Rabbit Model of Extending Knee Joint Contracture: Progression of Joint Motion Restriction and Subsequent Joint Capsule Changes after Immobilization

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Abstract

This study aimed to develop a rabbit model of knee contracture in extension and investigate the natural history of motion loss and time-dependent changes in the joint capsule after immobilization. We immobilized the unilateral knee joints of 32 rabbits by maintaining the knee joint in a plaster cast at full extension. Eight rabbits were euthanized at 2, 4, 6, and 8 weeks after casting, respectively, and the lower extremities were disarticulated at the hip joint. Eight control group rabbits that did not undergo immobilization were also examined. We assessed the progression of joint contracture by measuring the joint range of motion, evaluating the histologic alteration of the capsule, and assessing the mRNA levels of transforming growth factor $\beta 1$ (TGF- $\beta 1$) in the anterior and posterior joint capsules. After 2 weeks of joint immobilization, the knee joint range of motion was limited, the synovial membrane of the suprapatellar and posterior joint capsules was thickened, the collagen deposition was increased, and the mRNA levels of TGF- $\beta 1$ were elevated in the anterior and posterior joint capsules. These changes progressed rapidly until 6 weeks of immobilization and may advance slowly after 6 weeks. Joint contracture developed at the early stage of immobilization and progressed over time. The changes in the anterior and posterior joint capsules after joint immobilization may contribute to the limitation in flexion. The elevated mRNA expression of TGF- $\beta 1$ may be related to joint capsule fibrosis and may be one of the causes of joint contracture.

Keywords

- joint contracture
- animal model
- joint range of motion
- joint capsule
- rabbit

Joint contracture is defined as a decrease in joint range of motion (ROM) due to various reasons.^{1–3} The formation of joint contracture may limit the activities of daily living, and some patients eventually develop lifelong disabilities. Joint contracture is currently considered to be one of the most troublesome problems in orthopaedic trauma and rehabilitation medicine. Clinically, many diseases (including joint damage, joint immobilization, and some nervous system diseases) can result in the development of joint contracture, of which joint immobilization is considered to be the most common one.^{4,5} Joint immobilization is often used as an orthopaedic treatment

for patients with joint injuries and other musculoskeletal disorders.^{6,7} However, the long-term side effects of joint immobilization are incidental. After a joint was immobilized for a long time, connective tissue shortening, muscle atrophy, and weakening strength of muscles around the joint may be caused, inevitably leading to restricted joint activities and then influencing the patients' qualities of life.^{8–11}

The development of immobilization-induced joint contracture may be influenced by two anatomical components around the joint: arthrogenic components and myogenic components. The arthrogenic components are injuries to the bone, cartilage,

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capsule, and synovial membrane, while the myogenic components are lesions of the muscle, tendon, and fascia.^{6,12} Arthrogenic components, particularly regarding the joint capsule, are reportedly important factors in the formation of immobilization-induced joint contracture.^{6,13,14} Reported changes in the capsule after immobilization include proliferation of connective tissues within the joint space, and adhesions in the capsule and synovial membrane.^{15,16} Some studies reported that joint capsule fibrosis may occur in the posttraumatic joint contracture caused by joint immobilization in flexion in rabbit or rat models.^{17,18} Furthermore, Hagiwara et al¹² reported that the increased elasticity and structural changes of the posterior joint capsule may result in limited extension after a long period of immobilization in flexion. Most of studies involving joint contracture investigated the histological and molecular changes of joint contracture using the traumatic flexion joint contracture animal models, which involved two factors (including trauma and joint immobilization) that may influence the development of joint contracture. Nevertheless, some studies showed that patients with conditions limiting their mobility but without traumatic or inflammatory conditions are also at a high risk for joint contracture.^{6,13,19}

During the process of joint contracture formation, the expressions of many growth factors, cytokines, and matrix metalloproteinases may be changed, which can contribute to joint contracture.^{20,21} Hildebrand et al²² reported elevated expressions of collagen types I and III and transforming growth factor β 1 (TGF- β 1) in the experimental posterior joint capsules compared with control capsules in a rabbit posttraumatic contracture model. Monument et al²³ reported significant increase in the mRNA and protein levels of α -SMA, TGF- β 1, tryptase, and collagen types I and III in the operated contracture group compared with the control group. Among the cytokines that may play important roles in the formation of joint contracture, TGF- β 1 is considered to be the most crucial one affecting the development of fibrotic processes, as it modulates fibroblast function to promote matrix preservation.⁷

Extending nontraumatic immobilization-induced joint contracture is a challenging problem in clinical practice. However, few studies have investigated this type of joint contracture. The study of extending joint contracture is of great importance for the prevention and treatment of the disease. In the present study, we established a model of rabbit knee contracture in extension using plaster casting. Serial changes in the joint ROM, the thickening and fibrosis of the joint capsule, and the mRNA levels of TGF- β 1 in the joint capsule over time were then evaluated. We aimed to probe into the histological and molecular changes of anterior and posterior joint capsules evoked by immobilization, to gain a better understanding in regard to the underlying molecular cause of immobilization-induced joint contracture.

