

# Joint hemorrhage accelerates cartilage degeneration in a rat immobilized knee model

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## Research article

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# Abstract

**Background:** Joint hemorrhage is caused by trauma, ligament reconstruction surgery, and bleeding disorders such as hemophilia. Recurrence of hemorrhage in the joint space induces hemosiderotic synovitis and 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 joint hemorrhage has negative effects on the articular cartilage, there is no consensus on whether a reduction in joint hemorrhage would effectively prevent articular cartilage degeneration. The purpose of this study was to investigate the effect of joint hemorrhage combined with joint immobilization on articular cartilage degeneration in a rat immobilized 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 randomly divided into the following groups: immobilized blood injection (Im-B) and immobilized-normal saline injection (Im-NS) groups. The cartilage was evaluated in two areas (contact and non-contact areas). The cartilage was used to assess chondrocyte count, Modified Mankin score, and cartilage thickness. The total RNA was extracted from the cartilage in both areas, and the expression of metalloproteinase (*MMP*)-8, *MMP*-13, interleukin (*IL*)-1 $\beta$ , and tumor necrosis factor (*TNF*)- $\alpha$  was measured by quantitative real-time polymerase chain reaction.

**Results:** The number of chondrocytes in the Im-B group significantly decreased in both areas, compared with that in the Im-NS group. Modified Mankin score from 4–8 weeks of the Im-B group was significantly higher than that of the Im-NS group only in the contact area. The expression of *MMP*-8 and *MMP*-13 from 2 to 4 weeks and *TNF*- $\alpha$  from 2 to 8 weeks significantly increased in the Im-B group compared with those in the Im-NS group, but there was no significant difference in *IL*-1 $\beta$  expression.

**Conclusions:** The results showed that joint hemorrhage exacerbated immobilization-induced articular cartilage degeneration. Drainage of a joint hemorrhage or avoidance of loading may help prevent cartilage degeneration during joint immobilization with a 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*)-1 $\beta$  [6-8] and tumor necrosis factor (*TNF*)- $\alpha$  [6, 9] are produced in the hemosiderin-laden synovial tissue, and these cytokines trigger

catabolic programs, activating nitric oxide (NO) [1, 6] and matrix metalloproteinase (MMP) expression [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 that induce joint bleeding, including bone marrow stimulation, have been proven to be effective for treating cartilage defects of the knee through cartilage regeneration [11]. Additionally, a similar procedure was reported to enhance healing of the torn meniscus [12]. However, the beneficial effects have been shown only for damaged tissues, and its clinical outcome has been demonstrated only for a short term [7]. So far, the effects of hemorrhage on normal cartilage have not been considered.

Posttraumatic osteoarthritis after fracture or ligament injury occasionally occurs even with appropriate treatment or surgery [13]. According to previous studies, 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 the articular cartilage [15] and contributes to osteoarthritis development [16]. Blood in the joint is cleared rapidly within 48 h without joint immobilization [17]; however, a study reported protraction of residual blood in the joint-by-joint immobilization [18]. Thus, joint immobilization may exacerbate the harmful effect of blood on joint cartilage; although, this has not been investigated. Therefore, in this study, we investigated the effect of joint hemorrhage combined with joint immobilization on articular cartilage degeneration in a rat immobilized knee model.

## Methods

### *Animals*

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

### *Tissue preparation*

The specimens were prepared for evaluation according to a previous study [21]. After euthanasia by intraperitoneal injection of sodium pentobarbital overdose, the rats were fixed with 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 maintained 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 with ethanol and xylene solutions, the specimens were embedded in paraffin. They were cut into 5- $\mu$ m sagittal sections in the medial midcondylar region of the knee and used for evaluation [21].

### *Histological evaluation*

Hematoxylin and eosin staining and Safranin O staining were performed to assess cartilage degeneration, chondrocyte count, and cartilage thickness in the contact and non-contact areas of the femur and tibia, respectively (1 and 3 days, and 1, 2, 4, and 8 weeks after surgery; n = 6/each group) [20]. Cartilage degeneration was evaluated using the Modified Mankin histological grading scheme [22]. The chondrocytes were 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]. Synovitis (including pannus, synovial hyperplasia and sub-synovial inflammation) was evaluated using a synovial scoring system [23]. Additionally, hemosiderin deposition was evaluated by Perls' Prussian blue staining (2, 4, and 8 weeks after surgery).

### *Immunohistochemistry*

The sections at 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 [24]. Endogenous peroxidase was inactivated with 3% H<sub>2</sub>O<sub>2</sub> 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 the secondary antibody and incubated 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<sub>2</sub>O<sub>2</sub> as the chromogen. Counterstaining was performed using Carazzi's hematoxylin. For the negative control, normal mouse IgG (Dako, Copenhagen, Denmark) was used as the primary antibody. All slides were stained in one session. CD68-positive cells were enumerated at the posterior capsule including joint space and synovium [25].

### *Gene expression analyses*

After euthanasia, the cartilage in the contact and non-contact areas from the femur and tibia was obtained using a surgical knife and rongeur. The harvested cartilage samples were immediately immersed in 1 mL of QIAzol (Qiagen, Hilden, Germany). The samples were crushed and homogenized with a Polytron (Kinematica AG, Lucerne, Switzerland). The total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized using the Cloned AMV First-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The efficiency of polymerase chain reaction (PCR) and relative expression levels of *MMP-8*, *MMP-13*, *IL-1 $\beta$* , and *TNF- $\alpha$*  as a function of elongation

factor-1 $\alpha$ 1 (EF1 $\alpha$ 1) were calculated as described previously (2, 4, and 8 weeks after surgery; n = 6/each group) [26]. The primer sequences used in the PCR are listed in Table 1.

