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Local Vitamin-C Injection Reduced Tendon Adhesion in a Chicken Model of Flexor Digitorum Profundus Tendon Injury

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Background: Adhesion formation is a complication of hand flexor tendon repair. Normal gliding function of flexor tendons can be impaired by an excessive fibrotic response, which may be caused by intraoperative and postoperative hemorrhage. As tissue damage and hemorrhage can disturb redox regulation, thereby favoring fibrotic responses, the purpose of this study was to investigate if antioxidants can reduce tendon adhesion by antagonizing oxidative stress.

Methods: Flexor digitorum profundus tendon injury was induced in fifty-seven chickens. In twelve chickens, oxidative stress preinjury, immediately after injury, and two and six weeks postinjury (n = 3 at each time period) was estimated by measuring tissue levels of the reduced form of glutathione (GSH) and oxidized glutathione (glutathione disulfide [GSSG]) in the proximal interphalangeal joint. In the remaining chickens, 50 μL of saline solution or vitamin-C solution (5 or 50 mg/mL) was injected into the wound immediately after closure of the tendon sheath. Samples were harvested at two weeks (n = 6 in each group) or six weeks (n = 6 in each group) postinjury for a gliding test, ultrasound imaging, and histological examination. Three chickens from each group were killed at two weeks postinjury for GSH and GSSG measurements to evaluate the treatment effects on postoperative oxidative stress.

Results: The GSH level was significantly decreased at two and six weeks postinjury, and the GSSG level was significantly increased at six weeks postinjury. Both 5 and 50-mg/mL vitamin C led to higher tissue levels of GSH at two weeks postinjury, as compared with that in the saline solution group, but no significant change in the GSSG level was detected. Chickens with vitamin-C supplementation showed no significant improvement in gliding resistance and no significant reduction of the fibrotic size at two weeks postinjury, but they did show significant improvement in gliding resistance at six weeks postinjury and the 5-mg/mL vitamin-C group showed a significant reduction of the fibrotic size at six weeks. Histological examination showed less peritendinous adhesion in the vitamin-C groups.

Conclusions: Our results suggest that local injection of vitamin-C solution can reduce the extent of adhesion of healing tendons, probably by redox modulation, in a chicken model.

Clinical Relevance: It may be feasible to apply vitamin-C solution intraoperatively at the time of tendon repair to reduce restrictive tendon adhesion, but additional studies are needed to optimize the dose required.

Adhesion formation is a complication of tendon injury repair. The postoperative outcome of hand flexor tendon repair can be affected by restrictive adhesions that bind the flexor tendons to each other and to surrounding structures, interfering with their normal gliding function[1]. Surgical releases are commonly used to circumvent the functional loss related to restrictive adhesion, but recurrence is common[2]. Postoperative tendon adhesion still presents a major clinical problem in hand surgery despite several decades of research[3-5].

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Tendon adhesion results primarily from an excessive fibrotic response between the tendons and the synovial sheath, which is caused by intraoperative and postoperative hemorrhage and an inflammatory response[6,7], both of which lead to tendon adhesion. Modulation of the inflammatory response[8], anti-fibrotic strategies[9], and introduction of a physical barrier[10] have been proposed to prevent tendon adhesion. As postoperative inflammatory responses involve the generation of oxidative stress, it has been suggested that reactive oxygen species may play an important role in adhesion formation[11]. Reactive oxygen species are essential mediators of fibrogenesis[12]. It is possible that reactive oxygen species also play a role in the formation of tendon adhesion. If so, postoperative antioxidation strategies may have the potential to reduce the extent of tendon adhesion by reducing reactive oxygen species. A previous study by Greenwald et al.[4] demonstrated that dietary supplementation with antioxidant vitamins (A and E) can reduce tendon adhesion. It is possible that direct injection into the operative site is a better approach. We hypothesized that local supplementation with an antioxidant could reduce the extent of tendon adhesion. In the present study, we investigated the effects of local vitamin-C injection on the formation of tendon adhesion in a chicken model of experimental flexor digitorum profundus tendon injury, and the ability of vitamin C to modulate postoperative oxidative stress was evaluated by the measurement of reduced and oxidized forms of glutathione. Tendon adhesion was quantified mainly by measuring functional loss with a gliding test. The fibrosis that may contribute to tendon adhesion was quantified by ultrasound imaging, and peritendinous reactions were qualitatively examined in histological sections.

