

Original Article

Effect of culture complex of BMSCs and sodium hydroxide- and GRGDSPC-treated PET on the reconstruction of injured anterior cruciate ligament in a rabbit model

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Received March 4, 2015; Accepted April 30, 2015; Epub May 15, 2015; Published May 30, 2015

Abstract: Ligament reconstruction is an effective therapy for anterior cruciate ligament (ACL) rupture. Polyethylene terephthalate (PET) artificial ligaments have recently gained popularity in clinical ACL reconstruction for its advantage in the improvement of knee function. However, the application of PET in clinical treatment is limited by its poor bioactivity and biocompatibility. Recently, bone marrow-derived mesenchymal stem cells (BMSCs) have been widely studied in regenerative medical therapy due to their multi-lineage differentiation. Previous study also indicated that BMSCs may promote the healing of tendon-bone interface of injured ligament. We speculate that BMSCs may enhance the curative effect of PET artificial ligament on the tendon-bone-healing in ligament reconstruction. In this study, the PET materials were first modified with sodium hydroxide hydrolysis and GRGDSPC peptide which was able to improve its bioactivity and biocompatibility. Then, the effects of modified PET materials on the adhesion, proliferation and differentiation of BMSCs were examined. The *in vitro* co-culture of BMSCs and modified PET showed the modified PET promoted the adhesion, proliferation and differentiation of BMSCs. Further, the effect of culture complex of BMSCs and modified PET artificial ligament co-culture system on the injured ligament reconstruction was investigated *in vivo*. Results showed not only better growth and differentiation of BMSCs but also satisfactory healing of the injured ligament was observed after implantation of this culture complex into the injured ligament of rabbits. Our study provides a brand-new solution for ACL reconstruction.

Keywords: Anterior cruciate ligament rupture, reconstruction, ligament advanced reinforcement system, polyethylene terephthalate, bone marrow-derived mesenchymal stem cells

Introduction

Anterior cruciate ligament (ACL) rupture is a common soft tissue knee injury in sports activities. Under this condition, the ligaments are immediately disabling and their rehabilitation usually takes a significant amount of time. Moreover, ACL injury is often accompanied by articular injuries which may increase the risk for early-onset post-traumatic osteoarthritis [1].

The common treatments for ACL rupture include conservative treatment and surgical treatment. However, conservative treatment has some disadvantages such as long-term brace-wearing and an increased risk for osteo-

arthritis in clinical practice. In addition, it is difficult to obtain sufficient regeneration of the torn ACL [2, 3]. Thus, reconstructive surgery is the best choice for the treatment of ACL injury due to its potential benefit in better recovery of knee stability. The common reconstruction surgery for the ligament injury mainly refers to the implantation of graft to the injured ligament. The most common graft used in the ACL reconstruction is the synthetic ligament due to its abundant supply and strong support as well as its security in disease transfusion and immunological rejection [4, 5].

Among synthetic ligaments, the ligament advanced reinforcement system (LARS), a non-absorbable synthetic ligament device made of

polyethylene terephthalates (PET) [6], has recently gained popularity in the clinical treatment of ACL injury for its advantage in the improvement of injured knee function such as high-level activity and stability [7, 8]. However, fibrous scar tissues and synovitis are common complications of implantation of this PET prosthesis mainly due to its poor bioactivity and biocompatibility [5]. Consequently, some methods have been used for the surface modification of PET materials by grafting functionalized chemical groups like carboxylic, amide, sulfonate, and phosphate group to improve their biocompatibility and bioactivity [9-11].

Recently, with the development of PET prosthesis scaffold, increasing attention has been paid to the effect of bone marrow-derived mesenchymal stem cells (BMSCs) on the ligament reconstruction because they can differentiate into a wide range of tissues such as bone [12, 13], tendon [14] and muscle [15]. Lim et al [16] used BMSCs for ACL reconstruction in a rabbit model, and found well-demarcated cartilage belts grown into the graft at 8 weeks after implantation. Their study indicated that BMSCs promoted the healing of tendon-bone interface of the injured ligament. On the basis of available findings, we hypothesized that BMSCs may enhance the curative effect of PET artificial ligament on the tendon-bone-healing in ligament reconstruction.

In the present study, to make the surface of PET materials more suitable for the adhesion, growth and differentiation of BMSCs, PET surface was modified by hydrolysis with sodium hydroxide hydrolysis and binding with GRGDSPC peptide. Furthermore, *in vitro* cultured complex of modified PET and BMSCs was transplanted into rabbits with ACL injury and the healing of injured ligament was examined.

Materials and methods

Fourier transform infrared spectroscopy (FTIR)

The remote sensing fourier transform infrared spectroscopy (FTIR) technology is a branch of analytical technique and has been developed rapidly in recent years. By detecting and analyzing the infrared electromagnetic, FTIR technology has already been widely used in the quantitative analysis and discrimination of materials. Avatar FTIR 360 (USA, Thermo Nicolet) was

used, scanning was done 32 times, the resolution was 4 cm^{-1} , and the scan range was $4000\text{--}400\text{ cm}^{-1}$. Spectral data were processed using Omnic 7.0, and curve fitting was done using origin 7.0 software.

