

# Changes in joint components after knee immobilization associated with hindlimb unweighting in rats.

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## Abstract

The purpose of this study was to establish a method of knee immobilization associated with hindlimb unloading, and to investigate the histopathological changes of the knee joint components after immobilization and unloading.

Forty male Wistar rats were randomly divided into four groups: control group, unweighting group, knee immobilization group, and unweighting + joint immobilization group. The knee immobilization was performed using external fixation. For the hindlimb unweighting, a Kirschner wire was inserted at the rat's caudal vertebra and a stainless steel wire was attached to the Kirschner wire to allow hindlimb suspension by the tail.

After two weeks of intervention, the body weight and knee range of motion were measured, and the histopathological changes in the articular cavity and joint capsule were examined. There was no significant difference between the body weight and the range of joint motion among the 4 groups before the intervention. After the experimental period, the average body weight was no statistically significant between them.

The knee range of joint motion was no significant different between unweighting group and control group, however, immobilized groups had significant decrease in comparison with non-immobilized groups. In unweighting groups, the cartilage was directly exposed to the articular cavity, and the surface of the articular cartilage was smooth. Conversely, invasion and adhesion of the granulation-like tissue into the joint cavity were observed in joint immobilization groups. Joint capsule of control groups and unweighting groups was typically composed of coarse and relatively loose fibrous connective tissues. The immobilized groups presented dense collagen bundles with narrow interstitial spaces and congested blood vessels in all cases. In the knee immobilization associated with unweighting group, the increase in collagen fiber density was less obvious in comparison with that observed in animals of immobilized group.

Although the Kirschner wire insertion is an invasive intervention, the animals in our study showed increasing body weight compared to the baseline weight. In addition, no suspension failing episodes were observed across the study, thus there was no need to re-suspend or drop-out animals. Therefore, the tail suspension technique using Kirschner wire reported in this study, could be safer and a less stressful technique of hindlimb suspension compared to other conventional techniques.

We also concluded the method described in this study does not allow weight bearing during joint immobilization resulting in better simulation of the joint contracture observation in clinical practice.

## KEY WORDS

Joint immobilization, Hindlimb Unweighting, Tail suspension

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## Introduction

The mechanical causes of joint range of motion limitation can be due to decreased elasticity of muscle and skin and by changes of joint components<sup>1)</sup>, such as joint limitation caused by shortening and adhesion of joint capsules and ligaments.

In orthopedics, mechanical joint limitation caused by tissues localized external to the joint components is defined as contracture, and when the limitation origin is localized inside the joint components, the joint limitation is defined as ankylosis. On the other hand, in rehabilitation medicine, independently to the mechanical origin, joint limitation is defined as contracture.

Once the joint contracture develops, it causes major inconveniences in daily activities and the range of movement limitation is usually not the only issue, pains often occur during joint movement, which leads to decrease in movement, less activities and the deterioration of quality of life. Therefore, in Orthopedic and Physical therapy area, joint contracture is an important issue to be treated and prevented.

Numerous studies using various animal models have been performed for the purpose of clarifying changes in joint components after joint immobilization<sup>2-10)</sup>. These studies performed the joint immobilization using internal / external fixation or cast immobilization.

The majority of those animal studies using, including studies reported by our group<sup>11-16)</sup>, utilized invasive procedures to perform joint immobilization and with most of them allowing weight bearing on the joint during the immobilized period. However, in clinical practice, most of the joint contractures that needs clinical treatments are caused by immobilization due to bone or joint diseases and reduction of joint movement caused by long-term bed resting. In clinical practice, when lower limb joints are immobilized as part of treatment, weight bearing on the joints is usually not allowed. In addition, in cases of long-term bed resting joints cannot bear body weight due to the incapacity of patients to stand. Therefore, animal models conducted so far to assess the impact of prolonged immobilization on joint structure have not considered the effects of joint compression on joint components caused by weight bearing during immobilization and therefore the reported changes of the joint components in joint

limitation may not reflect the changes that are observed in clinical practice.

The purpose of this study was to establish a method of knee immobilization associated with hindlimb unloading, and to investigate the histopathological changes of the joint components after immobilization and unloading.

## Material and Methods

### Ethical issues

The protocols for these experiments were approved by the Animal Care Committee and Institutional Ethics Committee of Kanazawa University (AP-122480), 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.

### Intervention

Forty male Wistar rats (weighing 240-270g) were purchased from Sankyo Labo Service Corporation (Toyama, Japan) and kept under regular conditions for one week before the start of the experiments. They were housed, 1 per cage, in a room maintained under a 12-hour light-dark cycle. Food and water were given ad libitum as previously described<sup>15)</sup>. After getting used to the rearing environment for one week, the rats were randomly divided into 4 groups, with 10 animals per group.

