Reversibility of Immobilization-Induced Articular Cartilage Degeneration after Remobilization in Rat Knee Joints

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Joint immobilization is commonly used for the treatment of joint injuries, but it causes articular cartilage degeneration. The purpose of this study was to clarify the reversibility of immobilization-induced articular cartilage degeneration using rat knee joints. Immobilization of rat knee joints induces atrophic changes in the non-contact area, loss of chondrocytes in the contact area, and hypertrophy of chondrocytes in the transitional area of the articular cartilage. The unilateral knee joints of adult male rats were rigidly immobilized at 150° of flexion with a plate and screws for 1, 2, and 4 weeks. After the experimental periods, the fixation devices were removed and the rats moved freely for 16 weeks. Sham-operated rats were used as a control. Sagittal sections at medial midcondylar regions of the knee were assessed with histological and histomorphometric methods. Mechanical properties were assessed by measuring the sound speed with scanning acoustic microscope. Reduction of cartilage proteoglycan in the non-contact area was almost reversible, but hypertrophy of chondrocytes in the transitional area and loss of chondrocytes in the contact area were irreversible even at 1-week immobilization-remobilization group. Sound speed of the articular cartilage in the contact area was not restored. These results indicate that atrophic changes through decreased mechanical stress in the non-contact area were reversible, but loss of chondrocytes and hypertrophy of chondrocytes in the contact and transitional areas through increased mechanical stress by rigid immobilization were irreversible after remobilization. Clinicians should be aware that even a short-term rigid immobilization could cause irreversible articular cartilage damage.

Keywords: cartilage; degeneration; elasticity; immobilization; reversibility


Joint immobilization is commonly used for the treatment of joint injuries such as periarticular fractures and ligament injuries, but it also causes articular cartilage degeneration (Brandt 2003). Immobilization-induced articular cartilage degeneration is generally recognized as disuse atrophy caused by decreased chondrocytes activity (Palmoski et al. 1979; Jurvelin et al. 1986; Kiviranta et al. 1994; Buckwalter 1995; Setton et al. 1997). Palmoski et al. documented that joint immobilization caused reduced proteoglycan content and synthesis, decreased articular cartilage thickness, but did not change the number of chondrocytes in canine immobilized knee model with a cast (Palmoski et al. 1979, Palmoski and Brandt 1981). In our rat immobilized knee model with a plate and screws, the changes in the non-contact area was similar to the results by Palmoski, but the changes in the contact area (the area where the femur and the tibia contacts directly) and the transitional area (the area between the non-contact and contact areas) were totally different from disuse atrophy, and the following changes were observed; decreased number of chondrocytes in the contact area, and hypertrophy of chondrocytes in the transitional area (Hagiwara et al. 2009). Evans et al. (1960) showed using similar models like ours that rigid immobilization induced death of the chondrocytes in the contact area caused by compressive forces. These results have indicated that articular cartilage degeneration differs between non-rigid and rigid immobilization, and between non-contact and contact areas, based on mechanical forces at specific areas.

and the control group, respectively.

**Experimental design and tissue preparation**

Originated from duration of immobilization, methods of immobilization (non-rigid and rigid immobilization), measurement sites (non-contact, contact, and transitional areas), and animal species (dog, rat, and rabbit). Haapala et al. reported that the articular cartilage changes induced by 11-week of immobilization, such as decreases in proteoglycan content, synthesis, and articular cartilage thickness, rapidly returned to the normal level after remobilization, but full recovery was not obtained even after 50 weeks of remobilization in canine immobilized knees with a cast (Haapala et al. 1999, 2000). Behrens et al. compared the degree of recovery in degenerated articular cartilage induced by non-rigid immobilization with a cast and rigid immobilization with an external fixator in a canine knee model, and concluded that severely-degenerated articular cartilage by rigid immobilization did not recover after remobilization (Behrens et al. 1989). To clarify the reversibility of articular cartilage degeneration after immobilization will give us an insight into approaches to rehabilitation of immobilized joints.

