnight at 4°C. Primary antibodies used here were monoclonal human antibodies against MMP-9 (1:1000 dilution; Cell Signaling Technology, Boston, MA), and TNF-α (1:1000 dilution; Cell Signaling Technology, Boston, MA). After extensive washing with TBST buffer, the blotted membranes were then incubated for 1 h at room temperature with an HRP-conjugated secondary antibody directed against rabbit IgG (1:2,000; Cell Signaling Technology, Boston, MA). The proteins were detected using an enhanced chemiluminescence system (ECL kit, Pierce Biotechnology, Beijing, China) and
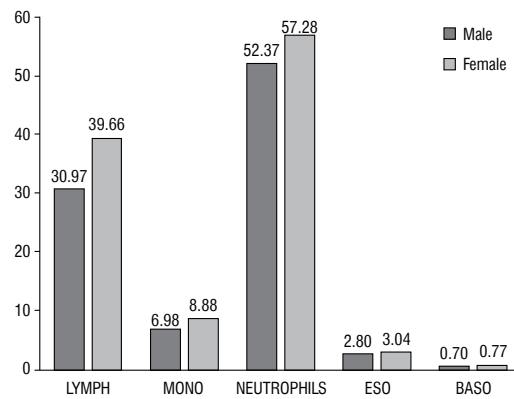
Graph 1. Blood cell counts in male and female patients; LYMPH — lymphocytes; MONO — monocytes; ESO — eosonophils; BASO — basophils.

captured on light sensitive X-ray film (AGFA, Belgium). Tissue densities were detected using ImageJ software.

Statistical analysis

IBM SPSS software ver. 21.0 for Windows was used for all analyses. Data are presented as the mean ± standard deviation. Categorical variables are shown as numbers with percentages (%). Between-gender comparisons were made with the aid of the Mann–Whitney U-test. All hypotheses were bidirectional and a p-value < 0.05 was considered to reflect statistical significance.

RESULTS

Blood and MRI findings

The lymphocyte counts were 30.97 ± 3.28 in males and 39.66 ± 1.59 in females, and the monocyte counts were 6.98 ± 0.73 in males and 8.88 ± ± 0.65 in females. The mean neutrophil count was 52.37 ± 3.33 in males and 57.28 ± 5.01 in females. The genders differed significantly in terms of lymphocyte, monocyte, and neutrophil numbers (all p-values < 0.001, Graph 1). In the male blood samples, the eosinophil number was 2.80 ± 0.37 and that of females was 3.04 ± 0.24; the basophil counts were 0.70 ± 0.24 and 0.77 ± 0.21, respectively, and the differences were not significant (both p-values > 0.05, Graph 1).