Group 1 was the control group with normal rearing and none intervention. Group 2 was subjected to hindlimb unweighting using tail suspension method. Group 3 had right knee immobilization intervention using external fixation. Group 4 had both interventions, hindlimb unweighting and right knee immobilization as described in Group 2 and 3.

Initially, all rats were anaesthetized using intraperitoneal pentobarbital sodium injection, (dose: 40 mg/kg) and operated for knee external fixation or sham operation under sterile conditions. Prior to the surgery, weight and passive knee range of motion were measured.

In this study, the Knee Range of Motion was determined as the measure of the extension limitation angle when hind limb was pulled in the caudal direction with 1 N force applied by a manual force gauge. The knee angles were measured using a stainless steel finger



Figure 1 : Using the external fixation, the rat hind limb knee joint was immobilized at flexion position.

goniometer as described in previous study<sup>15</sup>).

Group 3 and Group 4 rats were immobilized at 120 degrees of knee flexion using external fixation for right knee (Fig.1).

The animals of Group 2 and Group 4 had a Kirschner wire inserted at the rat's caudal vertebra. The wire was inserted approximately 2 ~ 3 cm from the tail base avoiding the intervertebral discs (Fig 2, 3). A stainless steel wire was attached to the Kirschner wire to allow hindlimb suspension by the tail. The length of the stainless steel wire was adjusted to suspend the hindlimb certifying that they would not touch the cage floor, however not too high to allow free movement and free access to food and water in the cage using the front limbs. The hindlimb suspension were kept throughout the experiment and no suspension failure occurred during the experimental period. The



Figure 2 : A Kirschner wire was pierced into the rat coccyx and a stainless steel wire was inserted. At this time, took care to avoid the intervertebral disc.

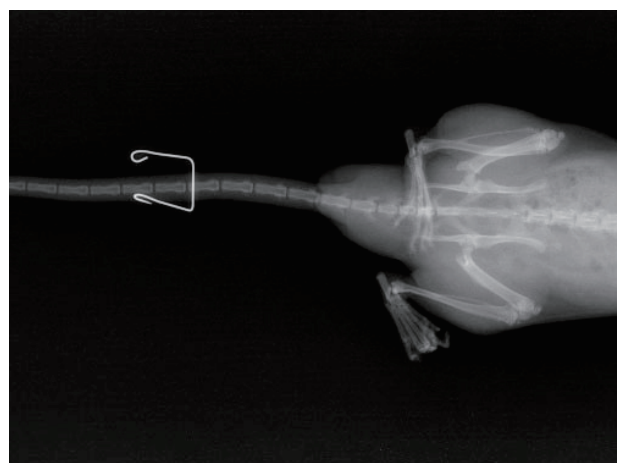


Figure 3 : X-ray findings of the rat's tail.

experimental period was 2 weeks for all groups. All animals had free movement and free access to food and water during the experimental period.

### **Specimen preparation**

After the experimental period, body weight and passive joint range of motion of rats in all groups were measured under anesthesia. Thereafter, the animals were euthanized with overdose of sodium pentobarbital intraperitoneal injection. Immediately after euthanasia, their right hindlimbs were disarticulated and dissected out from the hip joint for histopathological analysis. The area of the caudal vertebra where the Kirschner wire had been inserted, was also dissected for Group 2 and Group 4 rats. The dissected right hindlimbs and tails were fixed in 10% buffered formalin and decalcified. After decalcification, the knees were cut over the sagittal plane at the level of the anterior and posterior cruciate ligament and the tails divided at the level of Kirschner insertion. Following neutralization with 5% sodium sulfate solution. The specimen's fixation, decalcification, and neutralization were kept at 4°C for 72 hours. The specimens were embedded in paraffin, sectioned at 3  $\mu$ m, and mounted on microscope slides. All sections were stained with hematoxylin and eosin stains and used for observation in a histopathological examination. Histopathological analysis was performed to the articular cavity, and the posterior joint capsule.

### **Statistical Analysis**

The statistical analysis was performed using IBM SPSS Statistics for Windows (version 19.0.1, IBM Corp.,



Table 1: Changes in bodyweight and range of motion (\*:  $p < 0.05$ ).

