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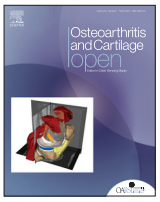

One session of 20 N cyclic compression induces chronic knee osteoarthritis in rats: A long-term study

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ABSTRACT

Objective: Mechanical stimulation is a risk factor for knee osteoarthritis. Non-surgical compression has been used to study the effects of mechanical stimulation *in vivo*. However, the long-term effects of low-force compression on knee joint had not been studied. Therefore, we sought to identify the long-term effects of low-force cyclic compression on the rat knee joint.

Design: In this study, we applied one session cyclic compression with a peak load of 20 N for 60 cycles to the rat knee joint in an approximately 140-degree flexion position (Wistar, male, 12 weeks old), followed by 1 year of observation (including data from 1 week, 2 weeks, 4 weeks, 8 weeks, 6 months, and 1 year after compression), and then performed a sub-regional analysis with hematoxylin-eosin, Safranin O and Fast Green, and MMP13 immunohistochemical staining.

Results: We observed osteoarthritis-like cartilage damage, synovial inflammation, and high expression of MMP13 within 1 year after compression. However, these changes progressed slowly, with obvious matrix cracks that did not appear until 1 year after compression. In the regional analysis, we found that low-force compression caused a much slower development of injury at the compression contact site, and no significant structural cartilage damage was observed after 1 year of compression. In contrast, the non-contact site during compression at tibial cartilage in the same joint was the first to show significant structural damage.

Conclusion: This study demonstrates that one session of 20 N cyclic compression induces a chronic osteoarthritis-like phenotype in the rat knee in the long term.

1. Introduction

Among the animal models of knee osteoarthritis (OA), non-surgical models have been proposed to avoid the possibility of unexpected bleeding, infection, and manipulation errors associated with surgical joint destabilization [1,2]. Machine-provided mechanical stimulation has been applied to the murine knee, without exposing of the joint cavity, to induce various OA features observed in humans. In the knees of mice, high loads of 9–50 N have induced intra-articular tibial plateau fractures, anterior cruciate ligament (ACL) ruptures, and avulsion fractures, resulting in degenerative changes in the articular cartilage (AC), synovitis, subchondral bone sclerosis, and ectopic chondrogenesis [3–7]. Mechanical stimuli, have also been applied, in the form of repetitive cyclic compression events, causing ACL ruptures and tibial fatigue fractures. Increased cortical bone thickness and gait changes have been reported as characteristic changes induced by this type of mechanical stimuli [3,8–10]. Compared with mice models, few non-surgical rat models have been reported, although they are more advantageous for imaging modalities and therapeutic interventions [2]. High-velocity mechanical stimuli have been applied to the knee joints of rats to cause ACL injury, inducing OA [11–13]. However, neither case explains the situation in patients without a definite history of knee trauma. Christiansen et al. concluded that mechanical loading using a single cycle may cause less damage [7]. One session of cyclic compression, resulting in no

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structural damage to the knee, has induced regional AC damage, although no deterioration was observed 2 weeks after compression [3]. These findings suggest that the extent of knee injury may be compression force-dependent. Thus, the effects of lower-force compression on the knee joint must be clarified.

In our previous experiment, we determined the lower-force cyclic compression in rats. In humans, the pressure on the knee joint during stair climbing can be up to six times the bodyweight [14]. We converted the average body weight of 12-week-old rats (approximately 300 g) and found the pressure to be approximately 20 N. One session of cyclic compression, with a peak load of 20 N and 60 cycles, was applied to the rat's knee [9]. We demonstrated that this one-session cyclic compression could lead to regional cytopenia and loss of glycosaminoglycan (GAG), which is an important functional component of the extracellular matrix (ECM) [15], and simultaneously, to no immediate structural damage. However, we did not observe further OA pathological progression of OA 8 weeks after compression. As OA in humans is often observed in the older population, and its pathological process may be slow [16], a long-term study is needed. Therefore, this study aimed to observe the rat knee joint after one session of 20 N cyclic compression for a period of 1 year.

2. Materials and methods

2.1. Experimental animals

A total of 42 healthy rats (Wistar, male, 12 weeks old, purchased from SHIMIZU Laboratory Supplies Co. Ltd., Kyoto, JP) were used in this study. After undergoing the compression described subsequently, the rats were housed in plastic cages with adequate space for activities (two per cage). All animals were maintained on a 12-h day/night cycle with adequate food (standard solid pellet feed) and water. This experiment was approved by the Animal Research Committee of Kyoto University (approval number: Medkyo21082). The experimental design, analysis, and reporting were performed in accordance with the ARRIVE guidelines (https://arriveguidelines.org/).

