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Research Article

The effect of collagenase clostridium histolyticum on adhesion reduction in a rat knee arthrofibrosis model

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ABSTRACT

Objective: The aim of this study was to investigate whether collagenase clostridium histolyticum (CCH) reduces intra-articular fibrotic adhesion formation in a rat model of arthrofibrosis.

Methods: A total of 24 male Wistar rats (7 months old, weighing 220 –275 g) were randomly and equally assigned to one of two groups: the collagenase group and the control group (n = 12 each). In each group, a partial capsulotomy, and synovectomy were performed in knee. After a partial capsulotomy and synovectomy were performed in each group, the collagenase group received intra-articular CCH of 0.008 mg in 0.1 mL saline solution while the control group received the equal volume of intra-articular saline solution alone. After 6 weeks of surgery, the rats were sacrificed by decapitation, and the following outcome measures were collected. Knee range of motion (ROM) was measured using with a goniometer under 20 g force. Adhesion formation was rated using the macroscopic visual scoring system after the knee joint was exposed through a lateral parapatellar approach. Histological evaluation was performed on samples including connective tissue and fibrotic adhesions, and fibroblast cell numbers were measured performed per square. Levels of interleukin 1 (IL-1) and fibroblast growth factor (FGF) were assayed by ELISA from the intra-articular fluid.

Results: The macroscopic visual scoring system was significantly lower in the collagenasegroup (median = 1, range = 0–2) than in the control group (median = 2, range = 1–3)(< 0.001). ROM was significantly higher in the collagenase group (102° ± 12.1°) than in the control group (77° ±8.94°) (P <0.001). The number of fibroblasts obtained from the scar tissue were considerably lower in the collagenase group (mean = 16.5 ± 2.74) compared tothe control group (mean = 30.1± 4.89) (P < 0.001). Levels of IL-1 andFGF were significantly lower in the collagenase group (mean = 18.6 ng/l ± 4.39,mean = 36.3 ng/l ± 2.03; respectively) compared to the control group (mean = 31.7ng/l ± 3.75, mean = 38.7 ng/l ± 2.19; respectively) (P < 0.001).

Conclusion: Evidence from this study has revealed that CCH injection can inhibit the development ofarthrofibrosis, decreasing the precursor inflammatory cytokines (IL-1 and FGF-1) and histologic fibrosis in a rat knee arthrofibrosis model.

Level of Evidence: Level IV, Therapeutic Study

Introduction

Arthrofibrosis is a disease characterized by the loss of Range of Motion (ROM) because of the painful stiffness of proliferated scar tissue.[1] This disease is seen in 1–13% people after Total Knee Arthroplasty (TKA), 0–4% people after ligament damage (such an anterior cruciate ligament), and 7% people after high-energy knee fracture.[2-4] Even the low level of extension loss prevents the knee from locking in the extension, which can lead to high-energy consumption and cause limp because of hamstring contracture and quadriceps inactivation.[5] Failure to inhibit the adhesion formation affects the post-operative recovery of the knee that is a risk factor for adverse long-term outcomes.[5,6]

The pathophysiology of arthrofibrosis has not been fully elucidated, but it appears to cause cytokines and mediators, especially Interleukin 1 (IL-1) and Fibroblast Growth Factor (FGF), stimulate immune cells. The resulting fibroblasts differentiated into myofibroblasts and increased local collagen[7,8] are caused to contract the knee capsule and fill joint with fibrosis.[9] Arthrofibrosis and other fibrotic diseases, such as Dupuytren's disease and Peyronie's disease, have similar pathogenic pathway. Collagenase Clostridium Histolyticum (CCH) is used in the treatment of Dupuytren's disease and Peyronie's disease.[8,10]

There are several clinical studies about the use of CCH with fibrotic diseases in the literature.[11,12] However, the use of CCH in knee arthrofibrosis is lacking in the literature. Wong et al. show that an intra-articular application of CCH into the rat knee prevented adhesion formation and reduced arthrofibrosis.[13] CCH cleave fibrillar collagen types 1-3 also affect inflammatory markers such as IL-1 and FGF. However, collagenase shows different inflammatory activities in

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normal and abnormal tissue.[14–16] Evaluation of the inflammatory effect of CCH in arthrofibrosis is not studied in the literature. To understand the precise mechanism of CCH in arthrofibrosis, studies evaluating fibrosis-related cytokines and growth factor are needed.

