Analysis for Osteoarthritis of the Ankle Joint in a Mouse Model of Chronic Ankle Instability

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Research Article

Keywords: ankle joint, osteoarthritis, Anterior talofibular ligament, Calcanoeoibular Ligament, animal model

Posted Date: September 30th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2110986/v1

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Abstract

Background
Ankle sprains are the most common orthopedic pathology experienced during sports and physical activity and often result in chronic ankle instability (CAI). The purpose of this study was to assess osteoarthritic changes in the ankle joint in a surgical CAI mouse model.

Method
The experiments were performed using 14-week-old ICR male mice (n = 19). Mice were randomly placed into the SH group (sham; control, n = 5), ATFL group (resected anterior talofibular ligament; mild ankle sprain, n = 7), or ATFL + CFL group (resected anterior talofibular ligament / calcaneofibular ligament; severe, n = 7) and housed individually. Behavioral analysis using the frequency of standing on the hind leg was performed. To evaluate the clinical severity of arthritis, bodyweight, paw thickness, and ankle thickness were assessed immediately before sacrifice. Immunohistochemical staining and micro-computed tomography were performed to analyze the arthritic changes of the ankle joint. Serological analysis of inflammatory cytokines and C-terminal telopeptide of type I bone resorption markers was performed using enzyme-linked immunosorbent assay (ELISA).

Results
Compared with the control group, the ATFL + CFL group significantly aggravated the clinical severity of arthritis. In the ATFL and ATFL + CFL groups, the number of mice standing on the hind leg was significantly decreased. ELISA confirmed that the inflammatory cytokines were significantly increased in the ATFL + CFL group. C-terminal telopeptide of type I levels were increased in the ATFL + CFL group but the difference was not statistically significant.

Conclusions
This study demonstrated that the surgical induction of chronic ankle instability (ATFL + CFL) in a mouse model results in the development of osteoarthritis of an ankle joint.

Introduction
Lateral ankle sprains are one of the most common sports injuries [1]. Although the initial ankle sprain is concerning, 10–30% of the patients can progress to chronic ankle instability (CAI) [2]. Previous researchers have examined multiple insufficiencies that likely lead to the development of CAI. A combination of mechanical instability and functional instability are the probable primary causes of CAI.
Consequently, if CAI is not treated adequately and neglected for a long period with continued unbalanced loading in the ankle joint, ankle osteoarthritis (OA) may develop [10].

Since a long-term prospective follow-up is required and confounding bias is easy to intervene during the study period, a few cohort studies on whether OA occurs in CAI were reported. Therefore, it is unclear whether an ankle sprain or CAI is the cause of ankle OA. The development of an animal model mimicking the pathologies that are observed in humans with CAI has the potential to lessen the challenges associated with prospective human research on CAI. Previous studies have introduced a surgical-induced ankle sprain model to evaluate the relationship between sprain and CAI or pain control in sprains [11, 12]. However, despite its high incidence and clinical importance, proper animal models of CAI on OA development and prevention are lacking.

Therefore, we aimed to assess osteoarthritic changes in the ankle joint in a surgical CAI mouse model. We hypothesized that several pieces of evidence associated with an ankle OA could be identified in surgical CAI mouse models on clinical, radiological, histological, and serological analyses.

**Patients And Methods**

### Animal group and experimental design

Fourteen-week-old male ICR mice ($n = 19$) were purchased from Samtako Co. Ltd. (Osan, Korea). All mice were fed a normal diet, provided free access to water, and maintained at an ambient temperature of 22–24°C and relative humidity of 55–60% under a 12 h:12 h light/dark cycle in a pathogen-free environment.

All the mice were randomly assigned to one of the following three groups: SH group (sham; control, $n = 5$), ATFL group (resected anterior talofibular ligament (ATFL); mild ankle sprain, $n = 7$), or ATFL + CFL group (resected ATFL/calcaneofibular ligament [CFL]; severe, $n = 7$), and ankle instability group; the mice were housed individually.

