Original article

Early treadmill running delays rotator cuff healing via Neuropeptide Y mediated inactivation of the Wnt/β-catenin signaling

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ABSTRACT

Background: Defining the optimal rehabilitation programs for rotator cuff healing remains a challenge. Early treadmill running may have negative effects on tendon-bone interface (TBI) healing with increased expression of Neuropeptide Y (NPY). However, the underlying mechanism is still unknown.

Methods: The mice were randomly assigned to four groups: control group, treadmill group, treadmill + BIBO3304 group and BIBO3304 group alone. Specifically, the control group was allowed free cage activity without any treatment after surgery. The treadmill group received early treadmill running initiated from postoperative day 2. The treadmill + BIBO3304 group received treadmill running combined with intra-articular injection of BIBO3304 postoperatively. The BIBO3304 group only received type 1 NPY receptor (Y1 receptor, Y1R) antagonist BIBO3304 postoperatively. Healing outcomes of the rotator cuff were evaluated by histological analysis, synchrotron radiation micro-computed tomography (SR-μCT) scanning, and biomechanical testing at 4 and 8 weeks after surgery. The expression of NPY and its Y1 receptor during the treadmill running were tested by immunofluorescence. In addition, the related signaling pathway of Neuropeptide Y among all groups was detected by immunohistochemistry and western-blot.

Results: Immunofluorescence results show that early treadmill training could lead to a significant increase in the expression of NPY at the healing site, and Y1R was widely expressed in both normal or injured rotator cuff without statistical difference. At the same time, early treadmill running delayed the healing of rotator cuff, as indicated with unsatisfactory outcomes, including a significantly lower histological score, decreased bone formation and inferior biomechanical properties at postoperative week 4 and 8. Moreover, the use of BIBO3304 could partly alleviate the negative effects of early treadmill running on the healing of rotator cuff and promote the natural healing process of rotator cuff, as evidenced by significant differences observed between the treadmill and treadmill + BIBO3304 groups, as well as observed between the control and BIBO3304 groups. On the other hand, the expressions of Wnt3a and β-catenin in the treadmill group were significantly lower compared with the other groups, while the expression in the BIBO3304 group was the highest, as evaluated by immunohistochemistry and western-blot.

Conclusions: Early treadmill running increased the expression of NPY at the RC healing site, which might burden the expression of Wnt3a/β-catenin and delay the healing process, inhibition of Y1 receptor with BIBO3304 could promote bone-tendon healing through the Wnt/β-catenin signaling.

The translational potential of this article: This is the first study to evaluate the specific role of the NPY-Y1R axis and its underlying mechanism by which early treadmill running delays bone-tendon healing. Further, our study may provide references of precise and individualized exercise-based rehabilitation strategies for TBI healing in clinic.

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1. Introduction

Tendon-bone interface (TBI) is a unique gradient structure connecting bone and tendon, which disperses stress concentration and facilitates load transfer between the tendon and bone [1]. Rotator cuff (RC), as a typical TBI tissue, is prone to a sports-related injury, resulting in shoulder pain and joint function deterioration. Approximately 250,000 RC repairs were performed in the United States each year [2]. However, RC is hard to regenerate owing to its inherent complexity and limited healing capacity [3]. Unfortunately, surgical intervention for RC tears sometimes leads to unsatisfactory outcomes: retear rates between 21% and 94% [4]. Therefore, rapid regeneration and function restoration after RC injury remains a significant challenge in clinic.

Mechanical stimulation is indispensable for TBI healing and the application of mechanobiological principles following RC injury is considered as a basis of rehabilitation protocols [5,6]. Whereas immobilization remains a common treatment after RC surgery in clinic, and studies have shown that reducing mechanical stimulation by paralyzing the supraspinatus muscle could benefit the RC healing [5]. However, complete removal of load (by paralyzing muscles and external immobilization) has been demonstrated detrimental to the healing RC [7,8], and not to mention the shoulder stiffness and loss of joint function caused by long-term immobilization after RC repair [9]. It has been addressed that appropriate exercise-related loading could enhance the mechanical properties of the repaired tendon [5,6]. Yet the outcome of inappropriate post-injury rehabilitation is unsatisfactory, for instance, the immediate postoperative passive motion would impair RC healing with decreased range of motion and increased risk of joint stiffness [6]. Camp CL et al. reported that both immediate and prolonged loading decreased the biomechanical strength of the healing TBI [10]. Wada S et al. showed that early tendon loading after injury eventually led to delayed healing of RC [11]. These studies indicate that immediate mechanical stimulation should be avoided after RC injury-repair. Treadmill has been widely utilized to generate extra muscle load as mechanical stimulation on tendon enthesis. And the effects of treadmill running on healing tendons have been systemically documented [12]. Although the negative outcomes of immediate treadmill running have been discussed, its specific effect and underlying mechanism of early tendon loading on the healing RC need further investigation.

The innervation of the periphery nerves has been observed in the human tendon, and emerging evidence suggested that periphery neuropeptides play a role in RC healing [13,14]. Neuropeptide Y (NPY) is a 36 amino acid peptide, mainly distributed in the central and peripheral nervous systems. NPY is also synthesized by osteoblasts, osteocytes and chondrocytes [15]. NPY immunoreactive fibers have been identified in osteogenic tissue including bone marrow and the periosteum [16]. Zhang T et al. found NPY acted as a mechanosensitive mediator involving in the healing of RC, but the specific role for healing remains unknown [17]. Previous studies have highlighted the roles of NPY played in the control of bone formation and healing through locally-expressed Y1 receptors (Y1R) [18–20]. BIBO3304 was identified as a specific antagonist for Y1R, which could furtherly facilitate bone formation and metabolism [21–23]. It has been reported that Wnt/β-catenin signaling is involved in the NPY-Y1R axis regulating the osteoblastic differentiation of stem cells [23]. Several prior studies have suggested that Wnt/β-catenin signaling was related to mechanical stress. Specifically, moderated loading could increase Wnt/β-catenin expression, resulting in enhanced bone regeneration [24,25]. The mechanosensitive expression of Wnt/β-catenin signaling has been observed in RC healing, which is related to healing quality [11]. We hypothesized that early treadmill running might affect RC healing through the NPY-mediated Wnt/β-catenin pathway.