Therefore, we evaluated the results of intra-articular CCH in the experimental arthrofibrosis model. Joint ROMs and histological effects of CCH were evaluated in the previous study.[13] Thus, we further evaluated the effect of CCH on the fibrosis-related cytokines and growth factor by evaluating the number of IL-1, FGF, and fibroblasts.

Materials and Methods

This study included 24 male 7-month-old Wistar albino rats with an average weight of 220-275 g. This experimental animal study was approved and confirmed that all experiments were performed in accordance with relevant guidelines and regulations by the Acıbadem University's Ethics Committee, Decision no: 2018/20. The animals were kept at 20–24°C, 50–55% relative humidity, and under a 12-hour light/12-hour dark cycle in a noiseless environment. They were fed with standard laboratory food without liquid or food restriction. Twenty-four rats were randomly divided into the collagenase group and the control group (n = 12 each).

A statistical power analysis was performed for sample size estimation. The Effect Size (ES) in this study was 0.50, considered to be large using Cohen's (1988) criteria. With an alpha = 0.05 and the power = 0.80, the projected sample size needed with this ES (GPower 3.1; Faul, Erdfelder, Lang, and Buchner, 2007; Germany) is approximately N = 21 for this simplest between group comparison. 10% of animal could die during an experiment, which may decrease the power of the study then, so we adjust this attrition in the calculated sample size.[17] For 10% attrition and because of two groups, we chose 24 rats.

Animal model of arthrofibrosis

The model of arthrofibrosis was used before it was described in the literature.[18,19] The rats were anesthetized with intramuscular xylazine hydrochloride (5 mg/kg, Rompun; Bayer, Germany) and ketamine hydrochloride (50 mg/kg, Ketalar; Pfizer, USA) at time zero. The depth of the anesthesia was assessed by monitoring the corneal reflex and responses to painful stimulation of the foot. After ensuring the anesthesia depth of the subject animals, each rat's right knee was shaved with electric clippers, prepped antisepsis with povidone (10% derivatized, Batticon, Adeka, Turkey), and sterile covering. The rats were followed by arthrotomy after an average 3-4 cm longitudinal incision, and a medial parapatellar approach was performed. A partial capsulotomy and synovectomy were performed. The joint was washed with sterile isotonic after the procedure. The wound layers were closed according to their anatomy with sutures using 3-0 non-absorbable thread. Then, an intra-articular injection of 0.008 mg (0.1 mL) CCH (Xiaflex, USA) was administered to the right knee joint in the collagenase treatment group, while the same volume of saline (isotonic sodium chloride solution) was given to the rats in the control group. Post-operative wound dressing and the external immobilization of the surgical limb were not performed. No food and fluid restrictions were applied. The rats did not show any wound infection or mortality after 6 weeks of free circulation (Figure 1).

Measurement of joint angle

The rats were sacrificed by decapitation at 6 weeks' post-surgery. Heavy duty cardstock blank cardboard 5mm was placed under the right leg after each rat was laid in a lateral decubitus position. The proximal shaft of the femur was fixed to the panel with a 1.2 pins k-wire and marked with a blue marker. Measurement was made with a goniometer under 20 g force.[18] The center of the goniometer was fixed with a 21 gauge syringe needle to be the middle part of the knee joint. Goniometer values were calculated parallel to femoral and tibial shafts.[20] The knee ROMs were measured three times, and the average value of these measurements was used for analysis (Figure 2).

