Effects of joint immobilization on changes in myofibroblasts and collagen in the rat knee contracture model

Ryo Sasabe, MS[1,2], Junya Sakamoto, PhD [3], Kyo Goto, MS[1,4], Yuichiro Honda, PhD[2], Hideki Kataoka,PhD[4], Jiro Nakano, PhD[3], Tomoki Origuchi PhD[1], Daisuke Endo, PhD[5], Takehiko Koji, PhD[5], Minoru Okita, PhD[1]

[1]Department of Locomotive Rehabilitation Science, Unit of Rehabilitation Sciences, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki, Nagasaki 852-8520, Japan

[2]Department of Rehabilitation, Nagasaki University Hospital, Sakamoto 1-7-1, Nagasaki, Nagasaki 852-8520, Japan

[3]Department of Physical Therapy Science, Unit of Physical and Occupational Therapy Sciences, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki, Nagasaki 852-8520, Japan

[4]Department of Rehabilitation, Nagasaki Memorial Hospital, 1-11-54 Fukahori, Nagasaki, Nagasaki 851-0301, Japan

[5]Department of Histology and Cell Biology, Unit of Basic Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki, Nagasaki 852-8520, Japan

Corresponding author:

Minoru Okita, PhD

Department of Locomotive Rehabilitation Science, Unit of Rehabilitation Sciences, Nagasaki University Graduate School of Biomedical Sciences,

Address: 1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8520, Japan

Telephone and Fax: +81-95-819-7919

E-mail: mokita@nagasaki-u.ac.jp

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ABSTRACT

The purpose of this study was to examine the time-dependent changes in the development of joint capsule fibrosis and in the number of myofibroblasts in the joint capsule after immobilization, using a rat knee contracture model. Both knee joints were fixed in full flexion for 1, 2, and 4 weeks (immobilization group). Untreated rats were bred for each immobilization period (control group). Histological analysis was performed to evaluate changes in the amount and density of collagen in the joint capsule. The changes in type I and III collagen mRNA were examined by in situ hybridization. The number of myofibroblasts in the joint capsule was assessed by immunohistochemical methods. In the immobilization group, the amount of collagen increased within 1 week and the density of collagen increased within 2 weeks, as compared with that in the control group. Type I collagen mRNA-positive cell numbers in the immobilization group increased at all time points. However, type III collagen mRNA-positive cell numbers did not increase. Myofibroblasts in the immobilization group significantly increased compared with those in the control group at all time points, and they increased significantly with the period of immobilization. These results suggest that joint capsule fibrosis with overexpression of type I collagen occurs and progresses within 1 week after immobilization, and an increase in myofibroblasts is related to the mechanism of joint capsule fibrosis. The findings suggest the need for a treatment targeting accumulation of type I collagen associated with an increase in myofibroblasts.

58 Key words: immobilization; joint capsule; fibrosis; myofibroblasts; collagen

INTRODUCTION

Joint contracture is characterized by loss of passive range of motion (ROM) and restriction of the activities of daily living[1], and is an important clinical problem in orthopedics and rehabilitation medicine. The most common cause of joint contracture is immobilization [1; 2], which is a common orthopedic treatment for patients with severe traumatic injuries of the joints or other musculoskeletal disorders [3-7]. In addition, several studies demonstrated that patients with conditions limiting their mobility are at a high risk for joint contracture[8-10]. Joint immobilization without traumatic or inflammatory conditions (e.g., bed rest after spinal cord injury or hemiplegia) may also be a factor in joint contracture.

Experimental research has shown two components contribute to the development of immobilization-induced joint contractures: arthrogenic (bone, cartilage, synovial membrane, capsule, and ligaments) and myogenic[11]. Arthrogenic components, particularly the joint capsule, play an important role in immobilization-induced joint contracture[9; 12; 13]. One study reported that the ROM on extension increased significantly after incision of the posterior capsule in knee joints with immobilization-induced flexion contractures[14]. Thus, the joint capsule is one of the main contributors to the development of arthrogenic contractures.

Previous studies suggested that immobilization induces joint capsule fibrosis [15-18]. Higher amounts of type I collagen have been reported in the capsules of immobilized knees[19], and synovial hypertrophy with fibrosis in the joint capsule has been observed after immobilization[20]. These studies suggest that joint capsule fibrosis may be a primary cause of

79 pathology in joint contractures. Several studies examined changes in the expression of type I
80 and III collagen—the major structural collagens of the joint capsule[21]—to determine the
81 pathology of joint capsule fibrosis[19; 22; 23]. Although these studies utilized
82 immunohistochemical, biochemical, and molecular biological methods, the pathology of
83 joint capsule fibrosis could not be conclusively determined, as the findings were not in
84 agreement[19; 20; 22; 24]. Other studies examined factors related to the development of fibrosis
85 (e.g., the role of cytokines, and inflammatory and hypoxic conditions) in a rat knee joint
86 contracture model. However, fibrotic change was not observed with upregulation of these
87 factors[25; 26]. The changes in types I and III collagen in the capsule after immobilization
88 remain unclear.
89 A number of studies have implicated myofibroblasts in various fibrotic disorders.
90 Myofibroblasts contribute to tissue repair during wound healing by migrating into damaged
91 tissue and synthesizing extracellular matrix (ECM)[27; 28]. In liver fibrosis, hepatic stellate cells
92 differentiate into myofibroblasts and contribute to the production of ECM protein[29]. The
93 myofibroblasts in renal fibrosis replace kidney parenchymal cells with scarring connective
94 tissue and ECM components[30]. In pulmonary fibrosis, myofibroblasts promote ECM
95 deposition by releasing inflammatory mediators[27]. In Dupuytren's contractures and
96 hypertrophic scarring after a burn injury, the number of myofibroblasts contributing to matrix
97 remodeling is increased[31; 32]. Immobilization after traumatic surgery reportedly increases the
98 number of myofibroblasts in the joint capsule[33-35]. In contrast, another study demonstrated that

only the reduction of cyclic mechanical stress increased the differentiation of lung fibroblasts into myofibroblasts[36]. Honda et al. reported that the myofibroblasts in the soleus muscle were increased after immobilization[37]. These previous findings suggest that the increase in myofibroblasts may be induced by immobilization, which leads to immobilization-induced joint capsule fibrosis; however, the influence of immobilization alone on myofibroblasts in the joint capsule is unknown.

Although alterations in the joint capsule after immobilization have been demonstrated, the pathology and mechanism of immobilization-induced arthrogenic contracture, and whether joint capsule fibrosis occurs after immobilization, remain unclear. Identification of the basis for immobilization-induced arthrogenic contracture would be useful for the development of treatments to prevent such contractures. The aim of this study was to examine the time-dependent changes in the development of joint capsule fibrosis and in the numbers of myofibroblasts in the joint capsule after immobilization, using a rat knee contracture model.