In this study, we firstly performed a murine RC injury-repair model to illustrate the expression of NPY and the distribution of Y1R at the bone-tendon healing site. Secondly, we investigated the role of NPY and the activation of the Wnt/β-catenin pathway using a treadmill running immediately after the operation, with/without intra-articular injection of BIBO3304. The purpose of the present study was to evaluate the effect of early treadmill running postoperatively on rotator cuff healing, and to elucidate the underlying mechanism in this intricate process.

2. Materials and methods

All study and animal care procedures were approved by the local Animal Ethics Committee and conducted in accordance with relevant guidelines and regulations.

2.1. Study design

A total of 224 male C57BL/6 mice underwent acute RC injury-repair procedure (Fig. 1). The animals were randomly allocated to four groups as follows: 56 mice allowed with a free cage activity after surgery (the control group), 56 mice only received intra-articular injection of Y1R antagonist BIBO3304 postoperatively (the BIBO3304 group), 56 mice received early treadmill running after surgery (the treadmill group), and 56 mice received early treadmill running combined with intra-articular injection of BIBO3304 (the treadmill + BIBO3304 group). Mice were euthanized to harvest supraspinatus (SS) tendon enthesis for evaluation at postoperative week 4 and 8 (n = 28 per group per time point). After sacrifice, the SS tendon samples were examined histologically, biomechanically and via synchrotron radiation-microcomputed tomography (SR-μCT). In addition, the collected specimens in control and treadmill groups were tested by immunofluorescence to detect the expression of NPY and its Y1 receptor. Next, the collected specimens were tested by immunohistochemistry and western blot to evaluate the activation of Wnt/β-catenin signaling. To reduce the request on animals, the contralateral side of mice RC without surgery was set as the normal reference group (the normal group). The sample size from each group was set according to previous studies in the murine rotator cuff injury model using power analysis [11,14,26].

2.2. Animal model and surgery

Unilateral microsurgical procedures in the left shoulder were performed under sterile technique according to a previous protocol [11,27]. In brief, the mice (8-week old, male, 22–24 g) were anesthetized by intraperitoneal administration of 0.3% pentobarbital sodium (0.6 mL/20 g; Sigma–Aldrich, St. Louis, MO), and positioned in a right lateral decubitus position. After the surgical site was shaved and disinfected, an incision was minimally cut over the deltoid muscle, and the muscle was split to expose the insertion site of the supraspinatus tendon. Then the SS was fixed using 6-0 PDS suture in an "8" figure fashion, and sharply detached from its native footprint on the greater tuberosity using a number 11 blade. The SS tendon was detached from the insertion site on the greater tuberosity and the remaining enthesis tissue (including cartilage and partial subchondral bone) was scraped off with a No.11 blade to expose the bellowing spongy bone. A bone tunnel was created transversely from the anterior to posterior extents of the humeral head using a 30G needle. The PDS suture was passed through the bone tunnel to ensure proper fixation of SS tendon tissue. The deltoid muscle was readapted and the skin was closed with Ethicon sterile sutures. Penicillin

G was administrated for antibiotic treatment once a day for 3 days after surgery.

2.3. Treadmill running

A custom-designed motor-powered treadmill was purchased from a biological instrument equipment company (ZH-PT; Anhui Zhenghua Co., Ltd., China) to induce mechanical loading to the SS tendon. The mice in all treadmill running groups underwent the first few days of exercise to be acclimated to the treadmill. Next, based on previous studies [11], the mice in all treadmill running groups maintained a 20 m/min rate run on a 5° declined lane for 60 min per day, 5 days per week, with initiative exercise 2 days after surgery.

2.4. Intra-articular injection of Y1R antagonist

Y1R antagonist BIBO3304 (Tocris, 2412) was dissolved and diluted at a final concentration of 2 μM, according to the manufacturer's instruction. The mice in the NPY + BIBO3304 group received a 5 μL intra-articular injection of BIBO3304 into the left shoulder joint once a week for 4 or 8 weeks. The other groups received an intra-articular injection of 5 μL PBS into the left shoulder joint. At the end of the experiments, the shoulder specimens were harvested for further analysis.

2.5. Histological evaluation

Samples were fixed in 4% neutral buffered formalin for 24 h immediately after sacrifice, embedded in paraffin after decalcification and sectioned in the coronal plane for a thickness of 5 μm using a microtome. Then, the representative sections of each group were stained with hematoxylin and eosin (H&E), safranin O and fast green (SO/FG) to analyze the characteristics of the repaired SS tendon insertion. The histological semiquantitative analysis was conducted by two blinded observers using a previously reported modified tendon maturation rating score. The scoring criteria includes cellularity, vascularity, continuity, fibrocartilage cells and tidemark ranked on a scale from 1 to 4 (Table S1) [17]. The interrater reliability of the two histology graders was measured by a consistency check. A higher score of the SS tendon insertion could indicate a more mature tendon-to-bone healing.

2.6. Synchrotron radiation-microcomputed tomography (SR-μCT) scanning

After sacrifice, the SS tendon insertion specimens of week 4 or 8 after surgery were scanned perpendicularly to the long bone axis using the SR-μCT at the Shanghai Synchrotron Radiation Facility (SSRF) in China. In brief, each sample was fixed in a table scanned with an angular step of 0.25° over an angular range of 180°. Then, the parameters of beam energy, exposure time and sample-to-detector distance in the current experiment were set as follows: 18.0 keV, 0.5 s and 10.0 cm respectively. A total of 720 radiographic projections of the SS tendon were captured by the charge-coupled device detector (CCD) under a spatial resolution of 3.25 μm per pixel. The initial projection images were performed with phase contrast retrieval, and sectioned into a series of 8 bit-slice images using PITRE software at SSRF. After removing the noises caused by the greyscale threshold filter, the bone was extracted from soft tissue. Consequent 8 bit-slice images of SS tendon enthesis were used to perform three-dimensional reconstruction and visualize with AMIRA software (Thermo Scientific, Waltham, MA). Next, a customized cylindrical region of interest (ROI) was selected surrounding the repaired tendon attachment site. Then morphological parameters of the newly formed bone, including bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp), were calculated.

