

Research article

VEGF and bFGF Expression and Histological Characteristics of the Bone-Tendon Junction during Acute Injury Healing

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Abstract

Bone-tendon junction (BTJ) injuries are common and may be caused by acute trauma and delayed healing during exercise or work. To understand the nature of the healing process of BTJ injuries would help to prevent injuries and improve treatment. Thirty-three mature female rabbit hindlimbs were assigned to normal control (CON, n = 7) and injury groups (n = 26). The acute injury was established by administering one 7 plum-blossom needle puncture. Specimens were harvested post injury at 1, 2, 4, and 8 weeks (ND1W, n = 6; ND2W, n = 6; ND4W, n = 7; and ND8W, n = 7). The injury existed in all of the injury groups. Compared with the CON group, all of the animals in the injury group showed poor cell profiles, an unclear or undetectable tide mark, a proteoglycan area and profile changes; the BTJ cell density diminished significantly in the ND1W ($p < 0.01$), ND2W ($p < 0.05$), ND4W ($p < 0.01$), and ND8W groups ($p < 0.01$); the fibrocartilage zone thickness in all injury groups was significantly thicker than in the CON group ($p < 0.05$), but no significant difference was found among the injury groups ($p > 0.05$). The basic fibroblast growth factor (bFGF) expression in the CON group was significantly less than in the ND1W group ($p < 0.01$), but no significant difference was found when compared with the ND2W, ND4W, and ND8W groups. The bFGF expression in the ND1W group was higher than that of the ND4W ($p < 0.05$) and ND8W groups ($p < 0.01$). The vascular endothelial growth factor (VEGF) levels were not significantly different among the groups ($p > 0.05$). The bFGF and VEGF expression levels indicated that the healing process stopped at 8 weeks post injury or was not activated, although the injury had not healed by histological examination. A repeatable animal model of BTJ acute injury was established in this study, and the results described the BTJ acute injury healing difficult concerned with the repairing stop.

Key words: Bone-tendon junction injury, puncture, histology, growth factor.

Introduction

Chronic bone-tendon junction (BTJ) injury is common in athletes and some professional workers (Torkki et al., 2002). The major causes of chronic BTJ injury include repeated micro-trauma during exercise and delayed healing of an acute injury (Qin et al., 2006; Wang et al., 2008). The injury may affect an athlete's routine training or result in an employee's work loss (Ergen, 2004; Fordham et al., 2004). Many researchers have established BTJ injury animal models for studying tendinosis or tendinopathy based on cyclical loading (Nakama et al., 2005; Wang et al., 2012), tendon enthesis section (Lu et al.,

2006; Soeki et al., 2000; Sonnabend et al., 2010), acute exercise (Solem et al., 2011), and injection of chemical agents (Hsu et al., 2004; Vineros et al., 2001). Although these models have provided a great deal of information regarding BTJ injury healing and treatment, a reproducible model that can be easily established is still needed for the study of BTJ injury. In cases of BTJ injury caused by acute injury, such as a micro-tear or a laceration of the BTJ, the overuse or delayed healing of the injury results in minor acute injuries accumulating (Riley, 2008). The poor treatment outcome may result from the poor regeneration capacity of the BTJ (Hamilton and Purdam, 2004; Wang et al., 2005) and the lack of understanding of the acute BTJ injury healing characteristics.

During the BTJ injury healing, the basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were two important growth factors that affected injury healing (Lu et al., 2008; Thomopoulos et al., 2005; Wurgler-Hauri et al., 2007). The bFGF was found to significantly increase the cell density, collagen diameter, stiffness and loading capacity in the early stage of tenotomy, but no significant improvement in the biomechanical properties was reported (Chan et al., 2000). The increased VEGF expression that is associated with the acute injury caused inflammation, tissue metabolism promotion and hypoxia (Wang et al., 2007). It contributed to the healing of acute BTJ injury, but high VEGF expression levels were also found in injuries from BTJ overuse (Pufe and Petersen, 2005). Therefore, the high expression levels of bFGF and VEGF had different roles in the different stages of injury healing. In this study, a simple and repeatable BTJ injury model was established by using 7 plum-blossom needles to vertically puncture the patella-patellar tendon junction. Post injury time points of 1, 2, 4, and 8 weeks were observed to histologically and immunohistologically investigate the natural healing process. The aim of this study was to reveal the BTJ acute injury healing characteristics and provide theory for the post injury training management.