MATERIALS AND METHODS

Animals

Sixty 12-week-old male SPF Wistar rats were obtained from Kyudo Laboratories (Saga, Japan); their weights were 350-450 g. All rats were housed in cages (2 or 3 per cage) at 22-24 °C with a 12-hour light-dark cycle and were allowed free access to food and water. The

experimental protocol was approved by the local ethics review committee for animal experimentation (approval number. 1404161137).

Experimental Design

The 60 rats were divided into an immobilization group (10 per time point) and a control group (10 per time point). Rats in the immobilized group had the knee joints of both hind limbs fixed in full flexion for 1, 2, and 4 weeks, using plaster casts placed under anesthesia with intraperitoneal pentobarbital sodium (40 mg/kg). The plaster casts were replaced at least every 2 to 3 days to prevent loosening of the casts and edema of the hind limbs. Rats were able to move freely in their cages using their forelimbs. When the immobilized rats were anesthetized, the controls were also anesthetized to avoid possible confounding by the aesthesia.

Measurement of ROM of Knee Joint Extension and Calculation of the Arthrogenic Contribution to Total Contracture

At 1, 2, and 4 weeks of immobilization, the rats were anesthetized with pentobarbital sodium (40 mg/kg) and the ROM of knee joint extension was quantified by measuring the angle (from -25° to -135°) between the line connecting the greater trochanter of the femur to the center of the knee joint and the line connecting the center of the knee joint to the lateral malleolus of the fibula, with the knee joint passively extended using a tension of 0.3 N generated by

ultra-small-capacity load cells (LTS-1KA, Kyowa Electronic Instruments Co., Tokyo, Japan). We confirmed that 0.3 N was the minimum tension required to achieve maximum extension in the knee joints of control rats. The measured ROM at each immobilization time point was used to calculate the arthrogenic (excluding the effect of skin and muscle) contribution to limitation of ROM using the following formulas: (1) Limitation in ROM (= total contracture) (°) = ROM (before immobilization) - ROM (after immobilization); (2) Limitation in ROM due to periarticular component (= arthrogenic contracture) (°) = ROM after skin excision (popliteal region) and myotomy (knee flexor) - Limitation in ROM. These procedures were carried out after measurement of the ROM in the immobilization group; (3) Arthrogenic restriction to total contracture (%) = arthrogenic contracture / total contracture × 100[13].

Tissue Sampling and Preparation

After each immobilization time period, the animals in each group were sacrificed by injecting pentobarbital sodium. The hind limb knee joints of each group were excised and fixed with 4% paraformaldehyde, then decalcified with either 10% ethylenediaminetetraacetic acid (EDTA) in 0.01 M phosphate buffer, pH 7.4, or Morse solution at 4°C. The specimens decalcified with 10% EDTA were used for histological and immunohistochemical analysis, while those decalcified with Morse solution were used for in situ hybridization. Each specimen was then dehydrated using a graded series of ethanol solutions and embedded in paraffin.

158

159 **Histological Analysis**

160 Longitudinal 6-μm serial sections were cut with a microtome, and 2 sections from each right
161 knee sample (medial and lateral) were stained with hematoxylin-eosin (H&E) to identify
162 pathological changes such as inflammation in the joint capsule. Three sections from each
163 sample (105 μm apart) were stained with picrosirius red (picrosirius red stain kit, Polyscience,
164 Inc., Warrington, PA, USA) to visualize collagen fibers in the posterior capsule; these sections
165 were used for semiquantification of collagen in the posterior capsule. The sections were
166 magnified 40×, and images including the entire posterior capsule were captured using a digital
167 camera (Nikon, Tokyo, Japan). Each image was overlaid with a lattice image (50 μm × 50
168 μm) using Adobe Photoshop ver. 6.0 (Adobe Systems, San Jose, CA, USA); the number of
169 intersection points with collagen fibers were then counted and the relative value based on the
170 control group was calculated for each immobilization period. To evaluate the changes in
171 collagen density, the posterior capsule was identified and 5 random points on each slide were
172 photographed at 400× magnification with a digital camera. Each image was then binarized
173 (red-stained areas of collagen made to appear black while other areas appear white) and total
174 area of black per field was calculated using Image J software (W. Rasband, National Institutes
175 of Health, Bethesda, MD, USA). The individual performing the analysis was blinded to the
176 group to which the rats belonged.

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In situ hybridization

Five rats per group at each time point were provided for in situ hybridization. Sections (6-μm thick) from each right hind limb knee sample were treated with 0.2 N HCl for 20 min and 10 g/mL proteinase K (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C for 15 min. After fixation for 5 min with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.4), sections were immersed in PBS with 2 mg/mL glycine and kept in 40% deionized formamide in 4× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) until hybridization. Hybridization was carried out at 37°C overnight with 1 μg/mL digoxigenin-labeled sense and antisense oligo-DNA dissolved in hybridization buffer containing 10 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 1 mM EDTA, 1× Denhardt's solution, 250 μg/mL yeast tRNA, 125 μg/mL salmon testis DNA, 10% dextran sulfate, and 40% deionized formamide. After hybridization, the slides were washed twice with increasingly dilute concentrations of SSC (0.5, 1, 2 × SSC/50% formamide) for 1 h at 37°C. Sections were reacted with the blocking solution for 1 h, reacted overnight with horseradish peroxidase (HRP)-conjugated goat anti-digoxigenin antibody (Roche, Indianapolis, IN, USA), and washed four times with 0.075% Brij 35 in PBS for 15 min. The HRP sites were visualized according to a method described in previous reports[38; 39]. The 28s rRNA probe signals were detected with HRP-conjugated mouse monoclonal anti-T-T dimer antibody (Kyowa Medex, Tokyo, Japan). The sequence of sense and antisense oligo-DNAs used is listed in Table 1; each consisted of 45 base pairs representing a portion of rat mRNA. Positive cells were

identified by measurement of the staining density over the negative control (anti-sense) probe using Image J software. The number of positive cells was counted and the ratio of positive cells to total cell number (excluding vascular endothelial cells) in the posterior capsule was calculated. More than 300 cells per section and 2 sections per rat were used for analysis. The individual performing the analysis was blinded to the group to which the rats belonged.

