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ORIGINAL PAPER

Cationised gelatin and hyaluronic acid coating enhances polyethylene terephthalate artificial ligament graft osseointegration in porcine bone tunnels

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Abstract
Purpose The aim of this study was to investigate whether cationised gelatin and hyaluronic acid (CH) coating could induce polyethylene terephthalate (PET) artificial ligament graft osseointegration in the bone tunnel.
Methods Surface modification of PET artificial ligament graft was performed by layer-by-layer (LBL) self-assembly CH coating. Six pigs underwent anterior cruciate ligament (ACL) reconstruction on the right knees, with three pigs receiving the CH-coated PET grafts and the other three pigs non-CH-coated PET grafts as controls. They were sacrificed at three months after surgery and the graft-bone complexes were acquired for computed tomography (CT) scan and histological examination.
Results CT scans showed a significant difference at the distal femoral site ($p=0.031$) or at the distal tibial site ($p=0.0078$), but no significant difference in the bone tunnel areas' enlargement at other sites ($p>0.05$) between the CH group and the control group. Histologically, application of CH coating induced new bone formation between graft and bone at three months compared with the controls at the distal site. The interface width of the CH group was significantly lower than that of the control group at the distal femoral site ($p=0.0327$) and at the distal tibial site ($p=0.0047$).
Conclusions The study has shown that CH coating on the PET artificial ligament surface has a positive biological effect in the induction of artificial ligament osseointegration within the bone tunnel at the distal site of the bone tunnel.

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Introduction

The LARS artificial ligament (Ligament Advanced Reinforcement System, Surgical Implants and Devices, Arc-sur-Tille, France), made of polyethylene terephthalate (PET), has been accepted as a graft choice for anterior cruciate ligament (ACL) reconstruction by many surgeons all over the world [1–3]. It is a valid alternative for treating some acute and serious cases [4]. As this type of PET ligament has hydrophobicity and chemical inertia, the graft-bone healing of the LARS artificial ligament is one of the most important concerns after implantation. By analysing ruptured ACL artificial ligaments, Guidoin et al. [5] observed a chronic inflammatory reaction with macrophages and giant cells in the polyester ligaments. In a three to five year follow-up multicentre study, Gao et al. [6] reported that seven of 156 cases of ACL reconstruction using the LARS ligament were noted to exhibit graft failure at the bone tunnel, and they found that an interposed layer of fibrous scar tissue appears at the graft-bone interface at the second arthroscopic revision surgery. It was presumed that the inflammation at the graft-bone interface might induce the scar tissue formation and hamper the graft osseointegration within the bone tunnel. This raises questions about whether we could improve the artificial ligament graft healing in the bone tunnel through alleviating the inflammation and promoting graft biocompatibility.

Hyaluronic acid has been used to make the artificial graft a more desirable biomaterial scaffold for ligament tissue engineering [7, 8]. As a component of the extracellular matrix, hyaluronic acid has been demonstrated to accelerate

the healing of rabbit flexor tendons [9]. Particularly, hyaluronic acid has the high capacity to suppress and alleviate inflammation [10–12]. Cationised gelatin, as a positively charged polymer, has long been used as a highly valuable carrier system as it provides a universally effective way to enhance biocompatibility of the polymers [13–15]. Previously, it has been demonstrated that the cationised gelatin and hyaluronic acid coating (CH coating) on PET artificial ligaments has a positive effect by inhibiting inflammatory cell infiltration and promoting graft biocompatibility [16].

As reported, the layer-by-layer (LBL) technique was very useful to make a surface coating on PET artificial ligaments [16, 17]. Therefore, the aim of this study was to promote graft osseointegration using LBL self-assembly CH coating. Moreover, a porcine ACL reconstruction model was applied to evaluate the effect of this CH coating in vivo. We hypothesised that the surface coating of CH would result in less fibrous scar tissue formation and better graft osseointegration at the graft-bone interface when compared with that of the blank controls at three months after operation.

