Range of motion exercise suppresses myofibroblast proliferation in the joint capsule in a rat joint contracture model

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Original Article

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Range of motion exercise suppresses myofibroblast proliferation in the joint capsule in a rat joint contracture model

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Abstract

Objective: Immobilization of the rat knee joint causes fibrosis of the joint capsule, and myofibroblasts have been implicated as the cause. This study aimed to perform range of motion exercises on a rat knee joint contracture model and clarify changes in the joint range of motion and changes in the joint capsule using α -SMA-positive cells.

Subjects and Methods: Eighteen male Wistar rats were used and randomly divided into the following three groups: control, immobilized, and exercise. The right hindlimb knee joints of rats in the immobilized and exercise groups were immobilized with external fixation at 120 degrees of flexion, and range of motion exercises were started for the animals in the exercise group the day after the joint immobilization. After a two-week experimental period, the knee joint extension restriction angles were measured, and the knee joints were collected as specimens. To observe the posterior joint capsules of the rat knee joints, hematoxylin and eosin staining and double immunostaining for α -SMA and CD34 were performed.

Results: Differences in knee extension restriction angles were significant between all groups, and differences in the number of α -SMA-positive cells were significant between the control and immobilization groups.

Conclusion: These results suggest that joint immobilization leads to the proliferation of myofibroblasts, and that range-of-motion exercises may inhibit the proliferation of myofibroblasts.

KEY WORDS

rat, joint immobilization, range of motion exercise, myofibroblast

Introduction

The joint capsule is a joint component that can be cited as a cause of limited joint range of motion (ROM). Several reports on experimentally creating joint contracture models using animals and investigating changes in joint components have been reported since the 1960s[1-3)]. We have immobilized rat hind limbs in various ways and observed joint structure changes[4-7)] and reported that collagen fibers become dense in the posterior joint capsule. In recent years, reports have focused on the myofibroblasts in joint capsule inducing the joint capsule fibrosis[8,9)]. These experiments indicate that joint immobilization leads to increased number of myofibroblasts in the joint capsule, suggesting this as the cause of joint capsule fibrosis. An important purpose of physical therapy is the prevention and treatment of joint ROM limitations. In the clinical environment, joint contracture treatment includes hyperthermia and therapeutic gymnastics in addition to ROM exercises (ROM Ex); however, the treatment effectiveness can only be determined by measuring the joint ROM. ROM Ex is a physical therapy treatment technique

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evaluated only by the joint ROM, and there are no reports on how the tissue changes. Joint immobilization causes decreased joint ROM, which is largely due to muscle shortening and rigidity up to 2 weeks, and, thereafter, due to many joint structures (cartilage, joint capsule, ligaments, etc.). Previous reports showed that myofibroblasts are found in the joint capsule due to joint immobilization or joint capsule trauma[8,10-12]. Since myofibroblasts are involved in tissue fibrosis, they are thought to cause physical hardening of the joint capsule[13, 14]. Therefore, this study aimed to perform ROM Ex on a rat knee joint contracture model and clarify joint ROM and myofibroblast changes in the joint capsule.

Materials and Methods

1) Experimental animals and animal care

The protocol for these studies was approved by the Animal Care Committee and Institutional Ethics Committee of Kanazawa University (approval no. 153504) and conducted in accordance with the ARRVE guidelines[15] and all procedures for animal care and treatment were performed in accordance with the guidelines for the care and use of laboratory animals at Kanazawa University. In total, eighteen 9-week-old Wistar male rats (body weight 243–275 g) were used in this study. The rats were purchased (Sankyo Labo Service Corporation, INC.; Toyama, Japan) at 8 weeks of age and placed in individual cages, and the experiment was started after acclimatization to the environment over a week. The breeding room temperature was kept constant, and the lighting was turned on and off every 12 h. Food and water were given ad libitum.

