The effects of an early return to training on the bone-tendon junction post-acute micro-injury healing

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Abstract
Bone-tendon junction (BTJ) overuse injuries are common athletic and occupational problems. BTJ injuries may sometimes be caused by resuming training too early after injury. To study the effects of post-injury resuming training within 48 hours on the acute injury healing process, as it is often the case for athletes. Twelve mature female rabbits were assigned to one of the following groups: acute injury (AI, n = 6), post-injury early return to training (PIERT, n = 6) and normal control (CON, n = 6). Tissue specimens were harvested at week 4. The radiological and histological characteristics of the AI and PIER group were compared among the groups. The trabecular thickness of the PIER group was significantly different from those of the AI and CON group. A histological evaluation revealed poor collagen fibre alignment, extensive scar tissue and lowered cell density in the AI and PIER groups compared with the CON group, but no significant differences were observed between the AI group and the PIER group. The fibrocartilage zone and proteoglycan area in the PIER group were significantly different from those in AI group. No differences were observed in the Total VOI volume (TV), Object volume (OBV), Percent object volume (BV/TV) and trabecular number (Tb.N) among the AI, PIER and CON groups. In conclusion, a repeatable animal model of bone-tendon junction acute micro-damage by puncture was established. Resuming training in 48 hours did not significantly deteriorate the BTJ injury healing, but improved bone remodelling and increased fibrocartilage zone thickness.

Key words: Bone-tendon junction, Patella, quantitative loading, early return to training, injury healing.

Introduction
Bone-tendon junction (BTJ) overuse injuries are common athletic and occupational problems (Kjaer et al., 2003; Panni et al., 2002; Torkki et al., 2002) that impede the routine training and performance of athletes (Ergen, 2004; Fordham et al., 2004). BTJ injuries in athletes are mainly caused by the accumulation of micro-tears or the repeated overload that occurs during training (Cook et al., 2000; Ergen, 2004; Fordham, Garbutt et al., 2004; Kujala et al., 2005; Knobloch et al., 2008), and some injuries might be caused by improperly resuming training too soon after injury. Many athletes attempt to resume to training as early as possible, both voluntarily or involuntarily, after acute injury. It is unclear if returning to training 24 to 48 hours after an acute injury improves or delays healing.

In the past, many studies have examined the treatment, rehabilitation and prevention of acute, chronic and delayed-healing BTJ injury (Hernandez et al., 2005; Kim et al., 2007; 2010; Kovacic and Bergfeld, 2005; Lu et al., 2008; Park et al., 2004; Wang et al., 2008). Nakama and colleagues established a tendon micro-tear animal model by cyclical loading. The enthesis of the flexor digitorum profundus (FDP) muscle was used to approximate the histology and morphology of the formation of a chronic injury (Nakama et al., 2005; 2006; 2007). Glazebrook reported an animal Achilles tendon overuse injury model established by uphill treadmill running (Glazebrook et al. 2008). Muscle loading is an important cause of BTJ injury and has the potential to either delay or improve healing (Benjamin et al., 2006; Hamilton and Purdam, 2004; Malaviya et al., 2000; Shaw and Benjamin, 2007; Xu and Murrell, 2008). Thomopoulos examined the effect of muscle loading on tendon-to-bone healing and found that the mechanical properties and range of motion were improved in the experimental group relative to those in a control group that was not subjected to loading (Thomopoulos et al., 2008). Frizziero et al. (2011) found that sudden detraining for 4 weeks from a 10 weeks moderate training caused negative effect on patellar tendon structure, no patella structure change was found. Our previous research established a delayed healing model that revealed that temporarily shielding the connection between tendon to bone for 4 weeks diminished the mechanical properties of BTJ and delayed healing in histology and bone remodelling (Qin et al., 2010; Wang et al., 2010). These studies implicated that sudden stop exercise or post injury no loading stimulation would delay the BTJ injury healing and bone remodelling.

There are several reasons why athletes have a higher incidence of BTJ chronic injury than non-athletes (Bedi et al., 2010a; 2010b), and repeated injury caused by improper post-injury training is accepted as one of the main reasons. In addition, the limited regeneration capacity of interface fibrocartilage is another reason for BTJ overuse injury (Liu et al., 1996; 1997; Hamilton and Purdam, 2004; Wang et al., 2005). Athletes have to resume training as soon as possible after an acute injury, and they often resume training within 24 to 48 hours following non-serious injuries that do not cause significant pain during training. Many researches described the mechanical effect on tendon enthesis (Benjamin et al., 2006; Shaw and Benjamin, 2007), but few histological or morphological reports examining the early return to training after acute injury have been published (Friizziero et al., 2011). In sport team, many doctors believed that athlete should not resume to training before an acute injury.