Materials and Methods

Experimental Design

The present study was approved by the Institutional Animal Care and Use Committee of Anhui Medical University. In total, the present study used 40 male New Zealand white

Fig. 1 Modeling of knee extension contracture. The rabbits underwent unilateral immobilization of the knee joint at full extension using a plaster cast from the groin to the proximal toes.

rabbits (obtained from the Laboratory Animal Center of Anhui Medical University, Hefei, China; age, 3–4 months; weight, 2–2.5 kg). Thirty-two of the 40 rabbits were anesthetized by intravenous administration into the ear vein of 30 mg/kg sodium pentobarbital. Joint interventions were performed on the left knee under general anesthesia. The 32 rabbits underwent unilateral immobilization of the left knee joint at full extension using a plaster cast from the groin to the proximal toes (→Fig. 1). The rabbits were then placed in cages, with unrestricted activity and free access to water and food. The plaster casts were removed at 2, 4, 6, and 8 weeks after immobilization, with eight rabbits in each time cohort. A control group of eight rabbits that did not undergo immobilization were examined at the beginning of the experiment.

Measurements of the Synovial Thickness of the Suprapatellar and Posterior Joint Capsule

In view of the possible influences of synovial thickening on the limitations of joint ROM, color Doppler ultrasonic diagnostic instrument with the frequency of 10 to 22 MHz was used for the measurements of the synovial thickness of suprapatellar and posterior joint capsules before the rabbits were euthanized.

Range of Motion Measurements

The rabbits were euthanized via a sodium pentobarbital overdose after the synovial thickness of the suprapatellar and posterior joint capsules was measured. A mechanical goniometer (arthrometer) was built and tested to measure the knee joint angle (→Fig. 2). The femur was secured using a metal clamp and a platform beside the disc. To avoid friction between the femur and the disc while the measurements were conducted, the platform and the middle part of the disc were designed to be a little higher than the periphery of the disc. Metal fixing clamps were used to fix the proximal and distal parts of the tibia on the disc. A precise digital force gauge was fixed on a pedestal which was placed on the slideway equipment. A string was used to connect the groove of the disc and the digital force gauge. All limbs started with 0 degree of extension before the force was applied. When the driving wheel

Fig. 2 The mechanical arthrometer used in the present study.

was turned, the disc was twisted, and the tibia was indirectly turned, while the femur remained static. The force can be read on the screen of the digital force gauge, and the angle variation between the femur and the tibia was calculated according to the scale on the disc as the measurements were performed. As the radius of the disc remained unchanged, the torque which was calculated via multiplying the force by the constant radius of the disc depended on the force applied. Before the former experiment, the ROM was measured in several hindlimbs of normal rabbits using the mechanical goniometer. We found that the knee joint can be pulled to ~ 140 degrees with a torque of 0.077 Nm, after that an increase in torque can result in very small angle increase. Consequently, in our formal experiments, 0.077 Nm was used as a standardized torque value to measure the knee joint ROM. To ensure experimental accuracy, two examiners independently conducted the ROM measurements, and repeated these measurements three times for each limb. The examiners were blinded to each other's scores. Reported values were the mean of the six measurements taken by both examiners. Thus, a larger flexion angle actually represented a less severe contracture or loss of motion. The lack of flexion was referred to as an extension contracture.

Tissue Preparation

The anterior and posterior joint capsules were harvested from the knee and partitioned into two equal samples after the ROM measurements at each time point. Some samples used for Masson staining were fixed with 4% paraformaldehyde overnight at 4°C, while other samples used for semiquantitative reverse transcription polymerase chain reaction (RT-PCR) were frozen in liquid nitrogen and then stored at -70°C .