Methods

Study design and operative procedure

In this study, PET artificial ligament grafts were modified by LBL self-assembly CH coatings. The detailed modification

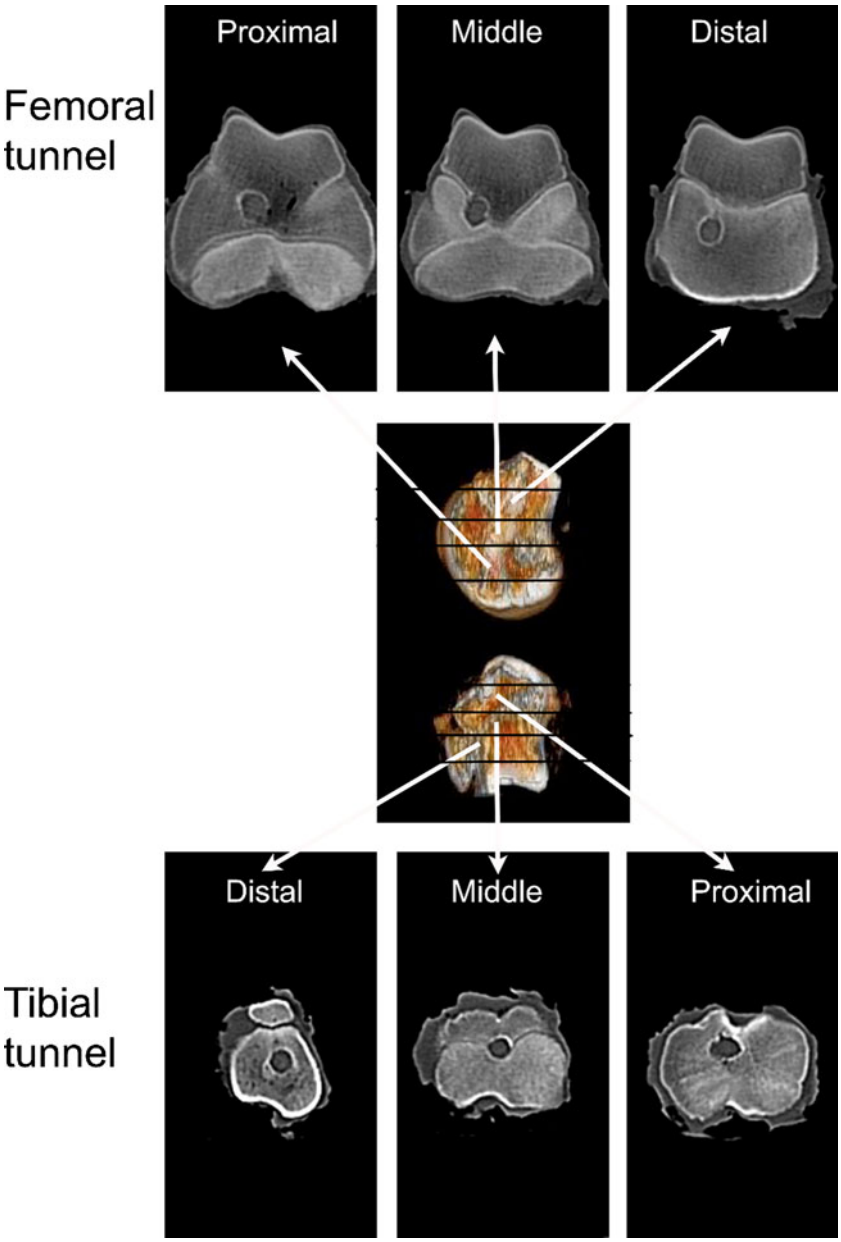

Fig. 1 CT scan of the knee joint. The bone tunnel of the femoral tunnel or the tibial tunnel was respectively divided into three parts according to the distance from the knee joint: distal part, middle part and proximal part

procedure has been illustrated in another report [16]. The pure PET graft without coating was used for the control group.