2) Joint immobilization and range of motion exercise

Rats were randomly divided into the following three groups (n = 6): control, immobilized, and exercise. The right hindlimb knee joints of rats in the immobilized and exercise groups were immobilized in a 120° flexion position using external fixation under general anesthesia, as in our previous study[16]. Joint immobilization was performed under clean condition with the sterilized instruments. After the intervention, the immobilized and exercise groups were able to move freely within the cage with their hindlimbs immobilized, and no drinking and eating problems were observed. The exercise group started the ROM Ex under general anesthesia from the day after that of the intervention. Prior to the ROM Ex, the rat trunk was fixed under general anesthesia and the external fixation was removed. Thereafter, a force gauge was attached to the foot of the animals, and the animal's foot was pulled through the force gauge at 1 N to the caudal direction for the first 5 seconds to extend the knee joint, and the next 5 s were held manually at 120° flexion. This exercise was performed once a day for 6 min for 7 days a week. The joints of the rats in the immobilized group were immobilized, and the animals were housed without any other intervention, whereas the control group was housed without any intervention. The experimental period lasted 2 weeks as in previous studies of our group[16-18]. At the end of the experimental period, all rats were euthanized, and knee joint ROM were measured with the hindlimb stretched at 1 N as in our previous study[18]. Knee joint ROM were measured under total anesthesia and the rats in the lateral supine position with the left side down and the trunk fixed. All knee joint ROM measurements were taken by the same examiner. Then the hip joint was cut off and the right hind limb was collected as a specimen. The hind limb was fixed with a neutral buffered formalin solution and then decalcified with a Plank Rychlo solution. After decalcification, the knee joint was sagittally sectioned in the middle and neutralized with 5% sodium sulfate solution. Tissue fixation, decalcification, and neutralization were all carried out for 72 h at 4℃. After neutralization, the sample was washed with water and degreased/dehydrated with ethanol and embedded in paraffin wax. Paraffin-embedded sections were sliced at 3 μm at a site where the posterior joint capsule could be observed, and tissue slides were prepared. To histopathological observation for the posterior joint capsule of the rat knee joint, specimens were stained with hematoxylin and eosin. Tissue images were captured using a light microscope (BX-51; Olympus Corporation, Tokyo, Japan) and a digital camera (DP-74 and cellSens (ver.2.3); Olympus Corporation), and the images were analyzed histopathologically.

3) Immunostaining

To detect myofibroblasts in joint capsule, immunostaining was performed using mouse anti-alpha smooth muscle actin (α-SMA) antibody (ab119952; Abcam plc, Cambridge, UK). However,

within the joint capsule, cells that reaction to α-SMA antibody include not only myofibroblasts but also vascular smooth muscle cells and vascular pericytes. To distinguish myofibroblasts from perivascular cells, vascular endothelial cells were stained with rabbit anti-CD34 antibody (ab81289; Abcam plc.) Tissue slides were deparaffinized, hydrated with graded alcohol, and washed with phosphate-buffered saline (PBS) for 5 min. Next, the tissue slides were placed in sodium citrate buffer (10 mM, pH 6.0) and heated for 60 min at 75℃ for antigen retrieval. After slow cooling to room temperature, endogenous peroxidase was inactivated with a 1% solution of hydrogen peroxide in PBS for 20 min at room temperature. To block nonspecific antibody binding, the sections were incubated with Protein Block Serum-Free (X0909; Dako Japan, Tokyo, Japan) for 15 min at room temperature. Then, the tissue slides were incubated overnight at room temperature with a mixture of mouse anti-α-SMA antibody (1:400 dilution) and rabbit anti-CD34 antibody (1:400 dilution). On the next day, after rinse the tissue slides with PBS, slides were incubated with the appropriate secondary antibodies. First, Histofine Simple Stain AP (M) (for mouse primary antibodies, labeled with alkaline phosphatase, 414241; Nichirei Biosciences Inc., Tokyo, Japan) was dropped on the slides and incubated for 60 minutes. Subsequently, α-SMA antibody positive cells were stained blue using the VECTOR® Blue alkaline phosphatase substrate kit III (Cat. No, SK-5300; Vector Laboratories). The Vector Blue staining solution was prepared by adding the reagent to 5 ml of 100 mM Tris-HCl buffer (pH 8.5) according to the instructions and stirring well. Then, the staining solution were dropped on the slide and incubated for about 10 minutes while confirming with an optical microscope. After rinsing tissue slides with PBS, Histofine Simple Stain Rat MAX PO (R) (for rabbit primary antibodies, labeled with peroxidase, 414181; Nichirei Biosciences Inc.) were used as secondary antibodies and dropped on the slides and incubated for 60 minutes. After incubation, rinse tissue slides in PBS, subsequently CD34 antibody-positive tissues were stained using the VECTOR® NovaRED™ peroxidase substrate kit (Cat. No. SK-4800; Vector Laboratories, California, USA). The NovaRED staining solution was prepared as instructed and stirring them well. The Working solution was dropped on the tissue slide, incubated for 10 minutes while observing with a light microscope, and then washed with PBS. The tissue was counterstained with Nuclear-fast-red, washed with water, dehydrated with ethyl alcohol, clarified with NEO-CLEAR® (Merck KGaA, Darmstadt, Germany), and then mounted with Neo-Mount™ (Merck KGaA, Darmstadt, Germany).