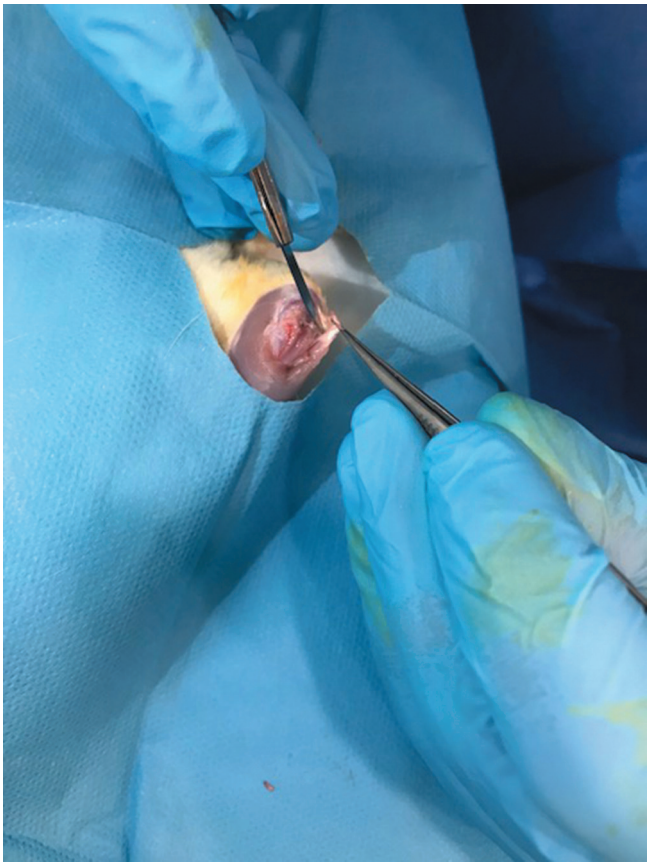

Figure 1. Animal arthrofibrosis modelling. After medial parapatellar approach, subpatellar Hoffa excised and total synovectomy were performed. Capsule exposed, partial capsulotomy was performed.

Gross observation

The rat's knee joints were exposed through a lateral parapatellar approach. Adhesion formation was classified by Rothkopf et al. that is name the macroscopic visual scoring system by a pathologist who was blinded to this study.[21] The severity of osteocapsular adhesion was scored as follows: 0, no adhesions; 1, the adhesion layer was thin and split, even minimal traction; 2, the adhesion layer was split manual traction; and 3, a thick adhesion membrane could not damage with traction that could only be removed by the pathologist.[21]

Histological evaluation

The knee joints including all the connective tissue and fibrotic adhesions were removed. The samples were further washed for three hours to clear the water and then placed in an automatic tissue monitoring equipment (Shandon Excelsior ES, USA) for 13 hours. The samples were fixed with 10% paraformaldehyde for 48 hours, decalcified (DDK) for 2 weeks, and embedded into the paraffin. A parallel section was taken from the area of fibrotic tissue to the femoral axis. Samples were stained with hematoxylin and eosin. Three areas in the scar tissue near the bottom of the decorticated areas were selected.[18] Fibroblast counts and the severity of fibrosis were evaluated in samples.