X-ray photoelectron spectroscopy (XPS)

X-ray photoelectronspectra (XPS) are recorded using a monochromatic Al K α X-ray source (15 keV, 25 mA emission current, VSW MX10 with 700 mm Rowland circle monochromator) and a 150-mm con-centric hemispherical electron energy analyzer (VSW Class 150) equipped with a multichannel detector operating at a constant energy analyzer mode. The photoemission angle was normal to the surface. The peaks were referenced to the aliphatic C (1 s) peak at 285.0 eV. Atomic percentages were determined from the peak areas by using sensitivity factors and transmission function for our analyzer (VSW). The atomic percentages reported were the average of three spectra with standard deviation.

Isolation and culture of BMSCs

Bone marrow was collected from the femur of healthy rabbits with a 12-gauge needle containing 200 U/ml heparin (Sigma, USA). The aspirates were depleted of mature blood lineages and purified by density gradient centrifugation in 1.073 g/ml Percoll (GIBCO, Grand Island, NY). Mononuclear cells were maintained in Dulbecco's modified Eagle's medium (α -MEM) (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO, Grand Island, NY). Non-adherent cells were removed 48 h later, and adherent cells were further cultured until cell confluence was observed.

Scanning electron microscopy (SEM)

First, samples were fixed in 2.5% glutaraldehyde for 24 h and then in 1% OsO $_4$ /0.1% uranyl acetate in acetone for 2 h. Dehydration was done in ethanol series (30% -50% -70% -85% -95% -100% (2 times) with 10-15 min at each concentration. Acetic acid (iso) amyl acetate: ethanol = 1:1 mixture soak 10 min, and isoamyl acetate soak 10 min, and then properly swing. The specimens were glued upwards on a regular scanning electron microscope (SEM) stub

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Table 1. Sequences of primers and length of products

Gene	Primers sequence (5'-3')	Length (bp)	T _m (°C)
OCN	5-GCGAGGTAGTGAAGAGACCC-3	156	59.94
	5-GCCGATAGGCCTCCTGAAAG-3		60.25
OPN	5-GCGTGGAAACCCAAAGTCAC-3	159	59.97
	5-TGGCTAGCTTGTCTTCGTGG-3		60.04
TGF-β	5-AATACACCACCAGCAGTCCG-3	125	59.59
	5-CATGAGGGTGCCTTGGGTT-3		59.96
Collagen I	5-GAGAAAAGGGTGCACCAGGA-3	202	59.89
	5-CCTGGAGAACCACGATCACC-3		60.11
GAPDH	5-GGAATCCACTGGCGTCTTCA-3	122	60.04
	5-GGTTACGCCCATCACAAAC-3		60.04

using a carbon adhesive tape and coated with a thin layer of gold prior to mounting in the vacuum chamber. The interface between PET and new bone was photographed using SEM (Hitachi TM3000) operated at 10 kV.

Cell proliferation and adhesion analysis

BMSCs and PET materials were co-cultured for 2 and 4 days. Cells were observed and counted after SEM.

Alkaline phosphatase activity analysis

Samples were processed according to the manufacturer's instructions of alkaline phosphatase (ALP) kit (Nanjing Jiancheng www.njjcbio.com No.: A059-1 50T/48), and the absorbance (A) was measured at 520 nm by spectrophotometry. The ALP activity was calculated according to the standard curve.

Western blot assay

Cells were harvested and lysed in RIPA buffer (Pierce, Rockford, USA) in the presence of Protease Inhibitor Cocktail (Pierce, Rockford, USA). In brief, protein samples were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). The membrane was blocked in 5% non-fat milk in Tris-buffered saline and then incubated with monoclonal primary antibody (Abcam, UK), followed by horseradish peroxidase-conjugated secondary antibody (Abcam, UK). The protein bands were visualized with an ECL Western blot assay system (Amersham Biosciences, UK) and images were captured using the GelLogic system (Eastman Kodak Company, Rochester, NY). GAPDH (Abcam, UK) served as a reference.

RNA isolation and qRT-PCR

Total RNA was extracted with TRIzol (Invitrogen, USA) and then reversely transcribed into cDNA by using the Reverse Transcription kit (Takara, Japan) according to the manufacturer's instructions. QRT-PCR was performed to determine the mRNA expression of each target gene by using SYBR green PCR master mix. "Two-step" method was used and PCR conditions were as follows: 95°C for 120 s, 95°C for 15 s, 65°C for 40 s, and 40 cycles. The expression of target

genes was normalized to that of GAPDH. The relative expression of target genes was calculated with $2^{-\Delta\Delta CT}$ method. The primers used in this study and the length of their products are shown in **Table 1**.

Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Comparisons were done using one- and two-way ANOVAs and Student's paired/unpaired t-test. Statistical analysis was performed using SPSS version 19.0 package. A value of $P < 0.05$ was considered statistically significant.

Results

Surface of sodium hydroxide- and GRGDSPC-treated PET

Given the poor bioactivity and biocompatibility, PET was first modified with sodium hydroxide hydrolysis as reported by Atthoff et al [17], after which its carboxyl group was exposed making it more hydrophilic. Then, GRGDSPC peptide was bound to it. FTIR spectrometry was performed to examine the surface of modified PET. Results (**Figure 1A**) showed a band at 1000-3000 cm^{-1} , a characteristic peak of hydrogen, which means the presence of N-H bond and C-O-N bond on the surface of sodium hydroxide- and GRGDSPC-treated PET, suggesting it was more hydrophilic when compared with untreated-PET and GRGDSPC-treated PET. To further determine the integration between GRGDSPC peptide and sodium hydroxide-treated PET, XPS was performed to analyze the content of nitrogen. Results showed (**Figure 1B**) that the content of nitrogen significantly increased in sodium

