Effects of corticosteroids and hyaluronic acid on torn rotator cuff tendons in vitro and in rats

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Running title

CS/HA Effects on RCTs

ABSTRACT

Purpose: Corticosteroids (CS) or hyaluronic acid (HA) is used in subacromial injection for the conservative treatment of rotator cuff tears (RCT); this study addresses the question of how CS and HA affect the tendon tissue and fibroblasts *in vitro* and *in rats*.

Methods: Cell proliferation assays were performed in human tendon fibroblasts from RCT. Rats underwent surgery to create RCT, and the surgical sites were injected with CS or HA. The rotator cuff tendons were subjected to biomechanical testing,

microscopic and immunohistochemical analysis of proliferating cell nuclear antigen (PCNA), and ultrastructural analysis.

Results: Cell proliferation was significantly decreased with CS *in vitro* ($P < 0.05$). Maximal load of CS-treated tendons was significantly decreased compared with that of HA-treated tendons ($P < 0.05$), as well as $PCNA^+$ cells at 2 weeks ($P < 0.05$). Ultrastructural observations of the CS-treated rats detected apoptosis of tendon fibroblasts 24 h after surgery. Histological and biomechanical data 4 weeks after surgery were not significant among the three groups.

Conclusion: Unlike HA, CS caused cell death and inhibition of the proliferation of tendon fibroblasts, leading to a delay of tendon healing involved and a subsequent decrease of biomechanical strength at the surgical site.

INTRODUCTION

Rotator cuff tears (RCT) occur in the middle-aged and elderly people and cause severe shoulder pain. The prevalence of RCT ranges from 5% to 40% in the general population,[1] increasing linearly with age from the third decade.[2–4] In clinical settings, initial treatment of RCT to alleviate shoulder pain and restore shoulder function

employs subacromial injection of corticosteroids (CS), administration of nonsteroid anti-inflammatory drugs, and physical therapy.[5]

Subacromial injection of CS is frequently administered to patients with RCT ,[6,7] because they reduce inflammation and provide long-term pain relief.[8-10] However, CS impair tendon healing and induce tendon ruptures.[11.12] Studies conducted *in vitro* have demonstrated that CS inhibit tenocyte proliferation[13,14] and deteriorate the biomechanical properties of the tendon involved, e.g., Achilles tendons of rabbits and rats[15,16] , rat peroneal tendons[17] and injured rat rotator cuff tendons.[18, 19] However, the mechanism of how CS affects deleteriously injured tendons *in vivo,* and the extent of its effects *in vivo* remains unclear.[20]

Hyaluronic acid (HA), a high molecular weight polysaccharide, is present in large amounts in the extracellular matrix of soft connective tissue and synovial fluids.[21] HA possesses various biological effects, e.g., chondrocyte protection, lubrication, an anti-inflammatory/adhesive effects.[22–24] HA inhibits the production of prostaglandin E2 by cyclooxygenase-2 by subacromial fibroblasts in a RCT through the CD44 receptor.[24] Consistent with these findings, subacromial injection of HA exerts a beneficial effect in patients with RCT.[25] Taken together with the data on CS, these results prompted us to compare the effects of these agents on the rotator cuff tendon with those of HA. We

hypothesize that unlike HA, CS weakens the biomechanical strength of the torn rotator cuff tendons, causing apoptosis of the tendon fibroblasts at the ruptured site. Thus, this study aimed to evaluate the effect of these agents on torn rotator cuff tendons *in vitro* and *in rats*.

MATERIALS AND METHODS

Study Design

Controlled laboratory study

The human study (*in vitro*) was approved by the authorized institutional review board of our institute (# 2456), and the animal study (*in vivo*) was approved by the Institutional Animal Care and Use Committee (#2014-189-1). Human specimens were acquired only after patients granted their informed consent. These specimens were used for in vitro experiments (cell proliferation assay and cell viability assay). Animal specimens were used for in vivo experiments (the biomechanical testing and histological, immunohistochemical, and electron microscopic analyses).