### Surgical procedures (Fig. 1)

Using 4% isoflurane gas and supplemental oxygen, each mouse was anesthetized before shaving their right ankle and disinfected with chlorhexidine. All mice remained under anesthesia while being moved to a sterile surgical field with a warming lamp. Using a microscope and sterile equipment, the skin overlying the anterolateral aspect of the ankle was incised longitudinally to expose the ankle joint. Then, the skin was gently retracted to expose the lateral structures of the ankle joint (Fig. 1A). To expose the ATFL, the peroneal tendons were identified and lifted using surgical forceps because the tendons were directly in line with and superficial to the ATFL in mice [11, 12]. For the ATFL group, the ATFL was resected (Fig. 1B). For the ATFL + CFL group, ATFL and CFL were identified and resected (Fig. 1C, D). In the SH group, no ligament was damaged. The incision was closed with simple sutures using Nylon 4−0. After the surgery was complete, the mouse was removed from the anesthesia and taken to the recovery area. Each mouse was allowed to recover under a warming lamp until it was freely mobile. Additional postoperative care
consisted of visual monitoring once at least every 24 h by the investigative team. Three days after surgery, all mice were housed individually in cages.

**Physical activity and clinical assessment**

According to a previous study that analyzed pain-like behaviors in animal models, hypersensitivity to mechanical stimulation of the hind legs was considered joint pain [20]. Therefore, behavioral ability was measured by standing on the hind leg for 60 min at the same time every four weeks from four weeks after surgery using video recording analysis [20]. In addition, to evaluate the clinical severity of arthritis, bodyweight, paw thickness, and ankle thickness were assessed immediately before sacrifice. Paw thickness and ankle thickness were measured using digital calipers (Mitutoyo, Andover, UK), and the average thickness of both hind limbs was used.

**Micro-computed tomography (micro-CT)**

Radiologic changes in the ankle OA were evaluated using a high-resolution micro-CT (SkyScan1173; Bruker-CT, Kartuizersweg 3 B 2550 Kontich, Belgium). After fixing the specimen to a jig for micro-CT measurement using parafilm, 800 image images were acquired using a tube voltage of 130 kV, a current of 66 µA, and a 1.0 aluminum filter; the cross-section was reconstructed using NRecon software (Bruker, Kontich, Belgium). The obtained cross-sectional images were aligned for each cross-section using DataViewer (Bruker, Kontich, Belgium), and the parameter values were calculated using CtAn software (Bruker, Kontich, Belgium).

**Histopathological assessment**

Ankle joint bone tissues were fixed with 10% neutral buffered formalin for 24 h, decalcified for one week in Calci-Clear Rapid® (National Diagnostics, 305 Patton Drive Atlanta, GA 30336, USA) and then embedded in paraffin. Sections (4 mm thick) were prepared using a Leica microtome RM2255 (Leica Microsystems, Bannockburn, IL, USA) and stained with hematoxylin and eosin (H&E), safranin O, or toluidine blue. Images were taken using a Pannoramic™ P250 Flash digital slide scanner (3DHISTECH Ltd., Budapest, Hungary), and a dedicated viewing program (CaseViewer; 3DHISTECH Ltd., Budapest, Hungary) was used for observation.

**Blood biochemical indicators**

Blood was collected and centrifuged at 1,300 ×g for 15 min at 4° C. Serum tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β (R&D Systems, Minneapolis, MN, USA), and mouse cross-linked C-terminal telopeptide of type I (CTX-1), a bone resorption marker (MyBioSource, San Diego, CA, USA), were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s guidelines.

**Statistical analysis**
All experimental tests were performed at least three times, and data were analyzed by one-way analysis of variance, followed by Tukey’s multiple comparisons test, using the SPSS 14.0 (IBM Corp., Chicago, IL, USA). Statistical significance was set at \( p < 0.05 \).

