Joint Hemorrhage Accelerates Cartilage Degeneration in a Rat Immobilized Knee Model

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Abstract

Background: Joint hemorrhage is caused by trauma, ligament reconstruction surgery, and bleeding disorder such as hemophilia. Recurrence of hemorrhage in the joint space induces hemosiderotic synovitis as well as oxidative stress, resulting in both articular cartilage degeneration and arthropathy. Joint immobilization is a common treatment option for articular fractures accompanied by joint hemorrhage. Although it is apparent that joint hemorrhage is harmful on the articular cartilage, there was no consensus whether reduction of joint hemorrhage is effective to prevent articular cartilage degeneration. The purpose of this study was to investigate the articular cartilage degeneration induced by a combination of joint hemorrhage and joint immobilization in a rat knee model.

Methods: The knee joints of adult male rats were immobilized at the flexion using an internal fixator from 3 days to 8 weeks. The rats were divided randomly into two groups: immobilized blood injection (Im-B) group and immobilized-normal saline injection (Im-NS) group. The cartilage was evaluated at two areas (contact and non-contact areas). The cartilage was assessed for the chondrocyte count, Modified Mankin score, and cartilage thickness. Total RNA was extracted from the cartilage in both areas, and gene expressions of metalloproteinase (MMP)-8, MMP-13, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α were measured by quantitative real-time polymerase chain reaction.

Results: The number of chondrocytes in the Im-B group significantly decreased in both areas, compared to the Im-NS group. Modified Mankin score from 4–8 weeks of the Im-B group was significantly greater than the Im-NS group only in the contact area. Gene expressions of MMP-8 and MMP-13 from 2–4 weeks and TNF-α from 2–8 weeks significantly increased in the Im-B group compared to the Im-NS group, while there was no significant difference in IL-1β.

Conclusions: This study showed that joint hemorrhage exacerbated immobilization-induced articular cartilage degeneration. Therefore, drainage of a joint hemorrhage and avoidance of loading are recommended to prevent cartilage degeneration during immobilization of a joint hemorrhage.

Background

Joint hemorrhage is often caused by trauma, major joint surgery, and bleeding disorders, such as hemophilia. Repeated joint bleeding has negative effects on cartilage degeneration directly and indirectly [1, 2]. Iron accumulation in the joint cavity by hemorrhage induces free radical formation near the cartilage, causing chondrocyte apoptosis and matrix deformation [1] [3]. Moreover, iron induces excessive neovascularization and proliferation of synoviocytes [4], and the subsequent synovial inflammation leads to arthropathy [5]. Although this devastating event becomes more severe through repeated bleeding, it is induced by a single blood exposure [1] [5], which causes long-lasting impaired matrix turnover [5] [6] and joint damage progression.

In hemophilic arthropathy, pro-inflammatory cytokines such as interleukin (IL)-1β [6] [7] [8] and tumor necrosis factor (TNF)-α [6] [9] are produced in hemosiderin-laden synovial tissue, and these cytokines

trigger catabolic programs, activating nitric oxide (NO) [1] [6], matrix metalloproteinases (MMPs) [6], osteoarthritis [10], and rheumatoid arthritis. These cytokines also increase iron uptake into monocytes and synovial fibroblasts and accelerate the vicious cycle of synovitis [7]. However, methods to cause joint bleeding, including bone marrow stimulation, have been proven to be effective treatments for cartilage defects of the knee through cartilage regeneration [11]. Additionally, a similar procedure has been performed to enhance healing of the torn meniscus [12]. However, the beneficial effects have only been shown for damaged tissue, and its clinical outcome has only been demonstrated in the short term [7]. Yet, the effect of hemorrhage on normal cartilage has not been considered.

Posttraumatic osteoarthritis after fracture or ligament injury occasionally occurs, even with appropriate treatment or surgery [13]. According to previous reports, trauma and surgery can induce degeneration of the articular cartilage, inflammation, and hypertrophy of synovial cells, resulting in posttraumatic osteoarthritis [13] [14]. Additionally, joint immobilization, which is a common treatment for traumatic injury to maintain rest or enhance recovery, causes degenerative changes in articular cartilage[15] and contributes to osteoarthritis development [16]. Blood in the joint is cleared rapidly within 48 hours without joint immobilization [17]; however, a report has described protraction of residual blood in the joint by joint immobilization [18]. Thus, joint immobilization may exacerbate the harmful effect of blood on joint cartilage; however, it has not been investigated. Therefore, this study investigated the effect of joint hemorrhage combined with joint immobilization on articular cartilage degeneration in a rat knee model.

Methods

Animals

Mature Sprague–Dawley male rats, aged 12 weeks old, were used (CLEA Japan Inc., Tokyo, Japan). In total, 108 rats were used (histological and immunohistological evaluation, n = 72; gene expression analysis, n = 36). The protocol of this study was approved by the Institutional animal research committee of Tohoku University prior to the experiments (approval number: 2013 MdA-360). Under anesthesia with intra-peritoneal administration of sodium pentobarbital (50 mg/kg), the unilateral knee joint was rigidly immobilized at 150 degrees of flexion with a plastic plate and two metal screws for various periods [19]. After surgery, the rats were divided randomly into two groups: immobilized blood injection (Im-B) and immobilized-normal saline injection (Im-NS) groups. After surgery, a single injection of 50 μL of blood obtained from the caudal vein was administered directly into the knees of the Im-B group [20]. An equal amount of normal saline was administered in the Im-NS group in the same manner.

Tissue preparation

According to the previous report [21], the specimens were prepared for evaluation. After euthanasia by intraperitoneal injection of an overdose of sodium pentobarbital, the rats were fixed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4 by perfusion into the aorta. Subsequently, the tissues around the knee joint were resected and kept in the same fixative for 24 h at 4 °C. The fixed specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 2 months

at 4 °C. After dehydration using ethanol and xylene solutions, the specimens were embedded in paraffin. They were cut into 5 μm sagittal sections in the medial midcondylar region of the knee and used for evaluation [21].

Histological evaluation

Hematoxylin and eosin stain and safranin O stain were used to assess cartilage degeneration, chondrocyte count, and cartilage thickness in contact and non-contact areas of the femur and tibia, respectively (1, 3 days, 1, 2, 4, and 8 weeks after surgery; n = 6/each group) [20]. Cartilage degeneration was evaluated by Modified Mankin histological grading scheme [22]. The number of chondrocytes was counted within a predefined field of view, and cartilage thickness was defined as the distance between the cartilage surface and osteochondral junction in each area [20]. Additionally, hemosiderin deposition was evaluated by Perls' Prussian blue staining (2, 4, and 8 weeks after surgery).

Immunohistochemistry

The sections in each period were deparaffinized and soaked in 0.3% hydrogen peroxide. Cluster of differentiation 68 (CD68) was used as a marker of macrophage-like type A synoviocytes [23]. Endogenous peroxidase was inactivated with 3% H_2O_2 in PBS for 20 min at room temperature. The slides were incubated with mouse anti-rat CD68 antibody (MCA341 R, AbD Serotec, Raleigh, NC, USA; dilution, 1:400) for 24 h at 4 °C. Goat anti-mouse immunoglobulin (IgG) (Nichirei, Tokyo, Japan) for CD68 was used as a secondary antibody for 30 min at room temperature. The final detection step was carried out using 3,30-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA) in 0.1 M imidazole and 0.03% H_2O_2 as the chromogen. Counterstaining was performed using Carazzi's hematoxylin. Negative control was performed using normal mouse IgG (Dako, Copenhagen, Denmark) as a primary antibody. All slides were stained in one session.

