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# Effects of *in vivo* cyclic compressive loading on the distribution of local Col2 and superficial lubricin in rat knee cartilage

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Running headline: Cyclic loading affects Col2, lubricin

## Abstract

This study aimed to examine the effects of an episode of *in vivo* cyclic loading on rat knee articular cartilage (AC) under medium-term observation, while also investigating relevant factors associated with the progression of post-traumatic osteoarthritis (PTOA). Twelve-week-old Wistar rats underwent one episode comprising 60 cycles of 20 N or 50 N dynamic compression on the right knee joint. Spatiotemporal changes in the AC after loading were evaluated using histology and immunohistochemistry at 3 days and 1, 2, 4, and 8 weeks after loading (n=6 for each condition). Chondrocyte vitality was assessed at 1, 3, 6, and 12 h after loading (n=2 for each condition). A localized AC lesion on the lateral femoral condyle was confirmed in all subjects. The surface and intermediate cartilage in the affected area degenerated after loading, but the calcified cartilage remained intact. Expression of type II collagen in the lesion cartilage was upregulated after loading, whereas the superficial lubricin layer was eroded in response to cyclic compression. However, the distribution of superficial lubricin gradually recovered to the normal level 4 weeks after loading-induced injury. We confirmed that 60 repetitions of cyclic loading exceeding 20 N could result in cartilage damage in the rat knee. Endogenous repairs in well-structured joints work well to rebuild protective layers on the lesion cartilage surface, which may be the latent factor delaying the progression of PTOA.

**Keywords:** *In vivo* cyclic compression; Post-traumatic osteoarthritis; Cartilage degeneration; Rat model; Type II collagen; Superficial lubricin

## Introduction

Post-traumatic osteoarthritis (PTOA) is a classification of clinical osteoarthritis (OA) that is common among patients with a history of articular cartilage (AC) damage and ligament injury. Animal models play an important role in understanding the pathophysiology of PTOA as well as developing novel therapies to treat this disease. Small animals such as rodents have the advantage of a faster pathological process and lower maintenance costs compared to large animals; hence, rodents are widely used as PTOA models for experimental purposes. The anterior cruciate ligament transection (ACLT) and destabilization of the medial meniscus (DMM) were the optimal conditions for short-term studies in the past. In recent decades, non-surgical models have been considered instead in order to avoid surgery-induced inflammation that could affect the evaluation. One of the most promising candidates is cyclic compression on knee AC[1].

The *in vivo* cyclic compression model was first designed for verifying trabecular bone adaptation to mechanical loading[2-4] and was developed as a nonsurgical model of OA in later studies[5-12]. However, there are doubts regarding the accuracy of the simulated pathologic progression of clinically relevant secondary OA in these models. One problem was over-frequent loading, which contributed to excessive subchondral bone reaction and the formation of disproportionately giant osteophytes, reported in many cases[7,8,12]. Ko et al[11] reported a single session of loading induced OA-like morphological destruction, where the regimen comprised 1200 cycles, roughly equal to the 5 days/week design in other studies. Poulet et al[5] confirmed that a loading episode of 60 cycles induced AC lesions without osteophyte formation, but after monitoring for 2 weeks found no loss in Safranin O staining. Therefore, further study on long-term tracking of low-dose loading effectiveness is necessary.

Although earlier studies using cyclic compression models showed visible osteophytes and reduced substrate staining, none reported irregular wearing of cartilage surface or subchondral bone porosity loss, which are important characteristics of OA progression in surgery-induced rodent OA models[13,14]. Thus, depending on the magnitude of compressive loading and the methodology of joint instability surgery, the mechanisms of repair or alleviation of the AC lesion in OA progression are still unclear.

The current study aimed to track the relatively long-term effect of *in vivo* low-dose cyclic loading on rat knee joints, the first such study in rat species. Further, we examined changes over time in loading-affected cartilage and investigated potential causes for the slower progression of OA development in a non-surgical model relative to a surgical model.

## Methods

### *Mechanical loading procedures and sample allocation*

All experimental procedures were approved by the animal research committee of Kyoto University (approval number: Med kyo 17616). Seventy-four 12-week-old wild-type male Wistar rats were used in this study. The animals were anesthetized with 5% isoflurane solution (Pfizer, Tokyo, Japan) before being injected intraperitoneally with 1 μg/g somnopentyl (Kyoritsu Seiyaku Corp., Tokyo, Japan). Each animal's right knee was fixed with a customized cup with approximately 140 degrees flexion, as previously described[6], and subjected to one session of dynamic loading in the daytime using a measuring compression instrument (Autograph AG-X, Shimazu, Japan). The loading regimen included a preload of 5 N and peak load of 20 N or 50 N, with approaching speed of 1 mm/s and 10 s rest intervals (Figure 1A-B). The load levels were set according to previous studies in other species[6,9], and were proportionately amplified based

on animal weight. Each session comprised 60 cycles that lasted approximately 12 min total. After loading compression, animals were returned to transparent plastic cages with a 12-h light/dark cycle and provided adequate feed and free space for movement. Experimental rats were randomly divided into three groups (peak load 20 N or 50 N, and control). The rats (n=6 for each condition, n=60 in total) were sacrificed for histological analysis at 3 days and 1, 2, 4, and 8 weeks after compression. Knee samples that underwent 20 N loading were harvested at 1, 3, 6, and 12 h for the live/dead assessment of chondrocytes (n=2 for each timepoint, n=8 in total). Normal 12-week old Wistar rat samples served as controls (n=4) for histological analysis and controls (n=2) for cell viability evaluation (Figure 1C). Randomization was performed using Excel functions, and animals in different experimental groups were treated in random order each time.

*Live/dead analysis of chondrocytes*

To evaluate the live/dead spatiotemporal changes of chondrocytes on lateral femoral condyles, calcein AM/ethd-1 staining (LIVE/DEAD Viability/Cytotoxicity Kit, Thermo Fisher Scientific, Tokyo, Japan) was performed immediately after specimens were dissected from the knee joints. Samples were treated with calcein AM (diluted 1:500) and Ethd-1 (diluted 1:4000) solutions in PBS for 20 min at room temperature. Samples were then rinsed in PBS and cut into two parts of the femoral intercondylar sulcus. The lateral half was then mounted on a transparent plate with the femoral condyle towards the camera (Supplementary figure 1). Fluorescence micrographs were taken using a fluorescence microscope (Fluoview FV10i, Olympus, Tokyo, Japan) with FITC (495/519 nm) and PI (535/617 nm) channels. Live cells were indicated by green fluorescence, and dead cells by red fluorescence. Contralateral limbs harvested at 12 h were used as controls.