Methods

Thirty-three female rabbits (aged 18 weeks, 2.8 ± 0.24 kg) were divided into post-injury groups at 1 (ND1W, n = 6), 2 (ND2W, n = 6), 4 (ND4W, n = 7), and 8 weeks (ND8W, n = 7) and a normal control group (CON, n = 7). This experiment only used the right hindlimbs of the rabbits; the left limbs were used in another study (Wang et al., 2012). Under general anesthesia with 0.25% pento-

barbital sodium (2 ml/kg, intraperitoneal injection) (Sigma Chemicals Co., St Louis, MO, USA), the patellar tendon enthesis (TE) area was damaged using 7 plum-blossom needles (0.1 mm diameter) via vertical puncture (Figure 1). The CON group had no injury. The animals were free in their cages and were provided with standard rabbit chow and water ad libitum. Animal research ethics approval was obtained from the China Agricultural University (ref: CUA4345/03M). The animals were euthanized with a 25% sodium pentobarbital overdose at weeks 1, 2, 4, and 8 post-injury. The patella–patellar tendon (PPT) complex of the knee was then harvested for the histological and immunohistological evaluations.

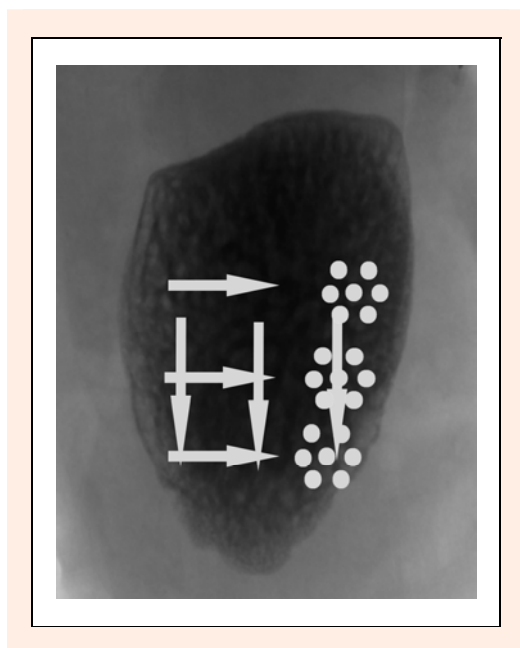


Figure 1. The plum-blossom needle vertically punctured the left-side surface and was then moved up to two other locations. The same process was performed on the middle and right side. Finally, the needle was scratched from right to the left at three levels.

Evaluation

Histological evaluation

The harvested PPT complexes were decalcified and embedded in paraffin. Subsequently, 5- μ m-thick sections from the mid-sagittal plane of the PPT complex were stained with Safranin O to examine the BTJ proteoglycan profile and with hematoxylin & eosin (H&E) to examine the general morphology; the latter analysis included an assessment of the tendon collagen fibers under a polarized microscope (Nikon Eclipse 50i, Nikon Inc., Japan).

Quantitative evaluation of the tendon cell density and the thickness of the fibrocartilage zone

The tendon cell density and fibrocartilage zone thickness (FZT) are measured using our established protocols (Fu et al., 2008; Wang et al., 2008). Briefly, the cell density was calculated by counting the number of cells in 5 random standardized rectangular fields (100 \times 100 μ m) along the junction of the calcified and uncalcified fibrocartilage within the H&E sections. The FZT of the sagittal sections

was calculated by dividing the sectional area of the fibrocartilage zone by the corresponding length. All of the quantitative evaluations were performed at a magnification of 100 using an image analysis system (Metamorph 7.1).