2.2. Compression procedures and sample allocation

On the day of the compression, the rats were anesthetized with 5% isoflurane solution, followed by injections with three types of mixed anesthetic agents (0.375 mg/kg medetomidine, 2.0 mg/kg midazolam, and 2.5 mg/kg butorphanol). Subsequently, random group assignment was conducted. The right knees of the rats in the experimental group were compressed at approximately 140° of flexion, as documented in a previous study [15] (Fig. 1A). All the rats in the experimental group underwent a single compression session, which included 60 cycles of cyclic loading, during the study. One cycle included a preload of 5 N and a peak load of 20 N, with an approaching speed of 1 mm/s following 10-s rest intervals [17] (Fig. 1B).

The rats were euthanized 1, 2, 4, and 8 weeks, 6 months, and 1 year post compression, respectively: PC-1wk, PC-2wks, PC-4wks, PC-8wks, PC-6mos, and PC-1yr (n = 6 for each group; Fig. 1C). Six additional 12-week-old uncompressed rats were euthanized to obtain baseline data. The right knee joints in the baseline and experimental groups and the contralateral knee joints in the PC-6mos and PC-1yr groups were collected.

2.3. Specimen preparation

The collected knee joints were fixed in 4% paraformaldehyde for 24 h and decalcified in 10% ethylenediaminetetraacetic acid solution for 25 days. Tissue sections were obtained from paraffin-embedded tissues. Serial sagittal sections of the knee joints were cut every 6 μm from the lateral compartment. Twelve sections were collected at 100-μm intervals and covered the entire area of the lesion on the lateral femur. Hematoxylin-eosin (HE), Safranin O (SO) and Fast Green (FG) with hematoxylin, and immunohistochemistry staining were performed.

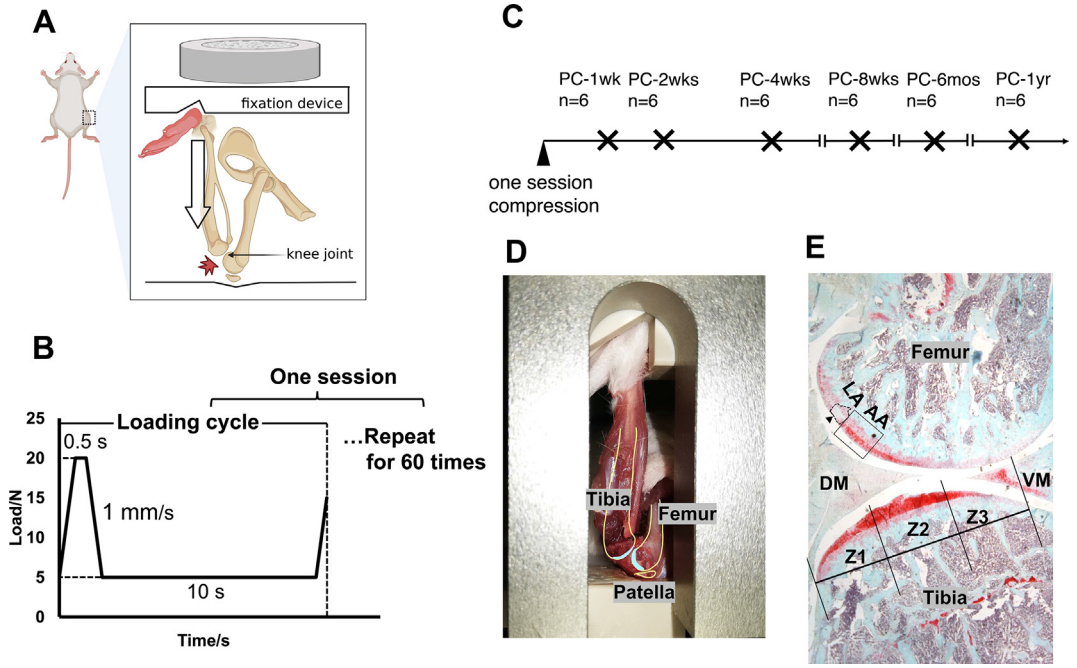

Fig. 1. Experimental materials and methods. (A) Schematic of a rat receiving cyclic compression. (B) Waveform plot of the compression regiment. The compression of one session consists of 60 pressure waves with a peak value of 20 N. (C) Distribution of experimental animals. The black cross indicates euthanasia of the animal and knee specimen collection at the corresponding time points. PC, post compression. (D) Confirmation of compression contact sites after skin removal. The yellow line represents the articular bone contour, and the blue area represents the cartilage. (E) Schematic diagram of cartilage partitioning. LA, lesion area; AA, adjacent area; DM, dorsal meniscus; VM, ventral meniscus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4. Histological analysis

Considering the locality of compression and its effects, a partition analysis was performed. In the pre-study, a rat was observed during compression after removing the skin of the knee joint (Fig. 1D), and a section was observed after the specimen was fixed at approximately 140° of flexion (Sup. 1). Thus, the AC contact sites during compression were determined. The lesion area (LA) represents the contact sites during compression and the cartilage portion where the superficial chondrocytes have a loss of spatial continuity, and the adjacent area (AA) represents an area adjacent to the LA within the field of view of a 200 × light microscope, which were then divided on the femur AC for analysis. Meanwhile, the tibial AC on the sagittal slice was equally divided into three zones (Z1, Z2, and Z3) from dorsal to ventral (Fig. 1E). Z1 included the contact site during compression. Z2 included the thickest part of the tibial AC and the site of AC degeneration in other surgical OA models. Finally, Z3 represented the uncompressed region.

