

Construction of tissue engineering bone with the co-culture system of ADSCs and VECs on partially deproteinized biologic bone *in vitro*: A preliminary study

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Abstract. Scaffold-based bone tissue engineering has therapeutic potential in the regeneration of osseous defects. The present study aimed to explore the adhesion and cell viability of a co-culture system composed of vascular endothelial cells PI/Annexin V⁺ represents early apoptotic cells, and PI⁺/Annexin V⁺ represents late apoptotic cells (VECs) and adipose-derived stem cells (ADSCs) on partially deproteinized biologic bone (PDPBB) *in vitro*, and determine the optimum time period for maximum cell viability that could possibly be used for standardizing the scaffold transplant into the *in vivo* system. VECs and ADSCs were isolated from pregnant Sprague-Dawley rats and confirmed by immunostaining with von Willebrand factor and CD90, respectively. PDPBB was prepared using standardized protocols involving coating partially deproteinized bone with fibronectin. PDPBB was incubated in a mono-culture with VECs or ADSCs, or in a co-culture with both of these cells at a ratio of 1:1. An MTT assay was used to assess the adhesion and cell viability of VECs and ADSCs on PDPBB in the three different cultures. Scanning electron microscopy was used to observe the adhesion, cell viability and morphology of the different types of cells on PDPBB. It was observed that the absorbance of each group increased gradually and peaked on the 10th day; the highest absorbance was found for the co-cultured cells group. The difference of cell viability between each cell group was statistically significant. On the 10th day, in the co-cultured cells group, several cells adhered on the PDPBB material and a nest-like distribution morphology was observed. Therefore,

the adhesion and cell viability of the co-cultured cells was higher compared with the mono-cultures of VECs or ADSCs. As cell viability was highest on the 10th day, this could be the optimal length of time for incubation and therefore could be used for *in vivo* experiments.

Introduction

Co-culturing cells with vascular endothelial cells (VECs) has the dual advantage of improving vascularization rates, and increasing the promotion of osteoblast and bone marrow mesenchymal stem cell differentiation (1). Osteoblasts secrete vascular endothelial growth factor (VEGF) to promote cell proliferation and differentiation of endothelial cells (ECs), while ECs can affect osteogenic differentiation by secreting bone morphogenetic proteins (BMPs) (2,3). This facilitates the interaction between osteoblasts and ECs to promote the formation of new bone and blood vessels, with VEGF being a key mediator for angiogenesis (4,5). Co-culture studies showed that a human bone marrow EC line increased the cell proliferation of human bone marrow-derived fibroblasts using gelatine-coated and hydroxyapatite-coated substrates (6). Adipose-derived stem cells (ADSCs) express VEGF and hepatocyte growth factor so a co-culture of VECs and ADSCs has the potential to differentiate into fat, bone, cartilage, and skeletal and smooth muscle cells; therefore, these cells could be useful sources for bone engineering (7). Increased melanocyte proliferation and migration, as well as reduced differentiation, was observed when they were co-cultured with ADSCs compared with melanocyte mono-cultures (8).

Various different scaffolds have been proposed for tissue engineering. Scaffolds are typically composed of natural or human-made polymers, bioceramics and hybrid materials (9). Partially deproteinized biologic bone (PDPBB) is a relatively novel scaffold used in bone tissue engineering that is prepared using fibronectin combined with partially deproteinized bone (PDPB) (10). PDPB is a natural bionderived bone scaffold material obtained by natural bone physicochemical treatment, which maintains the natural reticulated pore structure of the original bone (11). It is comprised of 22.4% protein, has a calcium-phosphorus ratio of 1:74 and contains hydroxyapatite for improved histocompatibility during osteogenesis (12).