Immunohistochemical Analysis

Five rats per group at each time point were provided for immunohistochemical analysis. Sections (6-μm thick) from left knee joint samples of each hind limb were subjected to an antigen retrieval step by incubating 2 mg/mL hyaluronidase from bovine testes (Sigma, St. Louis, MO, USA) at 37°C for 30 min. Endogenous peroxidase was inactivated with 0.3% H_2O_2 in methanol for 10 min at room temperature (RT; 22-25°C). The sections were blocked with 5% bovine albumin in PBS for 60 min and incubated overnight at 4°C with a mouse monoclonal anti-α-smooth muscle actin (α-SMA) primary antibody (1:1000; Exalpha Biologicals Inc., Shirley, MA, USA). The sections were rinsed in PBS and incubated with a biotinylated goat anti-mouse immunoglobulin G (1:1000; Vector Laboratories, Burlingame, CA, USA) for 60 min at RT. Each section was stained using avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories) for 60 min at RT. Sections were then visualized with a metal enhanced DAB substrate kit (Thermo Fisher Science Inc., MA, USA). After the final washing step, each section was stained with 1% methyl green. Each section

was examined with an optical microscope and the entire posterior capsule was photographed at 400× magnification with a digital camera. The number of total cells and α-SMA-positive cells in the posterior capsule were counted (10 areas per section). The ratio of α-SMA-positive cells to the total number of cells (excluding vascular endothelial cells) was then calculated. This analysis was performed using Photoshop v. 6.0. Vascular areas were omitted from the analysis. Two sections per rat and 10 microphotographs per section were used for analysis. The individual performing the analysis was blinded to the group to which the rats belonged.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). The difference between groups for each immobilization period was assessed using a non-paired t-test, whereas differences within the same group were assessed using 1-way analysis of variance (ANOVA), followed by Scheffe's post hoc test. Differences were considered significant at $p < 0.05$.

RESULTS

ROM and Arthrogenic Contribution to Total Contracture

ROM in knee extension in the immobilization group was significantly less than that in the control group for each time point and decreased significantly in a time-dependent manner.

Joint contractures due to immobilization developed and progressed with immobilization time. Arthrogenic contracture started at 11.5° at 1 week and progressed to 43.5° at 4 weeks. Arthrogenic contribution to total contracture was 40.0% at 1 week and increased to 63.0% at 4 weeks. Arthrogenic contracture and arthrogenic contribution to total contracture at 2 and 4 weeks of immobilization were significantly greater than those at 1 week (Table 2).

Histological Analysis

The results of microscopic observation of all H&E-stained specimens of hind limb knee joints did not reveal any abnormal findings such as cellular infiltration in either the control or immobilization groups (data not shown). In addition, the posterior capsule thickness was greater in the immobilization group compared to the control group, and this change progressed with the period of immobilization (Figure 1).

The number of intersection points with collagen increased 1.2 ± 0.1-fold at 1 week, 1.3 ± 0.1-fold at 2 weeks and 1.5 ± 0.2-fold at 4 weeks in the immobilization group (Figure 2A). At each time point, the number of intersection points with collagen fibers was significantly higher in the immobilization group than in the control group. The number of intersection points with collagen fibers at 4 weeks of immobilization was significantly higher than at 1 week and 2 weeks of immobilization. In the control group, the density of collagen per unit area ranged from 59.3 ± 7.2% to 59.9 ± 6.7%; in the immobilization group, the density of collagen per unit area was 63.4 ± 4.8% at 1 week, 74.8 ± 4.8% at 2 weeks, and 77.6 ± 5.8% at

4 weeks (Figure 2B). The density of collagen in the immobilization groups was significantly higher than in the control groups at the 2- and 4-week immobilization time points. Additionally, the density of collagen in the immobilization group increased significantly with increasing duration of immobilization (Figure 2).

In situ hybridization

First, we performed competitive testing to confirm each probe's specificity. In the presence of an excess of unlabeled antisense probe, the hybridization signal was essentially abolished for each type of collagen.

The ratio of type I collagen mRNA-positive cells to total cells in the posterior capsule ranged from 20.1 ± 7.1% to 22.2 ± 10.4% in the control group; in the immobilization group, the ratios were 25.2 ± 8.8%, 28.8 ± 11.5%, and 41.7 ± 12.7% at 1 week, 2 weeks, and 4 weeks of immobilization, respectively (Figure 3, 4A). The ratio of type I collagen mRNA-positive cells to the total cells in the posterior capsule was significantly higher in the immobilization group than in the control group at each time point. Furthermore, in the immobilization group, the ratio of type I collagen mRNA-positive cells to the total cells in the posterior capsule was significantly increased with prolongation of the immobilization period. On the other hand, there was no significant difference in the ratio of type III collagen mRNA-positive cells to total cells in the posterior capsule between the control and immobilization groups at any time point (Figure 3, 4B).

278

279 **Immunohistochemical Analysis**

280 The ratio of α-SMA-positive cells to total cells in the posterior capsule ranged from 3.3 ±
281 2.0% to 4.4 ± 2.8% in the control group. In the immobilization group, this ratio was 7.3 ±
282 2.6%, 10.2 ± 2.8%, and 20.1 ± 3.7% at 1 week, 2 weeks, and 4 weeks of immobilization,
283 respectively (Figure 5). The ratio of α-SMA-positive cells to total cells in the posterior
284 capsule was significantly higher in the immobilization group than in the control group for
285 each time point. In addition, the ratio of α-SMA-positive cells to total cells in the posterior
286 capsule of the immobilization group significantly increased with increasing periods of
287 immobilization.

288

289 **DISCUSSION**

290 Immobilization-induced contractures have been extensively studied. The joint capsule has
291 been considered an important contributor to joint contractures after prolonged
292 immobilization[13-15; 17; 40]. Reported changes in the capsule after immobilization include
293 proliferation of connective tissues within the joint space and adhesions between the synovial
294 folds and the surface of the articular cartilage[15-18; 41]. In addition, the changes in type I and III
295 collagen, which are the main components of the joint capsule, have been examined to
296 evaluate fibrotic changes induced by immobilization. Liu et al. reported that type I and III
297 collagens in the shoulders of rats were increased after immobilization for 2 and 4 weeks[42]. In

298 contrast, Hagiwara et al. demonstrated that not every type of collagen was increased in the
299 knees of rats after prolonged immobilization[22; 23]. Onoda et al. reported that
300 immunoreactivity of collagen types I and III did not change in the joint capsule of a rat
301 immobilized-bleeding model[43]. The results of each study were different and
302 immobilization-induced fibrotic changes in joint capsules have remained unclear. Nearly all
303 previous reports analyzed fibrotic changes with immunohistochemical scores of staining
304 intensity. Previous reports demonstrated that type I collagen accounted for 83% of the total
305 collagen in the synovial capsule[21], which means that an increase in the proportion of type I
306 collagen would be minor. Thus, it may be difficult to detect an increase in type I collagen
307 using immunohistochemical staining. In a reverse transcription polymerase chain reaction
308 (RT-PCR), contamination by other tissues (e.g., ligament, periosteum, and muscle) can occur,
309 because the joint capsule is too small to extract. This may be one of the reasons for
310 contradictory results. In the current study, we used a simple, semiquantitative histological
311 method to clarify immobilization-induced joint capsule fibrosis, using the amount and
312 density of collagen as indicators of fibrosis according to immobilization time. Additionally,
313 type I and III collagen mRNA-positive cells in the joint capsule were detected by in situ
314 hybridization.
315 Moreover, we immunohistochemically examined the change in the number of
316 myofibroblasts, which synthesize high levels of ECM, particularly type I and III collagen,
317 labeled by α-SMA in the joint capsule.