	Before intervention		After intervention		
	Body weight	Range of motion	Body weight	Range of motion	
Group1	293.7±16.7	28.4±3.4	321.1±28.5	31.8±3.8	] n.s.
Group2	287.6±27.8	26.6±3.1	307.5±20.0	32.6±3.8	
Group3	280.6±18.6	26.2±4.0	314.4±15.7	65.8±5.6	] *
Group4	284.9±19.1	30.0±2.3	293.8±38.9	58.6±3.1	

Armonk, NY, USA). Knee range of motion angles were analyzed using one-factor analysis of variance (ANOVA) with Bonferroni post-hoc multiple comparisons. A value of  $p < 0.05$  was accepted as statistically significant. All results are reported as mean  $\pm$  SD.

## Result

All animals survived the experimental period. Rat knees and caudal vertebra showed no visible swelling at the end of the intervention.

There was no significant difference between the body weight and the range of joint motion among the 4 groups before the intervention (Table 1). After the experimental period, the average body weight was  $321.1 \pm 28.5$  g,  $307.5 \pm 20.0$  g,  $321.1 \pm 28.5$  g,  $293.8 \pm 38.9$  g for Group 1 to 4, respectively, and no statistically significant difference was observed between them.

The mean of the range of joint motion was  $31.8 \pm 3.8$ ,  $32.6 \pm 3.8$ ,  $65.8 \pm 5.6$ ,  $58.6 \pm 3.1$  degree for Group 1 to 4, respectively. There was no significant difference between Group 1 and Group 2, however, immobilized groups (3 and 4) had significant decrease in the mean of the range of motion in comparison with non-immobilized groups (1 and 2). There was also a significant decrease difference between Group 3 and Group 4 ( $p = 0.001$ ).

## Histopathological changes in the joint cavity and posterior joint capsule.

In Control Group (Group 1), the cartilage was directly exposed to the articular cavity, and the surface of the articular cartilage was smooth (Fig. 4a). In Group 2 where hindlimb suspension was performed, there was no considerable changes in the joint cavity, compared to the Group 1 (Fig. 4b). Conversely, in Group 3 (joint immobilization), invasion and adhesion of the granulation-like tissue into the joint cavity was observed in all animals (Fig. 4c). In Group 4,

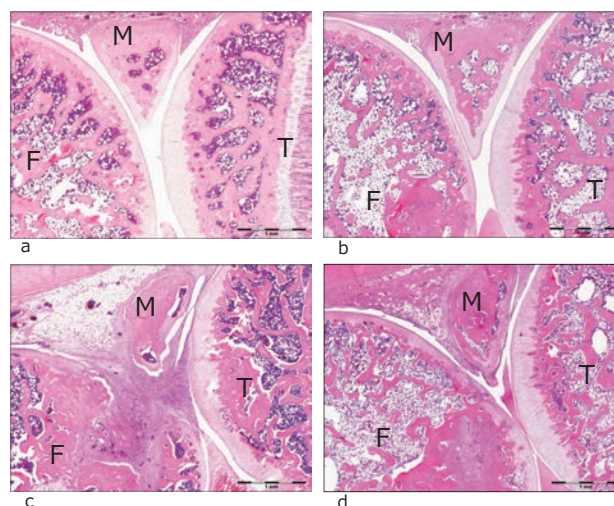


Figure 4: Control Group (Group 1), the cartilage was directly exposed to the articular cavity (Fig.4a). In Group 2, there was no considerable changes in the joint cavity, compared to the Group 1 (Fig.4b). In Group3, invasion and adhesion of the granulation-like tissue into the joint cavity was observed in all animals (Fig.4c). In Group 4, localized membrane-like structure of cartilage surface layer was observed (Fig.4d). F: femur, T: tibia, M: meniscus, Scale bar = 1 mm

(joint immobilization + hindlimb unloading), localized membrane-like structure of cartilage surface layer was observed (Fig. 4d), and adherence between granulation-like tissue and articular cartilage was found in 5 out of 10 animals (Table 2).

In addition, in group 1, the joint capsule was typically composed of coarse and relatively loose fibrous connective tissues (Fig. 5a). In Group 2, as in Group 1, gaps were observed between the collagen fibers, which was relatively loose (Fig. 5b). The immobilized groups (Group 3) presented dense collagen bundles with narrow interstitial spaces and congested blood vessels was observed in all cases (Fig. 5c).

However, in the joint immobilization and unweighting group (Group 4), the increase in collagen fiber density was less obvious in comparison with the observed in animals in Group 3 (Fig. 5d). Although no visual signs of inflammation were observed on the caudal vertebra

Table 2: Histopathological changes in the joint cavity

	Invasion of granulation-like tissue into the joint cavity	membrane-like structure of cartilage surface layer	Adhesion of granulation-like tissue to articular cartilage
Group1	0	0	0
Group2	0	0	0
Group3	10	10	10
Group4	4	7	5

The numerals represent the number of individuals.

where the Kirschner wire was inserted, histologically a mild neutrophil infiltration could be observed (Fig 6).

