Relationship of Superoxide Dismutase to Rotator Cuff Degeneration and Tear in a Rat Model

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ABSTRACT

Rotator cuff degeneration is one of the several factors that lead to rotator cuff tears. Oxidative stress and superoxide dismutase have been reported to be related to rotator cuff degeneration; however, the precise mechanism still remains unclear. In this study, we investigated the relationship of oxidative stress and superoxide dismutase to the degeneration of the rotator cuff using rat models. Eighty-four rats were used to create a collagenase-induced rotator cuff injury model (injury model) and a rotator cuff tear model (tear model). The controls were administered saline and had only a deltoid incision, respectively. We evaluated degeneration morphology of the rotator cuff using a degeneration score; dihydroethidium fluorescence intensity, which detects oxidative stress; gene expression; and superoxide dismutase activity. The rotator cuffs in the injury and tear models significantly increased degeneration scores and dihydroethidium fluorescence intensity. On the other hand, gene expression of superoxide dismutase isoform, superoxide dismutase 1, and superoxide dismutase activity were significantly decreased in the injury model but showed no significant difference in the tear model. These findings suggested that superoxide dismutase might not be associated with rotator cuff degeneration after tear but may be involved in degenerative rotator cuff without tear. However, we found that rotator cuff degeneration involves oxidative stress both with and without tear. Based on these findings, it is presumed that different treatments may be appropriate, depending on the state of rotator cuff degeneration, because the mechanisms of the degeneration may be different.

Key Terms: rotator cuff degeneration; rotator cuff tear; oxidative stress; superoxide dismutase; peroxiredoxin

INTRODUCTION

The factors contributing to rotator cuff tears fall under 2 main categories: extrinsic factors and intrinsic factors. Extrinsic factors include acromion morphology, subacromial spurs, trauma, and shoulder overuse. Intrinsic factors include inflammation, hypovascularity, aging, and degeneration.[1–3] Rotator cuff degeneration is one of the characteristics of tears and aging in rotator cuff entheses. Pathologic changes typical of degeneration have been reported to include thinning and disorientation of collagen fibers and loss of cellularity, vascularity, and fibrocartilage mass at the site of cuff insertion.[3,4] The precise mechanism of rotator cuff degeneration, however, remains unclear.

Recently, it was reported that rotator cuff degeneration is caused by oxidative stress,[3,5,6] an imbalance between oxidation, caused by reactive oxygen species, and reduction, induced by antioxidant systems.[7] It is still controversial whether oxidative stress can be a primary mechanism of age-related disease. Oxidative damage could lead to the hallmarks contributing to the ageing process, which include genomic instability, attrition of telomere, epigenetic alterations, and loss of proteostasis.[8] For example, the attrition of telomere, which accelerates aging and increases the risk of age-related diseases, can be caused by oxidative damage because they are highly sensitive to the damage and their repair capacity is less well than other parts of the chromosome.[9,10] Furthermore, it was reported that oxidative stress is thought to play a pivotal role in the pathological processes implicated in ageing and age-related diseases and the underlying biochemical mechanisms have been clarified in detail.[11] For intense, the mitochondrial DNA damages would lead to loss of redox homeostasis, perturbed Ca^{2+} homeostasis, damage to membrane proteins and lipids, as well as to abnormal mitochondrial energy transduction.[12] These modifications are the driving force for further mitochondrial

78 dysfunction and loss of integrity, which will affect cell viability and cellular function.[12,13]
79 In addition, organs, being high-energy consuming and sensitive to bioenergetic defects,
80 are strongly affected by mitochondrial dysfunctions, resulting in organ-specific
81 pathologies.

82 Superoxide dismutase (SOD) is a major antioxidant enzyme that scavenges
83 reactive oxygen species *in vivo*.[14] There are 3 SOD isoforms, each having a different
84 active center and locality: SOD1 (CuZn-SOD) is localized in the cytoplasm, with Cu/Zn
85 as its active center; SOD2 (Mn-SOD) exists in the mitochondrial matrix, with Mn as its
86 active center; and SOD3 (EC-SOD) is distributed in extracellular fluids, such as synovial
87 fluid, plasma, and lymph, with Cu/Zn as its active center.[14]

88 Previous studies showed that SOD1 deficiency induces age-related effects on
89 various tissues.[15] Morikawa et al.[16,17] found that excessive oxidative stress induced
90 degeneration of rotator cuff enthesis in SOD1-deficient mice. Yoshida et al.[18] reported
91 that SOD activity was not significantly different between rotator cuffs with tears and
92 those without tears although oxidative stress and tendon degeneration in humans were
93 greater in rotator cuffs with tears than in those without. As the reason for no significant
94 difference in SOD activity, Yoshida et al. described that the SOD might be inactivated
95 and not compensable in cases of advanced rotator cuff tears. Chen et al.[19] created an
96 animal rotator cuff injury model in which collagenase injected into the rotator cuff
97 enthesis caused biomechanical weakness and histological degeneration. Hashimoto et
98 al.[20] created animal rotator cuff tear models and showed that the rotator cuff was
99 histologically degenerated after the tear. However, the association of rotator cuff
100 degeneration with oxidative stress and SOD in these models has not been investigated.

The purpose of this study was to investigate and compare the relationship of oxidative stress and SOD with rotator cuff degeneration in rat injury models with and without tear. We hypothesized that oxidative stress is involved in the degenerative process in rotator cuff injury models with and without tear through different mechanisms of action of SOD.

METHODS

Animals

Eighty-four Sprague–Dawley (SD) rats were used in this study. The rats were maintained and housed with a 12-hour light-dark cycle and allowed free access to food and drinking water. We created two models with injured rotator cuff, the model without rotator cuff tear as “injury model” and the model with rotator cuff tear as “tear model”. This study was approved by the Animal Care Committee of the authors’ institutions.

Rotator Cuff Injury Model

All rats were anesthetized with inhalation of sevoflurane. The injury model was produced using a collagenase injection with some modifications to a previous study.[19] The acromion was located in the vertex of the right shoulder and 80 U/8μl of type II collagenase (C6885; Sigma-Aldrich, St. Louis, MO, USA) was injected near the supraspinatus tendon under the acromion using a 27-gage needle (NN-2719S; Terumo, Tokyo, Japan). The amount of collagenase was decided as the same amount as in the previous study[19] because we could make rotator cuff injury model without tear and with histological rotator cuff degeneration using the same amount in our preliminary experiments. The injection was performed for 3 consecutive days. On the third day, the

skin was incised by 5 mm and the injection was definitely administered (cuff-injury side). The left shoulder served as a control, and was injected with saline (no-injury side).