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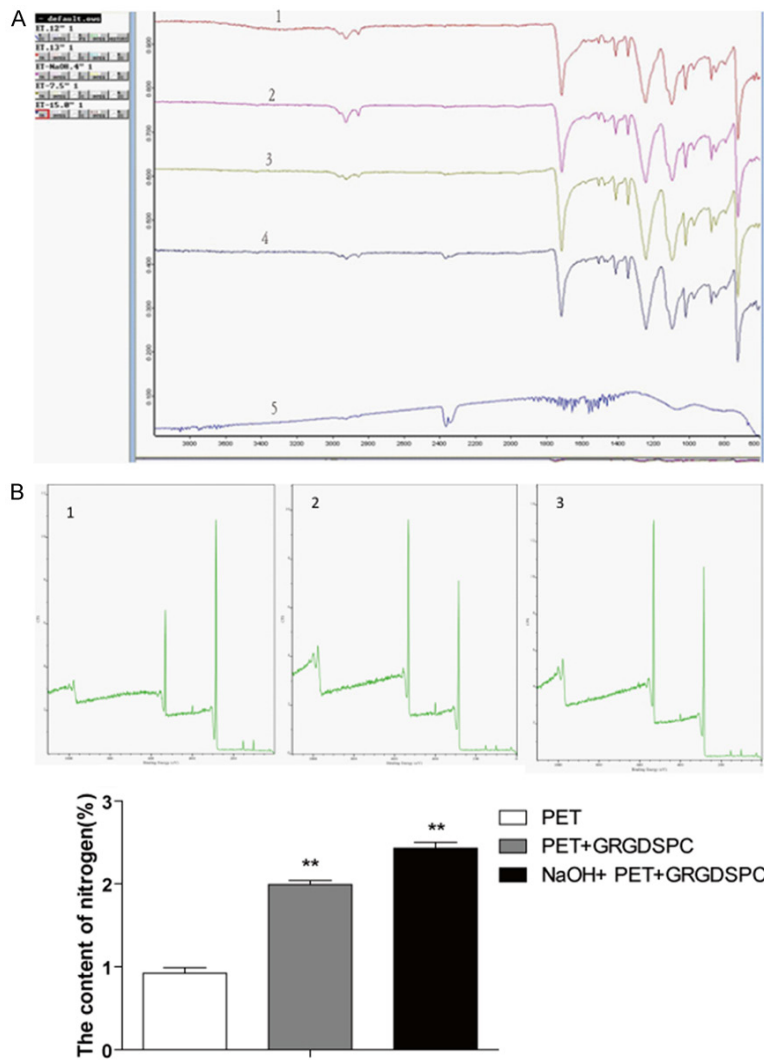


Figure 1. Examination of modified PET. A. The material content of the surface of binding GRGDSPC solution was determined by FTIR, and the groups were as follows: NaOH+GRGDSPC (15 mg/ml)+PET, NaOH+GRGDSPC (7.5 mg/ml)+PET, GRGDSPC (15 mg/ml)+PET, GRGDSPC (7.5 mg/ml)+PET, PET. B. Content of nitrogen was detected in PET, PET+GRGDSPC, NaOH+PET+GRGDSPC by XPS.

hydroxide- and GRGDSPC-treated PET which indicated more GRGDSPC peptides were integrated onto PET surface when compared with untreated PET.

In vitro culture of BMSCs

In order to investigate the growth of BMSCs *in vitro*, BMSCs were isolated from rabbit bone marrow and cultured *in vitro*. Light microscopy (Figure 2) showed cells were spindle-shaped on days 5 and 10 and formed nodular aggregates after first passaging. Our results indicated that the isolated BMSCs could be cultured *in vitro*.

Effect of sodium hydroxide- and GRGDSPC-treated PET on the induced differentiation of BMSCs into osteoblasts

In order to investigate the effect of sodium hydroxide- and GRGDSPC-treated PET on the differentiation of BMSCs to osteoblasts, BMSCs were treated with different PETs and the mRNA expression of TGF- β , collagen I, OPN and OCN, characteristic genes in osteoblasts, was examined by real-time PCR. Results (Figure 3A) showed that the mRNA expression of these genes was not changed after 7 days of co-cultured. However, the expression of these genes significantly increased in sodium hydroxide- and GRGDSPC-treated PET group on days 14 and 21. Furthermore, the protein expression of these genes was determined by western blot assay. Results revealed that the protein expression of these genes (Figure 3B and 3C) was similar with their mRNA expression. To further test the osteogenic differentiation of BMSCs on the surface of different PETs, the ALP activity was detected after OM treatment. Results showed that BMSCs cultured on the sodium hydroxide- and GRGDSPC-treated PET displayed significantly higher ALP activity when compared with cells on untreated PET or GRGDSPC-treated PET after 21-day culture. These findings indicated that sodium hydroxide- and GRGDSPC-treated PET was more effective to induce the differentiation of BMSCs into osteoblasts.