318 This study showed that ROM for knee extension in the immobilization group was
319 significantly lower than that in the control group at 1 week after immobilization, and
320 continued to decrease gradually with immobilization time. The arthrogenic contribution to
321 limitation of ROM increased in a time-dependent manner. These results indicate that
322 immobilization induces arthrogenic contractures that progress with immobilization time,
323 similar to the findings described in a previous report[13]. Furthermore, Richard and Wright
324 found that 47% of the total resistance to midrange movement in a normal cat wrist joint was
325 accounted for by the capsule, representing the highest contribution among the periarticular
326 soft tissues; in comparison, the tendons account for only about 10%[44]. Accordingly, we
327 surmise that the joint capsule contributes more to arthrogenic contracture than other
328 periarticular components.
329 On gross and microscopic observation, hypertrophic changes in the posterior capsule were
330 detected in immobilized knee joints, and this change progressed in a time-dependent manner.
331 Semiquantitative analysis showed that the number of intersection points with collagen fibers
332 in the posterior capsule increased as early as 1 week after immobilization and progressed until
333 4 weeks after immobilization. This result indicates that collagen fibers in the posterior capsule
334 increased as a result of immobilization. Previous studies [15-18] have shown an
335 immobilization-related increase in connective tissue, which is consistent with our results. The
336 reduction of ROM in the immobilized knee joint accompanied by synovial hypertrophy in the
337 mouse model has been demonstrated[20]. Moreover, previous studies found that the

338 extensibility of the joint capsule depends on its thickness[45; 46]. Thus, it is presumed that
339 hypertrophy of the joint capsule contributes to immobilization-induced arthrogenic
340 contracture. At 2- and 4-week time points, the density of collagen in the posterior capsule of
341 the immobilization groups was higher than that in the control groups. This change might have
342 resulted from an increase in collagen fibers; however, other factor might have been involved.
343 Previous research has shown that biochemical changes occur in the composition of
344 periarticular fibrous connective tissues including the joint capsule after immobilization, with
345 notable reduction of water and glycosaminoglycans[47-50]. These changes may then result in
346 narrowed collagen interfascicular spaces, altered plasticity and pliability of connective tissue
347 matrices, and reduced lubrication efficiency. We suggest that similar changes occurred in the
348 posterior capsule in the immobilization group. Although several previous studies have
349 analyzed various aspects of immobilization-induced joint capsule fibrosis, quantitative
350 changes in collagen with immobilization time have not yet been reported. In this study, we
351 showed longitudinal changes in the collagen content during development of
352 immobilization-induced joint capsule fibrosis. These results were the most important findings
353 in this study.

354 We observed that the ratio of type I collagen mRNA-positive cells in the posterior capsule
355 was increased at 1 week after immobilization and increased in a time-dependent manner. This
356 indicates that immobilization may evoke an increase in cells with the ability to produce type I
357 collagen. In contrast, the type III collagen mRNA-positive cells did not increase. These results

suggest that the increase in the amount of collagen in the posterior capsule may derive from overexpression of type I collagen. Schollmeier et al. found that type I collagen was increased at sites of fibrosis and connective tissue proliferation[24]. Furthermore, in other fibrotic conditions such as idiopathic pulmonary fibrosis and cirrhotic liver, type I collagen was found to be disproportionately increased, whereas there was minimal change in type III collagen[51; 52]. Thus, it is surmised that type I collagen plays a major role in fibrotic conditions in a variety of tissues. The same seems true of immobilization-induced joint capsule fibrosis; however, type III collagen mRNA and protein levels were increased in the joint capsule after immobilization following traumatic surgery[34]. This discrepancy of results for type III collagen increase after immobilization may be explained by the traumatic condition of the joint. In any case, the reasons for the increase in type I collagen mRNA-positive cells remains unclear and detailed studies are needed to fill in this gap in the knowledge.

In this study, the α-SMA-positive cells in the posterior capsule significantly increased in the immobilization group from 1 week after immobilization, and varied with immobilization time. We used α-SMA as a marker of myofibroblasts. Previous studies suggested that reduction in cyclic mechanical stimulation is a key factor promoting differentiation of fibroblasts into myofibroblasts[36; 37; 53]. Joint immobilization may reduce the mechanical stimulation to fibroblasts in the joint capsule, and this change may be a trigger of fibroblast differentiation into myofibroblasts. Another possible cause of an increase in myofibroblasts might be explained by hypoxia. Research has shown that α-SMA protein expression increases in

hypoxic fibroblasts, indicating that hypoxia promotes fibroblast differentiation into myofibroblasts[37; 54]. In addition, Yabe et al. reported that joint immobilization induced hypoxic conditions in the rat knee joint capsule[25]. Thus, hypoxia due to joint immobilization might promote fibroblast differentiation into myofibroblasts. Immobilization might have a direct and/or indirect influence on the increase in myofibroblasts in the joint capsule. Furthermore, myofibroblasts produce large amounts of collagen and play a pivotal role in tissue fibrosis[36; 55]. Our results indicate that type I collagen mRNA-positive cells and α-SMA-positive cells in the posterior capsule increased in a time-dependent manner; greater increases in α-SMA-positive cells compared to type I collagen mRNA-positive cells were noted at all time points from 1 to 4 weeks of immobilization. Thus, type I collagen mRNA-positive cells in the posterior capsule might be myofibroblasts that increased due to immobilization. Therefore, we believe that immobilization-induced increases of myofibroblasts are strongly related to the development of joint capsule fibrosis.

There are some limitations to this study. First, the changes in type I and III collagen protein concentrations were not investigated. The joint capsule of the rat knee is too small to facilitate the examination of collagen levels using biochemical and molecular biological methods. In addition, a large part of the capsule is composed of type I collagen, which makes it more difficult to perform semiquantitative analysis using immunohistochemistry. This limitation could be overcome by using larger animals such as rabbits or canines. Another limitation of this study is that we were unable to completely elucidate the underlying pathophysiology of

immobilization-induced joint capsule fibrosis; moreover, hypoxic conditions were not evaluated. Honda et al. reported that immobilization-induced muscle fibrosis was associated with hypoxia, as well as with upregulation of interleukin-1β and transforming growth factor-β[37]. Future studies are needed to determine the relationship between these factors and development of joint capsule fibrosis, as well as the pathophysiology of arthrogenic contractures.