Joint immobilization caused softening of the articular cartilage (Jurvelin et al. 1986, 1989; Setton et al. 1997; Haapala et al. 2000; LeRoux et al. 2001). We revealed in the previous study that sound speed, which strongly correlated with tissue elasticity, of the articular cartilage decreased after immobilization using a scanning acoustic microscope (SAM) (Hagiwara et al. 2009). Scanning acoustic microscope is a powerful tool for measuring tissue elasticity in situ on a slide glass (Hozumi et al. 2004). In the present study we used SAM to estimate the reversibility of immobilization-induced cartilage degeneration after remobilization.

**Materials and Methods**

**Experimental design and tissue preparation**

The protocols for the experiments were approved by the Animal Research Committee of Tohoku University. Forty-eight adult male Sprague-Dawley rats aged 12 weeks were prepared for histology. Unilateral knee joints of the rats were rigidly immobilized at 150° of flexion with a plastic plate and metal screws as previously described (Hagiwara et al. 2006; Ando et al. 2008). Sham-operated rats had holes drilled in the femur and tibia with metal screws inserted, but without a plate. After 1, 2, and 4 weeks, the plastic plate and screws of the immobilized rats and screws of the sham-operated rats were removed, and the rats were allowed to move freely in standard cages for 16 weeks \( n = 8 \) (each period). The immobilized rats and sham-operated rats made up the immobilized-remobilized (Im-Re) group and the control group, respectively.

At the end of the experimental period, the rats were killed with an overdose of sodium pentobarbital and fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) by perfusion through the ascending aorta. The knee joints were manually flexed at 150° and kept in position during fixation (Hagiwara et al. 2010). The knee joints were resected and kept in the same fixative for 24 hours and decalcified in 10% ethylenediaminetetraacetic acid in 0.01 M PBS for 2 months at 4°C. The decalcified specimens were then embedded in paraffin. Five-μm serial sections were obtained at the medial midcondylar region in sagittal plane as previously described (Hagiwara et al. 2006; Ando et al. 2008).

The other twelve rats were prepared for inspection of the gross appearance of the articular cartilage. After the experimental period \( n = 2 \) (each period), the capsule of the knee joint was cut with a surgical knife and the joint was opened. After resection of bilateral menisci, the gross appearance of the articular cartilage was inspected.

**Histology and Histomorphometry**

The sections were stained with hematoxylin-eosin (HE) and safranin O-fast green (SO) for histological and histomorphometric analyses. Six areas (non-contact area, transitional area, and contact area of the femur and tibia) were set as previously described (Hagiwara et al. 2009). Non-contact area is the area that does not contact the opposing articular cartilage and meniscus. Contact area is the area that contacts the facing articular cartilage directly. Transitional area is the area between the contact and non-contact areas, and contacts to the anterior meniscus. Modified Mankin’s histological grading scheme was used for evaluation of the articular cartilage degeneration (Sakakibara et al. 1994; Ando et al. 2008; Hagiwara et al. 2009). Modified Mankin’s histological grading scheme was used for evaluation of the articular cartilage degeneration (Sakakibara et al. 1994; Ando et al. 2008; Hagiwara et al. 2009). The grading scheme is composed of 5 items: I: structure, II: cell-tangential zone, II: cell-transitional and radial zone, III: safranin O staining, IV: tidemark, V: pannus formation) and each item is graded as I: 0-10, II-1: 0-2, II-2: 0-10, III: 0-4, IV: 0-3, V: 0-3. HE sections were used to assess the structure, cells, tidemark, and pannus formation. Loss of proteoglycans was assessed by SO sections. Light microscopic images in each area were captured by another observer at magnification ×200. The distance between the osteochondral junction and the articular cartilage surface were measured at the mid-portion of the captured images. Number of chondrocytes was counted in the rectangle 100 μm deep and 400 μm long in the non-calculated articular cartilage using the captured images.

