Antioxidant treatment with vitamin C attenuated rotator cuff degeneration caused by oxidative stress in Sod1-deficient mice

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ARTICLE INFO

Keywords: Oxidative stress Rotator cuff degeneration Enthesis Vitamin C administration Sod1-deficient mice Antioxidant

Level of evidence: Basic Science Study, Histology, Animal Model

Background: Rotator cuff degeneration is 1 of several factors that lead to rotator cuff tears; however, the mechanism of this degeneration remains unclear. We previously reported that deficiency of an antioxidant enzyme, superoxide dismutase 1 (Sod1), in mice induced degeneration in supraspinatus tendon entheses, a model that replicates human rotator cuff degeneration. In this study, we analyzed possible effects of vitamin C (VC), a major antioxidant, on the degenerative changes of supraspinatus entheses in Sod1−/− mice.

Methods: We administered VC or vehicle, distilled water, for 8 weeks to Sod1−/− and wild-type male mice beginning at 12 weeks of age (n = 5-8 per group). When mice were 20 weeks of age, we sectioned rotator cuff tissue samples and performed hematoxylin-eosin and toluidine blue staining for quantitative histologic evaluation.

Results: VC administration, compared with vehicle administration, attenuated the histologic changes, including a misaligned 4-layered structure, fragmented tidemark, and toluidine blue staining, in the supraspinatus entheses of Sod1−/− mice. In the quantitative histologic evaluation, all parameters were significantly decreased in Sod1−/− mice compared with wild-type mice, except for the number of nonchondrocytes.

Conclusion: We demonstrated that an antioxidant treatment, VC administration, attenuated rotator cuff degeneration, similar to that observed in humans, that is caused by oxidative stress in Sod1−/− mice. VC effects included improvements in quantitative histologic parameters and other histologic changes. These results suggest that VC treatment can prevent oxidative stress–induced degeneration of the rotator cuff.

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Methods

Animals

Sod1-deficient mice (Sod1−/−) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The Sod1−/− mice were back-crossed with C57BL/6NcRSlc mice (Nilson SLC, Shizuoka, Japan) 5-6 times. The mice were maintained and studied according to protocols approved by the Animal Care Committees of the authors’ institutions based on Guidelines for Proper Conduct of Animal Experiments.

Oral administration of the antioxidant vitamin C

To analyze effectiveness of antioxidant therapy against the degenerative changes of supraspinatus entheses in Sod1−/− mice, we administrated the antioxidant VC or vehicle orally to Sod1−/− and wild-type (WT) male mice. VC (sodium L-ascorbate; Sigma-Aldrich Chemicals, St. Louis, MO, USA) was dissolved in water at 1% (w/v). Oral administration began at the age of 12 weeks and continued for 8 weeks. The method of dosing was to provide the mice, ad libitum, with drinking water containing VC. The VC solution was replenished with fresh solution twice per week.

Tissue preparation

The Sod1−/− and WT mice were sacrificed with proper euthanasia procedures at 20 weeks of age (n = 5-8 per group). The complexes of the supraspinatus and infraspinatus muscles, tendons, and humeral head were removed together, fixed in 4% paraformaldehyde at room temperature overnight, decalcified with 10% ethylenediaminetetraacetic acid in 10mM of phosphate buffer (pH 7.4) for 1 week, and then embedded in paraffin blocks. The paraffin blocks were cut on a standardized frontal plane and stained with hematoxylin-eosin and toluidine blue (TB).

Histologic analysis of the supraspinatus enthesis

We analyzed sections under an optical microscope to assess overall histologic structure and microstructure of the supraspinatus entheses. This analysis included examination of the 4-layered structure, including the tendon, nonmineralized and mineralized fibrocartilage, and bone, and the tidemark, which forms a boundary between 2 fibrocartilages.

Histologic analysis of collagen fibers in the entheses

To analyze collagen fiber structure in the entheses, we observed the sections under a polarizing microscope. This analysis was based on the principle that polarizing light directed at spatially oriented collagen fibers in tissue sections is diffracted and shines brightly against a dark background. The slides were rotated for 360° on the microscope tray to select the position showing maximum brightness. In the intact enthesis, collagen fibrils are spatially aligned, conferring a high tensile strength to fibrocartilage.

Histologic evaluation of supraspinatus entheses

Quantitative histologic measurements were performed as described previously. Parameters analyzed were the number of chondrocytes, number of nonchondrocytes, percentage of aligned chondrocytes, spatial arrangement of collagen fibers, and area of metachromasia.