Rotator Cuff Tear Model

Anesthesia was performed in the same manner as in the injury model. The surgery was performed in bilateral shoulders according to previously described methods.[21] In the right shoulder, the supraspinatus tendon was exposed by splitting the deltoid muscle. The supraspinous tendon was sharply detached from the insertion using a scalpel. The tendon stump was retracted medially by contraction of the rotator cuff muscle so that a U-shaped medium-size tear was created naturally. The overlying deltoid muscle and skin were then closed (cuff-tear side). In the left shoulder, served as a control, the deltoid muscle was split to expose the supraspinatus tendon and the deltoid muscle and skin were immediately closed without rotator cuff resection (no-tear side). All rats were allowed unrestricted activity in the cage.

Tissue Preparation

Rats from the injury model and the tear model were euthanized with an overdose of sodium pentobarbital at 3, 7, or 14 days after the injection or surgery. The supraspinatus tendon entheses of the injury models and the stumps of the ruptured supraspinatus tendon of the tear models were harvested and used for analysis. Specimens used for histological studies were cut into 5-μm frozen sections and stained with hematoxylin-eosin (H&E), alcian blue (AB), or dihydroethidium (DHE) (Life Technologies Corporation, Gaithersburg, MD). We evaluated one section for each staining for each

specimen. Other specimens were used for gene expression analysis using quantitative real-time polymerase chain reaction (RT-PCR) and measurement of SOD activity.

Histological Evaluation

The H&E stain was used to investigate the morphology of the rotator cuff tissue, including cell form, collagen orientation, vascularity, and cell distribution. Mucin formation was evaluated using AB staining. We used kernechtrot staining as the counterstain for AB staining. Each sample was cut into 5-μm thick sections and individually stained with H&E or AB. Sections were viewed under a brightfield microscope (for H&E and AB) and/or a polarized light microscope (for H&E). The tissues were evaluated using a scale described as the "degeneration score." The score had been proposed in a past study,[18] based on previous reports.[22–24] The score comprises 7 factors: (i) cell morphology, (ii) collagen alignment, (iii) vascularity, (iv) cellularity, (v) ground substance, (vi) fiber structure, and (vii) collagen stainability. Collagen alignment, cellularity, ground substance, fiber structure, and collagen stainability were evaluated one field of view, at 100× total magnification. Cell morphology was evaluated four fields of view, at 200× total magnification. Vascularity was evaluated ten fields of view, at 400× total magnification.[24] All fields were set at the tendon 200-400 μm proximal to bone of the enthesis in injury models and no-tear side of tear models or 200 μm away from the margin of the stump in cuff-tear side of tear models.[25] Each factor was evaluated using a 4-point scale. The 7 scores were then combined and the total score for each sample was calculated. All slides were blindly numbered and independently scored by two researchers familiar with musculotendinous histopathology.

Measurement of Dihydroethidium Fluorescence Intensity

Dihydroethidium has been mainly used for superoxide detection.[26] In this study, DHE staining was performed on the optimum cutting temperature-preserved frozen tissues, as reported in previous studies.[27,28] Immediately after collection, the frozen samples were cut into 5-μm thick sections and placed on slides for DHE staining. The DHE was adjusted to 10 μmol/l and applied to each tissue section (~500–1,000 μl) so that the entire section was covered. The slides were then incubated in a light-protected, humidified chamber at 37°C for 30 min.

After incubation, the slides were washed twice with phosphate-buffered saline and covered with coverslips. All DHE-stained sections were then immediately photographed using a fluorescence microscope (BZ-9000; Keyence Corporation, Osaka, Japan) at settings of 20× magnification, 100% fluorescence excitation, and 0.5-s exposure time. The images were randomly numbered for blind evaluation and analysis using ImageJ (NIH, Bethesda, MD) to determine the fluorescence intensity of the DHE-stained cells. The region of interest was set as the standardized field (100 × 100 μm^2) at the tendon 200-400 μm proximal to bone of the enthesis in injury models and no-tear side of tear models or 200 μm away from the margin of the stump in cuff-tear side of tear models. Image analysis was performed within the standardized field on a color scale, and the value of the brightest portion in the region of interest was defined as the fluorescence intensity[29-31]. DHE fluorescence intensity was calculated based on an average fluorescence intensity of five fields made for each slide.

Gene Expression Analysis

In this study, the gene expression of SOD1 SOD2, and peroxiredoxin 5 (PRDX5), another antioxidant enzyme,[32] were examined. Total RNA from specimens was extracted with RNeasy Mini Kit (Qiagen, West Sussex, UK) according to the protocols provided by the manufacturer. First-strand cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). Quantitative real-time polymerase chain reaction (RT-PCR) studies were performed using TB Green Premix Ex Taq (Takara, Tokyo, Japan) and 400 nM of each primer, routinely in duplicate. The primers were designed based on the sequences in the GenBank database (Table 1). The fractional cycle number at which the fluorescence passes the threshold (Ct values) was used for quantification by using a comparative Ct method. Sample values were normalized to the threshold value for β-actin: $\Delta Ct = Ct$ (experiments) − Ct (β-actin). The Ct value of control was used as a reference. $\Delta\Delta Ct = \Delta Ct$ (experiment) − ΔCt (control). The fold change in mRNA expression was calculated by following the formula: $2^{-\Delta\Delta Ct}$.

Measurement of SOD Activity

The SOD activity is regulated both by the amount of SOD protein present and the post-translational modifications reducing the activity. Therefore, it is important to confirm the activity, when using SOD.[33] In this study, SOD activity was measured using a process that was based on a previous report.[18] We used an assay kit (Northwest Life Science Specialties, LLC, Vancouver, WA) and measured the total SOD activity at 7 days after the injection or the surgery according to the protocols provided by the manufacturer. The samples were examined soon after collection. These were mixed with beads and phosphate-buffered saline and crushed to prepare 10% tissue homogenates (mg/μl). The