Cell Culture

Tendon specimens were collected from patients with RCT during surgery (N = 16) and were cut into small pieces. Inclusion criteria were patients with repairable rotator cuff tears. Exclusion criteria were patients with irreparable tears, glenohumeral arthritis, revision surgeries, fractures around the shoulder, or systemic disease. Tendon fragments were transferred to T-75 flasks containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Culture flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until the culture reached 80% confluence. The cells were then subcultured and used for experiments after the first passage.

Reagents

DMEM and L-glutamine were purchased from Nissui Seiyaku (Tokyo, Japan) and FBS was purchased from Thermo Scientific (Tokyo, Japan). CS (dexamethasone, 4.0 mg/ml) was purchased from MSD Co. (Decadron, Tokyo, Japan), and HA (10 mg/ml) was purchased from Kaken Co. (Artz Dispo., Tokyo, Japan). CS and HA were added to monolayers at concentrations of 0.01, 0.1, 0.5, and 1.0 mg/ml in DMEM containing 10% FBS.

Cell proliferation assay

Tendon-derived fibroblasts were seeded in 96-well plates at 5,000 cells/well and cultured in DMEM containing 10% FBS for 24 h. The cells were exposed for next 24 h to CS or HA in DMEM containing 10% FBS; as control, the cells were cultured in the medium alone (without agents). The relative number of viable cells in each well was determined using WST-8 reagent (Nacalai Tesque, Kyoto, Japan). WST-8 solution was added to each well, including eight wells containing medium alone to determine background absorbance. The cells were incubated at 37°C for another 4 h. The absorbance at 450 nm of each well was measured using a microplate reader (BIO-RAD Model 550, CA, USA).

Analysis of cell viability

Tendon-derived fibroblasts were seeded in 6-well plates at 1.5×10^5 cells/well and cultured in DMEM containing 10% FBS for 24 h. The cells were exposed for the next 24 h to 1.0 mg/ml CS or HA and only medium as a control. Floating cells and trypsinized adherent cells were combined and diluted to 1×10^6 cells/ml using 1× PBS. The components of a LIVE/DEAD Cell Viability Assay Kit (Invitrogen, OR, USA), C12-Resazurin and SYTOX GREEN, were added to stain viable and dead cells. After incubation for 15 min at 37°C, the numbers of viable and dead cells were obtained using

FACS machine (BD Bioscience, USA).

Animals

Sixty adult Sprague–Dawley male rats (mean body weight 540 ± 11.0 g) were used according to the guidelines of the Institutional Animal Care and Use Committee. To reduce the number of rats sacrificed and, we used the bilateral shoulder of 60 rats; consequently, the following 3 combinations were inevitable: 40 shoulders treated by CS or HA, 40 by CS or PBS, and 40 by HA or PBS. These 120 shoulders were further divided into 3 groups: CS-treated shoulders (N = 40), HA-treated shoulders (N = 40), and PBS-treated shoulders (as control; N = 40). Finally, these specimens were randomly allocated to each experiment at every time point. (Fig. 1).

Surgical Procedure

Animals were anesthetized with isoflurane under a high flow rate of oxygen. Full-thickness tears were introduced in the midportion of the supraspinatus (SSp) tendon in both shoulders. Full-thickness tear of the SSP tendon was made as totally resected as possible, using #11 blade and protecting the cartilage part at its insertion. Then, the myotendinous unit was immediately retracted. Before closure, 0.1 μl/g body

weight of CS/HA at a dose equivalent of that given to humans or PBS as a control was immediately applied onto the torn site. The animals were allowed to move freely in their cage after surgery and were sacrificed at 24 h and at 2 and 4 weeks after the surgery and analyzed as follows: eight specimens for biomechanical testing at 2 and 4 weeks, six specimens for histological analysis, and two specimens for electron microscopy.