2.7. Biomechanical testing

Biomechanical testing was measured by a tensile testing machine (Instron, 3343 System, USA) to detect the failure load, ultimate strength and stiffness. Before the test, the width and thickness of the footprint were measured with a vernier caliper under a constant tensile load of 0.1 N. The cross-sectional area (CSA) was calculated as well. Subsequently, the tendon was secured in an upper clamp using sandpaper, while the humeral shaft was fixed to a lower clamp. The specimens were preconditioned with 0.1 N and then loaded to failure at a rate of 0.03 mm/s. During tests, 0.9% saline was applied to avoid drying of the sample.

2.8. Immunofluorescence staining for NPY and its receptor Y1R

To detect the expression of NPY and its Y1R at the bone-tendon healing site between the control and treadmill groups, sections of the control group and the treadmill group were treated with 0.25% trypsin for 1 h at 37°C for antigen retrieval, and then were blocked using 3% goat serum for 60 min at room temperature. Afterward, sections were incubated with primary antibodies NPY (ab10980, Abcam) or Y1R (sc-393192, Santa Cruz) overnight at 4°C, subsequently incubated with Alexa-Fluor 594 (ab150120; Abcam) or Alexa Fluor® 488 (ab150113, Abcam) conjugated secondary antibody at room temperature for 1 h, then counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad). Images were acquired using a Zeiss Axio Imager.M2 microscope equipped with an ApoTome.2 system. The positive staining areas of the immunofluorescence of NPY and Y1R were measured by defining an ROI at the SS tendon attachment site using × 10 magnification photomicrographs. The positive area (%) was calculated by dividing the positively stained area by the total area of the ROI using Image-Pro Plus software (Version 6.0.0; Media Cybernetics Inc).

2.9. Immunohistochemical analysis

After hydrated with PBS, boiled in citrate buffer to retrieve antigens and incubated with 5% normal goat serum, the sections were performed
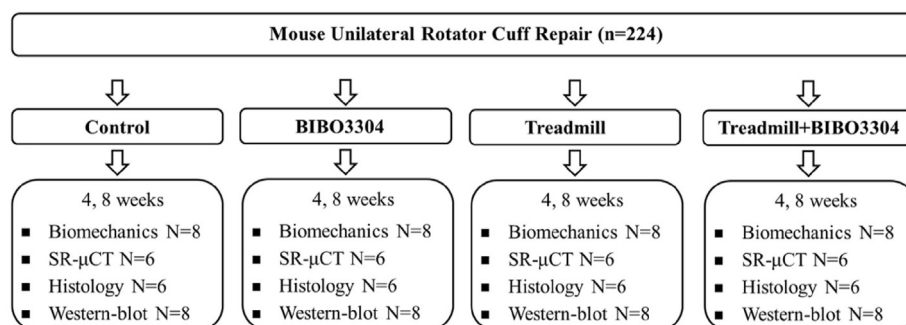
Figure 1. Experimental design flowchart. SR-μCT, synchrotron radiation micro-computed tomography.

with the anti-Wnt3a (ab219412, Abcam) or anti-β-catenin (ab264262, Abcam) at 4°C overnight. Subsequently, the sections were incubated with a biotinylated secondary antibody for 1 h at room temperature. Finally, HRP-streptavidin was performed for 15 min to visualize the immune reaction. All immunohistochemistry images were captured by a transmitted light microscope (CX31; Olympus) and the positive area (%) was calculated by Image-Pro Plus software (Version 6.0.0; Media Cybernetics Inc) according to the published literature [11].

2.10. Western blot

Before protein extraction, the humerus-supraspinatus samples were frozen in liquid nitrogen. Then we removed the muscle belly, kept the tendon (one millimeter in length) and the portion of the humeral head proximal to the growth plate near the tendon attachment. The weight of each sample was adjusted to be around 20 mg. Samples were collected and lysed in RIPA lysis buffer. The insoluble materials were sedimented by centrifugation, while the supernatants were collected for protein extraction. The protein concentration was determined by the BCA assay. Protein extracts were separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes (Immobilon P, Millipore, Billerica, USA). After being blocked with 5% non-fat milk, membranes were incubated with a specific primary anti-beta catenin antibody (ab264262, Abcam) at 4°C overnight and with the horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 h. Anti-β-actin and all the secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Finally, protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, USA). The reactions were detected by enhanced chemiluminescence assay using ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad, Hercules, CA, USA).

2.11. Statistical analysis

All quantitative data were shown as mean ± SD. Statistical analyses were performed using the SPSS 25.0 software (SPSS, USA). An unpaired two-tailed Student's t-test was used to determine statistical significance between every two groups. One-way ANOVA with *post hoc* test was used to analyze the differences above two groups, while the histological scores among all groups were performed using the Mann–Whitney test. $P < 0.05$ was considered statistically significant.

3. Results

All mice tolerated the surgery, treadmill running treatment and intra-articular injection, without perioperative complications and side effects. No gross failures or gaps were observed at the healing site of the SS tendon in all groups.

3.1. Expression of NPY at the healing site during the early treadmill running

We first analyzed the levels of NPY in control and treadmill groups. Remarkable elevation of NPY level was detected at the healing site of RC at postoperative week 4 and 8, when in comparison with the normal group. We furtherly evaluated the expression of NPY at postoperative week 4 and 8. As shown in Fig. 2, the control group has a minor expression of NPY, whereas the treadmill exhibited higher levels of NPY, with an NPY-positive area localized in the repaired insertion site. The above results suggested that NPY might have a very important effect on RC healing, especially in treadmill training groups.