218 homogenates were centrifuged (10,000 rpm × 4 min, 4℃) and the supernatant was
219 collected.
220 A microplate assay was performed using a plate reader (Wallac 1420 ARVOsx
221 Multi-label plate reader; Perkin Elmer, Inc., Waltham, MA). 230 µl of assay buffer was
222 added into each well used for testing. 10 µl of the sample or assay buffer (for the blank)
223 was then added, shaken to mix, and left undisturbed for 2 min. Thereafter, 10 µl of
224 hematoxylin reagent was added and immediately mixed with the shaker function of the
225 instrument. The absorbance at 560 nm was recorded every 10 s for at least 5 min.
226 The data were calculated using the following equations. The decomposition rate
227 (ΔAbs560 nm/min) was defined from the reaction rate, and the decomposition rates of
228 the sample ($Rate_s$) and the blank ($Rate_b$) were then calculated. The percentage for SOD
229 inhibition of the baseline (blank) reaction rate was calculated as follows: %Inhibition =
230 $(1-Rate_s/Rate_b) \times 100$ (%). The SOD activity was found to be approximately 1.25× the
231 measurable percent inhibition of hematoxylin auto oxidation, as established. The final
232 concentration after adjusting the sample dilution was calculated as follows: SOD activity
233 (units SOD/mg protein) = 1.25 × %Inhibition × (sample dilution factor).
234
235 **Statistical Analysis**
236 The Wilcoxon signed-rank test was performed to compare the degeneration score, DHE
237 fluorescence intensity, gene expression, and SOD activity with control sides. Data were
238 presented as mean values ± standard error of the mean. A value of $p < 0.05$ was
239 considered statistically significant. All analyses were performed with GraphPad Prism
240 (version 8; MDF, Tokyo, Japan).
241

RESULTS

Rotator Cuff Injury Model

Histological Evaluation

The H&E-stained tissue revealed signs of degeneration in the cuff-injury side (Fig. 1A–D). Collagen alignment was disrupted and broken down in the cuff-injury side under polarized microscopy (Fig. 1E–H). Mucin formation in the ground substance occurred in the cuff-injury side (Fig. 1I–L). The degeneration scores for each item and the total degeneration score were shown in the table (Table 2). The intra-examiner reliability of the degeneration score in the injury model by two examiners was satisfactory: ICC(2,1) = 0.89. Scores of cell morphology at 3 days, collagen alignment at 14 days, vascularity at 3 and 7 days, and fiber structure at 3 days showed a significant increase. The total scores at 3, 7, and 14 days were significantly higher in the cuff-injury side than the no-injury side (Fig. 2A).

Measurement of DHE Fluorescence Intensity

Comparison of the histological specimens from injury models showed that the DHE fluorescence intensity was higher in the cuff-injury side than the no-injury side (Fig. 1M–P). DHE fluorescence intensity of the cuff-injury side was not significantly different at 3 and 7 days in comparison with the no-injury side but was higher than the no-injury side at 14 days (Fig. 2B).

Gene Expression Analysis

In the gene expression profiles in injury models examined by RT-PCR at 3, 7, and 14 days after the injection, a decreased expression of SOD1 in the cuff-injury side was

observed at 7 days (Fig. 3A). No significant differences were found between the cuff-injury side and the no-injury side regarding either SOD2 or PRDX5 expression (Fig. 3B and C).

Measurement of SOD Activity

The SOD activity (Fig. 3D) was significantly lower in the cuff-injury side (64.4 ± 12.8 units/mg protein) than in the no-injury side (123.6 ± 0.3 units/mg protein; $p = 0.002$).

Rotator Cuff Tear Model

Histological Evaluation

In tear models, H&E and AB stained tissues showed signs of degeneration in the cuff-tear side (Fig. 4A–L). The degeneration scores for each item and the total degeneration score were shown in the table (Table 3). The intra-examiner reliability of the degeneration score in the tear model by two examiners was satisfactory: ICC(2,1) = 0.91. Unlike the injury model, scores of cellularity at 7 and 14 days, vascularity at 14 days, and fiber structure at 7 and 14 days showed a significant increase. The total scores at 3, 7, and 14 days exhibited significant increases in the cuff-tear side than no-tear side (Fig. 5A).

Measurement of DHE Fluorescence Intensity

Histological specimens from tear models also showed that the DHE fluorescence intensity was higher in the cuff-tear side than the no-tear side (Fig. 4M–P). The DHE fluorescence intensity in the cuff-tear side was increased at 3, 7, and 14 days compared with the no-tear side. (Fig. 5B)

290

291 ***Gene Expression Analysis***

292 Gene expression of SOD1 and SOD2 showed no significant difference at all points (Fig.
293 6A and B). On the other hand, a significant decrease of PRDX5 expression in the tear
294 side was recognized at 14 days (Fig. 6E).

295

296 ***Measurement of SOD Activity***

297 The SOD activity was 149.6 ± 10.4 units/mg protein in the no-tear side and 183.0 ± 21.2
298 units/mg protein in the cuff-tear side. No significant differences ($p = 0.24$) were found
299 between the two groups (Fig. 6D).

300

301 **DISCUSSION**

302 In this study, we had created the collagenase-induced rotator cuff injury model
303 and the rotator cuff tear model. We demonstrated that the rotator cuffs of both the models
304 showed tissue degeneration and that increased DHE fluorescence intensity detected
305 oxidative stress. There were significant differences in the involvement of SOD between
306 the two models, in that SOD was significantly decreased in the injury model and not
307 significantly different in the tear model.

308 Several previous studies suggested a relationship between oxidative stress and
309 rotator cuff degeneration.[3,5,6] These studies proposed a signal pathway to suggest that
310 oxidative stress causes rotator cuff degeneration secondary to oxidative stress via matrix
311 metalloproteinase and c-Jun N-terminal protein kinase.[3,5] These data indicated that
312 increased oxidative stress promoted rotator cuff degeneration through a variety of
313 signals, ultimately leading to the tear. Oxidative stress has been evaluated in many

studies with DHE fluorescence intensity, which can mainly quantify superoxide, as one indicator.[26] The results of the present study showed that DHE fluorescence intensity was significantly increased in both the injury and the tear models, demonstrating that these models caused an increase of oxidative stress in the rotator cuffs.