**Table 1:** Sequence of Primers Used for Polymerase Chain Reaction

Gene Name	GenBank	Nucleic Acid Sequence	
<b><i>MMP-8</i></b>	NM 022221	Upstream	tggaatccttgcccatgcct
		Downstream	aagcagccacgagaaacaggt
<b><i>MMP-13</i></b>	NM 133530	Upstream	tggctcttctggcacaacgctt
		Downstream	tggaagctgcttgtccaggtt
<b><i>IL-1<math>\beta</math></i></b>	NM 031512	Upstream	accgtggcaacattctgggtca
		Downstream	tcgacaaatgctgcctcgtga
<b><i>TNF-<math>\alpha</math></i></b>	NM 0126763	Upstream	actggcgtgttcaatccgttctctac
		Downstream	ccgcaaaatccaggccactacttc
<b><i>EF1<math>\alpha</math>1</i></b>	NM 175838	Upstream	tgatgccccaggacacaagagaact
		Downstream	gataccagcttcaaaattccaacaac

### *Statistical analyses*

Differences between the Im-B and Im-NS groups were assessed using unpaired *t*-test (histological evaluation: Mankin score, chondrocyte count, and cartilage thickness, CD68-positive cell count) and Mann–Whitney's *U* test (RT-qPCR). Data are presented as mean  $\pm$  standard deviation (SD). Tests were two-sided, and a value of  $p < 0.05$  indicated statistical significance. 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 using 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 Im-B and Im-NS groups. The degeneration progressed gradually after immobilization, and it was more significant in the contact area (Fig. 1 a-h). At 3 days after immobilization and intra-articular administration, swelling of the chondrocytes and disappearance of cells in the tangential zone were observed, 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, which was more severe in the Im-B group, decreased at 4 weeks (Fig. 1e and f). Cartilage surface irregularity was observed at 2 weeks in the

Im-B group (Supplementary Fig. 1). Cell cloning and cell loss occurred at 8 weeks (Fig. 1g and h). Degeneration of the non-contact area is shown in the lower row of Fig. 1 (i-p). Although a similar cellularity was observed in the contact and non-contact areas, cartilage degeneration was milder in the non-contact areas than in the contact areas. A reduction in Safranin O staining intensity was observed at 8 weeks in both groups, and it was more severe in the Im-B group than in the Im-NS group (Supplementary Fig. 2).

Mankin score of the Im-B group was significantly higher than that of 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 significant change in the non-contact area, and the difference between the Im-B and Im-NS groups was not significant (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 with that in the Im-NS group (Fig. 2b and e). In the non-contact area of the Im-B group, the number of chondrocytes 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 it was not affected by immobilization and intra-articular administration in both contact and non-contact areas (Fig. 2c and f, Fig. 3c and f). The synovitis score of the Im-B group was slightly but not significantly higher than that of the Im-NS group at all periods (Supplementary Fig. 3).

Iron deposition evaluated by Perls' Prussian blue staining is shown in Fig. 4. The injection of blood 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 the administration of blood in the Im-B group (Fig. 4b-d). CD68-positive cells were observed in both Im-B and Im-NS groups, and primarily, in the synovial membrane (Fig. 5). The number of CD68-positive cells was slightly but not significantly higher at 4 weeks in Im-B group and 8 weeks in Im-NS group (Supplementary Fig. 4).

### *Gene expression analyses*

The expression of genes related to collagenase (*MMP-8* and *MMP-13*) in the cartilage of the contact area is shown in Fig. 6. The expression of *MMP-8* and *MMP-13* in the femur at 2 weeks and that of *MMP-8* in the tibia at 2 weeks were significantly increased in the Im-B group compared with those in the Im-NS group. *MMP-8* and *MMP-13* expression in the non-contact area is shown in Fig. 7. The expression of *MMP-8* at 2 weeks and *MMP-13* at 4 weeks in both femur and tibia was significantly increased in the Im-B group compared with that in the Im-NS group. The expression of inflammation-related genes (*IL-1 $\beta$*  and *TNF- $\alpha$* ) in the cartilage in the contact and non-contact areas is shown in Figs. 8 and 9. There was no significant difference in *IL-1 $\beta$*  expression. *TNF- $\alpha$*  expression in the femoral contact area was significantly increased in the Im-B group compared with that in the Im-NS group at 2 weeks. In contrast, its 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 with that in 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. Iron deposition in and inflammation of the synovium persisted until 8 weeks after blood injection, and joint hemorrhage increased the expression of *MMP-8* and *MMP-13* throughout the cartilage. Furthermore, joint hemorrhage aggravated cartilage degeneration induced by joint immobilization in the contact area. The differences in the contact and non-contact areas of the immobilized knee model can be attributed to the degree of mechanical stress or residual mobility [15, 27, 28]. Patellar mobility was not restricted, and it could move slightly in the medial or lateral direction. In the femoral non-contact area adjoined to the patella, immobility rigidity was less than that in the contact area.

Mechanical stress on cartilage determines the delicate balance between cartilage growth and breakdown [29]. Some studies have reported that mechanical stress induces cartilage degeneration, whereas others have shown that mild and moderate loading can stimulate cartilage matrix synthesis [29-31]. Joint hemorrhage, when combined with mechanical loading, exerts harmful effects on cartilage matrix turnover and integrity [32]. In our immobilized knee model, 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 severe only in the contact area of the Im-B group. A previous study showed that iron-induced synovitis and hydroxy radicals cause chondrocyte apoptosis [7], but we did not assess it. The results of this study suggested that chondrocyte death by apoptosis or necrosis was stimulated by hemorrhage, regardless of mechanical stress. Thus, avoidance of mechanical stress may prevent the progression of cartilage degeneration. In clinical practice of hemophilic arthropathy treatment, articular cartilage is damaged symmetrically and broadly [8]. However, there have been no studies to evaluate the changes in cartilage thickness due to joint hemorrhage. A previous study assessed cartilage thickness using a rat immobilized knee model [15] and 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 the dual effects of cartilage regeneration and degeneration caused by joint immobilization and hemorrhage, immobilization periods, or portion of cartilage assessed in the study.