*Histological analysis*

Knee joints were fixed in 4% paraformaldehyde overnight and decalcified in 10% EDTA for 25 days. Samples were then embedded in paraffin. Twelve 6-μm sagittal sections for every 100-μm interval were prepared, covering the entire area of the lesion in the lateral femur for each sample. Safranin O, fast green, and hematoxylin staining were performed on each section, and the average modified Mankin score[15] was calculated to evaluate the degree of cartilage degeneration at the lateral femoral condyle. To assess the volume of degenerative cartilage, the lesion area was defined using ImageJ software, and the stacked volume was calculated by multiplying the total area by a 100-μm average thickness. The intensity of Safranin O staining was calculated on an inverted 8-bit grayscale image using ImageJ software. The relative intensity in lesion areas was calculated by dividing by the intensity in normal cartilage (Supplementary figure 2). In addition, the hematoxylin-stained nuclei of chondrocytes in the lesion cartilage were counted.

*Immunohistochemistry and semi-quantitative evaluation*

Immunohistochemical staining of type II collagen (Fine Chemical Co., Toyama, Japan; diluted 1:200), matrix metalloproteinase thirteen (MMP-13) (ab39012 Abcam Co., Tokyo, Japan; diluted 1:1000), A disintegrin and metalloproteinase with thrombospondin motifs five (ADAMTS-5) (ab185795 Abcam Co., Tokyo, Japan; diluted 1:50), and Lubricin/Proteoglycan 4 (EMD Millipore, Temecula, USA; diluted 1:1000) were performed as described below. Deparaffinized sections were treated with 3% hydrogen peroxide solution for 30 min. Sections were then stained for the anti-type II collagen reaction and treated with 1.25% hyaluronidase for 60 min at room temperature. Sections for the ADAMTS-5 reaction were

133 treated with HistoVT One solution (Nacalai Tesque, Inc., Kyoto, Japan; diluted 1:10) for 40 min at 65 °C.
134 After rinsing in PBS, nonspecific reactions were suppressed by blocking with 5% normal goat serum for
135 60 min. Subsequently, sections were treated with primary antibodies and incubated at 4 °C overnight.
136 Sections were then washed in PBS and treated with goat anti-rabbit IgG (MMP-13 and ADAMTS-5) or
137 goat anti-mouse IgG (type II collagen and lubricin) for 30 min at room temperature. Detection was
138 performed using the streptavidin–biotin–peroxidase complex technique with an Elite ABC kit (diluted
139 1:100; Vector Laboratories, Burlingame, CA, USA). Localization was detected using 3,3-
140 diaminobenzidine solution (Vector Laboratories) followed by counterstaining with hematoxylin.

141

142 Immunohistological staining in the cartilage matrix of type II collagen and lubricin was evaluated using
143 ImageJ software. Images were converted to grayscale (0–255) from dark to bright, and intensity was
144 calculated by subtracting the values in blank spaces. Details of ROI selection are described in
145 Supplementary figure 3. The number of MMP-13- and ADAMTS-5-positive immunostained
146 chondrocytes in the lesion area and the adjoining zone were counted and normalized by dividing by the
147 corresponding cartilage surface length. The adjoining zone was defined as the area in proximity to the
148 lesion cartilage in a 0.48 mm × 0.64 mm 200× histological image.

149

150 *Statistical analysis*

151 Statistical analyses were performed using SPSS software (version 22.0; SPSS Inc., Chicago IL). Two-way
152 analysis of variance was employed to analyze histological staining with loading as intragroup factors and
153 duration as intergroup factors. The normality of all continuous data was examined using the Shapiro–Wilk
154 normality test. The parametric variables of the modified Mankin score, volume of degenerative areas, and

155 semi-quantitative measurements of immunohistochemistry were included in the model directly, whereas
156 the nonparametric variables were first transformed into ranked data and then introduced into the model.
157 Comparisons between intergroup marginal means using Tukey HSD tests were performed only when the
158 main effects exhibited significant results. When the analysis showed interactional effects in addition to
159 the significant main effects, multiple one-way ANOVA tests with post-hoc comparisons for stratified
160 samples were conducted on each level to examine potential differences in interactional effects among the
161 levels. Additionally, Mann-Whitney U (2 groups) or Kruskal-Wallis H tests (3 groups) were applied to
162 compare the control and loaded samples. The required sample size was calculated based on our pilot
163 experimental data of lesion area size between groups. P-value $< 0.05$ was considered statistically
164 significant.

165

166 **Results**

167 *Vitality of chondrocytes after cyclic loading*

168 Samples that underwent 20 N cyclic compression were stained with calcein AM/ethd-1 and evaluated
169 (Supplementary figure 4). Representative images exhibited mixed distribution of red and green fluorescent
170 cells at 1 and 3 h after loading, whereas large areas without green-stained chondrocytes were observed at
171 6 and 12 h, indicating that complete cell death occurred within 6 h, even at the lower load level of 20 N.

172

173 *Degree and extension of the articular cartilage lesion*

174 Histology showed that AC in both groups was damaged, and one focal degenerative zone in the lateral
175 femoral condyle was confirmed for every subject (Supplementary figure 4A). However, the AC surface

remained intact except for a slight fibrillation present in several samples (data not shown). A clear boundary between the lesion cartilage and unaffected calcified cartilage was observed 2 weeks after loading. The average modified Mankin score per section increased after loading (Supplementary figure 4F) and differed between groups and observational durations (Supplementary figure 4B). As the peak load or interval time increased, the degree of degeneration tended to worsen at higher histological scores (Supplementary figure 4B). Although the lesion area volume did not change significantly throughout the duration of the study, it was significantly higher in the 50 N load group than in the 20 N group at all time points (Supplementary figure 4C). The relative Safranin O staining intensity in the lesion area declined with time after loading for both groups compared to the intact area (Supplementary figure 4D); however, there was no evident difference between groups with 20 N or 50 N peak loads. In addition, the number of hematoxylin-stained nuclei in the lesion area continuously decreased after loading in both groups (Supplementary figure 4E, 4G), whereas no significant changes were found in medium-term observation from 2 to 8 weeks.

*Expression of type II collagen in the lesion area*

Immunohistochemistry results showed focal type II collagen overexpression in the AC lesion. Enhanced staining was observed in each sample compared to adjacent intact substrates (Figure 3A), and the intensity in loaded samples was significantly higher than in the control group (Figure 3B). However, there were no notable effects on intensity in the lesion region with different load levels or time points (Figure 3C-D), regardless of whether raw values or relative percentage increments were used.

