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Silver nanoparticles alter proteoglycan expression in the promotion of tendon repair

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Abstract

This study demonstrates a novel method of using silver nanoparticles for Achilles tendon injury healing. In vitro results indicated a stimulatory effect on cell proliferation and collagen synthesis with silver nanoparticles. Biomechanical test on the 42-day post operation Achilles tendon sample exhibited a significant improvement in tensile modulus when compared to the untreated group. Histology suggested that silver nanoparticles promoted cell alignment and proteoglycan synthesis. The collagen deposition was also improved. An alleviation of tumor necrosis factor α, and an increase in fibromodulin and proliferating cell nuclear antigen expression were seen in silver nanoparticles group by immunohistochemistry. This study further corroborates the finding of our previous study that silver nanoparticles help to restore the functionality of injured connective tissues. We believe that the anti-inflammatory nature of silver nanoparticles has an important role in accelerating the healing process and reducing scarring, leading to better functional outcome.

Key words: Silver nanoparticles; Achilles tendon; Healing; Proteoglycan; Fibromodulin

Introduction

The healing of tendon after rupture remains a significant problem in modern medicine. The hurdle comes from the formation of scar tissue which leads to the weakening of the healed tendon, and consequently being prone to re-rupture. In addition, due to low metabolism in mature tendons, healing is usually a slow process in the adult population.[1,2] Clinically, the healing of Achilles tendon usually takes 4-8 weeks but a full return to sport activities is only recommended after a long span of 4-12 months. The treatment options for acute Achilles tendon rupture include surgical repair and conservative treatment. For surgical approach, the injured tendon is approximated and sutured. The affected leg is usually immobilized for a short period of time before motion is allowed in order to prevent early re-rupture. For conservative treatment, the tendon is immobilized in a cast for about 6 weeks to allow for natural healing. The surgical approach is notorious for its association with postoperative complications, but it remains the widely adopted method for Achilles tendon repair because conservative treatment is believed to yield an unacceptable re-rupture rate and long recovery time.[3] The trade-off between re-rupture rate and surgical complication rate has always been controversial. Indeed, a number of meta-analysis studies have been performed and there is currently no evidence favouring either method when functional rehabilitation is adopted following conservative treatment.[3,4] Hence, the holy grail in tendon healing remains a

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less invasive treatment approach, with improvement in the functional outcome while reducing healing time and complications. Many recent studies used growth factors,[5–7] gene therapy[8–10] and tissue engineering[11,12] as alternative methods, however, none of these emerging strategies are well validated.[13]

Silver has long been used in the history of medicine as an antimicrobial agent. With the advances in nanotechnology, silver can now be fabricated into the nanometer size range particles with novel physiochemical properties. Our previous studies have demonstrated that silver nanoparticles (AgNPs) is an excellent agent in wound healing. AgNPs not only exert antimicrobial effect, but are also capable of accelerating burn wound healing,[14] reducing wound inflammation[15] and modulating collagen deposition and alignment.[16] They also encourage fibroblast differentiation in vitro.[17] Since fibroblasts play an important role in the process of healing in both skin and tendon, it is hypothesized that AgNPs would help in tendon injury healing.

In this study, we aimed to investigate the effects of AgNPs in the early phase of the tendon healing process. The early phase of healing was emphasized because it is a critical stage that affects the healing quality and allows early load bearing. The objectives of this study were: 1) To investigate the in vitro response of primary tenocytes to AgNPs in proliferation and the production of collagen and proteoglycans (PGs); 2) To study the regeneration of rat Achilles tendon in vivo in terms of tensile property, histology; and 3) To examine the in vivo toxicity of AgNPs.