3.2. Expression of Y1R at the healing site of the control and treadmill groups

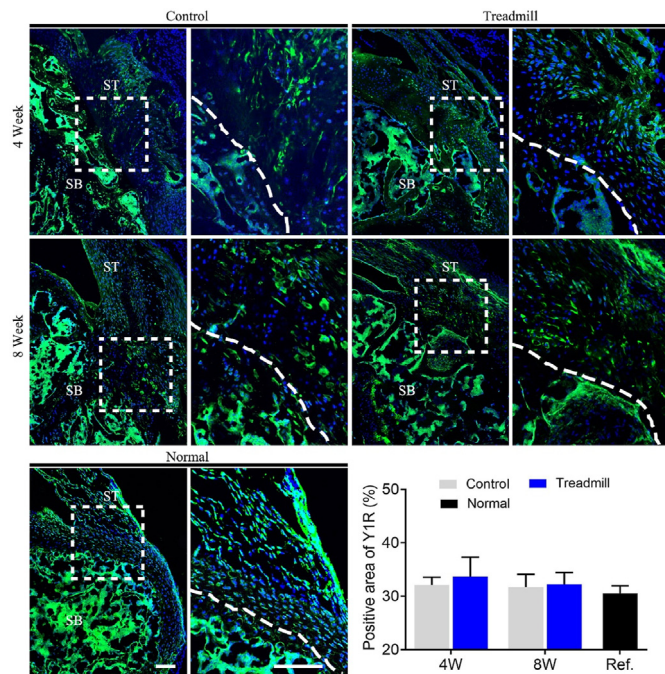Previous observations have shown that the local production of NPY
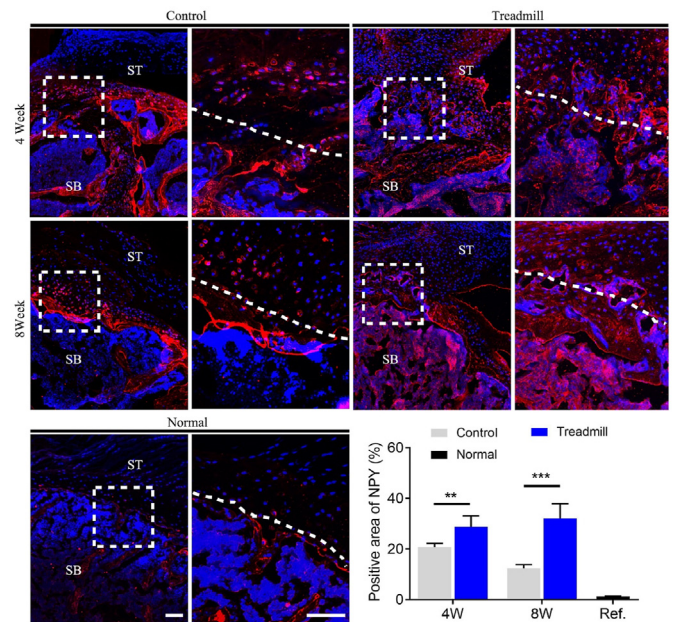
Figure 2. Representative immunofluorescence images and positive staining areas of NPY at the normal group and at the control and treadmill groups postoperatively 4 and 8 weeks. The area selected by the rectangle dashed line is the local magnified area. The dashed line denotes the normal or repaired insertion of the examples. SB, subchondral bone; ST, supraspinatus tendon; Scale bar = 50 μm, ** $P < 0.01$, *** $P < 0.001$.

and its receptor Y1 is involved in the regulation of peripheral musculoskeletal homeostasis [19,28]. To investigate the expression of NPY receptor at the RC healing site, we evaluated the expression of Y1R at the injury regions at week 4 and 8 postoperatively, the Y1R predominantly localized in the tendon, bone and repaired insertion of the control and treadmill groups, which were similar to their distribution of the normal enthesis of mice RC (Fig. 3). In addition, we semi-quantitatively analyzed the positive areas of Y1R from the normal, control and treadmill groups, and found that there was no significant statistical difference in the content of Y1R. The above results provided evidence for our injection of Y1R antagonist BIBO3304 in the study.

3.3. Histological analysis

As shown by H&E and SO&FG staining images (Figs. 4 and 5), no gap formations were found and the regenerated interfaces at the healing site were observable at 4 and 8 weeks for all groups. On the other side, along with the progress of the healing process, the healing morphology of the mouse RC injury tissue gradually improved.

Using the modified bone-to-tendon insertion maturing score, the histological scores for the repaired insertion in the treadmill group were significantly lower than those in the control group at postoperative week 4 and 8 (Figs. 4 and 5, $P < 0.05$), indicating that delayed or worse outcomes of SS enthesis healing in terms of morphology. In addition, the scores in the treadmill + BIBO3304 group were significantly higher than the treadmill group at 4 and 8 weeks after surgery ($P < 0.05$), indicating that the use of BIBO3304 could partly improve the morphology of the healing site to the treadmill group. Furthermore, the histological scores of the BIBO3304 group were significantly higher than the other groups at 4 and 8weeks postoperatively ($P < 0.05$). These results indicated that early postoperative treadmill running would impair TBI healing, while the added BIBO3304 might eliminate the negative effects of treadmill running thus enhancing TBI healing to some extent, which implied that the NPY-Y1R axis played an important role in early treadmill running after RC injury.

Figure 3. Representative immunofluorescence images and positive staining areas of Y1R at the normal group and at the control and treadmill groups postoperatively 4 and 8 weeks. The area selected by the rectangle dashed line is the local magnified area. The dashed line denotes the normal or repaired insertion of the examples. SB, subchondral bone; ST, supraspinatus tendon; Scale bar = 100 μm.

