Electrospun Resorbable Drug-eluting Nanofibers Promote Healing of Allograft Tendons

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Abstract

We developed biodegradable drug-eluting nanofibers employing an electrospinning technique, and evaluated their effectiveness on the healing of allograft tendon. Poly-D-L-lactide-glycolide was used as the polymeric material for nanofiber, while doxycycline was selected as the drug for delivery. The *in vitro* and *in vivo* drug release profiles were investigated. The biomechanical properties of allograft Achilles tendons repaired using the nanofibers were tested in euthanized rabbits at 2-, 4-, and 6-week time intervals. Histological examination was performed for the evaluation of tissue reaction and tendon healing. The level of postoperative animal activity was also monitored using an animal behavior cage. The experimental results showed that the degradable nanofibers as a vehicle could provide sustained release of doxycycline for over 40 days after surgery with very low systemic drug concentration. Allograft Achilles tendon reconstruction assisted with the drug-loaded nanofibers was associated with better biomechanical properties at 6 weeks post-surgery. In addition, the animals exhibited a better level of activity after surgery. Use of drug-eluting nanofibrous membranes could enhance healing in Achilles tendon reconstruction surgery with allograft.

Introduction

The Achilles tendon is the strongest and largest tendon in the human body. Achilles tendon rupture is a common injury of the foot and ankle, which has a large influence on an individual's level of sports activity.[1] The rate of misdiagnosis or delayed diagnosis can be as high as 25%.[2,3] Chronic injury may occur 4–6 weeks after initial injury.[4,5] Surgical treatment for primary repair is feasible in acute injury, but not in chronic injury due to the retraction of the injured tendon and superimposed fibrous tissue. Thus, chronic injury is linked to poor prognosis. Numerous different treatment options have been used in the therapy of neglected Achilles tendon rupture with large defects, including V-Y tendon plasty[6] and tendon transfer or reconstruction. Tendon grafts used for reconstruction include autograft, allograft, and synthetic material. Reconstruction with an autograft tendon[7] offers the advantage of a higher healing rate; however, the development of donor site morbidity cannot be ignored. Tendon reconstruction with allograft is free from the aforementioned disadvantage. Nevertheless, concerns regarding the healing between the graft and ruptured Achilles tendon exist due to the low vascularity and high weight-bearing force during the recovery process.[8,9]

Following tendon injury, matrix metalloproteinases (MMPs) play an important role in the healing process.[10,11] MMPs participate in the degradation and remodeling of the extracellular matrix of connective tissue.[11-14] The increase in MMP activity after tendon rupture further reduces the quality of collagen fibers[15-17] Tetracycline, an established antibiotic, can inhibit the action of MMPs, thus reducing excessive degradation and remodeling of the extracellular matrix.[18-20] Several studies have shown reduced tendon degeneration after local and systemic use of tetracyclines.[15,21-23] Doxycycline is the most potent MMP inhibitor among the tetracycline family, with the ability to enhance tendon healing in the rotator cuff and Achilles tendon.[24-29] Systemic and local administrations of doxycycline have been

shown to promote Achilles tendon healing after primary repair in rats.[24,27,28] However, currently, there are no studies investigating the effects of doxycycline on the healing process of Achilles tendons reconstructed with allograft.

The aim of this study was to evaluate the effects of doxycycline-loaded biodegradable nanofibrous membranes on the healing process and activity level in an animal model after Achilles tendon reconstruction with allograft. We hypothesized that the use of doxycycline-loaded biodegradable membranes improves tendon strength in the absence of severe inflammation during healing after tendon reconstruction with allograft. Doxycycline-loaded poly-D-L-lactide-glycolide (PLGA) nanofibers were prepared through an electrospinning process. We evaluated the *in vitro* characteristics of doxycycline-loaded PLGA nanofibrous mats and the *in vivo* efficacy of nanofibers using a rabbit model. Histological examination was also performed.

Materials And Methods

Fabrication of doxycycline-loaded nanofibers

Doxycycline-eluting nanofibers were fabricated using 50:50 PLGA (Sigma–Aldrich, St. Louis, MO, USA.) [33], while hexafluoroisopropanol (Sigma–Aldrich) was employed as solvent. Doxycycline (300 mg) and PLGA (75 mg) were initially blended with hexafluoroisopropanol (1 mL). Electrospinning experiments were completed on a lab-scale device, which involved a syringe and needle (with an internal diameter of 0.42 mm), a ground electrode, an aluminum sheet, and a high voltage supply. The PLGA/drug solution was delivered and electrospun by a syringe pump with a volumetric flow rate of 0.1 mL/hr. The travel distance between the tip of the needle and the ground electrode was 15 cm, and the voltage applied to polymer solutions was +15 kV. After electrospinning, the PLGA/doxycycline nanofibers were gathered on the aluminum sheet. All nanofibrous samples were prepared at ambient temperature (25°C). Nanofibrous membranes with a thickness of approximately 0.2 mm were thus obtained. All electrospun nanofibers were placed in a vacuum oven at 40^{o}C for three days to vaporize the residual solvents.

We used a field-emission scanning electron microscope (SEM; JSM–7500F; Joel, Tokyo, Japan) to evaluate the doxycycline-embedded fibers. Fifty randomly selected nanofibers were characterized from the SEM images for each sample to determine the diameter distribution of nanofibers.

The thermal properties of virgin PLGA, doxycycline, and doxycycline-loaded PLGA nanofibers were investigated using a TA-DSC25 differential scanning calorimeter (TA Instruments, New Castle, DE, USA). The scanning temperature ranged from 30°C–350°C, while the nanofibrous specimens were heated at 10°C/min.

In vitro release patterns of pharmaceuticals

The release pattern of doxycycline from the drug-loaded biodegradable nanofibrous membranes was obtained through an *in vitro* elution method. Samples cut from the electrospun mats (1 cm × 1 cm) were

placed inside glass tubes (one specimen per tube, $N = 3$) along with phosphate buffer solution of 1 mL in each tube. After the glass tubes were incubated for 24 h at 37°C, the eluent was collected and analyzed. During the next 24 h, 1 mL of fresh phosphate-buffered saline was added, and the procedure was repeated for 42 days. The levels of doxycycline in the mixtures were evaluated through high-performance liquid chromatography (L-2200R multi-solvent delivery system; Hitachi, Tokyo, Japan).