*Distribution of ADAMTS-5- and MMP-13-positive chondrocytes*

198 Superficial and intermediate zone chondrocytes in the control group moderately expressed MMP-13 and
199 ADAMTS-5 (Supplementary figure 5). In the loading groups, we found positively stained radial zone
200 chondrocytes under the lesion area (Supplementary figure 6, Figure 4) that were not observed in normal
201 samples (quantitative data not shown). The number of active cells in the area adjoining the lesion (no
202 direct contact) significantly increased after loading compared to normal AC (Supplementary figure 6B,
203 Figure 4B). Moreover, the results of semi-quantitative analysis revealed that the number of both MMP-
204 13- and ADAMTS-5-positive chondrocytes (in the lesion area or in the adjoining region) gradually
205 decreased during the 8-week observation period (Supplementary figure 6C-D, Figure 4C-D). However,
206 no significant main effects were generated by the load levels.

207

208 *Superficial lubricin response to cyclic loading*

209 Lubricin expression in the lateral femoral condyle AC is presented in Figure 5. The staining intensity in
210 the lesion area of substrates within the superficial cartilage declined one week after loading compared to
211 that in the intact area (Figure 5A). However, semi-quantitative analyses showed an increased
212 concentration of lubricin on the AC lesion over time, reaching the same level at 4 and 8 weeks compared
213 to the intact area (Figure 5B). No statistical load-level effect was found, whereas the main effects of
214 duration and interaction between observational duration and load effect were confirmed. Furthermore,
215 results of stratified analysis revealed that superficial lubricin in 20 N-loaded samples were more likely to
216 recover (more pairwise differences) in comparison to 50 N samples (Figure 5C-D). Additionally, we found
217 signs of stain aggregating around the site of degenerative chondrocytes or even lacunae from dead cells
218 lacking hematoxylin-stained nuclei.

219

## Discussion

The current study demonstrated for the first time that a single episode comprising 60 cycles of mechanical stimulus can induce AC lesions in the lateral femoral condyle of rats, consistent with results reported in smaller rodents such as mice[5-6]. Compared to surgical PTOA models of ACLT and DMM that gradually develop chondrocyte apoptosis and cartilage matrix loss[16], we did not find any apoptotic cells around the lesion area, even at 6 h after loading (data not shown). This was considerably different from previous results in mice showing that clustered active TUNEL-stained chondrocytes were retained in degenerative AC until 14 days after loading[6]. Our results from live/dead staining demonstrate that chondrocytes in the superficial lesion cartilage were dead within 6 h due to direct damage (Figure 2). The Mankin score[15] deteriorated over time, whereas structural destruction of AC cartilage did not progress as rapidly as the invasive models that showed a jagged cartilage surface and subchondral bone perforation within 4 weeks after the instability surgery[17,18]. Furthermore, there was a distinct difference in staining between AC above and below the tidemark (Supplementary figure 4A), consistent with *in vitro* results indicating that calcified radial zones of cartilage suffered less than 5% of the total mechanical stress[19,20]. Cell signaling studies have suggested that overloading activates the toll-like receptors expressed on chondrocytes, resulting in the release of inflammatory cytokines[21] and catalyzing ADAMTS-5 for aggrecan degradation[22], consistent with findings of the current study (Supplementary figure 6). According to our investigation, this non-surgical model may be superior for simulation of acute extensive AC damage, which is common in athletic injuries.

Expression of Col2 was found to be transiently increased within 1 h after *ex vivo* mechanical loading in several experiments using extracted cartilage explants[23,24]. A recently published tissue-engineering review[25] summarized the biochemical anabolism of synthetic substrate-seeded chondrocytes subjected to

*in vitro* dynamic loading, most of which demonstrated subsequent Col2 upregulation in response to various loading regimens. Ragan[23] reported transient upregulation of type II collagen within 4 h in extracted bovine cartilage explants subjected to static mechanical compression but did not check the chondrocyte survival rate. The current study, to our knowledge, is the first report on focal enhanced staining of type II collagen in lesion cartilage that has undergone *in vivo* cyclic loading, despite the complete death of the affected surface chondrocytes within 6 h. A previous study reported decreased Safranin O and Col2 staining in an osteochondral defect model[26], which was directly created on the AC surface using a 1-mm biopsy punch. However, our model showed a diametrically opposite type-II-collagen response to cyclic loading. Although one of the major collagenases, MMP-13, was overexpressed immediately after loading injury (Figure 4), the morphological degradation of AC did not progress extensively as in regular OA development. Further studies should focus on whether type II collagen proliferation is beneficial or harmful to AC protection.

Lubricin localized on the cartilage surface is reportedly a protective and lubricating component of the *O*-linked glycoprotein[27]. We found drastic decrease in superficial lubricin staining of the damaged area immediately after cyclic loading compared with the non-loaded region (Figure 5). Decreased superficial cartilage lubricin/proteoglycan 4 was confirmed in both *in vivo*[28] and *ex vivo*[29] experiments. Several studies have reported an increased coefficient of friction within a few hours of cyclic loading[30,31] and that lubricin in the cartilage surface was denuded by loading, even in the joint where most chondrocytes remained alive throughout the observation period[31]. Our study revealed similar results in a non-surgical model during early observation after *in vivo* cyclic compression. On the other hand, after tracking different time points for 8 weeks, we found that superficial cartilage staining with lubricin gradually recovered to the normal level (Figure 8). The results after 4 weeks indicated a different trend of OA progression

compared to that with joint instability-induced OA. Although lubricin expression on cartilage was found to be elevated in late-stage OA patients[32], mainstream results from posttraumatic OA of human[33] and animal joint instability models[28,34-36] in a long-term observation demonstrated that joint lubricin concentration decreased after injury or surgery. Combining our results with the results of decreased Prg4 expression in unstable joints after forced movement,[37,38] we hypothesized that instability-induced persistent motivation should play a more important role than magnitude of loading in determining irreversible lubricin loss. Several studies[35,39,40] involving delivery of exogenous recombinant lubricin to medial meniscectomized rats found that the cartilage surface protein was protected from depletion in the experimental group. In the current study, we observed a self-healing process in the loading-damaged AC without any lubricin supplementation, indicating that endogenous lubricin is an important repair mechanism in the post-traumatic knee and providing a plausible explanation regarding the slower cartilage degradation progression in the non-invasive loading model compared to that in the joint instability surgery models. Interestingly, although superficial cell death was confirmed within 6 h after loading, the locations of cartilage lacunae were strongly stained with lubricin even at 8 weeks after injury. Previous studies found that chondrocytes encapsulated in agarose[20,41] and cartilage explants[42] expressed higher levels of *prg4* when subjected to compressive strain and the intensified lacunae staining around cells was confirmed by immunofluorescence images[20], similar to our results. Further studies should focus on potential links between chondrocyte-derived lubricin and the mechanism of superficial cartilage repair.