3.4. SR-μCT analysis

The three-dimensional visualization images of tendon enthesis
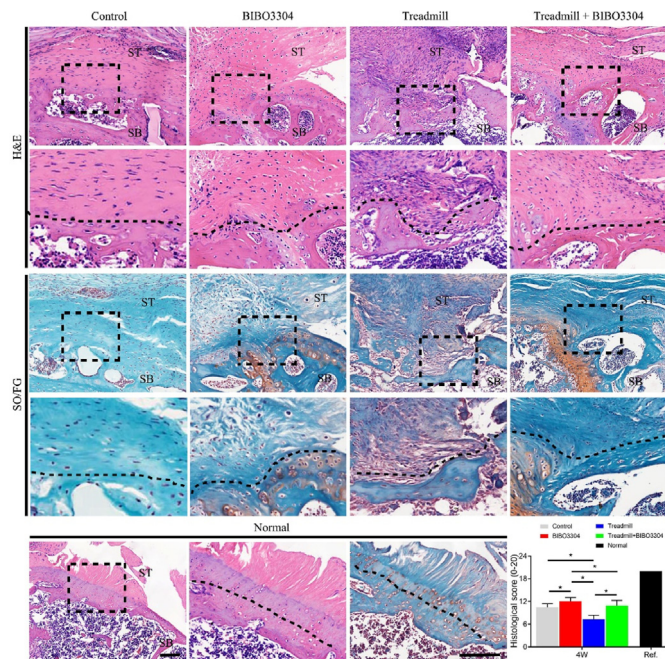
Figure 4. Representative histologic images of SS tendon enthesis at 4 weeks postoperatively for all groups. The area selected by the rectangle dashed line is the local magnified area. The dashed line denotes the normal or repaired insertion of the examples. SB, subchondral bone; ST, supraspinatus tendon; H&E, hematoxylin and eosin; SO/FG, safranin O and fast green; Scale bar = 500 μm, * $P < 0.05$.
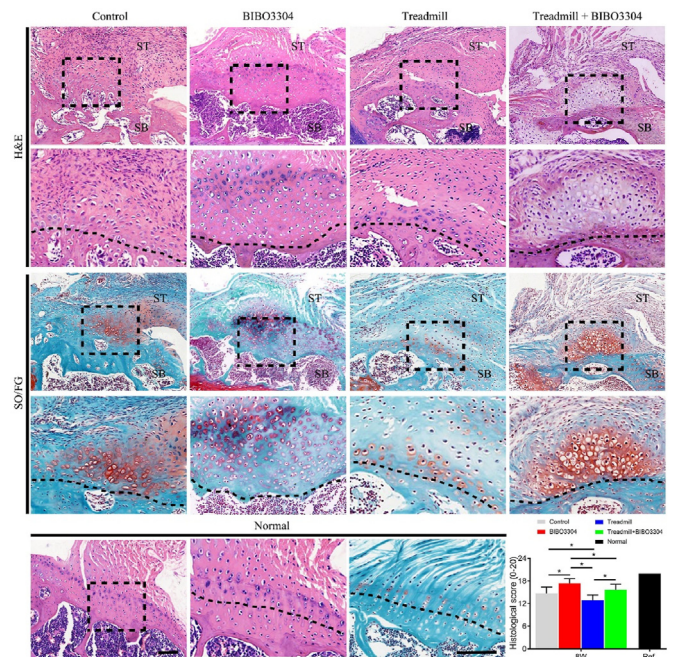

Figure 5. Representative histologic images of SS tendon enthesis at 8 weeks postoperatively for all groups. The area selected by the rectangle dashed line is the local magnified area. The dashed line denotes the normal or repaired insertion of the examples. SB, subchondral bone; ST, supraspinatus tendon; H&E, hematoxylin and eosin; SO/FG, safranin O and fast green; Scale bar = 500 μm, * $P < 0.05$.

showed the microarchitecture of the RC healing site. The bone parameters at the healing site were gradually increased with the healing process from all groups, which were shown in Fig. 6. At 4 and 8 weeks postoperatively, the healing interface of the treadmill group was shaped worse than that of the other groups. Quantitatively, BV/TV, Tb.N and Tb.Th of the disorganized bone in the treadmill group showed a significant decline in comparison with those of the control group ($P < 0.05$). Tb.Sp of the ROI surrounding the tendon attachment site had a significant increase ($P < 0.05$), showing that early treadmill running could impair the formation of new bone at the healing site. On the other hand, the treadmill group also showed a significantly lower BV/TV, Tb.N, Tb.Th and a significantly higher Tb.Sp, in comparison with the treadmill + BIBO3304 group at postoperative weeks 4 and 8 ($P < 0.05$). Meanwhile, 4 and 8 weeks after surgery, the BIBO3304 group showed higher values of BV/TV, Tb.N and Tb.Th among all groups, lower values of Tb.Sp at the regenerated interfaces than the other groups ($P < 0.05$). These results suggested that the NPY-Y1 axis might have an important function in regulating the quality of new bone formed on the injured tendon-bone insertion in a murine RC repair model during early treadmill running.