Biomechanical testing

A total of 48 shoulders (16 shoulders in each group) were immediately tested after sacrifice. The SSp tendon–humerus complexes were placed into a uniaxial testing machine (TENSILON RTE-1210, Orientec, USA). The tendon was secured in a screw grip using sandpaper and ethyl cyanoacrylate. The humerus was secured in a custom-designed pod using a capping compound. The SSp tendon was secured to a load cell and was preloaded at 0.1 N, followed by five cycles of loading and unloading with 0.5 N at a cross-head speed of 5 mm/min and then loaded until failure at 1 mm/min. The ultimate load to failure and linear stiffness were calculated from the resulting load elongation curve. This testing protocol was similar to that described by Gulotta *et al.*[26,27]

Histological analysis

The SSp tendon–humerus complex was fixed in 10% buffered formalin and then decalcified. The specimens were stained with hematoxylin and eosin.

Immunohistological protocol

Immunostaining was performed using a monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Abcam, Tokyo Japan). After deparaffinization, antigen retrieval was conducted for 20 min at 70°C. Endogenous peroxidase activity was inhibited by incubation for 30 min at room temperature with methanol containing 0.3% H_2O_2. The primary antibody was incubated overnight at 4°C the secondary antibody (DAKO, K4000) for 30 min at room temperature. After antibody staining, the sections were stained with hematoxylin. Normal mouse IgG1 (DAKO, X0931) was used as a negative control. The processed sections were visualized at 10× magnification (OLYMPUS BX50, Tokyo Japan), and images were recorded using an Olympus DP71 (Olympus, Tokyo, Japan). Five fields of healing sites were selected randomly across each SSp tendon section. Tendon sample were analyzed in random order, and all histomorphometric analyses were performed in a masked fashion by two independent investigators. The $PCNA^+$ cells were counted three times using ImageJ software (NIH, USA). The average fraction of $PCNA^+$ cells was calculated as the percentage of $PCNA^+$

cells based on the reactivity of total tendon cells.

Ultrastructural analysis by low voltage scanning electron microscope (LV-SEM)

Using the wide range observation of the flat block face by LV-SEM, morphology of the tendon fibroblasts was analyzed 24 h and 2 and 4 weeks after surgery. Sprague–Dawley rats were deeply anesthetized, transcardially perfused through the left ventricle with heparin-containing saline and subsequently fixed with 50% Karnovsky's solution. After perfusion, the SSp tendon–humerus complexes were harvested and immersed in the same fixative for 2 h at 4°C. After decalcification, the specimens were cut into small cubes and fixed with ferrocyanate and 1% OsO_4, treated with 1% thiocarbohydrazide, and then immersed in 1% OsO_4. For *en bloc* staining, the specimens were immersed in a solution of 4% uranyl acetate overnight and washed with distilled water. The specimens were then stained with Walton's lead aspartate solution, infiltrated with an epoxy resin mixture, and polymerized. The resin blocks were trimmed to 2 mm × 4 mm in size, and contained the entire surgical site. Ultrastructural photomicrographs of various regions at the surgical sites were obtained from the block surface of the same specimen using LV-SEM, as material contrast images resembled the transmission electron microscopic images referred to as the block face image.[28] The images were obtained at the following

conditions; accelerating voltage = 2 kV, dwell time = 30 μs and the image size = 2048 × 1768.

Statistical analysis

Statistical analysis was performed using JMP version 10 (SAS, USA). Cell proliferation of the experimental groups is reported as the mean and standard error of the mean (SE) percentage luminescence of the control. Cell viability data acquired using FACS is reported as the mean (SE) percentage of viable cells. The data were analyzed using analysis of variance (ANOVA) with Tukey's *post-hoc* analysis. Values of *in vivo* experiments are expressed as the mean and SE. Biomechanical testing data and semiquantification of the percentage of cells that expressed PCNA and comparisons of the groups were performed using the Mann–Whitney U or Kruskal–Wallis tests. Differences with $P < 0.05$ were considered significant.