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healed, but coaches believed that the athlete should start training as soon as possible after injury. In this study, the authors attempted to establish an easy and repeatable acute BTJ injury animal model by puncture, and to elucidate the effect of returning to training within 48 hours post-injury in acute injury healing.

Methods

Animals and model establishment
Twelve mature female rabbits (18 weeks old, 2.5 (0.3 kg)) were divided into an acute injury (AI) group (n = 6), a post-injury early return to training (PIERT) group (n = 6) and a normal control (CON) group (n = 6). The left legs of the rabbits were assigned to the PIERT group, and the right legs were assigned to the AI and CON groups. Under general anaesthesia with 0.25% pentobarbital sodium (2 ml/kg, intraperitoneal injection) (Sigma Chemicals Co., St Louis, MO, USA), the patellar tendon enthesis (TE) area of the AI group animals was damaged using 7 plum-blossom needles (0.1-mm diameter) via vertical puncture (Figure 1). In the PIERT group, the injury was similar to that of the AI group, but the animals started a post-injury training regimen after 48 hours. During post-injury training, the rabbits were placed in a supine position, and their hind limbs were secured to supports. Two acupuncture needles were vertically inserted in the 1/3 and 2/3 of both rectus. The rectus of the left hind limb was electrically stimulated to contract repetitively for 2 hours per day, 3 days a week, for 24 hours of cumulative loading in 4 weeks. The stimulation intensity was adjusted to maintain a mean contraction force of 30% of the peak tetanic force (Hung et al., 1993; Krueger-Franke et al., 1995; Liu et al., 1996; 1997). The muscle was stimulated with a 1-Hz pulse with a duration of 200 ms, pulse width of 2 ms, and a 100 pulses/sec pulse rate for a total of 7200 contractions every training day. In the control group, no electrical stimulation was used. Animals were free in cages and were provided with standard rabbit chow and water ad libitum. Animal research ethics approval was obtained from the China Agricultural University (ref: CUA4342/03M).

Animals were euthanised with a 25% sodium pentobarbital overdose in the fourth week post-injury. The patella–patellar tendon (PPT) complex of the knee was then harvested for subsequent radiological and histological evaluations.

Evaluation

Bone structure measurement
The bone microstructure of the PPT was scanned and analysed using a microCT system (SkyScan1076 High resolution in-vivo microCT scanner, SkyScan, Kontich, Belgium). The specimens were wrapped in cling film and scanned. Each X-ray projection acquired in a 16-bit gray level image, 2000×1150 pixel, 8.67µm image pixel sizes, with an aluminium filter and for a time of 45 minutes. The X-ray voltage and current were set at 60 kV and 149 mA. Continuous scans were taken with the camera moving trans-axially around the specimens with a 180-degree rotation and an angular step of 0.6 degrees. The region of interest was selected at the patellar tendon enthesis in patella, and a total of 200 slices were chosen above the mineralisation line for analysis. Three-dimensional reconstruction of the X-ray images was created using NRecon software version: 1.6.3.3, Belgium), and all the sections were cut across section of patella. The parameters, including total VOI volume (TV), Object volume (OBV), the fraction of OBV over TV (OBV/TV), trabecular number (Tb. N) and thickness (Tb. Th) were analysed via CTAn for comparison among the groups.

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

Quantitative evaluation of the tendon cell density, thickness of the fibrocartilage zone and area of proteoglycan
The tendon cell density and fibrocartilage zone thickness (FZT) were measured using our established protocols. Briefly, the tendon cell density was calculated by counting the number of cells in five random standardised rectangular fields (100×100 µm) within the H&E sections. The FZT of the sagittal sections was calculated by dividing sectional area by the corresponding length (Wang et al. 2008). The proteoglycan area (PA) was obtained by measuring the red-stained area in five random standardised rectangular fields (200×200 µm) in the fibrocartilage zone of a Safranin O section (Figure 2). All of the quantitative evaluations were performed at a magnification of 100 using an image analysis system (Image-Pro Plus version 5.1).