The current study has some limitations. First, it focused on investigating the medium-term changes over time from 3 days to 8 weeks after loading implementation. Since cell death, type II collagen biosynthesis, and superficial lubricin degradation occurred earlier than expected, further studies should more precisely design the unit of observation intervals in hours to reveal the full process of dynamic loading-induced

289 cellular reactions. Second, in experimental sheep[43,44] and horse[45,46] models, lubricin concentration in the
290 synovial fluid was upregulated transiently in the acute phase after injury, and synovial PRG4 (the gene
291 encoding lubricin) expression was positively correlated with TNFα and ADAMTS-5[46]. Future studies
292 should evaluate not only cartilage, but also synovium using quantitative techniques to specify the details
293 of lubricin recovery. Moreover, we did not record the activities of rats after loading that may have
294 influenced the progression of lubricin self-healing, which may have contributed to the large variation in
295 the data (Figure 5D). Third, in the current study, we assessed only the cartilage lesion on the lateral femur
296 condyle, which is not the main part of cartilage loaded during walking[47]. Considering that cartilage
297 heterogeneity could affect the results of injury assessment, cartilage damage on the other contact surface
298 should be examined in the future. Finally, we failed to compare the current model to any surgery-induced
299 model. As described above, the surface lubricin is reportedly diminished in many injury-induced OA
300 animals[28,34-36], whereas it is still unknown if joint instability independently affects the progression of post-
301 traumatic OA, especially in the lesion area. Further studies should combine invasive destabilization
302 surgery with preexisting lesions caused by cyclic compression, which could reflect the spatiotemporal
303 changes of cartilage in the non-contact area.

304 In conclusion, we found a specific, localized AC lesion in both the 20 N and 50 N groups that underwent
305 60 cycles of compression in rat knee joints. The local expression of type II collagen was increased after
306 repeated loading, whereas lubricin in the cartilage surface was lost in response to cyclic compression.
307 However, the distribution of superficial lubricin recovered 4 weeks after non-surgical injury (Figure 6).
308 These results indicate that dynamic loading exceeding 20 N damages the lateral femoral condyle AC in
309 rats. Although the damage caused localized chondrocyte death and upregulated expression of degrading
310 enzymes, endogenous repair in well-structured joints rebuilt the layer of protective proteins on the
311 superficial cartilage.

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## Author Contributions

XJ: conception and design of the study, acquisition, analysis, and interpretation of data, drafting of the article, revision of the article, final approval of the article.

AI: conception and design of the study, interpretation of data, drafting of the article, revision of the article, final approval of the article.

AN: conception and design of the study, interpretation of data, revision of the article, and final approval of the article.

KN: conception and design of the study, interpretation of data, revision of the article, final approval of the article.

HK: obtaining funding, conception and design of the study, interpretation of data, revision of the article, and final approval of the article.

TA: conception and design of the study, interpretation of data, revision of the article, and final approval of the article.

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## Figure legends

**Figure 1.** Schematic diagram of the non-surgical cyclic compression model. **A.** Right knee of anesthetized rat fixed on a customized apparatus with the patella embedded in a loading dent. The indexed knee angle was set at a deep flexion of 140°. **B.** A full cycle of the loading regimen contained a 0.5 s peak load and a 10 s rest interval with the loading cup approaching at a speed of 1 mm/s. The preload of 5 N and peak load of 20 N or 50 N were set for samples in corresponding groups. **C.** Flow chart of sample allocation.

**Figure 2.** Chondrocyte vitality in superficial cartilage after 20 N compressive loading detection by calcein AM/ethd-1 staining. Representative fluorescent images demonstrate spatiotemporal cell death from 1 to 12 h after loading. Green and red channels illustrate the distribution of live and dead chondrocytes, respectively. A well-defined focal region without green-stained cells was observed at 6 and 12 h. Pre-loaded normal rat limbs were used as controls. Scale bar: 100 μm.

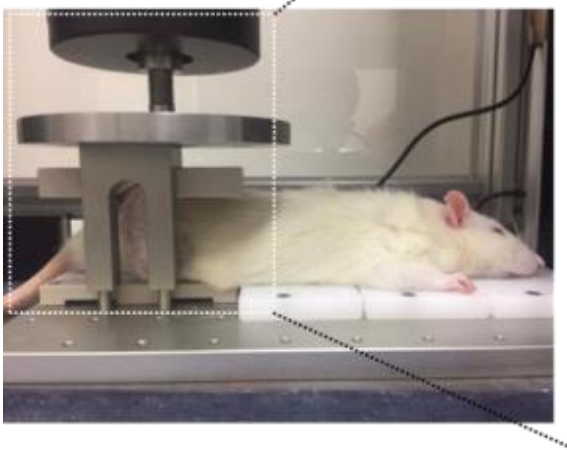
**Figure 3.** Changes in type II collagen expression in articular cartilage substrates after cyclic loading. **A.** Representative immunostained sections for type II collagen in the lesion areas of lateral condyle cartilage. The border of normal and degenerative cartilage is represented by a dashed, light-gray line. **B.** Differences in type II collagen expression in control and loaded samples. **C.** Average intensity of staining on the degenerative cartilage matrix was calculated on 8-bit grayscale images using ImageJ software. **D.** Percentage variation of intensity in the lesion area relative to the intact area on the same section. Relative intensity was calculated in an inverted 8-bit grayscale image by dividing the intensity in the intact area. Significant effect or interaction between time points or load levels; **p<0.01. No symbol: not significant. Scale bar: 100 μm.