4) Cell number counting

After staining, the slides were examined under an optical microscope, and a digital camera was used to capture 2 mm^2 area of the posterior joint capsule. The number of cells stained blue with α-SMA antibody in the captured images was then counted. When counting the number of cells, cells adjacent to possible blood vessels and CD34-positive cells (stained red) were not counted to exclude vascular pericytes from the cells

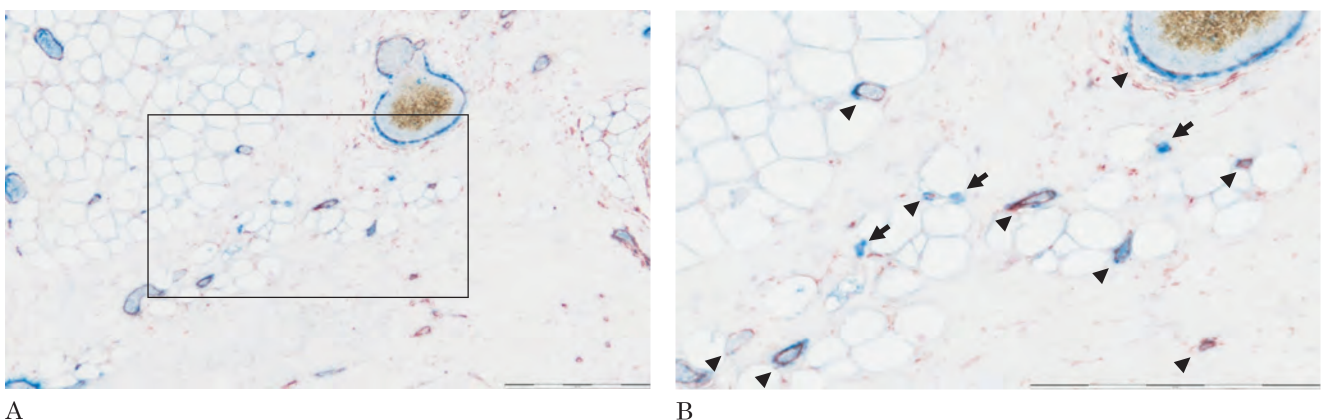
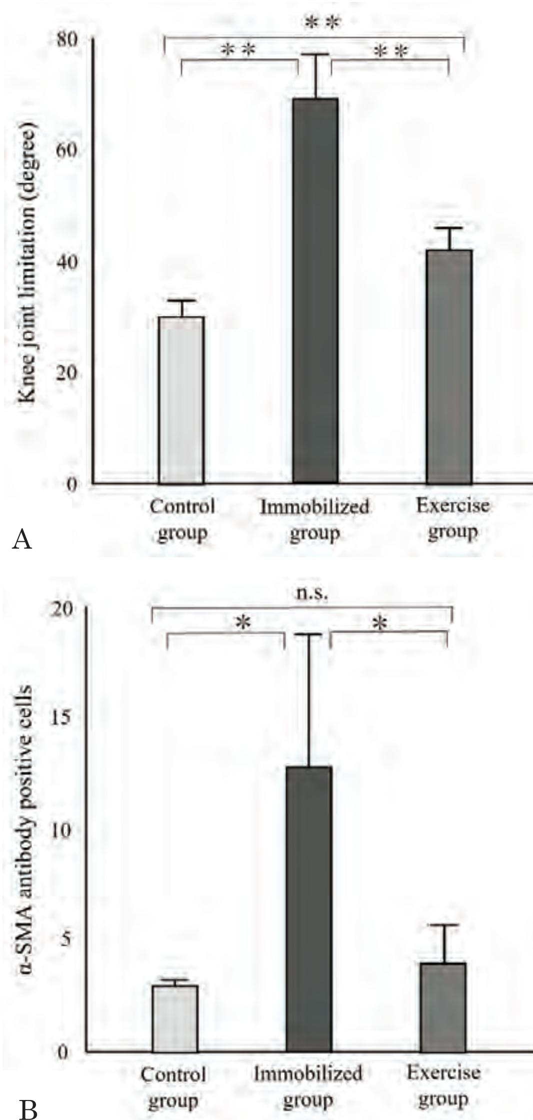


