PRP significantly promoted the adhesion and migration of vascular smooth muscle cells on the stent material

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Research Article

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Abstract

Background

The adhesion and survival state between cells and scaffold material is a major problem in the process of tissue engineered blood vessel (TEBV) culture. Platelet rich plasma (PRP) contains a large amount of biologically active factors and fibrin, which is expected to play an important role in the process of TEBV culture.

Purpose

To combine PRP with cells and scaffold material to promote cell adhesion and biological activity on the scaffold material.

Methods

The adhesion status and migration ability of SMC cells under the conditions of optimal concentration suitable for SMC growth and optimal concentration of PRP were examined by scanning electron microscopy, HE staining, CCK8, qPCR, WB and other experimental means, and compared with the situation of conventional culture (20% FBS); finally, the effect of PRP on the deposition of ECM in vascular tissue engineering culture was verified by three-dimensional culture.

Results

20% PRP is a more suitable concentration of SMC, compared with the control group, the 20% PRP group has better migration ability, the number of SMC adhesion is significantly higher than the control group, in addition to this, the collagen deposition in the experimental group is significantly higher than the control experimental group.

Conclusion

20% PRP can promote the ability of SMC adhesion, migration and collagen deposition on the scaffold material.

Introduction

The culture of tissue-engineered blood vessels consists of three basic elements: an adequate amount of seed cells, a suitable scaffold material, and a complex culture environment\cite{1,2}. Due to the long culture time, the adhesion status of seed cells on top of the scaffold material is directly related to the outcome of TEBV. PGA material is a biodegradable polymer hydrophobic material, which can obtain a porous three-dimensional structure through non-woven technology\cite{3}. We found in the process of culture, the traditional method for TEBV culture, smooth muscle cells on the non-woven PGA material adhesion is poor, cells in an uncontrollable proportion fall on the bottom of the reactor, fall on the bottom of the current technology
can not be completely removed for the time being, part of the residual seed cells will be in the bottom and co-culture with TEBV. On the one hand, the cells will take away the upper part of the TEBV nutrients. On the other hand, the bottom cells will die when they reach a certain density, and it is not known whether some factors will be released in this process to have uncontrollable effects on the upper TEBV layer.

To make the culture process smoother and to improve the biological activity of seeded cells on the scaffold material, many scholars have tried various methods[4], and some scholars combined gelatin and PLLA material and found that it could improve cell adhesion and migration on the material. LI electrospun keratin and PCL material to form a composite vascular tissue engineering scaffold material, and it was found that this biocomposite scaffold enhanced endothelial cell (EC) adhesion and growth.[5] Some researchers have also promoted cell adhesion on top of the material by adding collagen or both fibrin and gelatin[6,7]. These methods modify the properties of the material by altering its surface morphology and characteristics, but the techniques are more complex.

Growth factors play an important role in biological activities such as cell adhesion, migration, secretion and proliferation[8,9], Platelet Rich Plasma (PRP) contains a variety of growth factor components such as TGF, VEGF, PDGF, etc. PRP also contains fibronectin, a three-dimensional meshwork structure that can effectively store a variety of growth factors and cells[10]. PRP is widely available, easy to obtain, and is commercially available. It can be obtained repeatedly and multiple times with minimal trauma[11–13] and is already available in established commercial products. We promoted the adhesion and migration ability of SMC on top of the scaffold material by adding a certain concentration of PRP to the smooth muscle cell suspension and then putting it onto the PGA scaffold.

**Materials and methods**

All experiments were conducted in accordance with the ethical regulations of Guangdong Provincial People’s Hospital, Guangdong Academy of Medical Sciences, and the ethic number is No.GDREC2019285h.(RI).

Non-woven PGA material was purchased from the United States (Biomedical Structures, Rhode Island, Warwick, USA), PRP was obtained from six healthy volunteers (aged 25–31 years), all with informed signed consent, and smooth muscle cells were obtained from the residual human aortic tissue of the heart transplant donor.