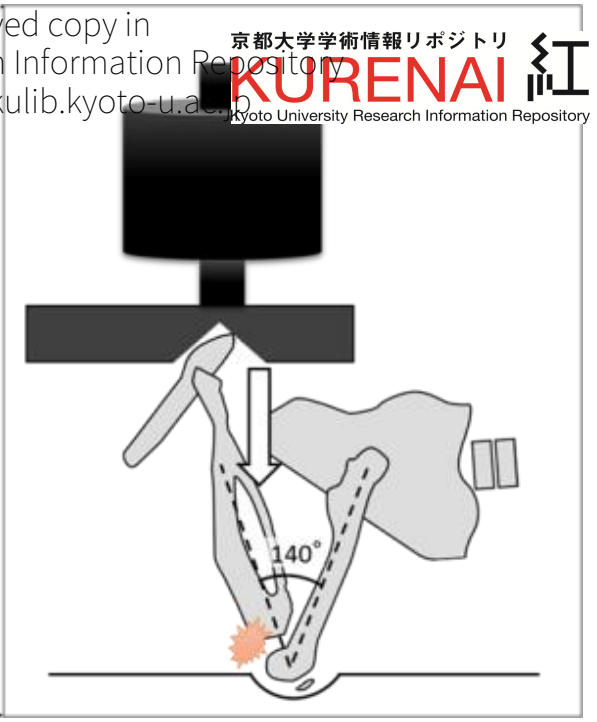
**Figure 4.** The distribution of MMP-13$^{+}$ chondrocytes in articular cartilage. **A.** Representative histological sections immunostained for MMP-13$^{+}$ in the lesion area and the adjoining zone. Black arrow heads indicate positive cells in the superficial and intermediate zone of cartilage, red arrow heads indicate positive cells under the tidemark. **B.** Comparison of positive cell number in intact cartilage with all loaded samples' adjoining area. **C, D.** Results of semi-quantitative analyses of positive cells within the adjoining (**C**) and under the lesion area (**D**). Results were normalized by dividing by the cartilage surface length (mm). Significant results ($p<0.05$) of ANOVA analysis ‡: main effect of duration was presented on the top of each chart. Marginal means of each observational point were compared when ‡ was found.*$p<0.05$, **$p<0.01$. Scale bar: 100 μm.

**Figure 5.** Localization of lubricin in articular cartilage subjected to dynamic loading. **A.** Representative Lubricin/Proteoglycan4 immunostained image in the lesion area of lateral condyle and intact area under the lateral meniscus. Staining was weakened in the superficial substrates, whereas it was enhanced in the cartilage lacunae. B. Differences between control (n=6), early observation (3 days and 1 week; n=24), and

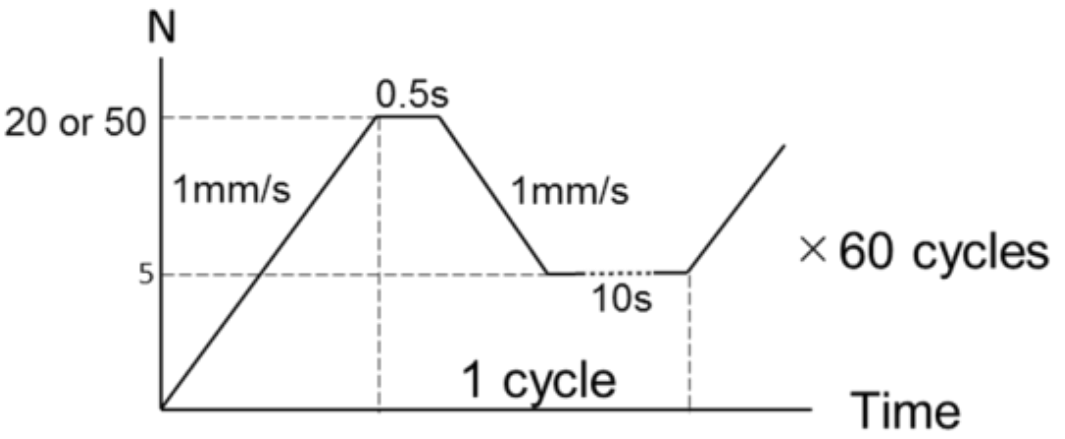
later observation (2, 4, and 8 weeks; n=36) of loaded samples were compared using Kruskal-Wallis H tests. **C.** Staining intensity in the superficial layer of cartilage substrates. The ROI of superficial cartilage were depicted with ImageJ software using the brush selection tool with 50 μm width (Supplementary figure 3). **D.** Relative intensity normalized with intact region staining in percentage. Significant results ($p$<0.05) of a two-way ANOVA are presented on the top of each chart; ‡: main effect of duration, Ψ: interaction effects. Stratified one-way ANOVA on each load level with multiple comparison were applied whenever Ψ was found. *$p$<0.05, **$p$<0.01. No symbol: not significant. Scale bar: 100 μm.

**Figure 6.** The illustration summarizes the findings of the current study. Localized Col2 was upregulated within 3 days after loading and stable during observation. Superficial lubricin decreased immediately after damage yet recovered gradually to the normal level. Safranin O staining in lesion cartilage weakened continuously after injury until week 4. Hematoxylin-stained nuclei in the damaged area dissolved completely 2 weeks after cyclic compression.