Number of chondrocytes

At the enthesis, the number of chondrocytes was counted in a standardized rectangle field on hematoxylin-eosin–stained section. Cells displaying 3 or 4 of the following were defined as chondrocytes: large nucleus, basophilic and shrunken cytoplasm, lacuna around the cytoplasm, and halo around the lacuna.

Number of nonchondrocytes

Non–chondrocytic cells were counted in the same fields as the chondrocytes. Non–chondrocytic cells indicated mesenchymal cells, fibroblasts, endothelial cells, or adipocytes.

Percentage of aligned chondrocytes

In the same rectangular field used for the number of chondrocytes, the number of chondrocytes forming rows was counted. A row was defined as 3 or more chondrocytes aligned longitudinally. The number of chondrocytes aligned in rows divided by the total number of chondrocytes provided the percentage of chondrocytes aligned in rows. In a normal mature enthesis, chondrocytes are aligned in rows in nonmineralized and mineralized fibrocartilage.

Area of metachromasia

Fibrocartilage binds basic blue dyes, such as TB, changing its color to reddish blue, a property known as metachromasia. Intensity of metachromasia staining with TB indicated proteoglycan content. The area of intense metachromasia was quantified using the image analysis software. On the TB-stained slides, a standardized field starting at the bone-tendon junction was captured. Intense metachromatic areas within the standardized field were measured automatically and interpreted as fibrocartilage.

Statistical analysis

Statistical analyses were performed using analysis of variance followed by Tukey test. All data are expressed as means ± standard deviation. P ≤ .05 was considered statistically significant.

Results

Vitamin C administration attenuated histologic changes of the supraspinatus entheses in Sod1−/− mice

According to histologic analyses under the optical microscope, the WT mice had a well-organized 4-layered structure (tendon proper, nonmineralized fibrocartilage, mineralized fibrocartilage, and bone) and a tidemark with a boundary between nonmineralized fibrocartilage and mineralized fibrocartilage (Fig. 1, A). In contrast, Sod1−/− mice had a misaligned 4-layered structure and a fragmented tidemark in the entheses (Fig. 1, B, arrowheads). VC administration (Fig. 1, D), compared with vehicle administration (Fig. 1, B), attenuated these histologic changes, improving the misaligned 4-layered structure and fragmented tidemark in Sod1−/− mice. According to TB staining, the WT mice had an area of reddish blue staining, known as metachromasia, in the supraspinatus entheses (Fig. 2, A). The Sod1−/− mice (Fig. 2, B) had weaker staining than the WT mice (Fig. 2, A). VC administration increased TB staining in the supraspinatus entheses of the Sod1−/− mice (Fig. 2, D).
Vitamin C administration attenuated deterioration of spatially aligned collagen fibers of the supraspinatus entheses in Sod1−/− mice.

We next evaluated the alignment of collagen fibers in the supraspinatus entheses using polarizing microscopy. The WT mice exhibited brightly diffracted light at the entheses along the tendon (Fig. 3, A). In contrast, the Sod1−/− mice had markedly less brightly diffracted light in the entheses compared with that observed in WT mice (Fig. 3, A and B). Furthermore, Sod1−/− mice treated with VC (Fig. 3, D) showed an increase in brightly diffracted light in the entheses compared with those receiving vehicle (Fig. 3, B). These results indicated that VC administration attenuated deterioration of spatially aligned collagen fibers in the supraspinatus entheses of the Sod1−/− mice.