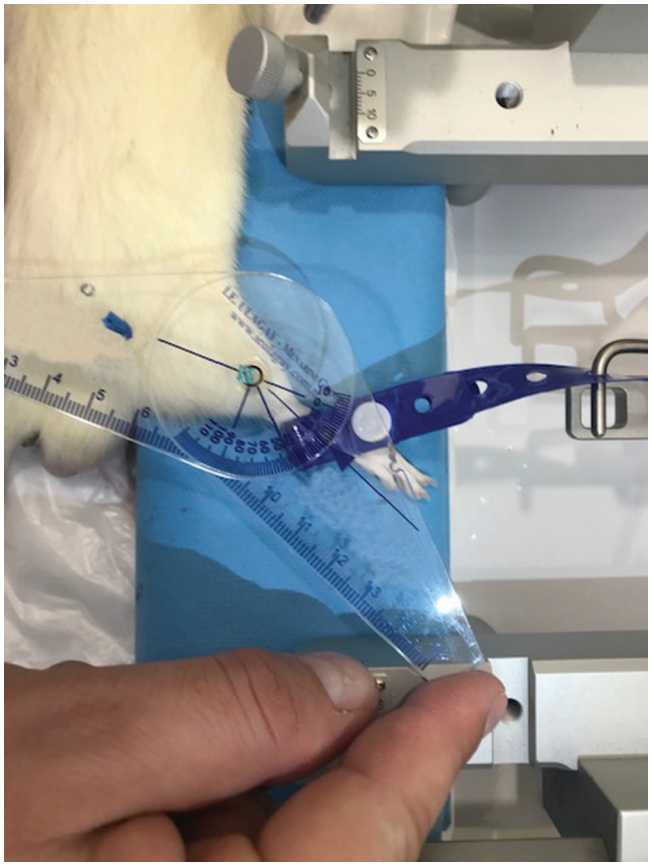

Figure 2. Measurement of range of motion. In the lateral decubitus position, the right leg of each rat was fixed, and the angle between the longitudinal axis of the femur and the tibia was measured by using a goniometer with 20 g of torque.

Intra-articular scar adhesions were evaluated under a light microscope at 400× magnification (Olympus microscope, CX41; Olympus, Tokyo, Japan). Three different forms were selected in the decorticated areas, and fibroblast counts were performed per square.[18]

Measurement of IL-1 and FGF

During the surgery, intra-articular fluid was taken with insulin injector after skin incision when the joint capsule was still intact and kept at −20 °C. The levels of IL-1 and FGF were assayed by ELISA according to the manufacturer's procedure and were calculated using a DuoSet ELISA Development Kit (R&D Systems, Minnesota, USA).[18]

Statistical analysis

The SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Descriptive statistical methods (mean, standard deviation, median, frequency, percentage, minimum, and maximum) were used to evaluate the study data. The normal distribution of quantitative data was tested with the Shapiro–Wilk test and graphical examinations. Student's *t*-test was used to compare the two groups with the normal distribution. Fisher's exact test was used for the comparison of qualitative data. *P* value of <0.05 was considered statistically significant.

Results

Gross observation

No infection, severe weight loss, or mortality were observed after the surgical intervention.

Table 1. Macroscopic Visual Scoring System of the Groups

Animal	CCH Treatment Group	Control Group
1	0	2
2	0	3
3	1	1
4	1	2
5	0	3
6	1	3
7	0	2
8	0	1
9	0	3
10	0	2
11	1	2
12	2	3
Median (*min–max)*	1 (0-2) 2	(1-3) (*$P < 0.001$)

*Fisher's exact test.

All the 12 rats of the collagenase group showed localized superficial fibrillation. No cartilage damage was observed in the control group. No ligament damage (such an anterior cruciate ligament, posterior cruciate ligament, or patellar ligament) was observed in either group. Some meniscal damage was observed in both groups.

A statistically significant difference was found between the groups according to the adhesion scores from the macroscopic visual scoring system. The adhesion scores of the collagenase group were found to be significantly lower than the control group ($P < 0.001$) (Table 1).

Measurement of joint angle

A statistically significant difference was found between the groups according to the ROM in the joint angle. The ROM of the collagenase group (102° ± 12.1°) was found to be significantly higher than the control group (77° ± 8.94°) ($P < 0.001$).

Histological evaluation

The most remarkable changes observed was the disappearance of the synovial fold and sub-synovial adipose tissue in the collagenase groups; however, the spindled fibroblasts replaced the sub-synovial adipose tissue in the control group. Infiltration of lymphoplasmacytoid cells was observed in the synovium in both groups (Figure 3).