Fig 1. Posterior joint capsule used for cell count measurement.
A: Double immunostaining was performed to distinguish between α-SMA-positive cells that adjacent or not adjacent to blood vessels. Anti-CD34 antibody positive cells (vascular smooth muscle cells and vascular pericytes) are stained red, and anti-α-SMA antibody positive cells (thought to be smooth muscle cells) are stained blue. Scale bar =200 μm
B: High-power view of A. Among the blue-stained anti-α-SMA antibody-positive cells, cells thought to vascular (▲) were excluded, and cells not adjacent to vascular (→) were counted. Scale bar = 200 μm



(*: p<0.01, **: p<0.001)
Fig. 2. Range of motion of the knee joints (A) and number of α-SMA antibody positive cells in each group (B)

stained blue with α-SMA antibody (Fig. 1). Cell counts were performed by a blinded examiner.

5) Statistical analysis

Knee joint limitation data and α-SMA-positive cell counts were each analyzed with one-way analysis of variance using IBM SPSS Statistics for Windows (version 25; IBM Corp., Armonk, NY, USA) and Bonferroni post hoc multiple comparisons. Significant differences were considered at $p < 0.05$, and all results were reported as mean ± SD values.

Results

1) Changes in knee joint ROM and α-SMA positive cells

All rats survived the experimental period without symptoms of infection. Fig. 2 shows the knee joint limitation and number of α-SMA-positive cells. The knee joint extension limitation angles were 30.3 ± 2.9 degrees, 69.0 ± 3.7 degrees, and 41.7 ± 7.8 degrees in the control, immobilized, and exercise groups, respectively. The differences in knee joint extension limit angles among all groups (shown as mean difference [95% CI]), were significant as follows: between the control and immobilized groups, 38.7 [28.8–48.6], p = 1.6 − 12e; between the control and exercise groups, 17.0 [8.2–25.8], p = 1.8 − 8e; and between the exercise and immobilized groups, 21.7 [11.0–32.4], p = 6.9 − 9e). Conversely, the number of α-SMA-positive cells was 3.7 ± 1.4, 12.7 ± 6.1, and 4.8 ± 1.7 in the control, immobilized, and exercise groups, respectively. Differences in number of α-SMA-positive cells (shown as mean difference [95% CI]) were significant between the control and immobilized groups, 9.0 [2.8–15.2], p = 0.002); and between the exercise and immobilized groups, 7.9 [1.6–14.2], p = 0.006). However, no significant difference was observed between the control and exercise groups (7.9 [− 1–3.4], p = 0.851).