Effect of sodium hydroxide- and GRGDSPC-treated PET on the adhesion and proliferation of BMSCs

BMSCs were maintained on the surfaces of different PETs and the adhesion of BMSCs to the surface of different treated-PETs was observed

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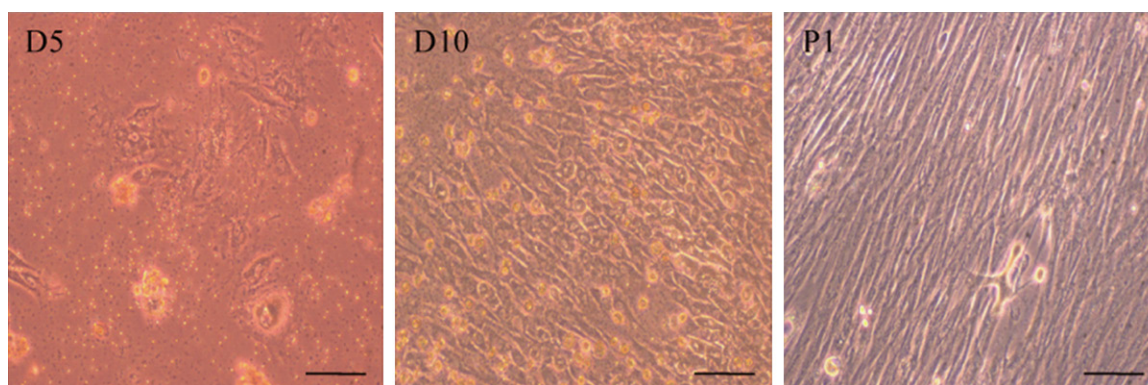


Figure 2. *In vitro* culture and characterization of rabbit BMSCs. Morphology of rabbit BMSC. BMSCs showed fibroblast-like shape and began to form cell colonies after 10-day culture. Scale = 0.5 μ m.

at 24 h and 72 h after culture. SEM showed that cells on the GRGDSPC-treated PET and sodium hydroxide- and GRGDSPC-treated PET were smooth and spherical (or flat spherical) (**Figure 4A**), which indicated that BMSCs were adherent to these PETs. Further, the proliferation of BMSCs on different PETs was investigated by hemacytometry. Results (**Figure 4B**) showed that BMSCs on untreated-PET did not proliferate after 48-h culture. However, the number of cells on GRGDSPC-treated PET increased after 48-h culture and that on sodium hydroxide- and GRGDSPC-treated PET significantly increased after 24-h and 48-h culture. Above findings indicated that sodium hydroxide- and GRGDSPC-treated PET was more suitable for the adhesion and proliferation of BMSCs.

Effect of culture complex of sodium hydroxide- and GRGDSPC-treated PET and BMSCs on the adhesion and proliferation of cells in vivo

The culture complex of sodium hydroxide- and GRGDSPC-treated PET and BMSCs was implanted into injured ligament of rabbits. 4 and 8 weeks after implantation, the growth of BMSCs on different PETs was examined by SEM and HE staining. SEM (**Figure 5A**) showed that the implanted BMSCs with GRGDSPC-treated PET displayed better growth than the only PET and a great number of cells grew along the surface of PETs and converged together in different PETs. However, the number of cells on sodium hydroxide- and GRGDSPC-treated PET was larger than that in other two groups. HE staining (**Figure 5B**) also showed obvious proliferation of BMSCs on sodium hydroxide-and

GRGDSPC-treated PET. These findings further indicated that sodium hydroxide- and GRGDSPC-treated PET was suitable for the adhesion and proliferation of BMSCs.

Effect of culture complex of sodium hydroxide- and GRGDSPC-treated PET and BMSCs on the healing of tendon-bone interface of injured ligament in rabbits

The culture complex was implanted into injured ligament of rabbits in an ACL reconstruction model, and the restoration of injured ligament was examined by masson staining at 4, 8 and 12 weeks after implantation. Results showed the regenerated ligament at 12 weeks after implantation mainly contained elastic fibrous tissues in sodium hydroxide- and GRGDSPC-treated PET group. On the contrary, collagen fibrous tissues accounted for a majority in PET group and GRGDSPC-PET group (**Figure 6**). These results indicated the culture complex of sodium hydroxide- and GRGDSPC-treated PET and BMSCs was more effective for the recovery of injured ligament.

Discussion

ACL is a dense band of connective tissues, comprising an anteromedial and posterolateral bundles based on the tibial insertion sites. ACL injury is very common among athletes and young individuals as a result of hyperextension injury in combination with valgus angulation and internal rotation of the knee. Nowadays, ligament reconstruction is the most common treatment for ACL rupture. However, the selection of grafts is of great importance for the liga-