**Results**

**CAI reduces physical activity and increases paw thickness**

To confirm the behavioral changes and clinical findings of OA in mice following the induction of CAI, we conducted a comparative analysis between the SH, ATFL, and ATFL + CFL groups. No significant differences in body weight changes were observed between the groups \( (p = 0.3367) \) (Fig. 2A). Physical activity was measured by the number of mice standing on the hind legs, and as a result, the ATFL and ATFL + CFL groups were significantly reduced at 18 W (SH vs. ATFL, \( p = 0.0063 \); SH vs. ATFL + CFL, \( p = 0.0037 \)), 22 W (SH vs. ATFL, \( p = 0.0027 \); SH vs. ATFL + CFL, \( p < 0.0001 \)), and 26 W (SH vs. ATFL, \( p < 0.0001 \); SH vs. ATFL + CFL, \( p < 0.0001 \)) compared to the SH group (Fig. 2B). The ATFL + CFL group developed severe swelling and increased paw thickness with inhibition of activity compared to the SH and ATFL groups (Fig. 2C and D). However, there was no significant change in ankle thickness even in the ATFL + CFL group \( (p = 0.6146) \) (Fig. 2D).

**CAI increases synovial inflammation and joint destruction**

Articular destruction and bone damage were confirmed in the ankle joint of the hind paw using micro-CT. While there was no significant change in ATFL, the ATFL + CFL group demonstrated increased inflammation and destruction of the tarsal metatarsal joint, calcaneus, or tarsal bone compared to the SH group (Fig. 3A). By examining the microstructural characteristics, it was observed that the total porosity value decreased in the ATFL and ATFL + CFL groups. In micro-CT analysis of the ankle bone, bone mineral density (BMD) and bone volume fraction (BV/TV) was significantly increased in the ATFL and ATFL + CFL groups (Fig. 3B). Histological assessment revealed inflammatory cell infiltrate, cartilage damage, and pannus formation in the ankle joints. The ankle joint in the ATFL + CFL group demonstrated increased inflammation and joint destruction compared to the SH and ATFL groups (Fig. 4). Histological analysis of the ankle joint using H&E staining revealed less cell infiltration and synovial proliferation in the ATFL + CFL group (Fig. 4).

**CAI stimulates proinflammatory cytokine production**

We investigated the underlying mechanisms of CAI-induced reduction in the incidence and severity of OA. Because various cytokines are involved in joint inflammation and osteoarthritis progression, we measured cytokine levels using ELISA. Significant increases in TNF-\( \alpha \), IL-6, and IL-1\( \beta \) were observed in the serum of the ATFL + CFL group compared to the SH group (Fig. 5A, B, and C). We determined the effect on blood markers related to bone resorption among the groups. Serum CTX-1 levels tended to increase in the ATFL + CFL group compared to the SH group, but the difference was not statistically significant (Fig. 5D).
Discussion

This study investigated the effects of CAI in a mouse model. The results demonstrated that the ATFL + CFL group, in which ATFL and CFL were resected with the defects, revealed significant differences in clinical severity, such as paw thickness, decreased number of mice standing on hind legs, erosion of the cartilage on three-dimensional micro-CT, and histological analysis. More specifically, quantitative analysis of arthritis-related inflammatory cytokines in serological analysis using ELISA indicated the arthritic change of the ankle joint in the gross instability induced ATFL + CFL group.

The recurrence rates of ankle sprains are high, leading to a large percentage of patients with ankle sprain developing CAI [13, 14]. The lingering ankle instability contributes to ongoing disability and sensorimotor control deficits, which were associated with decreased physical activity and quality of life [13, 14]. Not surprisingly, these residual impairments were believed to persist for the remainder of the patient's life, in part due to the link between CAI and posttraumatic ankle OA [13, 14, 15, 16]. However, a lack of lifelong prospective studies, for obvious logistical reasons, limits our understanding of whether CAI affects the development of ankle OA. The optimal prospective study design to enable the systematic determination of factors that contribute to the development of ankle OA would include an evaluation of individuals before and after CAI. Based on the time and difficulty associated with performing these systematic investigations of clinical and radiological evidence for arthritic changes after CAI, this design is currently not feasible in humans. However, longitudinal, live-animal experimental designs circumvent this concern and allow the application of systematic perturbations to the ankle joint to quantify the consequences of ankle OA related to CAI.