Materials and Methods

Animal Model

Fifty-seven female Kamei chickens (Hong Kong Poultry [Kamei Chicken] Development Limited, Hong Kong) were used in the current study. We utilized an experimental model of flexor digitorum profundus tendon injury as described previously[13]. In brief, the chickens were anesthetized with intravenous injection of 1 to 1.5 mL of ketamine/xylazine (1:1). The flexor digitorum profundus tendon was approached through a 10-mm medial incision at the proximal interphalangeal joint area of the long toe. The skin was mobilized by blunt dissection to expose the flexor sheath. An anular pulley equivalent to the A2 pulley in the human finger was located just distal to the proximal interphalangeal joint. An opening of 10 mm was made at the proximal interphalangeal joint area, proximal to the pulley, on the side of the flexor sheath. The deep tendon (flexor digitorum profundus equivalent) was withdrawn through the opening by flexing the toe. The superficial tendon (flexor digitorum superficialis equivalent) was left untouched. Three partial cuts at 4-mm intervals were made on the medial aspect of the tendon. Each cut amounted to 50% of the diameter of the tendon. The cuts were repaired with a single-strand stitch of 5-0 Ethilon nonabsorbable nylon suture (Johnson & Johnson, New Brunswick, New Jersey) passing through all three cuts simultaneously. The tendon was returned to the sheath, and the opening of the sheath and the skin were closed with the 5-0 sutures. The injured foot was immobilized for two weeks with a fiberglass cast (3M, St. Paul, Minnesota). The contralateral side served as an uninjured and free-moving control.

In the first part of the study, nine chickens that had undergone the operation were killed with an overdose of pentobarbital (25% w/v) at three time points (immediately after the operation and two and six weeks postinjury; n = 3 at each time point) for measurement of glutathione, and three age-matched chickens that had not undergone the operation served as controls. The chicken feet were harvested according to the sample preparation protocol for glutathione assay (see below). The remaining chickens were randomly assigned into