Gene expression analyses

After euthanasia, cartilage of the contact and non-contact areas from the femur and tibia was obtained with a surgical knife and rongeur. The harvested cartilage samples were immediately immersed in 1 mL QIAzol (Qiagen, Hilden, Germany). The samples were crushed and homogenized with a Polytron (Kinematica AG, Lucerne, Switzerland). The total RNA was purified using the RNeasy Lipid Tissue Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized using a Cloned AMV First-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). PCR efficiencies and relative expression levels of MMP-8, MMP-13, IL-1β, and TNF-α as a function of elongation factor-1α1 (EF1α1) were calculated as described (2, 4, and 8 weeks after surgery; n = 6/each group) [24]. The primer sequences used in the polymerase chain reaction are listed in Table 1.

Table 1
Sequences of Primers Used for Polymerase Chain Reaction

Gene Name	GenBank	Nucleic Acid Sequences	
MMP-8	NM 022221	Upstream	tggaatccttgcccatgcct
		Downstream	aagcagccacgagaaacaggt
MMP-13	NM 133530	Upstream	tggtcttctggcacaacgctt
		Downstream	tggaagctgcttgtccaggtt
IL-1β	NM 031512	Upstream	accgtggcaacattctggtca
		Downstream	tcgacaaatgctgcctcgtga
TNF-α	NM 0126763	Upstream	actggcgtgttcaatccgttctctac
		Downstream	ccgcaaaatccaggccactacttc
EF1a1	NM 175838	Upstream	tgatgccccaggacacaagagaact
		Downstream	gataccagcttcaaaattcccaacaac

Statistical analyses

Differences between the Im-B and Im-NS groups were assessed statistically by unpaired *t*-test (histological evaluation: Mankin score, chondrocyte count, and cartilage thickness) and Mann–Whitney's U test (qRT-PCR). Data are described as mean ± standard deviation (SD). Tests were two-sided, and a value of $p < 0.05$ was accepted as statistically significant. The inter-observer variabilities for Mankin score, chondrocyte count, and cartilage thickness were assessed using intraclass correlation coefficients, which were 0.92, 0.62, and 0.82, respectively. All statistical analyses were performed with IBM SPSS Statistics version 24.0 (SPSS Japan Inc., Tokyo, Japan).

Results

Histological evaluation

Histological features of the hematoxylin and eosin-stained femoral cartilage are shown in Fig. 1. Articular cartilage degeneration was observed in both the Im-B and Im-NS groups. The degeneration gradually progressed after immobilization and was more significant in the contact area (Fig. 1A-H). At 3 days after immobilization and intra-articular administration, swelling of the chondrocytes and disappearance of cells in the tangential zone were seen, especially in the Im-B group (Fig. 1A and B). The cell layers were irregular at 1 week (Fig. 1C and D). The intensity of cell staining decreased at 4 weeks, which was more severe in the Im-B group (Fig. 1E and F). Cells cloning appeared, and cell loss occurred at 8 weeks (Fig. 1G and H). Degeneration of the non-contact area is shown in lower row of Fig. 1 (I-P). Although similar cellularity was observed in contact and non-contact areas, cartilage degeneration was milder in non-contact than contact areas.

Mankin scores of the Im-B group were significantly higher than the Im-NS group at 4 weeks in the femur and 4 and 8 weeks in the tibia (Fig. 2A and D). There was no severe progression in the non-contact area, and no statistical difference between the Im-B and Im-NS groups (Fig. 3A and D). The number of chondrocytes in the contact area of the Im-B group significantly decreased at 3 days and 8 weeks in the femur and 3 days and 1 and 8 weeks in the tibia compared to the Im-NS group (Fig. 2B and E). In the non-contact area of the Im-B group, it was significantly decreased at 3 days and 1 week in the femur and 3 days in the tibia (Fig. 3B and E). Cartilage thickness did not change and was not affected by immobilization and intra-articular administration in both contact and non-contact areas (Fig. 2C and F, Fig. 3C and F).

Iron deposition evaluated by Perls' Prussian blue is shown in Fig. 4. Injection could induce joint bleeding, but there was no iron deposition in the Im-NS group (Fig. 4A). Iron deposition was observed in the synovium and meniscal surface, which lasted until 8 weeks after administration in the Im-B group (Fig. 4B-D). CD68-positive cells were observed in both Im-B and Im-NS groups (Fig. 5). It was observed mainly in the synovial membrane of the Im-B group until 4 weeks. At 8 weeks, there was a small difference in the number of CD68-positive cells between the two groups.

Gene expression analyses

Gene expression levels related to collagenase (MMP-8 and MMP-13) in the cartilage of the contact area are shown in Fig. 6. Gene expression of MMP-8 and MMP-13 at 2 weeks in the femur and those of MMP-8 at 2 weeks in the tibia were significantly increased in the Im-B group compared to the Im-NS group. MMP-8 and MMP-13 gene expression levels in the non-contact area are shown in Fig. 7. MMP-8 at 2 weeks and MMP-13 at 4 weeks in both the femur and tibia were significantly increased in the Im-B group compared to the Im-NS group. Inflammation-related gene expression (IL-1β and TNF-α) in the cartilage of contact and non-contact areas is shown in Figs. 8 and 9. There was no significant difference in IL-1β gene expression. TNF-α gene expression of the femoral contact area was significantly increased in the Im-B group compared to the Im-NS group at 2 weeks. By contrast, expression in the non-contact area of the femur at 8 weeks and tibia at 4 and 8 weeks was significantly increased in the Im-B group compared to the Im-NS group.

Discussion

Our study demonstrated that joint hemorrhage exacerbated articular cartilage degeneration in a rat immobilized knee model. Joint hemorrhage progression decreased the number of chondrocytes, independent of the cartilage area. Joint immobilization prolonged iron deposition and inflammation of the synovium, and joint hemorrhage increased gene expression of MMP-8 and MMP-13 in throughout the cartilage. Further, joint hemorrhage aggravated cartilage degeneration induced by joint immobilization in the contact area. Differences in the contact and non-contact areas of the immobilized knee model were considered due to the degree of mechanical stress or residual mobility [15, 25] [26]. Patellar mobility was

not restricted, and it could move slightly in the medial or lateral direction. For the femoral non-contact area adjoined to the patella, immobility rigidity was lesser than the contact area.

It is known that mechanical stress on cartilage determines the delicate balance between cartilage growth and breakdown [27]. Some studies have reported that mechanical stress induces cartilage degeneration, while others have shown that mild and moderate loading can stimulate cartilage matrix synthesis [27–29]. Joint hemorrhage exerts harmful influences on cartilage matrix turnover and integrity by mechanical loading[30]. In our immobilized knee model, more significant decreases in the number of chondrocytes were observed in both contact and non-contact areas of the Im-B group. However, cartilage degeneration was only severe in the contact area of the Im-B group, suggesting that chondrocyte death by apoptosis or necrosis was stimulated by hemorrhage, regardless of mechanical stress. Thus, avoidance of mechanical stress may prevent progression of cartilage degeneration. In clinical practice of hemophilic arthropathy, articular cartilage is damaged symmetrically and broadly [8]. However, there have been no basic research studies to evaluate the change in cartilage thickness due to joint hemorrhage. A previous study assessed cartilage thickness using a rat immobilized knee model [15], which reported that joint immobilization induced cartilage thickening due to cartilage regeneration induced by compressive and shear force at the joint. In this study, cartilage thickness was not altered by joint hemorrhage and immobilization. The difference might be due to dual effects of cartilage regeneration and degeneration caused by joint immobilization and hemorrhage, the immobilization periods, or the portion of cartilage assessed in the study.