In conclusion, this study demonstrated that joint immobilization induces an increase in the number of myofibroblasts in the joint capsule from early stages of immobilization in the absence of traumatic or inflammatory conditions. Upregulation of myofibroblasts may promote expression of type I collagen, which subsequently induces fibrosis in the joint capsule, resulting in an increase in the amount and density of collagen. These changes represent a portion of the pathophysiology underlying immobilization-induced arthrogenic contractures. In addition, this study suggests that treatment targeting accumulation of type I collagen by limiting the increase in myofibroblasts would be beneficial in preventing immobilization-induced contractures.

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433 **REFERENCES**

434 1. Wong K, Trudel G, Laneuville O. 2015. Noninflammatory Joint Contractures Arising
435 from Immobility: Animal Models to Future Treatments. Biomed Res Int 848290.
436 2. Kaneguchi A, Ozawa J, Kawamata S, et al. 2016. Development of arthrogenic joint
437 contracture as a result of pathological changes in remobilized rat knees. J Orthop Res.
438 3. Ahn JH, Lee SH, Choi SH, et al. 2011. Evaluation of clinical and magnetic resonance
439 imaging results after treatment with casting and bracing for the acutely injured posterior
440 cruciate ligament. Arthroscopy 27:1679-1687.
441 4. Kerkhoffs GM, van den Bekerom M, Elders LA, et al. 2012. Diagnosis, treatment
442 and prevention of ankle sprains: an evidence-based clinical guideline. Br J Sports Med
443 46:854-860.
444 5. Akeson WH, Amiel D, Abel MF, et al. 1987. Effects of immobilization on joints.
445 Clin Orthop Relat Res:28-37.
446 6. Gault SJ, Spyker MJ. 1969. Beneficial effect of immobilization of joints in
447 rheumatoid and related arthritides: a splint study using sequential analysis. Arthritis Rheum
448 12:34-44.
449 7. Partridge RE, Duthie JJ. 1963. Controlled Trial of the Effect of Complete
450 Immobilization of the Joints in Rheumatoid Arthritis. Ann Rheum Dis 22:91-99.
451 8. Clavet H, Hebert PC, Fergusson D, et al. 2008. Joint contracture following prolonged

452 stay in the intensive care unit. Canadian Medical Association Journal 178:691-697.

453 9. Moriyama H, Yoshimura O, Kawamata S, et al. 2007. Alteration of knee joint

454 connective tissues during contracture formation in spastic rats after an experimentally induced

455 spinal cord injury. Connect Tissue Res 48:180-187.

456 10. Dittmer DK, Teasell R. 1993. Complications of immobilization and bed rest. Part 1:

457 Musculoskeletal and cardiovascular complications. Can Fam Physician 39:1428-1432,

458 1435-1427.

459 11. Trudel G. 1997. Differentiating the myogenic and arthrogenic components of joint

460 contractures. An experimental study on the rat knee joint. Int J Rehabil Res 20:397-404.

461 12. Lee S, Sakurai T, Ohsako M, et al. 2010. Tissue stiffness induced by prolonged

462 immobilization of the rat knee joint and relevance of AGEs (pentosidine). Connect Tissue Res

463 51:467-477.

464 13. Trudel G, Uhthoff HK. 2000. Contractures secondary to immobility: is the restriction

465 articular or muscular? An experimental longitudinal study in the rat knee. Arch Phys Med

466 Rehabil 81:6-13.

467 14. Chimoto E, Hagiwara Y, Ando A, et al. 2007. Progression of an arthrogenic motion

468 restriction after immobilization in a rat experimental knee model. Upsala Journal of Medical

469 Sciences 112:347-355.

470 15. E. Burke Evans GWNE, James K Butler, Johanna Blumei. 1960. Experimental

471 immobilization and remobilization of rat knee joint. J Bone Joint Surg Am 42A:737-758.

472 16. T. H. Thaxter RAM, C. E. Anderson. 1965. Degeneration of Immobilizaed Knee

473 Joints in Rats. J Bone Joint Surg Am 567-585.

474 17. Enneking WF, Horowitz M. 1972. The intra-articular effects of immobilization on

475 the human knee. J Bone Joint Surg Am 54:973-985.

476 18. Finsterbush A FB. 1973. Early changes in immobilized rabbits knee joints : a liight

477 and electron microscopic study. Clin Orthop Relat Res 92:305-319.

478 19. Matsumoto F, Trudel G, Uhthoff HK. 2002. High collagen type I and low collagen

479 type III levels in knee joint contracture: an immunohistochemical study with histological

480 correlate. Acta Orthop Scand 73:335-343.

481 20. Sotobayashi D, Kawahata H, Anada N, et al. 2016. Therapeutic effect of

482 intra-articular injection of ribbon-type decoy oligonucleotides for hypoxia inducible factor-1

483 on joint contracture in an immobilized knee animal model. J Gene Med 18:180-192.

484 21. Kleftogiannis F, Handley CJ, Campbell MA. 1994. Characterization of extracellular

485 matrix macromolecules from bovine synovial capsule. J Orthop Res 12:365-374.

486 22. Hagiwara Y, Ando A, Onoda Y, et al. 2010. Expression patterns of collagen types I

487 and III in the capsule of a rat knee contracture model. J Orthop Res 28:315-321.

488 23. Hagiwara Y, Chimoto E, Ando A, et al. 2007. Expression of type I collagen in the

489 capsule of a contracture knee in a rat model. Ups J Med Sci 112:356-365.

490 24. Schollmeier G, Sarkar K, Fukuhara K, et al. 1996. Structural and functional changes
491 in the canine shoulder after cessation of immobilization. Clin Orthop Relat Res 310-315.
492 25. Yabe Y, Hagiwara Y, Suda H, et al. 2013. Joint immobilization induced hypoxic and
493 inflammatory conditions in rat knee joints. Connect Tissue Res 54:210-217.
494 26. Hagiwara Y, Chimoto E, Takahashi I, et al. 2008. Expression of transforming growth
495 factor-beta1 and connective tissue growth factor in the capsule in a rat immobilized knee
496 model. Ups J Med Sci 113:221-234.
497 27. Hinz B, Phan SH, Thannickal VJ, et al. 2007. The myofibroblast: one function,
498 multiple origins. Am J Pathol 170:1807-1816.
499 28. Gabbiani G. 2003. The myofibroblast in wound healing and fibrocontractive diseases.
500 J Pathol 200:500-503.
501 29. Seki E, Brenner DA. 2015. Recent advancement of molecular mechanisms of liver
502 fibrosis. J Hepatobiliary Pancreat Sci 22:512-518.
503 30. Zell S, Schmitt R, Witting S, et al. 2013. Hypoxia Induces Mesenchymal Gene
504 Expression in Renal Tubular Epithelial Cells: An in vitro Model of Kidney Transplant
505 Fibrosis. Nephron Extra 3:50-58.
506 31. Verjee LS, Midwood K, Davidson D, et al. 2009. Myofibroblast distribution in
507 Dupuytren's cords: correlation with digital contracture. J Hand Surg Am 34:1785-1794.
508 32. Nedelec B, Shankowsky H, Scott PG, et al. 2001. Myofibroblasts and apoptosis in

509 human hypertrophic scars: the effect of interferon-alpha2b. Surgery 130:798-808.