### Discussion

Several joint immobilization methods that cause changes in joint components have been reported so far, however, in those studies only immobilized the joints was performed, allowing the weight bearing on the limb. The allowance of weight bearing probably results in pressure applied to the immobilized joint during limb movement with floor contact.

In addition, various hindlimb unweighting animal models to study disuse muscle atrophy has been reported<sup>17-21)</sup>, however, few studies have reported the body weight change. Yamauchi and colleagues<sup>17)</sup> reported that rat's tail suspension for 3 weeks significantly decreased body weight in 4 month-old rats, however this decrease was not observed in 20 month-old rats. In another study that utilized pierced Kirschner wire into the rat's caudal vertebra to suspend the hindlimbs reported a significant weight loss in the suspension group<sup>19)</sup>, which contrasts with the observed in the present study with no statistical difference in body weight among all groups after the experimental period. This can be due to the hindlimb suspension height, which was set to be as minimum as possible, just the enough to avoid the hindlimb contact to the cage floor. This method also allows free trunk movement and less constrain maintaining animals closer to the normal physiological conditions and consequently resulting in less stress and less weight loss. As a result, although the Kirschner wire penetration is an invasive intervention, animals utilized in our study showed increase body weight compared to the baseline weight. In addition, no suspension failing episodes were observed across the study, thus there was no need to re-suspend or drop-out animals. Therefore, the tail suspension technique using

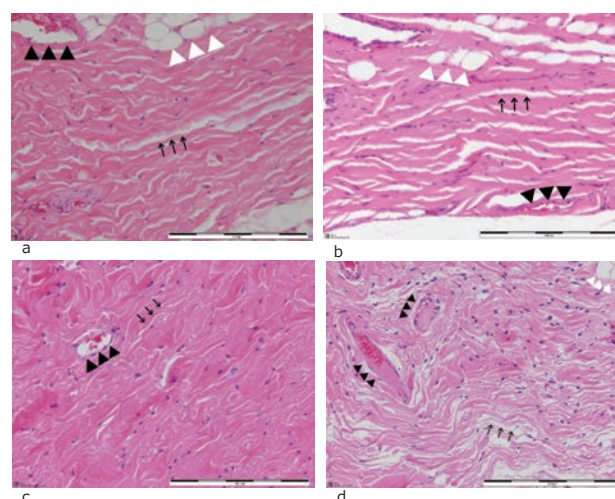


Figure 5: In Group 1, the joint capsule was typically composed of coarse and relatively loose fibrous connective tissues (Fig 5a). In Group 2, gaps were observed between the collagen fibers (Fig 5b). The immobilized groups (Group 3) presented dense collagen bundles with narrow interstitial spaces and congested blood vessels was observed in all cases (Fig.5c). In the joint immobilization and unloading group (Group 4), the increase in collagen fiber density was less obvious in comparison with the observed in animals in Group 3 (Fig.5d).  
▲▲▲ : vessel, △△△ : adipocytes, ↑↑↑ : Gap between collagen fibers (Typical examples), Scale bar =200 μ m

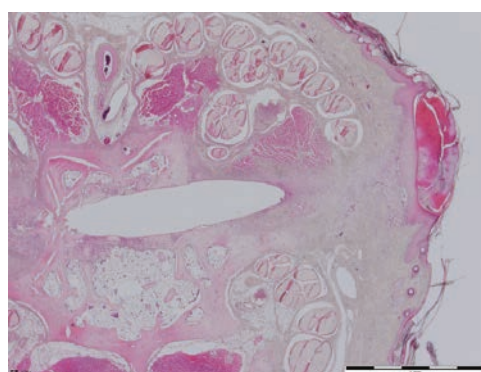


Figure 6: There were no visual signs of inflammation were observed on the caudal vertebra where the Kirschner wire was inserted. Scale bar=2mm

Kirschner wire reported here, could be safer and a less stressful technique of hindlimb suspension compared to other conventional techniques.

