Hydroxyapatite coating enhances polyethylene terephthalate artificial ligament graft osseointegration in the bone tunnel

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Abstract The purpose of this study was to investigate whether hydroxyapatite (HAp) coating could induce polyethylene terephthalate (PET) artificial ligament graft osseointegration in the bone tunnel. Twenty-four New Zealand white rabbits underwent artificial ligament graft transplantation in bilateral proximal tibia tunnels. One limb was implanted with HAp-coated PET graft, and the contralateral limb was implanted with non-HAp-coated PET graft as control. The rabbits were randomly sacrificed at four and eight weeks after surgery. The loads to failure of the experimental group at eight weeks were significantly higher than those of the control group ($p=0.0057$). Histologically, application of HAp coating induced new bone formation between graft and bone at eight weeks compared with the controls. Real-time polymerase chain reaction examination revealed significantly elevated messenger ribonucleic acid expression levels of osteopontin and collagen I in the grafts of the HAp group compared with the controls at four weeks ($p<0.05$). The study has shown that HAp coating on the PET artificial ligament surface has a positive effect in the induction of artificial ligament osseointegration within the bone tunnel.

Introduction

Although the LARS ligament (Ligament Advanced Reinforcement System; Surgical Implants and Devices, Arc-sur-Tille, France), made of polyethylene terephthalate (PET) material, has not been approved for use in the USA, it has acquired much favour in many other countries all over the world [10]. The LARS ligament has several potential clinical benefits in anterior cruciate ligament (ACL) surgery and other reconstructions of knee ligaments in severe injuries, such as avoiding the morbidity of autograft harvest and avoiding potential disease transmission with the allograft, and it appears to be a promising approach for rapid recovery, less pain, less swelling and a quicker return to sporting activity, especially in the early postoperative period [3, 11, 16]. However, the LARS ligament, since it is hydrophobic, seems to have poor integration into the surrounding bone [4]. Gao et al. [3] reported that seven of 156 cases of ACL reconstruction using the LARS ligament were noted to have graft failure at the bone tunnel with loose screw or loose graft within three to five years in a multicentre study. Instead of the fibrocartilage transitional zone found in the native ACL insertion site, an interposed layer of fibrous scar tissue appears at the interface between the graft and the bone tunnel after ACL reconstruction using the LARS artificial ligament graft. Long-term motion could possibly lead to graft laxity and failure. This raises questions as to how to improve the artificial ligament graft healing in the bone tunnel after implantation.

Hydroxyapatite (HAp), as the main component of skeletal bone, has been widely studied for its strong osteoconductive capacity. It has been successfully applied to treat bone defects or articular cartilage defects and to induce bone ingrowth into and/or onto the soft tissue or metal implants in large experimental animals [2, 8, 13, 23].

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Furthermore, it has also been shown that HAp powder, due to its osteoconductive property, can promote tendon osseointegration through increasing new bone formation in a tendon-to-bone healing model when compared with the controls [6]. It is not known, however, whether HAp application could enhance PET artificial ligament graft osseointegration in the bone tunnel.

We used HAp coating for the surface modification of PET artificial ligament sheets which was taken from the LARS ligament remnant during the ACL reconstruction procedure. The purpose of this study, therefore, was to investigate the effect of HAp coating on the PET artificial ligament in a rabbit extra-articular tendon-to-bone healing model. We postulated that the surface coating of HAp would result in less fibrous scar tissue formation and more new bone formation at the graft-bone interface when compared with that of the blank controls during eight weeks observation.

Materials and methods

Preparation of the PET sheet with HAp coating

PET sheets (2×0.9 cm) were taken from the LARS ligament. They were cleaned in 75% alcohol solution for four hours to remove foreign material and contamination, washed with a large amount of deionised water and then dried in air for 24 hours. The HAp solution was made with 0.6 g nano HAp powder (Aladdin Company, Shanghai, China) and 0.4 g gelatin powder (Porcine Gelatin Powder, Sigma, St. Louis, MO, USA). They were put into a test tube with 20 ml phosphate-buffered saline (PBS) and stirred for five minutes. The cleaned PET sheets were modified through plasma surface modification (HPD-100B Plasma Apparatus, Coronalab Co. Ltd., Nanjing, China) and immersed into the HAp solution for two hours. Then they were dried in air for 24 hours. Both non-treated PET sheets and HAp-coated PET sheets were sterilised by a conventional gas sterilisation technique using ethylene oxide gas [19]. In order to guarantee the same quality of HAp coating on the experimental group sheets, we only chose the HAp-coated sheets with the same weight of about 80 mg as our experimental sheets. These grafts were rolled to 2 cm in length and 3 mm in diameter.