The number of fibroblasts in the scar tissue of the collagenase group (16.5 ± 2.74) was found to be significantly lower than the control group (30.1 ± 4.89) ($P < 0.001$) (Table 2).

Enzyme-linked immunosorbent assay

The levels of IL-1 and FGF were found to be remarkably lower in the collagenase group (18.6 ± 4.39 and 36.3 ± 2.03, respectively) than in the control group (31.7 ± 3.75 and 38.7 ± 2.19, respectively) ($P < 0.001$) (Table 3).

Discussion

The present study supports CCH injection as a possible treatment option for arthrofibrosis. Our study results demonstrated that rats treated with CCH after induced experimental arthrofibrosis displayed a significantly low amount of fibrosis, IL-1, and FGF and increased ROM in rats' knee joint when compared with the treatment control group.

Treatment outcomes for arthrofibrosis vary according to treatment options. Physiotherapy is an important part of the treatment of arthrofibrosis, but it involves the potential complications, which include

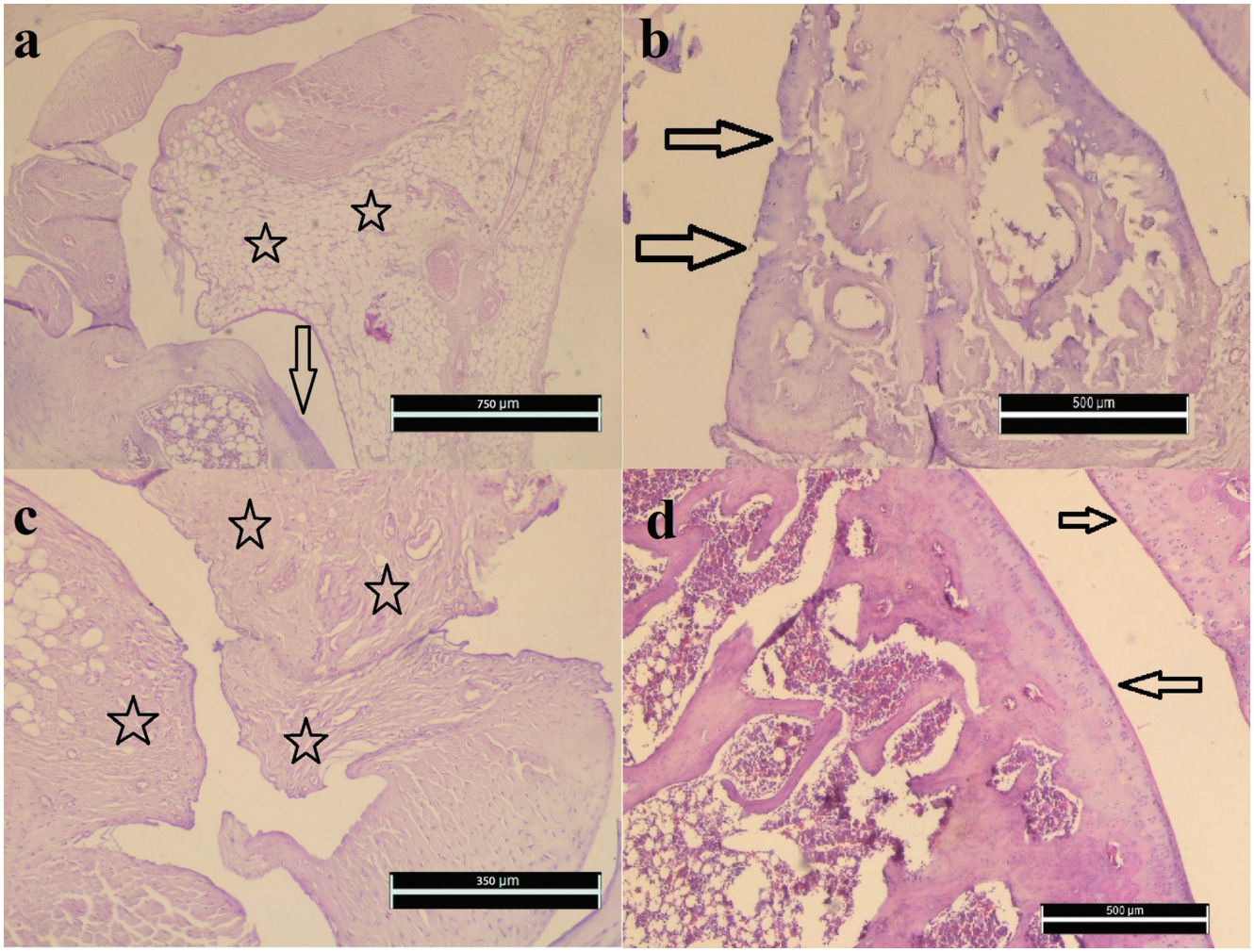

Figure 3. a-d. Histopathological finding of both groups. (a) In the treatment group, the appearance of fibrofatty tissue is shown with asterisks, and the mild irregular appearance on the cartilage surface is shown by arrows (Hematoxylin and Eosin × 40). (b) In the treatment group, the irregular appearance on the cartilage surface is shown by arrows (Hematoxylin and Eosin × 40). (c) Presence of dense fibroblasts in the control group is shown with asterisks (Hematoxylin and Eosin × 40). (d) In the treatment group, the regular appearance on the cartilage surface is shown by arrows (Hematoxylin and Eosin × 40).

Table 2. Range of Motion and the Number of Fibroblasts of the Groups

Measurements	Sample	Collagenase Group	Control Group	*P
Range of motion of the knee (degree)	12	102° ± 12.1°	77° ± 8.94°	<0.001
Number of fibroblasts	12	16.5 ± 2.74	30.1 ± 4.89	<0.001

*Student's *t*-test.

Table 3. Level of IL-1 and FGF in the Intra-Articular Fluid

Measurements	Sample	Collagenase Group (ng/l)	Control Group (ng/l)	*P
IL-1	12	18.6 ± 4.39	31.7 ± 3.75	<0.001
FGF	12	36.3 ± 2.03	38.7 ± 2.19	0.002