Statistical analysis
One-way ANOVA was used to analyse the differences in the TV, OBV, OBV/TV, Tb.N, Tb.Th, tendon cell density, FZT and PA among the PIERT, AI and CON groups.
Results

Micro-structure of patella
MicroCT scanning indicated that the Tb.Th of the AI group (0.67 (0.25) mm) was significantly thicker than that of the PIERT group (0.44 (0.15) mm), p < 0.01. No significant difference was found between the CON (0.52 (0.10) mm) and AI group (p > 0.05), and between CON and PIERT groups (p > 0.05). The TV, OBV, OBV/TV, and Tb.N were not significant different among the AI, PIERT and CON groups (Table 1). The Tb.N values in the PIERT group and AI group displayed a tendency towards a difference relative to the CON group, though no significant difference was found among these groups.

Morphological evaluation
In the AI group and PIERT groups, the patellar BTJ regions displayed a less organised alignment of collagen (Figure 3D, E), the distribution of cells was uneven and decreased, the tidemark became unclear, and scar tissue was observed (Figure 3A, B). The specimens in the CON group presented a clear tidemark and fibrocartilage zone structure, an even distribution of cells, and parallel collagen fibres that exhibited good alignment under polarised light microscopy (Figure 3C, F). The Safranin O staining in the AI group and PIERT groups indicated obviously discontinuous proteoglycan distributions (Figure 4A, B) compared with the CON group (Figure 4C).

Tendon cell density and thickness of the fibrocartilage zone
The tendon cell density (cellularity) was significantly higher in the CON group than in the PIERT and AI groups (31.84 (9.64) cells/100 µm² vs. 16.78 (7.52) and 14.3 (6.19) cells/100 µm², respectively) (p < 0.01), but there was no significant difference between the PIERT group and AI group (p > 0.05). The FZT was significantly different between the PIERT and AI groups (213.52 (57.42) µm vs. 140.68 (43.09) µm, p < 0.01) and between the PIERT and CON groups (160.22 (52.85) µm, p < 0.01) (Table 2).
Wang et al.

Figure 3. A week 4 representative sagittal section of the patella-patellar tendon junction after injury and training. H&E-stained sections from the AI (A) and PIERT (B) groups indicated an indistinct cell profile, lowered cell density, and unclear tidemark, but the CON group (C) presented well-aligned collagen fibres and a clear tidemark. Polarised microscopy images demonstrated poorer collagen alignment in the AI group (D) and PIERT group (E) than in the CON group (F).

Proteoglycan area
In the AI group, the amount of Safranin O-stained area was significantly less than that observed in the CON group (75.22 (39.17) µm², p < 0.01) and in the PIERT group (154.95 (41.09) µm², p < 0.01). No significant difference was found between the CON and PIERT groups (Table 2).

Discussion
BTJ injuries occur frequently in athletes and are difficult to heal. This type of athletic injury is mainly caused by long-term overload training and repetitive injury by improper post-injury training, as athletes are expected or required to return to training as early as possible to avoid functional loss and decreases in performance. However, a few studies indicate concern over the effect of an early return to training following acute injury (Lu et al., 2006; Qin et al., 2006; Wang et al. 2007; 2008; 2010). The present study successfully established an acute micro-damage model in the BTJ by puncture, and indicated a promotional effect on injury healing for returning to training within 48 hours post-injury through radiological, densitometrical, and histomorphological evidence. Muscle loading promoted significant patella and fibrocartilage zone remodelling even in the presence of an injury.

In this study, the loading effect was evidenced in the patellar micro-structure and histological parameters.

Figure 4. Safranin O staining indicated an indistinct proteoglycan profile and reduced area in the AI group (A, D) and PIERT group (B, E) relative to the CON group (C, F). (10× for A, B, C and 20× for D, E, F)
Patella microCT scanning was used to reveal the patellar micro-structure characteristics of the AI, PIERT and CON groups. The Tb.Th was the only parameter that demonstrated a significant difference between the AI and PIERT groups. The Tb.Th in the AI group was 152.37% of that in the PIERT group (p < 0.05). Compared with the CON group, the Tb.Th in the AI group was 128.85% of the CON group (p > 0.05). The Tb.Th of the PIERT group was 84.61% of the CON group (p > 0.05). The Tb.Th in the AI group and PIERT group shifted in opposite directions compared to the CON group, which was a result of the muscle contraction loading. Tb.Th was an important index to describe the bone microstructure remodelling and its change in relation to loading (Dalle Carbonare et al., 2001; Vandyke et al., 2010). Combined with the lack of significant differences of TV, OBV, and OBV/TV among the PIERT, AI and CON groups, the Tb.N in the PIERT group was expected to be greater than that in the AI group. Though there were no significant differences in Tb.N among the three groups, the Tb.N of the PIERT group was 4.2-fold greater than that of the AI group and 2.5-fold greater than that of the CON group. These results revealed that the trabecular bone remodelling in the PIERT group was superior to the remodelling in the AI group (Borius et al., 2010; Rees et al., 2008; Yamamoto et al., 2000). The post-injury training protocol promoted patella remodelling.

