Original Article

Preventing effects of joint contracture by high molecular weight hyaluronan injections in a rat immobilized knee model

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Abstract: Purpose: To elucidate preventive effects of high molecular weight hyaluronan (HMWHA) on the joint capsule of immobilized knees in rats. Materials and Methods: Unilateral knee joints of rats were immobilized with an internal fixator. Either 50 μl of HMWHA (Im-HA group) or 50 μl of saline (control group) was administered intrarticularly once a week after surgery. Sagittal sections were prepared from the medial midcondylar region of the knee joints and assessed by histological, histomorphometric, and immunohistochemical methods. Gene expressions related to inflammation, fibrotic conditions, and hypoxia were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Tissue elasticity of the capsule from both groups was examined using a scanning acoustic microscope (SAM). Results: CD68 positive cells decreased in adhesion areas of the synovial membrane after 1 week in both groups. The length of the superficial layer in the synovial membrane of the Im-HA group was significantly longer than those in the control group over a period of 4 to 8 weeks with significantly small numbers of CD68 positive cells. The gene expressions of IL-6, IL-1β, TGF-β, CTGF, COL1a1, COL3a1, SPARC, and HIF1-α were significantly lower in the Im-HA group compared to those in the control group. The sound speed of the anterior and posterior synovial membrane increased significantly (a reduction in elasticity) in the control group compared to those in the Im-HA group during weeks 1 to 4. Conclusions: This study demonstrated that HMWHA injections suppressed inflammatory, fibrotic, and hypoxic conditions observed in the immobilized joint capsule.

Keywords: Joint immobilization, high molecular weight hyaluronan, inflammation, fibrosis, hypoxia, joint contracture

Introduction

Though joint immobilization is commonly used as an acute treatment for musculoskeletal disorders to relieve joint pain and decrease inflammation, it often has a direct affect on joint contracture [1]. Joint contracture is defined as a limitation of range of motion (ROM) and causes permanent impairment and disability to patients [2]. Recent studies indicate that arthrogenic factors, especially the joint capsule, play an important role in a progression of limited ROM after joint immobilization [3-9].

Changes in the joint capsule and synovial membrane after joint immobilization were adhesions and shortening of them [2, 10]. Joint immobilization induced hypoxic and inflammatory conditions in the joint capsule [11]. Joint adhesion formation begins with an inflammatory response and can be regarded as tissue fibrosis [12-15]. Transforming Growth Factor-β1 (TGF-β1) is a primary cause of profibrotic cytokine that can induce collagen-secreting myofibroblasts in fibrotic tissues [16, 17]. Furthermore, TGF-β1 was shown to stimulate collagen type I synthesis and secreted protein, acidic and rich in cysteine (SPARC) expression [18, 19]. Several studies show that SPARC is closely related to various fibrotic diseases [20-22].

Hyaluronic acid (HA) is a high molecular weight polysaccharide and is an important comp-
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Component of synovial fluid and the extracellular matrix of the articular cartilage [23, 24]. HA acts as a fluid shock absorber, which helps to maintain structural and functional characteristics of the cartilage matrix. It also inhibits the formation and release of prostaglandins, induces proteoglycan aggregation and synthesis, modulates the inflammatory response [25, 26], and inhibits growth of blood vessels and sensory nerves [27]. Joint immobilization decreases HA concentration and molecular size in synovial fluid [28, 29] and causes advanced osteoarthritis with degeneration and/or atrophy of the articular cartilage [30, 31].

Intra-articular injections of HA have demonstrated a protective effect on the articular cartilage and have widely been used in the treatment and prevention of osteoarthritis [32-34]. The efficacy of intra-articular HA injections might be dependent on viscoelastic properties and a function of its molecular weight in osteoarthritis [35, 36]. Several studies have shown differential effects of high molecular weight HA (HMWHA) and low molecular weight HA (LMWHA) on different types of cells such as macrophages, dendritic cells, osteoclasts, and T cells [37, 38]. Lo et al. reported that the HMWHA was more effective for the treatment of knee osteoarthritis by a meta-analysis, however, the heterogeneity of the included trials limited the validity of such a definitive conclusion [39]. Recent studies have found that an intra-articular injection of LMWHA decreased adhesion formation as well as collagen content and increased ROM after prolonged immobilization in a rabbit knee injury model [40]. However, the influence of HMWHA on immobilized synovial tissue is still unknown.