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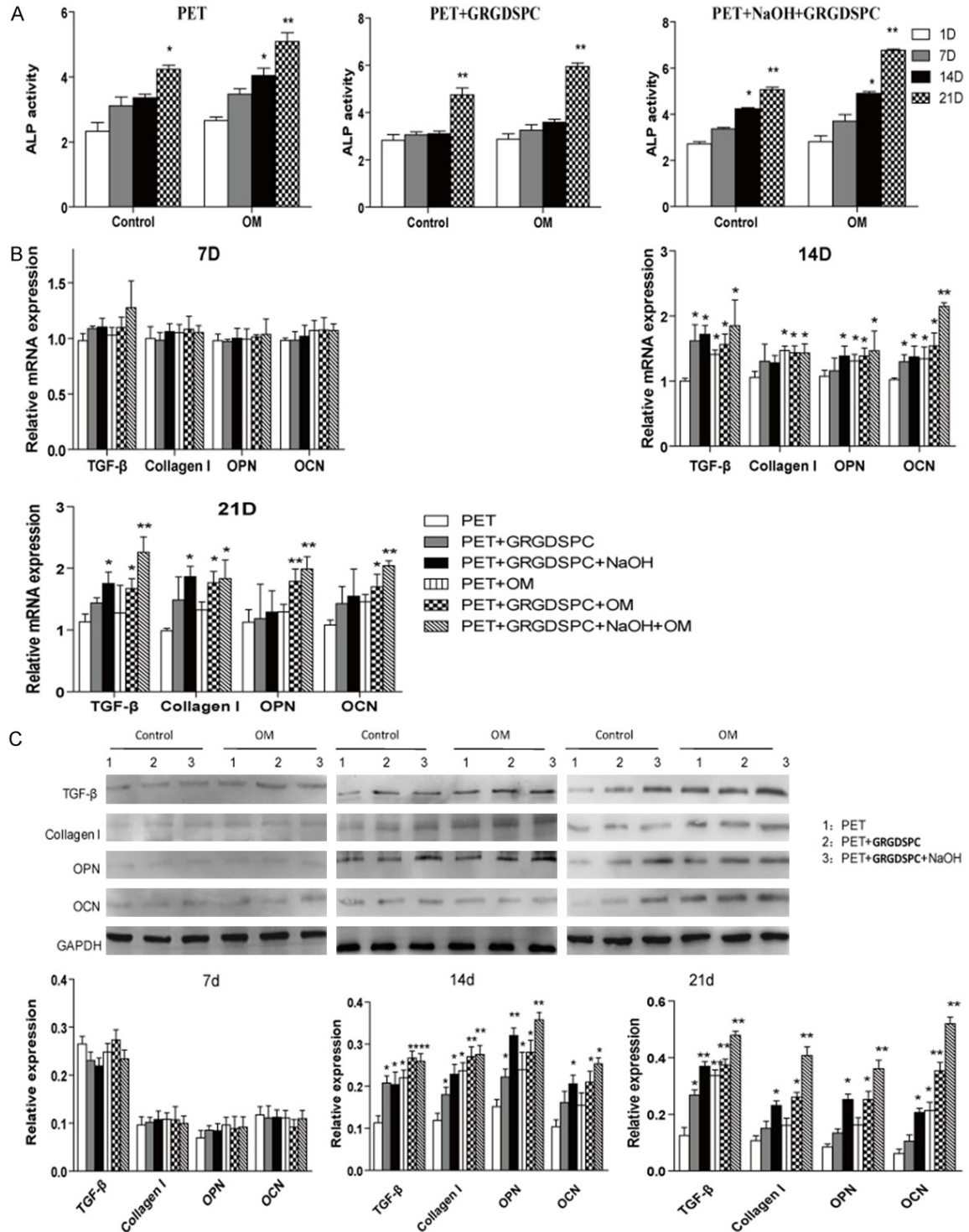


Figure 3. Effect of modified PET on the differentiation of BMSCs. A. ALP activity was measured in PET, PET+GRGDSPC, NaOH+PET+GRGDSPC (OM- and OM+ groups) on days 1, 7, 14 and 21 at 520 nm by with a Microplate Reader. * $P < 0.05$ vs 1 day, ** $P < 0.01$ vs 1 day. B. Total RNA was isolated from cells in each group and mRNA expression of TGF- β , collagen I, OPN and OCN was measured on day 7, 14 and 21 by real time PCR. * $P < 0.05$ vs PET, ** $P < 0.01$ vs PET. C. On days 7, 14 and 21, the protein expression of TGF- β , collagen I, OPN and OCN was detected by western blot assay. * $P < 0.05$ vs PET group, ** $P < 0.01$ vs PET group.

ment reconstruction. As far as grafts are concerned, there are mainly three categories:

autologous grafts, allografts, and synthetic ligaments. Although autologous grafts such as