**Preparation for PRP**

Two-step centrifugation method was used to obtain PRP, firstly, venous blood was taken with anticoagulation tubes containing sodium citrate (KWS, China), and two-step centrifugation was performed at 200g*15min and 400g*15min to collect PRP and PPP components, and whole blood and PRP were subjected to platelet counting, so that platelet concentration of 4–6 times that of whole blood
was qualified as PRP\(^{[14,15]}\). The platelet markers were stained using Richter- (Biosharp, China) staining method was used for platelet marker staining. The PRP was stored at -80° for 1 h and then resuscitated at room temperature for 1 h. The procedure was repeated three times and the PRP solution without cells was obtained by centrifugation for the following experiments and stored at -80°. \(^{[15]}\)

**Isolation and identification of SMC**

Human aortic tissue samples of 2 cm*2 cm were used for primary culture of smooth muscle cells by the applanation culture method. Briefly, the mesangial tissues were first evenly attached to a T25 culture flask (Corning, USA), reversed into a 37°C incubator (Thermo, USA) containing 5% CO2, and 4–5 ml of DMEM/F12 (Dulbecco's Modified Eagle Medium, Corning, USA) medium containing 20% FBS (Fetal Bovine Serum, Gibco, USA) was added to.\(^{[16]}\) After 4 hours, the culture is worth noting that the flask should not be shaken for one week to prevent the tissue from floating and the cells from climbing out. After two weeks, the cells were passaged until P3 generation, 5*10^4 cells were taken on cell crawlers (Biosharp, China), immunofluorescence staining was performed according to the instructions, and the cells were treated with antibody (Calponin, smoothlin, 1:500, Abcam, USA) and antibody DAPI working solution (Solarbio, China). ) were processed and then examined microscopically at.\(^{[17]}\)

**Electron microscopic detection**

PGA materials from different treatment groups (A: control group treated according to 20% FBS, B: experimental group treated with 20% PRP, C: control group with smooth muscle cells, D: experimental group with smooth muscle cells) were fixed with 3 ml of 2.5% glutaraldehyde, and then the samples were rinsed in 0.1 mol/L sodium dimethylcarbamate buffer (pH 7.4), and then the PGA materials were placed in buffer solution at 4°C overnight. 24 h later, the buffer solution was removed, and the PGA materials were soaked in 1% citric acid for 1 h. The PGA materials were repeatedly rinsed with the buffer solution. After that, the PGA material was subjected to gradient dehydration treatment and finally treated with isoamyl acetate alternate. Observations were performed with a scanning electron microscope (S-3500N, Japan).\(^{[18]}\)

**Growth factor release assay**

In order to determine the ability and time of PRP to release growth factors on top of PGA material, PDGF and TGF-β1 were selected as assay indicators. The above non-woven PGA material was taken 1cm*1cm, and PRP was pre-configured into 20% PRP solution with DMEM/F12. 100ul of 20% PRP solution was taken and injected dropwise onto the PGA material, fixed with a cotton swab and placed in a 24-well plate (Corning, USA) for incubation, and 1ml of PBS solution was added to each well, and the medium was collected at different time points and placed at -80 The medium was stored at -80°C at 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days according to the instructions (DuoSet®; R&D Systems, Minneapolis, MN, USA) and the concentrations of PDGF and TGF-β1 released from the assay material were quantified by ELISA.

**CCK8 experiment**
SMC in different groups (20% FBS, 10% PRP, 20% PRP, 30% PRP, 40% PRP, 50% PRP, 100% PRP) were inoculated in 96-well plates (n = 6) at a density of 3000/well for 1, 3, 5, 7, and 9 days, respectively, by adding 10% CCK8 reagent according to the CCK8 kit (Dojindo, Japan) and incubated for 2 h at 37°C, then the absorbance of the medium at 450 nm was measured by enzyme marker and compared [19].

Cell scratching experiments

SMC in different groups (20% FBS, 20% PRP) were plated in 6-well plates at 10*10^4/well (n = 6), and the cells were laterally scored in the middle of the well plate with a 20ul gun tip, photographed at 0-6-12-18 h, respectively, and the scored area was calculated using Image J [20].