Masson Staining

The fixed joint capsule specimens were dehydrated in graded alcohol and then embedded in paraffin. A series of 5.0- μm sections were cut, deparaffinized, and stained with the Masson method to assess the level of collagen deposition. The Masson-

stained sections were then observed under $100\times$ magnification, and six randomly selected fields were analyzed to determine the percentage of the blue area that indicated collagen deposition using Image ProPlus software (Media Cybernetics, Silver Spring, MD). The average percentage of the blue area from each slice was calculated and used as a measure of collagen deposition.

RNA Extraction and Semiquantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from the joint capsule specimens using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, San Jose, CA) according to the manufacturer's instructions. Rabbit-specific primers (GAPDH: F-TCA CCA TCT TCC AGG AGC GA and R-CAC AAT GCC GAA GTG GTC GT; TGF- $\beta 1$: F-CGG CAG CTG TAC ATT GAC TT and R-AGC GCA CGA TCA TGT TGG AC) were used as described previously.^{23,24} PCR products were then separated by electrophoresis on 2.0% agarose gel. Images were captured using the Gel-Doc image analysis system (Bio-Rad, CA). To ensure experimental accuracy, all reactions were performed in triplicate. GAPDH was used as an internal control, and the gray ratio of TGF- $\beta 1$ /GAPDH was used to reflect the relative expression levels. The results were expressed as the mean $\pm$ standard deviation (SD).

Statistical Analyses

Data were presented as the mean $\pm$ SD. All data were analyzed using analysis of variance (ANOVA) with a post hoc Student-Newman-Keuls' (SNK) test for comparison between individual groups. The ROM, synovial thickness of the joint capsule, deposition of collagen, and mRNA expressions of TGF- $\beta 1$ were compared among all groups via ANOVA. An α less than 0.05 was chosen as the significance level for these statistical analyses.

Fig. 3 Knee flexion range of motion (ROM) in each group. 0W = control group that did not undergo immobilization; 2W = rabbits that underwent 2 weeks of immobilization; 4W = rabbits that underwent 4 weeks of immobilization; 6W = rabbits that underwent 6 weeks of immobilization; 8W = rabbits that underwent 8 weeks of immobilization. * $p < 0.05$ versus the 0W group; # $p < 0.05$ versus the 2W group; & $p < 0.05$ versus the 4W group.

Results

The knee flexion ROM of the five groups is illustrated in ►Fig. 3 (0 week, 140.86 ± 4.88 degrees; 2 weeks, 80.43 ± 5.96 degrees; 4 weeks, 49.75 ± 6.96 degrees; 6 weeks, 29.05 ± 5.24 degrees; and 8 weeks, 25.21 ± 3.21 degrees). The ROM of the knee joints gradually decreased over time. After 2 weeks of immobilization, knee flexion ROM was decreased compared with the control group ($p < 0.05$). Furthermore, there was a significant difference between the ROM of rabbits immobilized for 4 weeks compared with those immobilized for 2 weeks ($p < 0.05$). There was also a significant difference in ROM between rabbits immobilized for 6 versus 4 weeks ($p < 0.05$). However, there was no significant difference in the ROM of the knee joints of rabbits immobilized for 8 versus 6 weeks ($p > 0.05$).

According to the B ultrasonic examination (►Fig. 4), the synovial membrane of both the suprapatellar and posterior

joint capsules gradually thickened after immobilization. Both the synovial thicknesses of the suprapatellar and posterior joint capsules were increased at 2 weeks after immobilization, and remained increased for the duration of the study. Regarding the effect of time, the experimental 2-week synovial thicknesses of the suprapatellar and posterior joint capsules were greater than the control 0-week values ($p < 0.05$). The experimental 4-week thicknesses of the suprapatellar and posterior joint capsules were greater than the experimental 2-week values ($p < 0.05$). The experimental 6-week thicknesses of the suprapatellar and posterior joint capsules were greater than the control 4-week values ($p < 0.05$). However, there was no significant difference in the thicknesses of both the suprapatellar and posterior joint capsules between the knee joints of rabbits immobilized for 8 versus 6 weeks ($p > 0.05$).

Masson staining showed that the collagen deposition in the anterior and posterior joint capsules gradually increased after immobilization (►Fig. 5). In both the anterior and posterior joint capsules, the collagen deposition in the synovial membrane was increased at 2 weeks after immobilization and remained elevated for 8 weeks. In regard to the impact of immobilization period, the experimental 2-week collagen deposition in the anterior and posterior joint capsules was greater than the control 0-week value ($p < 0.05$), the experimental 4-week collagen deposition in the anterior and posterior joint capsules was greater than the experimental 2-week value ($p < 0.05$), and the experimental 6-week collagen deposition in the anterior and posterior joint capsules was greater than the control 4-week value ($p < 0.05$). However, there was no significant difference in the collagen deposition in both the anterior and posterior joint capsules between the knee joints of rabbits immobilized for 8 versus 6 weeks ($p > 0.05$).