Iron deposition was observed for 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 the cartilage and synovial tissues [17]. Furthermore, 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 prolong hemosideric inflammation. The synovitis score was slightly but not significantly higher in the Im-B group than in the Im-NS group, and CD68-positive cells were primarily observed in the synovial membrane, indicating that iron deposition potentially induces synovial inflammation. Furthermore, CD68-positive cells tended to be more numerous in the Im-B group than in the Im-NS group at 4 weeks, and this number was similar in

both groups at 8 weeks. The effect of joint hemorrhage on inflammation reduced after 4 weeks, after which immobilization was considered as the main cause of inflammation [25, 33].

MMP-8 and MMP-13 act as collagenases to cleave type I collagen, which is the basis of articular cartilage [34]. MMP-8 is expressed in neutrophils, osteoarthritic chondrocytes, articular chondrocytes, and synovial fibroblasts [35]. It interacts with inflammatory cytokines and contributes to chronic inflammatory diseases. In contrast, MMP-13 is the only collagenase implicated in the degradation of collagenous matrices, and has a higher enzyme activity than other MMPs in osteoarthritis [36]. MMP-13 is known to be involved in hemophilic arthropathy and rheumatoid arthritis [3]. Our study revealed that joint hemorrhage significantly increased the expression of *MMP-8* and *MMP-13* compared with that in the control group, regardless of mechanical stress. The findings indicated that even a single joint hemorrhage could lead to a higher expression of *MMP-8* and *MMP-13*, which can cause cartilage breakdown. *MMP-13* expression increased at 2 weeks in the contact area and at 4 weeks in the non-contact area of the Im-B group compared with that in the Im-NS group. Mechanical stress and strain have been associated with *MMP-13* activation and synthesis [37, 38], and the difference in the time of expression in our study could be due to the influence of mechanical stress.

There are numerous studies on inflammatory cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$  in joint disorders, including osteoarthritis [10], rheumatoid arthritis, posttraumatic osteoarthritis [14], and hemophilic arthropathy [2, 6]. These cytokines are detected in the synovial fluid, synovial membrane, and cartilage [39], affecting chondrocytes and resulting in tissue destruction [10]. It is considered that TNF- $\alpha$  promotes acute inflammation, whereas IL-1 plays a pivotal role in sustaining inflammation and cartilage destruction [40]. IL-1 $\beta$  and TNF- $\alpha$  regulate MMP activation [38], and the activation of IL-1 $\beta$  and TNF- $\alpha$  originally precedes that of MMPs [41]. In our study, *TNF- $\alpha$*  expression in the contact area was higher in the Im-B group than the Im-NS group at 2 weeks, which could lead to the subsequent MMP expression.

Mechanical stress activates TNF- $\alpha$  expression [42]. Joint hemorrhage also increases *TNF- $\alpha$*  expression [6, 41], which might strengthen the effect of mechanical stress [41]. Conversely, *TNF- $\alpha$*  expressions in the non-contact area was higher in the Im-B group than in 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 [42]. Additionally, there was no significant difference in *IL-1 $\beta$*  expression between the hemorrhage and control groups. We speculate that the effect of immobilization on IL-1 $\beta$  gene expression might exceed that of hemorrhage.

The results of this study may have clinical implications. Joint hemorrhage exacerbated cartilage degeneration induced by joint immobilization. Drainage of a joint hemorrhage or avoidance of loading may help prevent cartilage degeneration during joint immobilization with a hemorrhage, and this should be assessed in future studies.

This study had some 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, gene expression was not evaluated within 2 weeks. Fourth, the synovium was not assessed by PCR, and

therefore, we could not show the active synovitis and its influence on cartilage. Fifth, the number of rats was not determined on the basis of the statistical power. Finally, we did not include a normally loaded control with blood injection, which made the difference between the effects of hemorrhage with or without loading unclear; this necessitates further studies.

## Conclusions

Joint hemorrhage exacerbated cartilage degeneration induced by joint immobilization. Drainage of a joint hemorrhage or avoidance of loading may help prevent cartilage degeneration during joint immobilization with a hemorrhage.

## Abbreviations

Im-B: immobilized blood injection

Im-NS: immobilized-normal saline injection

MMP: metalloproteinase

IL: interleukin

EF1 $\alpha$ 1: elongation factor-1 $\alpha$ 1

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 before 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.

## Funding

No funding was received.