The purpose of this study was to elucidate preventive effects of HMWHA on the joint capsule of immobilized knees in rats. We applied histological, histomorphometric, and immunohistochemical techniques to analyze these changes. In addition, we assessed tissue elasticity of the capsule using a scanning acoustic microscope (SAM). The gene expressions related to inflammatory, fibrotic, and hypoxic conditions were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Materials and methods

Experimental design and surgical procedure

Animals: Protocol for this experiment was approved by the Animal Research Committee of Tohoku University. Mature Sprague-Dawley male rats, aged 12 weeks old, were used (CLEA Japan Inc., Tokyo, Japan). A total of 84 rats were prepared for histological analysis (1, 2, 4, 6, 8, 12, and 16 weeks; n = 6/each period). Thirty six rats were prepared for qRT-PCR (1, 2, and 4 weeks; n = 6/each period). Under anesthesia with intra-peritoneal administration of sodium pentobarbital (50 mg/kg), the unilateral knee joints were rigidly immobilized at 150 degrees of flexion with a plastic plate and metal screws for various periods (1, 2, 4, 6, 8, 12, and 16 weeks: n = 6/each period) [41]. After the operation, the rats were divided into two groups: Immobilized-HA injection (Im-HA) group and immobilized-normal saline injection (Control) group. A shot of 50 μl of HA with a high molecular weight of 2700 kDa (Suvenyl; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was administered intra-articularly after the surgery in the Im-HA group. An identical amount of normal saline was administered for the Control group [42].

Tissue preparation

At the end of the experimental period, the rats were euthanized with an overdose of sodium pentobarbital and fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 by perfusion through the ascending aorta. The resected knee joints were kept in the same fixative state for 24 hours at 4°C. The fixed specimens were decalcified in 10% ethylenediaminetetraacetic acid in 0.01 M PBS for 2 months at 4°C. After dehydration through a graded series of ethanol solutions, the specimens were embedded in paraffin. At the medial midcondylar region of the knee, 5 μm sections were obtained [6].

Histology and histomorphometry

The sections were stained with hematoxylin and eosin, and morphological changes of the synovial membrane and capsule were observed in the anterior and posterior portion. The length

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Scanning acoustic microscope (SAM)

In general, sound speed is proportional to the square root of Young’s elastic modulus. SAM can measure the sound speed of tissues on slide glass in situ and the system used in this study has been reported elsewhere in detail [6, 43]. We set the region of the anterior and posterior synovial membrane (1, 2, 4, 8, 12, and 16 weeks: n = 6/each period) and their average sound speed was calculated with gray scale SAM images with image analysis software (Photoshop CC 2014, Adobe Systems Inc., San Jose, CA) [6].

Immunohistochemistry (IHC)

Number of the macrophage and number of blood vessels: The sections were deparaffinized and immersed in 3.0% hydrogen peroxide. The slides for Cluster of differentiation 68 (CD68), and Alpha smooth muscle actin (α-SMA), were incubated with 0.1% trypsin and 0.1% CaCl$_2$/Tris buffer. Endogenous immunoglobulins were blocked by incubation with normal goat serum (Nichirei). The slides were incubated with mouse anti-rat CD68 antibody (MCA341R, AbD Serotec, Raleigh, NC, dilution 1:400), which was a marker of the macrophage-like type A synoviocyte [44] or rabbit polyclonal α-SMA antibody (ab5694, Abcam, Cambridge, UK, dilution 1:100), which was a marker for myofibroblasts. However, α-SMA was also expressed abundantly in smooth muscle cells of blood vessels and pericytes [45]. The final detection step was carried out using 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO) in 0.1 M imidazole and 0.03% H$_2$O$_2$ as the chromogen. Counter-staining was done with Carazzi’s haematoxylin. Negative control was performed using normal mouse IgG (Dako, Copenhagen, Denmark) as a primary antibody. All slides were stained in one session [10, 11]. The number of CD68 positive cells in the posterior capsule was counted.

We divided the capsule into 4 areas, antero-superior, postero-superior, antero-inferior, and postero-inferior areas according to our previous report [10] for α-SMA. Blood vessels were defined as the lumen structure with strong immunostaining of α-SMA. The images of blood vessels in each area were captured at a magnification of 100 with light microscopy and the number of blood vessels were counted as previously described [11].