Animal experiments

Twenty-four New Zealand White rabbits (weight: 2,500–3,000 g) were included in the study; three animals were used for *in vivo* drug release testing, while the remaining 21 animals were employed for the tendon biomechanical and postoperative activity experiments. Three of the 21 animals did not undergo surgery and served as the healthy group in the activity test. Throughout the entire investigation, all animals-related procedures were carried-out according to the guidelines of the Department of Health and Welfare, Taiwan.The study was carried out in compliance with the ARRIVE guidelines. Besides, all animal related procedures were approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC Approval No.: CGU107-168).

For anesthesia, the rabbits were placed into a transparent chamber (40 cm × 20 cm × 28 cm) filled with vaporized isoflurane (Aesica-Queenborough, Queenborough, Kent, UK). After anesthesia, each rabbit was placed onto a sterilized disposable sheet with continuous administration of vaporized isoflurane through a mask.

After sterilizing one leg of each rabbit with 75% alcohol, local anesthesia was induced through local injection of 2% xylocaine (1 mL). A 4-cm longitudinal skin incision was made with a No. 10 blade over the posterolateral side of the right leg. Gentle dissection of the Achilles tendon was performed circumferentially with Mosquito (Fig. 1). In case of active bleeding, direct pressure with a sterilized gauze was applied to the wound for hemostasis. The Achilles tendon (0.5 cm) was harvested from the rabbit through a horizontal cut proximally and distally over the middle portion of the tendon for further transplantation to another rabbit. Subsequently, a tendon (0.5 cm) from another rabbit was transplanted to this harvest site with end-to-end repaired proximally and distally using 3-0 Dexon (Johnson & Johnson, USA) sutures. In the experimental group, a biodegradable nanofibrous membrane was wrapped around the transplanted/repaired tendon and fixed with 5-0 Dexon (Johnson & Johnson) sutures. Next, superficial skin was repaired with 3-0 Nylon sutures. In the control group, the surgical wound was repaired directly with 3-0 Nylon sutures without any membrane. In all rabbits, neomycin ointment was applied onto the sutured wound to prevent infection.

In vivo drug release

We evaluated the *in vivo* drug levels 1, 3, 14, 28, and 42 days post implantation of the nanofibrous membranes. At each time point, blood sampling (1 mL) was performed after euthanasia of the rabbits. Meanwhile, tissue sampling was performed from the tendon around the membrane. The concentration of doxycycline in tissue and blood was tested using high-performance liquid chromatography.

Post operation activity testing

Following operation, each rabbit was raised in an animal behavior cage (Fig. 2; manufactured in our laboratory) for 1 week to record the level of postoperative activity. The cage (120 cm × 120 cm × 60 cm) was divided into nine symmetric square areas. Nine diffusion-scan type photoelectric switch sensors were placed above each square area (HP100-A1; Azbil Corp., Tokyo, Japan). When the rabbit moved from one square area to another, the corresponding “arriving” sensor would be triggered. The number of times the sensor was triggered was monitored and recorded for 1 week. Thereafter, the rabbit was placed in its original cage for further animal care. The activities of three groups of animals were assessed ($N = 3$): control rabbits that underwent surgery only, drug rabbits that underwent surgery and implantation of doxycycline-loaded nanofibers, and healthy rabbits that did not undergo surgery.

Biomechanical strength testing

The rabbits were euthanized by intravenous injections of lidocaine (10 mL) 2, 4, and 6 weeks after surgery. The right Achilles tendons and the left healthy tendons were harvested for further biomechanical strength testing ($N = 3$). The biomechanical test was performed using a Lloyd tensiometer (AMETEK, USA), with the ASTM D638 standard. The tendon was clamped at both ends onto the tensiometer and pulled at a speed of 6 cm/min for 10 cm.

Histological analysis

Retrieved tendons were embedded in 10% formalin and fixed with paraffin. Next, the retrieved tendon was sectioned in the frontal direction (thickness: 4 μm). Hematoxylin and eosin staining was used for histological evaluation. The slides were sent to an independent pathologist for histological analysis.

Statistical analysis

We used the paired *t*-test for statistical evaluation between distinct groups with SPSS software (Version 12.0; SPSS Inc., Chicago, IL, USA). Statistical significance was set at *p*-values below 0.05.

Results

Assessment of spun doxycycline-loaded nanofibers

Doxycycline-embedded nanofibers were prepared using the electrospinning technique. Figure 3A shows the gross view of electrospun nanofibers, while Figure 3B displays the SEM image of the doxycycline-embedded nanofibers. The calculated diameter distribution of spun nanofibers was 104 ± 25.8 nm.

The thermal properties of isolated PLGA, doxycycline, and doxycycline-loaded PLGA nanofibers were assessed, and the results are displayed in Figure 4. The endothermal peak of doxycycline at 250°C disappeared after being incorporated into the PLGA matrix, while a new peak at 300°C was noted. This demonstrated the successful embedment of doxycycline into the PLGA nanofibrous mats.

Doxycycline release

Figure 5 displays the daily and cumulative discharge profiles of doxycycline from the nanofibrous membranes. The profile showed a triphasic release: a burst release at day 1, a second peak of discharge at day 17, and a progressive reduction in drug elution thereafter. Moreover, the drug-loaded nanofibrous membranes provided sustained *in vitro* release of doxycycline for over 40 days.

Figure 6 displays the elution profiles of *in vivo* doxycycline in blood and tissue. The local concentration of the drug at the tendon was high for 7 weeks, whereas the systemic drug concentration was markedly lower.

Biomechanical strength study

At 2 weeks (Fig. 7A), the maximum load of the control group (surgery only) and the doxycycline-loaded group was similar, and significantly lower than that of the healthy group. Similar results were noted at 4 weeks (Fig. 7B). The maximum loads of the doxycycline-loaded and control groups were lower than that of the healthy group. At 6 weeks after surgery, the maximum load was markedly lower in the control group (Figure 7C). The maximum loads of the doxycycline-loaded and healthy groups were comparable; although the absolute value of the maximum load in the doxycycline-loaded group was slightly lower than that recorded in the healthy group, the difference was not statistically significant ($p > 0.05$). This finding demonstrates the excellent capability of doxycycline-loaded nanofibers to enhance the healing of reconstructed Achilles tendons with allograft.

Animal activity

The activity of the animals post operation was evaluated using an animal behavior cage. The number of times the sensors were triggered in each group is shown in Figure 8, with higher numbers representing better animal activity. The photoelectric switch sensors were triggered 8,519 ± 3,235, 7,964 ± 510, and 6,437 ± 732 times in the healthy, doxycycline-loaded, and control groups, respectively. The difference between the doxycycline-loaded and control groups was statistically significant ($p < 0.05$). Nonetheless, there was no significant difference between the doxycycline and healthy groups. Therefore, rabbits undergoing allograft Achilles tendon reconstruction with a doxycycline-loaded biodegradable nanofibrous membrane had a similar level of activity as healthy rabbits and higher activity levels compared to control rabbits.