3.5. Biomechanical evaluation

Biomechanical analysis of the samples at 4 and 8 weeks after surgery are shown in Fig. 7, all specimens ruptured at the surgical repair site. There was no significant difference in CSA among the three groups at postoperative week 4 and 8. Failure load, stiffness, ultimate strength of all groups were furtherly obtained by biomechanical examination. At postoperative week 4 and 8, failure load and ultimate strength in the treadmill group showed significantly lower compared with the other groups ($P < 0.05$), while failure load and ultimate strength in the BIBO3304 group showed significantly higher compared with the other groups ($P < 0.05$). For stiffness, at 4 and 8 weeks after the operation, the value in the BIBO3304 groups was higher than the other groups
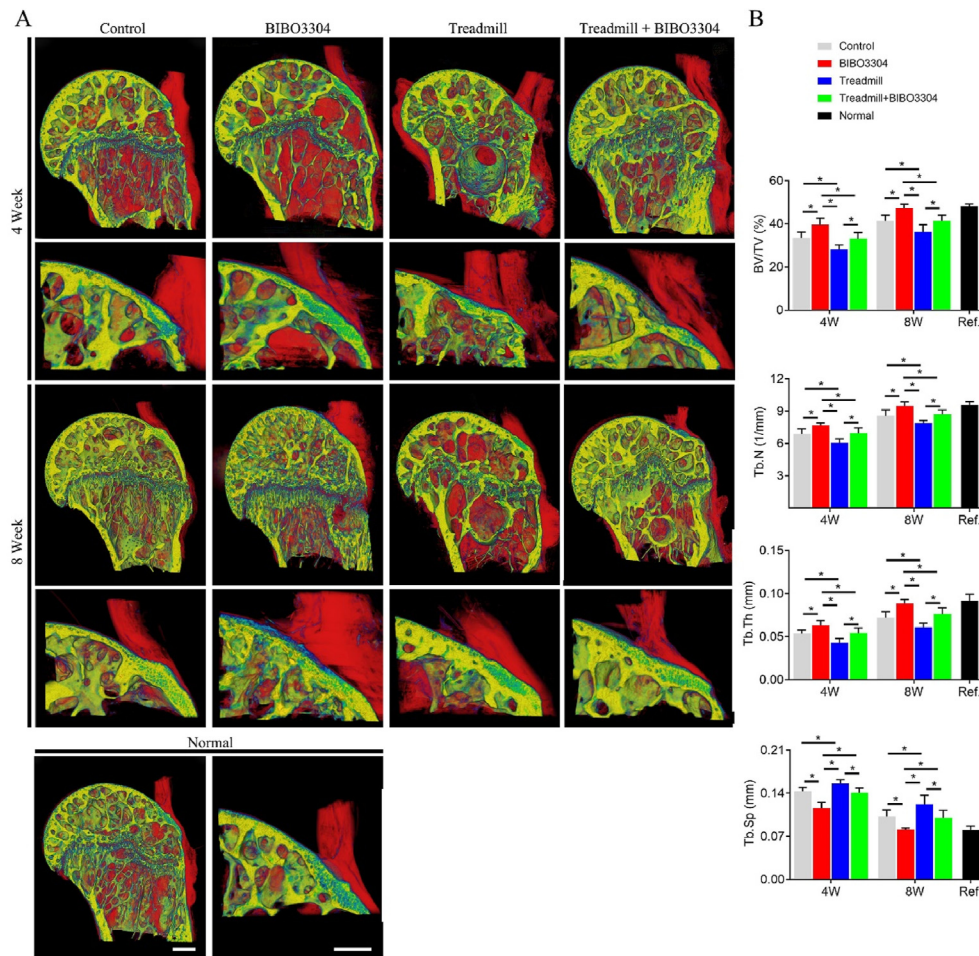
Figure 6. (A) Representative SR-µCT reconstruction images of the RC healing site for all groups at postoperative week 4 and 8, Scale bar = 500 µm. (B) Comparison of the bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) in newly formed bone among all groups at different time points after RC injury-repair. *$P < 0.05$.

($P < 0.05$), but no significant differences among the treadmill, control and treadmill + BIBO3304 groups. These results indicated that early treadmill running would hinder RC healing in biomechanical properties. And NPY-Y1 axis might influence the healing of RC stimulated with immediate exercise after surgery.

3.6. The activities of Wnt/β-Catenin signaling in vivo

The expression levels of Wnt3a and β-Catenin were assayed by immunohistochemical analysis (Fig. 8). At 4 and 8 weeks after surgery, the expressions of Wnt3a and β-Catenin were lower in the treadmill group than the other groups ($P < 0.05$). Meanwhile, the BIBO3304 group showed higher expressions of Wnt3a and β-Catenin at the healing site than the other groups ($P < 0.05$). Taken together, these data suggested that early treadmill running would inhibit the Wnt/β-Catenin signaling pathway, while the addition of Y1R antagonist BIBO3304 could alleviate the negative effects of early postoperative training and activate the Wnt/β-Catenin pathway. On the other hand, with the healing time increased, at 8 weeks after surgery, the expressions of Wnt3a and β-Catenin for all groups were lower than those of the same group at 4 weeks, which were consistent with the characteristics of healing of bone-tendon interface. These lower expressions of Wnt3a and β-Catenin might be beneficial for the formation of regenerated bone and cartilage in the early-middle stages after RC repair.

To investigate the mechanism underlying the results above, we furtherly evaluated the activities of Wnt/β-Catenin signaling by western-blot assay (Fig. 9). At 4 and 8 weeks after surgery, the results indicated that when compared to the other groups, the treadmill group showed a significantly lower protein expression level of β-Catenin, which revealed that early treadmill running inhibited the activation level of Wnt/β-Catenin signaling compared to the control group. The increased expression of β-Catenin in the treadmill + BIBO3304 groups showed significantly higher than those in the treadmill groups at postoperative week 4 and 8, which revealed that the use of BIBO3304 could partly alleviate the negative effects of early treadmill running on the healing of rotator cuff. Moreover, the expression level of β-Catenin in the BIBO3304 group was significantly higher compared with the other groups. Consistent with immunohistochemical analysis, these results also furtherly confirmed that inhibition of Y1R using BIBO3304 could potently increase the activation level of Wnt/β-Catenin signaling, and the NPY-Y1 axis might play an important role in the effects of early treadmill running on RC healing via the Wnt signaling.