Iron deposition was observed up to 8 weeks in the Im-B group. Jansen et al. described opsonization of injected red blood cells into the joint cavity and recognition as foreign by macrophages and synoviocytes [17]. Additionally, the concentration of red blood cells in the joint cavity decreased to less than 5% within 48 h; however, this time course was sufficient to adversely affect cartilage and synovial tissues [17]. Further, Onoda et al. reported iron deposition for 8 weeks in the synovial membrane and capsule, employing the same model used in this study [18]. The results were consistent with our findings, indicating that immobilization could inhibit hemosiderin absorption and could prolong hemosideric inflammation. CD68-positive cells were mainly observed in the synovial membrane of the Im-B group until 4 weeks; however, there were small differences between both groups at 8 weeks. The effect of joint hemorrhage on inflammation reduced after 4 weeks, after which immobilization was considered as a main cause of inflammation [31, 32].

MMP-8 and MMP-13 play roles as collagenase to cleave type Ⅱ collagen, which is the basis of articular cartilage [33]. MMP-8 is expressed in neutrophils, osteoarthritic chondrocytes, articular chondrocytes, and synovial fibroblasts [34]. MMP-8 interacts with inflammatory cytokines and is contributes to chronic inflammatory diseases. In contrast, MMP-13 is the only collagenase implicated in the degradation of collagenous matrices and has higher enzyme activity than other MMPs in osteoarthritis [35]. In hemophilic arthropathy, MMP-13 is known to be potentially responsible for disease, as well as for rheumatoid arthritis [3]. Our study revealed that the joint hemorrhage significantly increased gene expression of MMP-8 and MMP-13 compared with the control group, regardless of mechanical stress.

The findings indicated that even a single joint hemorrhage could lead to higher expression of MMP-8 and MMP-13, which can cause cartilage breakdown. MMP-13 gene expression increased at 2 weeks in the contact area, while at 4 weeks in the non-contact area of the Im-B group compared to the Im-NS group. Mechanical stress and strain were associated with MMP-13 activation and synthesis [36] [37], and the difference in the period in our study could be due to the influence of mechanical stress.

There are numerous studies investigating inflammatory cytokines such as IL-1α, IL-1β, and TNF-α in joint disorders, including osteoarthritis [10], rheumatoid arthritis, posttraumatic osteoarthritis [14], and hemophilic arthropathy [2, 6]. These cytokines were detected in synovial fluid, synovial membrane, and cartilage [38], affecting chondrocytes and resulting in tissue destruction [10]. It has been considered that TNF-α promotes acute inflammation, whereas IL-1 plays a pivotal role in sustaining inflammation and cartilage destruction [39]. IL-1β and TNF-α regulate MMPs activation [38], and the activation of IL-1β and TNF-α originally precedes MMPs [40]. In our study, TNF-α gene expression in the contact area was higher in the Im-B group than the Im-NS group at 2 weeks, which could lead to subsequent MMPs expression. Mechanical stress activates TNF-α expression [41]. Joint hemorrhage also increases TNF-α gene expression [6, 40], which might strengthen the effect of mechanical stress [40]. Conversely, TNF-α gene expressions in the non-contact area was higher in the Im-B group than the Im-NS group at 4 or 8 weeks. It might be due to chronic inflammation induced by prolonged deposition of hemosiderin without mechanical stress [41]. Additionally, there was no significant difference in IL-1β gene expression between hemorrhage and control groups. The effect of immobilization might exceed that of hemorrhage regarding IL-1β gene expression.

Thus, results of this study could have clinical implications. Joint immobilization exacerbated cartilage degeneration induced by hemorrhage. Therefore, drainage of the joint hemorrhage and avoidance of loading could be an important treatment option to prevent acceleration of articular cartilage degeneration with immobilization-induced joint hemorrhage.

Further, this study had several limitations. First, the actual amount of blood administered into the joint was not assessed. Second, we did not quantify protein expression related to the pathogenesis. Third, the gene expression was not evaluated within 2 weeks.

Conclusions

Joint hemorrhage exacerbated immobilization-induced articular cartilage degeneration. Therefore, drainage of a joint hemorrhage and avoidance of loading are recommended to prevent cartilage degeneration during immobilization of a joint hemorrhage.

Abbreviations

Im-B
immobilized blood injection injection

Im-NS
immobilized-normal saline injection
MMP
metalloproteinase
IL
interleukin
EF1α1
elongation factor-1α1
TNF
tumor necrosis factor
NO
nitric oxide
PFA
paraformaldehyde
EDTA
ethylenediaminetetraacetic acid
CD
cluster of differentiation
IgG
immunoglobulin G
DAB
diaminobenzidine tetrahydrochloride
cDNA
complementary DNA

Declarations

Ethical approval and consent to participate

The study protocol was reviewed and approved by the Institutional animal research committee of Tohoku University prior to the experiments (approval number: 2013 MdA-360).

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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No finding was recieved

Author’s contributions

YS participated in the design of the research, statistical analysis of the data, and wrote the manuscript. YH is the corresponding author of this study. YY and YH participated in the design of the research and helped to draft and edit the manuscript. TM and YO participated in the design of the research. TS, NI, SY, TY, KS, and TO participated in data collection, helped to analyze the data, and drafted the manuscript. EI conceived the study, collected the data, and helped to analyze the data and draft and edit the manuscript. All authors read and approved the final manuscript.