Immunohistochemical double staining of CD68/IL-6

The sections were deparaffinized and washed in PBS and double stained with Inter leukin-6 (IL-6) and CD68. After predigestion with elimination of nonspecific binding with 10% normal goat serum (Nichirei, Tokyo, Japan), the sections were incubated with a rabbit anti-rat IL-6 antibody (ab6672, Abcam, Cambridge, UK, dilution 1:400) and mouse anti-rat CD68 antibody (MCA341R, AbD Serotec, Raleigh, NC, dilution 1:400) at 4°C. Secondary staining with AlexaFluor-488 conjugated donkey anti-rabbit (Molecular Probes, Invitrogen) and Alexa-Fluor-594 conjugated goat anti-mouse secondary antibodies (Molecular Probes, Invitrogen) were carried out at room temperature for 60 min, followed by 4’,6-Diamidino-2-Phenylindole (DAPI) nuclear counterstaining for 10 min.

qRT-PCR

After administration of an overdose sodium pentobarbital, the capsule of the knee was cut with a surgical knife and the harvested capsule was immediately placed in a vessel containing 1 ml QIAzol (Qiagen, Hilden, Germany) as previously described [41]. The tissue was disrupted and homogenized with Polytron™ (Kinematica AD, Switzerland). The total RNA of the homogenate was purified using RNeasy Lipid Tissue Mini Kit (Qiagen) and complementary DNA (cDNA) was synthesized using Cloned AMV First-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). PCR efficiencies and relative expression levels of IL-6, IL-1β, Connective Tissue Growth Factor (CTGF), TGF-β1, SPARC, collagen type 1 (COL1a1), collagen type 3 (COL3a1), hypoxia inducible factor-1α (HIF-1α), and vascular endothelial growth factor (VEGF) as a function of elongation factor 1α (EF1α1) were calculated as previously described [41]. Primer sequences for expression analysis were shown in Table 1.
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Statistical analysis

Statistical analysis among the groups was performed using one-way ANOVA with Bonferroni/Dunn post hoc multiple comparisons. Differences between the Im-HA group and control group were compared at each time point by Mann-Whitney’s U test (qRT-PCR) and unpaired t-test (histomorphometry, SAM, the number of macrophages and vessels). Data was expressed as mean ± standard deviation (SD). A value of P < 0.05 was accepted as statistically significant.

Results

Histology and histomorphometry

In the histological appearance of the postero-superior synovial membrane and capsule, adhesion was observed primarily between the postero-superior synovial fold and the synovial membrane around the posterior horn of the medial meniscus after 2 weeks, both in the control and Im-HA groups (Figure 1A and 1D). After 4 weeks, the adhesion area extended to the posterior side, diminishing the residual joint space, especially in the control group (Figure 1B and 1E). The adhesion area was replaced by fibrous and hypocellular connective tissues after 8 weeks both in the control and Im-HA groups. These changes were more dominant in the control group than those in the Im-HA group (Figure 1C and 1F). The anterior synovial membrane and capsule showed similar changes compared to the posterior synovial membrane and capsule (Figure S1).

Statistical differences in the length of the antero-superior (Figure 2A), antero-inferior (Figure 2B), postero-inferior (Figure 2D), outside length of the posterior capsule (Figure 2E), or posterior capsular area (Figure 2F) between the groups.

The length of the postero-superior synovial membrane was significantly longer at 4 to 8 weeks in the Im-HA group than that of the control group (Figure 2C). There were no

Inflammatory conditions

CD68 positive cells were mainly located at the surface layer of the synovial membrane and the fibrous layer of the capsule at 1 week in both groups (Figure 4A and 4D). Positive cells were observed in the adhesion area after 2 weeks and gradually disappeared in both groups (Figure 4B, 4C, 4E and 4F). Though the Im-HA

Table 1. Polymerase chain reaction primer sequences

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**Control**

![Control images](image1.png)

**Im-HA**

![Im-HA images](image2.png)

Figure 1. Histological appearance of the posterior synovial membrane and capsule. Changes in the histological appearance of the postero-superior area at 2 weeks, 4 weeks, and 8 weeks in the control groups (A-C) and in the Im-HA groups (D-F). Adhesion (asterisk) was observed primarily between the postero-superior synovial fold and the synovial membrane around the posterior horn of the medial meniscus after 2 weeks in the control groups and the Im-HA groups. Adhesions were extended especially in the control group. The adhesion area (asterisk) was replaced by fibrofatty loose connective tissues. F: Femur, T: Tibia, M: Meniscus, JS: Joint space, Asterisks: Adhesion area. Hematoxylin and Eosin staining. Scale bars = 500 μm.