Quantitative histologic changes of the supraspinatus entheses

To quantify the histologic changes, we measured five parameters: the number of chondrocytes (Fig. 4, A) and nonchondrocytes (Fig. 4, B), percentage of aligned chondrocytes (Fig. 4, C), area of diffracted polarized light (Fig. 4, D), and area of metachromasia (Fig. 4, E). With vehicle administration, Sod1−/− mice showed significant reduction of these quantitative histologic measurements compared with WT mice, with the exception of the number of nonchondrocytes.
VC is not commonly used to address musculoskeletal problems but has been recommended to prevent and inexpensive treatment believed to be beneficial for many conditions including cancer, the common cold, and smoking-related problems.\(^\text{10,25}\) VC has two major biologic functions: as a scavenger of ROS, such as \(\text{O}_2^\cdot\) and \(\text{H}_2\text{O}_2\), and as a cofactor for collagen synthesis.\(^\text{20,36}\) Our data showed that VC administration attenuated the histologic changes of supraspinatus entheses (Figs. 1-3), improving quantitative histologic parameters in \(\text{Sod}1^{-/-}\) mice (Fig. 4). Yet, in the WT mice, VC administration for 8 weeks did not affect the histologic findings (Figs. 1 and 2), including the quantitative histology in supraspinatus entheses (Fig. 3). These results indicated that the protective effects of VC in \(\text{Sod}1^{-/-}\) mice were caused by its ROS scavenger activity rather than its actions as a cofactor for collagen synthesis. In 1 animal study, VC accelerated tendon healing, restoring normal structure.\(^\text{24}\) Other antioxidant treatments were reported to improve total collagen levels and collagen orientation as well as to increase strength during Achilles tendon healing.\(^\text{42}\) Moreover, previous data from our group showed that VC accelerated the healing and outgrowth of \(\text{Sod}1^{-/-}\) fibroblasts and a VC derivative increased cell viability during oxidative stress in vitro.\(^\text{42,38}\) Together, these findings suggest that redox balance regulation, especially through VC treatment, prevented the degeneration of supraspinatus entheses in \(\text{Sod}1^{-/-}\) mice.

In this study, VC administration improved the 4 histologic parameters of entheses, such as number of chondrocyte, chondrocytes aligned in rows, area of metachromasia, and collagen orientation, and collagen fibers are spatially aligned in the intact enthesis, conferring a high tensile strength to fibrocartilage.\(^\text{11}\) These indicated that histologic findings with VC treatment are more similar to those of intact enthesis compared with those with vehicle administration in \(\text{Sod}1^{-/-}\) mice.

**Limitations**

There were several limitations in this study. First, it is difficult to measure oxidative stress or to analyze oxidative stress-related genes in mouse rotator cuff tissues because the supraspinatus tendon is very small, with a tendon width of approximately 1 mm. Therefore, we did not confirm that the VC treatment decreased oxidative stress in supraspinatus tendon. However, \(\text{Sod}1\) deficiency causes several age-related changes attributed to oxidative stress in mice,

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**Figure 3**  Vitamin C (VC) administration attenuated deterioration of spatially aligned collagen fibers of the supraspinatus enthesis in \(\text{Sod}1^{-/-}\) mice. Polarizing microscopic images of the supraspinatus enthesis in wild-type (WT) mice with vehicle administration (A), \(\text{Sod}1^{-/-}\) mice with vehicle administration (B), WT mice with VC administration (C), and \(\text{Sod}1^{-/-}\) mice with VC administration (D). The \(\text{Sod}1^{-/-}\) mice displayed a decrease in brightly diffracted light in the enthesis compared with that observed in the WT mice (A and B). With VC administration, the brightly diffracted light was increased in the enthesis of \(\text{Sod}1^{-/-}\) mice (B and D). The scale bars indicate 50 μm.
and several studies have shown that VC has antioxidant effects in Sod1−/− mice.9,12,16,18,21,22,37,39 As a second limitation, we could not perform tensile testing because of the small size of the tendons. Third, our study used a relatively limited sample size. Finally, this study has been performed in only 1 protocol regarding VC concentration and duration of oral administration, meaning that we have not checked the dose-dependent or duration-dependent effects of VC administration. Furthermore, the exact concentration and in vivo kinetics of VC in tissues were unclear because these could not be monitored in this examination and depended on the amount of drinking and the timing of measurement. However, we measured total amount of daily drinking of VC and vehicle in WT and Sod1−/− mice and found no difference among the 4 groups (data not shown). Further analyses, including studies using human samples, will be

Figure 4 Histologic evaluation of supraspinatus enthesis in wild-type (WT) and Sod1−/− mice with vehicle and vitamin C (VC) administration. Quantitative histology measured 5 parameters: (A) the number of chondrocytes, (B) the number of nonchondrocytes, (C) the percentage of aligned chondrocytes, (D) the spatial arrangement of collagen fibers, and (E) the area of metachromasia. n = 5-8 per group. The error bars indicate standard deviation.
needed to fully clarify the protective role of VC against rotator cuff degeneration.

Conclusion
We have demonstrated that antioxidant treatment, through VC administration, attenuated histologic changes in the supraspinatus entheses induced by Sod1 deficiency. Our findings suggest that antioxidant treatment may prevent oxidative stress–induced degeneration of the rotator cuff.

Disclaimer
The authors, their immediate families, and any research foundations with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

Acknowledgments
The mice were maintained and studied according to protocols approved by the Animal Care Committees of the authors’ institutions.

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