Importantly, the lateral ligaments of the mouse ankle have anatomic locations and functions similar to those in humans, so the mouse ankle is a useful model for studying neuromuscular adaptations after an ankle injury [11, 12, 13, 14, 15, 16, 17]. While mouse models have been well established for an ankle sprain, a surgically induced CAI mouse model has not been used [11, 12, 13]. In a previous study of a rat model of post-traumatic OA, it was significantly different from the pathophysiology of CAI because the tendon and ligament tissues were excised [21]. In a previous study, when the group with simple transection of ATFL (n = 7), the group with ATFL + CFL transection (n = 7), and the group with the sham operation (n = 5) were analyzed using the same method as in the current study, and there were no differences in behavioral, histological, or serological analyses among the three groups. Therefore, in our study, unlike the previous surgically induced mouse model that transected ATFL and CFL, a CAI in a mouse model was created by resection with few defects of the ATFL and CFL to prevent ligament healing. As a result, OA changes of the ankle joint in this CAI mouse model were verified through clinical, radiological, histological, and serological analyses. Therefore, the strength of this study is that it demonstrated that posttraumatic OA is induced by CAI in an animal model. Furthermore, this study could serve as a basis for subsequent studies to evaluate the drugs and genetic pathways that prevent or alleviate OA caused by CAI.
CAI was described as an isolated ATFL tear in 80% of cases, or an ATFL and CFL tear in 20% of cases [18]. The ATFL is essentially a thickening of the lateral joint capsule, and is the most anterior, the weakest, and the most easily injured structure of the lateral ankle ligamentous complex [18, 19]. An isolated injury of the CFL is rare, and the CFL is commonly torn followed by the ATFL tear [19]. In this study, the isolated ATFL group demonstrated inferior outcomes compared to the control group in histological analysis, and TNF-α in serological analysis. However, OA changes were significantly higher in the ATFL + CFL group than in the ATFL group. These results might provide indirect evidence that the gross instability of the ATFL and CFL is more likely to induce posttraumatic OA than the instability of ATFL alone in humans.

This study has several inherent limitations that warrant review. However, few functional differences should be considered. In the mouse ankle, the tibiotalar joint is more flexed and the calcaneus bears less weight than the human ankle. These differences may limit research on the present mouse models, as true for human ankle OA [21]. Second, due to the limitations of the experimental schedule and the research scale, the life span of mice during the experiment does not correspond to adult age. Therefore, it is difficult to predict OA changes in elderly mice, and it is difficult to estimate the life-long outcome in the isolated ATFL resection group. Nevertheless, the fact that ankle OA occurred in the ATFL + CFL group within a period of 12 weeks is meaningful in that it confirmed that the insufficiency of the lateral ligament complex contributed to the development of ankle OA. Third, we used ICR mice instead of rats; they were larger than normal mice and were easy to perform surgery on. When performing surgery, the use of a microscope enables precise and delicate surgeries. In addition, we plan to study the molecular pathways involved in CAI causing OA in follow-up studies using transgenic mice, such as knot-out mice.

**Conclusion**

This study demonstrated that the surgical induction of CAI (ATFL+CFL) in a mouse model can result in the development of osteoarthritis of the ankle joint through behavioral analysis, clinical severity, micro-CT, histological, and serological analysis. This model fits within the framework of contemporary theoretical models of posttraumatic OA due to CAI and highlights the need to use this model to gather preclinical data regarding the effectiveness of the intervention.