Immunohistochemistry

For the immunostaining of the VEGF and bFGF protein, mouse anti-VEGF (Beijing ZhongYuan Ltd. (Abcam agency), Beijing, China) and anti-bFGF (Beijing biosynthesis biotechnology CO., LTD, Beijing, China) were used. Immunohistochemistry was performed according to the manufacturers' instructions. Briefly, 5 μ m sections were mounted on slides coated with a poly-L-lysine solution (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, Beijing, China). These sections were dewaxed and rehydrated before immersion in 3% hydrogen peroxide (to block the endogenous peroxidases) followed by rinsing in phosphate buffered saline (PBS). The sections were then digested with 0.1% trypsin for 30 min at 37°C and washed in PBS. Non-specific sites were saturated with normal goat serum (Beijing Zhong Shan - Golden Bridge Biological Technology CO., LTD, Beijing, China) for 10 min at room temperature. The sections were incubated with the specific antisera 1 (VEGF: rat, anti-rabbit 1:50; Abcam, Cambridge, UK; bFGF: rabbit, anti-rabbit, 1:300; Biosynthesis biotechnology CO., LTD, Beijing, China) overnight at 4°C. In the next day, sections were placed for 30 min at room temperature. After washing in PBS, the biotinylated goat anti-mouse/rabbit IgG (VEGF: 40111, Beijing Sinopept Biotechnology Co., LTD, Beijing, China; bFGF: SP-0023, Beijing Biosynthesis Biotechnology CO., LTD., Beijing, China) was applied for 30 min at room temperature. After rinsing the slides in PBS and developing them in diaminobenzidine (DAB, Beijing Zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China) for 10 min, the VEGF and bFGF expression of the cells in whole images of each BTJ were measured and described based on the integral optical density (IOD). Two sections of each specimen were used to calculate the average. The average IOD of VEGF- and bFGF-positive staining was used for the quantitative analysis.

Statistical analysis

A one-way ANOVA was used to analyze the differences in the tendon cell density, FZT, bFGF, and VEGF between the injury and CON groups. The significance level was set at $p < 0.05$. The results are presented as the mean (\pm SD). All of the data were analyzed using SAS version 6.0 (SAS Institute Inc., Cary, North Carolina, USA).

Results

Morphological evaluation

The specimens in the CON group presented an even distribution of cells, a clear tidemark and fibrocartilage zone structure (Figure 2A) and parallel collagen fibers that exhibited good alignment under polarized light microscopy (Figure 2B). Compared with the CON group, the injury groups showed significant structural changes. The

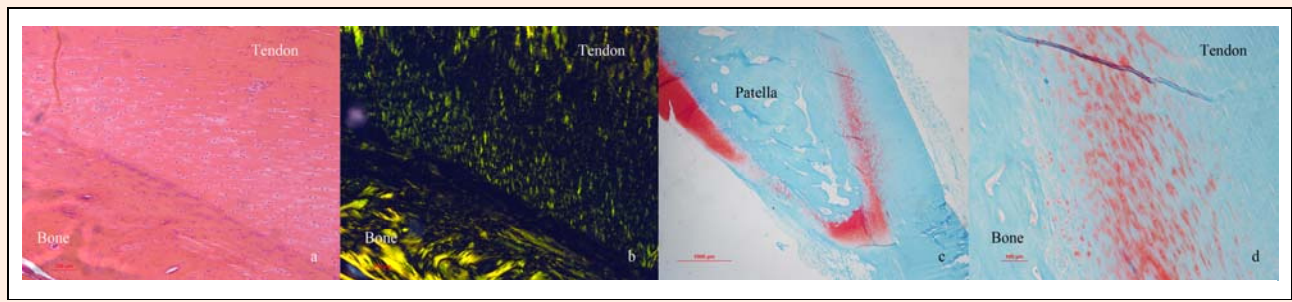


Figure 2. A sagittal section of the patella-patellar tendon junction of the CON group. The H&E staining of a section from the CON group shows good alignment of the cell profile and a clear tidemark (arrow) (A). The polarized light image revealed well-aligned collagen fibers, and a dark band was found in the fibrocartilage zone (B). The proteoglycan profile located in the fibrocartilage zone was even and continuous (C, D) (100× for a, b, and d; 20× for c).

cell density decreased; the cell profile was uneven, and the tidemark became discontinuous or undetectable (Figure 3A, B, C and D). Chondrocyte proliferation and poor alignment were found in the ND4W group (Figure 3C). The collagen fiber alignment in the ND1W group showed no obvious change (Figure 3E) compared to the CON group, but the collagen fibers of the ND2W, ND4W, and ND8W groups showed a significantly different diameter and poor alignment (Figure 3F, G and H). The Safranin O staining showed a significantly different proteoglycan profile between the CON group and the injury groups. The proteoglycan profile of the CON group was even and continuous (Figure 2 C and D); the area of proteoglycan obviously increased in the ND2W and ND4W groups and then recovered in the ND8W. This result indicates that proteoglycan proliferation reached the maximum at 2 weeks post-injury, and the profiles of the ND2W, ND4W, and ND8W groups were much more uneven than in the CON group (Figure 4).