Histological study

Figure 9 shows images obtained after hematoxylin and eosin staining. There were no significant differences noted in terms of tendon cell maturation, proliferation, and arrangement between the doxycycline-loaded and control groups at any timepoint. However, at 4 weeks post operation, the images revealed severe inflammation with extensive accumulation of white blood cells in the control group and only mild inflammation in the doxycycline-loaded group.

Discussion

Surgical intervention is indicated for complete Achilles tendon rupture with gap formation. Conservative treatment in this patient group leads to poor recovery and limping. Surgery for primary repair is the gold standard treatment in the acute phase, with cast immobilization for at least 1 month postoperatively. However, the treatment of chronic Achilles tendon rupture varies, and more complex surgery is indicated. Achilles tendon reconstruction with allograft leads to relatively satisfactory outcome without donor site morbidity compared to reconstruction with autograft.

The healing between the allograft and ruptured tendon is a concern for surgeons. The role of MMPs in the healing process has been widely studied.[10,11] Increased MMP levels lead to poor collagen quality during tendon healing.[17,30] Doxycycline, as the most widely studied drug among the MMP family, enhances healing in the primary repair of Achilles tendon. In our previous study,[31] use of a doxycycline-embedded biodegradable nanofibrous membrane enhanced tendon healing after primary repair of Achilles tendons in rats, particularly at 3 weeks after surgical repair. The present study further proved that doxycycline-embedded biodegradable nanofibrous membrane is effective at enhancing tendon strength 6 weeks after Achilles tendon reconstruction with allograft in rabbits. There were no drug-related or major complications observed in this analysis.

Currently, there is no gold standard in terms of the route of administration (systemic or local), duration of treatment, or concentration of doxycycline. Studies concerning the effect of doxycycline on tendon healing have mostly yielded positive results. Kessler et al[24] found that administration of doxycycline through oral gavage for 4 weeks significantly inhibited MMP activity by 60% in rats after Achilles tendon transection and repair. Improved collagen fibril alignment and better biomechanical properties were noted in the extended doxycycline treatment group. In their study, the serum level of doxycycline was 6 μg/mL. Nguyen et al[27] used oral doxycycline (10 mg/kg daily) for the treatment of surgically repaired and unrepaired Achilles tendons in rats. They found that doxycycline improved the biomechanical properties of Achilles tendons at 3–6 weeks after surgical repair. However, the serum concentration of doxycycline was not evaluated in that study. Pasternak et al[32] used a rat model with Achilles tendons transected and left unrepaired. Oral doxycycline was administered at a mean serum concentration of 3.4 μg/mL. Decreased force at failure was noted at 5, 8, 14 days post-surgery in the doxycycline group. However, there was no significant difference noted in stiffness or stress at failure at all three time points. Besides the systemic use of doxycycline, Pasternak et al[26] employed doxycycline-coated sutures for Achilles tendon repair in rats, demonstrating improved holding capacity.

According to our previous study,[31] use of a doxycycline-embedded biodegradable membrane improved the mechanical strength at 6 weeks after primary Achilles tendon repair. In this study, similar results were found in Achilles tendon reconstruction with allograft, with doxycycline enhancing tendon healing. The biodegradable nanofibrous PLGA vehicle provides longer release duration and higher concentrations of doxycycline at the target site compared with those observed in other studies, while maintaining low systemic drug concentration. Other studies demonstrated that the effect of doxycycline on tendons

becomes significant from at least 3 weeks after surgery. It appears that doxycycline requires a longer period of time to achieve a statistically significant influence on the healing process of allograft tendons. In the literature,[24,27] oral administration of doxycycline has been reported as the only way to maintain an effective drug concentration over a long period of time. There were no drug-related side effects observed in these animal experiments. Nevertheless, there is a risk of adverse effects (eg, gastritis, esophageal erosion, heartburn, nausea, or vomiting) after long-term systemic use of doxycycline. Pasternak et al[26] used doxycycline-coated sutures to improve the holding capacity; however, only 73% and 96% of the doxycycline had been released from the surface of sutures after 24 and 72 h, respectively. In contrast, using the PLGA-based membrane, we can reach a local concentration of at least 1 μg/mL doxycycline for 42 days and a systemic concentration of doxycycline under 0.1 μg/mL after 3 days. These findings indicate that the biodegradable nanofibrous membrane can maximize the positive effect of doxycycline on tendon healing, while minimizing the side effects associated with the long-term usage of doxycycline.

The limitations of this study should be acknowledged. Firstly, we did not examine membranes with different amounts of doxycycline to determine the relationship between drug concentration and tendon healing. Secondly, the relevance of the present findings to tendon reconstruction with allograft in humans remains uncertain. These will be the topics of our future research.

Conclusions

We used PLGA as a drug carrier and evaluated the efficacy of doxycycline-loaded biodegradable nanofibrous membranes in Achilles tendon reconstruction with allograft in rabbits. The *in vitro* and *in vivo* drug release profiles showed persistent drug release for over 40 days. The concentration of doxycycline was markedly higher in local tendon tissue than in blood, indicating a very low systemic drug effect. Rabbits undergoing Achilles tendon reconstruction with allograft and doxycycline-loaded nanofibers exhibited stronger tendons and higher levels of activity after surgery. Therefore, the doxycycline-loaded nanofiber membrane promotes healing in both autologous-to-autologous and autologous-to-allogeneic tendons.

Declarations

Author Contributions: Conceptualization, S.J.L.; methodology, C.J.W, S.J.L.; data curation, software and validation, Y.C.W, M.Y.H; writing—original draft preparation, C.J.W.; writing—review and editing, F.P.C, S.J.L; supervision, S.J.L.; funding acquisition, C.J.W, S.J.L. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figures