Magnetic resonance imaging of a 35-year-old female patient revealed a degenerative horizontal tear of the medial meniscus rim of the left knee (Fig. 1a). Imaging of the posterior horn of the same patient revealed that the rupture was oval in shape (Fig. 1b) and dislocated. MRI of the left knee revealed a degen-
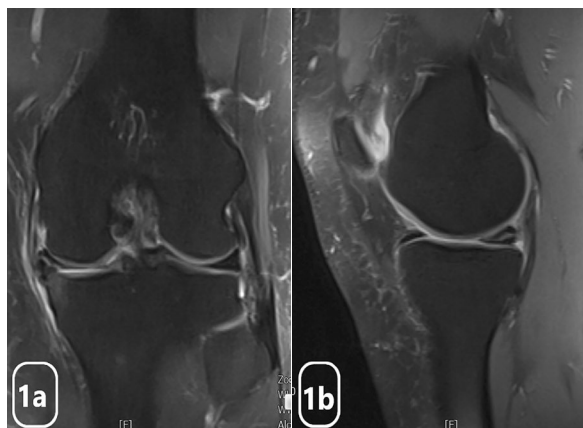
Figure 1. a. A coronal T2 weighted magnetic resonance imaging (MRI) showing a degenerative horizontal tear of the medial meniscus rim of the left knee (a 35-year-old female patient); **b.** A sagittal T2 weighted MRI showing oval-shaped rupture (a 35-year-old female patient).
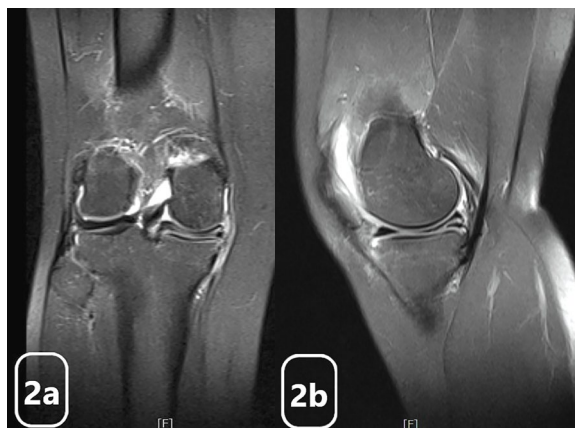
Figure 2. a. A coronal T2 weighted magnetic resonance imaging (MRI) showing a degenerative horizontal tear (caused by trauma) in the medial meniscal region (a 19-year-old female patient); **b.** A sagittal T2 weighted MRI showing horizontal rupture of the posterior horn (a 19-year-old female patient).
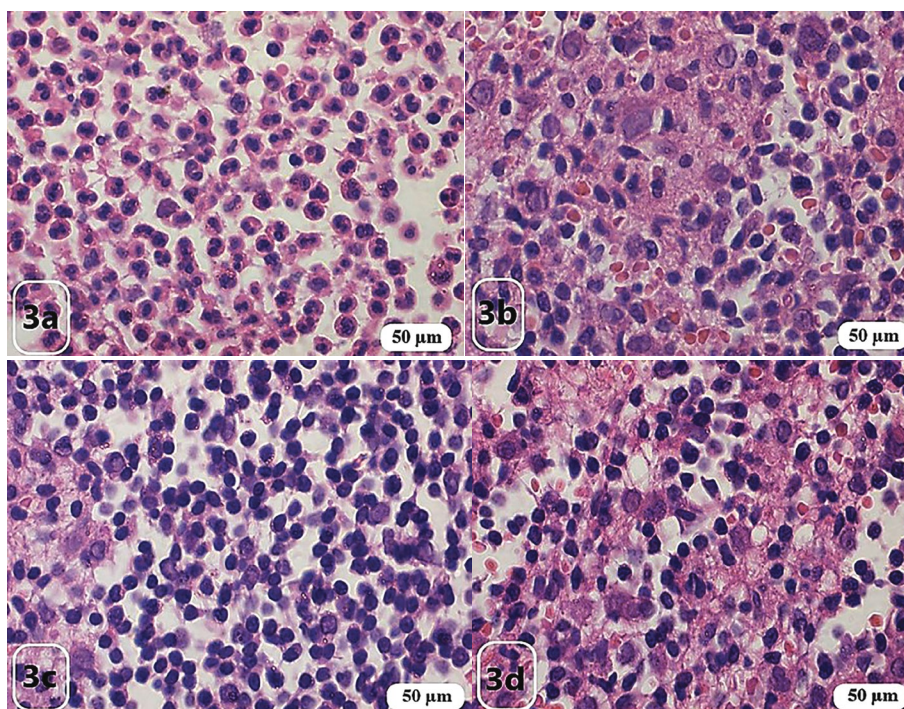
Figure 3. a. Haematoxylin and eosin (H&E) staining. The lymphocytes were dense, neutrophils were absent, and no chromatin bridges were apparent between polymorphic nuclei. Scale bar = 50 μm; **b.** H&E staining. Plasma cells were prominent, fibroblasts were hyperplastic, and fibroblast and erythrocyte numbers were increased. Scale bar = 50 μm; **c.** H&E staining. Lymphocytes of various sizes increased in number. Scale bar = 50 μm; **d.** H&E staining. Plasma cells were prominent, as were fibroblasts, and scattered hyperplastic erythrocytes were evident. Scale bar = 50 μm.

erative horizontal tear (caused by trauma) in the medial meniscal region of a 19-year-old female patient (Fig. 2a), and horizontal rupture of the posterior horn (Fig. 2b).