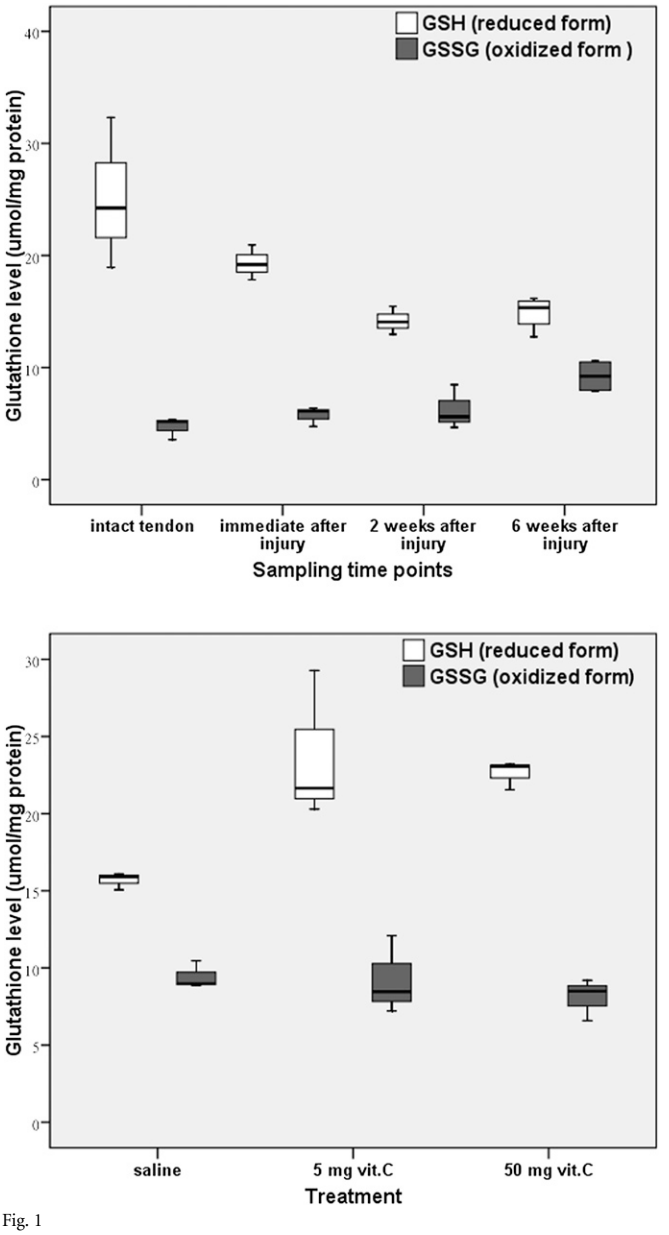

Fig. 1

A decrease in the level of the reduced form of glutathione (GSH) at two and six weeks postinjury and an increase in the level of oxidized glutathione (GSSG) at six weeks postinjury, compared with the levels in the intact tendon, suggested the presence of oxidative stress during the development of restrictive tendon adhesion (**Fig. 1-A**). The local administration of vitamin C immediately after the operation significantly increased the level of GSH, whereas no significant change was observed in the level of GSSG, when compared with that in the saline solution group (**Fig. 1-B**). In the boxplot, the top and bottom of the rectangles indicate the 75th percentile and the 25th percentile, respectively; the horizontal lines within the rectangles indicate the median; and the top and bottom of the I bars indicate the 95th percentile and the 5th percentile, respectively.

three groups that received a local injection of saline solution or 5-mg/mL or 50-mg/mL vitamin-C solution (Sigma-Aldrich, St. Louis, Missouri) after the tendon sheath was sutured closed. A total volume of 50 μL was injected. No leakage of solution was observed after the injection. Two dosages of vitamin C were used to determine the effective dose for reducing tendon adhesion. Three chickens from each group were killed at two weeks postsurgery to assess the effects of antioxidant supplementation on the tissue glutathione levels. The remaining chickens were killed at two or six weeks postoperatively (n = 6 in each group at each time point) for ultrasonographic evaluation, the gliding test, and histological examination. Both the injured and the contralateral chicken feet were sampled, kept in saline-solution-moistened gauze at −20°C until the gliding test was performed (at less than one month after death) (see Appendix). The above animal experiments were approved by the Animal Research Ethics Committee, The Chinese University of Hong Kong (Reference number CUHK4500/06M).

Determination of Reduced and Oxidized Glutathione

Immediately after the animals were killed, proximal interphalangeal joint segments (~2 cm) were harvested with the skin removed, rinsed with phosphate-buffered saline solution (pH 7.4), and kept frozen in liquid nitrogen. The proximal interphalangeal joint segments were weighed, ground to a powder under liquid nitrogen, and reconstituted in cold extraction buffer (50-mM phosphate buffer [pH7] containing 1-mM ethylenediaminetetraacetic acid per gram of tissue). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured with a glutathione assay kit (Catalog Number 703002; Cayman Chemical Company, Ann Arbor, Michigan) on the basis of the enzymatic recycling method[14]. The protein content in the tissue homogenate was measured with the Bradford test (Bio-Rad, Hercules, California). Tissue levels of GSH and GSSG were expressed as micromoles of glutathione per milligram of protein. As the reduced form of glutathione (GSH) is an essential molecule that participates in the reduction of hydrogen peroxides (H_2O_2), GSH has a critical role in the defence against oxidative stress. A decrease in GSH with an increase in GSSG indicates the presence of oxidative stress.