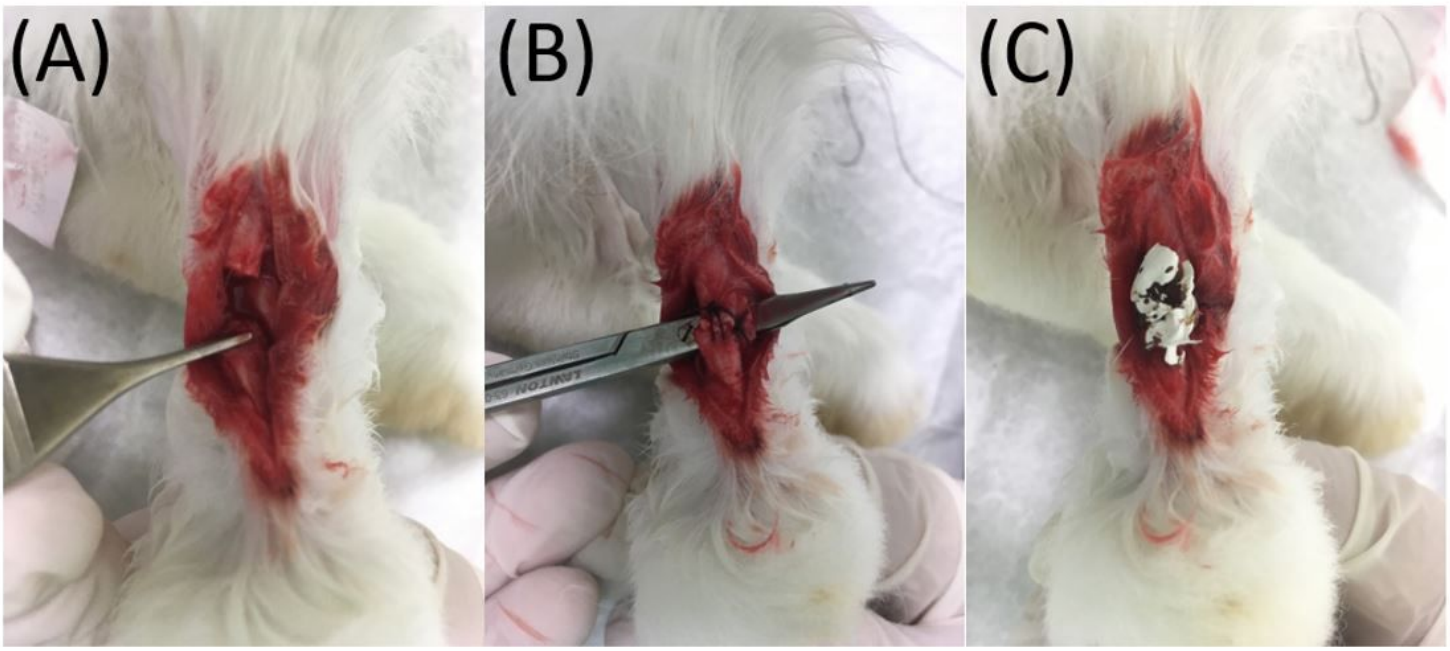

Figure 1

(A) The Achilles tendon was cut using a blade. (B) The tendon was primarily repaired with Dexon sutures and (C) enveloped with doxycycline-embedded nanofibers.

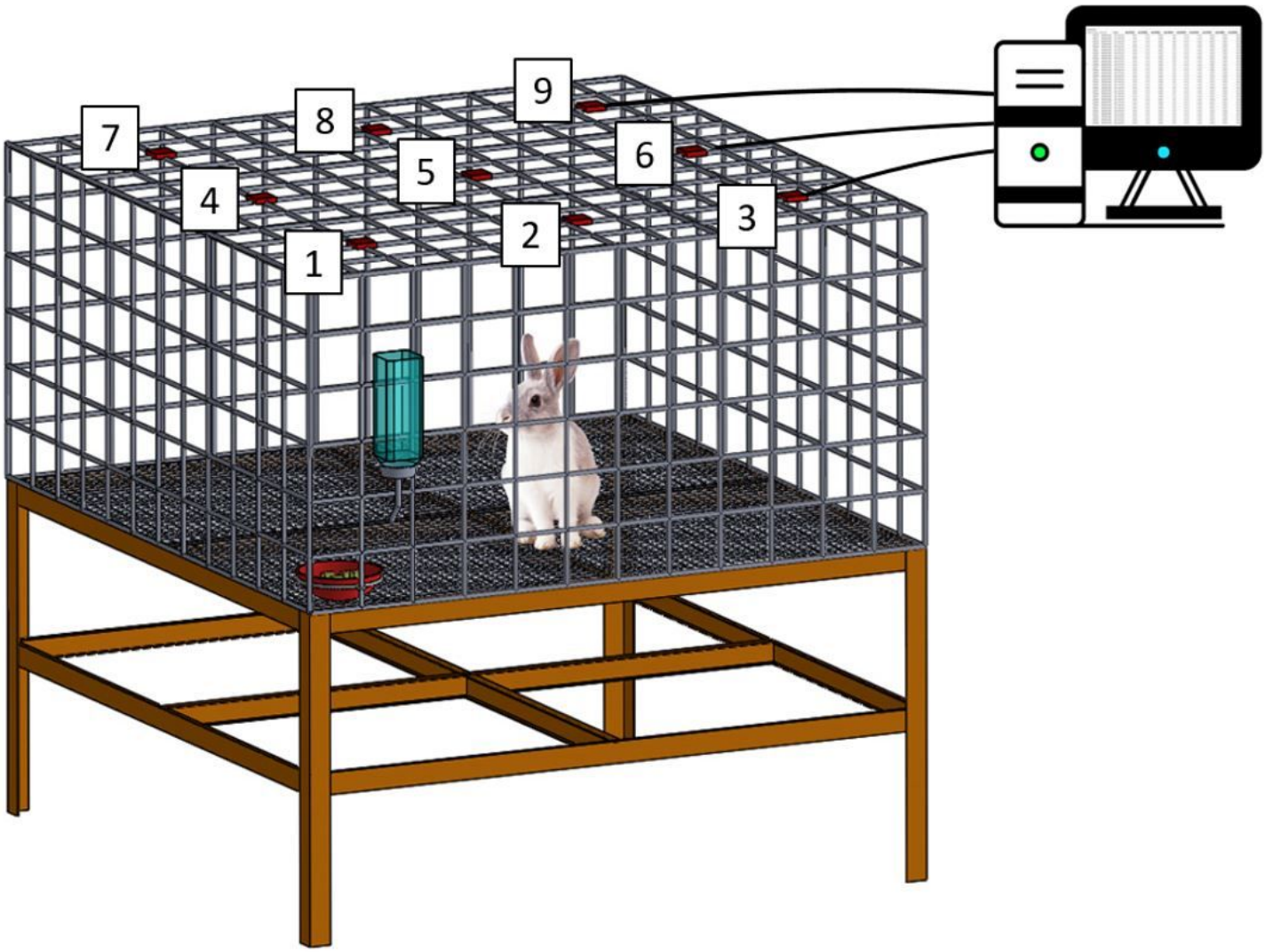

Figure 2

Schematic of the animal behavior cage.