Histological findings

The increased levels of abnormal cells in the synovial fluid reflected the severity of meniscal tearing. On microscopic examination of synovial fluid cells from females with meniscal ruptures, the lymphocytes were dense and the neutrophils markedly polynucleated, but no nuclear chromatin bridges were apparent. Plasma cells were evident; the fibroblasts exhibited hyperplasia; and erythrocyte numbers were high (reflecting a hematoma) (Fig. 3a, b). On microscopic

examination of the synovial fluid cells of males, increased numbers of lymphocytes (of various sizes) were noted. Plasma cells were evident, and the fibroblasts were hyperplastic. Plasma cells are generally abundant in regions of chronic inflammation. The erythrocytes were scattered (Fig. 3c, d).

Immunohistochemical findings

Immunohistochemical examination revealed that MMP-9 expression was increased in lymphocytes, fibroblasts, and neutrophils of both males and females, and plasma cells of females (Fig. 4a, b). Following inflammation, MMP-9 is thought to modulate matrix degradation, collagen synthesis, and angiogenic development (Fig. 4c, d). In females, TNF-α was expressed by neutrophils, lymphocytes, and phagocytic monocytes (Fig. 5a, b). Notably, TNF-α expression was increased in polymorphic nucleated cells, plasma cells, fibroblasts, and neutrophils of males, and in some lymphocytes (Fig. 5c, d).

Western blot findings

In a total of 80 patients with meniscus tears, MMP-9 and TNF-α expression in synovial cells of 4 patients were evaluated by Western blot analysis. The picture showing total protein weight and expression of TNF-α and MMP-9 were included in the study (Fig. 6).

DISCUSSION

In this study, meniscus tears which were caused by trauma in 80 patients were determined by MRI and the MMP-9 and TNF-α expressions in synovial fluid samples were examined by immunohistopathology and Western blot analysis. This study found that the expression of these proteins in synovial fluid samples increased immunohistochemically. In parallel with this increase in expression, it was determined that the molecular weights of these proteins increased in the Western blot analysis.

Clinically, meniscus tears have been reported to be both acute and chronic forms [38]. Anatomically, the posterior horns of the medial and lateral menisci transmit more load than the anterior horns while the leg is at 90° flexion [10]. For this reason, posterior medial meniscus tears were the main focus of the studies [19, 35]. Meniscal damage increases the risk of knee OA. Meniscal surgery following radial and oblique tearing of the middle and posterior third of the medial meniscus can significantly increase cartilage tension in the tibial and femoral condyles, possibly because the extent of tissue resorption following radial or oblique tearing may be less than that after longitudinal tearing. Radial tearing will cause more damage to peripheral fibrils running from the inner to outer surface [6, 15, 16, 37, 40]. MRI affords a 90–95% diagnostic success rate, although MRI data should be combined with clinical findings [12, 44]. We showed a 35-year-old male patient with a degenerative horizontal tear of the medial meniscus rim of the left knee (Fig. 1). The MRI also revealed degenerative horizontal rupture (a medial meniscal tear) in the left knee of a 19-year-old female patient after trauma (Fig. 2). Acute inflammation is usually of sudden onset, developing over minutes or hours; the classical symptoms are heat, pain, redness, and swelling. Chronic inflammation develops over a longer period of time, and may persist for days, weeks, or months [28].