Methods

Silver nanoparticles fabrication

The AgNPs was fabricated by sodium borohydride reduction of 0.1 mM silver nitrate and 0.7 mM sodium citrate. The mixture was stirred overnight before concentrating into a 50 ml of 1 mM AgNPs solution by rotary evaporation. 100 mg/ml of polyvinylpyrrolidone (PVP) was added to stabilize the solution.[18] The size of the AgNPs ranged between 5 and 10 nm and was confirmed by transmission electron microscopy (FEI Tecnai G2 20 S-TWIN).

Primary cell culture

Primary tenocytes were harvested from the Achilles tendon of 4 week-old Sprague Dawley rats. Briefly, after sacrifice, the tendons were cut into small pieces and digested in 2.5 mg/ml collagenase I (Worthington) for 3 h at 37 °C. The cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% fetal bovine serum and were discarded after passage 5. Medium was changed every 3 to 4 days.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Primary tenocytes were seeded at a density of 5×10^3 on a 96 well plate. AgNPs of 6 different concentrations (100 μM, 40 μM, 20 μM, 10 μM, 1 μM and 0.1 μM) were added after 24 hours and cultured until experimental time point on day 1, 3 and 7. 0.5 mg/ml of MTT solution (Sigma-Aldrich, USA) was added to the culture on day 1, 3 and 7 and was incubated for 4 hours before adding sodium dodecyl sulphate for solubilization. Reading was taken at a wavelength of 570 nm with reference at 650 nm after overnight incubation.

Table 1
Surgical and treatment procedure of different experimental groups.

Treatment group	Tendon transection	AgNPs injection
Sham	No	No
AgNPs	Yes	0.1 ml of 1 mM, s/c, 5-day interval
Untreated	Yes	No

Sirius red fast green staining of collagen content

2×10^4 of primary tenocytes were seeded in triplicate on a 24 well plate. Sirius red fast green staining was performed on day 7 and 14 to determine the collagen content. Silver nanoparticles of different concentrations (20 μM, 10 μM and 1 μM) were added to the medium 24 hours after seeding and replenished at 7-day intervals. Cells were stained with the Sirius red/fast green dye (Sigma-Aldrich, USA) for 30 min and the dye was then extracted with 0.1 N sodium hydroxide (Sigma-Aldrich, USA) and methanol and read at OD540 and OD605 with the Multiskan GO microplate spectrophotometer (Thermo Scientific, USA).[19] The collagen (Sirius red) to non-collagen proteins (Fast green) ratio was then calculated.

Bromodeoxyuridine (BrdU) labeling assay

5×10^3 of primary tenocytes were seeded in triplicate on a 96 well plate and AgNPs were added 24 hours later in the concentrations of 20 μM, 10 μM, 1 μM and 0.1 μM. The assay was performed on day 1, 3 and 7. On the appropriate day, 10 μM BrdU was added to the culture and incubated for 4 hours. Cell proliferation was detected with the BrdU ELIZA (colorimetric) kit (Roche, CH).

In vivo rat Achilles tendon injury model

4 week-old Sprague Dawley rats were obtained from the Laboratory Animal Unit, the University of Hong Kong. The experimental protocol was approved by the Committee of the Use of Live Animals in Teaching and Research, the University of Hong Kong (CULATR 2320–11). The surgical procedures were as follows: Skin preparation was done before surgery. A 1 cm longitudinal cut was made on the right leg above the calcaneus and the Achilles tendon was exposed. The Achilles tendon was transected at 0.5 cm from its insertion. The wound was closed with 4/0 non-absorbable nylon sutures. The injured tendon was then treated with either 0.1 ml of 1 mM AgNPs injection at 5-day intervals or left untreated until sacrifice on day 10, 21 and 42. Skin incision was done on the left leg without transecting the tendon as the sham control. The grouping is shown in Table 1. The sample size of each group was four for statistical significance.

Tensile test

The tensile property of the day 42 samples (n = 4) was measured using the MTS 858 Mini Bionex with 100 N load cell at a strain rate of 1 mm/min. The cross sectional area of the samples was assumed to be rectangular and dimensions of the samples