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Original article

Effect of eccentric exercise on healing process of injured patellar tendon in rats

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Abstract

Background. Earlier studies have reported positive results from eccentric training in patients with tendon disorders. The reasons for the beneficial clinical effects of eccentric training are not known. Vascularization followed by regression of the vasculature enhances the healing response of injured tendons. Eccentric exercise induces a more beneficial healing response than concentric exercise.

Methods. Sixty rats with patellar tendon injuries were divided into three groups: nonexercise controls (group N; $n = 20$); concentric exercise group (group C; $n = 20$); eccentric exercise group (group E; $n = 20$). Each rat was taught to run uphill or downhill for 14 days. Patellar tendons were removed 1, 4, 7, 10, and 14 days following injury. Vascular endothelial growth factor (VEGF), angiopoietin-1, and angiopoietin-2 were measured by reverse transcription polymerase chain reaction.

Results. In group C, VEGF mRNA was increased 1 and 4 days following injury but was decreased on days 7, 10, and 14. In group E, VEGF mRNA was elevated only on day 1. In group N, VEGF mRNA remained at a low level throughout all 14 days. The angiopoietin-2/angiopoietin-1 ratio was higher for group C than for group E.

Conclusions. In the presence of VEGF, angiopoietin-1 promotes vessel stability, whereas angiopoietin-2 has the opposite effect. Eccentric exercise contributes to stabilized angiogenesis during the early phase of tendon injury. Conversely, concentric exercise, which induces destabilized angiogenesis, leads to a delayed healing response. Initiation of eccentric exercise immediately after tendon injury may help improve healing by reducing vascularity.

Introduction

Injury of the tendon is particularly evident in activities that involve repeated impact loading of the lower limbs, especially running and jumping activities. The treatment of tendon injury has long been disputed. Tendon injury and tendinosis are often resistant to conservative treatment. Several articles have recommended an eccentric training program for tendon disorders and have demonstrated positive results with eccentric training for patients with tendon disorders. The causes of the good clinical effects achieved with eccentric training are not known. Restoration of physiological structure of the tendon, including normal vasculature, is essential for healing of a tendon injury or tendinosis. However, increased vascularization of the injured tendon is essential during the early phase of healing. We surmised that vascularization followed by the regression of the vasculature enhances the healing response of injured tendons. We hypothesized that eccentric exercise would induce a better healing response than concentric exercise. The purpose of this study was to investigate the effect of eccentric and concentric exercise on injured tendons during the early phase of tendon healing and to confirm the effectiveness of eccentric exercise on the healing of injured tendons, especially with regard to tendon vascularity.

Methods

Tendon injury model

Female Wister rats (200–250 g, Charles River Laboratories, Yokohama, Japan) were used in this study. Their average age was 7 weeks. The rats were anesthetized by intramuscular injection of ketamine (25 mg/kg). A previous report about patellar tendinosis concluded that it was caused by dissociation of fiber rather than partial fiber rupture.[1] Therefore, we used a fiber dissociation model to mimic patellar tendon injury. To create a fiber dissociation model of patellar tendon injury, we made small incisions in the skin to expose the tendon and

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created two longitudinal splits in each tendon.[2] The rats were given a full day to recover before beginning the exercise training program. Rats were divided into three experimental groups with 20 rats in each group: nonexercise controls (group N); concentric exercise group (group C); eccentric exercise group (group E). The protocol was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Exercise training

While running down an incline, extensor muscles primarily perform eccentric contractions in which the muscles lengthen while they are actively developing tension. The major function of these extensor muscles during downhill running is to decelerate the animal's center of mass to maintain a constant average running velocity. On the other hand, the extensor muscles perform concentric contractions during uphill running.[3] The concentric exercise group ran on a treadmill up a 15° incline. The eccentric exercise group ran down a 15° incline. Rats exercised once daily on a motor-driven rodent treadmill (Muromachi Kikai, Tokyo, Japan) for 1 h at 15 m/min in room air. To study the time course of angiogenic growth factor expression in response to training, rats followed the training program for 1, 4, 7, 10, and 14 days (four animals for each time point). On the last day of training, patellar tendons were collected 1 h after the final exercise bout. We used hindlimb elevation as a non-weight-bearing and non-exercise control model ($n = 20$). These control rats were also euthanized at, 1, 4, 7, 10, and 14 days after injury (four animals in each group).