## Author's contributions

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

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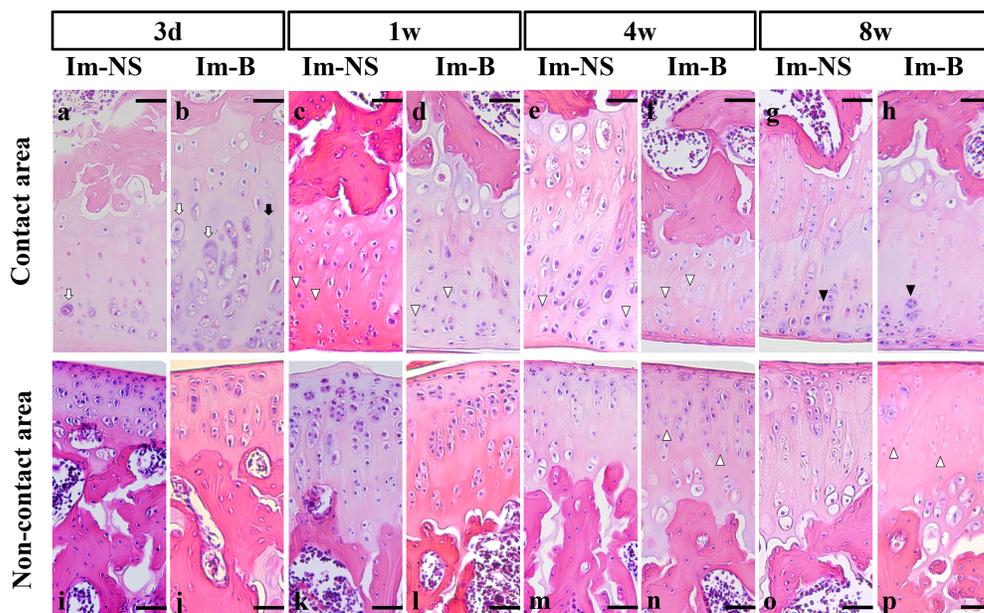
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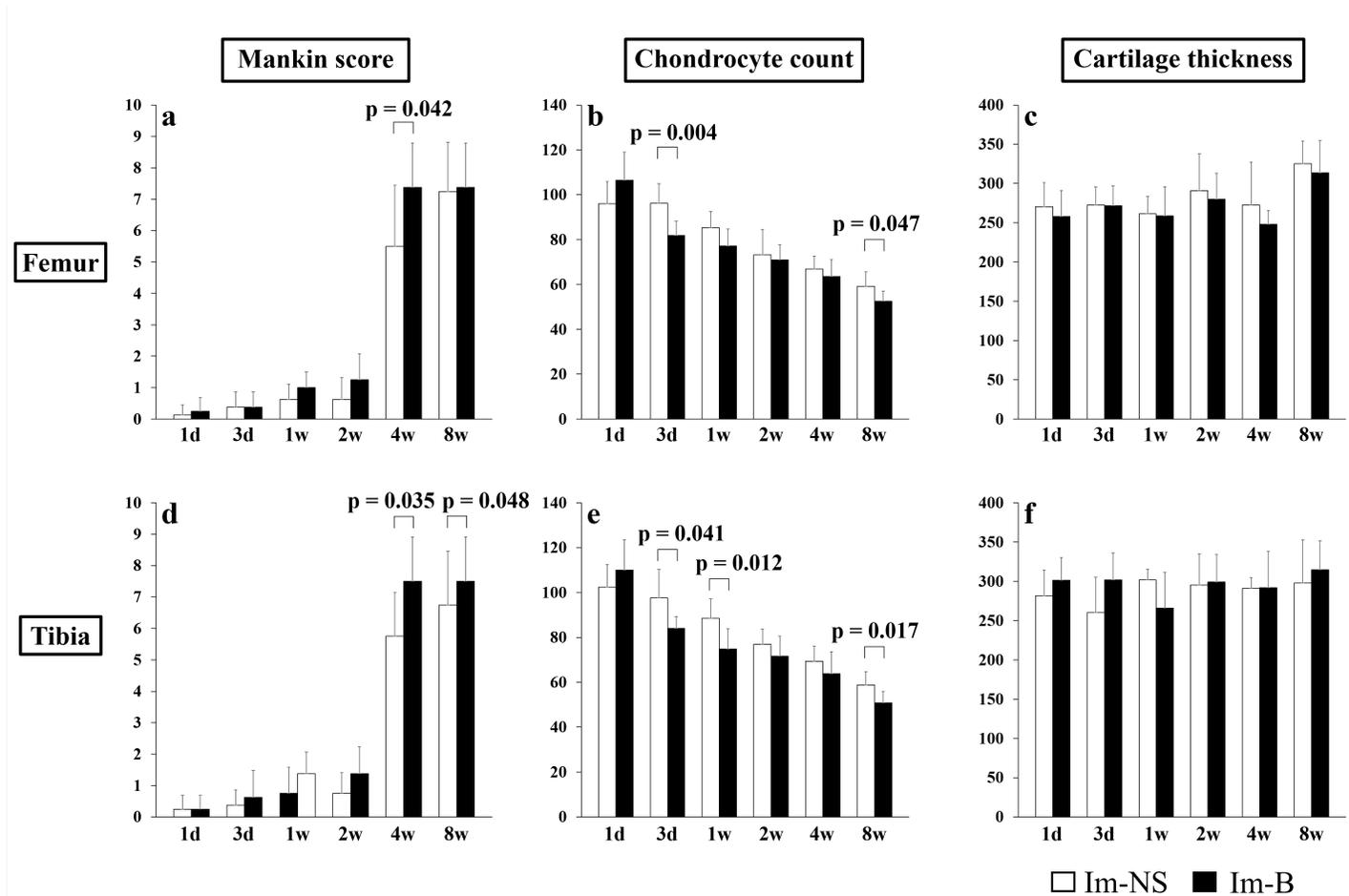
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## Figures