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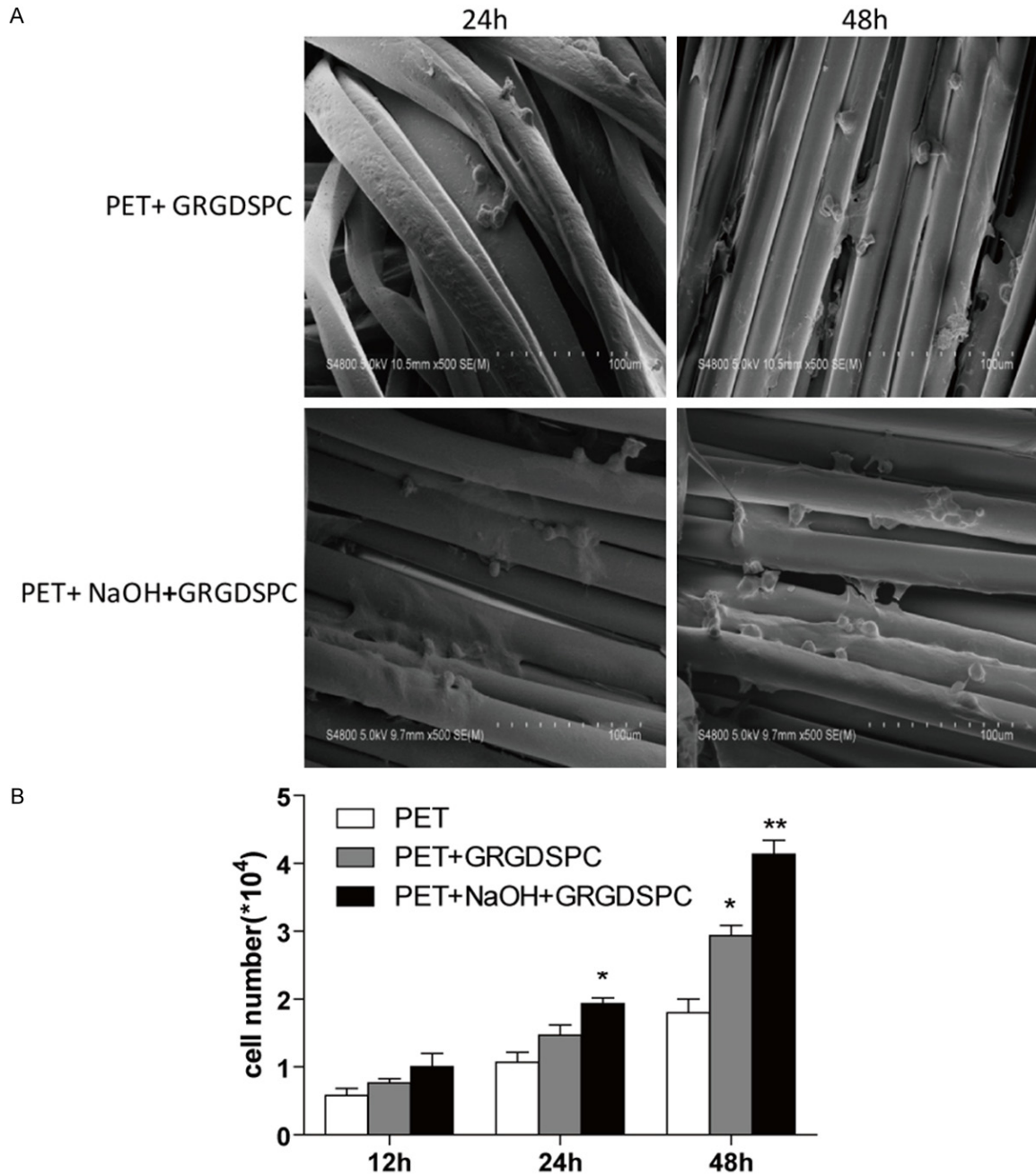


Figure 4. Effect of modified PET on the adhesion and proliferation of BMSCs. A. BMSCs were maintained with modified PET and cell adhesion was observed at 24 h and 48 h by SEM. B. BMSCs were maintained with modified PET and cell proliferation was measured by hemacytometry. * $P < 0.05$ vs PET, ** $P < 0.01$ vs PET.

hamstring and bone-patella tendon grafts are widely used due to low cost and no risk for graft infection [18, 19] they have disadvantages in site morbidity harvest and longer avoidance of activities after operation, which limits its wide application in clinical practice [20]. Allografts are less used due to the risk for complications such as potential viral infection [19]. Recently,

with the development of synthetic materials, PET artificial ligaments are used for the ACL reconstruction due to their abundant supply, significant strength and satisfactory efficacy in early postoperative period [7]. However, in long-term follow-up, fibrous scar tissues are frequently observed at the interface between tendon and bone of restored ligament. The main

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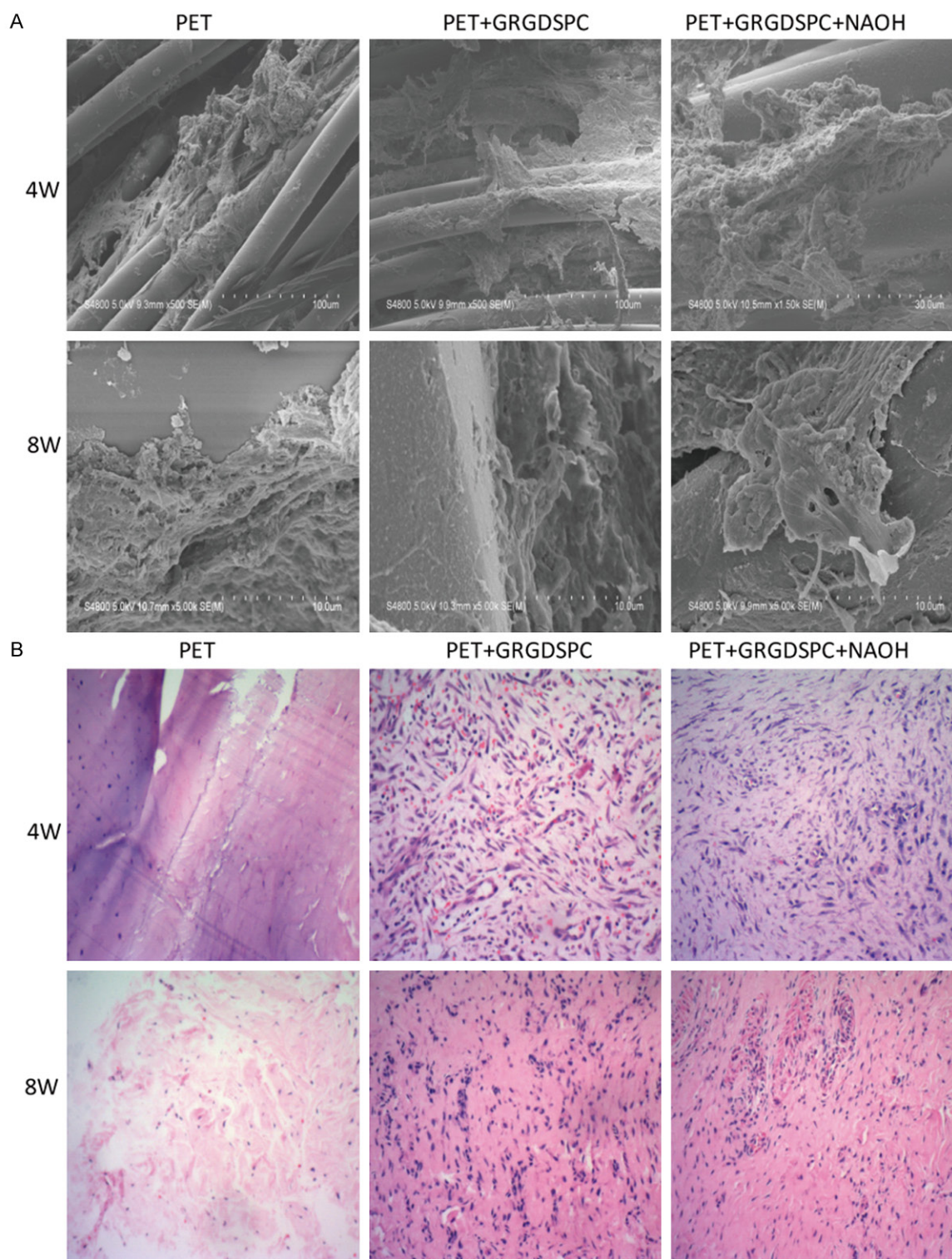