Quantitative evaluation of cell density in the fibrocartilage zone and the fibrocartilage zone thickness

Cell density was significantly higher in the CON group than in the ND1W ($p < 0.01$), ND2W ($p < 0.05$), ND4W ($p < 0.05$), and ND8W groups ($p < 0.01$). No significant differences were found among the ND1W, ND2W, ND4W, and ND8W group ($p > 0.05$) (Table 1).

Compared with the FZT of the CON group, the FZTs in the injury groups showed obvious changes. The FZT increased significantly to the highest level in the ND1W group ($p < 0.01$) and then gradually decreased in the ND2W group ($p < 0.05$), the FZT had not returned to normal in the ND8W group ($p < 0.05$). No significant difference in the FZT was found among the ND1W, ND2W, ND4W, and ND8W group ($p > 0.05$) (Table 1).

The effect of acute injury on bFGF and VEGF expression

The bFGF expression increased in the ND1W group, and

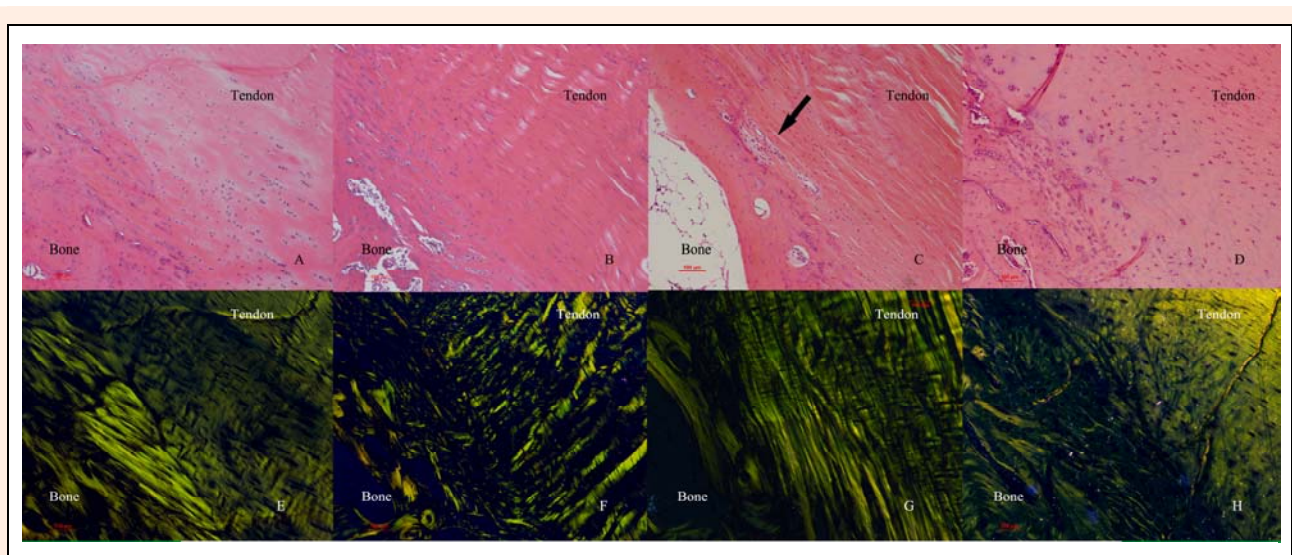


Figure 3. The histological characteristics of post-injury in BTJ. Compared with the CON group, the ND1W group exhibited decreased cell density, an unclear tidemark, and some scar tissue (A). The collagen alignment was normal, but the waviness of the collagen fibers was diminished (E). The ND2W group showed a poor cell profile, no detectable tidemark, and a different collagen fiber diameter (B, F). The cell profile in the ND4W group was poor, and the chondrocytes proliferated in the fibrocartilage zone. A chondrocyte island formed (arrow), and the collagen fiber diameter increased; the waviness decreased (C, G). The ND8W group showed a poor cell profile, decreased cell density, scar tissue formation, and diminished waviness of the collagen fibers (D, H). (100 ×).