Tissue collection and RNA isolation

Under deep anesthesia with high-dose ketamine, patellar tendons were collected from both hindlimbs. Samples were immediately flash-frozen in liquid nitrogen after harvest and stored at −70°C. Total cellular RNA was isolated from each sample according to the manufacturer's instructions using Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). RNA preparations were quantitated by absorbance at 260 nm.

Primer design

The genes examined in this study were vascular endothelial growth factor (*VEGF*), angiopoietin-1 (*Ang1*), angiopoietin-2 (*Ang2*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers for all genes were designed using gene sequences published on the NCBI website. The sequence of each primer was as follows: *VEGF* (GenBank accession no. L20913): 5′-GTC TAC CAG CGC AGC TAT TG-3′ (sense), 5′-ACA GTG AAC GCT CCA GGA TT-3′ (antisense); *Ang1* (GenBank accession no. AF311727): 5′-TCA GTG GCT GGA AAA ACT TG-3′ (sense), 5′-TTT GTC TGT TGG AGA AGC TG-3′ (antisense); and *Ang2* (GenBank accession no. AY052400): 5′-AGA GTA CAA AGA GGG CTT CG-3′ (sense), 5′-GTG GGT AGT ACT GTC CAT TC-3′ (antisense).

In addition, *GAPDH* was used as an internal control (cat. no. SP-10241; Maxim Biotech, South San Francisco, CA, USA). The primers used were as follows: 5′-GGG TGG TGC CAA AAG GGT C-3′ (sense), 5′-GGA GTT GCT GTT GAA GTC ACA-3′ (antisense).

Semiquantitative RT-PCR

Expression of these angiogenic mRNAs was quantitated by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) using the TaqMan One-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). The thermal cycling program consisted of an initial denaturation step of 95°C for 2 min, followed by 35 cycles for VEGF, Ang-2, and GAPDH and 40 cycles for Ang-1 consisting of 95°C for 15 s, a 30-s annealing step at 58°C, and a 1-min extension step at 72°C. Aliquots of each RT-PCR product were electrophoresed on 1.5% agarose gel containing ethidium bromide. The bands were visualized and analyzed quantitatively by densitometric scanning. These values were normalized to GAPDH RNA expression.

Histology and immunohistochemistry

Patellar tendons in each exercise group were harvested at 1, 4, 7, 10, and 14 days after injury and were fixed for histological analysis in 10% formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E) by routine methods. For assessment of type III collagen and smooth muscle α-actin (α-SMA) deposition, sections were stained with rat monoclonal antibody to α-SMA (1:200 dilution) (Nichirei, Tokyo, Japan) or rat monoclonal antibody to type III collagen (1:200 dilution) (Nichirei). Localization of the antibody was detected immunochemically according to the manufacturer's instructions using the Histofine Simple Stain kit (cat. no 414191; Nichirei).

Statistics

Analysis of variance (ANOVA) with Fisher's post-hoc analysis was used to determine if significant differences in mRNA levels existed among the experimental periods (at 1, 4, 7, 10, and 14 days after injury). Significance was taken as $P < 0.05$.