The animal experiment was approved by the Animal Care and Use Committee of our college. ACL reconstruction experiments were also performed in six healthy GuangXi male pigs (mean weight 43.4±8.5 kg). After successful anaesthesia (induced by 25 mg/kg sodium pentobarbitone), the native ACL was exposed and removed from the insertion sites by sharp dissection. Next, a 4.5-mm diameter tunnel was drilled in the femoral and tibial insertion sites of the ACL. The graft was pulled manually into the bone tunnel. The graft with LBL coating was implanted into the right knees of three pigs, while the graft with no coating was implanted into the right knees of the other three pigs. At the bone portal, the graft ends were sutured with the adjacent periosteum and soft tissue with no. 5 Ethibond sutures. The wounds were closed in layers. Post-operatively, the animals were then returned to the animal care facilities. Three pigs in each group were sacrificed at three months after surgery for the following examinations.

Computed tomography

Immediately after sacrifice, the graft-tibia complex samples (n=3) underwent computed tomography (CT, SOMATOM Sensation 64, Siemens, Forchheim, Germany) scan to measure the area of bone tunnel (bone window data, width 1,500; level 450). The tunnel areas on the axial plane were estimated and measured with Image-Pro Plus 6.0 software (Media Cybernetics Corp, Rockville, MD, USA). The sections of each sample were averaged for statistical estimation. The bone tunnel of the femoral tunnel or the tibial tunnel was respectively divided into three parts according to the distance from the knee joint: distal part, middle part and proximal part (Fig. 1).

Histological examination

Graft-bone complexes (n=3 limbs for each group) were prepared for histological analysis of the graft-bone interface. Immediately after sacrifice, the graft-bone complex specimens were fixed in 10 % formalin for 48 h, then decalcified in ethylenediaminetetraacetic acid (EDTA) solution and then embedded in paraffin wax. The samples were sectioned perpendicular to the longitudinal axis of the tibial tunnel with a thickness of 5 μm using a microtome (SM2500, Leica, Wetzlar, Germany). These sections were stained with haematoxylin and eosin (H&E) for routine histological evaluation. The slides were examined to visualise the graft-bone interface with inverted light microscopy (IX71SBF-2, Olympus Co., Tokyo, Japan). Digital images were taken using a DP Manager (Olympus Optical Co., Tokyo, Japan). The two investigators performing the histological analysis were blinded to animal treatment.

Histomorphometric analysis

Each section was divided into four quadrants to determine the graft-bone interface width. In each of the four quadrants, the interface width was measured as the distance between the edge of the bone tunnel and the outer graft determined under ×200 magnification. Four separate measurements were made in each of the quadrants for a total of 16 measurements for each specimen. The average interface width for each specimen was then determined by averaging these values obtained from each specimen. The two investigators who performed the histomorphometric analysis were blinded to the type of animal treatment.

Statistical analysis

Data analysis was performed using Stata 9.0 software (StataCorp, College Station, TX, USA) and then data were reported as mean and standard deviation for description. A statistical