The antioxidant enzyme SOD activates and decomposes in the presence of reactive oxygen species.[14] In studies using SOD deficient mice, Morikawa et al.[16,17] found that SOD1 is a crucial gene that inhibits rotator cuff degeneration due to oxidative stress. Fillipin et al.[34] showed in rat models that SOD activity in the Achilles tendon after a tear was not significantly different from that with no tear. Yoshida et al.[18] reported that the SOD activity was not significantly different between patients with and without rotator cuff tear; however, degeneration score and DHE fluorescence intensity of the tear group were significantly higher than in the no tear group. The SOD activity is regulated by the amount of all three SOD isoforms, and SOD isoform specificity cannot be examined. Therefore, SOD1 and SOD2 gene expressions which were important for the rotator cuff degeneration[16] and cartilage of the osteoarthritis[31,35] were investigated using the RT-PCR. Additionally, no studies have examined changes in SOD using animal rotator cuff injury models without tear and tear models. We found that in the injury model, SOD1 expression and SOD activity were significantly decreased at 7 days and DHE fluorescence intensity was significantly increased at 14 days. Scott et al.[36] reported that the expression of all three SOD enzymes decreased with increased collagenase gene expression in osteoarthritis cartilage. In the present study, it is possible that SOD1 expression was decreased by collagenase administration. It seems that decrease of SOD1 may be one of the reasons for the decrease in SOD activity. Collagenase may also have caused post-translational changes in SOD, but it was not investigated and further study

should be needed. In any case, the loss of redox balance due to decreased SOD may cause oxidative stress, leading to an increase in DHE fluorescence intensity at 14 days. On the other hand, the tear models showed no significant difference in SOD1,2 expression and SOD activity and showed a significant increase in DHE fluorescence intensity at all points. Rotator cuff tissue degeneration and oxidative stress were caused in both models, but the tear model did not involve SOD. Therefore, rotator cuff degeneration without tear and rotator cuff degeneration after tear are supposedly different in the involvement of SOD for the loss of redox balance.

Antioxidant enzymes have been reported to include glutathione peroxidase, catalase, and PRDX, in addition to SOD.[37] There are 6 PRDX isoforms, all of which participate directly in eliminating hydrogen peroxide (H_2O_2) and neutralizing other oxidizing chemicals.[38] Previous studies reported the association between rotator cuff degeneration and PRDX5, which is widely expressed in mammalian tissues and cellularly localized to mitochondria, peroxisomes, and the cytosol. Nho et al.[3] reported that cell apoptosis played an important role in the pathway of tendon degeneration from oxidative stress, and Yuan et al.[39] noted an overexpression of PRDX5 via transfection in cultured tendon reduced cell apoptosis. Wang et al.[40] reported that PRDX5 was significantly upregulated in the degenerative rotator cuff compared with the normal rotator cuff, suggesting a compensated response to oxidative stress in the degenerative tendon. On the other hand, a previous study reported that mitochondrial dysfunction contributes to oxidative stress and cell apoptosis during the aging process.[41] Furthermore, Takayama et al.[42] showed that the increase in oxidative stress can also be caused by mitochondrial dysfunction in tendon tissues. In the tear model of our study, SOD was not significantly different; however, DHE fluorescence intensity was

362 significantly increased from 3 days and PRDX5 was significantly decreased at 14 days.
363 It is possible that mitochondrial dysfunction and cell apoptosis may increase oxidative
364 stress in the rotator cuff after tear, and then PRDX5 in mitochondria may be reduced.
365 Previous studies reported that various antioxidants are effective for treating
366 musculoskeletal tissue degeneration. Kim et al.[43] showed that oxidative stress induces
367 autophagic cell death in rotator cuff tenofibroblasts, and cyanidin inhibits cell death.
368 Morikawa et al.[16] reported that rotator cuff degeneration in SOD1-deficient mice could
369 be suppressed with vitamin C. Thus, antioxidants could be helpful for the treatment and
370 prevention of rotator cuff tears in patients with oxidative stress. We showed that
371 degenerative rotator cuffs without tear involved SOD1 in the injury model, and
372 degenerative rotator cuffs with tear in the tear model involved a different antioxidant
373 enzyme. It is possible that antioxidants related to SOD may be useful for suppressing
374 rotator cuff degeneration without tear, and that antioxidants related to antioxidant
375 enzymes other than SOD are useful for suppressing rotator cuff degeneration after tear.
376 Which antioxidant might be useful may depend on the state of the rotator cuff
377 degeneration.
378 There were several limitations in this study. First, we investigated the
379 shoulders of SD rats. It is unclear whether the injury model in this study is the same as
380 the degenerative rotator cuffs of humans. A rat rotator cuff injury model was reported by
381 Chen et al.[19] in which rotator cuff degeneration continued until 14 days after collagenase
382 injection and improved at 21 days. We also found rotator cuff degeneration at 3, 7, and
383 14 days in the injury model, with results similar to those in the study. In addition, the
384 control of the injury model was also injected with saline in order to eliminate the effect
385 of the injection based on previous studies.[19,44] However, the injection of the control

potentially causes mechanical trauma. Second, rotator cuff tear models mimic traumatic ruptures. The tear model in our study was different from that in humans, which is caused by the progress of degeneration due to aging. Because our tear model cannot mimic this, we followed the same method as many previous studies that used animal models. Additionally, in the tear model, the time course was limited to two weeks after the tear. The previous studies reported that in the rat rotator cuff tear model, the rotator cuff tears reattached spontaneously unlike in humans.[45,46] Because the space between the bony insertion site and the tendon stump mostly filled with scar tissue in approximately one week after the tear,[45,46] we measured in two weeks after the tear. Third, it is still controversial whether DHE is an accurate detector. Although it has been used as a traditional superoxide detector in many studies, some papers have claimed that DHE is oxidized by various molecules, such as H_2O_2, ONOO–, and Fenton's reagent and it is not an accurate indicator of superoxide concentration.[26] However, the detection of oxidative stress by DHE may be still considered useful. A previous study showed that DHE was especially oxidized by ROS intracellularly,[26] and in this study we eliminated the effect of extracellular staining by measuring the brightest intensity of a field, which would be indicated by the intracellular intensity. Therefore, DHE, which can detect intracellular ROS, may be useful. Fourth, we used β-actin as a housekeeping gene for gene expression analysis. It was reported that ROS negatively regulate actin polymerization by driving actin glutathionylation[47] although other studies have showed that ROS increased actin polymerization[48,49] and superoxide production induce an increase in filamentous-actin.[50] β-actin may be affected by ROS, therefore we need further investigation using other housekeeping genes. Fifth, SOD activity was investigated only at 7 days after the injection or the surgery. The previous study[19] reported that the tissue

degeneration has been observed from 3 to 14 days. Therefore, we chose at 7 days as an intermediate time point. However, the results of SOD activity at 3 and 14 days, and before 3 days were not evaluated and further investigation is needed.

CONCLUSIONS

Tissue degeneration and oxidative stress of the rotator cuff were observed in both the rotator cuff injury and tear models; however, SOD was significantly decreased only in the rotator cuff injury model. These results suggest that SOD might not be associated with rotator cuff degeneration after tear but is involved in the degenerative rotator cuff without tear, although rotator cuff degeneration involves oxidative stress both with and without tear.