Cell counting was performed using Fiji (v2.1.0) and photographs of HE-stained sections. The relative area of each region was calculated using Fiji with the freehand and area-measurement tools. Relative cellular density was calculated using the number of nuclei and relative area of the cartilage in each region.

Each region in SO and FG-stained sections was viewed under a microscope and scored using the Histologic/Histochemical Grading System (HHGS) and the Cartilage Histopathology Assessment System (OOCHAS) [18–20].

Synovial tissue was observed in the HE-stained sections. The synovial score (synovial enlargement plus synovial inflammatory cell infiltrate) [21] was calculated.

All scoring was performed in a blinded manner by at least two observers (ZZ, AN, and/or CT). With a review of the histological findings for which there were disagreements among the observers, a final consensus score was reached.

2.5. Immunohistochemistry and semi-quantitative analysis

The paraffin sections were dewaxed in water and treated with a 3% hydrogen peroxide solution for 30 min. For antigen retrieval, the sections were heated at 65 °C for 30 min in HistoVT One (Nacalai Tesque, Inc., Kyoto, Japan; diluted at 1:10) and washed three times with phosphate-buffered saline. Normal goat serum (5%) was used as a blocking solution for 30 min at room temperature (approximately 25 °C). Subsequently, the sections were incubated overnight at 4 °C with a matrix metallopeptidase 13 (MMP13) primary antibody (Abcam, ab39012; diluted at 1:1000); the MMP13 primary antibody recognizes the latent proenzyme, active form, and intermediate activation forms of MMP13 and does not cross-react with the other MMP family members). All the sections were then rinsed and treated with goat anti-rabbit IgG for 30 min at room temperature (approximately 25 °C). Antigen-antibody complexes were detected using the avidin-biotin complex reagent (ABC kit PK-6100, Vector Laboratories, Burlingame, CA, USA) enhanced with 3, 3 -diaminobenzidine (DAB kit SK-4105, Vector Laboratories, Burlingame, CA, USA). The positive cells were counted in each region. The percentage of positive cells was calculated as the ratio of the number of positive cells to the total cell number of cells in each region.

2.6. Statistics

The sample sizes were determined according to previous experience and a previous study [15]. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). The parameters were compared at each time point, with time as the variable. The relative cell density and percentage of MMP13-positive cells were normally distributed and passed the homogeneity of variances test. One-way analysis of variance was used, followed by a post-hoc Tukey's honestly significant difference test. The non-parametric Kruskal–Wallis test was used for data in the form of scores. Comparison of data from both limbs in the PC-6mos and PC-1yr groups was performed using the *t*-test (when the data were normally distributed) or Mann–Whitney *U* test (when the data were not normally distributed or were in the form of scores). Various parameters of the uncompressed knees of 12-week-old rats were collected, and their means were used as the baseline. Statistical significance was set at $P < 0.05$. Data are expressed as means; errors bars represent 95% confidence intervals.

3. Results

3.1. Cartilage damage is heterogeneous across regions in the long term

Owing to the direct effect of compression, the LA showed a significant reduction in cellular and ECM staining, while preserving the AC form structure (Fig. 2A). Both the HHGS and OOCHAS scores in this region stabilized in the long term owing to the absence of significant structural damage (Fig. 2C, Sup. 2B). In the AA (Sup. 2A), the injury mainly manifested as an enlargement of the area involved in the injury ("stage" program of OOCHAS), without significant structural changes (Fig. 2D, Sup. 2C). In the tibia, the Z1 (Sup. 2A), which was directly affected by the compression, showed no characteristic damage similar to the LA; only slight superficial AC damage and cellular changes were observed, and it was stabilized in the long-term damage score (Fig. 2E, Sup. 2D). In a subset of samples (3 of n = 6), Z2 showed significant cell clustering and cyst formation in the PC-6mos group (Fig. 2A), resulting in cavities in the AC. In the PC-1yr group, Z2 showed clefts in all the samples (n = 6; Fig. 2A, F). Z3 showed no significant damage (Sup. 2F, G).

3.2. Chondrocytes decrease after compression

After compression, the LA cell density decreased and tended to decline in the long term (Fig. 3A). In contrast, the cell density of AA did not show a trend of change, but a lower cell density was observed at all time points after compression (Fig. 3B). There was a clear trend of low cell density at both Z1 and Z2 in the tibial AC (Fig. 3C and D).

3.3. Compression causes mild synovitis

From the PC-4wks group on wards, the lining layer was hyperplastic, with multiple cell layers, accompanied by cell proliferation in the sublining layer (Fig. 4A). Synovial scores were higher in the PC-6mos and PC-1yr groups than the PC-1wk group (Fig. 4C).