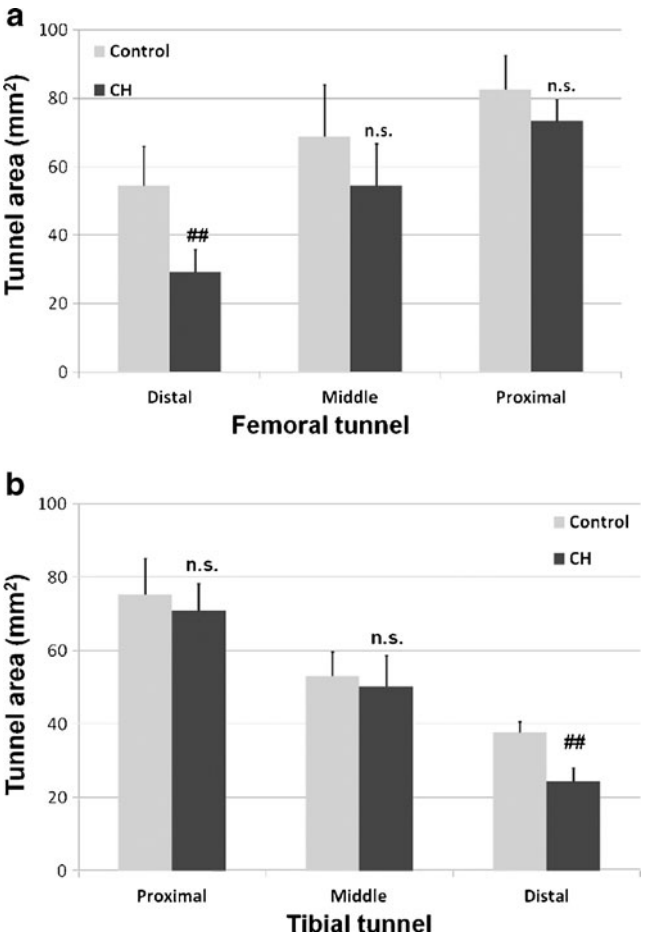

Fig. 2 **a** The average bone tunnel area of the femoral tunnel between the CH group and the control group. **b** The average bone tunnel area of the tibial tunnels between the CH group and the control group. ## significant difference, *NS* no significant difference, *CH* cationised gelatin and hyaluronic acid coating

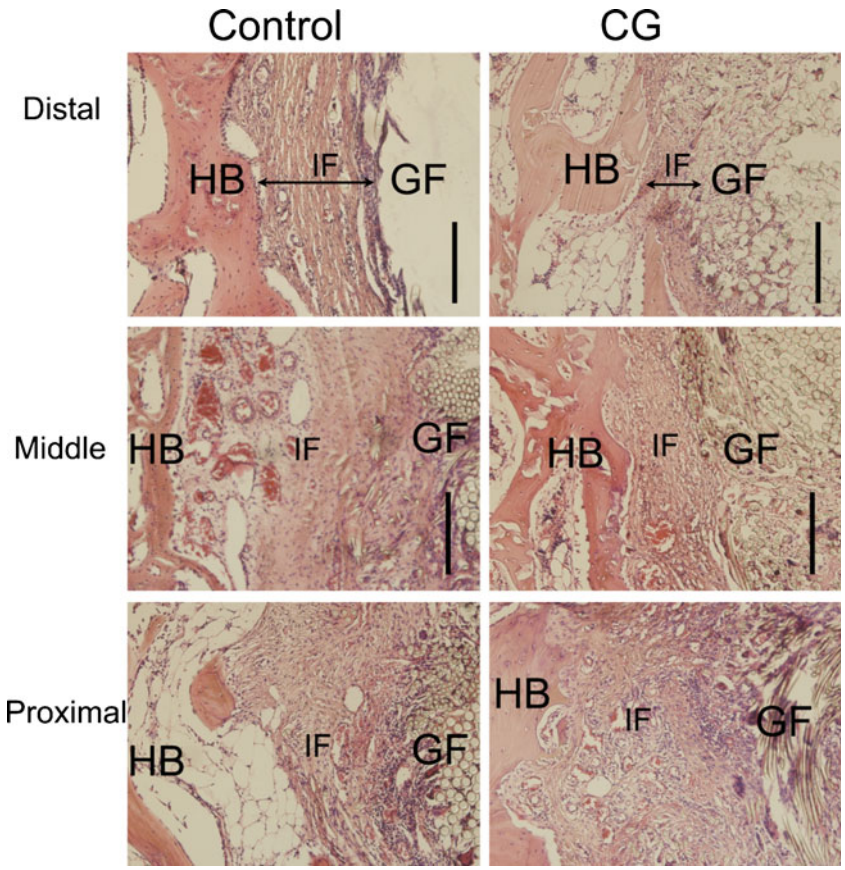

Fig. 3 Histological characterisation of the femoral graft-bone sample between the control group and the CH-coated group at each site. *HB* host bone, *IF* interface, *GF* graft fibres. *Bar*=200 μm