Quantitative Real-time PCR

SMC in different groups (20% FBS, 20% PRP) were plated in 6-well plates at 10*10^4/well (n = 3), and incubate in DMEM with 20% FBS for 3 days. Then total RNA was extracted using the TRIZOL kit (Takara, Japan) according to the instructions, and total RNA was reverse transcribed using the reverse transcription kit (Takara, Japan), and the cDNA obtained by reverse transcription was used with the SYBR Premix Ex Taq (Takara, Japan) according to the instructions [21]. The conditions for real-time PCR were 40 cycles at 95°C for 10 s, 60°C for 30 s, and GADPH was used as a reference. The following is the base pair composition of the primers used (Table 1):

| Table 1. Real-time PCR primers and product sizes. |
|-----------------|-----------------|-----------------|
| **Gene**         | **Sequence(5’-3’)** | **Size(bp)**    |
| GSK3B            | sense           | TATGGTCTGCTGGCTGTGTG | 120     |
|                  | antisense       | GCTCCCTTGGGAGGTCCC |         |
| CTNNB1           | sense           | GTACCGGAGCCCTTCACATC | 165     |
|                  | antisense       | CAGCTTCCTTGTCTGTAGCA |         |
| MMP2             | sense           | AGGATGGCAAATCGGCTTC | 185     |
|                  | antisense       | CTTCTTGGCGGGTCGTAGT |         |
| MMP9             | sense           | GGACAAGCTCTTGCGCTTCT | 126     |
|                  | antisense       | TCGCGGGTACAGGGTGAGTA |         |
| GADPH            | sense           | TTCGTCATGGGTGTGAACCA | 170     |
|                  | antisense       | GTCCTTGGGTGGCAGTGAT |         |
Western-Blot (WB) experiment:

SMC processing as above. Prior to the start of WB experiments, proteins were lysed using a protein extraction kit containing protease inhibitors, RIPA (Solarbio, China), and the concentration of total protein was detected using a BCA kit (biosharp, China) according to the kit’s instructions\(^{[22]}\). The spiked volume was calculated in advance and the samples were added on 12% SDS polyacrylamide gels, keeping the concentration of the spiked samples consistent, and the total volume of the spiked samples did not exceed 25 ul. Electrophoresis was performed at a voltage of 100-120v for 40–60 min, followed by removal of the upper part of the dentate gel and the lower part of the excess gel components, and the gel was transferred to a polyvinylidene difluoride membrane (Millipore) at 140-160v for 30min, followed by 5% skim milk in Tris-buffered saline containing 0.05% and 1% Tween 20 (TBST) for closure, followed by the following primary antibody incubations: anti- CNN (1:500; Abcam, USA), anti-SMTN (1:2000; Abcam, USA), anti-GSK-3\(\beta\) (1:1000; Abcam, USA), anti-p-GSK-3\(\beta\) (1:500; Abcam, USA), anti-Catenin (1:500; Abcam, USA), anti-COL.I (1:1000; Abcam, USA), and anti-GAPDH (1:10000, Abcam, USA), anti-Histone (1:10000; Abcam, USA), and finally Millipore was subjected to chemiluminescence detection using ECL protein blotting substrate (Pierce). Chemiluminescence detection was performed, followed by development of the film using an exposure machine (Tanon, ShangHai, China).

Three-dimensional culture and Masson staining experiments:

The PGA material was processed into 1cm*1.95cm squares, soaked in 1M NaOH solution alkaline for 1min, and rinsed in sterile water 3 times for 5min each time, then the PGA material was sutured on wooden sticks containing silicone tubes with 6 – 0 sterile sutures, sterilized by ethylene oxide, and cell inoculation was performed on the PGA material the next day\(^{[23]}\), with a cell density of 1*10^7/ml. The assembled material was cultured in DMEM/F12 containing 20% fetal bovine serum for 4 weeks, and the vascular matrix material was removed after 4 weeks for WB experiments and Masson staining\(^{[24]}\). Masson staining Masson trichrome staining was performed according to the instructions (PhyEasy, China) and can be briefly summarized as sectioning - dewaxing - Masson trichrome staining - dehydration, transparency, and sealing. Collagen will be stained blue.