Ultrasound Imaging and Video-Assisted Gliding Test

The chicken feet were brought to room temperature before further processing for ultrasound imaging and the gliding test. The skin and the subcutaneous adipose tissue over the second phalanx of the long toe were removed, exposing the flexor sheath over the A2 pulley, and coupling gel was applied on top for ultrasound imaging. A Vevo 770 high-resolution ultrasound imaging system (Visualsonics, Toronto, Ontario, Canada) with an RMV 711 scan head (6-mm focal length and 30-μm axial resolution) was used to capture three-dimensional (3-D) ultrasound images for a total length of 12 mm with a step size of 32 μm. The acquired images were later examined with the built-in software to identify the A2 pulley, and the cross-sectional area of the flexor digitorum superficialis plus the flexor digitorum profundus was measured at 4 mm proximal to the A2 pulley by two independent sonographers according to methods described in our previous study[13]. The fibrotic response, tendon adhesions, and peritendinous reactions near the injured sites in all groups were also examined on the 3-D ultrasound images[13]. Histological examination was later performed to confirm the ultrasound observations. The chicken feet were then mounted to a custom-made testing jig for the gliding test with a mechanical testing machine (H25KM; Tinius Olsen, Salfords, United Kingdom) as described previously[13]. The maximum flexion angle and the gliding resistance were calculated from the force displacement curve. The extent of tendon adhesion in the injured flexor digitorum profundus tendon was presented as a percentage of the maximum flexion angle and the gliding resistance in the contralateral control. The chicken feet were kept for histological processing after the gliding test.

Histological Analysis

A joint segment (~20 mm long) including the A2 pulley was dissected from the long toe after the gliding test. The joint segments were fixed in 10% buffered formalin overnight, decalcified in 9% formic acid for two weeks, and embedded