**B**

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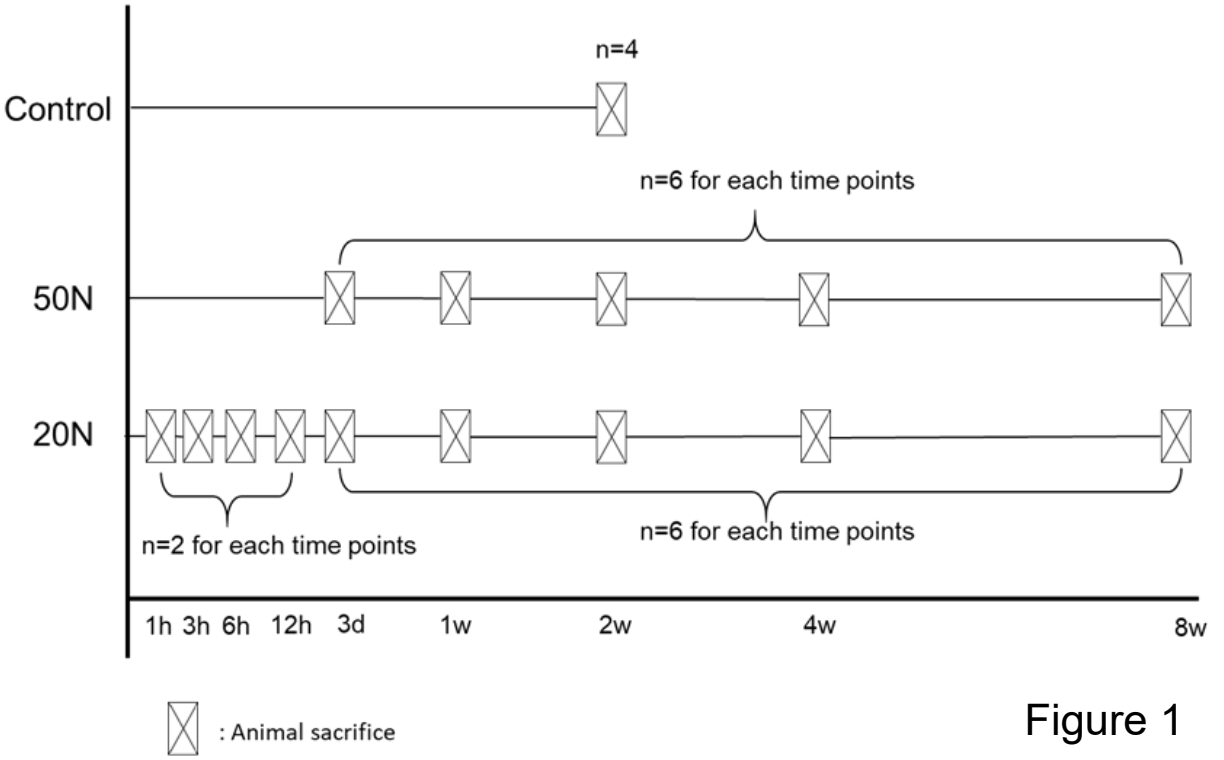
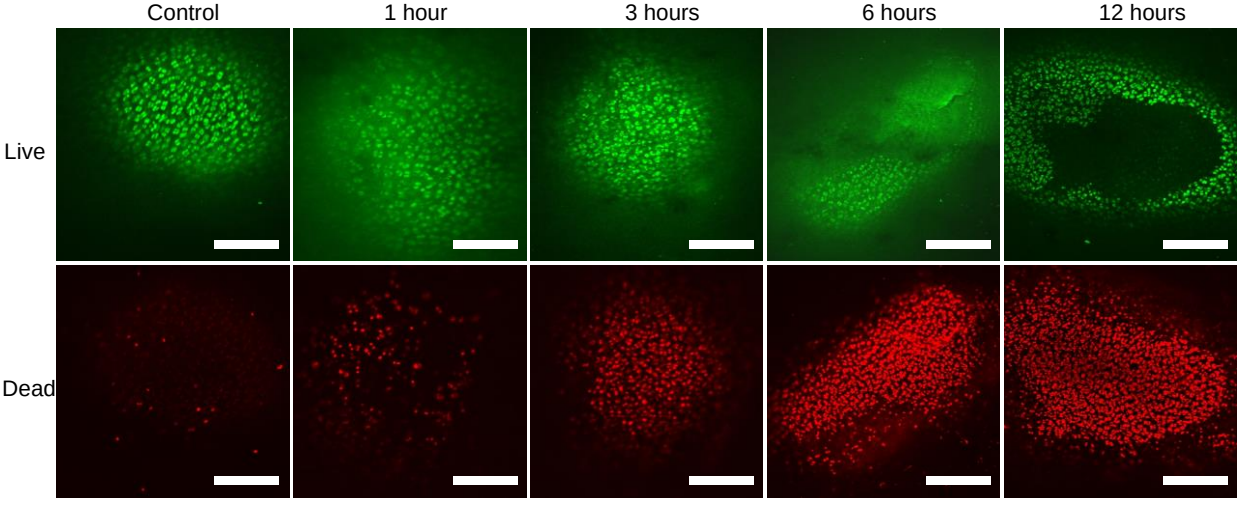