RESULTS

Cell proliferation assays

CS caused a significant decrease in the proliferation of human tendon fibroblasts in a

dose-dependent manner ($P = 0.64$ at 0.01 mg/ml, $P = 0.36$ at 0.1 mg/ml, $P = 0.0017$ at 0.5 mg/ml, $P < 0.001$ at 1.0 mg/ml). Significant differences were also detected between CS and HA in the percentage of proliferating cells except at 0.01 mg/ml (Fig. 2). There was no significant difference between HA and the control at all concentrations ($P = 0.96$ at 0.01 mg/ml, $P = 0.89$ at 0.1 mg/ml, $P = 0.90$ at 0.5 mg/ml, and $P = 0.86$ at 1.0 mg/ml).

Analysis of cell viability

The percentage of viable human tendon fibroblasts was significantly decreased in CS-treated cells (60.9 ± 9.2%) compared with HA-treated cells (87.5 ± 2.6%) and controls (86.9 ± 2.9%) ($P = 0.014$, $P = 0.021$, respectively) (Fig. 3). There was no significant difference between the viability of cells in the HA-treated and control cultures ($P = 0.998$).

Biomechanical testing

All the specimens failed biomechanical tests at the tendon-bone interface. The ultimate load to failure 2 weeks after surgery was significantly decreased in the CS group (11.3 ± 1.1 N) compared with the HA (19.8 ± 1.8 N) and control groups (20.9 ± 0.8 N) ($P =$

0.001), although no significant difference was detected between the HA and control groups ($P = 0.563$) (Fig. 4A). Four weeks after surgery, there was no significant difference among the three groups as follows: CS (19.0 ± 5.8 N), HA (20.1 ± 1.7 N), and control (22.5 ± 0.7 N) ($P = 0.350$).

On stiffness after the surgery, there was no significant difference among the three groups (CS: 10.9 ± 1.9 N/mm, HA: 16.9 ± 2.3 N/mm, control: 20.8 ± 3.0 N/mm at 2 weeks, $P = 0.067$) (CS: 16.5 ± 5.5 N/mm, HA: 17.2 ± 1.8 N/mm, control: 18.4 ± 3.3 N/mm at 4 weeks, $P = 0.627$) (Fig. 4B).

Microscopic analysis

Twenty-four hours after surgery, membranous tissue covering the torn site, and the edge of the torn tendon in the CS group showed edema in the stroma. Collagen bundles were irregularly arranged, indicating collagen necrosis in the tendon (Fig. 5). These effects were not detected in the HA and control groups, although the cellularity appeared to be similar among the three groups. Two weeks after surgery, the torn sites were consistently covered with abundant fibrovascular tissue at the extra-articular side, and tendinous continuity was noted in the three groups; however, the healing of tendinous tissue was more organized in the HA and control groups compared with the CS group,

suggesting a delay of the healing process in the latter group. Four weeks after surgery, the tendinous tissue was matured with a normal arrangement of the collagen bundles in all three groups.

Immunohistochemical analysis

Twenty-four hours after surgery, no significant difference in the numbers of $PCNA^+$ cells was observed among the three groups (data not shown), which was consistent with the corresponding histological cellularity described above. Although at 2 weeks, the number of the $PCNA^+$ cells at the injected site significantly decreased in the CS group compared with that in the other two groups (Fig. 6, $P = 0.005$). Four weeks after surgery, improvement was noted, and there was no significant difference in immunohistochemical reactivity among the three groups ($P = 0.796$).

Ultrastructural analysis

Twenty-four hours after surgery, collagen necrosis and apoptotic tendon fibroblasts (indicated by condensed nuclei) were observed at the edge of the torn tendon where CS was directly injected (Fig. 7A). Concurrently, apoptotic cells with disrupted membranes (secondary necrosis[29]) were observed at the same site (Fig. 7D). In the midtendon

distant from the torn site that was shielded from direct exposure, normal fibroblasts were present that were not apoptotic or necrotic and lacked collagen necrosis (Fig. 7C). Two and 4 weeks after surgery, no apoptotic or necrotic changes were observed in the CS group. Cell death and collagen necrosis as observed in the CS group were not detected at any time in the HA and control groups.