Before the animal experiment, both non-treated sheets and HAp-coated PET sheets were scanned by scanning electron microscope (SEM) to observe the surface morphology of the sheets. Samples were finally vacuum coated with gold/palladium and examined by SEM (Tescan TS5136MM, Brno, Czech Republic) at 20 kV accelerating voltage.

Animal study design and operative procedure

The animal experiment was approved by the Animal Care and Use Committee of our college. Twenty-four mature male New Zealand rabbits (mean weight 3.1 ± 1.3 kg) underwent an operative procedure using an extra-articular graft-to-bone healing model (Fig. 1). The graft coated with HAp was implanted into one limb, while the graft with no coating was implanted into the contralateral limb as the control.

Anaesthesia was induced by the intravenous administration of 3% pentobarbital (30 mg/kg). The animal was placed supine on the operative table. After skin preparation and disinfection, a midline knee incision was made. The tibialis anterior muscle was retracted laterally. A 3-mm diameter tunnel was drilled in the proximal tibial metaphysis at a 60° angle relative to the long axis of the tibia. The graft was pulled manually into the bone tunnel. The medial graft

Fig. 1 **a** Optical photograph of artificial ligament graft. The graft, with a size of 2×0.9 cm, was rolled to 2 cm in length and 3 mm in diameter (indicated by the *black arrow*). **b** Surgical schematic diagram of extra-articular tendon-bone healing model. The artificial graft was pulled through the tunnel; about 0.5 cm length of graft was left out of the lateral tunnel entrance for later biomechanical testing, and the graft end at the medial tunnel entrance was sutured to the adjacent periosteum and soft tissue

Fig. 2 Scanning electron micrograph of the surfaces of the PET graft without and with HAp coating. **a** Surfaces of the PET fibres without HAp coating are quite smooth and clear. **b** With HAp coating, many HAp particles are deposited on the fibre surface, with a size from 2 to 20 µm in diameter. Bar=20 µm

end was sutured into the adjacent periosteum and soft tissue. About 0.5 cm length of graft was left out of the lateral tunnel entrance for later biomechanical testing. The retinacular incision and wound were closed in layers. Postoperatively, the animals were returned to their cages and allowed free cage activity without immobilisation. Buprenorphine (0.05 mg/kg) was used subcutaneously for three days for pain control [5].

Mechanical testing

The graft-tibia complex ($n=6$ limbs in each group at each time point) was harvested from each knee after sacrifice and prepared for mechanical testing immediately without being frozen. All scar tissue and sutures at the tibia tunnel exits were carefully removed. All mechanical testing was conducted using an Instron materials testing system

machine (8874, Instron Co., Norwood, MA, USA). The graft out of the lateral tunnel entrance was sutured by a No. 5 Ethibond suture for traction. The tibial part was fixed firmly in a clamp. Care was taken to keep the bone tunnel oriented parallel to the testing axis. Immediately after preconditioning, the ultimate load to failure was performed with an elongation rate of 2 mm/min. The load-deformation curve was recorded, from which the ultimate failure load (N) and the stiffness (N/mm) were measured. The failure mode was characterised as the following: the graft pulled out of the bone tunnel, the graft ruptured out of the tunnel or the graft ruptured inside the tunnel. For each specimen, testing was completed when the graft ruptured or was pulled out of the bone tunnel.

Histological examination

Graft-tibia complexes ($n=4$ limbs for each group at each time point) were prepared for histological analysis of the graft-bone interface. Immediately after sacrifice, the graft-bone complex specimens were fixed in 10% formalin for

Fig. 3 Mechanical examinations for graft-bone healing in a rabbit model of the control group and HAp group at each time after surgery. **a** Comparison of maximal failure load. **b** Comparison of stiffness at failure (*indicates significance with $p<0.05$ level when compared with the control group; N.S. indicates there is no significant difference between the HAp group and the control group)

48 hours, then embedded undecalcified in methyl methacrylate compound according to established protocols. The samples were sectioned perpendicular to the longitudinal axis of the tibial tunnel with a thickness of 5 μm using a microtome (SM2500, Leica, Nussloch, Germany). These sections were stained with haematoxylin and eosin (H&E) for routine histological evaluation. The slides were examined to inspect the graft-bone interface with an inverted light microscope (IX71SBF-2, Olympus Optical 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 as to animal treatment.