Neutrophils have been found to be the most inflammatory cells in patients with acute synovitis. On the other hand, it has been reported that many macrophages are frequently accompanied by lymphocytic infiltrates in OA chronic synovitis patients [23]. Meniscal damage (caused especially by degenerative and traumatic tearing) is accompanied by both acute and chronic inflammation. Such a response is noted following rupture of the anterior cruciate ligament (ACL), traumatic meniscus tearing, and in those with premature OA [9]. Shortly after ACL rupture, the joint levels of inflammatory cytokines, particularly IL-1 and TNF-α, rise [21]. Several parameters including obesity, diurnal rhythm, diet, medications, sample collection/storage procedures, time of sample collection, physical activity level, trauma, and gender affect the measured levels of these cytokines [14, 39, 48].

High levels of TNF-α and IL-6 in traumatic areas indicate the level of cartilage damage and the presence of a local inflammatory response that triggers early knee OA [32]. Cuellar et al. [8] reported a correlation between synovial fluid levels of IL-6 and the pain score in patients with ACL tears, but no effect on the level of TNF-α was apparent. By contrast, Orita et al. [33] found a positive correlation between the synovial fluid TNF-α level and the pain score in OA patients. In patients with meniscal tears, elevated levels of cytokines in synovial cells and increased OA activity change the osteoclastic numbers and cartilage tissue morphology. The levels of soluble TNF receptors in the sera of OA patients correlated positively with pain, joint stiffness, and increased radiographic severity of disease [41]. Aging is correlated with increased systemic inflammation and can trigger mitochondrial dysfunction and an inability to eliminate oxidatively damaged proteins
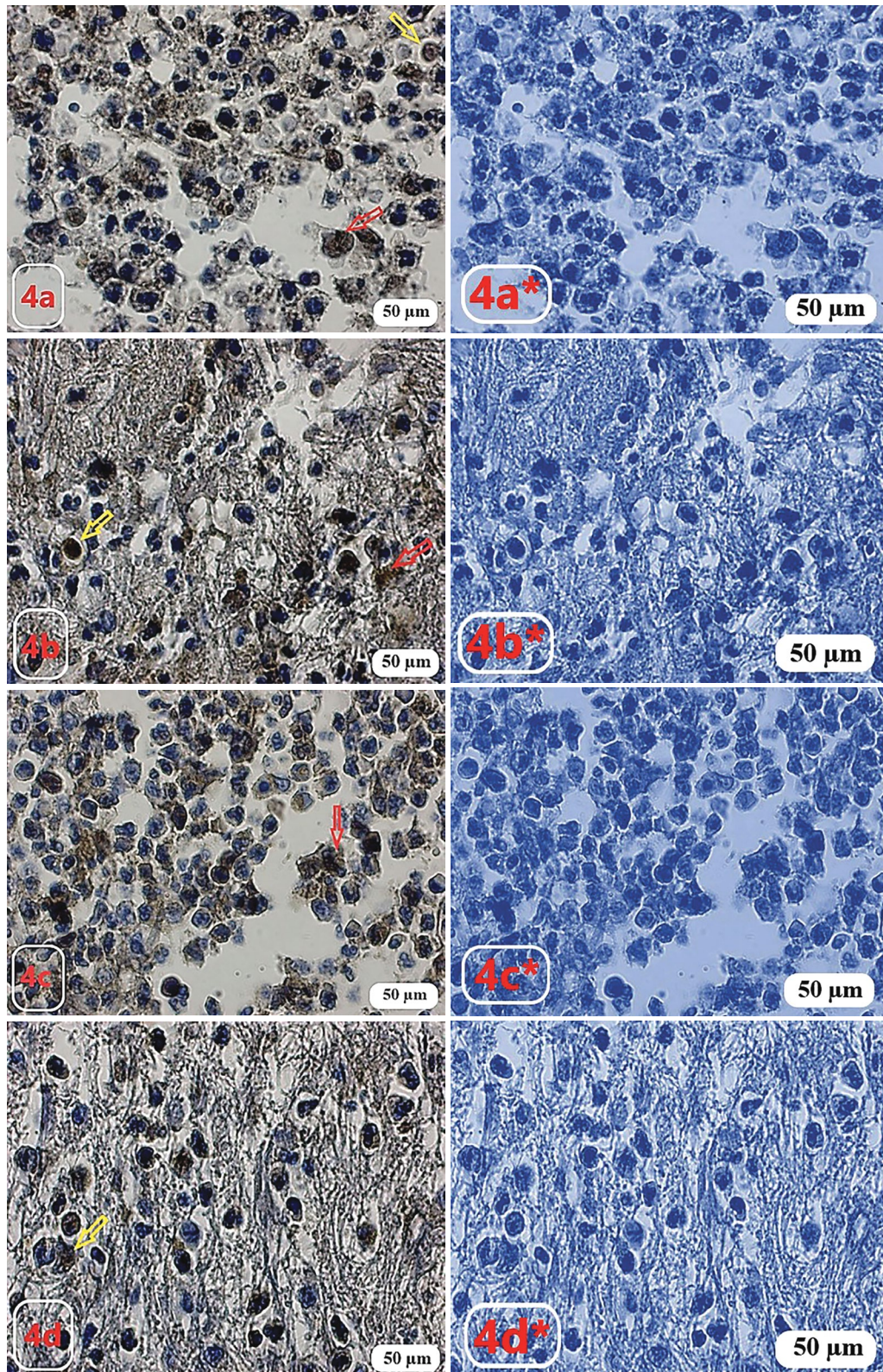
Figure 4. a. Matrix metalloproteinase 9 (MMP-9) immunostaining. Increased MMP-9 expression in lymphocytes (yellow arrow), plasma cells (red arrow), fibroblasts, and neutrophils of females. Scale bar = 50 μm; **a*.** Negative control, haematoxylene staining. Scale bar = 50 μm; **b.** MMP-9 immunostaining. MMP-9 expression in lymphocytes (yellow arrow), fibroblasts (red arrow) and neutrophils of males. Scale bar = 50 μm. **b*.** Negative control, haematoxylene staining. Scale bar = 50 μm; **c.** MMP-9 immunostaining. MMP-9 expression in plasma cells (red arrow) and neutrophils. Scale bar = 50 μm; **c*.** Negative control, haematoxylene staining. Scale bar = 50 μm; **d.** MMP-9 immunostaining. MMP-9 expression in hyperplastic fibroblasts (yellow arrow). Scale bar = 50 μm; **d*.** Negative control, haematoxylene staining. Scale bar = 50 μm.