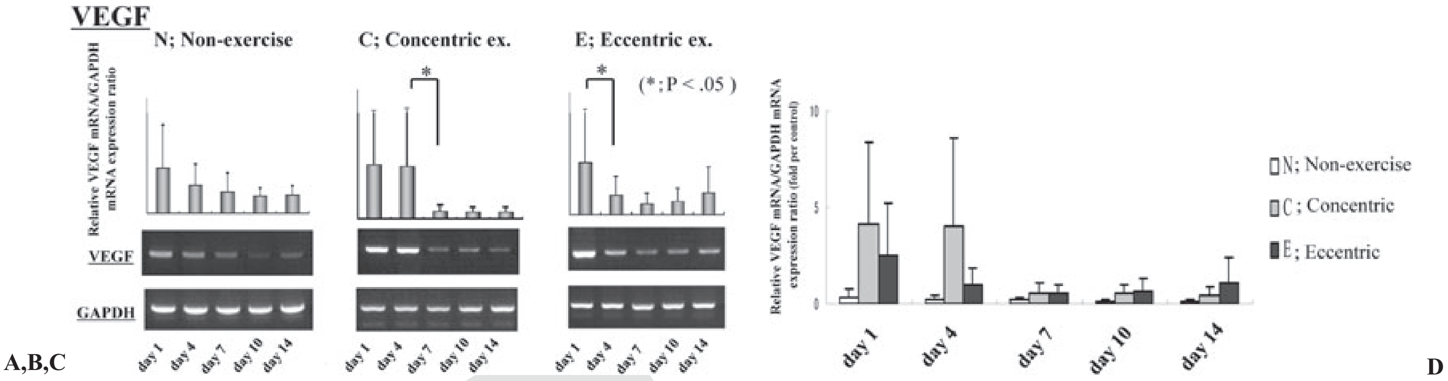

Fig. 1. Time course of expression of vascular endothelial growth factor (*VEGF*) messenger ribonucleic acid (*mRNA*). VEGF mRNA expression was initially at the highest level and decreased thereafter in all groups. **A** In group N, VEGF mRNA decreased gradually. **B**,**C** In the exercise groups, VEGF mRNA was up-regulated by exercise training during the initial phase of tendon injury, and then rapidly decreased by day 4 (group E) or by day 7 (group C) fter the injury. **D** In contrast, in group N, VEGF mRNA did not change and remained at a low level (**D**). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in each lane served as an internal control

Results

Expression of angiogenic mRNAs

VEGF

In all groups, VEGF mRNA was observed to be at its highest level 1 day following injury, and it decreased at each time point thereafter. In group N, VEGF mRNA gradually decreased (Fig. 1A). In the exercise groups, the pattern of expression in response to exercise was different in each exercise group. In group E, VEGF mRNA was sharply increased and rapidly decreased at day 4. In group C, on the other hand, VEGF mRNA was maintained at high levels for 4 days after injury and decreased thereafter (Fig. 1B,C) ($P < 0.05$). In the exercise groups, VEGF mRNA was up-regulated by exercise training during the initial phase of tendon injury. However, compared to groups C and E, VEGF mRNA in group N was maintained at low levels, with no changes in its level observed at any time point (Fig. 1D).

Angiopoietin-1

In each group, Ang1 mRNA expression was not changed at any time point. In group C, Ang1 mRNA was maintained at low levels during the training period. In group E, Ang1 mRNA levels were higher than those of group C (Fig. 2). These data indicate that concentric exercise of the injured tendon may suppress expression of Ang1 mRNA.

Angiopoietin-2

No obvious differences in Ang2 mRNA expression were observed among the exercise and control groups. In all experimental groups, Ang2 mRNA expression was observed to be at the highest level during the early phase after injury and decreased gradually thereafter (Fig. 3).

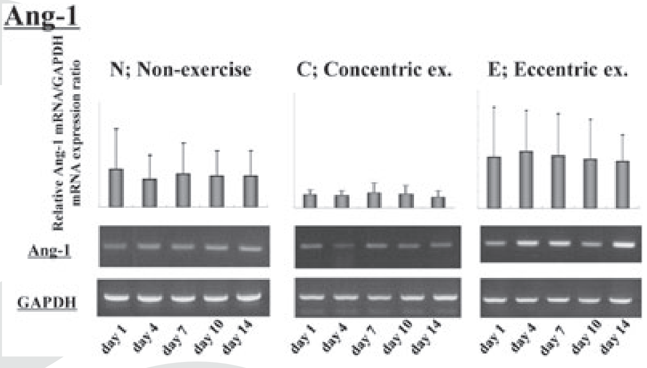

Fig. 2. Time course of angiopoietin-1 (*Ang-1*) mRNA expression. In each exercise group, Ang1 mRNA expression did not change at each time point. In group C, Ang-1 mRNA was down-regulated at every time point in comparison to groups E and N

Angiopoietin-1/angiopoietin-2 ratio

The Ang1/Ang2 ratio was higher in group E than in group C for all experimental days (Fig. 4). These data indicate that eccentric exercise induced Ang1 dominance, whereas concentric exercise induced Ang2 dominance. The Ang2 dominance in group C was primarily due to lower levels of Ang1 expression for all experimental time points. The Ang1 dominance in group E was due to higher levels of Ang1 expression.