2) Histopathological changes in the posterior joint capsule

Each joint capsule is shown in Fig. 3. Fig. 3A shows the control group, in which articular cartilage is exposed in the joint cavity, and the arthrodesis of the lower part of the meniscus is observed. Fig. 3C shows the immobilized group, in which articular cartilage is partially visible, but the capsule is thickened and hypertrophied, invading the joint cavity and narrowing it. Fig.3E shows an exercise group, in which the articular capsule is thickened but the joint space is still preserved. The posterior area of the joint capsule in the control group was characterized by collagen fiber bundles arranged homogeneously in the same direction, relative loose bundles with gaps between. On the other hand, in the immobilized group, the same posterior area of the joint capsule showed a tendency of an increased number of fibroblasts and infiltration of activated fibroblasts (indicating cell plumping) were observed. The collagen fiber bundles in this group were dense and the spaces between them were relatively narrow. In the exercise group, collagen fiber bundles were kept loose, but the fiber orientation was not uniform, and the cell

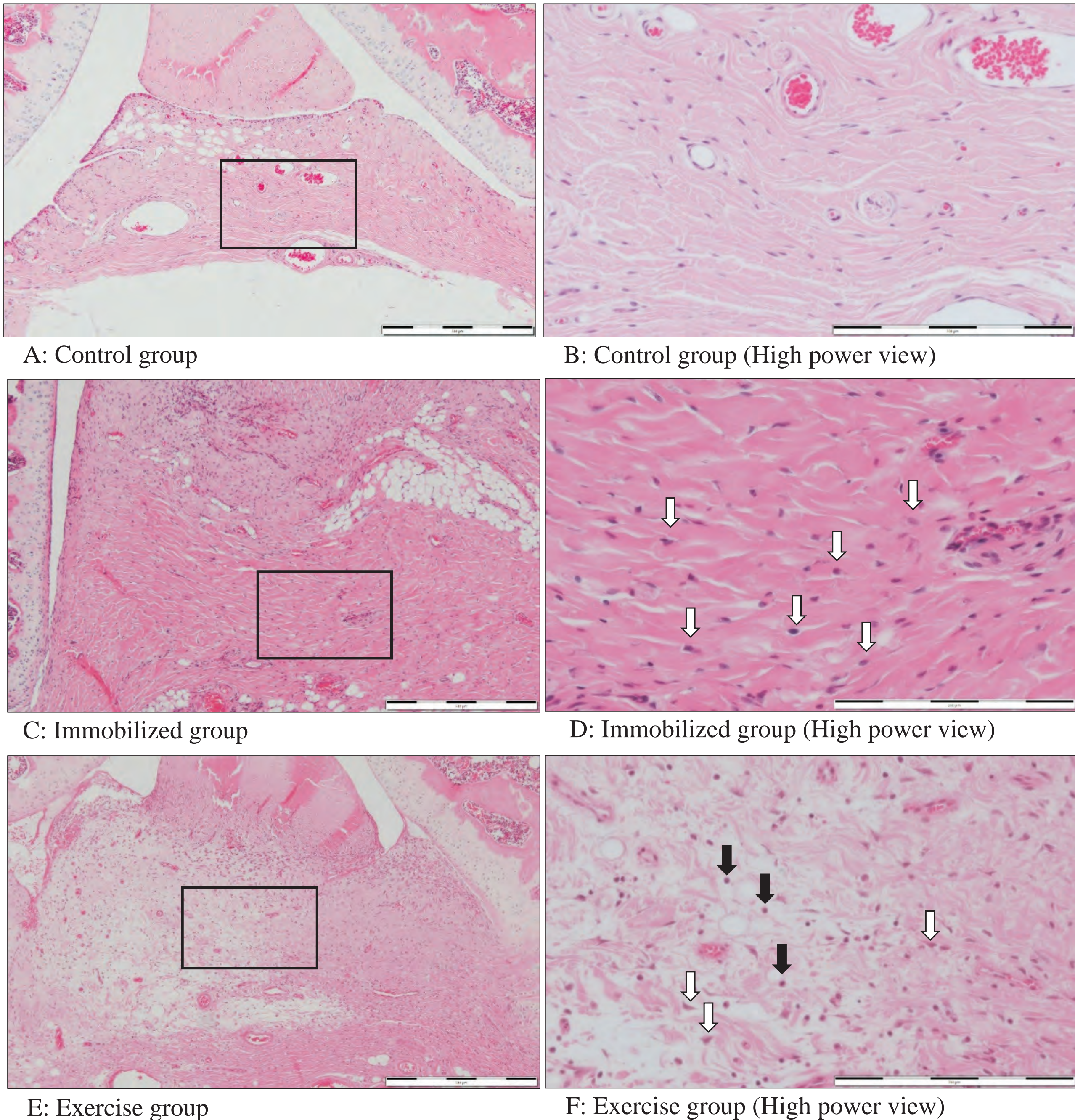


Fig 3. Histopathological assessment of regions in the posterior joint capsule.
In the control group (Fig. 3A, 3B), the collagen fiber bundles overlap in the same direction. There are gaps between the fiber bundles and no densification is observed. In the immobilized group (Fig. 3C, 3D), coarse fibrous tissue, loss of interfiber spaces, and activated fibroblasts (showing cell plumping) were observed in the joint capsule (white arrows). Also, inflammatory cells (neutrophils, black arrows) infiltration was observed in the tissue. In the exercise group (Fig. 3E, 3F), collagen fiber densification was also observed, but it was milder than that of the immobilization group. Activated fibroblasts were observed and inflammatory cells (neutrophils, black arrows) infiltration was observed in the tissue. Scale bar= 500 (Fig. 3A, 3C, 3E) and 200 μm (Fig. 3B, 3D, 3F)

density tended to be high. An increase in fibroblasts was observed compared to the control group, but not as much as in the immobilized group. Infiltration of inflammatory cells (neutrophils) was also observed in the tissue.

Discussion

Joint contracture is one of the common disorders associated with joint Immobility, which often interferes with daily activities. And physiotherapist often perform ROM Ex as a treatment for joint contractures. To clarify its pathology, several studies have been conducted by fixating the joints of animals and