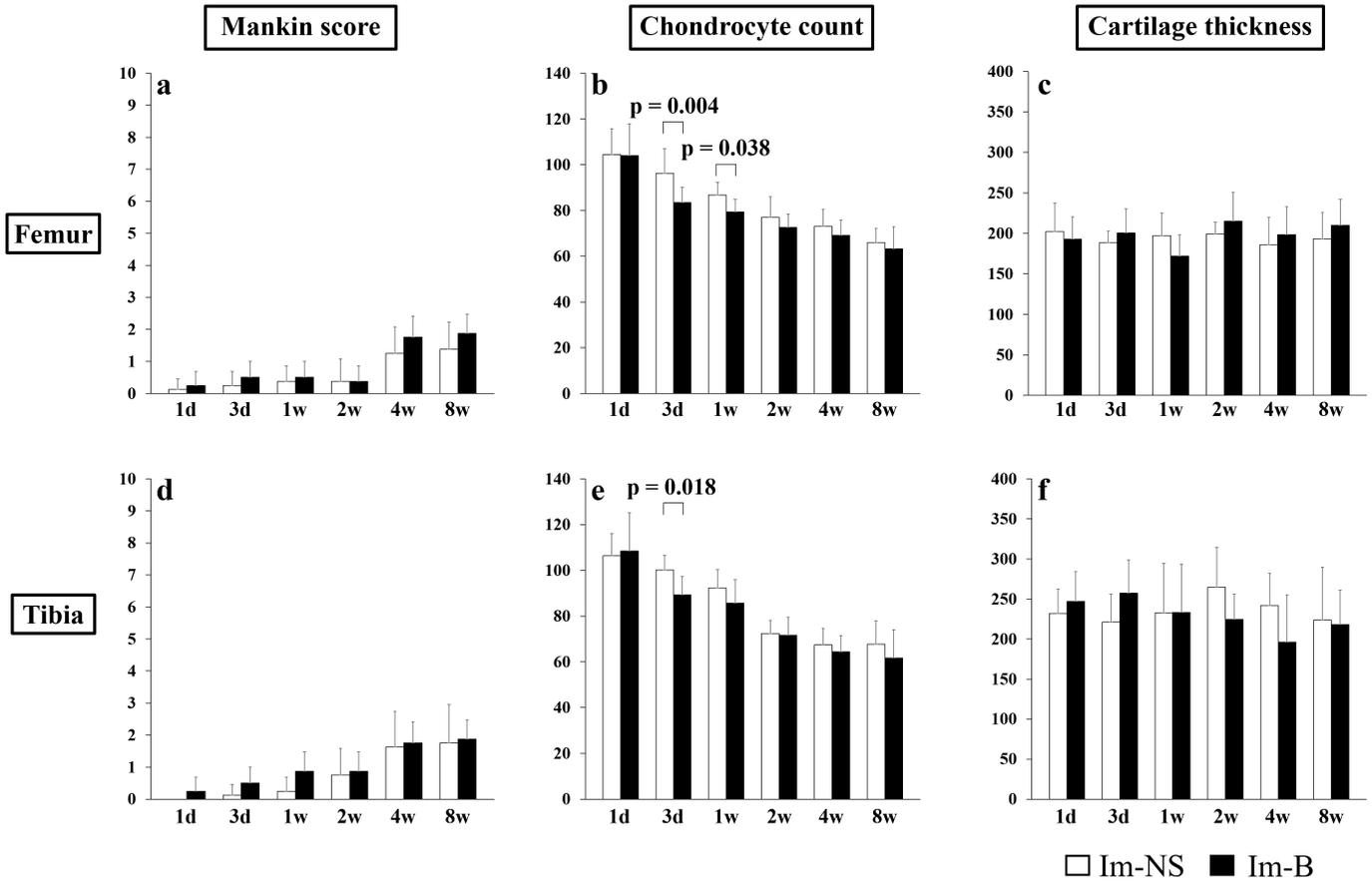
**Figure 1**

Histological features of femoral articular cartilage. The changes were observed 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 the changes in cartilage in the contact area. Cartilage degeneration and decreasing number of chondrocytes gradually progressed after immobilization. Degeneration of the non-contact area is shown in the lower row (i-p). Although similar cellularity was observed in the contact and non-contact areas, cartilage degeneration was milder in the non-contact areas. Hematoxylin and eosin staining. The white arrows indicate the swelling of chondrocytes and the black arrows indicate the disappearance of the cells. The white arrowheads indicate cells with decreased cell staining intensity. The black arrowheads indicate cloning cells. Scale bar = 50  $\mu$ m



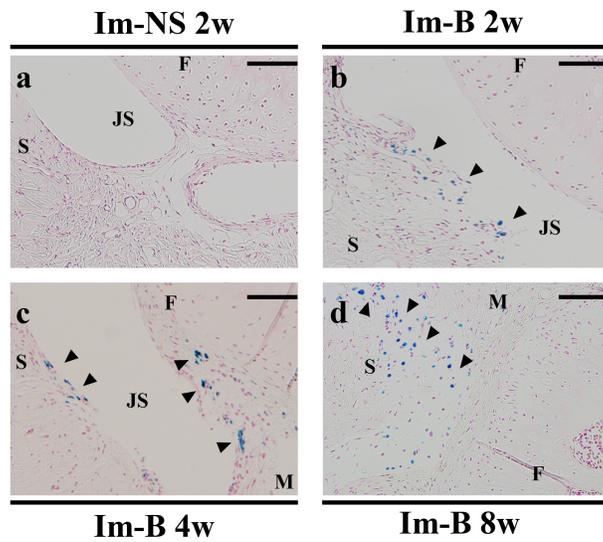
**Figure 2**

Time-lapse changes in the femoral and tibial cartilage in the contact area. Mankin score of the Im-B group was significantly higher than that of the Im-NS group at 4 weeks in the femurs and 4 and 8 weeks in the 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 with that in 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).