Figure 5. *In vivo* effect of modified PET on the adhesion and proliferation of BMSCs. Ligamentum cruciatum anterius injury model was established in New England rabbits at both knee joints. Animals were randomly divided into three groups: rabbits in Group A received PET transplantation; rabbits in Group B received transplantation of PET+GRGDSPC into the knee joints; rabbits in Group C received transplantation of NaOH+PET+GRGDSPC into the knee joints. A. SEM scanning for of ligaments 4 and 8 weeks after implantation. B. H&E staining of ligaments 4 and 8 weeks after implantation.

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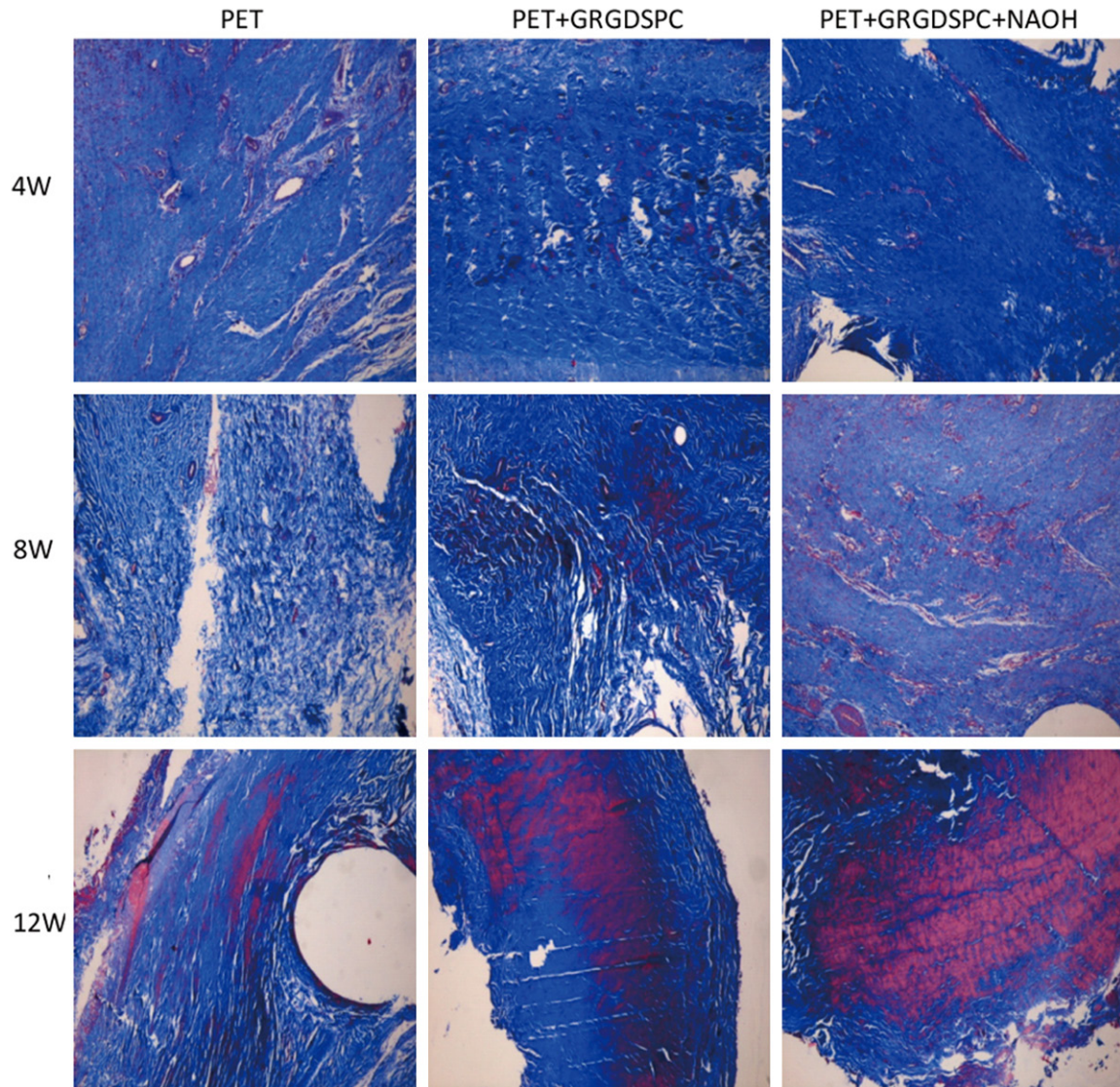