examine the joint histology. Various methods such as cast fixation[23-26], surgery with internal fixation[1,3,25,26], and external fixation[2,26,27] have been used to perform joint immobilization; however, in this study we used external fixation. External fixation facilitates easy joint immobilization or fixation removal to move the joint; however, a risk of infection is present. Therefore, sterilization should be carefully performed during the intervention, and the experiments should be performed while preventing infection. In our experiments, infection appeared to be well controlled.

The myofibroblast was initially identified by means of electron microscopy in granulation tissue of healing wounds as a modulated fibroblast, exhibiting features of smooth muscle cells[28,29]. Moreover, they are observed in normal and pathological tissues. They are considered to be present in fibrous lesions such as wound healing and cirrhosis in pathological tissues[30]. In these experiments, the α-SMA antibody that stains smooth muscle actin was used. Among the tissues of the joint capsule, vascular pericytes are known as tissues having smooth muscle actin in addition to myofibroblasts. To rule out vascular pericytes, vascular endothelial cells adjacent to vascular pericytes were stained with CD34 antibody. We excluded cells adjacent to CD34 antibody-positive cells among α-SMA antibody-positive cells. The results distinguished myofibroblasts, which have been shown to play an important role in post-injury tissue reconstruction and pathological changes characterized by fibrosis[31]. Hildebrand et al. reported that immobilization of the hind limb of rabbit increases myofibroblast production in the joint capsule[8], and reported that myofibroblasts increased in the joint capsule of the human elbow joint with a limited ROM due to trauma[32]. This result indicates that joint immobilization leads to myofibroblast hyperplasia and generic healing mechanism.

In the present study, densification of collagen fiber bundles and activation of fibroblasts were observed in the immobilization group compared to the control group, and densification of collagen fiber bundles was also observed in the exercise group, but to a lesser extent compared to the immobilization group. This suggests that ROM Ex stretching exercise had an inhibitory effect on fiber densification.