Statistical analysis

All reported values were averaged (n = 6) and expressed as mean ± standard deviation (SD), and statistical differences were analyzed with two-sample t-test assuming equal variances. The value of P < 0.05 was considered statistically significant.

Results

Figure 1A shows the schematic diagram of the production and application of PRP. A large number of platelet particles stained blue were seen on the PRP smear by Richter's staining (Fig. 1: B, C). By selecting five different fields of view for platelet counting, it was found that the number of PRP platelets was 4.96
times higher than that of whole blood (Fig. 1: D), which was in accordance with the preparation criteria of PRP.

The optimal concentration of PRP for use was tested by CCK8 assay, and smooth muscle cell phenotypes were identified in cells from control and experimental groups. Calponin and smoothlin were markers of contractile phenotypes of smooth muscle cells, and the phenotypic expression of both smooth muscle cells was found to be close to 100% by immunofluorescence detection (Fig. 2: A-G). The CCK8 results showed that 20%, 30% and 40% PRP all had a pro-proliferative effect on SMC, with the 20% PRP group showing the most prominent pro-proliferative effect, and the differences were statistically different at days 5, 7, 9 and 11 (Fig. 2: J). Therefore, subsequent experiments were conducted using 20% PRP as the experimental group and 20% FBS group as the control group for subsequent experimental studies. WB experiments revealed that the cells used in both experimental and control groups expressed Calponin and Smoothlin proteins (Fig. 2: H,I).

Compared with the control group, it can be seen under the electron microscope that the PGA material in the PRP group contains a large number of loose fibrin filaments in the middle, as shown by the arrows (Fig. 3: B), the cells in the control group adhered to the PGA scaffold, the PGA gap due to the large pores, a small number of cells attached to the PGA filaments, the cells attached to the PGA filaments have a single morphology, fewer tentacles, tightly attached to the PGA filaments exist. In general, the amount of smooth muscle cells visible in the field of view was significantly less than that of the experimental group (Fig. 3: A, C), and in the experimental group, due to the presence of fibronectin, smooth muscle cells could be seen in addition to the PGA filaments, and smooth muscle cells could also be seen between the PGA pores attached to fibronectin, with a variety of cell morphology and wide tentacles (Fig. 3: D). (Fig. 3: E) shows the general picture of PGA, which is generally a multi-hollow mesh structure; the water absorption of the two materials was measured, and it was found that the PRP group was more absorbent, and the difference was statistically significant compared with the control group (Fig. 3: F). The release of growth factors was found to be sustained for one month after PRP treatment (Fig. 3: G,H).

The results of cell adhesion experiments showed that the PGA material treated with PRP had better adsorption ability to smooth muscle cells, and the observation of dropped cells at the bottom after inoculation and resting revealed that the control group had more dropped cells at the bottom, reaching 5% of the inoculation number, while almost no loss of dropped cells could be seen at the bottom of the experimental group (Fig. 4: A-C). The content of genes CTNNB1 and GSK3β and their regulatory proteins GSK-3β and Catenin were detected by Qpcr and WB, and it was found that the gene expression was up-regulated in the experimental group, and the protein content of both P-GSK-3β and Catenin increased, the results indicated that PRP also promoted the protein expression of GSK-3β-activated Catenin by up-regulating adhesion-related genes thereby promoting cell adhesion (Fig. 4: D-F).

The results of the cell scratching assay study showed that there was no significant difference in the cell migration rate between the experimental and control groups at hour 4. At hour 8, the cell migration rate was faster in the PRP group and the difference was statistically significant (Fig. 5: A-K). qPCR detected
that the cell migration-related genes MMP-2 and MMP-9 were both upregulated in the experimental group versus the control group (Fig. 5: L,M).