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Figures

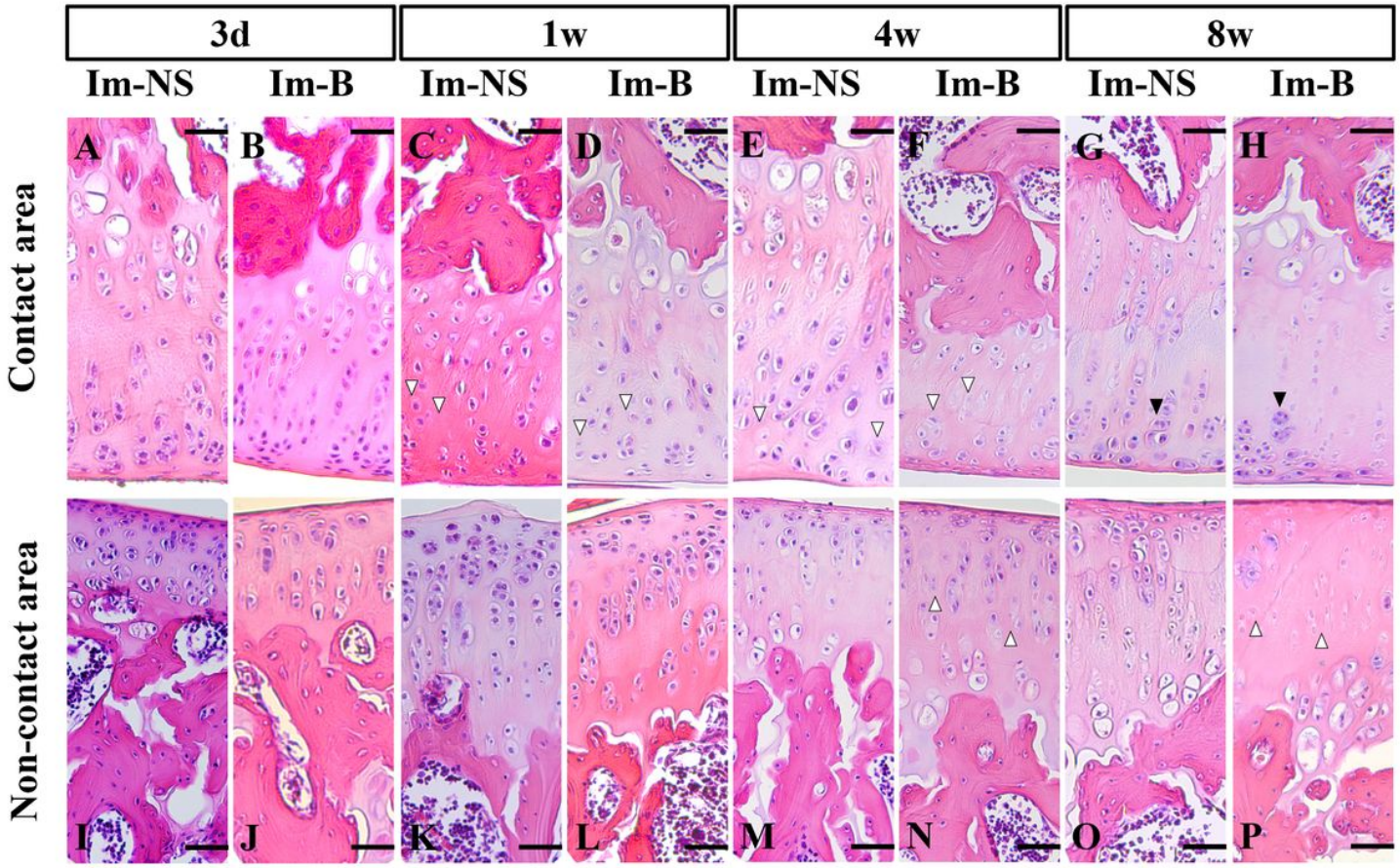

Figure 1

Histological features of femoral articular cartilage after immobilization and intra-articular administration of blood (Im-B) or normal saline (Im-NS) from 3 days to 8 weeks. Upper row (A-H) showing cartilage changes in contact area. Cartilage degeneration and decreasing number of chondrocytes gradually progressed after immobilization. Degeneration of the non-contact area is shown in lower row (I-P). Although similar cellularity was observed in contact and non-contact areas, cartilage degeneration was milder in non-contact areas. Hematoxylin and Eosin staining. White arrowheads indicate cells with decreased cell staining intensity. Black arrowheads indicate cloning cells. Scale bars = 50 μm

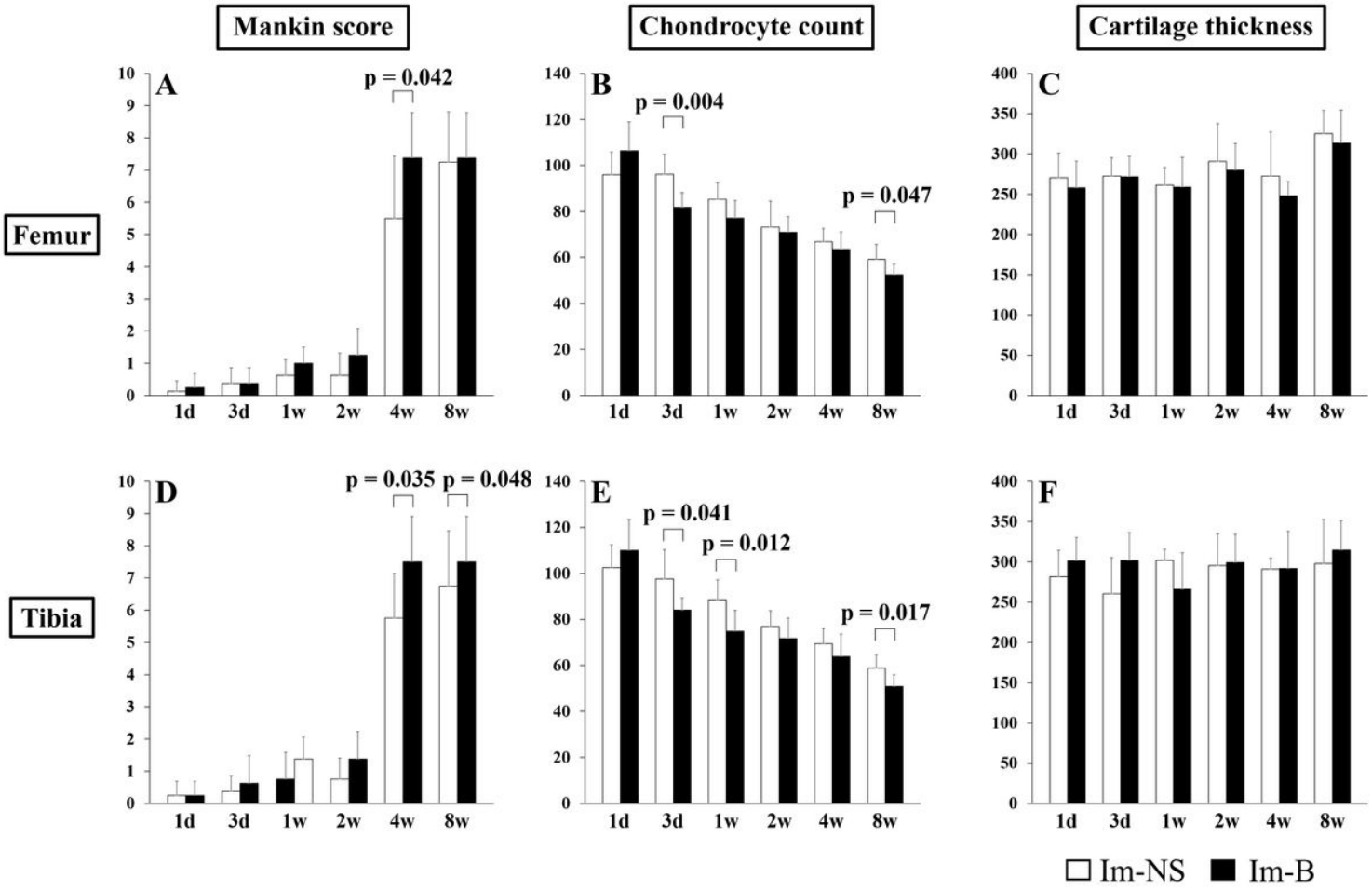

Figure 2

Time-lapse changes in femoral and tibial cartilage of contact area by Mankin score, chondrocyte count, and cartilage thickness. Mankin scores of the Im-B group were significantly higher than the Im-NS group at 4 weeks in femurs and 4 and 8 weeks in tibias (A and D). The number of chondrocytes in the Im-B group significantly decreased at 3 days and 8 weeks in the femur and 3 days and 1 and 8 weeks in the tibia compared to the Im-NS group (B and E). Cartilage thickness did not change and was not affected by immobilization and intra-articular administration (C and F, Fig 3C and F).