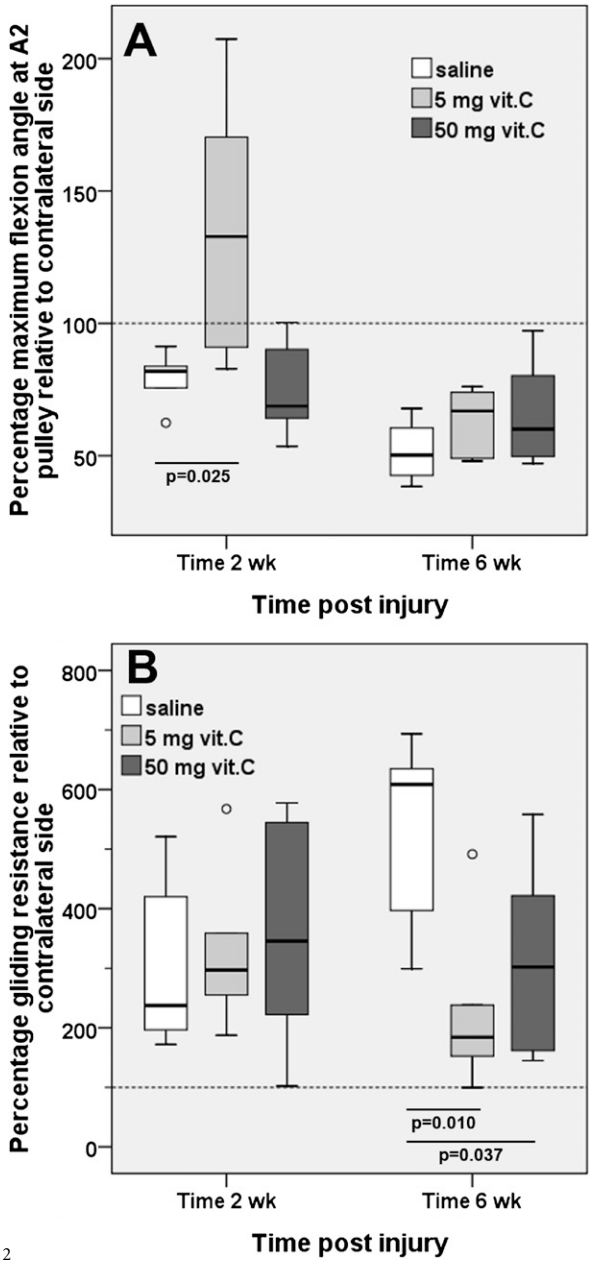

Fig. 2

The maximum flexion angle of the proximal interphalangeal joint and the gliding resistance were calculated to evaluate the extent of restrictive tendon adhesion. Local injection of 5-mg/mL vitamin C led to a significant increase in the maximum flexion angle at two weeks postinjury, whereas there was no significant difference among the groups at six weeks postinjury (**Fig. 2-A**). There was no significant difference in gliding resistance among the groups at two weeks postinjury, but a significant decrease in resistance was observed in association with both vitamin-C doses, as compared with the saline solution group, at six weeks postinjury (**Fig. 2-B**). In the boxplot, the top and bottom of the rectangles indicate the 75th percentile and the 25th percentile, respectively; the horizontal lines within the rectangles indicate the median; the top and bottom of the I bars indicate the 95th percentile and 5th percentile, respectively; and the circles indicate outliers.

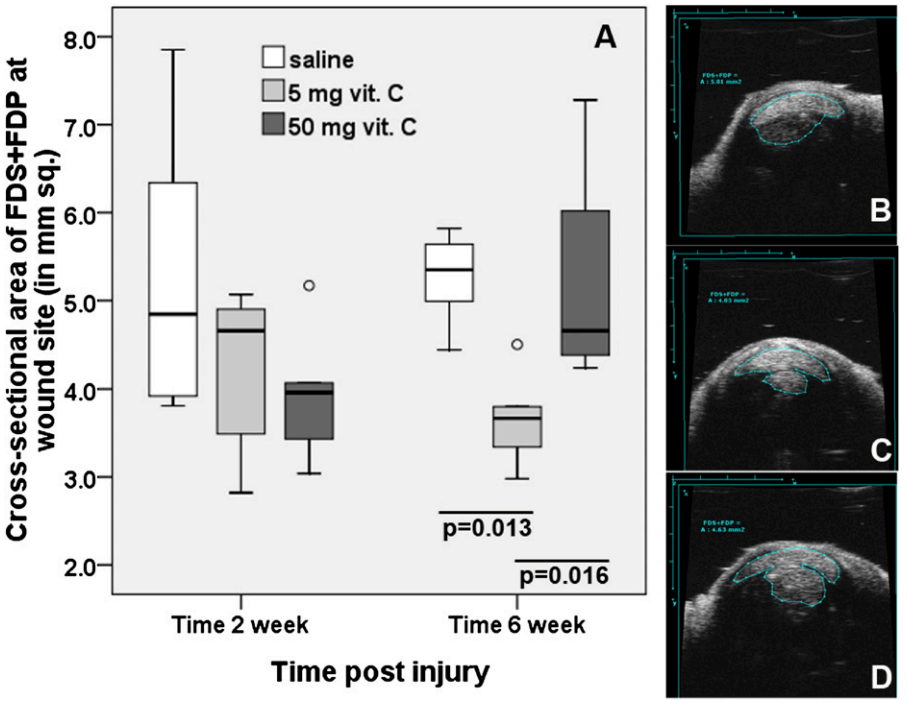

Fig. 3

The cross-sectional area of the flexor tendons (flexor digitorum superficialis and flexor digitorum profundus [FDS+FDP]) at the injured site was measured with 3-D ultrasound imaging to evaluate the magnitude of fibrosis. No significant difference was observed among the groups at two weeks postinjury, but there was a significant decrease in the 5-mg/mL vitamin-C group at six weeks postinjury, compared with both the saline solution group and the 50-mg/mL vitamin-C group. In the boxplot, the top and bottom of the rectangles indicate the 75th percentile and the 25th percentile, respectively; the horizontal lines within the rectangles indicate the median; the top and bottom of the I bars indicate the 95th percentile and 5th percentile, respectively; and the circles indicate outliers (**Fig. 3-A**). Substantial fibrotic tissues between the flexor digitorum superficialis and the flexor digitorum profundus were observed on the ultrasound images of the saline solution group (**Fig. 3-B**) and 50-mg/mL vitamin-C group (**Fig. 3-D**) but not in the 5-mg/mL vitamin-C group (**Fig. 3-C**).

in paraffin to prepare 10-μm sagittal sections for hematoxylin and eosin staining. The severity of tendon adhesion was qualitatively evaluated by examining the cellularity and fibrotic response of the healing tendon itself and the peritendinous region around the tendon.