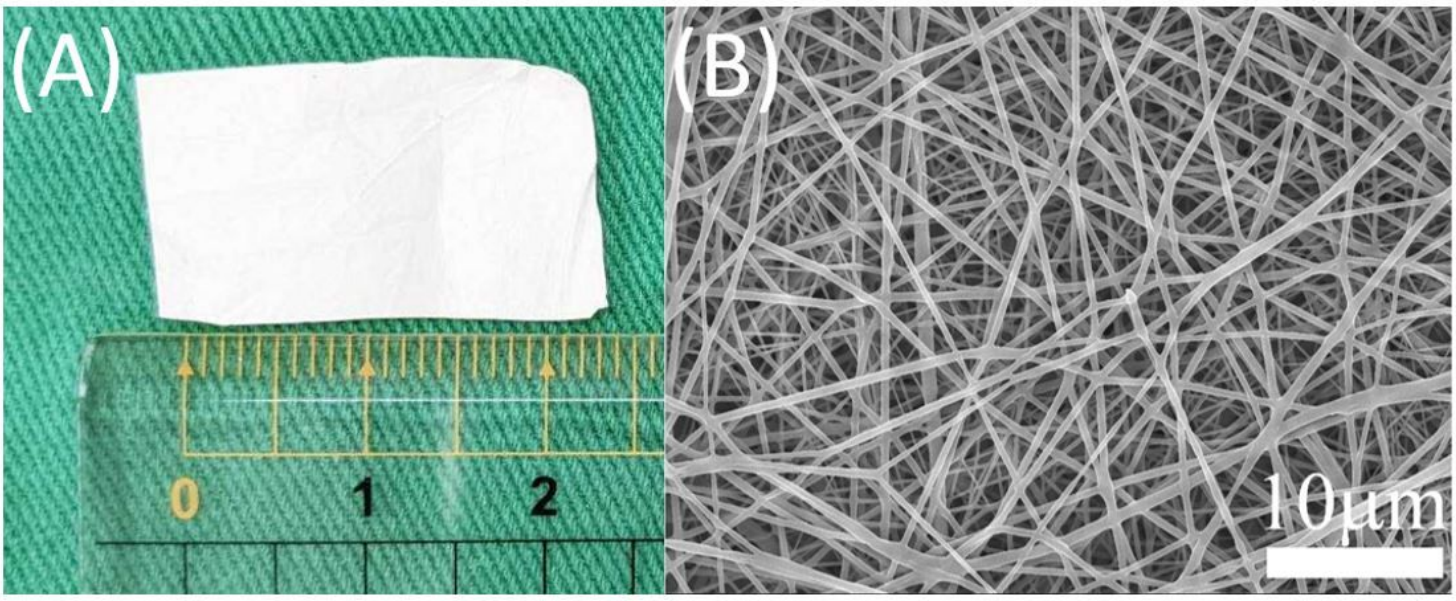

Figure 3

(A) Gross view and (B) SEM image of doxycycline-loaded PLGA nanofibrous membranes. Abbreviations: PLGA, poly-D-L-lactide-glycolide; SEM, scanning electron microscope

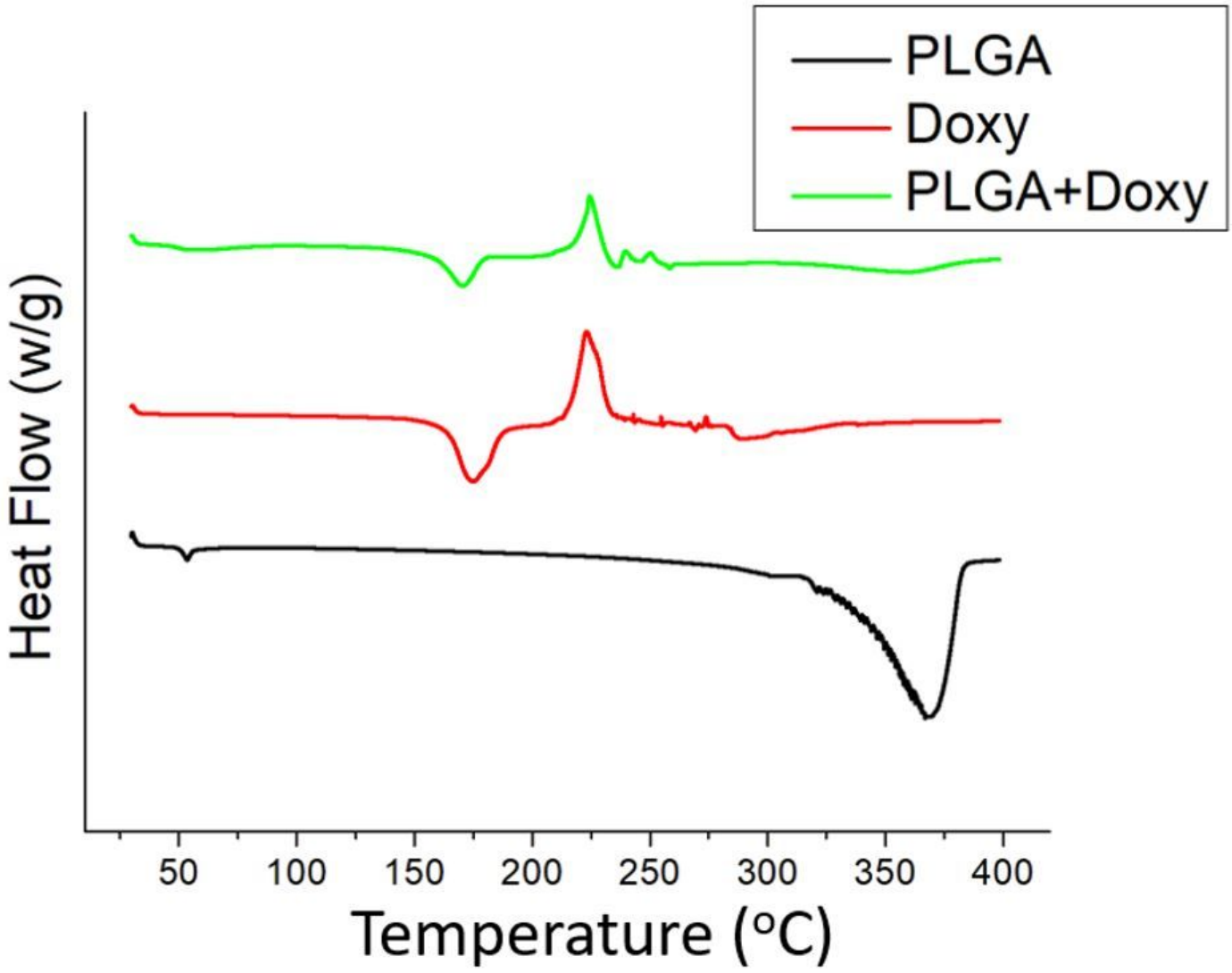

Figure 4

DSC assay of pure PLGA, doxycycline, and doxycycline-embedded PLGA nanofibrous membranes. Abbreviations: Doxy, doxycycline; DSC, differential scanning calorimetry; PLGA, poly-D-L-lactide-glycolide; SEM, scanning electron microscope