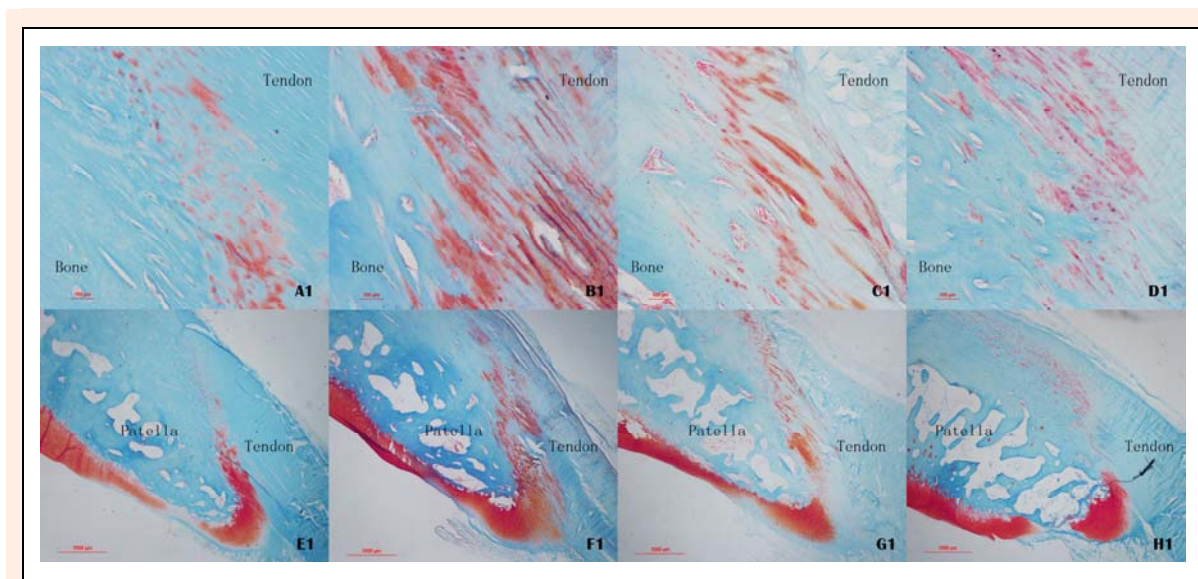


Figure 4. The results of the Safranin O staining revealed that the proteoglycan profiles of the injury groups were altered when compared with the CON group. The proteoglycan profile of ND1W group was uneven, and the area of red staining decreased (A1, E1). The ND2W group showed an obviously uneven profile and an increase in the area of red staining (B1, F1). The red staining area of the ND4W group was still larger than that of the CON group, but the area and density were decreased compared with the ND2W group (C1, G1). The red staining area in the ND8W group was decreased compared with the ND4W group, and the profile was significantly uneven (D1, H1) (100× for A1, B1, C1, D1; 20× for E1, F1, G1, H1).

it was significantly higher than it was in the CON, ND4W, and ND8W groups. The bFGF expression in the ND1W was 258.26% that of the CON group ($p < 0.01$), 172.26% that of the ND4W group ($p < 0.05$), and 207.70% that of the ND8W group ($p < 0.01$). No significant difference was found among the CON, ND2W, ND4W, and ND8W groups. The bFGF expression level tended to decrease from the ND1W group to the ND8W group (Table 2). The VEGF expression level showed no significant difference among the CON and all injury groups ($p > 0.05$); however, its tendency was to increase in the ND1W and ND2W groups and then decrease in the ND4W and ND8W groups (Table 2).

Table 1. Cell density and fibrocartilage zone thickness (FZT) in each group. Data are means (\pm SD).

Group	n	Cell density (cell/10000 μm^2)	FZT (μm)
CON	7	19.9 (4.04)	307.86 (27.62)
ND1W	6	10.76 (3.82) **	443.21 (57.98) **
ND2W	6	12.7 (4.53) *	395.33 (46.54) *
ND4W	7	13.4 (4.08) *	383.7 (89.44) *
ND8W	7	9.51 (1.86) **††‡	379.55 (59.51) *

CON: normal control; ND1W: post-injury 1 week; ND2W: post-injury 2 weeks; ND4W: post-injury 4 weeks; ND8W: post-injury 8 weeks.

* and ** denote $p < 0.05$ and 0.01 , respectively, compared with the CON group. †† denotes $p < 0.01$ compared with the ND1W group. ‡ denotes $p < 0.05$ compared with the ND4W group.