3.4. Compression leads to high expression of MMP13 with no trend of change

MMP13 was expressed in the deep layer of the LA as well as in the AA, Z1, and Z2 after compression (Fig. 5A, Sup. 3A). Meanwhile, MMP13 was not expressed in the superficial layer of the LA because of the loss of chondrocytes. Importantly, although high levels of MMP13 expression were maintained in the AA, Z1, and Z2, no differences in MMP13 expression were observed at any time point in any region (Fig. 5C–F).

3.5. Cartilage damage, synovial inflammation, and high MMP13 expression are not spontaneous

To exclude the possibility of spontaneous long-term OA, the compressed side in the PC-6mos and PC-1yr groups was compared with the uncompressed contralateral limb. The contralateral side showed a few damaging manifestations of OA (Fig. 2B). Regional AC damage (Fig. 2G–J), reduced cell density (Fig. 3E–H), synovitis (Fig. 4B, D), and high MMP13 expression (Fig. 5B, G-J) can be considered the results of compression.

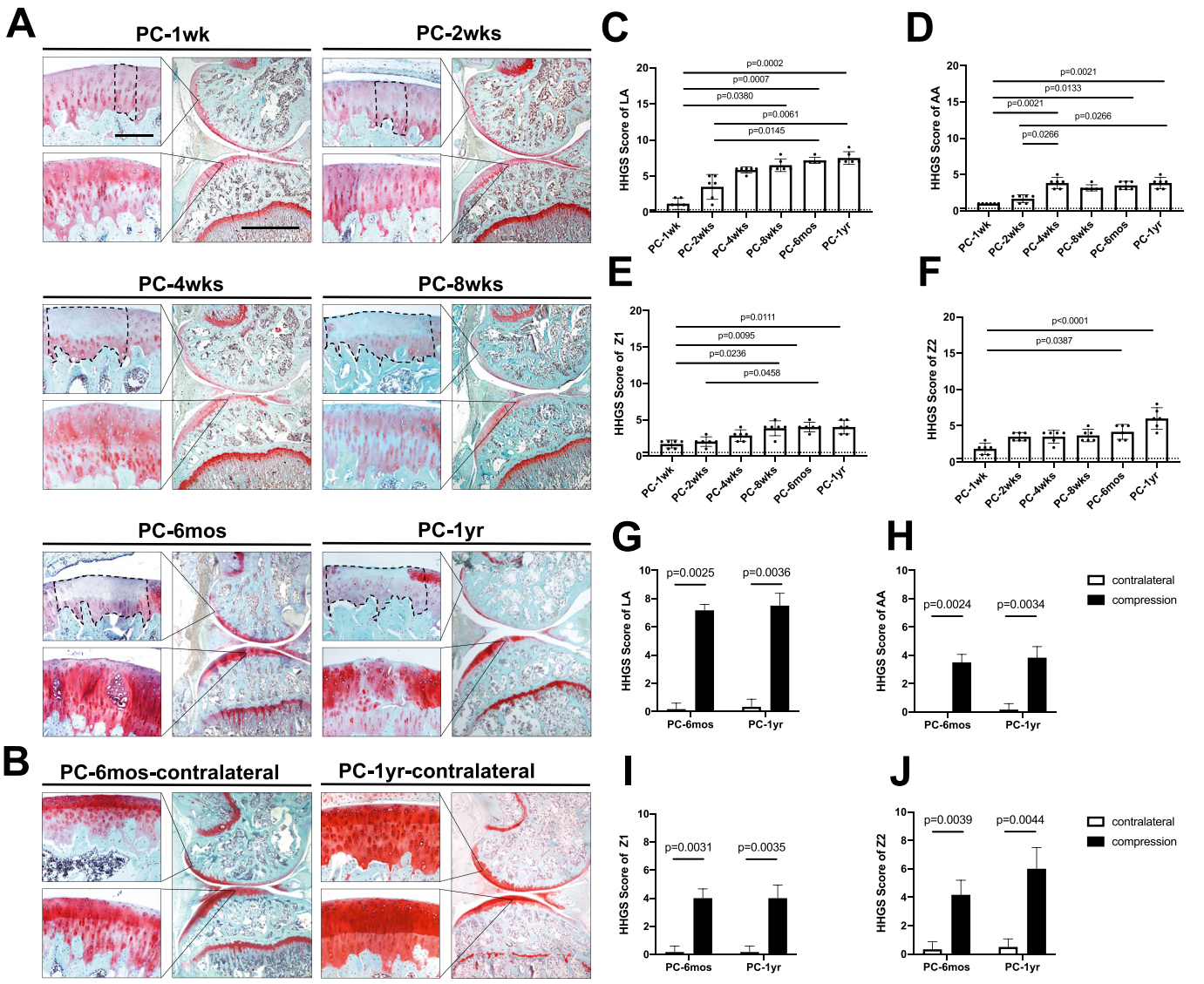

Fig. 2. Histological observations and analysis. (A) SO & FG staining results by time point. LA with part of the AA region, and the Z2 region are shown in the upper and lower left of each time point, respectively, scale bar, 100 μm; the right side shows the whole knee joint in low magnification view, scale bar, 1000 μm. The dashed line indicates the LA region. (B) Results of SO & FG staining of the uncompressed contralateral limb at PC-6mos and PC-1yr time points. (C–F) HHGS scores for LA, AA, Z1, and Z2 at each time point. The dashed line indicates the mean of the baseline group. (G–J) Comparative results of HHGS scores of the compressed and uncompressed contralateral limbs in each region of LA, AA, Z1, and Z2 at the PC-6mos and PC-1yr time points.

4. Discussion

Here, we demonstrated that one-session cyclic compression, with a peak of 20 N and 60 cycles, resulted in slow OA-like degeneration in the rat knee joint. In this study, long-term observation of the rat knee joint after 20-N cyclic compression was performed for 1 year. Significant chondrocyte death and GAG loss were observed at the