DISCUSSION

We show here that CS induced the death of tendon fibroblasts and inhibited their proliferation, decreasing biomechanical strength at the injected site with a delay of tendon healing in a rat model of RCT. Interestingly, the present study found cells exhibiting features typical of apoptosis and secondary necrosis with a disruption of the cell.[29] Although decreased cell proliferation and delayed healing in the CS group were noted 2 weeks after surgery, no significant differences were found among the three groups 4 weeks after surgery.

CS induces apoptosis of monolayer tendon fibroblasts *in vitro*.[30-32] An ultrastructural study shows that 0.1 nM dexamethasone induces the apoptosis of human tendon fibroblasts.[30] Analyses using fluorescence microscopy detected the apoptosis of tendon fibroblasts after exposure to 0.1 mg/ml triamcinolone acetonide[31] and 0.1–50 μg/ml

dexamethasone.[32] However, there are no similar studies conducted *in vivo*. Utilizing LV-SEM to analyze a wider area with higher resolution by block face imaging, the present study successfully detected tendon fibroblasts undergoing apoptosis at the edge of torn tendons with normal fibroblasts in the midtendon remote from the edge of the torn site. Further, these apoptotic changes were observed 24 h after surgery but not after 2 weeks. Thus, these results indicate that apoptosis-induced cell death is an adverse effect of CS that occurs early after its injection.

Tillander *et al.* demonstrated focal inflammation, necrosis, and fragmentation of collagen bundles in the rat tendon after five triamcinolone injections.[33] Balasubramanian *et al.* showed that after hydrocortisone injection into rabbit calcaneal tendons (5 mg/0.2 ml), collagenous necrosis and cell death of tendon fibroblasts were detected as early as 45 min after injection.[34] However, these studies did not address the mechanism of cell death. The present study demonstrates that CS induced apoptosis and secondary necrosis. The mechanism of action of CS that induces these lethal effects should be analyzed in future studies.

PCNA, which serves as an auxiliary protein of DNA polymerases, δ and ε, is essential for DNA replication and repair.[35] Inhibiting PCNA expression inhibits cell division, indicating that PCNA plays a pivotal role in cell proliferation.[36] CS interferes

with the early inflammatory phase of tendon healing as well as in the regenerative and remodeling phases.[15,18] An *in vitro* study revealed that decreased PCNA mRNA and protein expression is associated with the inhibition of tendon fibroblast proliferation by PCNA.[37] However, this was not addressed *in vivo*. In the present study, a significant decrease of $PCNA^+$ cells was detected 2 weeks after surgery and was accompanied by a significant decrease of biomechanical strength at the site. Taken together with the histological findings, we conclude that the inhibition of tendon healing by CS continued for at least 2 weeks after its injection.

In vitro studies demonstrate that HA possesses anti-inflammatory and anti-adhesive activities in tendon and synovial fibroblasts derived from RCT,[24,38,39] although they have not been studied *in vivo*. In contrast to CS, HA caused no adverse effects in the present study. Rather, a previous study found that HA accelerated tendon-bone healing in a rotator cuff model in rabbits (in Japanese). Therefore, HA appears to be relatively safe compared with CS when administered by intra-articular injection into RCT.

Limitations of the present study are as follows. First, the acute injury model employed weight-bearing forelimbs of rats, which differs from humans with chronic tendinopathy. However, the rat shoulder serves as *in vivo* model for studying human rotator cuff disease because of the close anatomical similarities of the rat and human

shoulders.[18, 19] Second, bilateral surgeries were performed in the present study, so that local CS injection may have caused systemic effects. However, the present study demonstrated a significant difference between CS and HA groups. In addition, the amount of CS used was calculated to those applied in humans; therefore, we thought the systemic effect may have been minimal. A strength of the present study is the evaluation of the effects of CS and HA on tendon fibroblasts *in vitro* and *in rats*. Third, "tear and subsequent healing" as seen in this study does not occur clinically. However, we previously showed healing capability of the torn tendon edge in patients with rotator cuff tears, detecting procollagen types I &III gene expression at the site.[40,41] These procollagen gene expression at the edge of the torn tendon was significantly correlated with the re-tear after surgery.[42] It is well known that CS/HA is usually administered preoperatively and not postoperatively. The present study therefore focused on the effect of CS/HA on the healing capability in rotator cuff tear without "repair." Although the animal model used in this study may have not fully represented the situation in clinical settings, we were able to demonstrate the clear difference between CS/HA, regarding the healing capability at the torn site. Fourth, power analysis was not performed in this study, although we demonstrated a certain significant difference between CS and HA biologically and biomechanically. Fifth, we only used male rats and had no information

on the response of female rats.