Figure 6. Effect of culture complex of sodium hydroxide- and GRGDSPC-treated PET and BMSCs on the healing of tendon-bone interface of injured ligament in rabbits. MASSON staining of ligaments with or without transplantation was performed in PET, PET+GRGDSPC, NaOH+PET+GRGDSPC groups 4 w, 8 w and 12 w after implantation.

reason for the formation of fibrous scars after treatment with PET artificial ligament is ascribed to the poor bioactivity and biocompatibility, which leads to low tissue integration. To overcome the inherent disadvantages of PET, much effort has been done to improve the immobilization of biomolecules by precise modifications. In this study, PET was modified by hydrolysis with sodium hydroxide and binding to GRGDSPC peptide. The modified PET has improved bioactivity and biocompatibility as shown by FITR spectrometry and XPS.

Common methods used to improve the biomedical applicability of PET are the immobilization

of their surfaces with biologically active components like short-peptide which may interact with integrins on cell membrane and promote cell adhesion to PET [21]. At present, the fragment commonly used in the synthesis short-peptide is Arg-Gly-Asp (RGD) which has been identified as having higher biocompatibility and less cytotoxicity. In BMSCs, Gly-Arg-Gly-Asp-Ger-Pro-Cys (GRGDSPC) has been identified as being the most suitable for BMSCs adhesion [22]. Except for biocompatibility, hydrophobicity is another characteristic of PET affecting the cell adhesion. In order to solve this problem, Atthoff et al [17] hydrolyzed PET with sodium hydroxide, the resultant PET was more hydro-

philic and the proportion of adhesive cell increased to 70% on sodium hydroxide-treated PET surface as compared to untreated PET. In our study, PET was bound to GRGDSPC and hydrolyzed with sodium hydroxide. In order to determine the change in the surface property of PET treated by GRGDSPC and sodium hydroxide, FITR and XPS analysis were performed. Results indicated that the modified PET was more roughness and more proteins were found on its surface. As compared to other modifications, our method is more convenient and effective in operation and has no influence on the mechanical property of artificial ligament.

In order to test the effect of sodium hydroxide- and GRGDSPC-treated PET on the differentiation of BMSCs, the expression of genes (TGF- β , collagen I, OPN and OCN) was detected in these cells. Results showed the expression of these genes in BMSCs cultured with hydroxide- and GRGDSPC-treated PET in the presence or absence of OM increased significantly after 14-d and 21-d culture (**Figure 2**). This indicated that BMSCs become mature in their osteoblastic lineage [23], and the sodium hydroxide- and GRGDSPC-treated PET with enhanced bioactivity and biocompatibility was more effective for the differentiation of BMSCs.

Then, the effect of sodium hydroxide- and GRGDSPC-treated PET on the adhesion and proliferation of BMSCs was investigated. Results showed an increased number of BMSCs on the surface of sodium hydroxide- and GRGDSPC-treated PET which indicated enhanced cell adhesion and proliferation on PET following modification. The increased adhesion is probably ascribed to the rougher surface after sodium hydroxide hydrolysis. Our result was consistent with that from the study of Keller et al [24], who found a significantly increased cell attachment on rough surface than on smooth surface. Another reason for the increased adhesion and proliferation may be the GRGDSPC peptide bound to PET because tissue engineering has found that GRGDSPC peptide may promote cell adhesion [22, 25]. In addition, *in vivo* experiment also revealed enhanced cell adhesion and proliferation in injured ligament at 8 weeks after implantation of culture complex of sodium hydroxide- and GRGDSPC-treated PET artificial ligament and BMSCs. Our findings suggested that sodium

hydroxide- and GRGDSPC-treated PET artificial ligament has better bioactivity and biocompatibility and may improve the long-term efficacy of LARS.

Reconstruction of injured ACL is always a hot topic in tissue engineering. Herein, we proposed a new strategy for the reconstruction of injured ACL. The culture complex of modified PET artificial ligament and BMSCs was implanted into injured ligament of rabbits and results showed the *de novo* synthesized elastic fibrous tissues increased in the injured areas indicating a good healing of injured ligament. In the clinical application of stem cells, a major obstacle is the low survival rate of grafted cells, which are attributed to the mechanical damage, acute inflammation, immunological rejection, and lack of trophic factors [26]. In our study, sodium hydroxide- and GRGDSPC-treated PET artificial ligament was found to provide a suitable scaffold for BMSCs to support their attachment and proliferation. This technique combined with stem cell therapy and modified artificial ligament therapy may provide a promising strategy for the enhancement of tendon-bone healing in ACL reconstruction.

In summary, the sodium hydroxide and GRGDSPC peptide modified PET has increased bioactivity and biocompatibility, and implantation of culture complex of BMSCs and modified PET artificial ligament may promote the healing of injured tendon-bone interface in the reconstruction of injured ACL in a rabbit model. This provides a new strategy for the therapy of ACL injury.

Acknowledgements

This study was supported by the Medical Science and Technology Innovation Foundation of Nanjing Military (No. ZX19, 12Z21) and 1255 Subject Characteristics Breeding Programs of Shanghai Changhai Hospital (NO. 20102066).

Disclosure of conflict of interest

None.

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