direct contact site of compression. In contrast, structural AC destruction was observed at the nondirect contact site (Fig. 2A), with no significant cartilage damage in the uncompressed contralateral limb (Fig. 2B). More importantly, the damage at the direct contact site showed no signs of recovery in the long term; however, simultaneously, the degeneration progress was slow (Fig. 2A, C).

At 20-N cyclic pressure, in addition to the human data for conversion, ACL rupture was observed in rats under a pressure of 60 N (data not shown). Meanwhile, we noticed that even a peak pressure of 50 N did not cause any damage to the ACL after 60 cycles [15]. We also observed changes in menisci during the experiment (Sup. 4) to ensure the compression force. Four weeks after compression, no structural damage and only scattered cellular changes were observed, and from the PC-8wks group onwards, the meniscus showed changes, such as fibrous disorganization and areas of apparent cellular deficiency. Overall, one session of cyclic compression at 20 N for 60 cycles resulted in a relatively low compression force without leading to immediate structural knee injuries.

Repeated loading that acts directly on the AC causes nitric oxide expression [22] and oxidation-dependent mitochondrial dysfunction in chondrocytes [23], eventually leading to chondrocyte death [24]. Chondrocytes are the only cell type present in the AC. Thus, after regional chondrocyte loss, accumulation of cellular metabolites no longer occurs in the region; in contrast, type II collagen accumulates [15], leaving the region in a quiescent state of injury. We believe that one session of 20 N cyclic compression promotes rapid cell death without harming the compressed part of the AC structure and is responsible for preserving that part. This is in line with a study in which the progression of OA was delayed by the rapid elimination of superficial cartilage cells [25]. A study using a peak load of 1 N compression as a post-surgical destabilization of the medial meniscus treatment in mice suggested that low-force compression inhibits the OA process [26]. Our results suggest that

