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Long-term anti-inflammatory efficacy in intestinal anastomosis in mice using silver nanoparticle-coated suture

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Abstract

Background
In our previous study, we coated silver nanoparticles (AgNPs) onto the surface of absorbable braided suture using layer-by-layer deposition and demonstrated significant anti-inflammatory property during the early phase of intestinal anastomosis healing in mice. The present study aimed to further investigate the long-term anti-inflammatory efficacy.
Methods
AgNP-coated suture, antibiotic coated suture, and normal suture were respectively used for single layered, interrupted intestinal anastomosis. The anastomotic segments in each group were harvested on day 14, day 21, and day 28 postoperation and investigated for the degree of inflammation by cell infiltration and expression of cytokines as well as collagen deposition.
Results
When compared with the control groups, the AgNP-coated suture group showed better histological appearance in the intestinal anastomotic segments at each time point. Immunohistochemistry staining and quantitative evaluation further indicated less macrophage infiltration and decreased production of IL-6, IL-10, and TNF-α ($p < 0.05$). Masson staining showed normal collagen deposition and remodeling at intestinal anastomotic tissue in the AgNP-coated suture group.
Conclusion
Our study shows that AgNP-coated suture provides better long-term anti-inflammatory efficacy and ideal tissue remodeling in intestinal anastomosis. Despite these findings, clinical trials are still needed for evaluation before medical application.

Key words

Silver nanoparticlesSuturesAnti-inflammatoryRemodelingIntestinal anastomosis

Silver nanoparticles (AgNPs) are nanoscale size pure silver containing 20–15,000 silver atoms and measuring less than 100 nm in diameter [1], [2]. Many studies have suggested they have excellent in vitro anti-inflammatory properties. Furthermore, the anti-inflammatory action induced by AgNPs was found to accelerate tissue repair and regeneration in various animal models, including ulcerative colitis and skin contact dermatitis [3], [4]. Our previous studies also supported these findings in skin wound healing [5], and in the reduction of peritoneal adhesions [6], because of the inhibition of inflammatory cell infiltration and suppressed production of proinflammatory cytokines.

Based on these, we further tested the coating of Vicryl sutures (Polyglactin 910) (Ethicon, Somerville, NJ) with AgNPs using layer-by-layer method. We found that AgNP-coated suture had more prolonged in vitro antibacterial effect when compared to commercial antibiotic-coated suture. AgNP-coated suture was shown also to have excellent anti-inflammatory action with better mechanical strength at anastomotic site in an intestinal anastomosis model in mice [7]. Despite these exciting findings, the efficacy during the late phase of intestinal tissue healing was not known. The aim of the present study was thus to further investigate the long-term anti-inflammatory efficacy induced by AgNP-coated suture.

1. Materials and methods

1.1. Preparation of silver nanoparticles

Polymethacrylic acid (PMA), polydiallyldimethylammonium chloride (PDADMAC), silver nitrate (AgNO3), and sodium chloride were purchased from Sigma-Aldrich Ltd. (St. Louis, MO). All solutions were adjusted to a value of pH 7 with 1 mM sodium acetate and stored at room temperature.

The preparation of silver nanoparticle (AgNPs) solutions was same as previously described [7]. Briefly, equivalent volume of AgNO3 and PMA solutions were mixed followed by photoinduced reduction under UV lamp for 4 h to prepare solution A. The color of solution A would change from pink to red because of the formation of AgNPs. PDADMAC solution was diluted into 1 mM working solution with 1 mM sodium acetate and set as solution B. Solutions A and B were used for fabrication of AgNP-coated sutures.

1.2. Fabrication of AgNP-coated sutures

6-0 Vicryl® suture (Ethicon) and Vicryl Plus® (with antibiotics) were purchased from Ethicon Ltd. and set as controls. Layer-by-layer deposition method was used to fabricate AgNP-coated Vicryl sutures as previously described [7], [8]. The AgNP-coated sutures were dried overnight after 20 circles of coating. The amount of AgNPs coated was measured with spectrophotometer and the distribution of AgNPs immobilized on suture was studied by scanning electron microscope. The three groups of sutures were stored at room temperature before animal experiments.