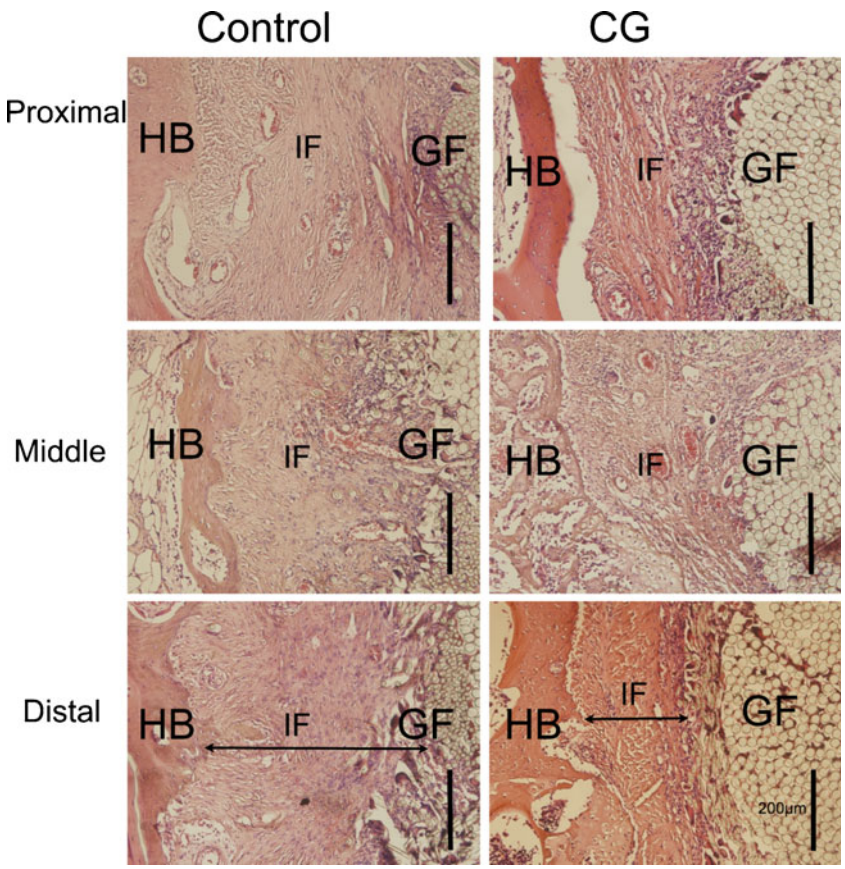

Fig. 4 Histological characterisation of the tibial graft-bone sample between the control group and the CH-coated group at each site. *HB* host bone, *IF* interface, *GF* graft fibres. *Bar*=200 μm

analysis of quantitative results was carried out with the paired Student's *t* test or two-sample Wilcoxon rank sum test. The statistical significance level was set at 0.05.

Results

Computed tomography

For the femoral tunnel, the average bone tunnel areas of the control group were 54.4±11.6 mm^2 (distal site), 68.9 ±15.1 mm^2 (middle site) and 82.7±9.8 mm^2 (proximal site), while the average bone tunnel areas of the CH group were 29.1±6.7 mm^2 (distal site), 54.4±12.4 mm^2 (middle site) and 73.3±6.4 mm^2 (proximal site) (Fig. 2a). In the femoral tunnel, there were significant differences of tunnel area at the distal site between the control group and the CH group (p=0.031), and there were no differences of the tunnel area between the control group and the CH group at the proximal site (p>0.05) or at the middle site (p>0.05). For the tibial tunnel, the average bone tunnel areas of the control group were 75.2± 9.8 mm^2 (proximal site), 53.1±6.5 mm^2 (middle site) and 37.8±2.9 mm^2 (distal site), while the average bone tunnel areas of the CH group were 70.9±7.4 mm^2 (proximal site), 50.3±8.4 mm^2 (middle site) and 24.4±3.7 mm^2 (distal site) (Fig. 2b). There was a significant difference of the bone tunnel area between the control group and the CH group at the distal tibial site (p=0.0078). No significant difference of tunnel area was detected between groups at the proximal site (p>0.05) or at the middle site (p>0.05).