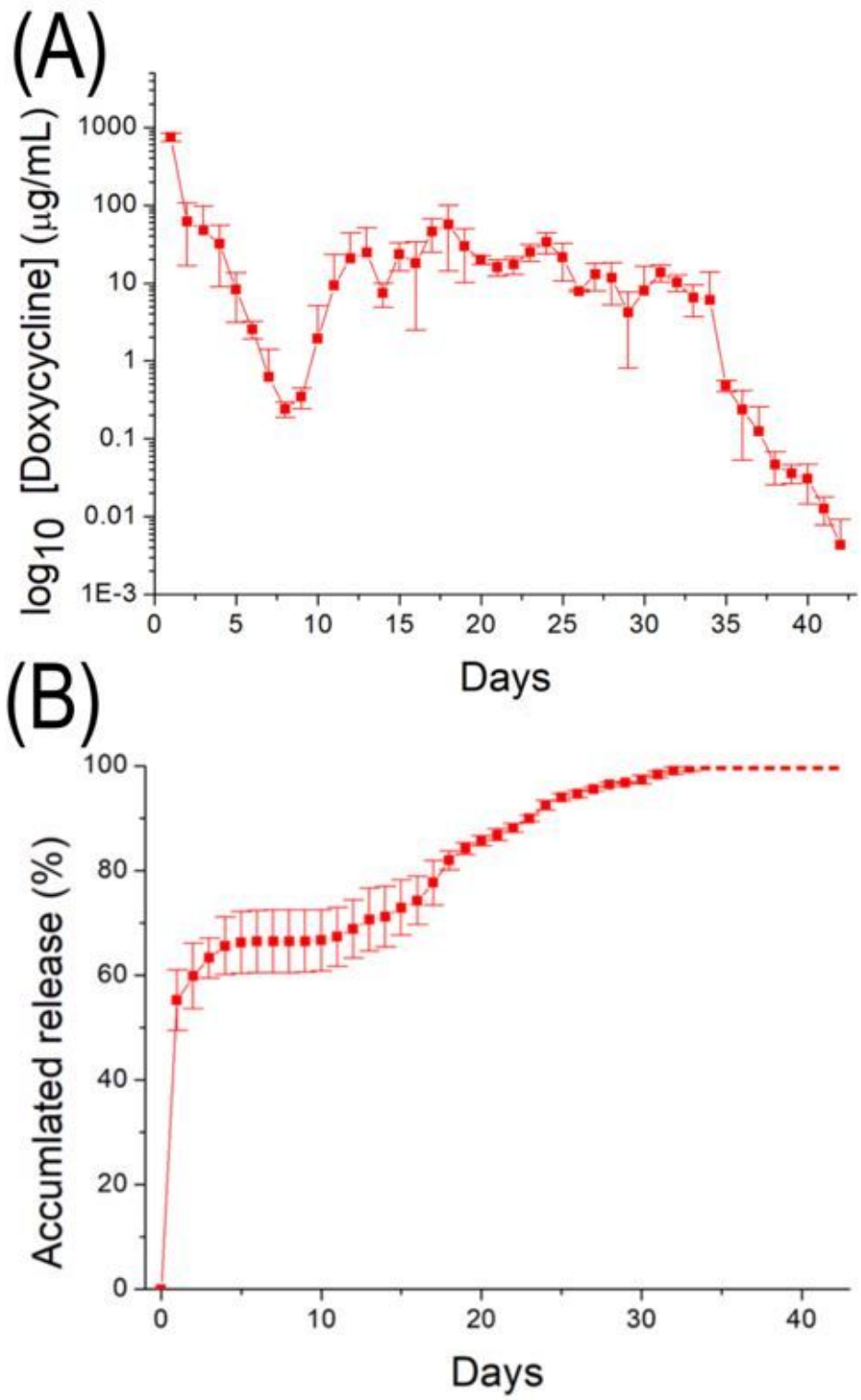

Figure 5

In vitro release of doxycycline from the nanofibers.