The group showed similar changes, the number of positive cells were fewer than those in the control group. CD68 positive cells at 4 to 8 weeks in the control group were significantly higher than that in the Im-HA group (Figure 4G). In double staining of CD68 (red) and IL-6 (green), IL-6 were especially observed at the early phase in the control group (Figure 4H and 4I), which existed around the CD68 positive cells and extended adhesion area of the posterior synovial membrane (Figure 4J). The gene expressions of IL-6 at 2 and 4 weeks (Figure 4K) and IL-1β at 1 week (Figure 4L) were significantly higher in the control group compared with those in the Im-HA group.

**Fibrotic conditions**

The gene expressions of SPARC at 1 week (Figure 5A), TGF-β1 at 1 week and 2 weeks (Figure 5B), COL1a1 at 1 week (Figure 5C), COL3a1 at 2 weeks (Figure 5D), and CTGF at 1 week (Figure 5E) were significantly higher in the control group compared with that in the Im-HA group.

**Hypoxic conditions**

The number of blood vessels in both groups gradually decreased in each area (Figure 6A-D). The number was significantly lower in the Im-HA group at 16 weeks in the antero-superior (Figure 6A), at 6, 12, and 16 weeks in the postero-superior (Figure 6C), and at 16 weeks in the postero-inferior areas (Figure 6D) compared to the control group. There was no statistical difference in the number of vessels in the antero-inferior area (Figure 6B). Small vessels were observed in the adhesion area, especially in the control group (Figure 6E and 6F).
Discussion

The present study examined the effects of HMWHA on the synovial membrane and joint capsule from immobilized knees in rats. The most important finding of this study was that intra-articular HMWHA injections suppressed inflammation, fibrosis, and vascularization in the synovial membrane and joint capsule, which led to joint contracture. Our results indicate clinical benefits of HMWHA injections for prevention of joint contracture.

Adhesion and shortening of the synovial membrane is a key to joint contracture and induces limitation in the ROM [1, 41]. The shortening of the synovial membrane was explained by atrophy or adhesions of the synovial membrane and the obliteration of joint spaces between the synovial membrane and articular cartilage [2]. In our previous study, significant shortening of the synovial membrane occurred at 2 weeks of immobilization in the posterior capsule [10]. Joint contracture rapidly progressed until 8 weeks and advanced slowly after 8 weeks of immobilization [10]. Several studies suggested that HA prevent postoperative adhesions [46-48]. Subacromial injections of HA after arthroscopic rotator cuff surgery tended to produce faster recovery results [46]. Hyaloglide, a hyaluron-based gel, demonstrated better recovery of finger motion after tenolysis surgery of flexor tendons in zone II [47]. The postero-superior length of the synovial membrane in the control group was significantly shorter compared with the Im-HA group from early to chronic phases, but no statistical difference was observed between the two groups in the outside length of the posterior capsule and posterior capsular area. This result suggests that the area of proliferated adhesions did not induce an increase in the total capsular area in both groups, and only the inner layer (synovial membrane) adhered to the facing capsule. Our data
indicate that HMWHA injections may prevent adhesion and shortening of the synovial membrane after joint immobilization. No statistical difference was observed between the two groups in the antero-superior or antero-inferior lengths of the synovial membrane. These results may be related to the methods of immobilization in the hyper-flexed position in our study.

The sound speed of the anterior and posterior synovial membrane and capsule in the control group was significantly higher than that in the Im-HA group at the early phase, especially around the adhesion area. This result suggests that HMWHA injections decreased the elasticity of the synovial membrane and capsule by reducing adhesions in the early experimental periods. The sound speed of the control group was higher in this study before 4 weeks compared with our previous studies [6, 43]. These differences may be due to different series of the experiment including injection methods.

Several prior studies have identified concerns regarding inflammation after joint immobilization [11, 49]. Michelson et al. reported inflam-
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reports, which suggested that HMWHA could be an anti-inflammatory solution [50-54]. HMWHA acted directly on macrophages to inhibit phagocytosis and active oxygen formation in chronic inflammation [54] and demonstrated significant decreases of IL-1β, IL-6, and macrophage infiltration in the early inflammatory phase in the epidural space and around the nerve root in a postlaminectomy rat model [52]. Roth et al. reported that HA inhibited histological signs of acute inflammation and cartilage degeneration, but promoted joint swelling, inflammation and cartilage degeneration at...
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29 days in experimental rat antigen-induced arthritis [55]. These contradictory results might be caused by a difference in animal species, experimental model and/or the molecular weight of HA.