4. Discussion

In the current practice, the restoration of RC function largely depends on the regeneration of its native structure. For decades, efforts have been employed to improve RC healing through the application of biophysical therapies [1]. Notably, despite the fact that such mechanical stimulation-based rehabilitation protocols have been considered effective, there remains a challenge to optimize the current treatment without better outcome measurements [3]. In this study, we investigate the effects of early treadmill running on RC healing and its underlying molecular mechanism, using a murine RC injury-repair model. Specifically,
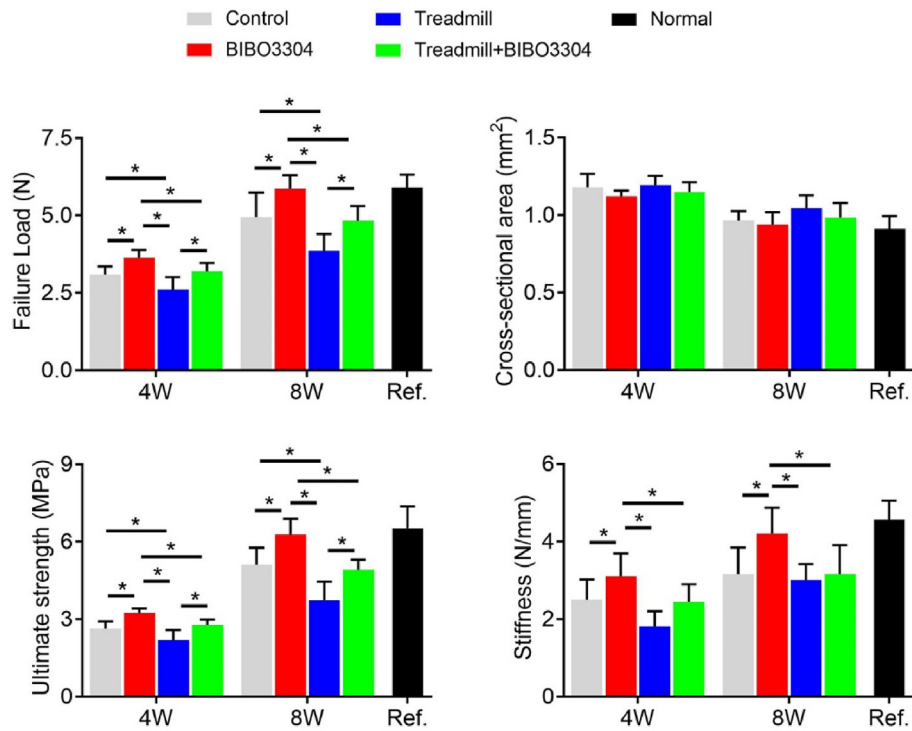
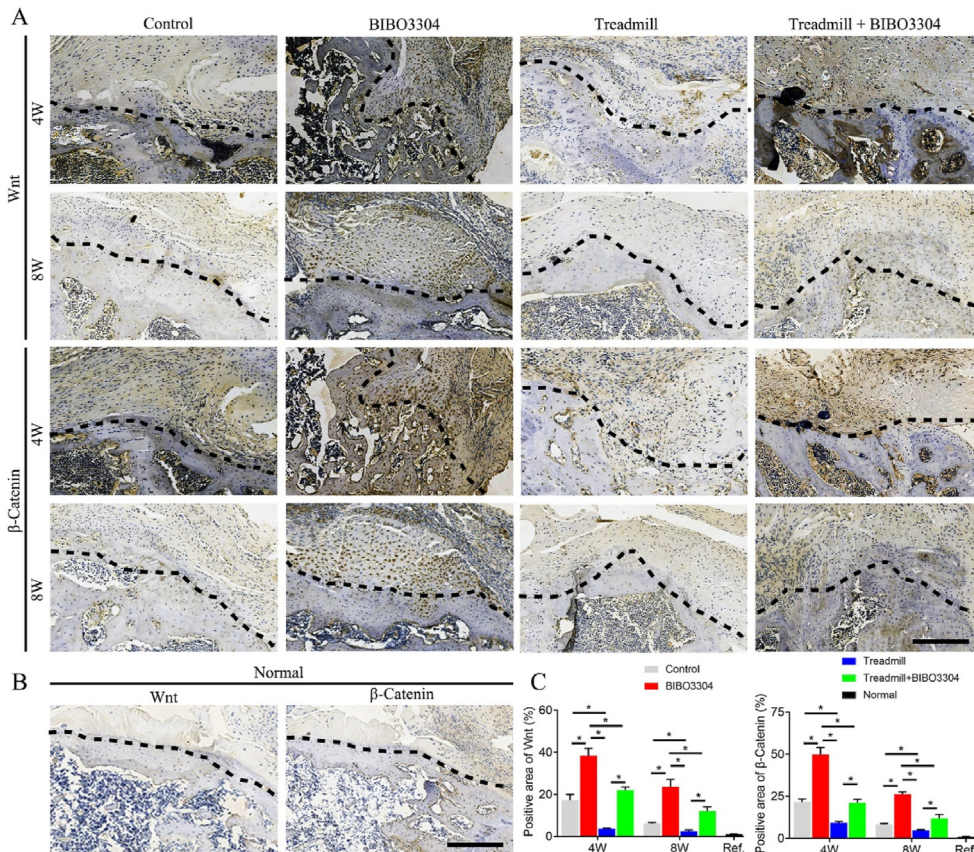**Figure 7.** Biomechanical properties of supraspinatus tendon specimens for all groups. *P < 0.05.
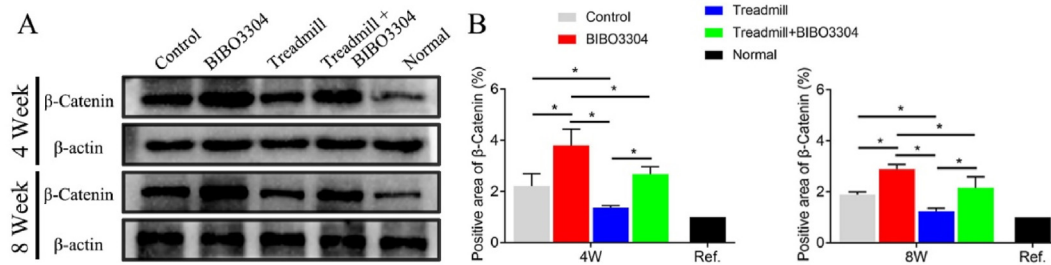

Figure 8. (A) Immunohistochemical staining images of Wnt3a and β-Catenin in the regenerated interface of RC at different time points after surgery. Scale bar = 300 μm. (B) Positive staining area of Wnt3a and β-Catenin for all groups at different time points after RC injury-repair. The dashed line denotes the normal or repaired insertion of the examples. * P < 0.05.