Histology and immunohistochemistry

Tissue samples were harvested at each time point (day 1, 4, 7, 10, 14). On H&E-stained sections, there was little D2 evidence of new capillary formation or inflammatory reaction cell accumulation in the collagen fibers or injury sites for all experimental periods in group N (Fig. 5). In group C, new capillaries were noted in specimens obtained at 4 and 7 days after injury. The capillaries were also observed at 10 days after injury (Fig. 6). In group E, the presence of capillaries was observed 4 days after injury, and a decrease in the number of capillaries was observed at each time point that followed. In groups C and E, aggregation of inflammatory cells was observed at the injury site 7 days after injury. These localized aggregates of cells were observed until 10 days after injury in group C, whereas in group E only a few aggregates of inflammatory cells were noted at the injured site on day 10.

Immunohistochemistry for α-SMA at day 14 after injury confirmed an increase in α-SMA deposition at the injured site in group C. The deposition of α-SMA was subdued in groups N and E (Fig. 7). Immunohistochemistry for type III collagen at day 14 after injury showed moderate deposition in the exercise groups. Deposition of type III collagen was lower in group N than in group C or E (Fig. 8). Deposition of type III collagen was not apparently different between groups C and E at day 14.

Discussion

Our results support the hypothesis that eccentric exercise can elicit a better healing response in regard to vascularization and regression of vasculature. For the healing of injured tendons, vascularization is essential to provide extrinsic cells, nutrients, and growth factors to the injured area. During tendon injury, as with damage to any tissue, there is a requirement for cell infiltration from the blood system to provide the necessary reparative factors for tissue healing.[4,5] Tendon healing is impaired if there is diminution of the blood supply.[6] If there is an impoverished vascular supply to the injured site, a portion of the injured site may die, resulting in poor healing. In our study, VEGF mRNA expression was up-regulated by exercise during the initial phase of the exercise training program compared to the nonexercise controls; the lower VEGF expression in the nonexercise controls suggests that this treatment may lead to poor vascularization and result in poor healing. The VEGF mRNA response to increases in exercise intensity has been shown previously.[7,8] Exercise is essential to accelerate neovascularization and thereby elicit a healing response in the injured tendon. Increased levels of angiogenic growth factors, such as VEGF,

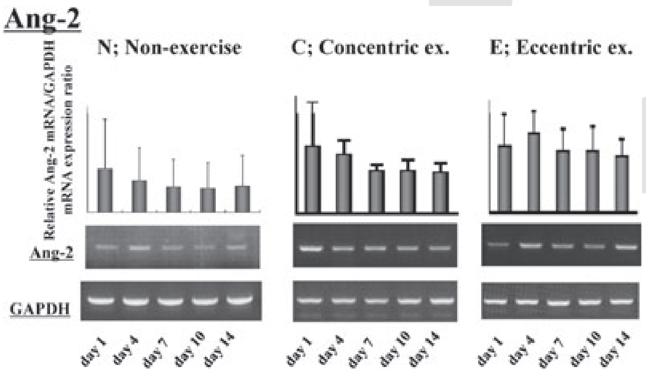

Fig. 3. Time course of angiopoietin-2 mRNA expression. In all groups, Ang2 mRNA expression was up-regulated during the early phase after injury and decreased gradually thereafter