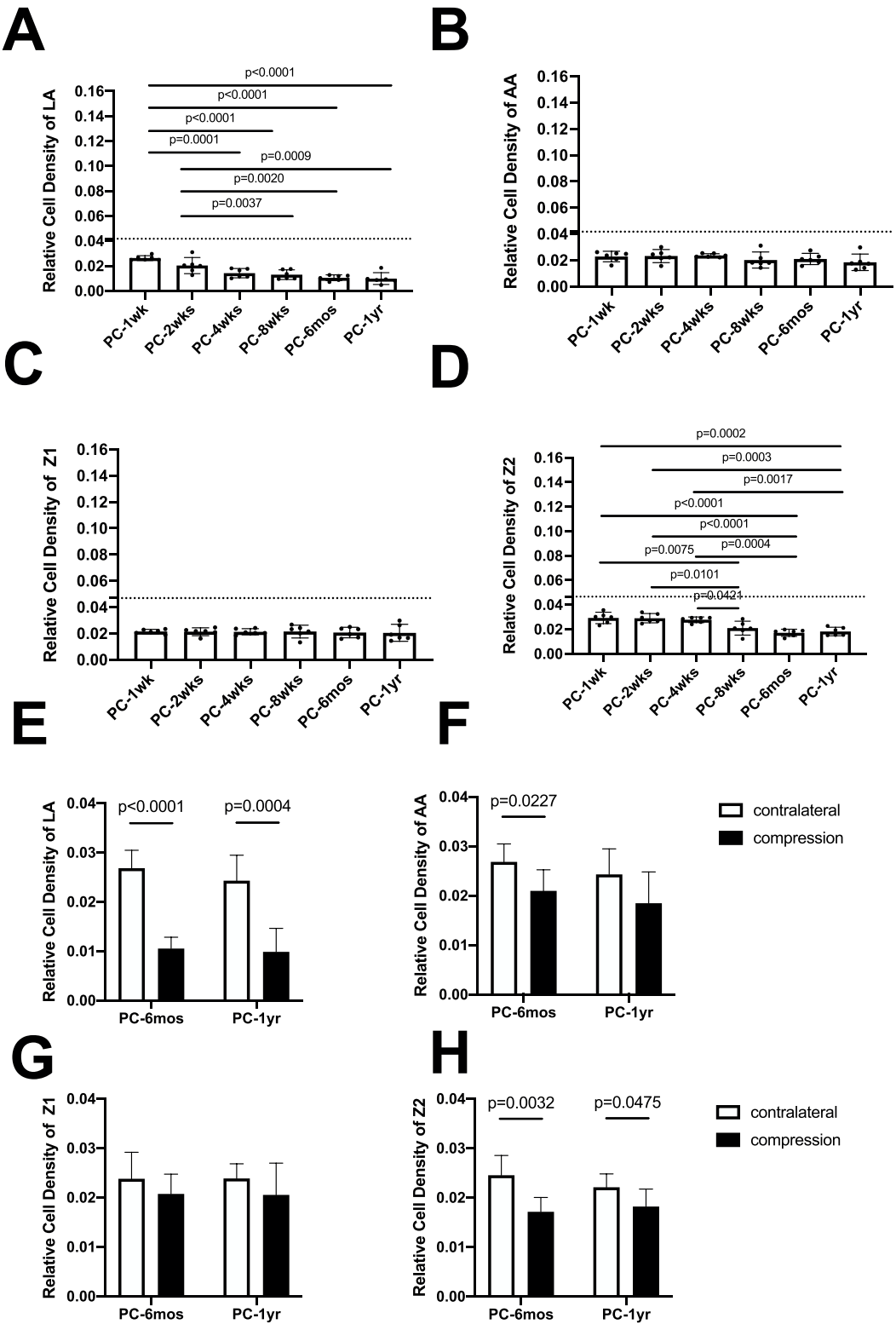

Fig. 3. Relative cell density. (A–D) Relative cell density at each time point in each region of LA, AA, Z1, Z2. The dashed line indicates the mean of the baseline group. (E–H) Comparative results of cell density between compressed and uncompressed side limbs of LA, AA, Z1, and Z2 at PC-6mos and PC-1yr time points.

low-force compression may only provide a local quiescent effect on cartilage injury development (we are currently unsure whether it is an ameliorative effect), but it would still induce OA in the whole joint in the long term.

During compression, the cartilages should only be in partial contact with each other (Fig. 1D, Sup. 1). Interestingly, Z2, the site not directly subjected to compression, was the first to show significant AC structural damage in a prolonged low-inflammatory environment (Figs. 2A and 5A, H-J). Our previous study in rats after surgery for meniscal instability in the knee showed that cartilage degeneration was localized in the area that received mechanical loads during knee walking [27]. This suggests that in the present study, Z2 was more susceptible to cartilage damage as