Discussion

Chronic BTJ injuries occur frequently in athletes and are difficult to heal. This difficulty comes from the poor regeneration capability of the BTJ and improper post injury training regimens that may cause repetitive trauma or delayed healing of the acute injury (Maganaris et al., 2004; Nakama et al., 2005). In this study, an animal model of BTJ injury was established by puncture, and it

was used to systematically delineate the nature of the healing process of the BTJ acute injury at 1, 2, 4, and 8 weeks post-injury. This model was easier to establish and more reproducible than our previous delayed healing model (Wang et al., 2010).

Table 2. The basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) expression in each group. Data are means (\pm SD).

Group	n	bFGF IOD	VEGF IOD
CON	7	114.68 (39.39) ††	131.26 (17.12)
ND1W	6	296.17 (180.47)	140.58 (48.07)
ND2W	6	202.35 (37.94)	161.29 (16.76)
ND4W	7	171.93 (75.4) †	144.07 (47.44)
ND8W	7	142.6 (12.26) ††	117.74 (41.78)

CON: normal control; ND1W: post-injury 1 week; ND2W: post-injury 2 weeks; ND4W: post-injury 4 weeks; ND8W: post-injury 8 weeks.

† and †† denote $p < 0.05$ and 0.01 , respectively, compared with the ND1W group.

The establishment of this BTJ delayed healing model was based on histological and immunohistological parameters. A typical BTJ healing process includes inflammation and angiogenesis, newly formed bone with regenerating fibrocartilage zone-like structure, maturity in the fibrocartilage junction and remodeling (Liu et al., 1997; Lu et al., 2006; Wang et al., 2006; Wong et al., 2003). This puncture injury model showed some differences compared to previous reports. No typical inflammatory cells or angiogenesis were found in the injury groups, which indicated that this injury did not cause an obvious inflammation reaction, and the healing procession showed much less scar tissue formation than the section models (Krivic et al., 2008). It was different from the injury model that was established by tendon enthesis sectioning.

Chronic BTJ injury often results in poor collagen fiber alignment, tendon cell degeneration, hypervascularity and changes in the cell density and FZT (Nakama et

al., 2005; Pecina et al., 2010; Wang et al., 2010). Compared with the CON group (Figure 2), the ND1W, ND2W, ND4W, and ND8W groups showed poor cell profiles, loss of the parallel arrangement of the collagen fibers, and an unclear or undetectable tidemark (Figure 3A, B, C and D); the poor collagen fiber alignment and structural changes were also confirmed in the polarized images (Figure 3E, F, G and H). The puncture injury caused the wavy BTJ collagen fibers to flatten in some injury groups (Figure 3E, G and H), which would diminish the BTJ's cushioning capacity. The chondrocyte islands in the fibrocartilage zone were found in the ND4W group (Figure 3C), which was a symptom of pathological change. The organization of the collagen fibers and the fibrocartilage zone of proliferation of the local chondrocytes would diminish the stress capacity of the tissue and increase the susceptibility of the BTJ injury. The Safranin O staining revealed changes in the proteoglycan profile in the injury groups. The proteoglycan area in the ND1W group (Figure 4A1 and E1) did not obviously change compared with the CON group (Figure 2C and D), but the ND2W group showed obvious proteoglycan proliferation and profile changes. The structural changes were the result of injury healing and adaptation in the BTJ (Figure 4B1 and F1). In ND4W and ND8W groups, the proteoglycan area gradually decreased, but the area was still large and the profile was still uneven compared to the CON group. Proteoglycans were the major content of the fibrocartilage zone and existed in the chondrocytes (Shimpuku et al., 2007). The fibrocartilage zone is responsible for transmitting mechanical forces from the tendon to the bone. The change in the proteoglycan profile would be affected by the stress experienced by the BTJ. This change could cause an injury, or it may have been caused by trauma (Rees et al., 2009; Scott et al., 2008; Screen et al., 2005; Wang et al., 2012).