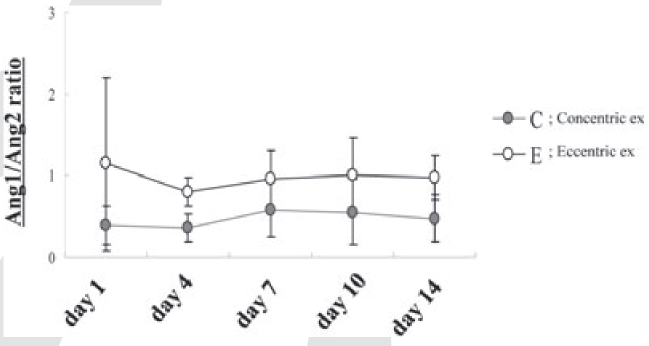

Fig. 4. Angiopoietin-1/angiopoietin-2 expression ratio. *Open circles*, group E; *filled circles*, group C. the Ang1/Ang2 ratio was higher in group E than in group C for 14 days. Eccentric exercise induced Ang1 dominance, whereas concentric exercise induced Ang2 dominance

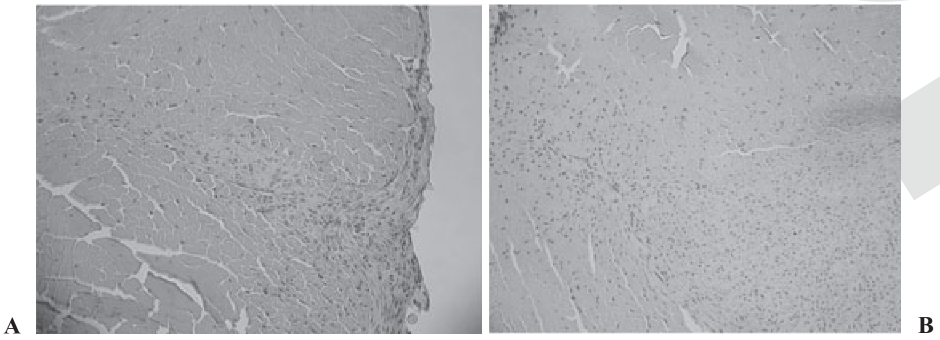

Fig. 5. Group N at day 7 (**A**) and day 10 (**B**). There was little evidence of new capillary formation or inflammatory cell accumulation in the collagen fibers and injured sites. H&E ×200