*Student's *t*-test.

fracture, patellar tendon strain or rupture, quadriceps avulsions, heterotopic bone formation, wound dehiscence, and hemarthrosis.[22,23] Surgery is performed to avoid these complications if the patient is more than 3 months or in advance cases[24]; however, high complication rates, such as neurovascular damage, are seen after these surgeries. Surgery-related complications increase with the severity of the initial contracture and time.[2-4] The intra-articular collagenase administration could decrease surgery, and it could be a treatment step between physical therapy and surgery.

CCH is a proteinase that hydrolyzes collagen fibers, especially degrades triple-helical type 1 and type 3 collagens. It destroys collagen fibers and also suppresses their adhesion sites. CCH causes a decrease in Extracellular Matrix (ECM) and cytokines and stimulates growth factors.[25] Histological and Western blot analyses showed significant dose-dependent decreases in the expression of type 1 and type 3 collagens, but CCH could affect collagen type 4 at the highest dose and at longer incubation times. Thus, important major structural collagen component such an artery, veins, and nerves are involved in collagen type 4.[26] In our study, hematoma or other prominent neurological problems was not observed.

The etiology of arthrofibrosis is multifactorial, and a number of risks have been identified. IL-1 and FGF are important inflammatory cytokine mechanism of arthrofibrosis and increase the expression of FGF.[27] These factors are triggered cytokines that cause an imbalance between the ECM production and degradation. These ECMs involved

a higher ratio of collagen type 1 to stretchy elastin, compared to normal tissues.[28] Fibrotic ECM content is involved mostly in cross-linking and that converted irreversible collagen accumulation because of levels of hydroxylysine.[29] In vitro study showed that CCH decreased inflammatory cytokine.[30] We observed that the level of IL-1 and FGF is low. These data show that CCH inhibits arthrofibrosis not only hydrolyzes collagen fibers but also decreases precursor inflammatory cytokines.