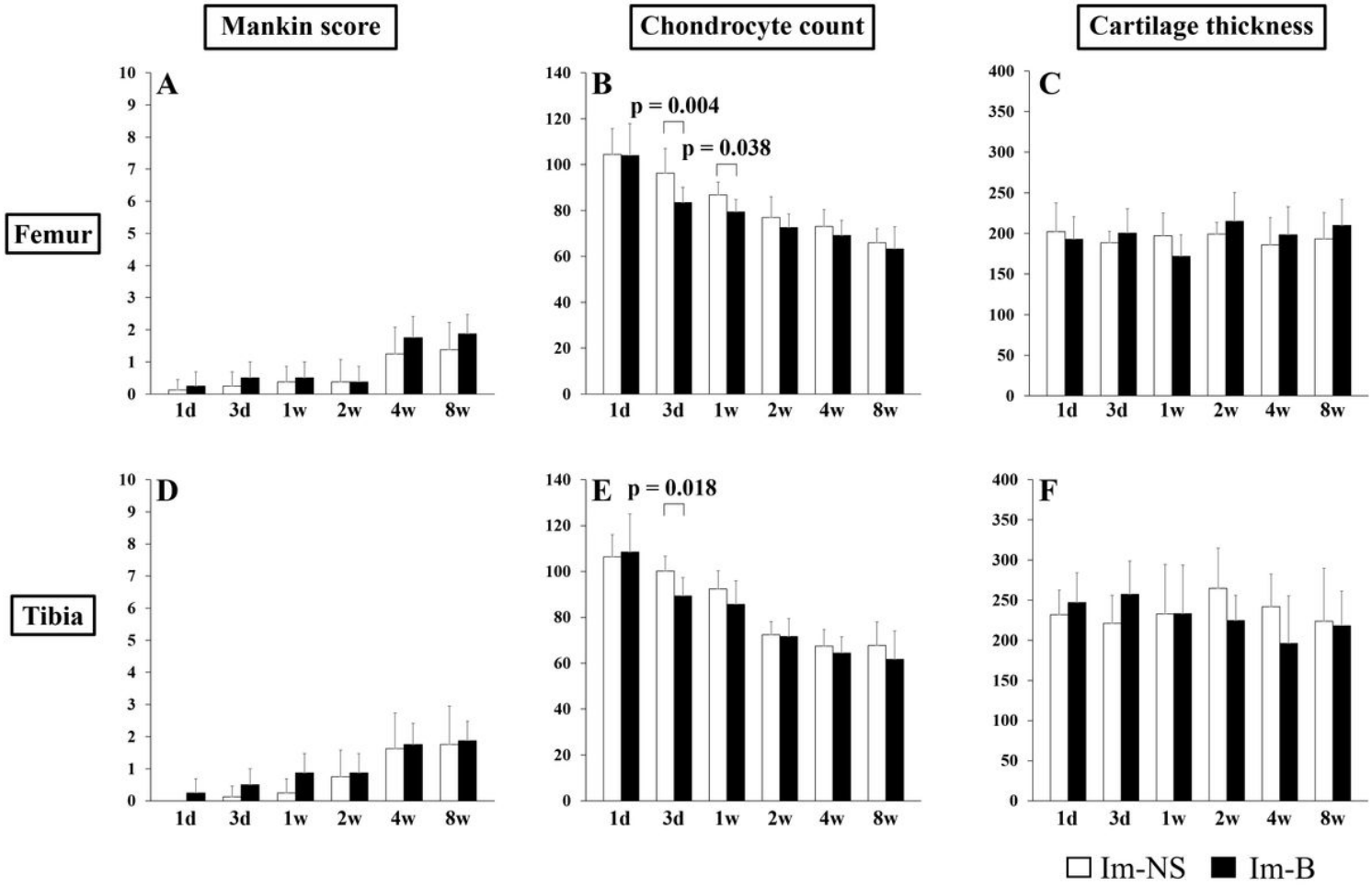

Figure 3

Time-lapse changes in femoral and tibial cartilage of non-contact area by Mankin score, chondrocyte count, and cartilage thickness. Mankin scores did not severely progress, and there was no statistical difference between Im-B and Im-NS groups (A and D). The number of chondrocytes in the Im-B group significantly decreased at 3 days and 1 week in the femur and 3 days in the tibia (B and E). Cartilage thickness did not change and was not affected by immobilization and intra-articular administration in both contact and non-contact areas (C and F).