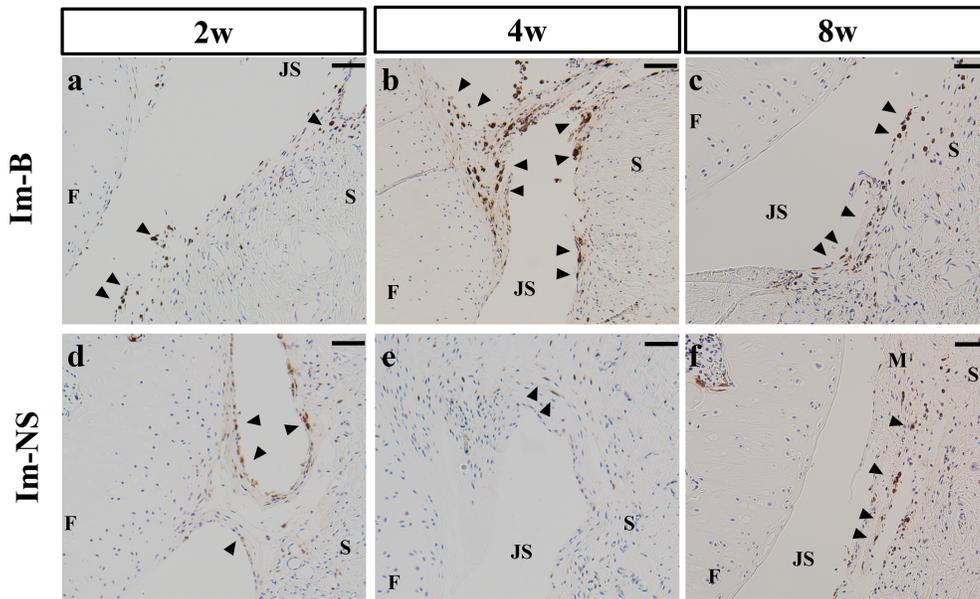
**Figure 3**

Time-lapse changes in the femoral and tibial cartilage in the non-contact area. Mankin score did not change significantly, and there was no statistical difference between the 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 it was not affected by immobilization and intra-articular administration in both contact and non-contact areas (c and f).



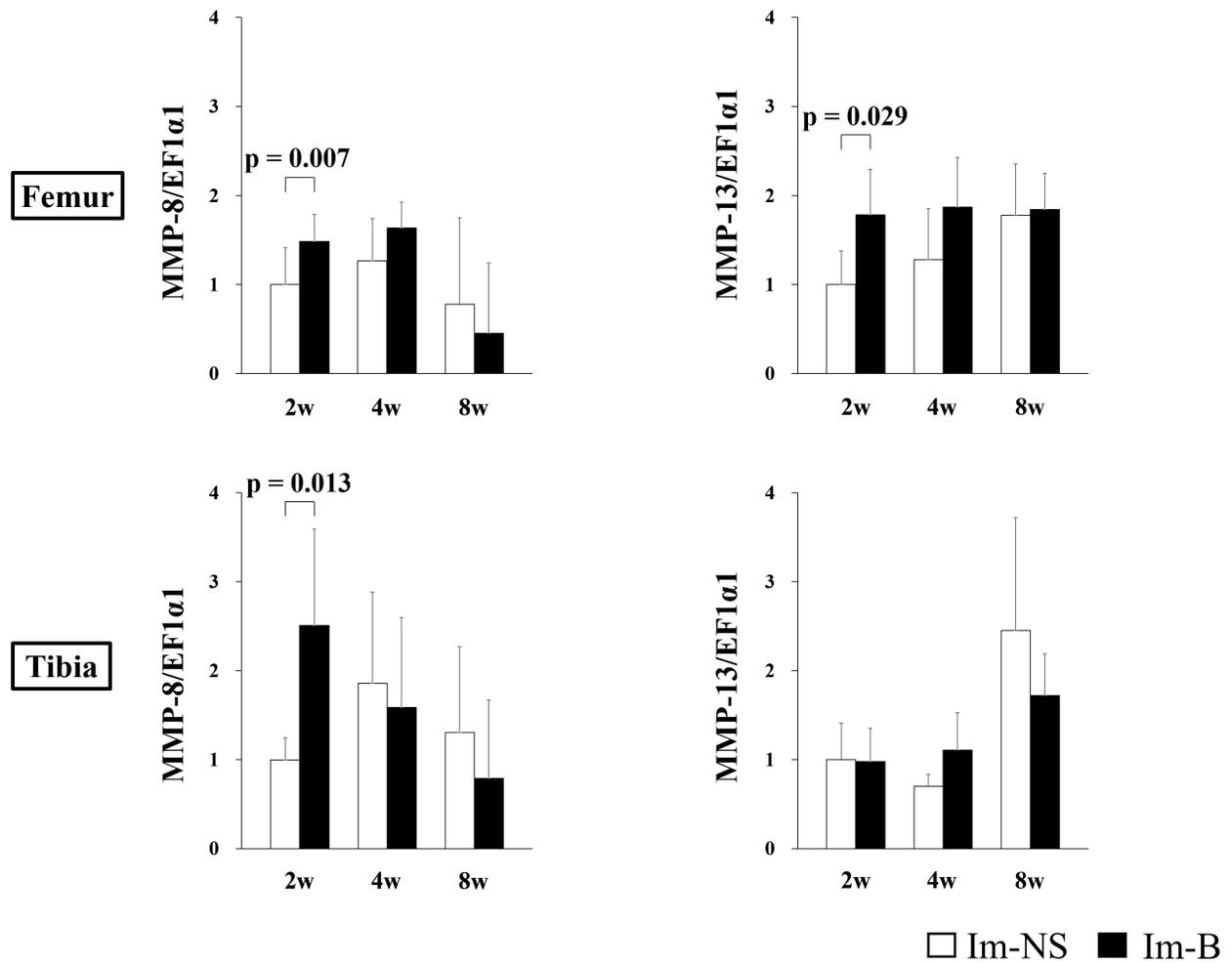
**Figure 4**

Iron deposition in the synovial membrane and meniscal surface. There was no iron deposition in the Im-NS group (a). Iron deposition was observed in the 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. The black arrowheads indicate iron depositions. Scale bar = 100  $\mu$ m