Fibroblast plays a key role in arthrofibrosis. It produces fibers of α-smooth muscle actin together with collagen type 1, deposits dense fibrotic collagen, and secretes specific inflammatory cytokines. The number of fibroblasts used evaluated the severity of arthrofibrosis in the recent study.[18] We observed less fibroblasts in the CCH group in our study.

Articular cartilage is a connective tissue consisting of a specialized ECM that involves mostly collagen and aggrecan.[31] In a previous study, even 1 U of collagenase injection causes chondrocytes clustering in the superficial zone of articular cartilage at week 1. Vertical fissures and localized deformed articular surfaces were observed later.[11] However, there is a study showing that collagen has no effect on articular cartilage in a experimental arthrofibrosis model.[13] We observed chondral injury in all samples in the CCH group. However, our observation is only the gross pathological examinations. More histological studies are needed to show at what dose chondral injury occurred. Similarly, anterior cruciate ligaments, posterior cruciate ligaments, patellar tendon, and quadriceps tendon involve mostly collagen and aggrecan. It suggests that there may also be damage to these tissues. However, even intralesional CCH injection for Dupuytren's disease was observed only for two tendon ruptures, one pulley rupture, and one complex regional pain syndrome.[32] We did not observe any ligamental injury in our study, such as an anterior cruciate ligament and a patellar tendon in the groups during the gross pathological examinations.

In the literature, intra-articular CCH applications are performed in rats for various purposes.[11,33,34] However, it is not clear at what dose and in which posology it will be applied in arthrofibrosis modeling. In Dupuytren's disease, a single dose of 0.58 mg CCH is administered to improve finger joint movements.[35] Animal equivalent dose is calculated based on body surface area and treatment of Dupuytren's disease.[36] The maximum amount of liquid that can be given to the knee without causing joint damage in rats was calculated as 0.1 mL.[37] Therefore, the calculated amount of CCH was applied in 0.1 mL. However, further studies are needed to determine the appropriate dosage and posology.

The design of arthrofibrosis in mice and rats is limited and includes variable formats. Basically, there are four types of design models: intra-articular injury models, rigid joint immobilization, rigid joint immobilization with intra-articular injury models, and gene delivery model that induces arthrofibrosis.[38–40] There is not enough study to compare experimental arthrofibrosis models in the literature. The model of arthrofibrosis induced by intra-articular injury has been shown to be well tolerated by animals. Studies have shown that all arthrofibrosis-induced groups have arthrofibrotic changes in the joint.[18,19] Similarly, we observed the adhesions of all samples in the control group. We chose an experimental design by using intra-articular injury because it was simple and similar to the arthrofibrosis etiology.

This study has several limitations. First, limited usage area of CCH is due to side effects of joint cartilage. Second, ROM is one of the indirect measurement methods used to evaluate the level of arthrofibrosis.[37] Although there is a study in the literature that evaluates with ROM with adhesion modeling,[18] it is not an adequate indicator to evaluate ROM.[19] Therefore, in our study, in addition to this evaluation, histolopathological and enzyme-linked immunosorbent was made. Third, when we measure ROM, skin and muscles were not extracted, and postmortem muscle contractions may have an impact on the results, since it is performed after the animal is sacrificed. Fourth, rat knee anatomy and mechanics might be different from the human knee; rats are quadrupeds, which means that the forelimb is load-bearing. Fifth, arthrofibrosis may develop with a different etiology. The arthrofibrosis model could not be similar to all of these etiologies.

In conclusion, CCH injection increases the ROM and decreases fibrosis in a rat model of arthrofibrosis. CCH inhibits arthrofibrosis and also decreases precursor inflammatory cytokines. These findings suggest a potential role for CCH as a therapeutic option for arthrofibrosis patients and warrants further investigation.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Acıbadem University (Decision no: 2018/20).

Informed Consent: N/A.

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