Chronic BTJ injury often results in poor collagen fibre alignment, tendon cell degeneration, hypervascularity, changes of cell density and FZT (Nakama et al., 2005; Pecina et al., 2010; Wang et al., 2010). In the AI and PIERT groups, the histopathological results indicated poorer collagen fibre alignment and lowered cell density (p < 0.05) compared to the CON group (Figure 3). Compared with the CON group (Figure 3C, F), degeneration and scar tissue formation in the BTJ area was found in the AI and PIERT groups. In addition, the tidemark became unclear or disappeared (Figure 3A, B), and the poor collagen fibre alignment and structure change were also approved in the polarised images (Figure 3D, E). The proteoglycan profile of the AI and PIERT groups indicated differences from the CON group (Figure 4); the proteoglycan distributions in the CON group (Figure 4C, F) were more orderly and distinct relative to the AI and PIERT groups (Fig 4A, B). Sections of the AI and PIERT groups indicated some local chondrocyte proliferation (Figure 4D, E). The AI and PIERT group history revealed the presence of injury, but no significant difference in the injury healing process was noted.

Injury and scar tissue formation caused a cell density decrease and a change in structure (Wang et al., 2008; 2010). The cell densities in the AI and PIERT groups were only 52.70% and 44.91%, respectively, of that in the CON group (p < 0.05), but it was difficult to identify which group was more seriously injured or exhibited altered healing because no significant differences were found between the AI and PIERT groups (p > 0.05). The FZT in the AI group was 87.80% of that in the CON group (p > 0.05), but the FZT in the PIERT group was 133.27% of that in the CON group (p < 0.01) and 157.78% of that in the AI group (p < 0.01). The proteoglycan area examination indicated similar results as the FZT. In the BTJ, proteoglycan mainly existed in the fibrocartilage zone, and its profile was mainly influenced by loading (Isberg et al. 2006). The proteoglycan area of the AI group was only 50.6% of that observed in the CON group (p < 0.01) and 48.5% of that in the PIERT group (p < 0.01). The decreased proteoglycan area in the AI group should be influenced by the loading reduction via a self-protective reaction following injury; this effect occurs because the training in the PIERT group decreased the reduction. The much wider FZT and thinner Tb.Th in the PIERT group relative to the AI group indicated that the subjects in the AI group used their injured hind limbs less than normal, and the decreased loading resulted in an increase in Tb.Th but decreases in the FZT and proteoglycan area (Koike et al., 2006). This tendency was reversed by the loading of muscle contraction in the PIERT group, but the loading was also one of the major reasons underlying the BTJ injury (Archambault et al., 2007). The intensity could promote injury healing with a correctly selected starting time point. According to the histological results, the training not only did not diminish healing but also promoted BTJ remodelling (Nakama et al., 2005; Pecina et al., 2010; Wang et al., 2010).

Conclusion

The results of this study indicated that the patella-patellar tendon junction trauma following plum-blossom needle puncture existed for four weeks. It was an easy and repeatable BTJ micro-damage model. The accumulative 24 hours of low-intensity, post-injury training improved the bone structure and fibrocartilage zone remodelling, and did not deteriorate the injury. In addition, 48 hours appears to be an acceptable time delay before resuming training. The training intensity and post-injury resuming time point are important variables on athletic injury healing that require further study.

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**Key points**

- An easy and repeatable bone-tendon junction injury model was established in this study, it will provide a platform to the injury research.
- Post-injury resuming training in 48 hours did not delay the acute bone-tendon junction injury healing process, it provided a basic theory for the post-injury training.
- To find the proper post-injury training intensity will help athletes to train scientifically, it is the destination of our next research.