Histological findings

For the femoral tunnel (Fig. 3), the graft-bone interface of the control group was filled with granulation tissue at the proximal and middle site, and a thick fibrous scar tissue was formed at the graft-bone interface at the distal site. In the group with CH coating, the graft-bone interface also appeared somewhat messy with fibrous tissue at the proximal site. At the middle site in the CH group, interface width appeared much narrower and host bone grew onto to the graft. When it comes to the distal site of the CH group, there was less scar tissue formation at the graft-bone interface, and the interface between the graft and host bone was very narrow.

In the tibial tunnel (Fig. 4), both the control group and CH group had fibrous scar tissue at the interface of the proximal site and the middle site, potentially arousing an inflammatory response. In the distal site of the control group, there was apparent scar tissue formation at the interface, whereas there was much progressive new matrix formation and bone ingrowth at the distal tibial site of the CH group. Treatment with the CH-coated grafts resulted in less apparent scar tissue formation at the graft-bone interface than did the non-coated grafts.

Histomorphometric analysis

In the femoral tunnel, there were no differences of the interface width between the control group and the CH group at the proximal site (p>0.05) or at the middle site (p>0.05). However, there was a significant difference of interface width between the control group (279.7±37.6 μm) and the CH group (167.0±47.8 μm) at the distal site (p=0.0327). Likewise in the tibial tunnel, the mean interface width of the CH-coated group was significantly lower than that of the control group at the distal site (238.7±37.0 μm vs 407.7±35.7 μm, p=0.0047). No significant difference of interface width was detected between groups at the proximal site (p>0.05) or at the middle site (p>0.05) (Fig. 5).

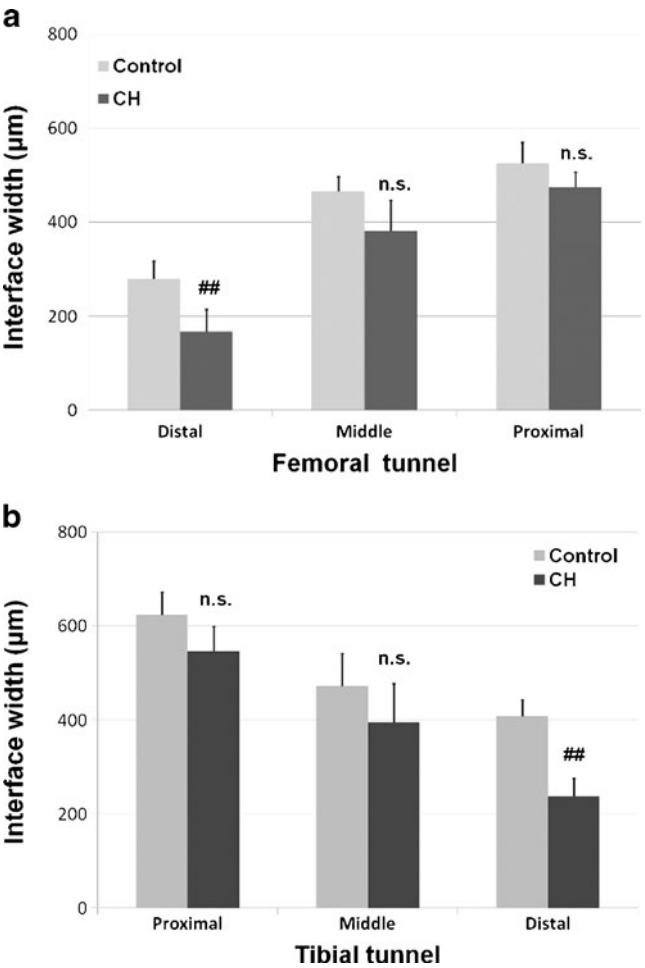

Fig. 5 Comparison of the graft-bone interface width between the control group and the CH group in the femoral tunnel (**a**) and in the tibial tunnel (**b**). ## significant difference, *NS* no significant difference, *CH* cationised gelatin and hyaluronic acid coating