1.3. Animal experiment

The experimental protocol in this study was approved by the Committee of the Use of Live

Animals in Teaching and Research, The University of Hong Kong (CULATR 1599-08). C57BL/6 N mice, weighing between 20 ± 2 g and ranging in age from 6 to 8 weeks, were provided by the Laboratory Animal Unit, The University of Hong Kong. Mice were randomized into three groups (AgNP-coated suture, antibiotic-coated suture and normal suture groups, 7 mice/group). Intraperitoneal injection of pentobarbital sodium solution (Abbott Laboratories, Abbott Park, IL) at a dose of 50 mg/kg was used for anesthesia. All animal experiments were performed by the same investigator. For the intestinal anastomosis, the ileum (2 cm from the cecum) was cut with scissors after the abdomen was opened. The ends were then closed with single layer, interrupted anastomosis using 6-0 suture in each group. The abdomen was then closed by mass closure. All the mice were given analgesia and allowed free access to water and diet after surgery.

1.4. Histological staining

The animals were sacrificed on day 14, day 21 and day 28 postoperation and the anastomotic site in each group was harvested. The specimens were formalin-fixed and embedded in paraffin. 4 μm-thick sections were taken, dewaxed and rehydrated for hematoxylin and eosin (H&E) staining.

For immunohistochemistry staining, endogenous peroxidase was quenched by 3% hydrogen peroxide/methanol after sections were deparaffinized and rehydrated, followed by incubation at room temperature with blocking solution containing 5% normal goat serum (Dako Bioresearch, USA). For antigen retrieval in tissues, the sections were blocked for nonspecific binding solutions containing 5% concentration of normal goat serum before primary antibody was added [7]. The sections were incubated overnight before rinsing in phosphate-buffered saline (PBS), and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Positive signals were developed using DAB (3,3′-diaminobenzidine) and counterstained in hematoxylin solution, followed by photographing microscope. The degree of inflammatory cell infiltration was assessed by a researcher who was blinded to the experimental groups.

1.5. Masson staining

The anastomotic segments from each group were collected and processed as above. Masson trichrome staining was performed to observe density and distribution of collagen at anastomotic tissue. In brief, the rehydrated sections were mordant in preheated Bouin's solution at 56 °C for 10–15 min, followed by washing in tap water to remove extra dye. Slides were immersed in working Weigert's iron hematoxylin solution for 5 min followed by rinsing. They were in tandem stained with Biebrich scarlet-acid Fucshin solution, phosphotungstic/phosphomolybdic acid solution and Aniline Blue solution for 5 min respectively. Finally slides were put in acetic acid (1%) for 2 min, followed by rinsing in tap water, dehydration with alcohol (from 70%, 95% to 100%), clearance in xylene and mounted.

1.6. Morphometric evaluation

Digital software (Image-Pro plus 6.0; Media Cybernetics) was used to quantify immunohistochemistry staining in anastomotic intestinal tissues. The average optical density in

stained area was determined in five random images with 400 × magnification. The numeric data obtained from the image analysis were exported for statistical analysis. The ratio between average optical density of tissue in each group and normal ileum was calculated for evaluation.

1.7. Statistics

Statistical analyses were conducted using Student's paired t test. A p value < 0.05 was considered significant. The results showed the average value ± standard deviation.

2. Results

2.1. Anastomosed intestinal tissue using AgNP-coated suture showed better macroscopic appearance at the late phase of healing

As edema and hyperemia are the most frequent pathological reactions after tissue inflammation, we assessed and recorded the gross morphology of the intestinal suture site at each time point when the abdomen was reopened. Although there was no significant peritoneal adhesion in each group, we did find less edema and hyperemic reaction in the AgNP-coated suture group in each time point when compared with the antibiotic suture and control groups, especially at postoperative day 28 time-point. Better macroscopic morphology with appearance resembling normal intestinal tissue could be observed in the AgNP-coated suture group in contrast to other two groups (Fig. 1). These would suggest, at least at the macroscopic level, that the AgNP-coated suture group had resulted in reduced inflammatory responses at the late phase of anastomosis and could contribute to “more physiological” intestinal tissue healing after wounding.

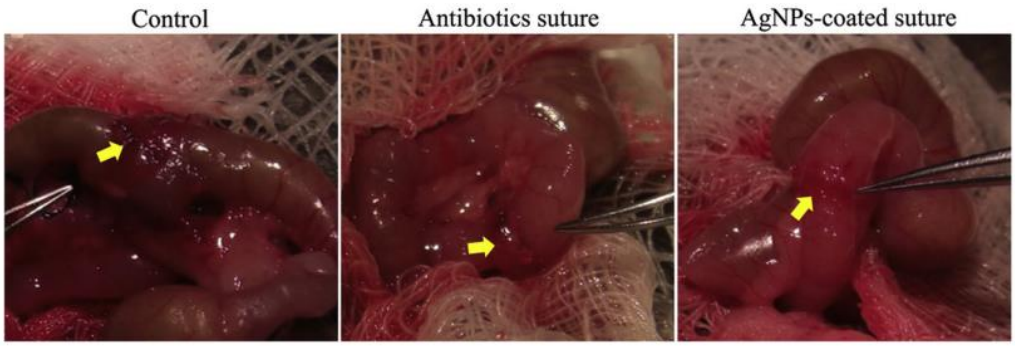

Fig. 1. Photographs showing macroscopic appearance of intestinal anastomosis (arrow) in the AgNP-coated suture group, antibiotic suture group and control group, on postoperative day 28.