Also, inflammatory cells (neutrophis) infiltration was observed in the in the immobilization and exercise groups tissue. Neutrophils account for approximately 50-70% of leukocytes and increase when infection or inflammation occurs[33]. In this experiment, the hindlimb knee joint was stretched to measure the limitation of knee joint extension, which may have caused damage to the joint capsule tissue.

Regarding ROM Ex in these experiments, the ROM limitation was significantly smaller in the exercise group than in the immobilized group, suggesting that ROM Ex was effective. Trudel et al. reported that within 2 weeks after joint immobilization, the ROM is limited by muscles due to joint components[34], suggesting that the ROM Ex protocol conducted may not have been sufficient as a treatment for muscle shortening. Conversely, the number of α-SMA-positive cells in the joint capsule was not different between the exercise and control groups, and a significant increase was observed only in the immobilized group. These results suggest that joint capsule immobilization leads to myofibroblasts proliferation; however, mild exercise aiming to maintain the joint ROM may inhibit myofibroblasts proliferation.

The limitations of this experiment were the short-term experiment (only 2 weeks), and other periods of time were not considered, and the fact that only one ROM Ex protocol was performed; therefore, factors that induce changes in strength or frequency were not investigated. Darby et al. reported that microfilaments in fibroblastic cells increased from day 6 to day 15 after injury in a rat wound healing model, but progressively regressed thereafter, and no long presented by day 30[35]. For this reason, the duration of this experiment seems appropriate; however, changes at weeks 1 or 4 should still be determined. In addition, the mechanism of myofibroblasts proliferation when joints are immobilized remains to be elucidated; the reason why exercise can suppress their proliferation is still unclear. In conclusion, this experiment suggests that joint movement may inhibit joint capsule fibrosis during immobilization, and that joint movement is necessary to maintain the ROM.

Conflicts of interest

There are no conflicts of interest to declare. The authors are solely responsible for the content of the paper.

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関節可動域運動はラット関節拘縮モデルにおける関節包の筋線維芽細胞増生を抑制する

松﨑　太郎 [1)]，吉田　信也 [2)]，細　正博 [1)]

Abstract

【目的】ラット関節不動モデルでは関節包の線維化が生じ，その原因として筋線維芽細胞の関与が指摘されている。本研究は，ラット後肢膝関節拘縮モデルに対し他動的な関節可動域運動を行い，関節可動域の変化および関節包内の変化をα -SMA 陽性細胞を指標として検討することを目的に行った。

【対象と方法】Wistar 系雄性ラット 18 匹を無作為に対照群, 不動群, 運動群の 3 群に分け，不動群と運動群のラット右後肢膝関節を 120 度屈曲位で不動化した。運動群の動物は関節不動化の翌日から麻酔下で関節可動域運動を開始し，不動群は関節を不動化したのみで可動域運動を行わずに飼育し，対照群は介入を行わず通常飼育のみとした。2 週間の実験期間終了後，膝関節の伸展制限角度を計測し，右後肢を採取して標本とした。ラット膝関節の後部関節包を病理組織学的に観察するために HE 染色を行い，α -SMA 抗体陽性細胞から血管平滑筋細胞および血管周皮細胞を除外するため，血管内皮細胞を CD34 抗体を用いて免疫二重染色を行い，CD34 抗体陽性の細胞に隣接しないα -SMA 陽性細胞の数を計測した。

【結果】膝関節伸展制限は全ての群間で有意差を認め，α -SMA 陽性細胞の数は対照群と不動群，運動群と不動群において有意差を認めたが対照群と運動群では有意差が見られなかった。

【考察】CD34 陽性細胞に隣接しないα -SMA 陽性細胞は筋線維芽細胞と考えられ，外傷による関節可動域制限の原因とするものや関節の不動化により筋線維芽細胞の増生が生じるとされているが，今回の実験から関節可動域運動は筋線維芽細胞の増生を抑制できる可能性が示唆された。