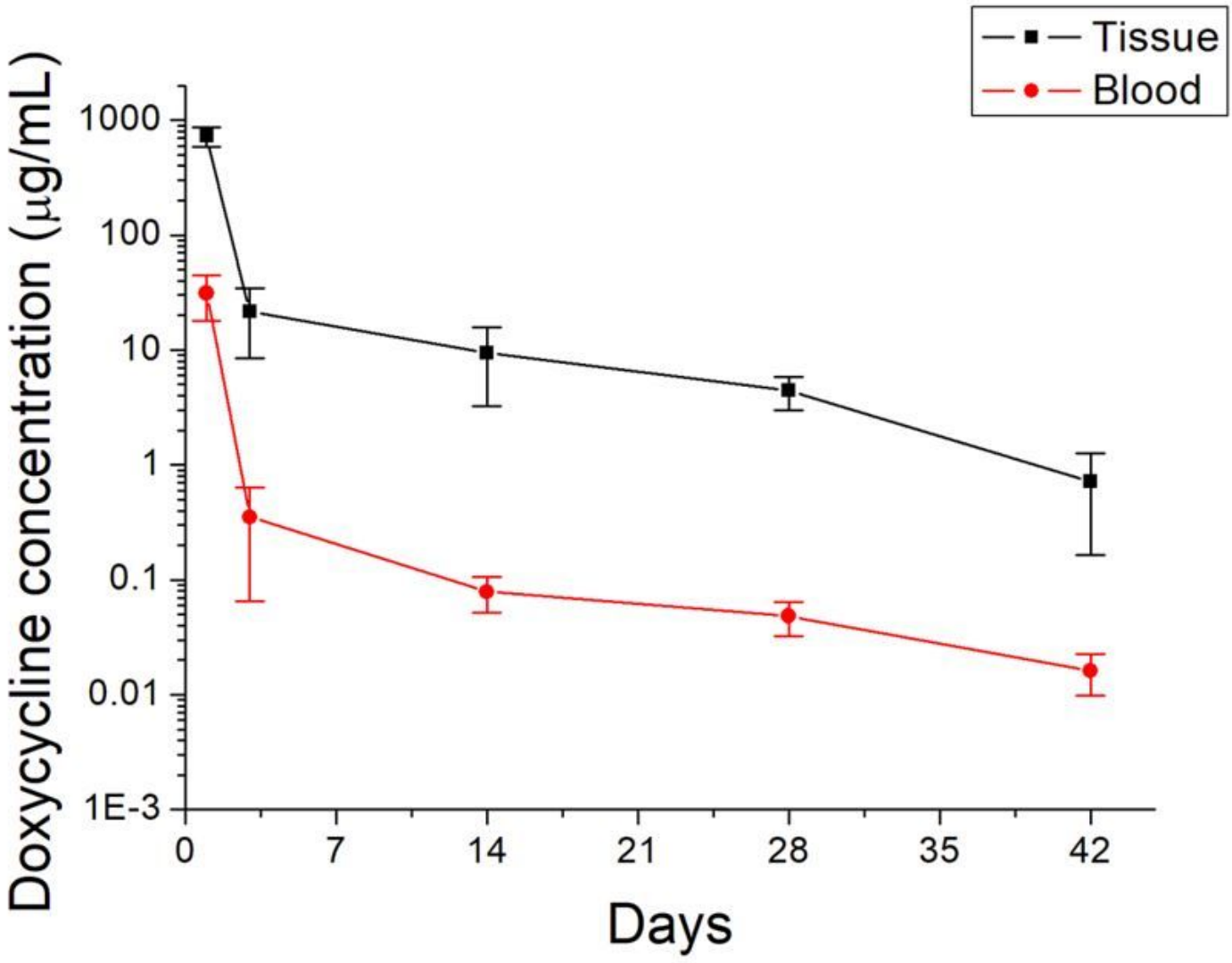

Figure 6

In vivo release of doxycycline over time.

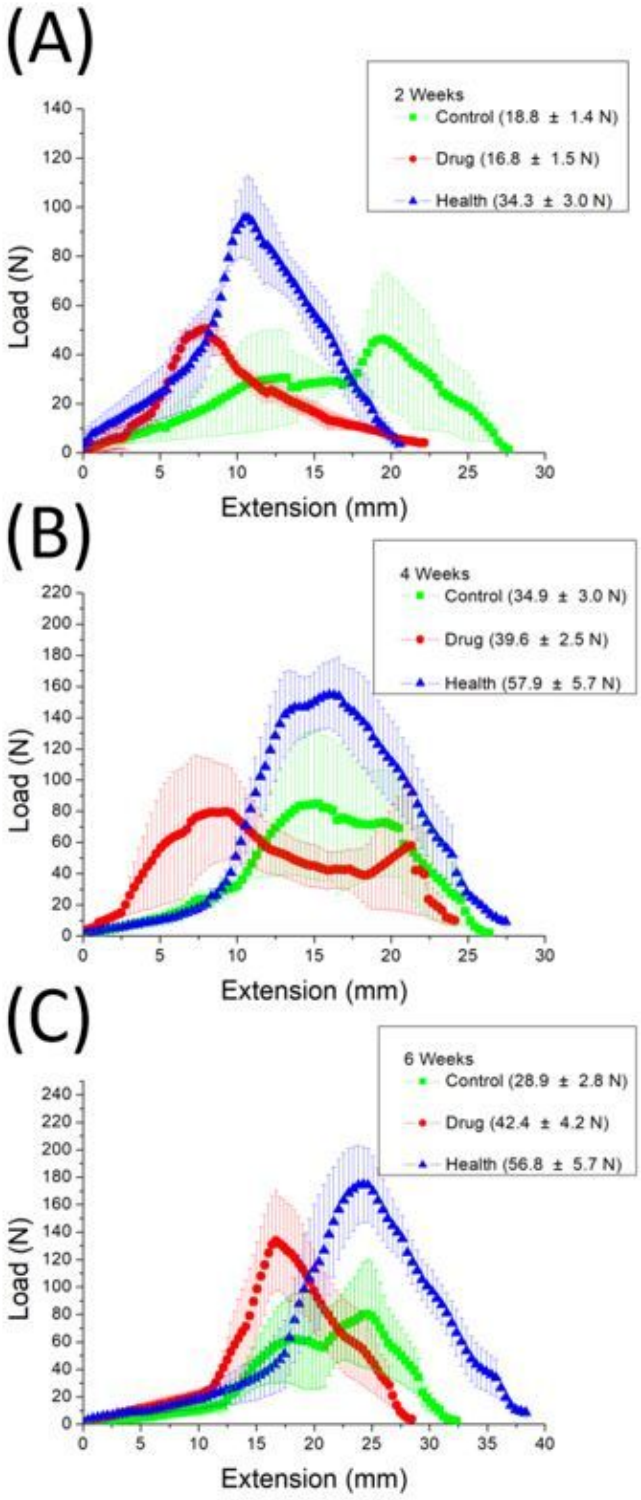

Figure 7

Tendon strengths at 2, 4, and 6 weeks post-surgery.