510 33. Hildebrand KA, Sutherland C, Zhang M. 2004. Rabbit knee model of post-traumatic

511 joint contractures: the long-term natural history of motion loss and myofibroblasts. J Orthop

512 Res 22:313-320.

513 34. Hildebrand KA, Zhang M, Germscheid NM, et al. 2008. Cellular, matrix, and growth

514 factor components of the joint capsule are modified early in the process of posttraumatic

515 contracture formation in a rabbit model. Acta Orthop 79:116-125.

516 35. Hildebrand KA, Zhang M, Hart DA. 2007. Myofibroblast upregulators are elevated

517 in joint capsules in posttraumatic contractures. Clin Orthop Relat Res 456:85-91.

518 36. Blaauboer ME, Smit TH, Hanemaaijer R, et al. 2011. Cyclic mechanical stretch

519 reduces myofibroblast differentiation of primary lung fibroblasts. Biochem Biophys Res

520 Commun 404:23-27.

521 37. Honda Y, Sakamoto J, Nakano J, et al. 2015. Upregulation of

522 interleukin-1beta/transforming growth factor-beta1 and hypoxia relate to molecular

523 mechanisms underlying immobilization-induced muscle contracture. Muscle Nerve

524 52:419-427.

525 38. Koji T, Brenner RM. 1993. Localization of estrogen receptor messenger ribonucleic

526 acid in rhesus monkey uterus by nonradioactive in situ hybridization with digoxigenin-labeled

527 oligodeoxynucleotides. Endocrinology 132:382-392.

528 39. Hikiji H, Endo D, Horie K, et al. 2014. TDAG8 activation inhibits osteoclastic bone
529 resorption. FASEB J 28:871-879.
530 40. Trudel G, Jabi M, Uhthoff HK. 2003. Localized and adaptive synoviocyte
531 proliferation characteristics in rat knee joint contractures secondary to immobility. Archives
532 of Physical Medicine and Rehabilitation 84:1350-1356.
533 41. Ando A, Hagiwara Y, Onoda Y, et al. 2010. Distribution of Type A and B
534 Synoviocytes in the Adhesive and Shortened Synovial Membrane during Immobilization of
535 the Knee Joint in Rats. The Tohoku Journal of Experimental Medicine 221:161-168.
536 42. Liu YL, Ao YF, Cui GQ, et al. 2011. Changes of histology and capsular collagen in a
537 rat shoulder immobilization model. Chin Med J (Engl) 124:3939-3944.
538 43. Onoda Y, Hagiwara Y, Ando A, et al. 2014. Joint haemorrhage partly accelerated
539 immobilization-induced synovial adhesions and capsular shortening in rats. Knee Surg Sports
540 Traumatol Arthrosc 22:2874-2883.
541 44. Richard J J, Wright. V. 1962. Relative importance of various tissues in joint stiffness.
542 J Appl Phisiol 17:824-828.
543 45. Itoi E, Grabowski JJ, Morrey BF, et al. 1993. Capsular properties of the shoulder.
544 Tohoku J Exp Med 171:203-210.
545 46. Stewart KJ, Edmonds-Wilson RH, Brand RA, et al. 2002. Spatial distribution of hip
546 capsule structural and material properties. J Biomech 35:1491-1498.

547 47. Akeson WH, Amiel D, LaViolette D, et al. 1968. The connective tissue response to
548 immobility: an accelerated ageing response? Exp Gerontol 3:289-301.
549 48. Akeson WH, Woo SL, Amiel D, et al. 1973. The connective tissue response to
550 immobility: biochemical changes in periarticular connective tissue of the immobilized rabbit
551 knee. Clin Orthop Relat Res 356-362.
552 49. Woo SL, Matthews JV, Akeson WH, et al. 1975. Connective tissue response to
553 immobility. Correlative study of biomechanical and biochemical measurements of normal and
554 immobilized rabbit knees. Arthritis Rheum 18:257-264.
555 50. Amiel D, Akeson WH, Harwood FL, et al. 1980. The effect of immobilization on the
556 types of collagen synthesized in periarticular connective tissue. Connect Tissue Res 8:27-32.
557 51. Aycock RS, Seyer JM. 1989. Collagens of normal and cirrhotic human liver. Connect
558 Tissue Res 23:19-31.
559 52. Zhang K, Rekhter MD, Gordon D, et al. 1994. Myofibroblasts and their role in lung
560 collagen gene expression during pulmonary fibrosis. A combined immunohistochemical and
561 in situ hybridization study. Am J Pathol 145:114-125.
562 53. Peters AS, Brunner G, Blumbach K, et al. 2012. Cyclic mechanical stress
563 downregulates endothelin-1 and its responsive genes independently of TGFbeta1 in dermal
564 fibroblasts. Exp Dermatol 21:765-770.
565 54. Robinson CM, Neary R, Levendale A, et al. 2012. Hypoxia-induced DNA

566 hypermethylation in human pulmonary fibroblasts is associated with Thy-1 promoter
567 methylation and the development of a pro-fibrotic phenotype. Respir Res 13:74.
568 55. Kuhn C, McDonald JA. 1991. The roles of the myofibroblast in idiopathic
569 pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active
570 extracellular matrix synthesis. Am J Pathol 138:1257-1265.

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573

574 FIGURE LEGENDS:

575 Figure 1. Picrosirius red staining of the knee joint. The arrowhead shows the posterior capsule.
576 The posterior capsule in the immobilization group was thicker than that in the control group.
577 This change progressed as the immobilization period lengthened. F, femur; T, tibia; scale bar
578 = 1 mm.

579

580 Figure 2. Semiquantitative results for collagen in the posterior capsule. The number of
581 intersection points with collagen fiber (A) and the density of collagen in the posterior capsule
582 (B). Data are expressed as mean ± SD. *$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. 1-wk
583 immobilization group; $^{\dagger}P < 0.05$ vs. 2-wk immobilization group.