►Fig. 6 illustrates the average mRNA levels of TGF- β 1 relative to the average band intensity of GAPDH in the five rabbit groups. A significant increase in mRNA levels was seen for TGF- β 1 in the joint capsule after immobilization. The mRNA levels of this growth factor in the experimental joint capsules were greater than those in the control group from 2 weeks after joint immobilization, and remained elevated until 8 weeks.

Fig. 4 (A, B) The synovial thicknesses of the suprapatellar and posterior joint capsules in each group. 0W = control group that did not undergo immobilization; 2W = rabbits that underwent 2 weeks of immobilization; 4W = rabbits that underwent 4 weeks of immobilization; 6W = rabbits that underwent 6 weeks of immobilization; 8W = rabbits that underwent 8 weeks of immobilization. * $p < 0.05$ versus the 0W group; # $p < 0.05$ versus the 2W group; & $p < 0.05$ versus the 4W group.

Fig. 5 (A, B) Collagen deposition area of the anterior and posterior joint capsules in each group. 0W = control group that did not undergo immobilization; 2W = rabbits that underwent 2 weeks of immobilization; 4W = rabbits that underwent 4 weeks of immobilization; 6W = rabbits that underwent 6 weeks of immobilization; 8W = rabbits that underwent 8 weeks of immobilization. a, anterior joint capsule; p, posterior joint capsule. * $p < 0.05$ versus the 0W group; # $p < 0.05$ versus the 2W group; & $p < 0.05$ versus the 4W group.

Fig. 6 Graphical representation of the transforming growth factor $\beta 1$ (TGF- $\beta 1$) mRNA levels relative to GAPDH in the anterior and posterior joint capsules (A), and a photograph of the TGF- $\beta 1$ and GAPDH mRNA band intensities (B). 0W = control group that did not undergo immobilization; 2W = rabbits that underwent 2 weeks of immobilization; 4W = rabbits that underwent 4 weeks of immobilization; 6W = rabbits that underwent 6 weeks of immobilization; 8W = rabbits that underwent 8 weeks of immobilization. * $p < 0.05$ versus the 0W group; # $p < 0.05$ versus the 2W group; & $p < 0.05$ versus the 4W group.

Furthermore, there was a significant difference between the mRNA levels at 4 versus 2 weeks ($p < 0.05$). There was also a significant difference between the mRNA levels in the anterior and posterior joint capsules of rabbits immobilized for 6 versus 4 weeks ($p < 0.05$). The mRNA levels of TGF- β 1 in the anterior and posterior joint capsules of rabbits immobilized for 8 weeks tended to be increased compared with the values in rabbits immobilized for 6 weeks, but this difference failed to reach statistical significance ($p > 0.05$).

Discussion

Joint contracture is a pervasive complication of many orthopaedic or neurologic diseases. Once joint contracture is established, functional disturbances such as restricted joint activities, decreased strength of the muscles around the joint, and abnormal gait may be caused.^{2,12,25} Hence, quality of life in patients with the disease can be seriously affected. In clinical practice, joint immobilization is usually performed after the occurrence of a limb fracture or damage to other periarticular tissues to promote healing of the fracture and other joint tissues, decrease pain, and avoid further joint damage.¹⁶ However, joint contracture may also result from a long immobilization time and a fixed position of a single limb joint.

Animal models are established to study the processes that cannot be studied in humans, and to examine the effects of experimental interventions.^{2,26} An ideal animal model is of prime importance to the study of pathophysiological mechanism of a disease, and to the investigation of treatment selection and optimization. Up till the present moment, many researchers investigated joint contracture with animal model of flexion joint contracture. Chimoto et al²⁷ immobilized the knee joints of rats with an internal fixator with the knee joint flexed at 150 degrees to develop animal model of flexion joint contracture and investigated the progression of an arthrogenic motion restriction after immobilization. The study indicated that joint contracture progressed rapidly until 8 weeks and then advanced slowly. Sasabe et al⁶ immobilized the knee joints of rats in full flexion using plaster casts to create animal model of flexion joint contracture and then examined the time-dependent changes in the development of joint capsule fibrosis and in the number of myofibroblasts in the joint capsule after immobilization. The results suggested that joint capsule fibrosis with overexpression of type I collagen occurred and progressed within 1 week after immobilization, and an increase in myofibroblasts was related to the mechanism of joint capsule fibrosis. Nevertheless, extending joint contracture, which usually occurs in the knee joint, is the most common contracture type in clinic. To be close to clinical practice, we established an animal model of extending knee joint contracture with plaster cast in our study. The clinical characterization of joint contracture is the loss of joint ROM. The present study investigated joint contracture secondary to immobility longitudinally over 8 weeks using standard experimental conditions and quantitative tools. The present results demonstrated that rabbits that underwent joint immobilization experienced loss of joint ROM. A decrease in ROM was measurable after only 2 weeks of immobilization, and this constantly progressed for