Statistical Methods

Statistical analysis was performed with use of the Statistical Package for the Social Sciences (SPSS) 16.0 (IBM, Armonk, New York). Significance was set at $\alpha = 0.05$. The nonparametric Kruskal-Wallis test was used to perform multiple-group comparisons, and p values are reported. Two-group comparisons were performed with the Mann-Whitney U test with Bonferroni correction when necessary ($\alpha = 0.05/2 = 0.025$).

Source of Funding

Funding from the General Research Fund under the University Grants Committee in Hong Kong was used for expenses of this study.

Results

GSH and GSSG Assay

A decrease in the level of GSH at two weeks ($p = 0.050$) and six weeks ($p = 0.034$) postinjury and an increase in the level of GSSG at six weeks postinjury ($p = 0.034$) were detected in the tissue homogenates of the proximal interphalangeal joint segments with an injured flexor digitorum profundus, suggesting the presence of oxidative stress (Fig. 1-A). Local injection of vitamin C (5 or 50 mg/mL) significantly increased the GSH level at two weeks postinjury as compared with that in the saline solution group ($p = 0.050$), but the tissue level of GSSG was not affected (Fig. 1-B).

Gliding Test

At two weeks postinjury, the maximum flexion angle at the proximal interphalangeal joint was higher in the 5-mg/mL vitamin-C group as compared with that in the saline solution group ($p = 0.025$), but the 50-mg/mL vitamin-C group did not show significant improvement (Fig. 2-A). At six weeks postinjury, the maximum flexion angle was not found to have been significantly affected by vitamin-C treatment ($p = 0.173$). The vitamin-C treatments did not significantly affect gliding resistance at two weeks postinjury ($p = 0.692$). At six weeks postinjury, both the 5-mg/mL and the 50-mg/mL vitamin-C group had reduced gliding resistance as compared with the saline solution group ($p = 0.010$) (Fig. 2-B).

Ultrasound Imaging

Estimation of the fibrotic size of the injured tendons with ultrasound imaging did not reveal significant differences between the saline solution group and the vitamin-C-treated groups at