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563 **TABLES**

564 Table 1. Primers for quantitative real-time polymerase chain reaction (RT-PCR)

Gene	Primers
β-actin	F: 5′-TGACAGGATGCAGAAGGAGA-3′
	R: 5′-TAGAGCCACCAATCCACACA-3′
SOD1	F: 5′-TGCGTGCTGAAGGGCGACGGTC-3′
	R:5′-AATCCCAATCACACCACAAGCCAAGC-3′
SOD2	F: 5′-AATCCCAATCACACCACAAGCCAAGC-3′
	R: 5′-CCCAGCAGTGGAATAAGGCCTGTGG-3′
PRDX5	F: 5′-AAAGGAGCAGGTTGGGAGTG-3′
	R: 5′-GCAGATGGGTCTTGGAACAG-3′

565

566 SOD1, superoxide dismutase isoform 1; SOD2, superoxide dismutase isoform 2;

567 PRDX5, peroxiredoxin 5

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569

Table 2. The Degeneration Scores of the Injury Model for Each Item and the Total Degeneration Score

	Day3			Day7			Day14		
	control	injury	*p* value	control	injury	*p* value	control	injury	*p* value
Cell morphology	0.5±0.3	2.0±0.0	<0.05	0.5±0.3	1.5±0.5	0.169	0.8±0.5	1.5±0.5	0.343
Collagen alignment	0.5±0.3	2.0±0.4	0.052	0.8±0.3	1.0±0.3	0.052	0.8±0.3	2.0±0.0	<0.05
Cellularity	0.5±0.3	1.0±0.0	0.180	0.5±0.3	1.0±0.0	0.453	0.8±0.3	1.3±0.3	0.257
Vascularity	0.0±0.0	1.3±0.3	<0.05	0.0±0.0	1.5±0.6	<0.05	0.3±0.3	1.3±0.5	0.160
Ground substance	1.0±0.4	1.3±0.3	0.739	0.3±0.3	1.5±0.5	0.069	0.8±0.5	1.3±0.3	0.436
Fiber structure	0.2±0.2	2.5±0.3	<0.05	1.0±0.4	2.5±0.5	0.095	1.0±0.4	2.5±0.5	0.095
Collagen stainability	0.8±0.3	1.5±0.3	0.134	0.8±0.3	1.3±0.3	0.134	0.8±0.3	2.3±0.5	0.063
Total	3.5±0.9	11.5±0.6	<0.05	4.0±1.1	11.8±2.0	<0.05	5.0±1.3	12.0±1.4	<0.05

Table 3. The Degeneration Scores of the Tear Model for Each Item and the Total Degeneration Score

	Day3			Day7			Day14		
	control	injury	*p* value	control	injury	*p* value	control	injury	*p* value
Cell morphology	0.3±0.2	0.7±0.2	0.311	0.2±0.2	1.0±0.4	0.086	0.5±0.2	1.2±0.3	0.137
Collagen alignment	1.2±0.4	1.5±0.3	0.735	1.3±0.5	2.2±0.5	0.240	1.8±0.6	2.0±0.5	0.931
Cellularity	0.7±0.3	1.5±0.3	0.125	0.5±0.2	1.3±0.2	<0.05	0.8±0.3	1.8±0.2	<0.05
Vascularity	0.2±0.2	0.5±0.2	0.282	0.3±0.2	1.3±0.4	0.070	0.0±0.0	0.8±0.3	<0.05
Ground substance	0.8±0.4	1.7±0.4	0.203	0.3±0.3	0.7±0.4	0.461	0.3±0.3	0.8±0.5	0.527
Fiber structure	0.2±0.2	1.3±0.5	0.073	0.3±0.3	1.7±0.4	<0.05	0.2±0.2	1.8±0.3	<0.05
Collagen stainability	0.2±0.2	0.3±0.2	0.595	0.3±0.3	0.3±0.3	0.900	0.3±0.3	0.3±0.3	0.900
Total	3.7±0.4	6.8±0.8	<0.05	2.6±0.7	8.3±1.0	<0.05	3.8±0.9	8.7±1.5	<0.05

FIGURE LEGENDS

Figure 1. Histology of tissue samples from the injury model. A–D, Hematoxylin-eosin (H&E) stain under light microscopy; E–H, H&E stain under polarized microscopy; I–L, alcian blue (AB) stain under light microscopy; M–P, dihydroethidium (DHE) stain under fluorescence microscopy. Bars = 100 μm.

Figure 2. Comparison of the injury side and no-injury side of the rotator cuff injury model. A, Degeneration score; B, fluorescence intensity with dihydroethidium (DHE) stain. ⋆; $p < 0.05$.