the first 8 weeks. Furthermore, our study indicated that the loss of ROM progressed rapidly in the first 6 weeks after immobilization, and then may progress slowly.

The formation of joint contracture results in stiffness of the capsule, synovial atrophy, fibrosis, and adhesion, which may be the causes of the limited ROM.^{12,28,29} In our study, synovial thickening of both the anterior and posterior joint capsules was found as early as 2 weeks after joint immobilization, and progressed throughout the 8-week study period. The thickening of the joint capsule progressed quickly in the first 6 weeks, and then seemed to progress more slowly. These results were consistent with the measurements of joint ROM, which indicated that the synovial thickening of the suprapatellar and posterior joint capsules may be related to the limited joint ROM. Furthermore, our study indicated that B ultrasonic examination can be a useful method for measuring the severity of joint contracture.

After the formation of joint contracture, histologic preparations revealed increased collagen synthesis and deposition in the contracted joint capsule.²³ Hildebrand et al²² found that the mRNA and protein levels of collagen types I and III were both elevated in the experimental capsules compared with the control capsules in a rabbit knee contracture model. In our study, the collagen deposition in both the anterior and posterior joint capsules increased throughout the 8-week study period. Furthermore, the collagen deposition progressed quickly in the first 6 weeks, and then appeared to progress more slowly. The results were consistent with the measurements of joint ROM, which indicated that the collagen deposition in the anterior and posterior joint capsules may be related to the limited joint ROM to some extent.

TGF- β 1 is regarded as an important cytokine in the process of joint contracture, and is related to joint capsule fibrosis.^{23,30} Previous research investigating immobilized normal rabbit knees has shown alterations in TGF- β 1 expression.^{22,23} In our study, the mRNA expression of TGF- β 1 changed very early within the joint capsule in this rabbit model of joint contracture. The mRNA levels of TGF- β 1 in the anterior and posterior joint capsules altered within 2 weeks after joint immobilization, and progressed throughout the 8-week study period. The mRNA expression levels increased quickly in the first 6 weeks, and then appeared to progress slowly, which coincided with the other measured variables. The increase in the mRNA levels of TGF- β 1 may be related to the collagen deposition in the anterior and posterior joint capsules. Joint capsule fibrosis may be concerned with the occurrence of joint contracture.

There are some limitations in our study. First, we only measured the mRNA levels of TGF- β 1; further work is required to evaluate the protein levels of TGF- β 1, and the mRNA and protein levels of other molecules and enzymes, to explore the possible mechanisms of the progression of joint contracture. Second, the apparatus used for ROM measurements in our study has some deficiencies. When the measurements were conducted, the force was not directly applied to the tibia but applied to the disc and then the tibia was indirectly turned. The force applied in the device was not always perpendicular to the tibia when the measurements were done, and the angle between the direction of force applied and the tibia changed

as joint ROM changed. We will try to improve the methodology in our following studies. Furthermore, we only investigated the progression of joint contracture and the subsequent changes in the joint capsule; the changes in other periarticular tissues such as muscles and ligaments should be studied. Finally, we only investigated the progression of joint contracture for 8 weeks; thus, the longer term changes in periarticular tissues after joint immobilization are still uncertain. In our next work, we will investigate joint contracture after joint immobilization for a longer time period.

Conclusion

Our results indicated that rabbit knee joint immobilization can result in joint contracture; the severity of joint contracture progressed quickly in the first 6 weeks, and then may progress more slowly. The treatments evaluated in our study in this particular animal model might be considered to be geared toward the period of joint immobilization. Next, we should investigate the reversibility of joint contracture and explore the proper methods to prevent and treat joint contracture.

Conflict of Interest

None declared.

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