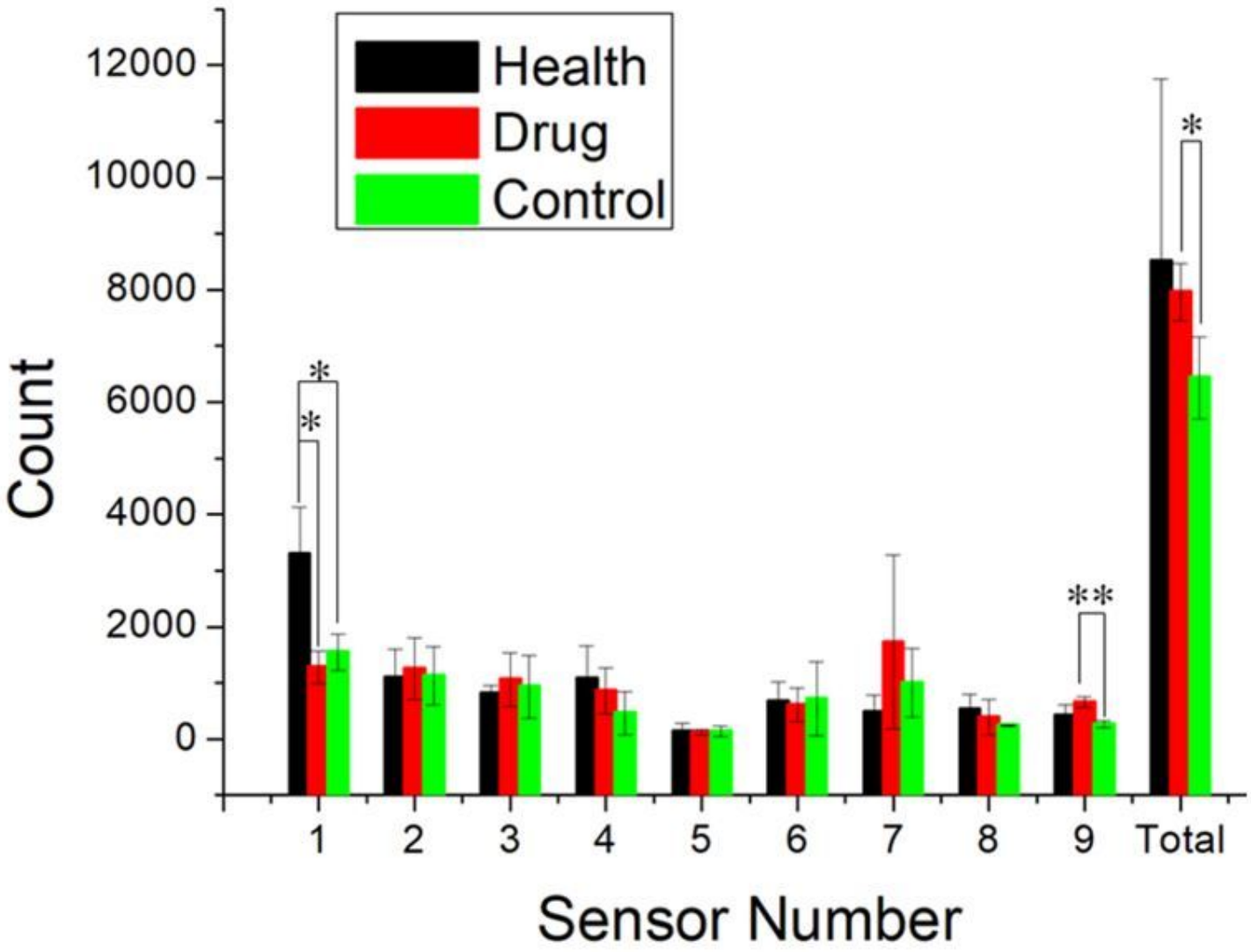

Figure 8

Activity counts of the rabbits (*p < 0.05; **p < 0.01).

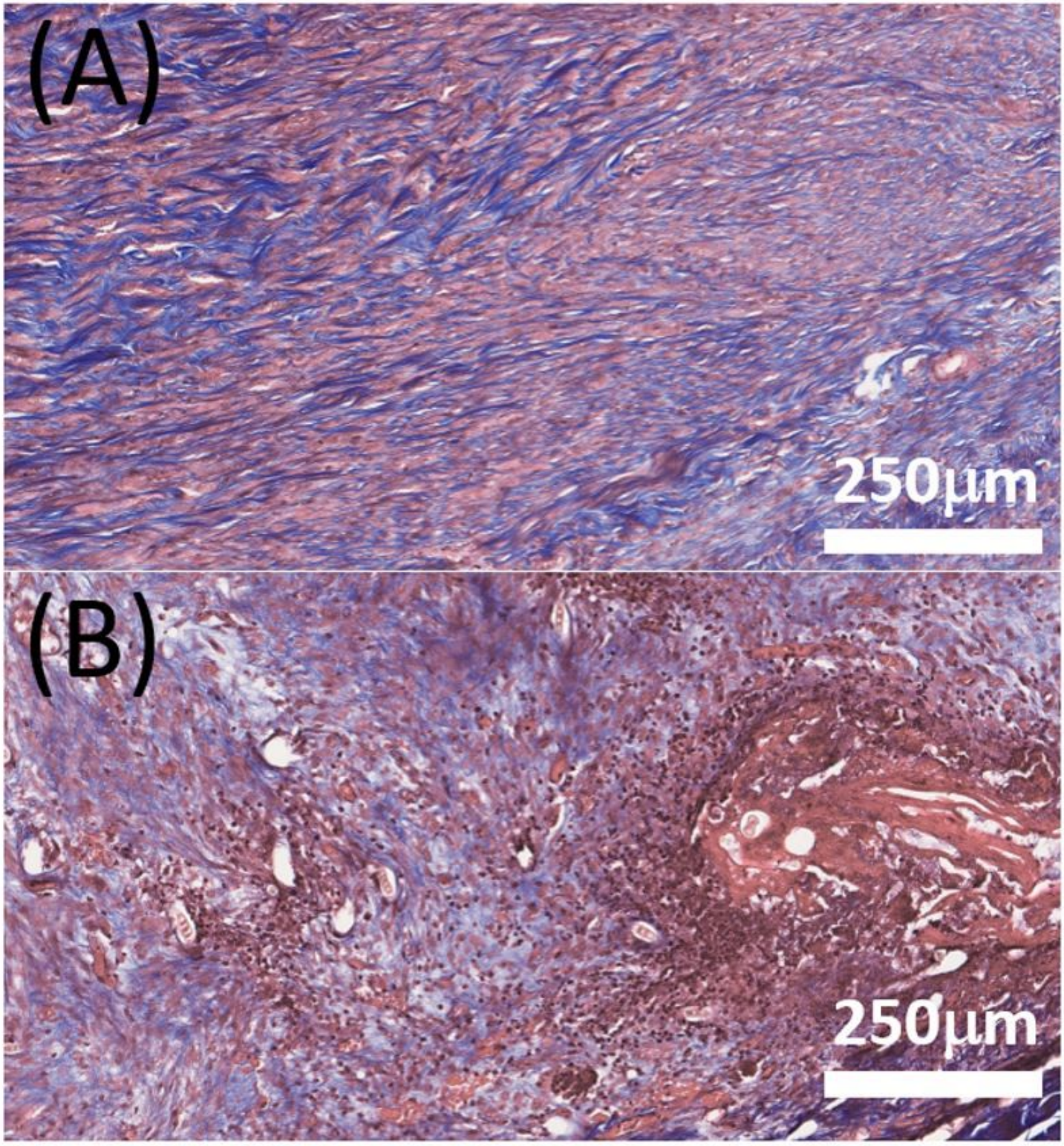

Figure 9

H&E staining of the Achilles tendon at 4 weeks post-surgery. (A) Doxycycline group. (B) Control group. Abbreviation: H&E, hematoxylin and eosin