**List Of Abbreviations**

Chronic ankle instability (CAI), ATFL (Anterior talofibular ligament), CFL (Calcaneofibular ligament), ELISA (enzyme-linked immunosorbent assay), OA (osteoarthritis), BMD (bone mineral density)

**Declarations**

**Competing interests**

The authors declare that they have no competing interests.

**Ethics approval and consent to participate**
The study was approved by the Institutional Animal Care and Use Committee of Wonkwang University (WKU19-63), Republic of Korea, and the study was conducted according to standard guidelines. The mice were monitored daily to assess their health status.

Consent for publication
Not applicable.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding
This study was supported by a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2020R1G1A1102304).

Acknowledgements
Not applicable

Authors’ contributions
SH Lee contributed to study conception and design. JY Kim and SY Eun contributed to acquisition of data. JY Kim and SY Eun contributed to data analysis and interpretation. SH Lee drafted manuscript. SH Lee, DK Kim, BM Yoo involved in manuscript preparation. All authors revised the manuscript critically for important intellectual content and approved the final submitted manuscript.

References


Figures

**Figure 1**

Anatomical structure and location of the ankle ligament in mice. (A) ATFL was identified between EDL and EDQ on anterolateral view after longitudinal skin incision and retraction. (B) ATFL was resected for the ATFL group and ATFL+CFL group. (C) CFL was identified under the distal part of fibula. (D) CFL was resected for the ATFL+CFL group.
ATFL: anterior talofibular ligament, CFL: calcaneofibular ligament, EDQ: extensor digiti quinti, EDL: extensor digitorum longus

Figure 2

Analysis of physical activity and clinical changes of osteoarthritis in a chronic ankle instability mouse model. (A) Bodyweight change of each group was determined on the indicated days. (B) Physical activity was measured as the number of hindlimb standing at 4, 8, and 12 weeks after surgery. (C) Representative photographs of SH, ATFL, and ATFL+CFL group mice. (D) Ankle thickness and paw thickness were measured at 12 weeks after surgery. Statistically significant differences are indicated by **p < 0.01 or ***p < 0.001 versus the SH group.

SH group: sham; control, ATFL group: resected anterior talofibular ligament; mild ankle sprain, ATFL+CFL group: resected ATFL/calcaneofibular ligament; severe, W: weeks
Figure 3

Micro-computed tomography (CT) analysis in a chronic ankle instability mouse model. (A) Micro CT images of the ankle joints of the SH group (A1), ATFL group (A2) and ATFL+CFL group (A3). Osteophyte and loose bodied were identified in the ATFL group and ATFL+CFL group (A, right). Red arrow indicates an osteophyte and loose bodied of ankle joint. (B-D) Quantitative analysis of bone mineral density (BMD) (B), bone volume fraction (BV/TV (C), and total porosity (D) of the mouse ankle joints. ***$p<0.001$ versus the SH group.

SH group: sham; control, ATFL group: resected anterior talofibular ligament; mild ankle sprain, ATFL+CFL group: resected ATFL/calcaneofibular ligament; severe
Figure 4

Histopathological changes in a chronic ankle instability mouse model. The ankle joint was sectioned and stained with H&E, TRAP, Safranin O, and toluidine blue. The pathological severity scores of inflammation, pannus formation, and cartilage damage in the ankle joint. Scale bar, 200 μm.

Figure 5
Analysis of proinflammatory mediators and bone resorption marker in serum of a chronic ankle instability mouse model. The expression levels of (A) interleukin (IL)-1β, (B) IL-6, (C) tumor necrosis factor-α (TNF-α), and (D) C-terminal telopeptide of type I (CTX-1) were determined using murine ELISA assay. Statistically significant differences are indicated by **$p < 0.05$, ***$p < 0.001$ versus the SH group.

SH group: sham; control, ATFL group: resected anterior talofibular ligament; mild ankle sprain, ATFL+CFL group: resected ATFL/calcaneofibular ligament; severe