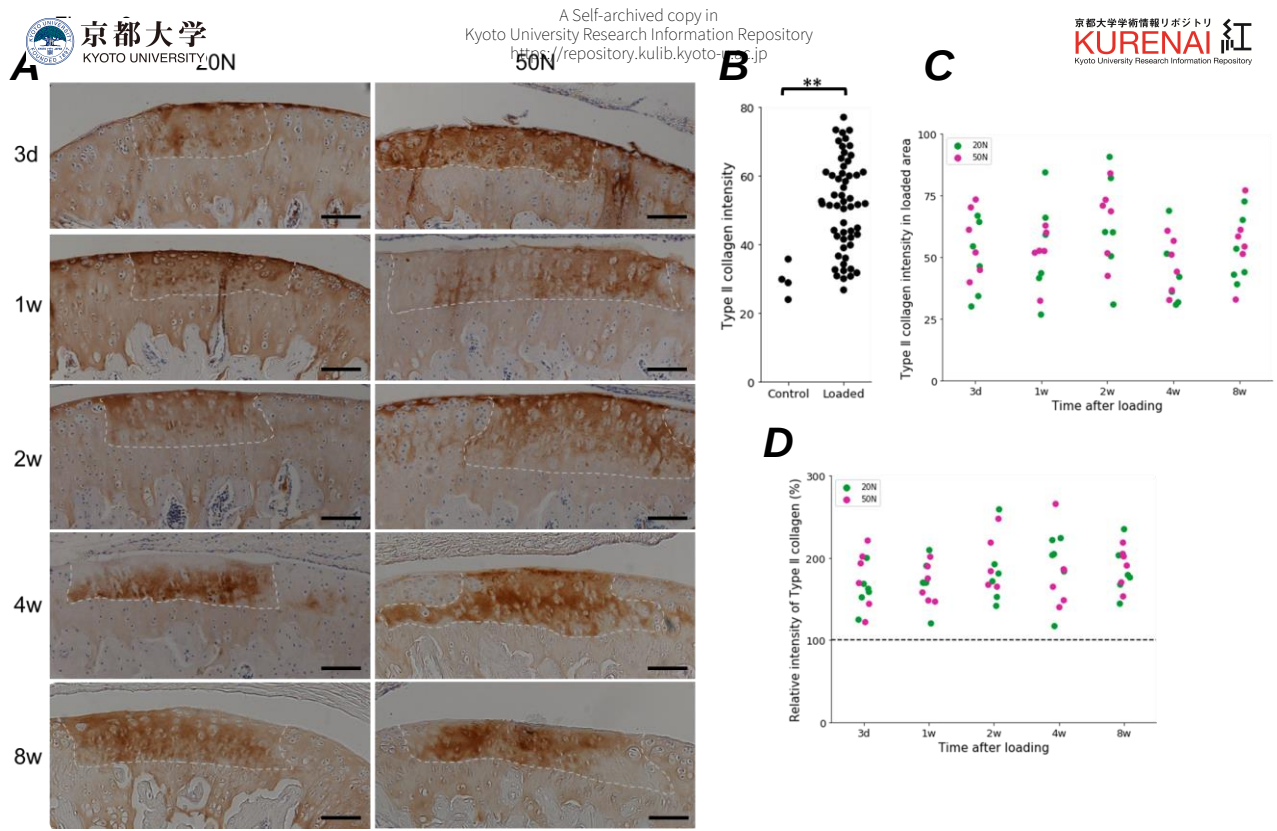
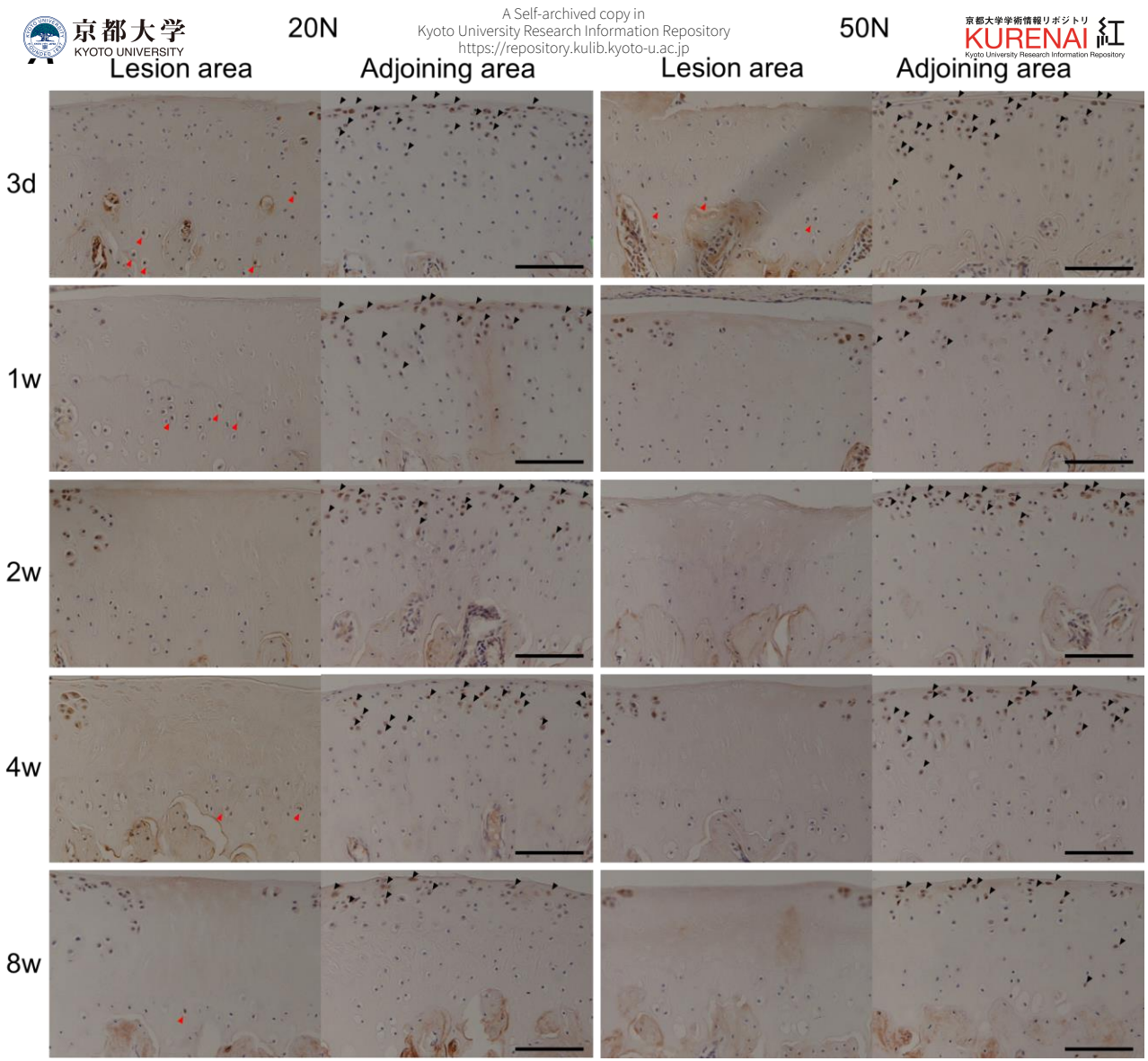
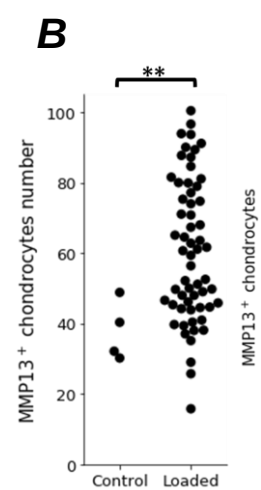