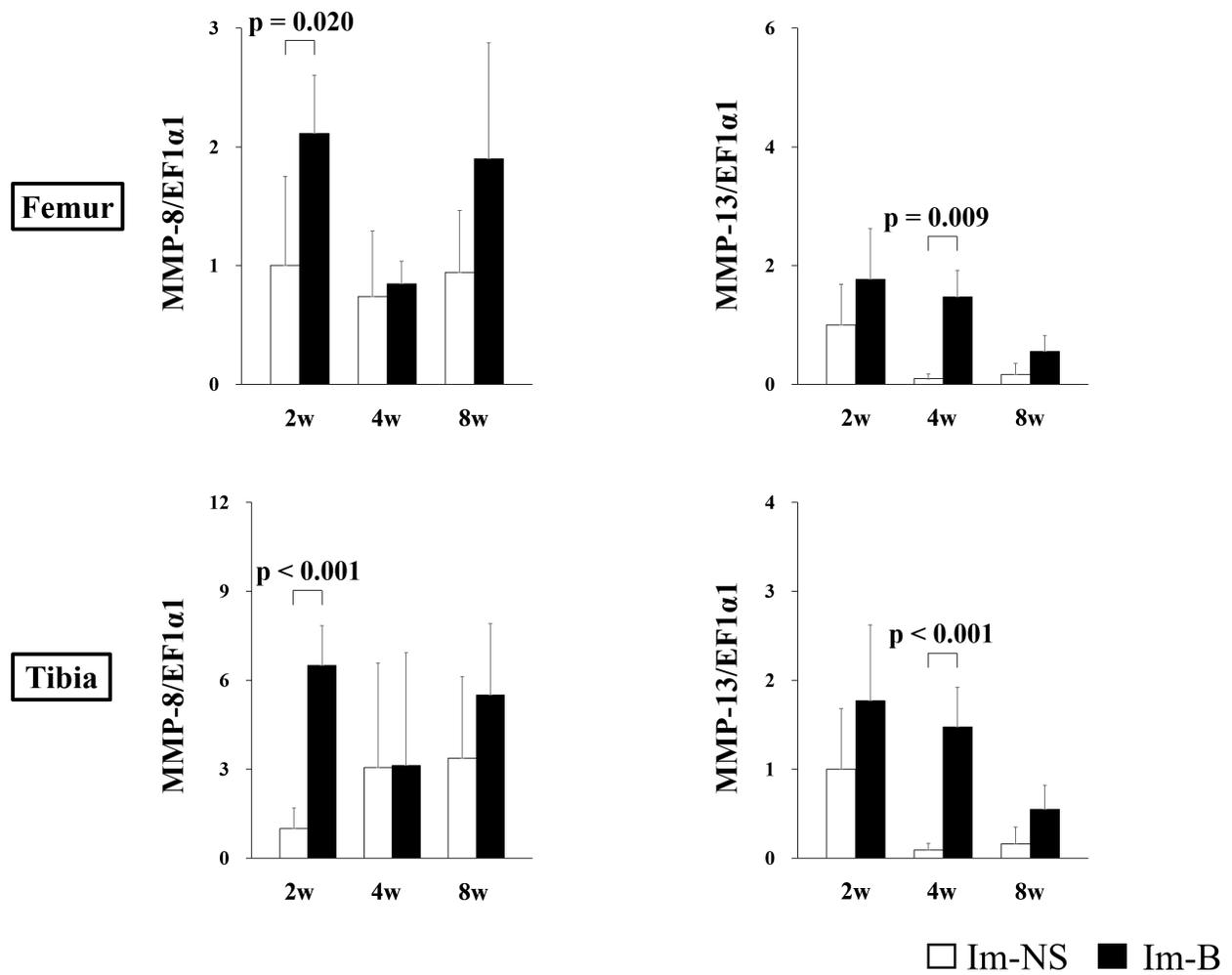
**Figure 5**

CD68 immunostaining in the Im-B and Im-NS groups. CD68-positive cells were observed in both Im-B (a-c) and Im-NS groups (d-f). They were 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. The black arrowheads indicate CD68-positive cells. Scale bar = 100  $\mu$ m