2.2. Reduced inflammatory response with less macrophage infiltration seen in the AgNP-coated suture group

Segments of intestinal anastomosis in each group were harvested and serial sections were subjected for histological evaluation. H&E staining showed that proliferated serosal tissue was found in the control, antibiotics suture and AgNP-coated suture groups. Nonetheless, the proliferative tissue response was less in the AgNP-coated suture group in each time point, in contrast to the control or antibiotics suture groups (Fig. 2A) (images from postsurgical day 14 and day 28 were not shown).

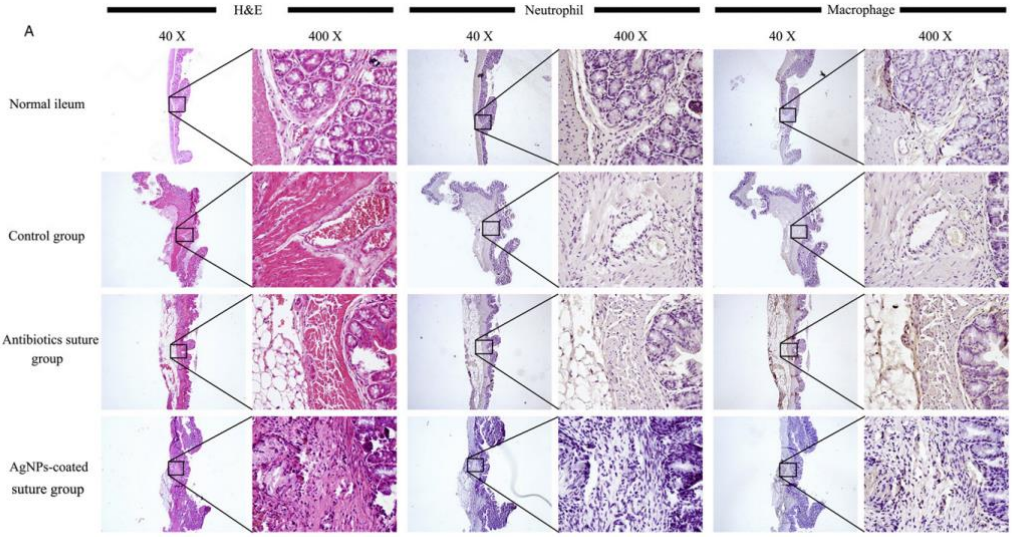

Fig. 2. Anti-inflammatory efficacy of AgNP-coated suture. (A) H&E and immunohistochemistry staining were done for neutrophil and macrophage infiltration around the anastomotic site on postoperative day 21. (B) Calculated optical density ratio of tissue in each group when compared to normal ileum on postoperative days 14, 21, and 28.

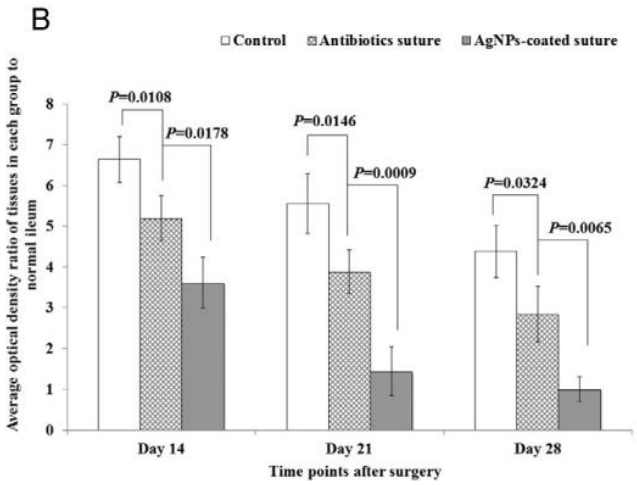

Fig. 2. (*Continued*)