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Preparation of the scaffold material removes the antigenicity of the material, and also removes the matrix necessary for cell and scaffold material adhesion (13). It has been found that when PDPB is co-cultured with cells *in vitro*, cell activity decreases gradually, and PDPB ages and sheds from the scaffold over a period of time (14). Therefore, in order to improve the efficacy of PDPB, fibronectin, which exists in normal bone matrix and is secreted by osteoblasts, has been used to prepare PDPBB (15). It is hypothesized that PDPBB is biocompatible because fibronectin improves the histocompatibility of PDPB. It has been demonstrated that PDPBB seeded in a co-culture with bone marrow stromal cells and endothelial progenitor cells accelerates bone healing by promoting vascularized biological bone regeneration (16).

To the best of our knowledge, there are no studies that examine the advantages of using PDPBB scaffolds co-cultured with VECs and ADSCs. Therefore, the current preliminary study aimed to assess the adhesion and cell viability of the co-culture of VECs and ADSCs *in vitro* on PDPBB scaffolds, and determine the optimal time period for maximum cell viability that could be used as a point of reference for *in vivo* experiments.

Materials and methods

Materials. A total of two female 18-week-old Sprague-Dawley (SD) rats (weight, 200±10 g) at full-term pregnancy were provided by Animal Section of Kunming Medical University (animal production license no. SCXK; approval no. 2005-0008). The experimental protocols of this study were approved by Kunming Medical University and the study was carried out in accordance with the recommendations presented in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (17). The rats were housed at 25°C and 45-60% humidity in standard housing, with a 12-h light/dark cycle and access to food and water *ad libitum*.

Instruments and reagents. Biosafety clean benches and a constant-temperature CO₂ incubator were purchased from Thermo Fisher Scientific, Inc.. The low temperature automatic balance centrifuge was from Beijing Medical Centrifuge Factory. An inverted microscope was purchased from Olympus Corporation. Electro-thermal constant water tanks were supplied by Shanghai Medical Instruments Factory. Ultrasonic tissue pulverizer (Sonics & Materials, Inc.). An automated reader for enzyme plate was purchased from Bio-Rad Laboratories, Inc. A scanning electron microscope (S-3000N; Hitachi, Ltd.), automatic biochemical analysis instrument (Olympus 2700) and a fluorescence microscope (Applied Imaging; Molecular Devices LLC) were used. The following reagents were used: Low sugar DMEM (Gibco; Thermo Fisher Scientific, Inc.); trypsin (Sigma-Aldrich; Merck KGaA); EDTA (Sigma-Aldrich; Merck KGaA); newborn calf serum (NBCS; Gibco; Thermo Fisher Scientific, Inc.); fetal calf serum (Abcam Bioleaf); MTT (Amresco, LLC); SDS (Sigma-Aldrich; Merck KGaA); isobutanol (Tianjin Fucheng Chemical Reagent Factory); VEGF (PeproTech, Inc.); insulin-like growth factor-1 (IGF-1; PeproTech, Inc.); basic fibroblast growth factor (bFGF; PeproTech, Inc.); and epidermal growth factor (EGF; PeproTech, Inc.). The following

antibodies were purchased: Anti-von Willebrand factor (vWF) antibody (cat. no. SC-365712; Santa Cruz Biotechnology, Inc.); mouse anti-CD90 antibody (cat. no. SC-53116; Santa Cruz Biotechnology, Inc.); cy3-labeled goat anti-mouse IgG (cat. no. BA1031; Wuhan Boster Biological Technology, Ltd.).

Preparation and scanning electron microscopy (SEM) of PDPBB. Following the method described previously (14), fresh pork vertebrae from Diannan small-ear pigs, obtained from the Experimental Animal Center Department of Laboratory Animal Science, Kunming Medical University, were used to make bone strips with a cross-sectional area of 0.5 cm². After washing repeatedly with distilled water, PDPB was prepared according to the method described in Table I. PDPB was dipped in distilled water. The pH value of the 30% H₂O₂ was adjusted to 7.0-7.2, and PDPB was air-dried in a drying box; then, the PDPB was cut to obtain bone pieces of 0.5x0.4x0.3 cm and bone fragments of 0.5x0.5x0.1 cm, which were rinsed in saline using an ultrasonic cleaning tank.