**Scanning Acoustic Microscope (SAM)**

Mechanical properties of the articular cartilage were assessed by measuring the sound speed using the scanning acoustic microscope (SAM). A total of forty-eight samples were prepared and the changes in each area were evaluated. The system of SAM has been reported elsewhere (Hozumi et al. 2004; Hagiwara et al. 2006), and thus, is briefly described here. A single pulse ultrasound with 5-ns pulse width was emitted and received by the same transducer above the specimen. The reflections from the deparaffinized tissue surface and from the interface between the tissue and the glass were received by the transducer and were introduced into a digital oscilloscope. Finally, two-dimensional distributions of the ultrasound intensity, sound speed and thickness of the 2.4 by 2.4 mm specimen area were visualized with 300 by 300 pixels. SAM images with a gradation color scale were produced for clear visualization of the sound speed. The optical and acoustic images were compared to ensure morphological congruence in the analysis.
**Statistical analysis**

Differences between the Im-Re and control groups were compared at each time point by Mann-Whitney’s U test (histological scores) and by unpaired t-test (thickness and cell density). Data were expressed as mean ± standard deviation. A value of \( p < 0.05 \) was considered as statistically significant.

**Results**

**Gross Appearance and Histology**

The articular cartilage surface in the control group was smooth and no apparent degeneration was observed (Fig. 1A). The articular cartilage surface in the Im-Re group was also smooth but subchondral bone was seen through the articular cartilage in the contact area (Fig. 1B). Apparent degeneration was not observed in the non-contact area both in the Im-Re and control groups. Synovial adhesions around the cruciate ligaments were observed in the Im-Re group.

The articular cartilage in the control group showed no apparent degeneration in all of the specimens (Fig. 2A). Chondrocytes in the contact area of the non-calcified cartilage decreased and disappeared in the 1-week and 2-week Im-Re groups both in the femur and tibia (Fig. 2B, C). Apparent fibrillation and cleft formation were not observed. In the transitional area, hypertrophy and cloning of chondrocytes in the transitional and radial zones were observed, and chondrocytes in the tangential zone were also hypertrophic or disappeared in the 2-week Im-Re group (Fig. 2D). In the non-contact area, morphology of the articular cartilage was almost normal, but ingrowth of connective tissues on the articular cartilage surface was observed especially on the tibial side in the 4-week Im-Re group (Fig. 2E).

The articular cartilage in the control group was well stained with SO and apparent loss of staining intensity was not observed (Fig. 2F). In the Im-Re group, a marked reduction of SO staining intensity was observed at the area of chondrocytes disappearance in the contact area both in the femur and tibia (Fig. 2G, H). A slight or no reduction of SO staining intensity was observed in the calcified articular cartilage. In the non-contact area, alteration of the SO staining intensity in the non-calcified articular cartilage was slight or absent, but the reduction of the staining intensity was observed around the tidemark (Fig. 2I). In this area, the tidemark was double- or multi-layered. In the transitional area, apparent changes of SO staining intensity were not observed.

**Histomorphometry**

The modified Mankin’s score in the non-contact area was significantly higher in the 2-week and 4-week Im-Re groups compared to that in the control group both in the femur and tibia (Fig. 3A, D). Scores of the safranin O, tidemark, and pannus mainly contributed to the significant difference in the non-contact area. In both transitional and contact areas, the scores in the 1-, 2- and 4-week Im-Re groups were significantly higher than the control group (Fig. 3B-F). Score of the cells in the transitional and radial zone mainly contributed to the significant difference in the transitional and contact areas.