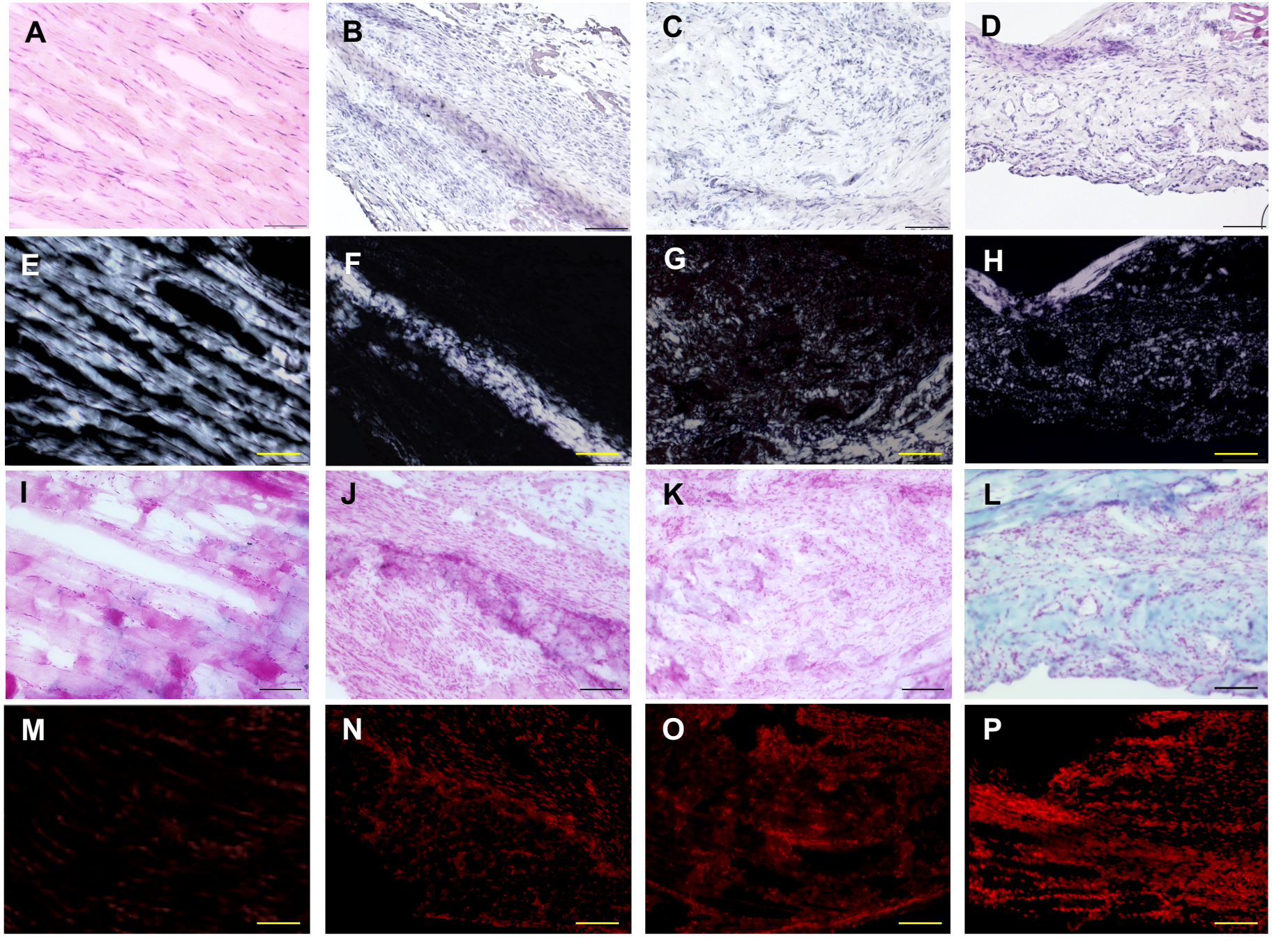
Figure 3. Comparison of the injury side and no-injury side of the rotator cuff injury model. A–C, Fold change of SOD1, SOD2, and PRDX5; D, SOD activity. ⋆$p < 0.05$, ⋆⋆$p < 0.01$. SOD1, superoxide dismutase isoform 1; SOD2, superoxide dismutase isoform 2; PRDX5, peroxiredoxin 5

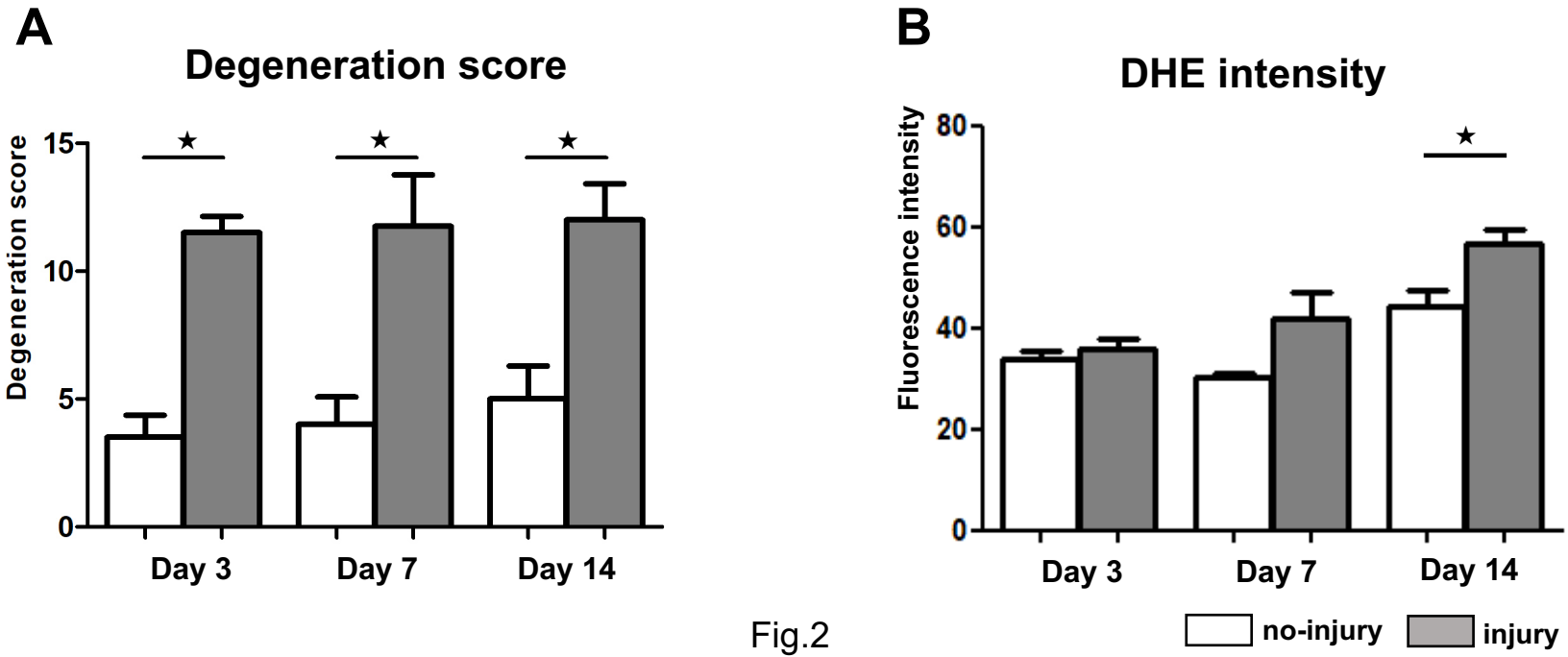
Figure 4. Histology of tissue samples from the tear model. A–D, Hematoxylin-eosin (H&E) stain under light microscopy; E–H, H&E stain under polarized microscopy; I–L, alcian blue (AB) stain under light microscopy; M–P, dihydroethidium (DHE) stain under fluorescence microscopy. Bars = 100 μm.

Figure 5. Comparison of the tear side and no-tear side of the rotator cuff tear model. A, Degeneration score; B, fluorescence intensity with dihydroethidium (DHE) stain. ⋆ $p < 0.05$.