We next investigated the anti-inflammatory efficacy of AgNP-coated suture. Immunohistochemistry staining was conducted on the three time points (postsurgical day 14, day 21 and day 28) for inflammatory cell infiltration. We did not find neutrophil infiltration around anastomotic tissues in any of the time points for each group. However, the AgNP-coated suture group had the least macrophage infiltration when compared to the control and antibiotics-suture group, and showed the most resemblance to normal ileum tissue (Fig. 2A). These results suggested that AgNP-coated suture could effectively decrease inflammatory cell infiltration over the long term. In order to specifically quantify the differences in each group, we calculated the average optical density ratio of tissue in each group to normal ileum. Our results confirmed that macrophages were the least in the AgNP-coated group ($p < 0.05$) (Fig. 2B).

2.3. Decreased proinflammatory cytokine expression at the late phase of healing in the AgNP-coated suture group

In order to further confirm the anti-inflammatory efficacy of AgNP-coated suture, we next looked at the proinflammatory cytokines expression. Here, we observed a higher amount of

IL-6 around anastomotic tissues in the control and antibiotics suture groups, as compared to the AgNP-coated group (Fig. 3). We next investigated expression of IL-10 and TNF-α, which showed similar trend to IL-6 expression, with more IL-10 and TNF-α in the control group and least in the AgNP-coated group (Fig. 3).

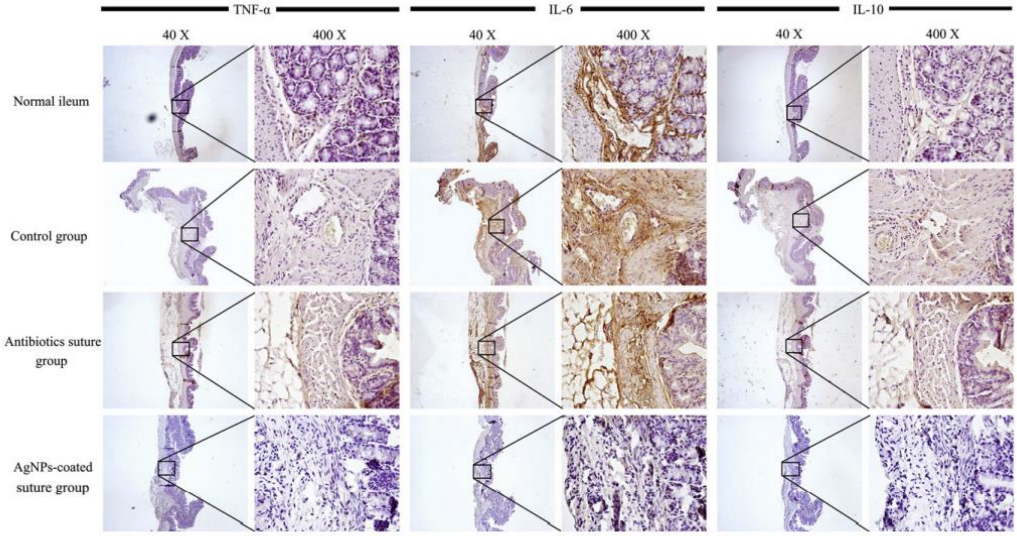

Fig. 3. Decreased proinflammatory cytokine expression at the late phase of healing in the AgNP-coated suture group. Immunohistochemistry staining was done for IL-6, IL-10 and TNF-α on postoperative day 21, comparing the AgNP-coated suture group, antibiotic suture group, control group and normal ileum.

Similar to the previous section, we calculated the average optical density ratio of tissue in each group to normal ileum and compared the differences in each group. The optical density results mirrored that of immunohistochemistry, with more IL-6, TNF-α and IL-10 seen around anastomotic tissue in the antibiotics suture group when compared with that in the AgNP-coated suture group, but less than in the control group at all time points ($p < 0.05$) (data not shown). Taken together, this would further support that AgNP-coated suture could effectively reduce the expression of proinflammatory cytokines up until the late phase of intestinal tissue healing.

2.4. AgNP-coated suture group showed better collagen deposition in the late phase of healing

As we showed that AgNP-coated suture could induce better long term in vivo anti-inflammatory efficacy, we next investigated if more extracellular matrix (ECM) would be produced during the late phase of healing. Our results showed that more collagen deposition in the AgNP-coated suture group was observed (Fig. 4). This trend could be seen even in the postsurgical day 28 specimens (data not shown).