Real-time polymerase chain reaction (RT-PCR) analysis

Immediately after sacrifice at the fourth week, four graft samples from each group were harvested for RT-PCR analysis. Total RNA from interfacial samples between graft and host bone tunnel were extracted using TRIzol reagent (10296010, Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. The cDNA was generated using reverse transcriptase M-MLV (D2640A, Takara, Beijing, China) according to the manufacturer's protocol. Quantitative PCR was carried out with SYBR Premix Ex Taq (DRR041A, Takara, Beijing, China), detected by a real-time PCR system (TP800, Takara, Kyoto, Japan), and the gene expressions of osteopontin and collagen I were normalised to that of the β -actin in all samples.

The primers for real time-PCR were described as follows: for β actin, forward 5'-CCA AGG CCA ACC GCG AGA AGA TGA-3' and reverse 5'-GCA GCG CGT AGC CCT CGT AGA TGG-3'; for osteopontin, forward 5'-GTG GAC AGC GAG GAC TTG GAT G-3' and reverse 5'-GGC CTC GCG CTT ATA TTG TCT GG-3'; for collagen I, forward 5'-TGA GGG CCA AAG CGA AGA CAT CC-3' and reverse 5'-TTC GGG GCA GAC AGG GCA GCA CTC-3'.

Statistical analysis

Data analysis was performed using Stata 9.0 software (StataCorp, College Station, TX, USA) and reported as mean and standard deviation for description. The data were compared between the experimental and control limbs using a paired Student's *t* test. Comparisons of groups at different time points were performed with an independent two-sample Student's *t* test. The level of statistical significance was set at 0.05.

Results

SEM analysis

As shown in Fig. 2, the PET grafts remained smooth and uniform, and no cracks could be identified. However, after surface modification with HAp coating, many crystal

Fig. 4 Histological characterization of the control group (**a**, **c**) and the HAp group (**b**, **d**) at 4 weeks (**a**, **b**) and 8 weeks (**c**, **d**) after surgery. This shows the interface between the artificial ligament graft and the host bone (hematoxylin-eosin staining, magnification-200 $\times$). At the HAp group at 8 weeks after surgery, new bone growth into the interface was observed (as indicated by black arrows). HB, host bone; G, artificial ligament graft; IF, interface. HAp, hydroxyapatite. Bar=200 μm

particles were deposited on the fibre surface. The HAp particles had approximately a size from 2 to 20 μm in diameter.

Mechanical findings

All specimens failed by pullout from the tunnel at four and eight weeks; no graft rupture occurred. At four weeks, there was no statistically significant difference in the load to failure between the two groups ($p=0.2009$). At eight weeks the mean load to failure for the experimental group was higher than that of the control group ($90.7\pm13.5\text{ N}$ vs $60.5\pm9.1\text{ N}$ for controls; $p=0.0057$). At four weeks, there was no statistically significant difference in the stiffness values between the two groups ($p=0.2895$, respectively). At eight weeks, the mean stiffness for the experimental group was higher than that of the control group ($9.3\pm2.0\text{ N/mm}$ vs $5.7\pm1.2\text{ N/mm}$; $p=0.0181$) (Fig. 3).

Histological findings

In the control group, thick fibrous scar tissue was formed at the graft-to-bone interface at four weeks and at eight weeks. No obvious new bone formation was observed in any of the control specimens. In the group with HAp coating, newly formed bone grew from host bone to the graft at four weeks. At eight weeks, the interface width appeared much narrower, and there was less scar tissue formation. Some protruding new bone tissue formation was found at the interface between host bone and graft (Fig. 4).

RT-PCR

At week four, the HAp group showed significantly higher osteopontin gene expression than the control group (HAp group, 0.1380 ± 0.040 ; control group, 0.02402 ± 0.0031 , respectively; $p=0.0091$). The type I collagen mRNA level at week four was also significantly higher in animals receiving HAp-coated graft compared with the control group (HAp group, 0.00135 ± 0.00029 ; control group, 0.00067 ± 0.00006 , respectively; $p=0.0107$) (Fig. 5).