Macrophages are critically involved in both the induction and resolution of fibrosis. Macrophages produce profibrotic mediators that directly activate fibroblasts, including TGF-β1 [56, 57]. TGF-β1 is one of the most significant factors in tissue fibrosis [16]. We demonstrated that the potency of the synovial membrane and capsule to produce TGF-β1 and CTGF after immobilization in a rat knee [58]. Previous studies have shown strong immunostaining of TGF-β1 in frozen shoulder [59], and an increase of TGF-β1 in post-traumatic contracture of human elbows [60]. Additionally, because a TGF-β response element was found in the CTGF promoter [61], CTGF may be a potential downstream mediator for TGF-β signaling in fibroblasts and some of the actions of TGFβ1 in wound healing may be due to CTGF induction and action [62]. The gene expression of CTGF is overexpressed in a large number of fibrotic conditions [63-65]. Subcutaneous injection of CTGF alone had little effect but co-injection of TGF-β1 and CTGF resulted in persistent fibrosis [66]. TGF-β1 and CTGF are likely to play an important role in causing and maintaining joint contracture [58].

It has been shown that expression of SPARC is regulated by TGF-β in several types of fibroblast [67]. It has also been reported that SPARC regulates the expression and activity of TGF-β [19]. SPARC is a multifunctional glycoprotein that exemplifies the multicellular class of proteins [68]. Increased production of SPARC has been shown in wound healing, at sites of angiogenesis, and during human cancer progression [69-71]. SPARC also binds directly to fibrillar collagen type I, III, and V, and to basement membrane collagen IV (Sage et al., 1989; Sasaki et al., 1998; Sasaki et al., 1999). Several studies have shown that inhibition of SPARC expression decreases inflammation and fibrosis in several animal models [20, 22]. These data support that SPARC is a mediator of tissue remodeling such as fibrosis. However, the role of SPARC in the joint capsule after immobilization has been controversial. Importantly, HMWHA has been shown to have antifibrotic properties [72, 73]. Ma et al. reported that HMWHA was effective in decreasing pleural fibrosis in a rabbit model [72], and Akeson et al. presented that topical

Figure 5. Fibrotic gene expressions in the posterior capsule. The gene expressions of SPARC, TGF-β, COL1a1, COL3a1, and CTGF in the posterior capsule (A-E). The gene expressions of SPARC at 1 week (A), TGF-β at 1 week and 2 weeks (B), COL1a at 1 week (C), COL3a at 2 weeks (D), and CTGF at 1 week (E) were significantly higher in the control group when compared with the Im-HA group. Data were expressed as mean ± SD. * = P < 0.05, ** = P < 0.01 versus control.
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HMWHA gel decreased epidural fibrosis in rat laminectomy models [73]. The gene expressions of SPARC, TGF-β1, and CTGF in the control group increased after immobilization, and it was significantly higher compared to the Im-HA group in our study. On the basis of these data, HMWHA strongly prevent fibrosis progression, with regulating SPARC and TGF-β1.

The major structural collagens of the capsule are collagen types I and III, and the former made up 83% of the collagen present [74]. Collagen type I is a major component of the capsule [74, 75]. Collagen type III is of particular significance in the development and differentiation of mesenchymal tissue, and is present in large quantities in tissues requiring high levels of mechanical compliance [76] and in wound healing [77]. Determination of structural collagens is a key to understanding the elastic changes and pathology of the synovial membrane and capsule after immobilization [75]. Some researchers reported expressions of collagen types I and III as follows; high level of collagen type I and low level of type III [75], high levels of collagen types I and III [78], no changes of collagen types I or III [79, 80]. In this study, the gene expressions of COL1a1 and COL3a1 in the Im-HA group were significantly lower compared with that in the control group. HMWHA may suppress expression of collagen type I and type III. However, changes in collagen

Figure 6. Hypoxic conditions in the capsule. The number of blood vessels in the antero-superior area (A), antero-inferior area (B), postero-superior area (C), and postero-inferior area (D), immunohistochemistry of α-SMA in the postero-superior area at 6 weeks in the Im-HA group (E) and the control group (F), and the gene expression of HIF-1α (G) and VEGFα (H). The number of blood vessels in both groups gradually decreased in each area (A-D). The number was significantly lower in the Im-HA group at 16 weeks in the antero-superior (A), at 6, 12 and 16 weeks in the postero-superior (C), and at 16 weeks in the postero-inferior areas (D) when compared to the control group. There was no statistical difference in the number of vessels in the antero-inferior area (B). Small vessels were observed in the adhesion area especially in the control group (E and F). The gene expression of HIF-1α and VEGFα at 1 week (G) was significantly higher in the control group when compared with the control group. M: Meniscus, Asterisks: Adhesion area. Arrow head: Small vessels. Scale bars = 200 μm. Data were expressed as mean ± SD. * = P < 0.05 versus control.
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types I and III of COL1a1 and COL3a1 in the synovial membrane and capsule after immobilization are still controversial. Abnormal cross-linking, such as pentosidine, may play an important role for capsular stiffness [81].