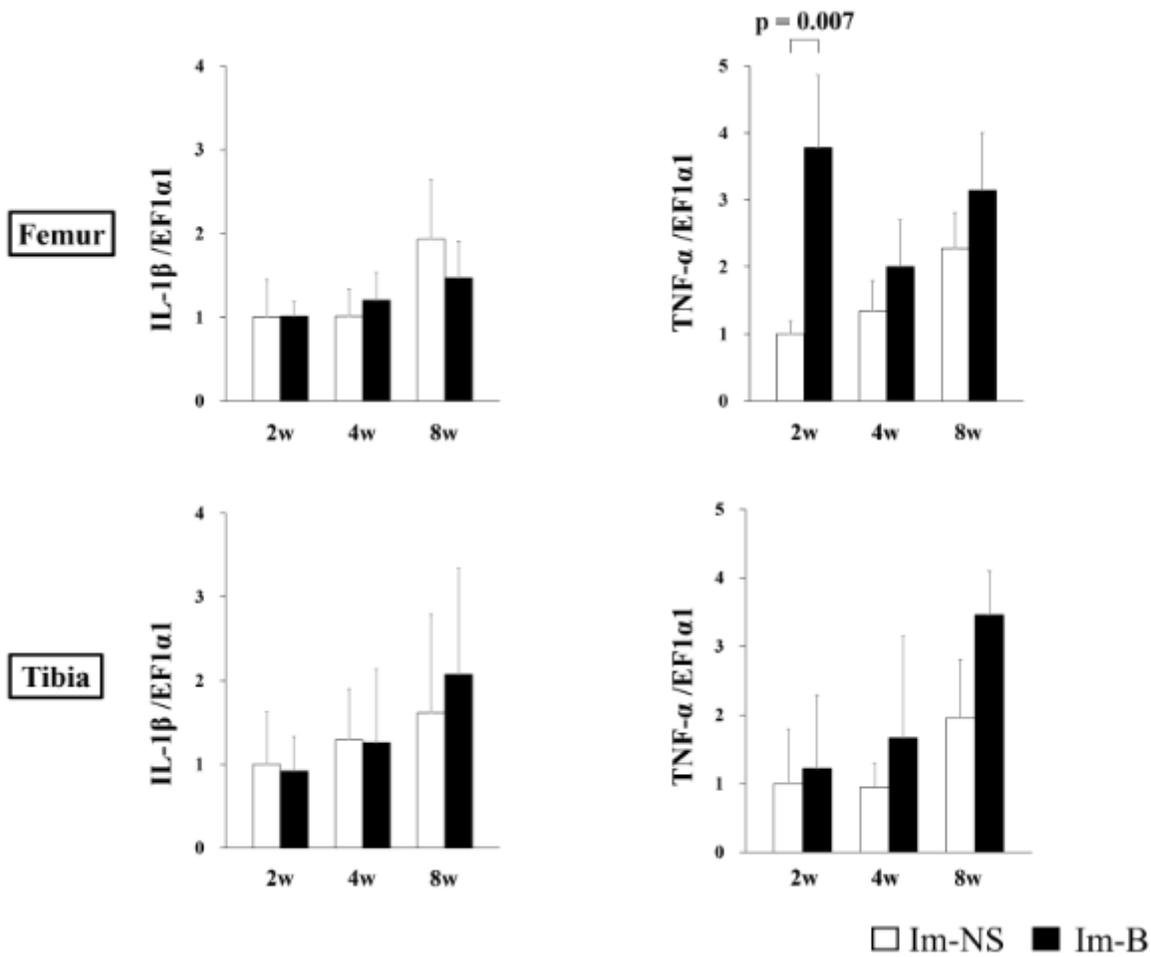
**Figure 6**

Expression of matrix metalloproteinase (MMP)-8 and MMP-13 in the cartilage in the contact area. MMP-8 and MMP-13 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 with those in the Im-NS group.



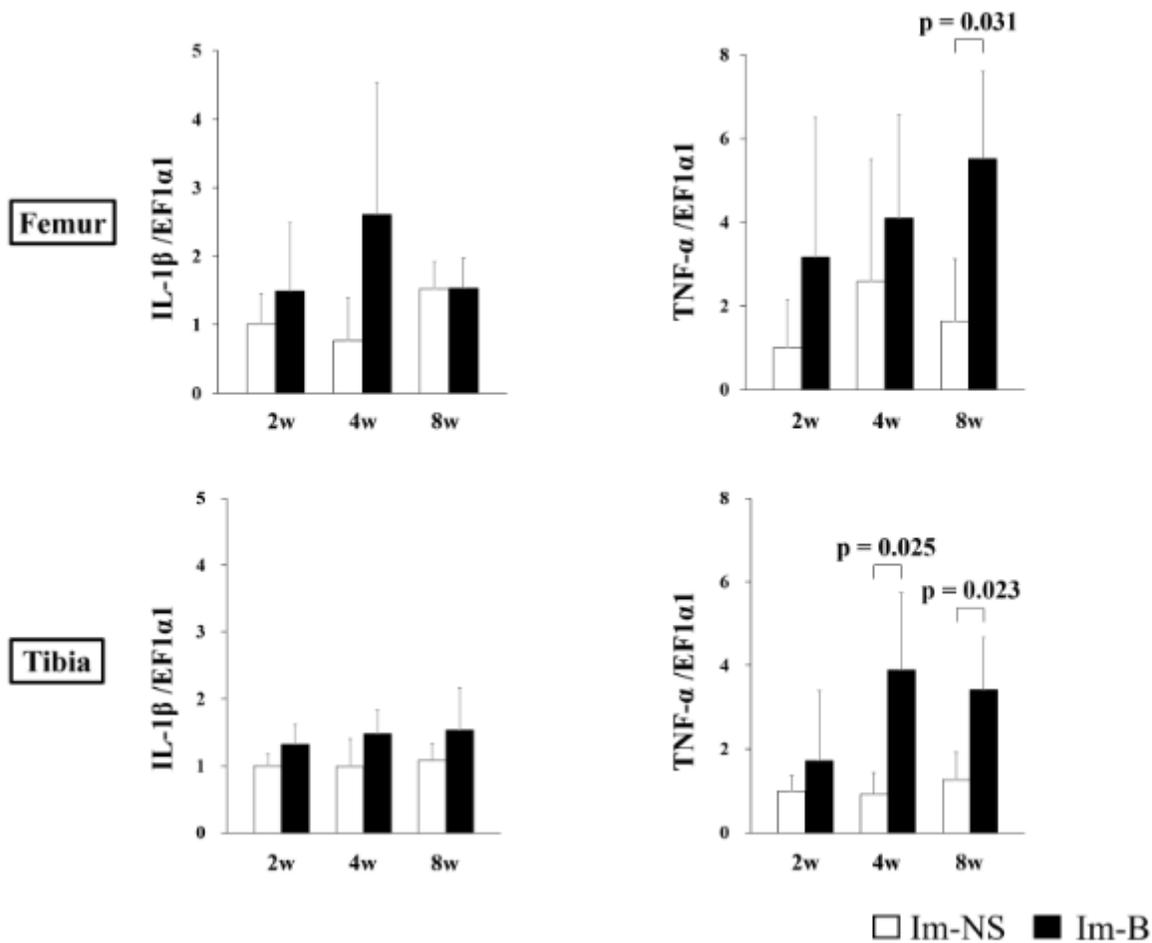
**Figure 7**

Expression of matrix metalloproteinase (MMP)-8 and MMP-13 in the cartilage in the non-contact area. MMP-8 expression at 2 weeks and MMP-13 expression at 4 weeks in both femur and tibia were significantly increased in the Im-B group compared with those in the Im-NS group.



**Figure 8**

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



**Figure 9**

Interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression in the cartilage in the non-contact area. There was no significant difference in IL-1 $\beta$  expression. TNF- $\alpha$  expression in the femur at 8 weeks and tibia at 4- and 8- weeks was significantly increased in the Im-B group compared with that in the Im-NS group.

## Supplementary Files

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