The cells of the experimental group and the control group were placed in 6-well plates for three-dimensional culture (Fig. 6: A), and the cultures were removed after 4 weeks of static culture, and the cultures were subjected to Masson staining and WB analysis of collagen content, and the results of Masson staining showed that the cells of the experimental group were more closely arranged, and the collagen was the blue-stained part, and the blue-stained collagen content of the experimental group was significantly higher than that of the control group as seen by the naked eye. Image j statistics also found this pattern and the difference was statistically significant (Fig. 6: B-D), and WB results showed that the collagen content of the experimental group was significantly higher than that of the control group (Fig. 6: E-F).

**Discussion**

PRP is widely used in material-assisted applications\[25\], however, its application in tissue-engineered vessels is rarely addressed. Does PRP form a connecting bridge between the seed cells of tissue-engineered vessels and the scaffold material, promoting the biological state of adhesion and secretion of SMC on top of the scaffold material? We do not know. To address this question, we introduced PRP during the inoculation of smooth muscle cells into the scaffold and found that PRP could release growth factors PDGF-BB and TGF-β continuously on the scaffold material for up to 30 days by detecting the content of growth factors in the culture medium. We investigated the effect of PRP on smooth muscle proliferation and migration by designing different experimental groups, determined the optimal concentration of PRP to use, and studied the adhesion behavior of smooth muscle cells after the addition of PRP and the secretion of collagen under three-dimensional culture conditions.

PRP has been used clinically for decades, and the preparation and distribution of PRP tend to be diverse\[26\]. The main reason is that PRP is rich in many growth factors\[27\], among which PDGF-BB and TGF-β are closely related to the biological activity of cells\[28\]. PDGF and TGF-β were found to significantly promote the proliferative activity of smooth muscle cells\[29, 30\], and Perrault R et al. showed that PDGF-BB could promote the conversion of smooth muscle cells to a secretory phenotype\[31\], and PDGF was also shown to promote the migration ability of smooth muscle cells by regulating the gene expression of MMP2 and MMP9\[32\], although PRP contains a large number of growth factors. Although PRP contains a large amount of growth factors, the hydrogel formed after activation of PRP is unstable and it is difficult to achieve stable release, so many researchers have used it together with other materials, such as PRP combined with sodium alginate for cartilage repair experiments\[33\], PRP combined with GelMA gel for 3D printing for rabbit cartilage repair experiments\[34\], or PRP combined with filament proteins using\[35\]. Although these methods solve the problem of PRP hydrogel stability well, they are more cumbersome to prepare. We added PRP mixed with smooth muscle cells directly to the surface of PGA material, and the electron microscopic results found that the fibrin filaments inside PRP evenly covered and filled the whole
PGA fibers and fiber pores, and the adhesion of smooth muscle cells to PGA material because of PRP was not only. The adhesion of smooth muscle cells in PGA material is not only dependent on PGA fibers, but also can be present in a large number of adhesions between PGA pores. It was found that PRP promoted the adhesion of smooth muscle cells inside the PGA material not only by this physical action, but also by qPCR results, which showed that PRP promoted the expression of CTNNB1 and p-GSK-3β in smooth muscle cells, thus promoting the adhesion function of smooth muscle cells, and promoted the migration ability of smooth muscle cells by promoting the gene expression of MMP2 and MMP9. In addition to this, the results showed that PRP promoted the water uptake of PGA materials.

It is well known that collagen I is a major component of the extracellular matrix[36]. To verify that the addition of PRP can ultimately enhance the extracellular matrix deposition in vascular tissue engineering, we cultured the experimental and control groups with PRP addition for 4 weeks and found that the vessels in the experimental group had more collagen deposition, and this result was fully confirmed in the WB experiments. In addition, the technique of repeated freeze-thawing of PRP to obtain growth factors is quite mature, and in the future, PRP can be obtained by processing frozen plasma for better application in the direction of tissue engineering vessel culture, which provides great convenience for future clinical applications[37]. Although the experiments showed that the addition of 20% PRP increased the adhesion function and migration ability of smooth muscle cells on top of PGA material, and the increase of collagen secretion after the addition of PRP was confirmed by three-dimensional culture, the specific mechanism of action remains to be confirmed for future application in tissue-engineered vascular culture.