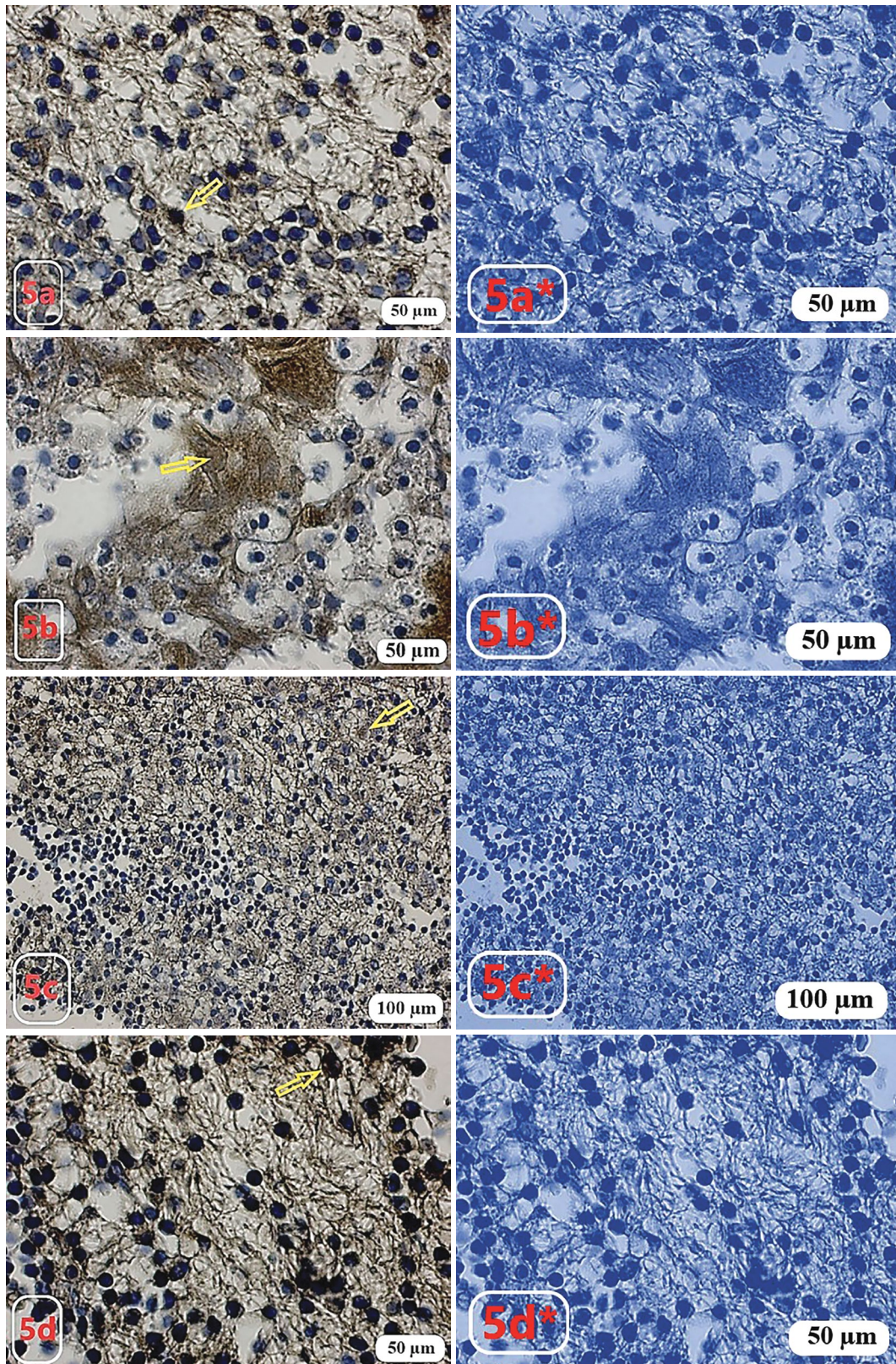
Figure 5. a. Tumour necrosis factor alpha (TNF-α) immunostaining. TNF-α expression in neutrophils, lymphocytes (yellow arrow), and phagocytic monocytes. Scale bar = 50 μm; **5a*.** Negative control, haematoxylene staining. Scale bar = 50 μm; **b.** TNF-α immunostaining. Increased TNF-α expression in polymorphic nuclear cells (yellow arrow). Scale bar = 50 μm; **b*.** Negative control, haematoxylene staining. Scale bar = 50 μm; **c.** TNF-α immunostaining. TNF-α expression in plasma cells (yellow arrow), and neutrophils of males. Scale bar = 100 μm; **c*.** Negative control, haematoxylene staining. Scale bar = 100 μm; **d.** TNF-α immunostaining. TNF-α expression in fibroblasts (yellow arrow) and some lymphocytes. Scale bar = 50 μm; **5d*.** Negative control, haematoxylene staining. Scale bar = 50 μm.
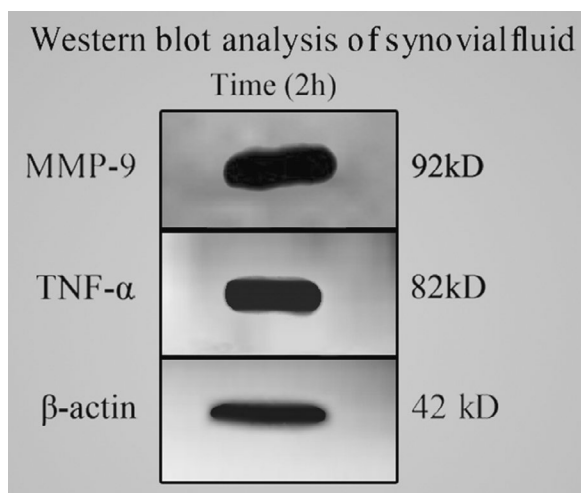
Figure 6. The expression of tumour necrosis factor alpha (TNF-α) and matrix metalloproteinase 9 (MMP-9) on synovial tissue was dramatically increased in patients with meniscal tears. Equal amounts of total proteins were run on the gel and analysed by Western blotting using anti-TNF-α, anti-MMP-9 and anti-β-actin antibodies. β-actin was used as a loading control. The position of molecular weight markers (kD) is shown (n = 4).