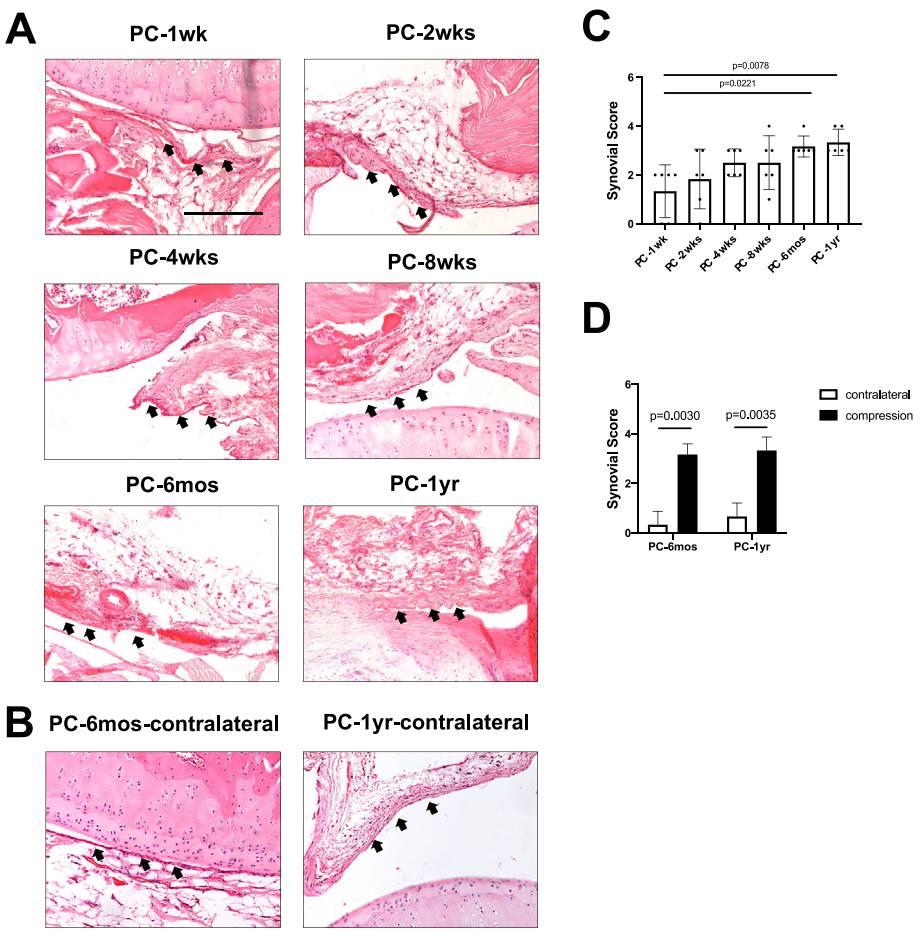

Fig. 4. Observation and analysis of the synovial tissue. (A) HE staining results of synovial tissues at each time point. Black arrows indicate synovial tissues. Scale bar, 100 μm. (B) HE staining results of synovial tissue of the contralateral uncompressed limb at time points PC-6mos and PC-1yr. Black arrows indicate synovial tissue. (C) Synovial tissue scores at each time point. (D) Comparison of synovial scores of the compressed and uncompressed contralateral limbs at the PC-6mos and PC-1yr time points.

an area of daily activity loading. Meanwhile, the thick cartilage, which is equivalent to the Z2 region in this study, is also considered to be the site of mid-zone cyst formation [20]. Although the cartilage on the surface of Z2 did not show obvious clefts in the PC-6mos group, the apparent cysts and cell clustering led us to determine its OOCHAS rating as grade 4. We suggest that the ultimate structural changes in the Z2 region may originate from cavities created by the cysts. The variability in damage in different areas was also consistent with the results in humans. The deformation of the tibial AC is more pronounced than that of the femur after impact exercise in humans [28]. This may be due to the inherent differences in the mechanical properties of knee AC [29,30].

Synovial inflammation is thought to occur first after a traumatic knee event. Consistent with our findings (Fig. 4A, C), OA synovial tissue is histologically referred to as mild to moderate inflammation [31], and its severity depends on that of OA [32]. This suggests that the development of subsequent intra-articular damage caused by compression at the 20 N level is sufficiently slow, which may be similar to the experience of many patients with OA who claim no significant traumatic events.

Mechanical loading not only leads to joint surface wear but also induces the activation of mechanosensitive signaling pathways that drive proteases that initiate cartilage breakdown processes [33]. AC degradation and AC clefts are caused by the upregulation of matrix-degrading enzymes, many of which are members of the MMP family [34]. Inhibition of MMP13 activity decelerates ECM loss and other OA-like phenotypes in a surgically induced OA model [35,36]. In our study, MMP13 was significantly upregulated and maintained high expression from 1 week after compression (Fig. 5A, C-F) and was expressed at low levels in the PC-1yr group in the uncompressed left knee (Fig. 5B, G-J), which did not show a significant OA phenotype (Fig. 2B). In the LA without further structural damage, MMP13 expression was relatively low, whereas in the Z2 without compression but with structural damage, MMP13 was highly expressed. These results suggest that compression can lead to a high regional expression of MMP13, resulting in structural damage to the AC. The local clearance of senescent cells attenuates the development of post-traumatic OA and creates an environment that promotes regeneration [37]. Similarly, in our study, a rapid loss of chondrocytes in the superficial layer of the LA was observed, and only MMP13 had a relatively low expression (Fig. 5A, C, G); progression of OA in this region was halted (Fig. 2A).

This study had several limitations. First, because the compression was applied directly outside the body, direct pressure on the cartilage could not be calculated. Therefore, we are not sure whether the 20-N compression of the rat knee cartilage is representative of the stresses that may occur in daily life. Notwithstanding this limitation, this study suggests that even low-force compression that does not cause any direct structural damage can lead to AC degeneration and structural cartilage damage in the long term. Second, we only performed a subregional evaluation of the lateral knee compartment with significant GAG loss, and to ensure consistency, only one section of each sample with the largest LA area was selected for evaluation, and we performed only one OA-related biomarker (MMP13) analysis. Other methods should be used in the future studies to evaluate changes in the overall AC in a non-invasive manner and further analyze the molecular mechanism changes. Third, although we randomly divided the cages and provided ample space for movement, because of the individual nature of OA development, the degree of cartilage damage varied among the same group of samples at long-term time points, and we do not know how the activity level varies between individuals. Further analysis of the causes of the development of OA in the uncompressed portion are needed.