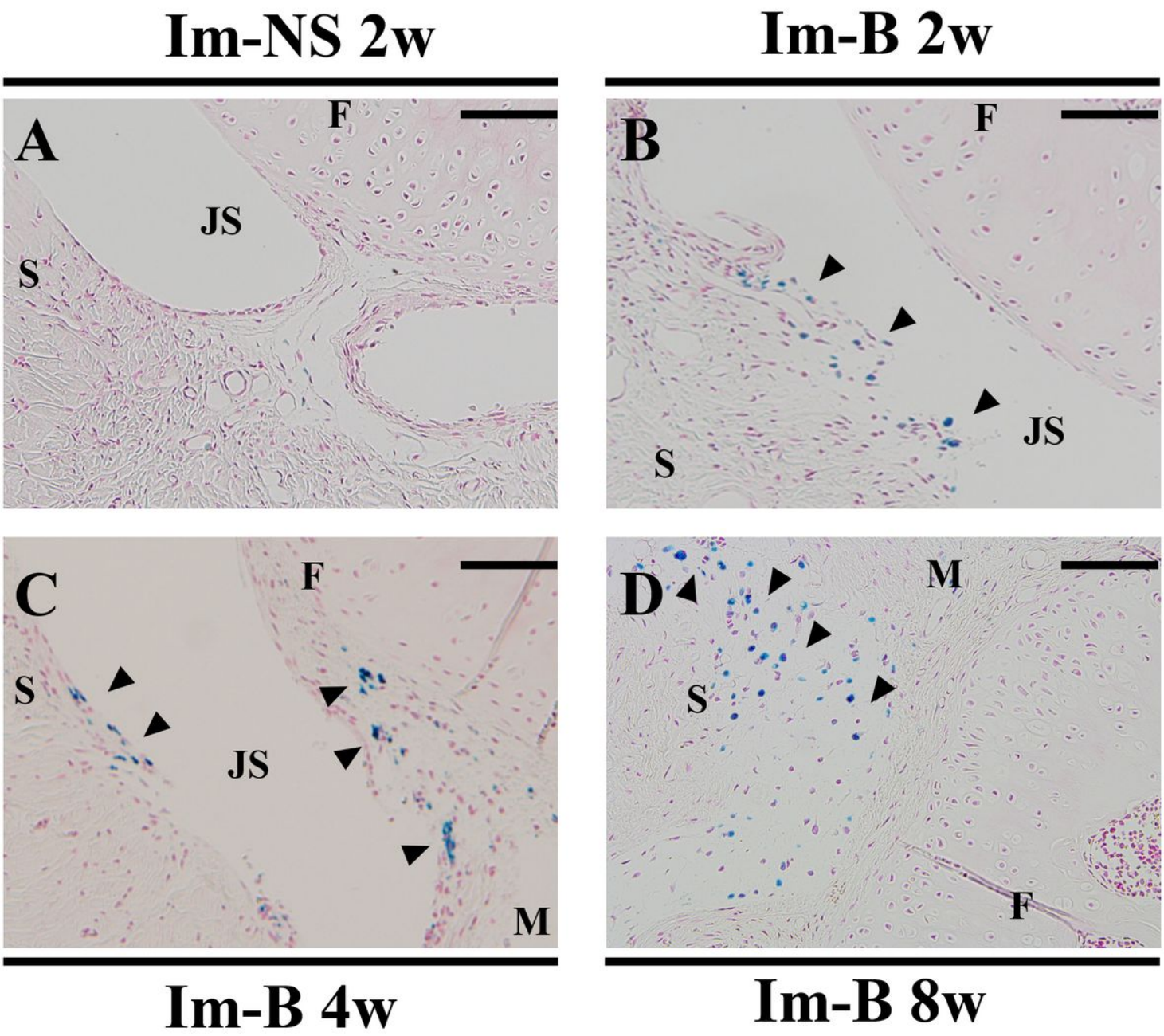

Figure 4

Iron deposition in synovial membrane and meniscal surface. There was no iron deposition in the Im-NS group (A). Iron deposition was observed in synovium and on the meniscal surface, lasting until 8 weeks after administration in the Im-B group (B-D). F: Femur, S: Synovium, JS: Joint Space, M: Meniscus. Perls' Prussian blue staining. Black arrowheads indicate iron depositions. Scale bars = 100 μm

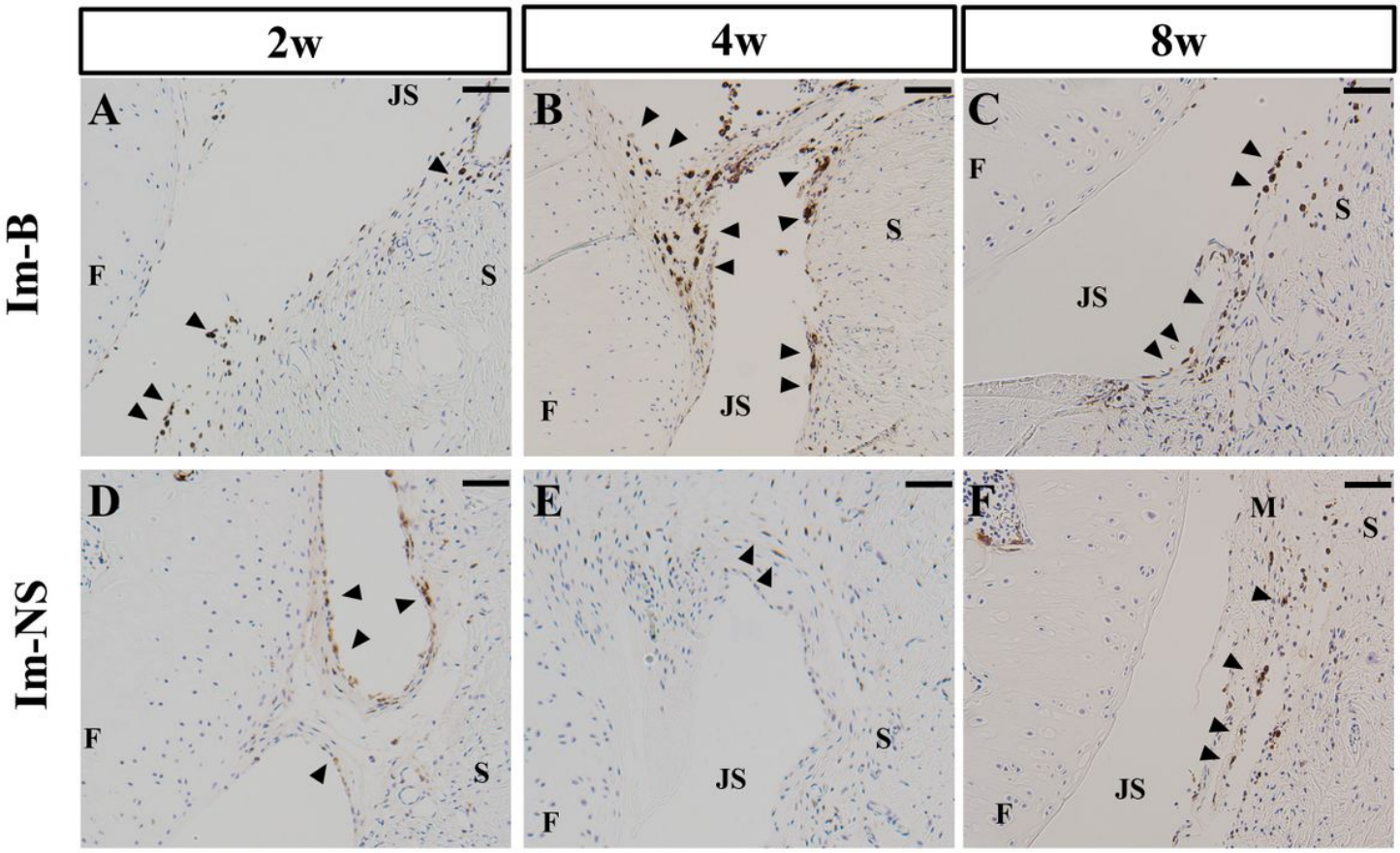

Figure 5

CD68 immunostaining in Im-B and Im-NS groups. CD68-positive cells were observed in both Im-B (A-C) and Im-NS groups (D-F). It was observed mainly in the synovial membrane of the Im-B group. After 8 weeks, there was a small difference in the number of CD68-positive cells between the two groups. F: Femur, S: Synovium, JS: Joint Space, M: Meniscus. Black arrowheads indicate CD68-positive cells. Scale bars = 100 μm