Discussion

It has been widely reported that the healing potential of grafts in a bone tunnel is extremely poor since a layer of fibrovascular scar tissue develops in the interface between the graft and the bone tunnel [5, 19]. This fibrovascular scar tissue becomes the weakest attachment of the tendon graft which could possibly hamper early rehabilitation and return to sports. Thus, several approaches have been investigated to augment tendon-to-

Fig. 5 Relative expression levels of genes 4 weeks after surgery. Real-time PCR data on two different samples were included to compare a series of bone-associated genes, such as collagen type I and osteopontin. The mRNA expression in the tibial bone tunnel was compared between the control and HAp groups. Specimens in the HAp group had significantly elevated **a** osteopontin to β -actin (<0.05) and **b** collagen type I to β -actin ($p < 0.05$) mRNA ratios versus controls (*indicates significance with $p < 0.05$ level when compared with the control group)

bone healing, including HAp [6], bone cement [21], bone morphogenetic protein-2 (BMP-2) [12, 18], bone morphogenetic protein-7 (BMP-7) [14], platelet-derived growth factor (PDGF) [22], transforming growth factor- β 1 (TGF- β 1) [24], granulocyte colony-stimulating factor (G-CSF) [19] and periosteum [7, 9]. These experimental agents have been demonstrated to be effective in the acceleration of tendon-to-bone healing by increasing new bone or fibrocartilage formation and decreasing fibrous scar tissue at the interface between tendon and bone. At present, very few in vivo studies have examined PET artificial ligament healing in the bone tunnel after using an agent to augment healing.

In this study, we investigated the ability of HAp coating to promote healing of PET artificial graft in the tibial bone tunnel using a rabbit extra-articular tendon-to-bone healing model. Through plasma surface modification, the HAp powders were successfully deposited on the fibre surface of PET sheet, which was observed by SEM. The gelatin used here could become a carrier or vehicle for HAp particles adhering to the PET fibres. We found that this HAp coating

on the PET sheet of the artificial graft could improve the biomechanical properties of the graft in the bone tunnel. HAp coating had a positive effect on the healing of the artificial ligament.

With respect to soft tendon graft implant, Ishikawa et al. [6] investigated the effects of HAp powder in the interface between graft and bone using a rabbit extra-articular tendon-to-bone healing model. Histologically, they found more new bone formation in the HAp group postoperatively, which is possibly due to the osteoconductive property of HAp. Moreover, Mutsuzaki et al. [15] hybridised calcium phosphate (CaP) with tendons using an alternate soaking process. They demonstrated that tendons with low-crystallinity apatite could clearly enhance graft healing at the tendon-bone interface by promoting new bone formation around the tendon even within three weeks of surgery. The mechanism is associated with CaP which was easily resorbed by osteoclasts, and subsequently after the resorption of CaP new bone tissue was formed on the tendon surface by osteoblasts.

In this study, histological results showed that the HAp-coated group had new protruding bone tissue formation at the interface eight weeks after implantation in comparison to the control group. Meanwhile less fibrous scar tissue was observed between the graft and the host bone in the HAp-coated group when compared with the controls. This is possibly due to the osteoconductivity of HAp in vivo and also the surface hydrophilicity of the coated fibre. Because of its biocompatibility and osteoconductive property, HAp has long been used to augment bone ingrowth across the interface between implant and bone [17, 20]. The graft with HAp coating had more osseointegration with the host bone at the eight week time point compared to the pure PET graft without coating.

Finally, we admit there are limitations in our study. We used a rabbit extra-articular tendon-to-bone healing model to investigate the effect of this HAp-coated PET sheet in tendon healing. It is different from the intra-articular tendon-to-bone healing required for ACL reconstruction. However, this model of graft implantation has been well established; several tendon-to-bone healing studies have been done with this rabbit model [1, 7]. Another limitation is that the HAp powders have an agglomeration phenomenon, which may influence the size effect of nano HAp. There are other ways to apply HAp coating on the artificial ligament surface, such as biomimetic deposition and electrochemical deposition, which may be a consideration for future studies.

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Conflict of interest The authors report no conflict of interest.

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