Fibronectin was dissolved in PBS to get a final stock solution of 150 µg/ml. PDPBB was prepared by soaking PDPB in the fibronectin solution for 12 h; it was then air-dried in a drying box and radioactively sterilized with cobalt-60. Then, two pieces of PDPBB were fixed with 3.5% glutaraldehyde for 6 h at 25°C and rinsed three times with distilled water (15 min each time), following which conductive staining with 4% tannic acid and 3.5% glutaraldehyde was performed for 48 h at 25°C. After this, they were rinsed three times with distilled water (30 min each time), fixed with 1% citric acid for 4 h at 25°C, rinsed four times with distilled water (20 min each time), rehydrated using 30-100% ethanol and subsequently tert-butanol, and finally pieces were freeze-dried. After platinum-palladium alloy was sprayed onto the samples, the morphology of fibronectin attached to the stent material, as well as the morphology and behaviour of the cells grown in the PDPBB, were observed using a scanning electron microscope (SEM S-3000N; Hitach, Ltd.). All samples were analyzed at 15 kV (18).

Isolation of cord blood mononuclear cells and induced differentiation culture of VECs. The two full-term pregnancy SD rats were administered with anesthesia intraperitoneally using 3% pentobarbital sodium (30 mg/kg) and a 1.5±0.5 cm skin incision was made in the middle of the abdomen to open up the abdominal cavity; the incision was cleaned with 75% alcohol for 10 min to ensure sterile conditions. Umbilical cord blood mononuclear cells were isolated according to the method described previously, under aseptic conditions (19,20). Following skin incision, the peritoneal cavity was opened, and 3 ml of rat umbilical cord blood was collected in heparin-coated tubes, and mixed with PBS at a ratio of 1:1 and with 0.5% methylcellulose at a ratio of 4:1. This was left at room temperature for 30 min for the sedimentation of red blood cells to take place, following which the supernatant was collected, layered on to the rat lymphocyte separation solution (MP Biomedicals, LLC) with a density of 1.803 g/ml and then centrifuged at 241.5 x g for 20 min at 25°C. PBS was added to the interface layer to resuspend cells for washing to remove lymphocyte separation medium and platelets. Then, 5 ml low sugar DMEM (L-DMEM; Gibco; Thermo Fisher Scientific,

Table I. Preparation of partially deproteinized biologic bone.

Reagents	Time	Temperature (°C)
30% H ₂ O ₂	72 h (switch every 24 h)	38
Cleaning with distilled water	30 min	25
Ethyl alcohol	24 h	25
Cleaning with distilled water	30 min	25
Acetone	24 h	25
Cleaning with distilled water	30 min	25
Air-dried in drying box	8 h	25

Inc.) containing VECs inducing solution [10% fetal bovine serum (Abcam Bioleaf), 1% penicillin, 1% streptomycin, 20 µg/l VEGF, 2 µg/l IGF-1, 2 µg/l bFGF and 20 µg/l EGF] was added. Afterwards, cells were seeded into a culture flask with a bottom area of 25 cm² and incubated at 37°C, with 5% CO₂ in saturated humidity. When the primary cells were fused and sufficiently confluent to cover >90% of the culture flask, they were digested with 0.25% trypsin and 0.01% EDTA, passaged and maintained for 6 weeks (20,21). Lastly, the morphology of the cells was observed under an inverted microscope (magnification, x40).

Isolation and culture of ADSCs. The two SD rats were administered anesthesia intraperitoneally with 3% pentobarbital sodium (30 mg/kg) and sacrificed by cervical dislocation. ADSCs were isolated from adipose tissue according to the method described previously (22-24), and were placed in DMEM containing 10% NBCS. The cells were cultured to the third generation at 37°C with 5% CO₂ in saturated humidity to observe adipogenesis.