from chondrocytes, in turn causing cell senescence and increased production of inflammatory cytokines [3, 25]. Rutgers et al. [42] concluded that inhibition of 40 ng/mL TNF-α via intravenous injection enhanced cartilage metabolism. We found that TNF-α levels were increased in meniscal tears, suggesting that TNF-α contributes to the pathogenesis of joint damage in chronic OA and rheumatic diseases, perhaps triggering cartilage and bone resorption.

Normal meniscal cells exhibited a marked increase in MMP expression 6 h after stimulation, but OA cells responded less rapidly, perhaps due to differences in receptor concentrations or relevant inflammatory pathways [45]. MMPs degrade and process many components of the ECM, including the basement membrane, and are thought to be important in terms of tumour spread and angiogenesis [31, 34]. The ECM is a complex mesh of structural and signalling molecules affording dynamic support to cells and tissues and modulating cell behaviour [30, 36]. MMP-9 is a proteolytic enzyme that plays a key role in tissue remodelling during pathological processes, initiating ECM degradation. And, we found that MMP-9 expression was increased in the lymphocytes and plasma cells of both male and female patients, inducing chronic inflammation and ECM deterioration. In addition, MMP-9 expression in fibroblasts may affect the synthesis of collagen fibres.

Liu et al. [24] observed that the total MMP activity in synovial fluid correlated negatively with the levels of MMP-3, MMP-9, and MMP-10 in patients with meniscal tears, indicating that these enzymes do not increase overall MMP activity. The levels of MMP expression in synovial cells have been reported to be modulated by different extracellular signals in patients with rheumatoid arthritis. Synovial fibroblasts directly stimulate the expression of both inflammatory cytokines and disease-associated MMPs [43].

There are important limitations to be considered in this immunohistochemical study. Increase in MMP-9 [5, 17] and TNF-α [5] expressions in patients with meniscal tear has been shown only in mRNA levels in a limited number of studies. In this study, the increase in expression of these proteins is shown immunohistochemically in patients with meniscus tear. In parallel with the increase in MMP-9 and TNF-α expressions, it has been shown that the molecule levels of these proteins increased by western blot analysis. In addition, we observed that MMP activity was increased in synovial fluid cells of meniscal tear patients exhibiting cartilage tension. After tearing, synovial cells act on the adjacent cartilage matrix, inducing tension by hyperactivating matrix-degrading enzymes. Thus, interactions among tissues trigger prolonged episodes of signalling.

CONCLUSIONS

We found that the increased fluid levels in inflamed joints with meniscal tears, and the inflammation per se, disrupted the cartilage matrix. As increases in the levels of TNF-α and MMP-9 disrupted cells that synthesize such materials (i.e., fibroblasts), it is possible that anti-TNF-α treatment would prevent meniscal tearing.

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