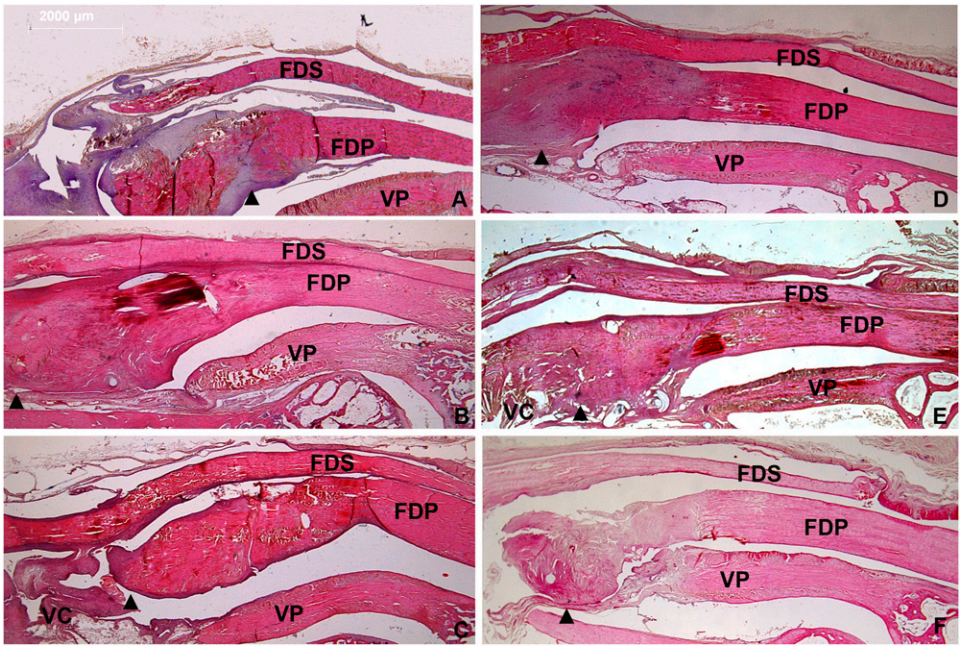

Fig. 4

Histological sections at two weeks postinjury reveal tendon swelling at the injured site in both the saline solution (**Fig. 4-A**) and the 5-mg/mL vitamin-C (**Fig. 4-B**) group. The swelling of the injured tendon at two weeks was less evident in the 50-mg/mL vitamin-C group, but more rigorous peritendinous reactions were associated with the vinculum (VC) in that group (**Fig. 4-C**). At six weeks postinjury, fibrosis and tendon adhesions were found between the flexor digitorum superficialis (FDS) and the flexor digitorum profundus (FDP) and between the flexor digitorum profundus and the volar plate (VP) in the saline solution group (**Fig. 4-D**). In contrast, fibrosis and tendon adhesion were not obvious at six weeks in the 5-mg/mL vitamin-C group, but the peritendinous reactions to the vinculum and volar plate were still noticeable (**Fig. 4-E**). Substantial fibrosis was also observed in the 50-mg/mL vitamin-C group at six weeks, but peritendinous reactions were less obvious (**Fig. 4-F**). Arrowheads = injured site.

two weeks ($p = 0.136$), but the 5-mg/mL vitamin-C group showed significantly reduced fibrotic size at six weeks postinjury ($p = 0.013$) (Fig. 3-A). Coronal sections extracted from 3-D ultrasound images at six weeks postinjury revealed extensive fibrosis and adhesions between the flexor digitorum superficialis and flexor digitorum profundus in the saline solution group (Fig. 3-B) and the 50-mg/mL vitamin-C group (Fig. 3-D), but not in 5-mg/mL vitamin-C group (Fig. 3-C).

Histological Examination

Histological examination showed that, at two weeks postinjury, there was swelling at the ruptured end in the saline solution group (Fig. 4-A) and 5-mg/mL vitamin-C group (Fig. 4-B) but to a lesser extent in the 50-mg/mL vitamin-C group (Fig. 4-C), in which tissue reactions were more notable in the vinculum. At six weeks postinjury, fibrosis and tendon adhesions were seen between the flexor digitorum superficialis and the flexor digitorum profundus and between the flexor digitorum profundus and the volar plate in the saline solution group (Fig. 4-D). In the 5-mg/mL vitamin-C group, the extent of fibrosis appeared to be reduced; tendon adhesions between the flexor digitorum superficialis and the flexor digitorum profundus were not obvious but the peritendinous reaction to the vinculum and volar plate was still observed on 3-D ultrasound imaging (Fig. 4-E). Treatment with 50 mg/mL of vitamin C also resulted in fibrosis, but peritendinous reactions were less evident (Fig. 4-F).

Discussion

Effects of Vitamin C on Tendon Adhesion

To our knowledge, this study is the first to test the ability of local supplementation with an antioxidant immediately postoperatively to reduce tendon adhesion. We showed that local vitamin-C injection immediately after surgical repair can reduce the development of restrictive tendon adhesion in the chicken model. As reactive oxygen species generated in the inflammatory phase may contribute to postoperative adhesions[15], we speculate that the ability of vitamin C to reduce tendon adhesion may be attributed to its antioxidant effect. Both 5-mg/mL and 50-mg/mL vitamin C restored tissue GSH levels in the vicinity of the injured tendons, but the lower dose (5 mg/mL) was more effective than the higher dose (50 mg/mL) in reducing the fibrotic size and gliding resistance. It is possible that too much vitamin C may remove the reactive oxygen species that are necessary for wound-healing, such as recruitment of reparative cells and angiogenesis[16]. This observation may appear to contradict the findings of a recent study by Omeroğlu et al.[17], who showed that a "high" parenteral dose of vitamin C (a single dose of 150 mg/day for rats with a body weight of 200 g) may improve tendon healing as characterized with histological analysis, but the dose response and bioavailability of vitamin C to the injured tendon were not reported. In fact, this "high" parenteral dose of vitamin C may not be sufficient to build up as high a local dose of vitamin C in the tendon wound as we reported in the current study because a