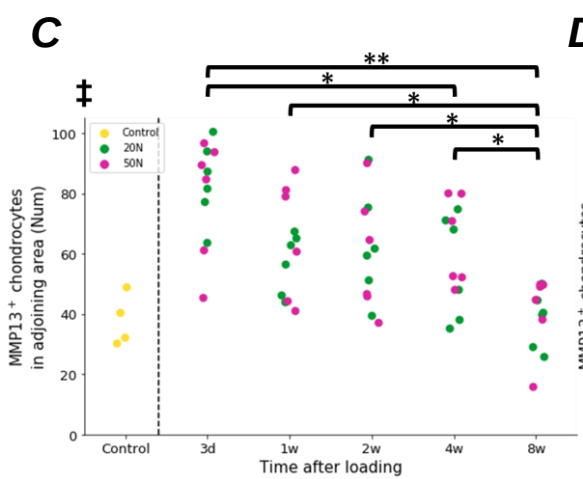

Figure 1







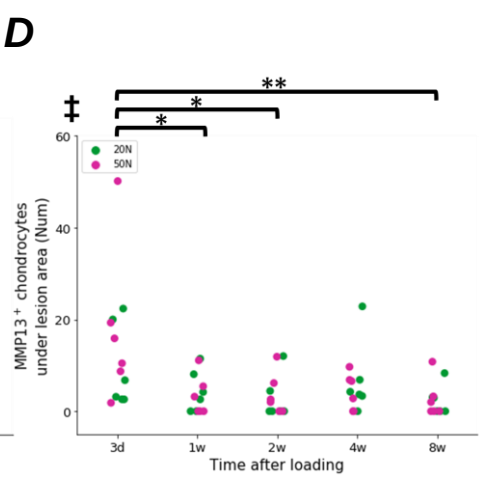
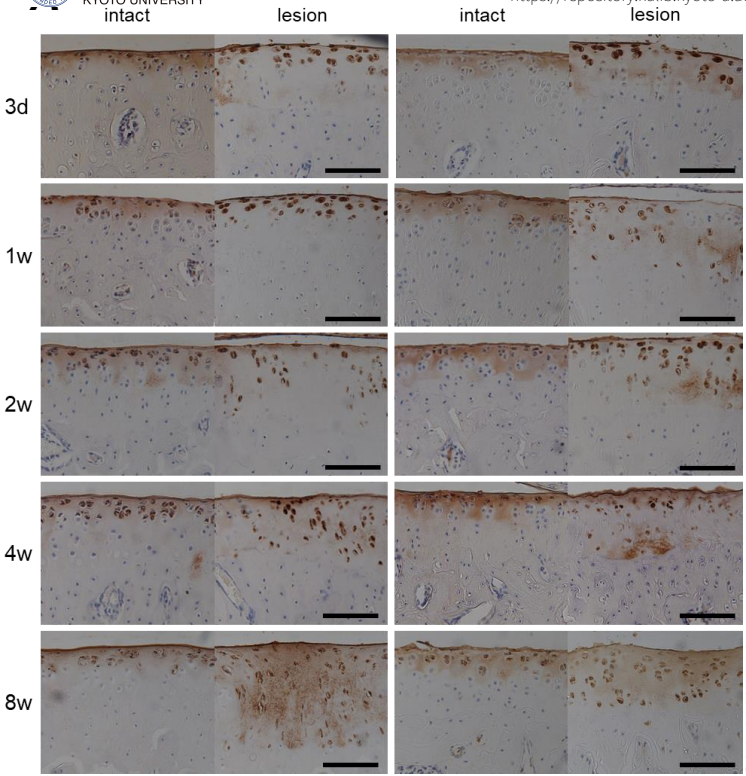
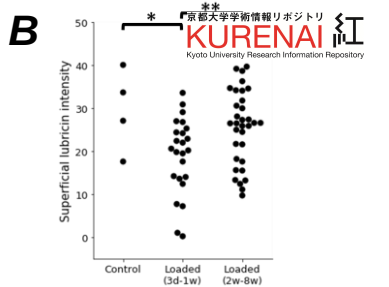
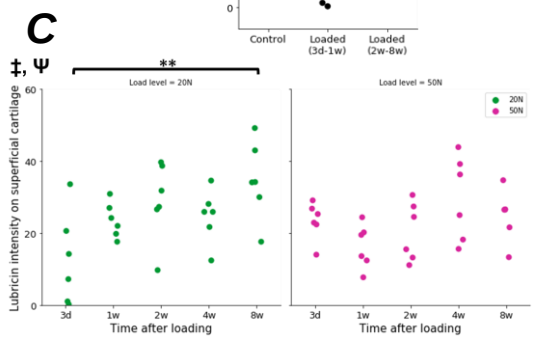




Figure 4



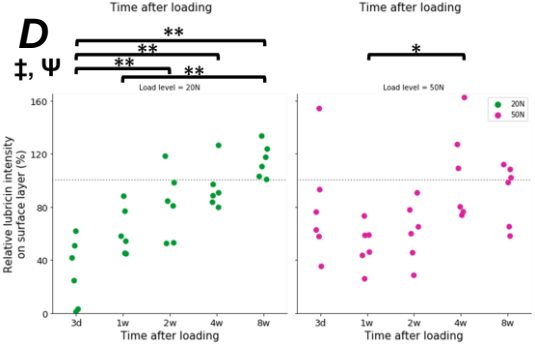




Figure 5

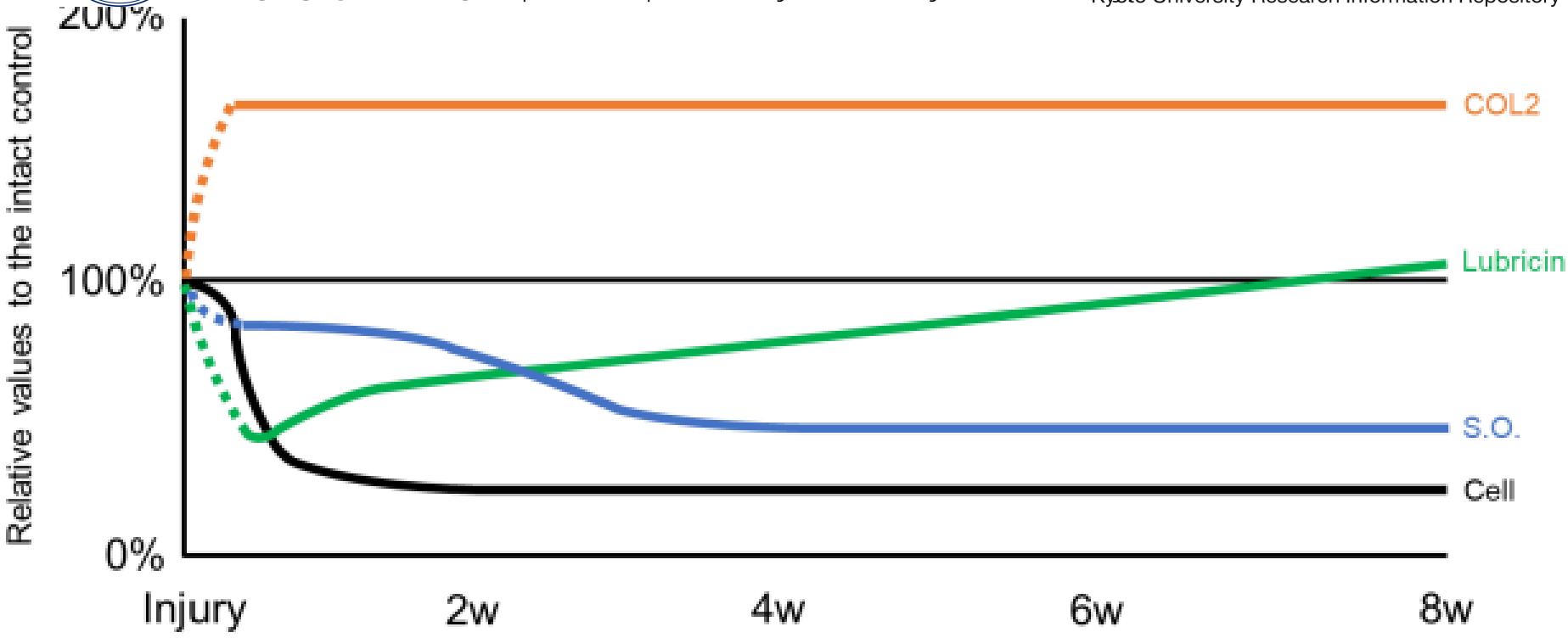



Figure 6