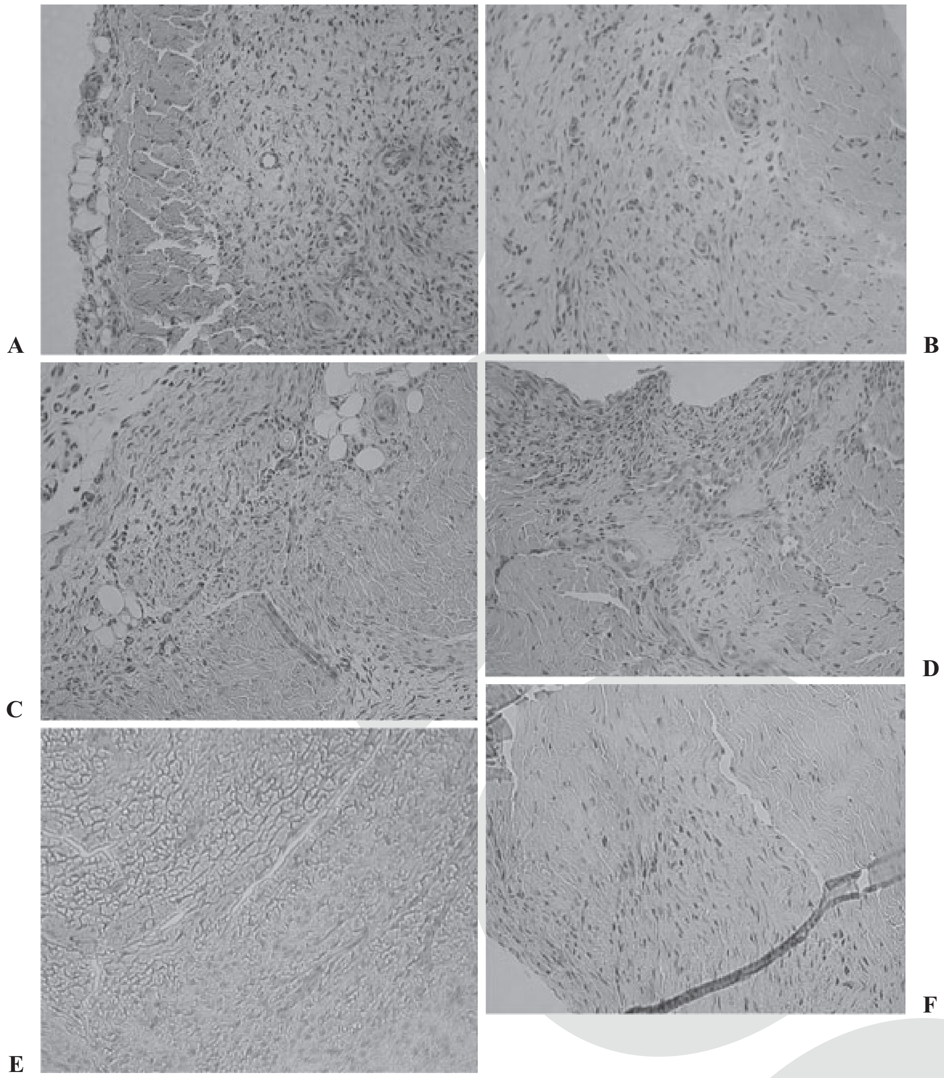

Fig. 6. Groups C and E. New capillaries were observed for 10 days after treatment in group C (**A** day 4, **B** day 7, **C** day 10), whereas capillaries were observed at day 4 and decreased thereafter in group E (**D** day 4, **E** day 7, **F** day 10). H&E ×200

within an injury site are correlated with a well-defined pattern of vascular ingrowth toward the site of repair. The abundance of VEGF mRNA and protein and its up-regulation during exercise training appear to vary with vascular density and active angiogenesis.[9]

However, the presence of hypervascularity cannot be regarded as an indicator of optimal tissue healing. Prolonged vascularization may lead to an unfavorable healing environment, such as persistent inflammatory changes and increased scar tissue. We think that regression of the vasculature following vascularization during the early phase of healing in tendons is essential to obtain a normal structure of the injured tendon. In this study, changes in VEGF mRNA expression are most dramatic during the initial phase of training and are tempered as the training progresses. In group C, VEGF mRNA increased immediately and remained at high levels for 4 days after injury, whereas the increase was observed only on the first day after injury in group E.

In addition to the effect on VEGF mRNA expression, exercise training also altered the expression of angiopoietin mRNAs. Our data indicate that eccentric exercise induces angiopoietin 1 dominance throughout the experimental period, whereas concentric exercise leads to angiopoietin 2 dominance. Recent evidence suggests that the angiopoietins play crucial roles in the modulation of VEGF-induced angiogenesis. VEGF is absolutely critical for the earliest stage of angiogenesis and is also important during the later stages of angiogenesis as well as for vessel survival.[10,11] However, the angiopoietins do not participate in the initial vasculogenic phase of vascular development; rather, they play critical roles in angiogenic outgrowth, vessel remodeling, and maturation.[12] Ang1 plays an important role in a later stage of angiogenesis subsequent to VEGF and is involved in vessel maturation and stabilization. A previous report suggested that endothelial cells in Ang1-deficient mice appear to be poorly associated with the