Articular cartilage thickness in the non-contact area did not show any statistical differences between the Im-Re and control groups at all time points (Fig. 4A, D). In the transitional area, thickness in the femur in the Im-Re group did not show any statistical differences at 1 and 2 weeks, but significantly higher at 4 weeks compared to that in the control group (Fig. 4B). The thickness in the transitional area in the tibia showed similar tendency, but no statistical differences were observed (Fig. 4E). The thickness in the contact area did not show any statistical differences between the Im-Re and control groups at all time points (Fig. 4C, F).

The number of chondrocytes in the non-contact area did not show any statistical differences between the Im-Re and control groups at all time points (Fig. 5A, D).
number in the transitional area did not show any statistical
differences at 1 week, but it was significantly smaller at 2
and 4 weeks in the Im-Re group compared to that in the
control group both in the femur and tibia (Fig. 5B, E). The
number in the contact area in the Im-Re group was signifi-
cantly smaller at 2 and 4 weeks in the femur and at 1, 2, and
4 weeks in the tibia compared to that in the control group
(Fig. 5C, F).

**Scanning Acoustic Microscope**

The gradation color images of the articular cartilage in
the Im-Re group apparently differed from those in the con-
trol group (Fig. 6). In the contact area, the articular car-
tilage in the control group was yellow-green, however, the
articular cartilage in the Im-Re group was almost blue (low
sound speed) (Fig. 6A, B). The low sound speed area cor-
responded to the area with cell disappearance and proteo-
glycan depletion (see Fig. 2G). Apparent sound speed dif-
fferences were not observed in the non-contact and the
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Fig. 3. Time lapse changes in the femoral and tibial articular cartilage by histomorphometric examination. A, Femoral non-contact area. B, Femoral transitional area. C, Femoral contact area. D, Tibial non-contact area. E, Tibial transitional area. F, Tibial contact area. In the non-contact area, the score was significantly higher in the 2- and 4-week Im-Re group both in the femur and tibia (A, D). In the transitional and contact areas, the score was significantly higher in the 1-, 2-, and 4-week Im-Re group (B, C, E, and F). Data were expressed as mean ± standard deviation. White bars, control group. Gray bars, Im-Re group. ** = P < 0.005, * = P < 0.05 versus control.

Fig. 4. Articular cartilage thickness of the femur and tibia. A, Femoral non-contact area. B, Femoral transitional area. C, Femoral contact area. D, Tibial non-contact area. E, Tibial transitional area. F, Tibial contact area. Articular cartilage thickness in the non-contact and contact areas did not show any statistical differences between the Im-Re and control groups at all time points both in the femur and tibia (A, C, D, and F). The thickness of the transitional area in the femur was significantly higher in the 4-week Im-Re group compared to that in the control group (B), but the thickness in the tibia did not show any significant differences (E). Data were expressed as mean ± standard deviation. White bars, control group. Gray bars, Im-Re group. ** = P < 0.005, * = P < 0.05 versus control.
transitional areas between the Im-Re and control groups.

**Discussion**

We clarified in the present study that reduction of proteoglycan in the non-contact area was almost reversible, but hypertrophy of chondrocytes in the transitional area and loss of chondrocytes in the contact area were irreversible even after 16 weeks of remobilization. Many authors reported that reduction in the synthesis and content of proteoglycan occurred after immobilization rapidly recovered after remobilization (Finsterbush and Friedman 1975; Palmoski et al. 1979; Behrens et al. 1989; Setton et al. 1997; Haapala et al. 1999). We also reported in the previous study that immobilization induced loss of SO staining intensity in the non-contact area (Hagiwara et al. 2009). Though the loss of staining intensity was almost restored...
after remobilization, the staining intensity around the tidemark was not completely restored in the 4-week Im-Re group. Incomplete recovery of SO staining around the tidemark, tidemark irregularity, and synovial adhesion on the articular cartilage mainly contributed to the significant differences of the Mankin’s score. Because nutrition of the articular cartilage is derived from the synovial fluid (Hodge and McKibbin 1969), cartilage degeneration in the non-contact area may be due to the lack of pumping action created by joint motion, and because remobilization may resume the pumping action, the recovery in the non-contact area might prove that immobilization does not induce irreversible damage on the articular cartilage and chondrocytes in the non-contact area. Adhesive tissues on the articular cartilage in the non-contact area and cruciate ligaments were not removed after 16 weeks of remobilization. We clarified in the previous study that immobilization induced synovial adhesions to the opposing synovial membrane and the articular cartilage in the non-contact area after 4 weeks (Ando et al. 2010). These changes may affect the property of the synovial membrane and synovial fluid production. Adhesions of synovial membrane and reduction in synovial fluid production may contribute to the incomplete recovery of proteoglycan content around the tidemark.