597 Figure 6. Comparison of the tear side and no-tear side of the rotator cuff tear model. A–
598 C, Fold change of SOD1, SOD2, and PRDX5; D, superoxide dismutase (SOD) activity.
599 ⋆; $p < 0.05$. SOD1, superoxide dismutase isoform 1; SOD2, superoxide dismutase
600 isoform 2; PRDX5, peroxiredoxin 5

Fig.1


HE staining
Polarized microscopy by HE staining
Alcian blue staining
DHE staining
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
no-injury
Day3
Day7
Day14


A
Degeneration score
Degeneration score
15
10
5
0
★
★
★
Day 3
Day 7
Day 14
B
DHE intensity
Fluorescence intensity
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60
40
20
0
★
Day 3
Day 7
Day 14
no-injury
injury

Fig.2

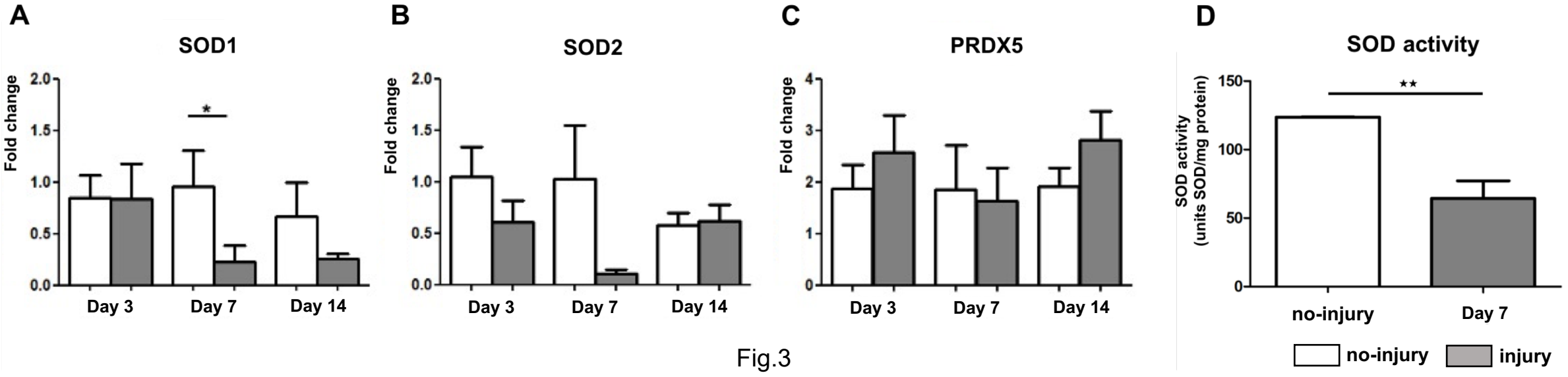
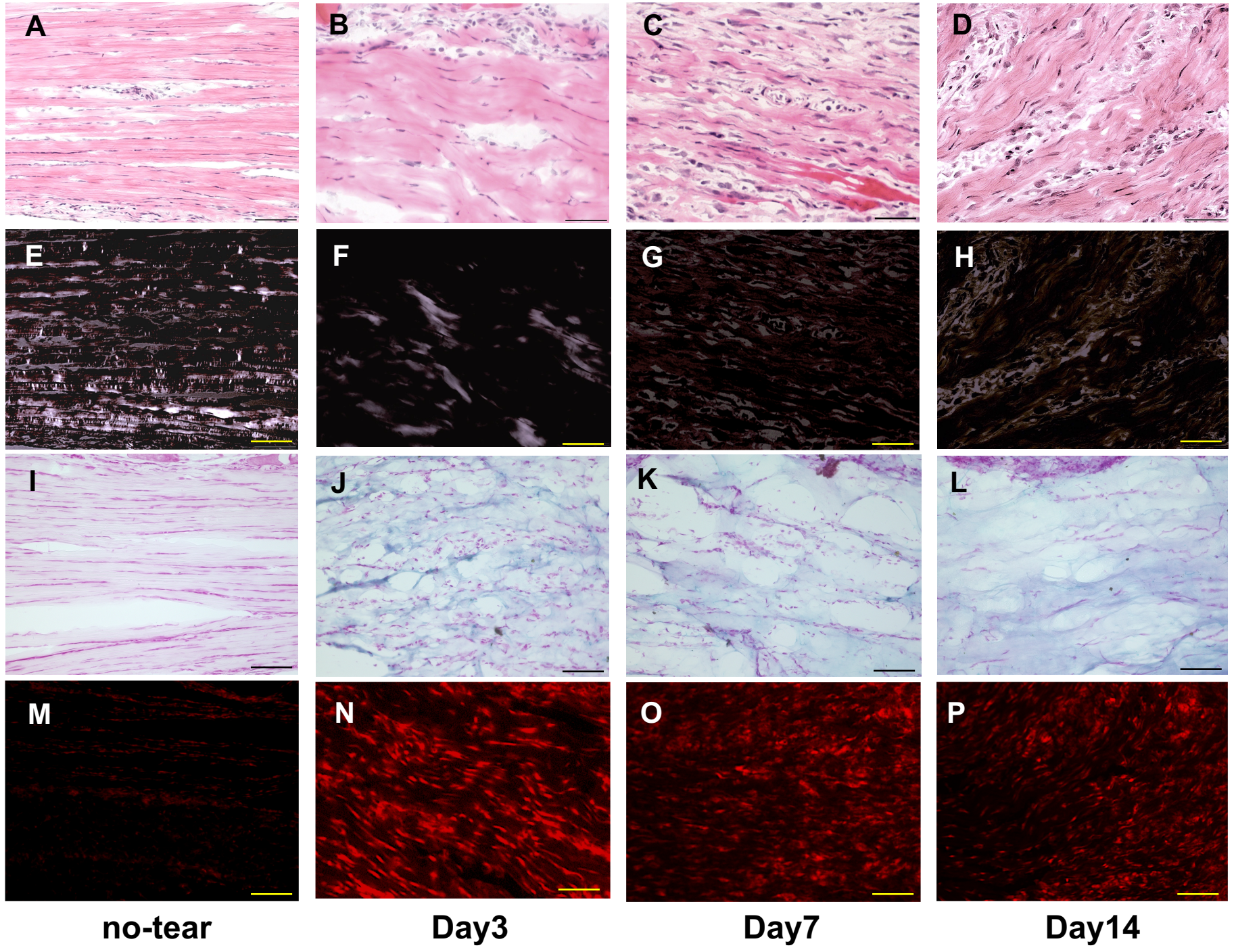