The quantitative parameters showed that the BTJ cell density was significantly decreased in all of the injury groups; the cell densities of the ND1, ND2W, ND4W, and ND8W groups were 53.58% ($p < 0.01$), 63.25% ($p < 0.05$), 66.73% ($p < 0.01$), and 47.36% ($p < 0.01$), respectively, of the CON group, which means that the puncture injury decreased the BTJ cell density, and the decrease persisted for more than 8 weeks. The cell density in this model was different compared to the tendon section models, and there was no abundant scar tissue formation, no cell proliferation, and no angiogenesis (Kaeding et al., 2007; Lu et al., 2006; Wang et al., 2008; 2010). The local swelling, scar tissue maturation, collagen fiber proliferation, and atrophy of the BTJ decreased the cell density and cell maldistribution, and caused an unclear or undetectable tide mark. In the injury healing process, the cell density gradually increased from the ND1W group to the ND2W and ND4W groups, but it was still significantly lower in these groups than in the CON group. The decrease in cell density that was observed in the ND8W group cannot be explained in this study (Table 1). The fibrocartilage zone reconstruction and thickness were used as important indicators to describe the healing process of the BTJ injury (Zhang et al., 2011; Zhao et al., 2010). The FZT of the CON group was significantly less

than that of the injury groups. The FZT of the ND1W group was 143.97% that of the CON group ($p < 0.01$), and it gradually decreased in the ND2W, ND4W, and ND8W groups. The FZT of the ND8W was 123.29% of the CON group ($p < 0.05$). The FZT changes indicated that the natural healing process of the BTJ was unsuccessful, and the change was caused by the chondrocyte proliferation (Sakane et al., 2012). As no other interruption was performed in the injury groups, the changes in histology, the proteoglycan profile, cell density, and FZT were caused by the puncture; these changes persisted at 8 weeks post injury. Therefore, the puncture injury did not completely heal within an 8-week period.

The bFGF and VEGF were used to describe the delayed healing mechanism for the BTJ injury. The bFGF is an important growth factor that promotes tenocyte proliferation and tendon injury healing, but it also induces adhesion through over expression (Sheng et al., 2007; Tang et al., 2008). The bFGF expression pattern has been reported in different studies. Wurgler-Hauri et al found that the bFGF expression reached a peak in the first week and then decreased to a normal level in the sixteenth week of post tendon section surgery (Jain et al., 2006). Liu et al. (2011) reported that bFGF-positive cells appeared at the end of the first week following a suture injury and peaked by the end of the second week. The ND1W group was the only group in which the bFGF expression was significantly higher compared with the CON group ($p < 0.01$), which indicated that the BTJ post-injury healing of this study reached its highest level faster and the recovery was earlier than in the surgery model because the puncture caused less tissue damage and less scar tissue formation than the surgery (Liu et al., 2011; Tolonen et al., 2006). However, high bFGF expression has been shown cause adhesion in the later period of injury healing, thereby delaying injury healing or leading to chronic injury (Tang et al., 2008). The decreased bFGF expression in the ND4W and ND8W groups revealed a normal healing process. VEGF was involved in acute tendon injury repair. The up-regulation is triggered by mechanical stimulation and injury (Aspenberg, 2007; Nakama et al., 2006; Tsubone et al., 2004). VEGF expression in the injury groups showed no significant difference compared to the CON group, and no differences were found among the injury groups, indicating that the puncture did not trigger an up-regulation of the VEGF expression. This finding may indicate that the puncture did not stimulate the healing activity of VEGF in a manner similar to the surgery. Combined with the bFGF and VEGF expression patterns, our findings revealed that the role of bFGF and VEGF had ended by 8 weeks post injury. The bFGF and VEGF expression patterns in this model revealed a different healing process compared to the tendon section model. In this study we found that the bFGF and VEGF expression could not clearly indicate the healing result of BTJ injury, as the high expression of bFGF and VEGF in the post injury 4 and 8 week might or might not be benefit for the injury healing, so the ratio of collagen I/III could be much better to reveal the healing.

Conclusion

In conclusion, the results of this study systematically described the acute BTJ injury healing process over 8 weeks and confirmed that the injury had not healed after 8 weeks. The bFGF expression returned to normal, and the VEGF expression did not exhibit any significant change, indicating that the BTJ injury healing process had nearly stopped in the post-trauma two weeks, which resulted in a difficult healing process. The establishment of this BTJ acute injury model could be used as an animal model for overuse or chronic BTJ injury research, and provided theory for post-acute injury training arrangement.

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Key points

- This study described the bone-tendon junction acute injury nature healing process.
- The bone-tendon junction acute injury could not be repaired naturally in 8 weeks.
- The bFGF and VEGF expression revealed that the bone-tendon junction acute injury delayed healing concern with the repairing stop.

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