Reversibility of the articular cartilage in the contact area was controversial, because animal species, duration and methods of immobilization were different from study to study (Evans et al. 1960; Sood 1971; Finsterbush and Friedman 1975; Akeson et al. 1977; Palmoski et al. 1979; Setton et al. 1997). Evans et al. reported that rigid immobilization with a plate and screws like ours led to articular cartilage ulceration and necrosis in the contact area because of an increase in compressive force (Evans et al. 1960). On the other hand, numerous reports documented that non-rigid immobilization with a cast rarely caused irreversible articular cartilage degeneration in the contact area (Palmoski et al. 1979; Behrens et al. 1989; Jurvelin et al. 1989; Säämänen et al. 1990; Kiviranta et al. 1994; Schollmeier et al. 1996; Setton et al. 1997; Haapala et al. 1999; Trudel et al. 2008). In our previous study, we clarified that 4-week immobilization induced significant loss of chondrocytes in the contact area, hypertrophy of chondrocytes in the transitional area, and proteoglycan loss in the non-contact area (Hagiwara et al. 2009). Because the degree of the articular cartilage degeneration was progressed with immobilization periods in our model, there remains less possibility of restoration after remobilization in prolonged immobilized knees (8 and 16 weeks). Therefore, we did not include longer duration of immobilization than 4 weeks in this study. Haapala et al. reported that 50 weeks of remobilization was insufficient for complete restoration of immobilization-induced cartilage degeneration in canine model, but the duration is inappropriate for rat (Haapala et al. 1999, 2000). Trudel et al. reported reversibility of joint contracture after 4 weeks of remobilization in 8 weeks of immobilized knee with the rat model (Trudel et al. 2008), but 4 weeks of remobilization was thought to be insufficient, therefore we set 16 weeks as duration of remobilization.

We clarified in the present study that sound speed of the articular cartilage in the contact area was not restored after remobilization. The low sound speed area corresponded to the area of cell disappearance and proteoglycan depletion. It indicates that loss of chondrocytes and proteoglycans may affect the elasticity of the articular cartilage. To our knowledge, this is the first report of sound speed changes of the articular cartilage after remobilization. Fixation method influences tissue elasticity, therefore we set the time for fixation in this study. In the control group, the immersing time was not different from that in the experimental group. That’s why influence of fixation on the articular cartilage was not taken into consideration. Data from SAM are to be evaluated quantitatively. Because of remobilization, the boarders of the non-contact and transitional areas, the transitional and contact areas are unclear compared with our previous study of immobilization. This is why we could not evaluate the sound speed quantitatively.

In conclusion, we clarified in the present study that
loss of proteoglycan in the non-contact area was almost reversible, but hypertrophy of chondrocytes in the transitional area and decreased number of chondrocytes in the contact area were irreversible even after 16 weeks of remobilization. Clinicians should be aware that even a short-term rigid immobilization could cause irreversible articular cartilage damage.

Acknowledgments

We wish to thank Mr. Katsuyoshi Shoji and Mrs. Michiko Fukuyama, Department of Orthopaedic Surgery, Tohoku University School of Medicine, for their excellent assistance.

Conflict of Interest

The authors report no conflict of interest.

References