HIF-1α acts as a master regulator for the expression of genes involved in the hypoxia response of most mammalian cells [82-84]. Hypoxia has been shown to express increased amounts of HIF-1α and HIF-1 target genes in synovial lining cells and articular chondrocytes, which aggravate joint inflammation in RA joints [85, 86]. Previous studies have examined joint immobilization induced prolonged inflammation and hypoxia in the joint capsule [11]. Additionally, there are a few reports examining the role of macrophage-specific HIF-1α in vascular inflammation and remodeling [87, 88]. Therefore, HIF-1α is identified as a key player in the pathogenesis of inflammation as it is related to hypoxia. To the best of our knowledge, only one study reported that HA suppressed HIF-1α, which demonstrated that early intervention of HA suppressed the expression of HIF-1α in the synovium of the tibiotarsal joint in a RA rat model [89]. HIF-1α leads to an increased expression of angiogenetic factors such as VEGF [90]. VEGF is a potent stimulator of vascular angiogenesis, permeability, and remodeling that also plays important roles in wound healing and tissue cytoprotection [91]. HIF and VEGF signaling is essential for the maintenance of vascular density and oxygen supply in tissue with hypoxia. In our study, the gene expressions of HIF-1α and VEGFα in the Im-HA group were significantly lower at 1 week in the posterior capsule. These results showed that HMWHA might suppress HIF-1α, which might induce inflammation.

Immobilization has been found to decrease the number of blood vessels in the synovial membrane and joint capsule, which may be a cause of hypoxia [11, 92]. However, hypoxic conditions induced newly formed blood vessels [93]. Tang et al. showed that intra-articular HA injections suppressed myofibroblasts and blood vessels in rat knees [27]. In our study, the number of blood vessels gradually decreased and small blood vessels were observed in the anterior and posterior synovial membrane and capsule in both groups, as previously reported [11]. Recent studies have found that the degree of hypoxia is associated with the gene expressions of collagen type I and α-SMA, and hypoxia induces profibrotic states [94]. In addition, the gene expression of SPARC was induced by blood vessels close to the lesion and in blood vessels which developed the following injury [69]. The vascular basement membrane was disrupted and vascular function was enhanced in tumors grown in SPARC-null mice [95]. These data support that SPARC modulates angiogenesis. The number of blood vessels in the posterior synovial membrane and capsule in the control group was significantly higher than that in the Im-HA group after 6 weeks. These data suggests that joint immobilization induced newly formed blood vessels caused by hypoxia, particularly in the control groups. This study showed that immobilization induces hypoxic condition and HMWHA might suppress vascularization, which leads to decreasing inflammation and fibrosis.

There were several limitations in this study. The study had a small sample size and did not assess LMWHA or dose effect. Further study is needed to clarify the effect of HMWHA on the synovial membrane and capsule after immobilization, both in animal and human samples.

In conclusion, the present study demonstrated that Intra-articular HMWHA injection might be beneficial for the treatment of joint contracture by suppressing the inflammatory, fibrotic, and hypoxic changes in the capsule after immobilization in the early phase.

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Disclosure of conflict of interest

None.

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**Figure S1.** Histological appearance of the antero-inferior SM and capsule of the immobilized knee joint at 2 weeks, 4 weeks, and 8 weeks in the control groups (A-C) and in the Im-HA groups (D-F). Adhesion was observed primarily after 2 weeks in both groups. The adhesion area (asterisk) was replaced by fibrofatty loose connective tissues. T: Tibia, M: Meniscus, Asterisks: Adhesion area. Hematoxylin Eosin staining. Scale bars = 500 μm.
Figure S2. SAM of the anterior SM (A-F), graduation color table (G), and sound speed changes of the anterior SM (M). The low sound speed (blue) area gradually decreased and high sound speed area (yellow to red) increased with time in both groups, especially in the control groups (A-F). The average sound speed of the anterior capsule at 2 weeks to 6 weeks in the control groups was significantly higher than that in the Im-HA group (H). Data were expressed as mean ± SD. * = p < 0.05, ** = p < 0.01 versus control.