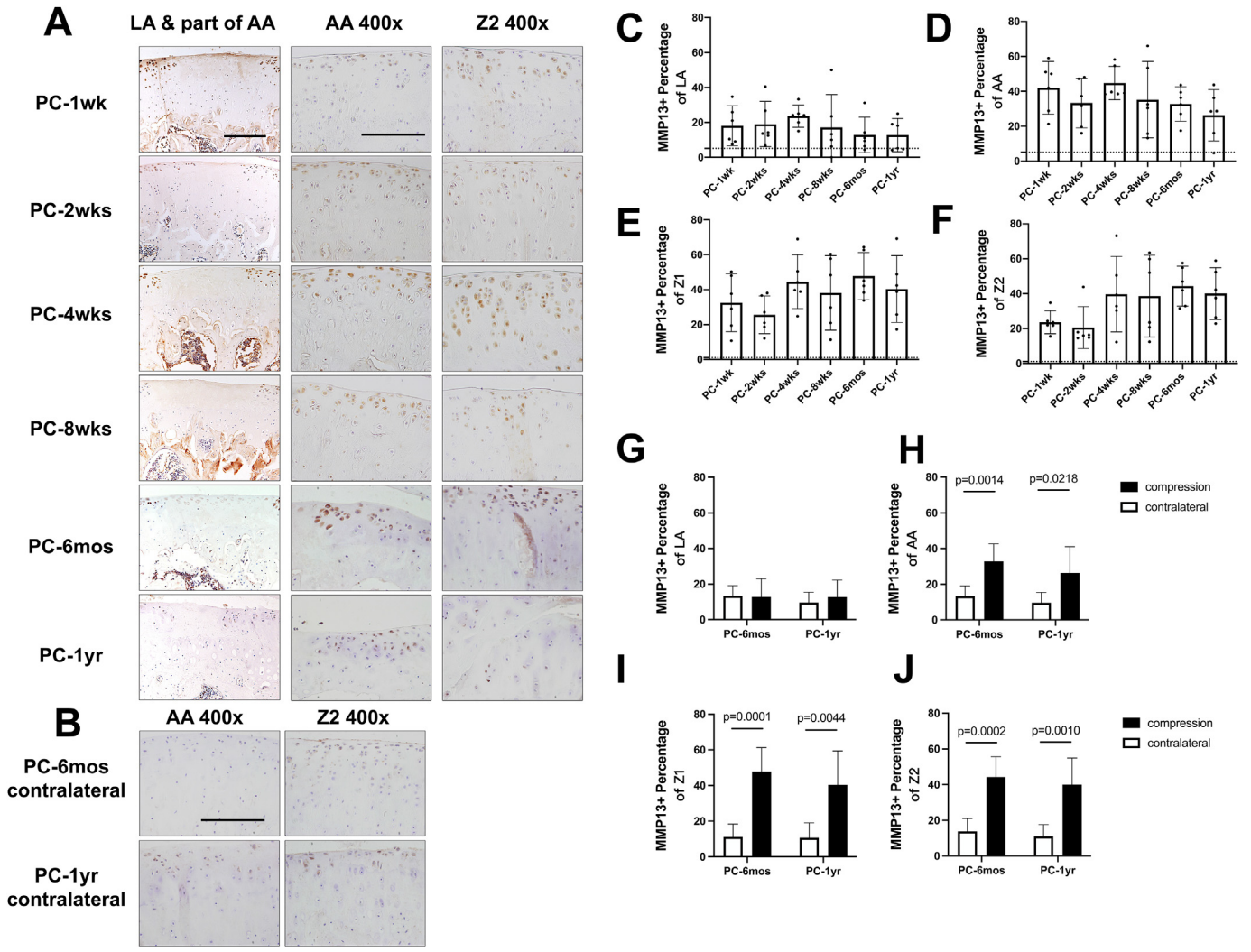

Fig. 5. Observation and analysis of MMP13 expression. (A) Results of MMP13 staining in LA with part of AA regions, AA and Z2 regions at each time point. Images of AA and Z2 regions are the results under high magnification view, magnification of images is 400x. Scale bar, 100 μm. (B) MMP13 staining results of AA and Z2 at the time points of PC-6mos and PC-1yr under high magnification. Scale bar, 100 μm. (C–F) Results of MMP13-positive cell percentage in each region of LA, AA, Z1, and Z2 at each time point. The dashed line indicates the mean of the baseline group. (G–J) Comparative results of the MMP13-positive cell percentage in the compressed and uncompressed contralateral limbs at the PC-6mos and PC-1yr time points in each region of LA, AA, Z1, and Z2.

Author contributions

AI (ito.akira.4m@kyoto-u.ac.jp) and ZZ (zhao.zixi.52s@st.kyoto-u.ac.jp) were responsible for the integrity of the work as a whole, from inception to the finished article. All authors made substantial contributions to (1) the conception and design of the study, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted.The specific contributions of the authors are as follows: Conception and design of the study: ZZ, AI, XJ, AN, and HK; Analysis and interpretation of the data: ZZ, AI, AN, and CT; ZZ and AI; Critical revision of the article for important intellectual content: ZZ, AI, AN, XJ, CT, MS, KN, TA, and HK; Final approval of the article: ZZ, AI, KN, TA, and HK; Statistical expertise: ZZ, AI, AN; Obtaining of funding: AI and HK; Collection and assembly of data: ZZ and XJ.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2022.100325.

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