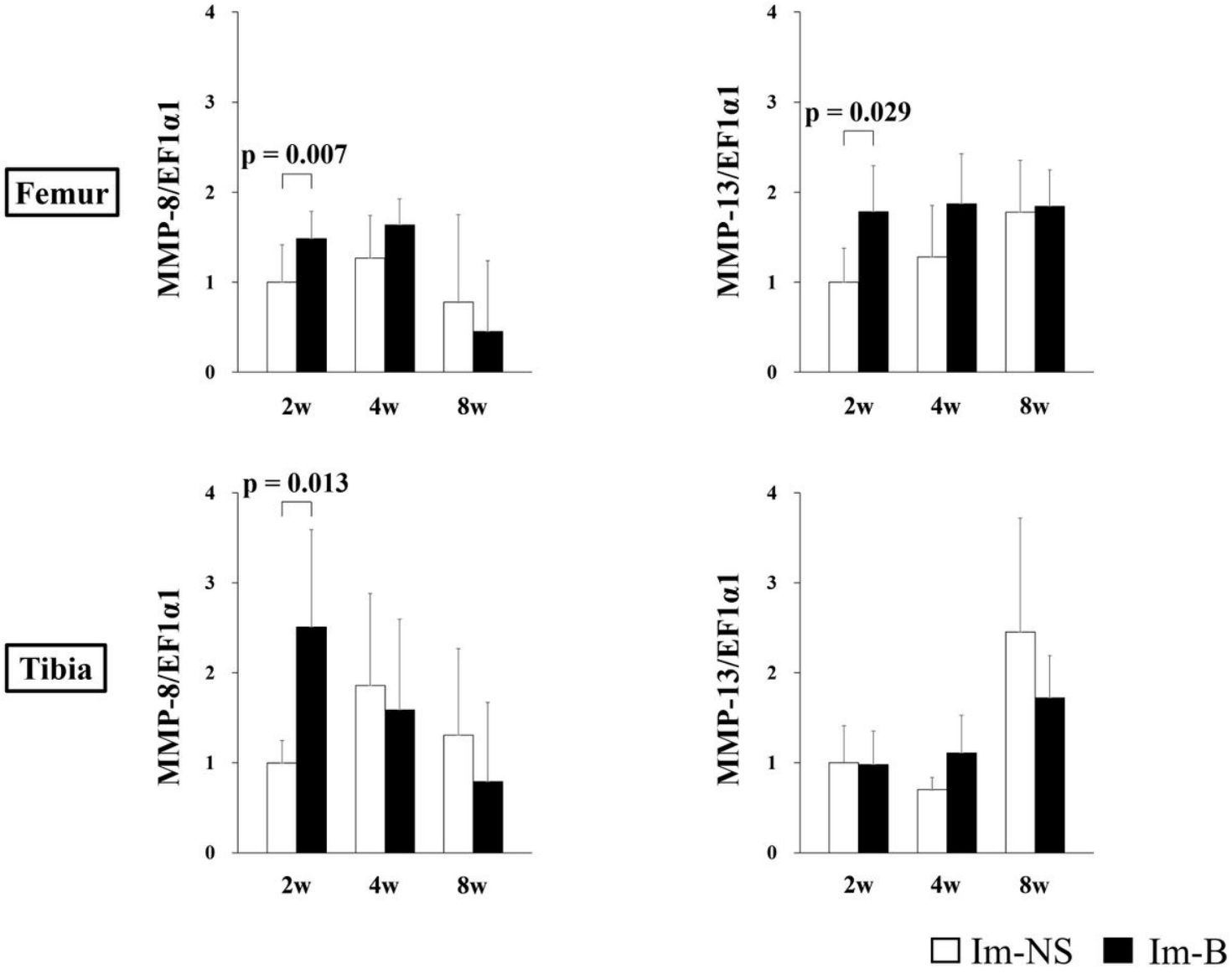

Figure 6

Gene expression levels of matrix metalloproteinases-8 (MMP-8) and MMP-13 in cartilage of the contact area. MMP-8 and MMP-13 gene expression at 2 weeks in the femur and MMP-8 expression at 2 weeks in the tibia were significantly increased in the Im-B group compared to the Im-NS group.

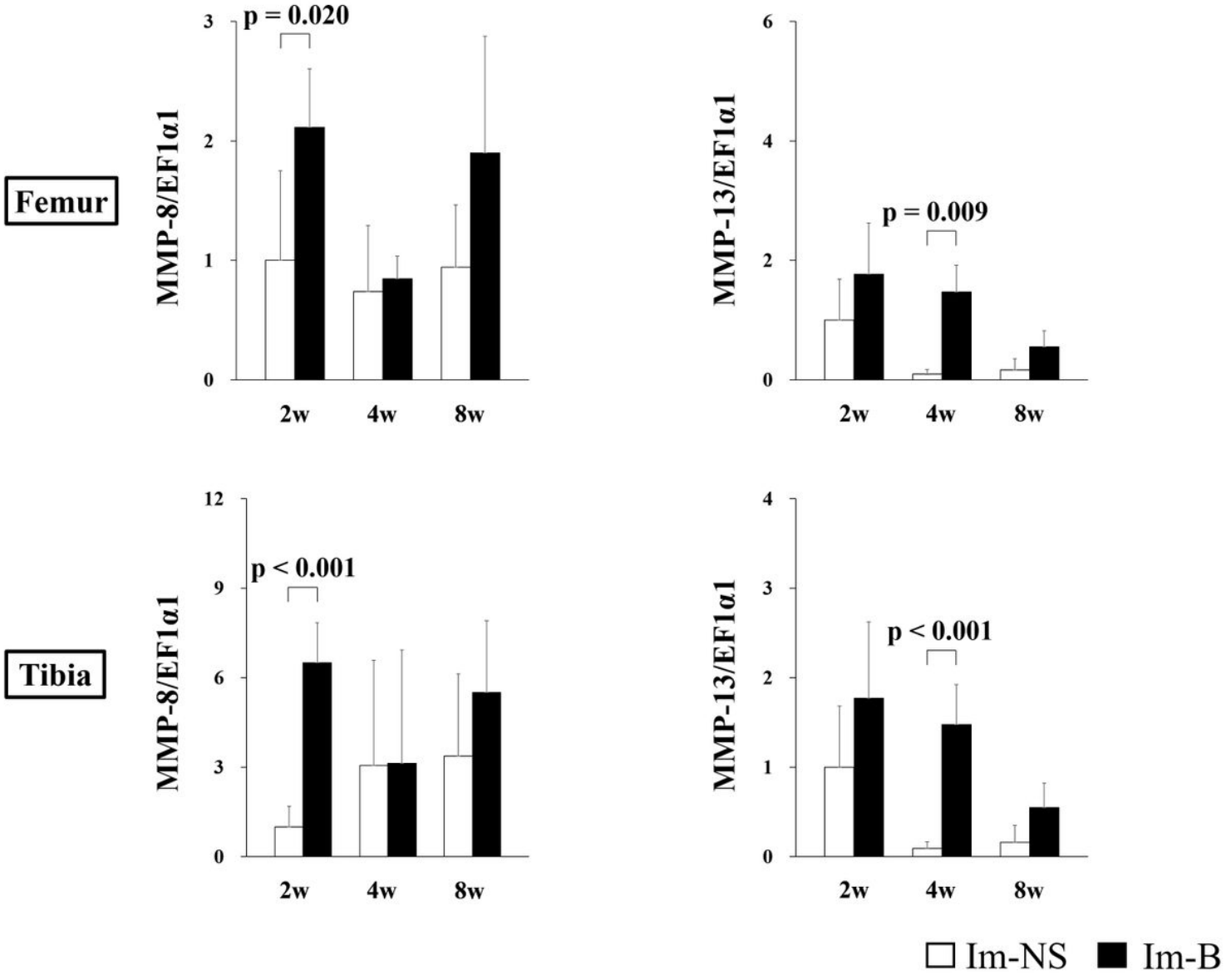

Figure 7

Gene expression levels of matrix metalloproteinases- 8(MMP-8) and MMP-13 in cartilage of the non-contact area. MMP-8 gene expression at 2 weeks and MMP-13 expression at 4 weeks in both the femur and tibia were significantly increased in the Im-B group compared to the Im-NS group.