In a previous study using immobilize by internal fixation, the cartilage matrix disappeared at the contact surface, and no change was observed on the matrix of the noncontact surface, but some fibrosis was observed in the surface layer<sup>2)</sup>. In another study that used external fixation of rabbit hindlimbs for the purpose of increasing pressure on articular cartilage, articular cavity lost, thin cartilage on load surface, and decreased staining of chondrocyte nucleus were observed<sup>3)</sup>. In our previous study, casting and unloading induce **d** invasion of the granulation-like tissue into the cartilage<sup>16)</sup>. In the present study, load and pressure were applied to the joints during the immobilization. Similar findings such as joint articular cavity lost and cartilage matrix lost were found in the Group 3 (immobilization and no hindlimb suspension). Thaxter reported that joint immobilization using internal fixation resulted on soft tissue proliferation and adhesion, bone necrosis or ulceration, however when immobilization was associated with unweighting, bone necrosis or ulceration was not observed and those joint histological images were similar to the initial changes observed in joint immobilization<sup>22)</sup>. These findings can be considered similar to findings observed in our experiments (Group 4 findings). The articular cartilage does not contain blood vessels and nerves and the nutrition is considered to rely on diffusion of synovial fluid which flow is assisted by joint movement<sup>23)</sup>. Therefore, nutrition supply to cartilage and disposal of metabolites requires intermittent load and regular joint movement. This nutritional and metabolic characteristics can explain the absence of change in articular cartilage in Group 2 which had no joint immobilization intervention in spite of unweighting. On the other hand, after pressuring the joint and cartilage, decrease in the staining property of cartilage nucleus and cartilage matrix, and cartilage fibrosis were observed in Trias study<sup>4)</sup>. Our results suggest that the unweighting state despite joint immobility (Group 4), the changes in the joint components are mild and localized, and the sustained load on the cartilage affects greater causing cartilage major changes (Group 3). Those results lead to the need to reanalyze the conventional methods that have

not been considering the weight bearing effects during joint immobilization once it could be an important factor influencing the greater changes of articular cartilage.

In the posterior joint capsule, findings of fibrous densification and blood congestion were observed after joint immobilization (Group 3). Same findings were found in our previous studies<sup>11-14)</sup>. However, in the unweighting associated group (Group 4), the changes in the joint capsule were too mild and no noticeable differences such as the articular cartilage changes were observed compared to the free loading group (Group 3). These facts suggest that induced changes in articular cartilage and joint capsule after joint immobility may have different causes and mechanisms.

Considering that the conventional research methods to study the effects of immobilization allow weight bearing during the experimental intervention, the method described here does not allow weight bearing during joint immobilization and resulted in better simulation of the joint contracture seen in clinical practice. We believe the findings reported here can contribute to further understanding in joint contractures.

### Limitation

In this experiment, Kirschner wire was inserted into the rat's tail and it was able to suspend the hindlimbs for 2 weeks, but we do not confirm how long it can last in place. This is important because the increased body weight can result in overloading the tail during suspension, which may result in inflammation. It is also necessary to keep in mind the importance of infection prevention and others potential complications that can develop in longer periods of tail suspension.

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## 非荷重でのラット後肢膝関節の不動による関節構成体の変化

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### 要 旨

今回の実験の目的は、ラット後肢を非荷重としたまま飼育する方法を確立すること、および荷重の有無が関節不動による関節構成体の変化にどのように影響するのかを調査することである。

40匹のWistar系雄性ラットを使用し、無作為に4群に分けた。それぞれ対照群、非荷重群、関節不動群、不動と非荷重群とした。関節不動は創外固定を用いて後肢膝関節を屈曲120度で不動化した。非荷重群はラットの尾骨にKirschner鋼線を刺し、ステンレスワイヤを用いて尾部を懸垂し、足部が接地しないようにした。

介入の2週後、体重と関節可動域を測定した後に関節腔と関節後方の関節包を病理組織学的に観察した。

実験後の体重には各群に差は見られなかった。関節可動域は対照群および非荷重群に対し不動群、関節不動と非荷重群で有意に減少していた。また、不動と非荷重群と比較して関節不動群では有意な可動域の減少が見られた。関節腔では対照群、懸垂群は軟骨が直接関節腔に露出していたが、関節不動群、不動と非荷重群では肉芽様組織の関節腔内の侵入、関節軟骨表層の膜様組織との癒着が観察され、不動と非荷重群では軟骨表層の膜様組織は限局的であった。関節包は対照群、非荷重群ではコラーゲン線維間に間隙を認め、比較的疎性であったが、関節不動群ではコラーゲン線維束はやや組硬化し、線維素区間が狭まり密生化しており、全例でうっ血像が観察された。不動と非荷重群では線維の密生化は見られたものの関節不動群と比較して軽度であった。

今回の手技は侵襲はあるものの懸垂が外れることはなく、より臨床での関節拘縮に近い研究を行う一助となると考えられる。