584

585 Figure 3. In situ hybridization for type I collagen mRNA (upper panels) and type III collagen
586 mRNA (lower panels). Control was a 16-wk-old rat and immobilization was for 4 wk. Arrows
587 indicate normal cells and arrowheads indicate collagen mRNA-positive cells.
588 The bars next to each photograph show the threshold level; cells were classified as collagen
589 mRNA-positive based on this threshold level. S, sense probe; AS, antisense probe; scale bar =
590 50 μm.

591

592 Figure 4. The ratio of type I collagen (A) and type III collagen (B) mRNA-positive cells to

593 total cells. Data are expressed as mean ± SD. *P < 0.05 vs. control; #P < 0.05 vs. the 1-wk

594 immobilization group; †P < 0.05 vs. the 2-wk immobilization group.

595

596 Figure 5. Immunohistochemical staining for α-SMA in the posterior capsule (upper panels).

597 Controls are 16-wk-old rats. Arrowheads indicate α-SMA-positive cells. Scale bar =100 μm.

598 The figure under the panel shows the ratio of SMA-positive cells to the total number of cells.

599 Data are expressed as mean ± SD. *P < 0.05 vs. control; #P < 0.05 vs. the 1-wk

600 immobilization group; †P < 0.05 vs. the 2-wk immobilization group.

TABLE 1: Sequences of probes used for in situ hybridization

Object gene	Arrangement	Gene bank No.
CoL1α1		
Sense	5’-AGACTGGCAACCTCAAGAAGTCCCTGCTCCTCCAGGGCTCCAACG-3	BC133728.1
Antisense	5’-CGTTGGAGCCCTGGAGGAGCAGGGACTTCTTGAGGTTGCCAGTCT-3’	
CoL3α1		
Sense	5’-ACTGTCCCGCGGAAGCACTGGTGGACAGATGCTGGTGCTGAGAAG-3	BC087039.1
Antisense	5’-CTTCTCAGCACCAGCATCTGTCCACCAGTGCTTCCGCGGGACAGT-3’	
28S rRNA		
Sense	5’-GCCGCCGCAGGTGCAGATCTTGGTGGTAGTAGCA-3’	NR046246.1
Antisense	5’-TGCTACTACCACCAAGATCTGCACCTGCGGCGGC-3’	

TABLE 2: Range of motion and arthrogenic restriction

	ROM (°)		Total contracture (°)	Arthrogenic contracture (°)	Arthrogenic contribution to total contracture (%)
	Control	Immobilization			
1 wk	−27.5 ± 2.6	−54.0 ± 6.2*	29.0 ± 6.5	11.5 ± 2.5	40.0 ± 10.3
2 wk	−28.5 ± 2.4	−79.5 ± 12.1 * #	54.5 ± 12.1	31.0 ± 9.1 #	56.9 ± 6.9 #
4 wk	−28.0 ± 3.5	−94.0 ± 10.2 * # †	69.0 ± 10.7	43.5 ± 7.5 #	63.0 ± 6.3 #

Data are expressed as mean ± SD. *P < 0.05 vs. control; #P < 0.05 vs. 1 wk immobilization group; †P < 0.05 vs. 2 wk immobilization group.

Figure 1

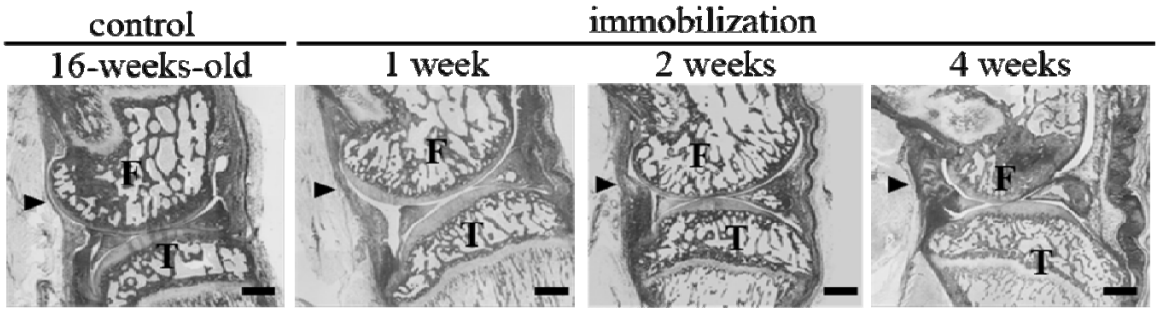

control
immobilization
16-weeks-old
1 week
2 weeks
4 weeks
F
T
F
T
F
T
F
T

Figure 2

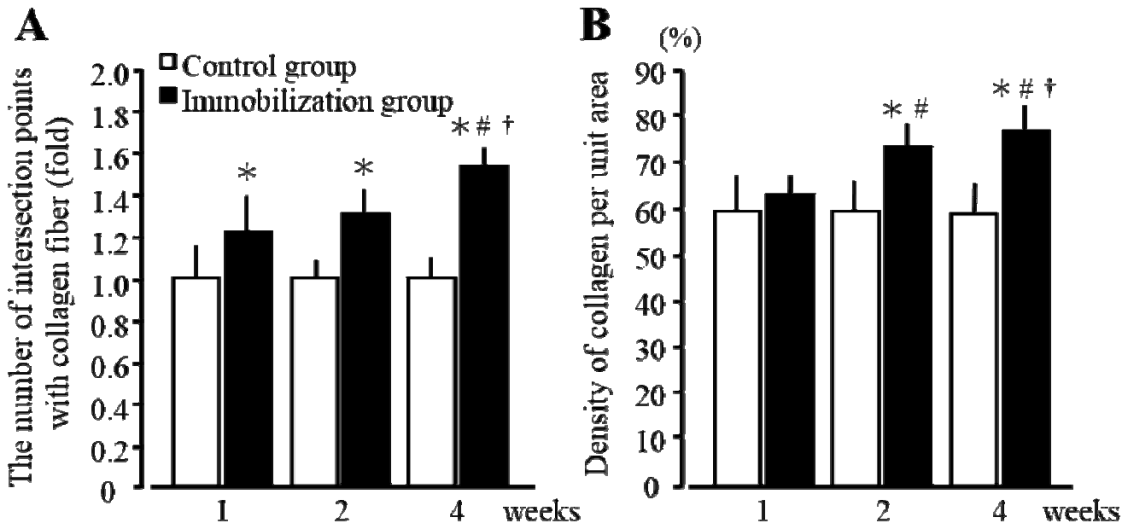

A
Control group
Immobilization group
The number of intersection points with collagen fiber (fold)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
* * * # †
1
2
4
weeks
B
(%)
Density of collagen per unit area
90
80
70
60
50
40
30
20
10
0
* #
* # †
1
2
4
weeks