systematically administered megadose of vitamin C will probably circulate in the blood and be stored in various tissues[18]. In our chicken model, the high dose (50 mg/mL) corresponds to 280 mM vitamin C, which is much higher than the normal plasma level (40 to 80 μM) and tissue storage level (22 mg/kg).

Redox Modulation and Tendon Adhesion

Oxidative stress is defined as an imbalance between oxidative agents and antioxidants (redox status) by which a stressful oxidative condition is presented to biological systems. Injuries can lead to overproduction of reactive oxygen species and impose an oxidative stress to the injured area. Redox regulation conceivably plays a role in the healing of tendons with degenerative injuries, as shown by the oxidative stress induced by fluoroquinolones[19], overexpression of the antioxidant enzyme peroxiredoxin in degenerated human tendon[20], and in vitro studies on the vulnerability of tendon cells to oxidative stress[21]. It appears that a sufficient antioxidant defense is essential for tendon healing. Fillipin et al. showed that oxidative stress and fibrosis were reduced by laser therapy in a rat model of Achilles tendon injury[22]. In the present study, we observed a decrease in the GSH level at two and six weeks postinjury as well as an increase in the GSSG level at six weeks postinjury in the joint segment with the injured flexor digitorum profundus. This might reflect the presence of oxidative stress during fibrogenesis, as fibroblasts are capable of generating reactive oxygen species under the stimulation of phagocyte stimulants[23]. Vitamin C injected immediately after surgical repair prevented a decrease in the GSH level at two weeks after the operation and improved tendon gliding, indirectly suggesting that the formation of restrictive adhesion may be associated with altered redox modulation. Because vitamin C can be actively taken up by most cells in the forms of ascorbate or dehydroascorbate[24], the supplemental vitamin C can form a reserve to cope with oxidative stress at later time points. Alternatively, it is possible that vitamin-C supplementation may differentially affect cell survival or cell recruitment at early stages of tissue injury, which results in accumulation of different cell types with differing redox status. For example, phagocytes such as neutrophils and macrophages are reported to migrate to injured tendons[25], and reactive oxygen species can affect recruitment and survival of inflammatory cells to sites of injury[26]. Additional study is necessary to delineate the mechanisms of vitamin-C supplementation for modulation of restrictive tendon adhesion.

Limitations

There are several limitations of the current study. First, it is difficult to measure oxidative stress directly in healing tendons by measuring free radicals and/or oxidative damage, as the former may change transiently and the latter may occur only with excessive oxidative stress. As glutathione is central to many cellular antioxidant systems, GSH and GSSG levels can be used to reflect changes in redox status. Second, the location of oxidative stress was not identified. As formation of restrictive adhesions involves infiltrated cells, tendon cells, and peritendinous tissues in the vicinity of the injured sites, it is difficult to dissect and analyze the contribution of various tissues to the overall oxidative stress. Moreover, because a sham operation was not performed, it is difficult to distinguish whether the changes in glutathione levels were associated with the tendon injury or the wound in the tendon sheath and the skin; yet there is no doubt that the healing tendons were exposed to a local change in glutathione status during the formation of restrictive tendon adhesion. Finally, we did not assess the effect of vitamin-C supplementation on the mechanical properties of the healing tendon such as load to failure. As shown by histological examination, high-dose vitamin C may exert a negative effect on tendon healing. Further investigation, including measurement of ultimate strength and stiffness, is needed to identify the safe and effective dose range of postoperative vitamin-C supplementation.

Appendix

eA A figure demonstrating the animal grouping in the study is available with the online version of this article as a data supplement at jbjs.org. ■

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