A
SOD1
Fold change
2.0
1.5
1.0
0.5
0.0
*
Day 3
Day 7
Day 14
B
SOD2
Fold change
2.0
1.5
1.0
0.5
0.0
Day 3
Day 7
Day 14
C
PRDX5
Fold change
4
3
2
1
0
Day 3
Day 7
Day 14
D
SOD activity
SOD activity
(units SOD/mg protein)
150
100
50
0
**
no-injury
Day 7
no-injury
injury

Fig.3

Fig.4


HE staining
Polarized microscopy by HE staining
Alcian blue staining
DHE staining
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
no-tear
Day3
Day7
Day14

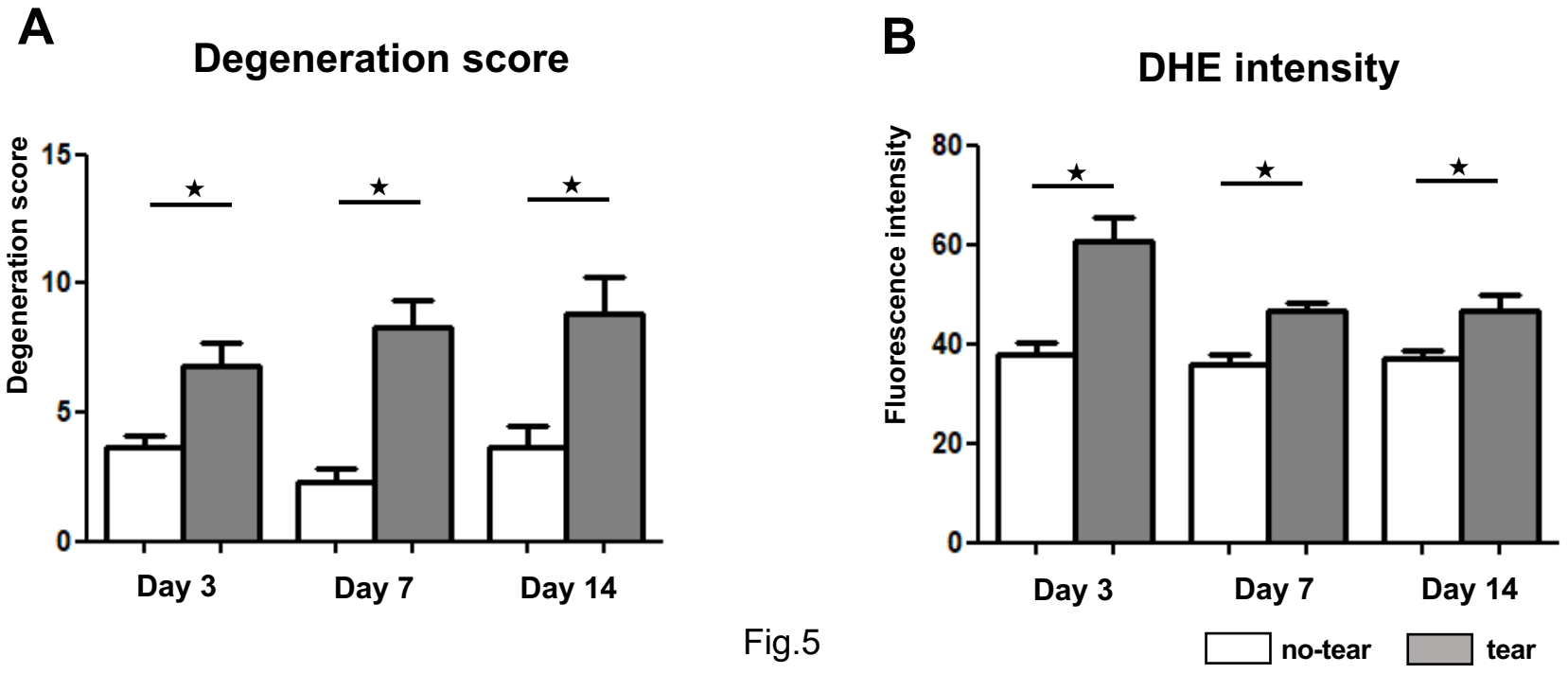

A
Degeneration score
Degeneration score
15
10
5
0
Day 3
Day 7
Day 14
B
DHE intensity
Fluorescence intensity
80
60
40
20
0
Day 3
Day 7
Day 14
no-tear
tear

Fig.5

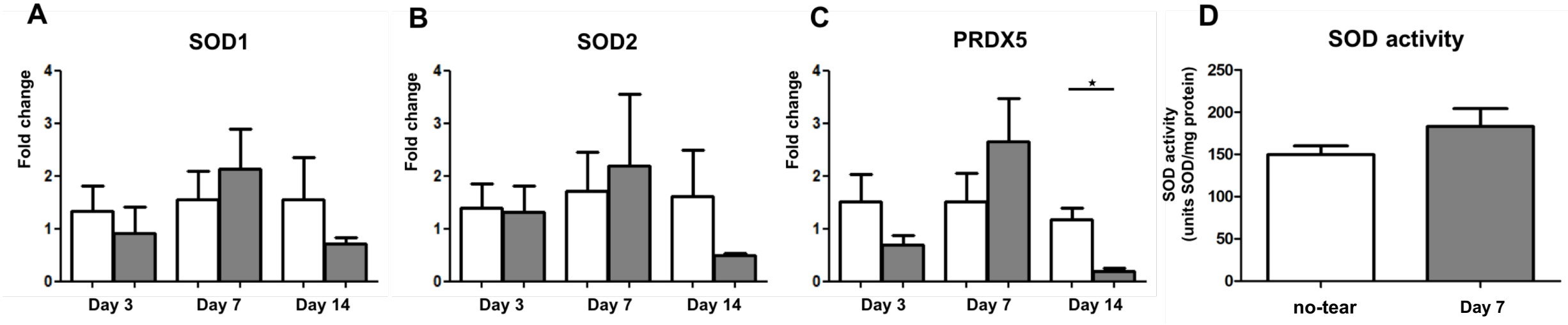

A
SOD1
Fold change
0
1
2
3
4
Day 3
Day 7
Day 14
B
SOD2
Fold change
0
1
2
3
4
Day 3
Day 7
Day 14
C
PRDX5
Fold change
0
1
2
3
4
★
Day 3
Day 7
Day 14
D
SOD activity
SOD activity
(units SOD/mg protein)
0
50
100
150
200
250
no-tear
Day 7
no-tear
tear

Fig.6