Figure 3

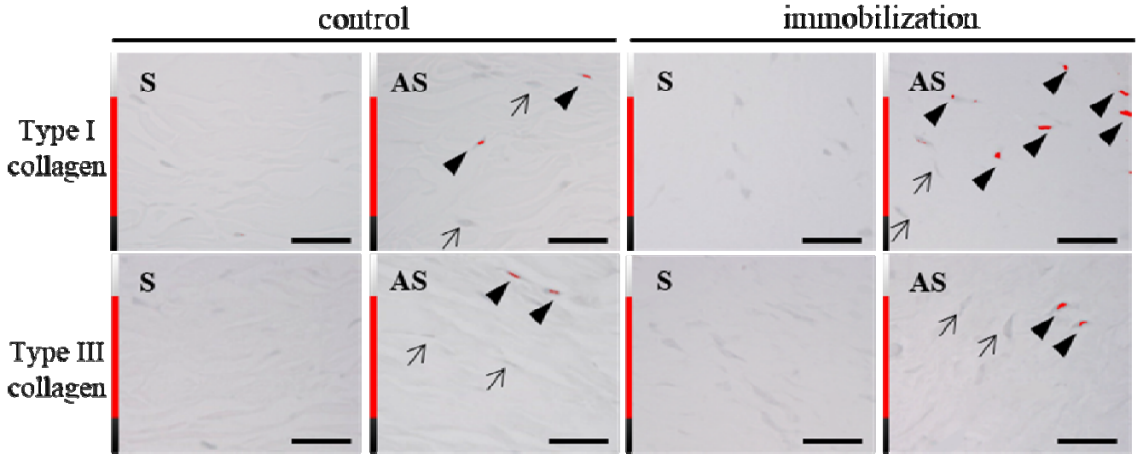

control
immobilization
S
AS
S
AS
Type I collagen
S
AS
S
AS
Type III collagen

Figure 4

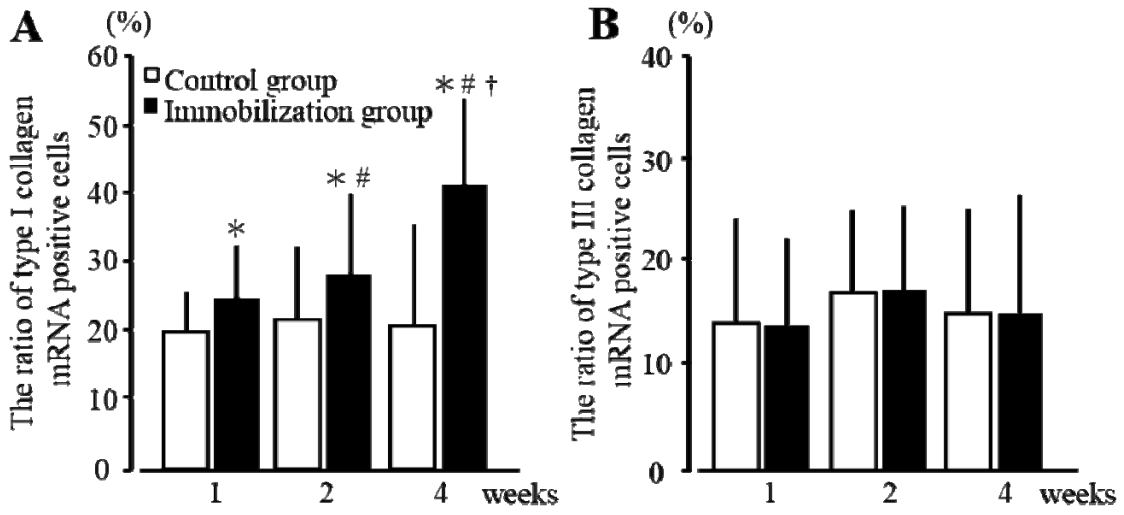
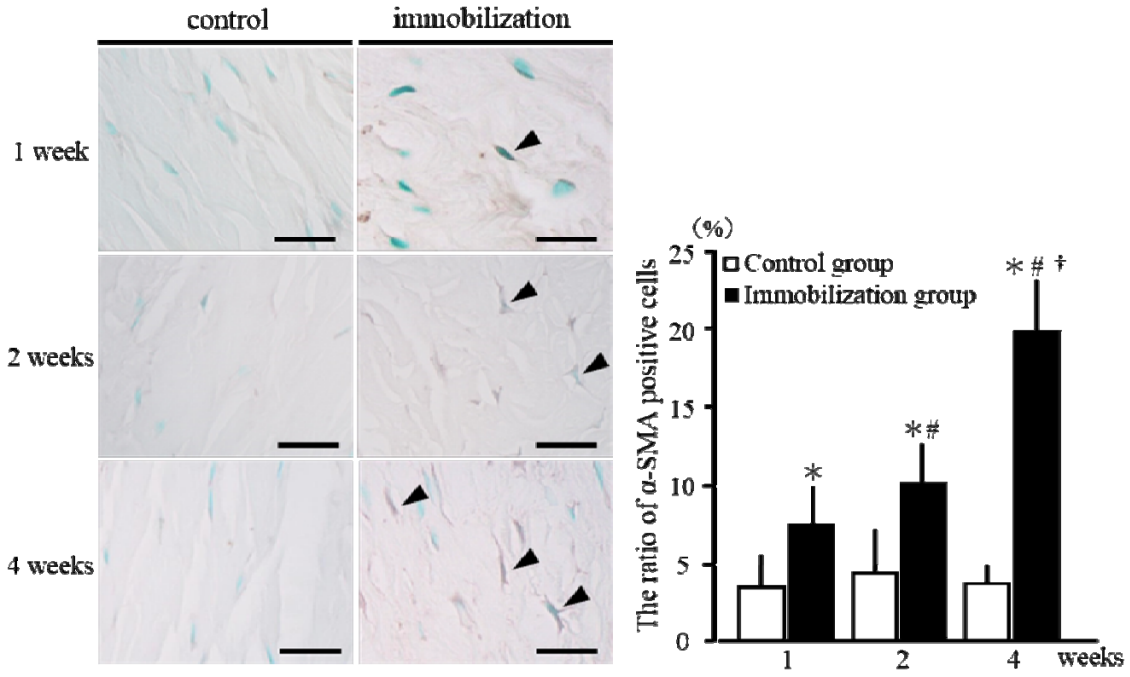

A
(%)
60
50
40
30
20
10
0
The ratio of type I collagen mRNA positive cells
Control group
Immobilization group
* # †
* #
*
1
2
4
weeks
B
(%)
40
30
20
10
0
The ratio of type III collagen mRNA positive cells
1
2
4
weeks

Figure 5


control
immobilization
1 week
2 weeks
4 weeks
(%)
Control group
Immobilization group
The ratio of α-SMA positive cells
25
20
15
10
5
0
1
2
4
weeks
*
*#
*# †