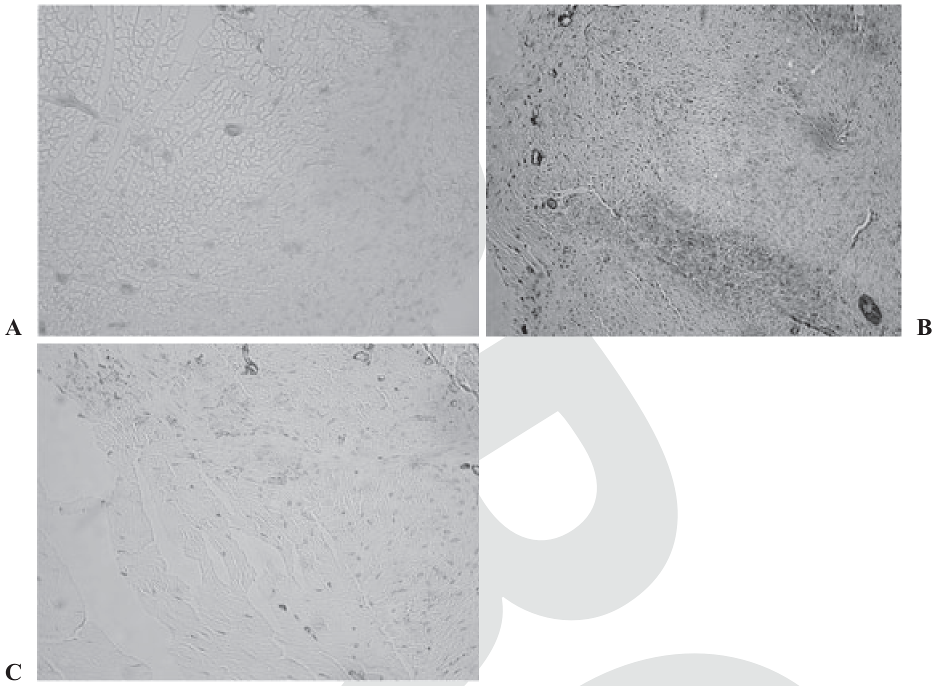

Fig. 7. Immunohistochemistry for α-smooth muscle antibody (α-SMA) at day 14 after injury. α-SMA-positive spots were prominent at the injured site in group C (**B**) compared to that in group N (**A**) and group E (**C**). ×200

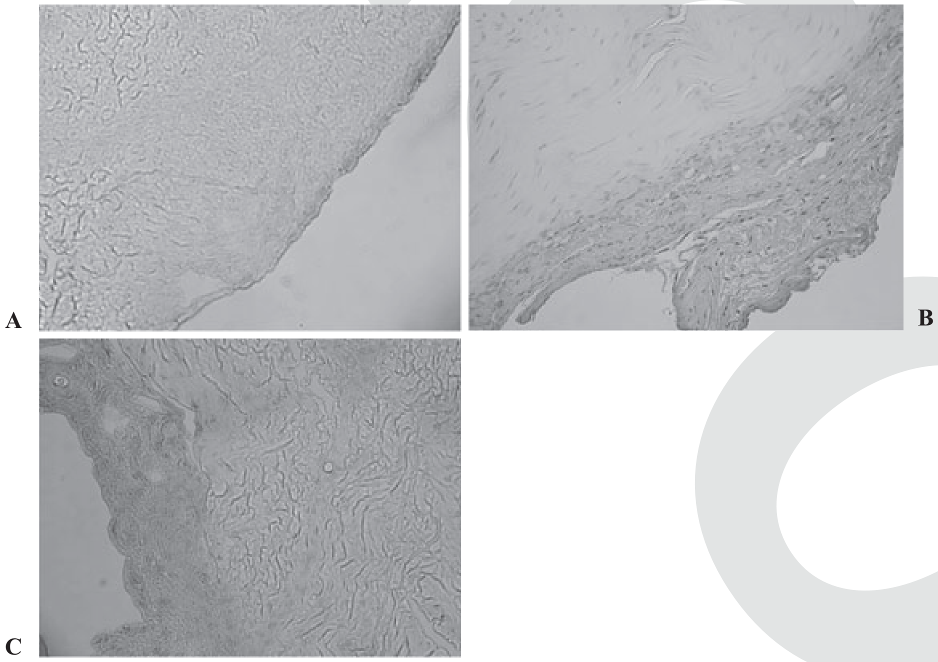

Fig. 8. Immunohistochemistry for collagen type III. Expression of type III collagen was at lower levels in group N (**A**) than in group C (**B**) or group E (**C**). ×200

basement membrane and with perivascular cells.[13] Ang1 increases endothelial cell proliferation and survival and contributes to more stabilized vessel formation. Ang1 and VEGF appear to act on distinct signaling pathways for vessel growth.