Discussion

Tendon healing in a bone tunnel is a priority concern in the orthopaedic and biomedical field after ACL reconstruction [18–23]. Fibrous scar tissue formation has been shown to be an incomplete healing response [24]. This kind of immature granulation fibrous layer is extremely weak and will significantly influence the graft stability in the bone tunnel. Thus, the importance of understanding and improving graft-bone healing, particularly in ACL reconstructions, has fuelled a great increase in research on the topic.

In this study, the PET artificial ligament was modified with a multiple layer of CH coating in order to promote the graft osseointegration in the bone tunnel. Both the CT scan and the histological examination revealed that the CH group had a smaller bone tunnel area and a thinner interface width than the control group in the distal site of the bone tunnels. These results indicated that CH coating had a positive effect by enhancing the graft osseointegration in the distal site of the bone tunnel though promoting the graft biocompatibility and enhancing new bone formation at the graft-bone interface.

Generally speaking, the tendon-bone healing process is divided into four stages: (1) inflammation, (2) proliferation, (3) matrix synthesis and (4) matrix remodelling. During this process, mechanical and biological factors are involved in the healing process of the reconstructed tendons [25–29]. In the distal part of the bone tunnel, the mechanical effect was relatively low and the graft-bone healing was influenced mainly by the biological environment. CH coating is able to exert a biological effect, promoting the graft biocompatibility and alleviating inflammation in the bone tunnel, thus decreasing the formation of fibrous tissue at the graft-bone interface of the distal bone site. Therefore, it was presumed that the CH coating came into play at the distal bone site with not much mechanical influence.

Iorio et al. [30] also used CT to analyse bone tunnel enlargement and concluded that a less aggressive rehabilitation process influenced the amount of tunnel enlargement after ACL reconstruction. Vadalà et al. [31] also used CT to exactly determine the diameters of both femoral and tibial tunnels at various levels of the lateral femoral condyle and proximal tibia and demonstrated that early post-operative knee motion increases the diameters of the tibial and femoral bone tunnels. Interestingly, in the proximal site of the femoral tunnel or the tibial tunnel in our study, we found no significant difference of the bone tunnel area between groups on the CT images. Both groups revealed enlarged bone tunnels at the proximal site. Tunnel enlargement indicated that the natural graft-bone healing is not satisfactory with a fibrous scar tissue layer formation at the graft-bone interface [24, 32, 33]. As demonstrated, tunnel widening or enlargement frequently occurred due to excessive graft-tunnel motion, so as to delay graft incorporation in the bone tunnel [34, 35]. The effect of mechanical load influences the cellular and molecular cascade of tendon-to-bone healing. It has been illustrated that the closer the fixation to the joint, the lower the incidence of tunnel widening [36, 37]. The graft motion influenced the proximal site of the graft more greatly than the distal site. Therefore, the proximal site had poorer graft healing in the bone tunnel compared with the distal site, although the CH coating might afford some positive biological effect.

Conclusion

This study demonstrated that the CH coating had a positive biological effect by enhancing graft-bone healing at the distal site of the bone tunnel. This biological technique of LBL self-assembly CH coating may open a new door for orthopaedic surgeons to accelerate artificial ligament graft healing after implantation in the bone tunnel, thereby promoting rapid recovery and a quicker return to sports activity. To be noted, the biological effect of CH coating might be influenced by the biomechanical factors. Investigation of the biomechanical effect should be undertaken in a larger sample size before the CH coating is used in clinical application.

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Conflict of interest The authors declare that they have no conflict of interest.

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