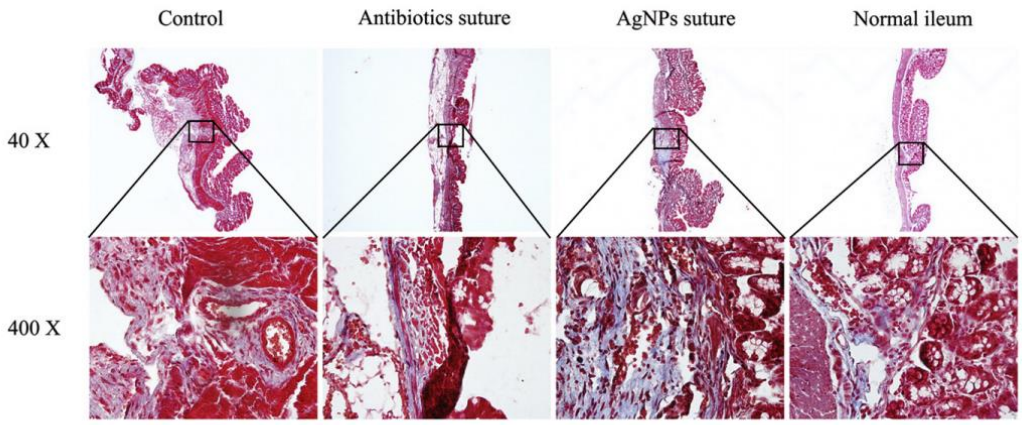

Fig. 4. AgNP-coated suture group showed better collagen deposition in the late phase of healing. Immuunohistochemical staining for collagen was done for the AgNP-coated suture group, antibiotic suture group and control group during the late phase of healing on day 28.

3. Discussion

The tissue healing process is characterized by coagulation, inflammation, formation of granulation and regeneration, as well as tissue remodeling. Inflammation plays an essential role in all the events [9], [10]. Nonetheless, a persistent inflammatory response will delay wound healing because of inflammatory cell infiltration and up-regulation of proinflammatory cytokines. Thus, some anti-inflammatory agents, including silver nanoparticles (AgNPs), have been used to promote healing [2], [5], [6], [11], [12], [13]. Indeed, our previous study provided evidence that AgNP-coated suture had superior anti-inflammatory efficacy on intestinal anastomosis, at least in the early phase of healing [7]. This study continued on the same theme, but investigated the late time points in tissue healing. As our results showed, we again confirmed the anti-inflammatory action induced by AgNP-coated suture. We also provided evidence that the long-term action of AgNP-coated suture had superior efficacy on intestinal tissue healing.

Our previous study showed that AgNP-coated suture could effectively inhibit both neutrophils and macrophage at the early phase of healing, while the present study indicated the reduction of macrophages at the late phase of healing only. This difference in inflammatory cell infiltration seems to correlate well with the temporal expression of neutrophils, which are seen in the early phase during the inflammatory response [14], [15]. Furthermore, the persistent anti-inflammatory action induced by AgNP-coated suture in the late phase was also proven by the suppression of inflammatory cytokines.

Taken together the results of our early study and this study, we propose two possible mechanisms which may explain the long-term anti-inflammatory efficacy of AgNP-coated suture. First of all, the silver release of AgNP-coated suture might only occur during the early phase of healing, but the powerful reduction of local inflammation contributed to a better local microenvironment for tissue repair and regeneration. Another possibility is that AgNP-coated suture released pure silver in a sustained fashion, which led to powerful anti-inflammatory action in local anastomotic tissue. To answer which hypothesis is correct, we need to perform drug release studies in the future. We may also need to evaluate the amount of AgNPs in the intestinal tissue at different time points.

Our in vivo experiments provided an unexpected finding of increased collagen production in the AgNP-coated suture group. This, on one hand, would suggest that excessive inflammation could indeed impair tissue healing. On the other hand, controlled inflammation would also contribute to tissue repair and regeneration, as well as tissue remodeling. Taking these in mind, the use of AgNP-coated suture may provide an effective solution in the clinical setting.

As we highlighted in our previous study, so far there is no report regarding the in vivo toxicity induced by AgNPs in the literature. Nonetheless, we have not found any evidence of cytotoxicity induced by AgNPs [1], [7], [9]. This, on one hand, could be attributed to the very small amount of silver used. On the other hand, any AgNPs applied would be rapidly diluted by the surrounding body fluids and thus any potential toxicity reduced.

In summary, we have shown that AgNP-coated suture provides better anti-inflammatory efficacy in the long term, and may be an ideal suture product to be used for anastomosis and tissue repair. This study should provide a solid basis for future clinical trial.

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