Angiopoietin-2 plays a facilitative role at the site of vascular remodeling by blocking the constitutive stabilizing action of Ang1, allowing the vessels to enter a more plastic and unstable state.[12] In the presence of abundant VEGF, Ang2 can promote the vessel to mount a strong angiogenic response that may lead to immature vasculature. Ang2 plays an active role in blood vessel remodeling.[14]

In this study, eccentric exercise induced high levels of VEGF for only 1 day after injury and Ang1 dominance for 14 days after injury, contributing to the formation of nonleaky, stabilized, functional capillaries only during the initial phase after injury. On the other hand, concentric exercise induced high levels of VEGF for at least 4 days and Ang2 dominance during the entire experimental period, eliciting elongation of the vascular remodeling phase, which allowed the vessels to revert to a more plastic, unstable state, leading to the formation of ineffective vasculature.

Vascularization can play a role in the development of scar formation. Abnormal, unstable vessels have excessive permeability and are at risk for easy structural disruption. Abnormal vascularization can be involved in the development of hypertrophic scars and keloids.[15] The presence of scar tissue in the healing tendon and muscle is recognized clinically to be a risk factor for injury recurrence and alteration of normal musculoskeletal structure. Scar tissue production in the healing tendon inhibits complete tendon tissue regeneration and may lead to decreased tendon strength.[16] Therefore, concentric exercise, which may cause the formation of ineffective vasculature and elongation of vascular remodeling, may induce more scar formation during the reparative phase after injury. On the other hand, eccentric exercise, which causes formation of a stabilized vasculature and shortening of vascular remodeling, may contribute to a healing response with less scar formation.

Recent studies have found that α-SMA is commonly expressed in healing wounds and scars. It is not observed in small wounds that heal without scars but is expressed in all wounds that heal with scars.[17] Myofibroblasts may facilitate early wound closure, but the persistence of these cells may lead to scar formation. A hallmark of myofibroblasts is the expression of α-SMA.[18,19] Therefore, α-SMA may be a marker for fibrosis and scar formation. We analyzed α-SMA expression by immunohistochemistry at day 14 after injury and found that deposition of α-SMA in the eccentric exercise group was more subdued than that in the concentric exercise group. These data, including the RT-PCR data described above, indicate that eccentric exercise may lead to healing with less scarring than concentric exercise.

To confirm the remodeling response of the injured tendon, we analyzed the expression of type III collagen. The expression of type III collagen in the non-exercise group was subdued at day 14. These data indicate that exercise contribute to the remodeling response.

Recent evidence suggests that eccentric training is more suitable for tendon injury and tendinosis.[20] Eccentric exercise is beneficial in evoking gains in muscle power and muscle spring stiffness,[21,22] increasing stiffness in the tendon, preventing injury to the muscle-tendon unit and the tendon itself,[21] and reducing pain associated with tendon disorders.[21,23,24] The advantages of eccentric exercise compared to concentric exercise are reduction of metabolic costs and O_2 consumption,[3,25] production of much greater force (i.e., two- to threefold) than that of concentric exercise,[26] and a better remodeling response. Ischemia and local hypoxia are thought to be major stimuli for VEGF-induced angiogenesis. The primary initiators of VEGF expression are undoubtedly hypoxia and oxidative stress. Therefore, we confirmed that eccentric exercise may induce less hypoxia in the injured tendon, regression of VEGF accumulation immediately, and shortening of the vascular remodeling response during the early phase of injury, leading to diminished scar formation and more favorable healing.

Conclusion

Although our investigative model does not provide a completely accurate clinical description of tendon injury and tendinosis, our data suggest that the application of eccentric exercise immediately after tendon injury facilitates healing by reducing vascularity. In this study, we examined only the early phase of healing and not the correlation between vascularization and remodeling. It would be useful to investigate whether the eccentric exercise may also contribute to the late phase of tendon healing.

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