In conclusion, we demonstrated *in vitro* and *in rats* that unlike HA, CS caused cell death and inhibited the proliferation of tendon fibroblasts, leading to a delay of tendon healing and a subsequent decrease of biomechanical strength in the rat model; however, these changes improved 4 weeks after surgery. Therefore, clinicians should recognize the different characteristics of these agents when treating RCT.

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FIGURE LEGENDS

Figure 1. Flow diagram showing how the rats were divided into three groups and the evaluations at three times. CS: corticosteroids; HA: hyaluronic acid; Histo: histological analysis; SEM: scanning electron microscopy; MT: mechanical testing.

Figure 2. Histogram showing mean fibroblast proliferation (%) (error bars represent the standard error) after treatment with CS and HA at various concentrations. Significant differences were also noted between CS and HA ($P = 0.42$ at 0.01 mg/ml, $P < 0.001$ at 0.1–1.0 mg/ml). The asterisk indicates a significant difference ($P < 0.001$). CS: corticosteroids; HA: hyaluronic acid.

Figure 3. Histogram showing mean fibroblasts viability (%) (error bar represents the standard error) after treatment with 1 mg/ml CS and HA. The number of viable cells were significantly decreased in the CS-treated cultures (viable cells: 60.9 ± 9.2%) compared with the controls (86.9 ± 2.9) ($P = 0.0206$). No significant difference between HA and control was noted ($P = 0.998$). The asterisk indicates a significant difference ($P < 0.05$). CS: corticosteroids; HA: hyaluronic acid.

Figure 4. (A) Histogram showing mean ultimate load to failure (N) of the healing tendon after treatment with CS, HA, and PBS (N = 8). The ultimate load to failure 2 weeks was significantly decreased in the CS group ($P = 0.001$) compared with the other groups, although no significant difference was observed among the three groups 4

weeks after surgery ($P = 0.35$). The asterisk indicates a significant difference ($P < 0.05$). (B) Histogram showing mean stiffness (N/mm) of the healing tendon after treatment with CS, HA, and PBS (N = 8). There was no significant difference among the three groups 2 and 4 weeks after surgery. CS: corticosteroids, HA: hyaluronic acid.

Figure 5. Histological findings at the surgical site after 24 h, and 2 and 4 weeks after exposure to CS, HA, or PBS (hematoxylin and eosin stain, 10×). E: Extra-articular side; I: Intra-articular side; P: Proximal side; and D: Distal side. In the CS-treated group, the irregularities of the tendon surface and collagen bundle was observed 24 h after surgery, and the healing process appeared to be delayed compared with the other groups 2 weeks after surgery.

Figure 6. Histogram showing the mean percentage of $PCNA^+$ cells (error bar represents the standard error) of the healing tendinous tissue after treatment with CS, HA, or PBS (N = 6). The percentage of $PCNA^+$ cells was significantly lower in the CS group compared with the other groups 2 weeks after surgery ($P < 0.05$). CS: corticosteroids; HA: hyaluronic acid.

Figure 7. LV-SEM ultrastructural micrographs of surgical sites 24 h after surgery. (A) Collagen necrosis and tendon fibroblasts with condensed nuclei (black arrow) indicating apoptosis was observed at the edge of the torn tendon where CS was directly injected. (B) Entire view of surgical site observed. Empty square indicates the edge of torn tendon. (C) In the midtendon remote from the torn site that was protected from direct exposure, normal fibroblasts were observed without apoptotic or necrotic cells or collagen necrosis (from B). (D) Apoptotic cells with membrane rupture (secondary necrosis) at the same site (white arrow) (from B).