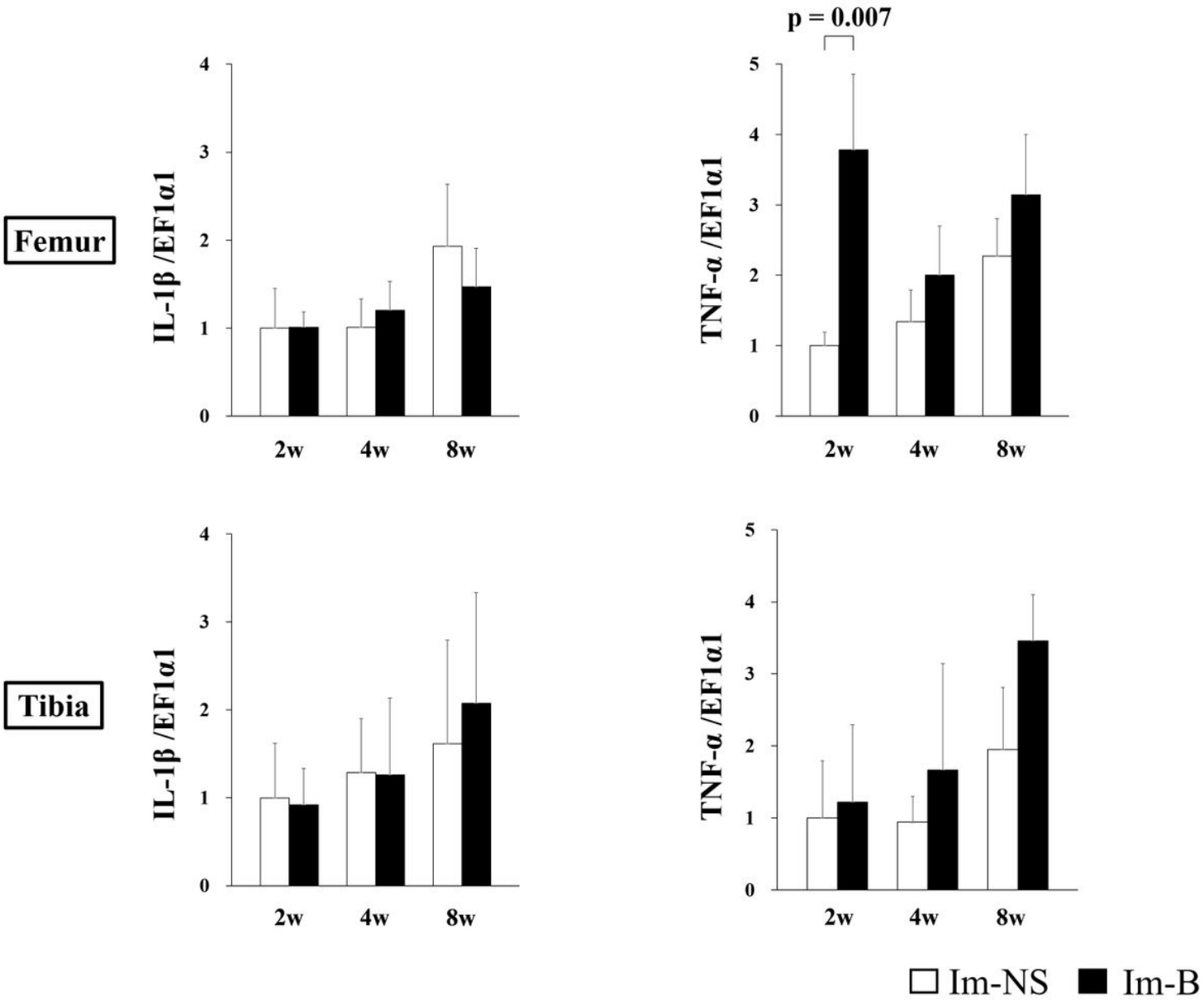

Figure 8

Gene expression of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in cartilage of the contact area. There was no significant difference in IL-1β gene expression. TNF-α expression in the femur was significantly increased in the Im-B group compared to the Im-NS group at 2 weeks.

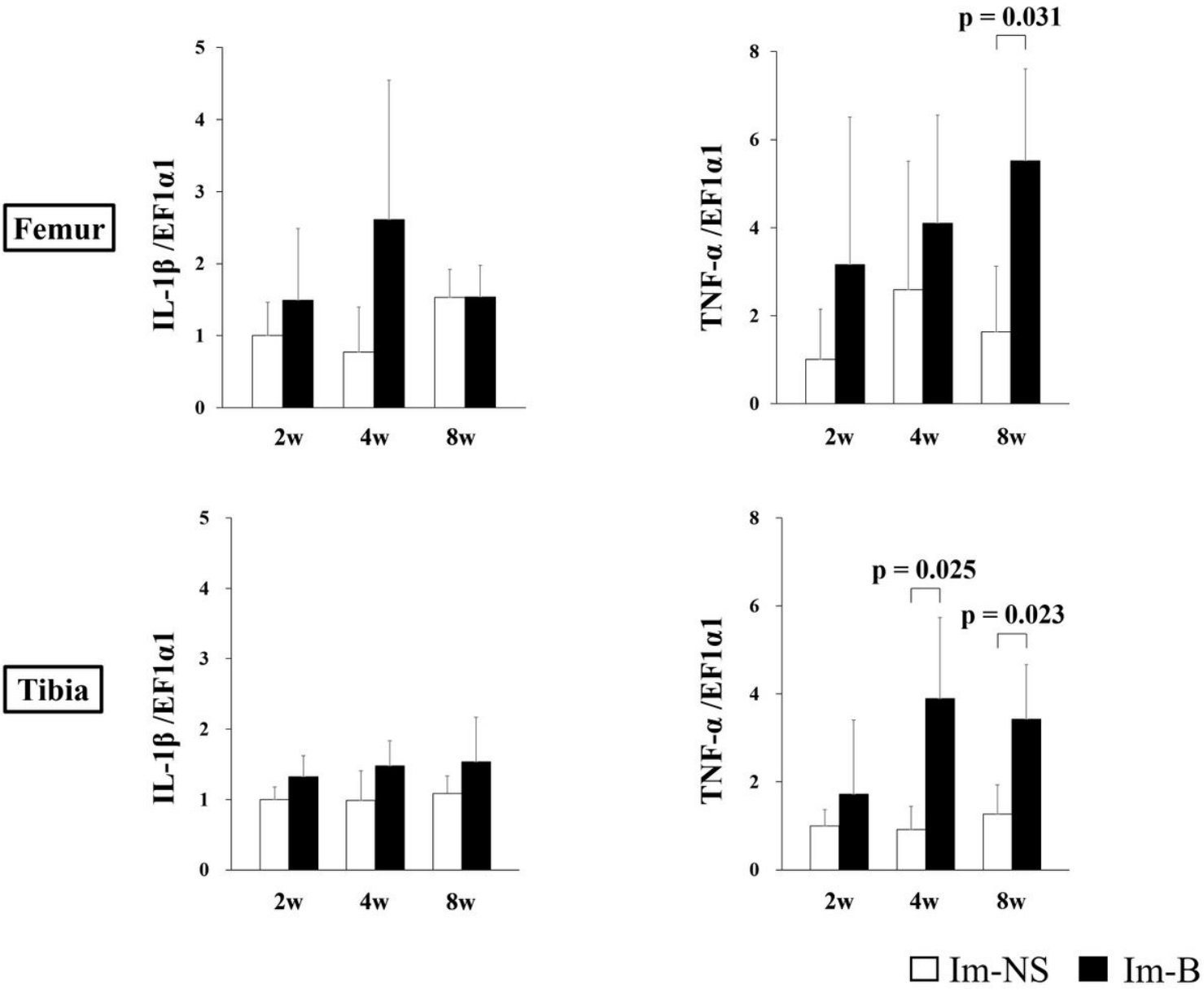

Figure 9

Gene expression of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in cartilage of the non-contact area. There was no significant difference in IL-1β gene expression. TNF-α expression levels in the femur at 8 weeks and tibia at 4 and 8 weeks were significantly increased in the Im-B group compared to the Im-NS group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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