Figure 9. (A) Western-blot analysis of the expression of β-Catenin for all groups at postoperative week 4 and 8. (B) Comparison of the relative expression for all groups at different time points. *P < 0.05.

the histological evaluation, SR-μCT analysis, as well as biomechanical test were utilized, and we found early treadmill running could impair RC healing, when in comparison with a free-cage healing group. We furtherly found the blockade of the NPY-1R axis in the early treadmill running group resulted in superior bone–tendon junction repair with more new bone formed and better mechanical properties, compared with the early treadmill running group alone.

Mechanical stimulation is essential for both development or injury-repair in the musculoskeletal system, engagement of mechanical cues throughout the injury-repair process is indispensable to RC healing [5, 17]. However, to what extent the postoperative exercise (or rehabilitation methods) translates into muscle traction, that would benefit the tendon-bone interface healing is difficult to address. It is partly owing to the sophisticated cell behavior or secreted factors within the microenvironment under the condition of mechanical stress exerting effects on musculoskeletal tissues.

Luckily, a growing number of mechanosensitive cytokines or growth factors related to skeleton homeostasis and injury-repair have been reported. Wang et al. demonstrated that the matricellular protein secreted protein acidic and rich in cysteine (SPARC) contributed to tendon mechanobiology and regeneration [29]. Chang SH et al. indicated that exposure of articular cartilage to excessive mechanical loading would promote degeneration through a mechanical inducible factor gremlin-1 [30]. Of note, the role of periphery neuropeptides as mechanosensitive mediators involving in skeleton repair has lately been systemically documented [31]. Specifically, peripheral neuropeptide Y (NPY) has been found to increase in both tendon injury and tendinopathy, either in animal models or clinical specimens, with the presence of mechanical stress [17,31]. Given that NPY has been regarded as the most abundant periphery neuropeptide expressed in bony cells which inhibits osteoblastic activity [32]. This gives us a hint that NPY might be a negative factor under the condition of early treadmill running that delays RC healing. The specific role of NPY in RC healing and its underlying mechanisms needs further investigation.

The expression of Y1R has been confirmed in the motor system including bone and muscle [22]. In the skeleton, peripheral NPY exerts its biological function predominantly through activation of the Y1 receptor [19,28]. Consistent with prior observations, our immunochemistry results showed that Y1R was widely present at the healing site of RC. Moreover, loss of Y1R has been found to induce a high bone mass phenotype with increased deposition of mineral and extracellular matrix [33]. Sousa DM et al. confirmed that oral administration of the selective Y1R antagonist BIBO3304 increased bone mass in mice in a dose-dependently manner [21]. Meanwhile, Xie W et al. reported that BIBO3304 played an anti-osteoporotic effect and regulated gut microbiota, suggesting Y1R antagonist could be a novel treatment for postmenopausal osteoporosis [34]. To our knowledge, the use of Y1R antagonist BIBO3304 partly alleviates the bone loss in RC healing under the condition of early treadmill running, evidenced by histological and biomechanical results. It suggests that the unsatisfactory healing outcomes of TBI in RC could be altered by the manipulation of the NPY-Y1R axis.

Wnt/β-catenin signaling plays a vital role in tissue healing and regeneration as well as development. A previous study found that Wnt-mediated mechanotransduction is essential to maintain bone metabolism and keep the dynamic balance between bone formation and resorption [35]. Moreover, except for the ability to promote bone regeneration, Wnt3a is furtherly involved in the signaling pathway mediating stem cells participating in tissue renewal and regeneration [36]. It has been well-documented that the expression level of Wnt3α/β-catenin is related to mechanical stress, specifically that moderate loading may increase Wnt/β-catenin signaling [24,25,35]. According to Wada et al., early tendon loading after surgery by treadmill running delayed the expression of Wnt/β-catenin signaling pathways, as well as impaired RC healing [37]. Based on that, we furtherly believe that early treadmill running increased the expression of NPY at the RC healing site, which might burden the expression of Wnt3a/β-catenin and delay the healing process, as evidenced by our results: the use of BIBO3304 had a local regulation of Wnt/β-catenin signaling in RC with early treadmill running, and furtherly facilitated the healing of the bone-tendon insertion.

There are some limitations in the current study. Firstly, given that inevitable anatomic, kinetic and biochemical differences exist between humans and rodents, the experimental results might not be fully applicable to clinical practice. Secondly, sustained-release drug delivery to the RC tissue of mice is very difficult. The methods we tried before were not satisfying, including microinjection pumps, fibrin gels, etc. Optimized doses or delivery systems in small animals need further study. At last, future studies can be warranted to confirm the role of the NPY-Y1R axis in RC healing by utilizing conditional knockout mice.

5. Conclusion

Early treadmill running increased the expression of NPY at the RC healing site, which might burden the expression of Wnt3a/β-catenin and delay the healing process. The inhibition of the Y1 receptor with BIBO3304 could promote bone-tendon healing through the Wnt/β-catenin signaling. The results appear to prove, at least in part, the negative role and underlying mechanism of NPY in the RC healing during early treadmill running.

Author contributions

H.L. and J.H. designed experiments. Y.C. and T.Z. performed experiments. Z.W. and S.L. analyzed data. Y.C. wrote the manuscript. L.Y. and D.X. assisted in the experiments and preparation of the manuscript.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2021.08.004.

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