**Conclusion**

In this study, we used PRP mixed with smooth muscle cells and added non-woven PGA material on top of the PRP before it formed a gel using a drop-in method. MMP9 expression was also increased. Three-dimensional culture results were observed with more collagen deposition. Our study provides a convenient and effective way to promote high cell adhesion and low cell utilization in tissue-engineered vascular cultures in the future.

**Abbreviations**

PRP, Plate Rich Plasma; FBS, Fetal Bovine Serum; SMC, Smooth Muscle Cell; ECM, Decellularized Extracellular Matrix; PGA, Polyglycolic Acid; PLLA, Poly-L-lactic Acid; PCL, Polycaprolactone; DMEM, Dulbecco's Modified Eagle Medium; TGF-β, Transforming Growth Factor; PDGF-BB, Platelet-Derived Growth Factor; COLI.1 (collagen type I), CNN (calponin), SMTN (smoothelin), MMP2 (matrix metalloproteinase 2), MMP9 (matrix metalloproteinase 9), CTNNB1 (recombinant human catenin beta-1) and GSK3β (glycogen synthase kinase 3β), GADPH, Glyceraldehyde-3-Phosphate Dehydrogenase.

**Declarations**

**Author Contributions**
YDW contributed to manuscript writing;

HJJ, YHW and QL contributed to the Figure 2-4;

HHZ and XHS contributed to software analysis;

All authors have read and agreed to the published version of the manuscript.

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None

Disclosure

The authors report no other conflicts of interest in this work

Availability of data and materials

Not applicable.

Ethical declaration

All experiments were conducted in accordance with the ethical regulations of Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, and the ethic number is No.GDREC2019285h.(RI).

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References


**Figures**
Figure 1

Schematic diagram of the process of PRP treatment and application, diagram of the process of PRP from treatment to use (A), graphs of Richter staining of whole blood and PRP (B,C), bar graphs of platelet count statistics (D), comparison with blank group ***P<0.001. all scale bars = 50 µm (scale bars = 50 µm).
Figure 2

Graphs of cell phenotype identification and results of CCK8 experiments, graphs of cellular immunofluorescence results in control and experimental groups (A-F), graphs of Calponin and Smoothlin WB protein assays and their gray-scale value measurements (G,H), graphs of the effect of different PRP concentrations on proliferative activity of smooth muscle cells by CCK8 assay (I). *p<0.05, **p<0.01, ***p<0.001 compared to blank group. All scale bars = 50 µm (scale bars = 50 µm).
Figure 3

Electron microscopic detection plots, bulk plots and growth factor release curves over time for PGA materials treated with control and experimental groups. Electron micrographs of different PGA treatments (A, B), electron micrographs of cells added after different PGA treatments (C, D), PDGF and TGF-β after PRP treatment of PGA material for one month (release graphs (G, H)). → Indicating the location of fibrin, *p<0.05 compared to blank group, **P<0.001. All scale bars = 100 µm (scale bars = 100 µm).

Figure 4

Cell adhesion assay, cell loss in control and experimental groups (A-C), Qpcr and WB assays for gene CTNNB1 and GSK3β and their regulatory proteins GSK-3β and Catenin content (D-F). **p<0.01 vs. blank group, ***p<0.001. all scale bars = 100 µm (scale bars = 100 µm).
Figure 5

Graphs of cell scratch assay and gene expression of MMP2 and MMP9, comparative graphs of cell migration ability of control and experimental groups (A-K), graphs of cell migration-related gene expression of MMP-2 and MMP-9 (L,M). **p<0.01 vs. blank group, ***p<0.001. all scale bars = 100 µm (scale bars = 100 µm).
Figure 6

3D culture results and gross plots, 3D culture for both treatments, plots of cultures after 4 weeks of incubation (A), plots of Masson staining and collagen staining for quantitative analysis of cultures (B-D), WB assay for protein collagen I content and its grayscale value conversion (E,F). **p<0.01 compared to blank group, all scale bars = 250 µm (scale bars = 250 µm).