Identification of umbilical VECs and ADSCs. The umbilical VECs and ADSCs (1:1, 1:3 and 3:1) were seeded into two 6-well cover slips; each population was seeded into 6 wells. Four wells of cord blood-derived VECs were identified using anti-vWF antibody immunofluorescence, 4 wells of ADSCs were identified using anti-CD90 antibody immunofluorescence and 2 wells of each population were used as negative controls (only secondary antibodies were added). Briefly, the adherent cells of each group were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min at 25°C, dried for 10 min and incubated with 3% newborn serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 20 min at 25°C. Cells were then incubated for 60 min at 25°C with anti-CD90 (1:100), anti-vWF (1:500) or without a primary antibody for the negative control. Following the primary antibody incubation, the cells were washed three times with PBS (5 min each time), then incubated with an anti-mouse cy3 fluorescein-labeled secondary antibody (1:500) at 37°C for 30 min, washed three times with PBS (for 5 min each time) and counterstained with DAPI for 1 min at 25°C. Following this, they were mounted by 50% buffered glycerol and observed under a fluorescence microscope (magnification, x630).

Determination of the transplantation time of the co-culture system combined with PDPBB in vitro. A total of 144 PDPBB bone pieces (0.5x0.5x0.1 cm) were randomly divided into the following four groups (with 36 pieces per group): group A, PDPBB and ADSCs; group B, PDPBB and VECs; group C, PDPBB, and the co-culture of VECs and ADSCs in the ratio of 1:1; and group D, PDPBB without cells as a control group. The PDPBB pieces were added to each group with a 20-µl solution at a concentration of 5x10⁶ cells/ml (25).

The cells were incubated for 4 h at 37°C and 5% CO₂ with saturated humidity, and then transferred to 96-well plates, to each of which 24 pieces of PDPBB were added. The cells were cultured at 37°C and 80 µl DMEM, which was changed on alternate days.

On the 2nd, 4, 6, 8, 10 and 12th day, 10 µl MTT (0.5%) was added to one 96-well plate and 100 µl MTT formazan solution (10 g of SDS containing sodium lauryl sulfate, 5 ml of isobutanol, 120 µl of 0.01 µmol/l hydrochloric acid per 100 ml of triple solution) was added after 4 h; after 12 h, the absorbance value of each well was measured by an enzyme labeling detector at a wavelength of 570 nm. The optimal time for maximum cell viability was recorded.

Observation of the co-cultured cells and PDPBB under SEM. When the cell viability on the scaffold was the highest, 2 pieces of PDPBB in each group were selected, fixed with 3.5% glutaraldehyde at 25°C for 6 h and rinsed 3 times with distilled water (15 min each time). Then, conductive staining with 4% tannic acid and 3.5% glutaraldehyde was performed for 48 h at 25°C, PDPBB was rinsed 3 times with distilled water (30 min each time), fixed with 1% citric acid for 4 h at 25°C, rinsed 4 times with distilled water (20 min each time), rehydrated using 30-100% ethanol and subsequently tert-butanol, freeze-dried and sprayed with platinum-palladium alloy. Finally, the adhesion of the cells to the PDPBB was observed by SEM.

Statistical analysis. Data were expressed as the mean ± SD, and the data was analyzed using SPSS 17.0 statistical software package (SPSS, Inc.). Two-way mixed ANOVA was performed to determine statistically significant differences between the cell viability of treatment groups, with post hoc pairwise comparisons performed using Bonferroni's test, with α=0.05. P<0.001 was considered to be statistically significant.

Results

SEM showing the structure of PDPBBs. The fibronectin-modified PDPBB was observed by SEM. The lacunas among the materials were interconnected internally until the blood vessels and cells on normal bone tissues disappeared. A large amount of flaky protein crystals were loosely attached to the surface of the scaffold (Fig. 1). The protein was distributed non-uniformly and was not attached stably to the stent material (Fig. 2).

Structure of umbilical cord blood mononuclear cells. Umbilical cord blood mononuclear cells were induced to grow in a vortex structure for 6 weeks. It was observed that the majority of cells were shortened from long spindles and had

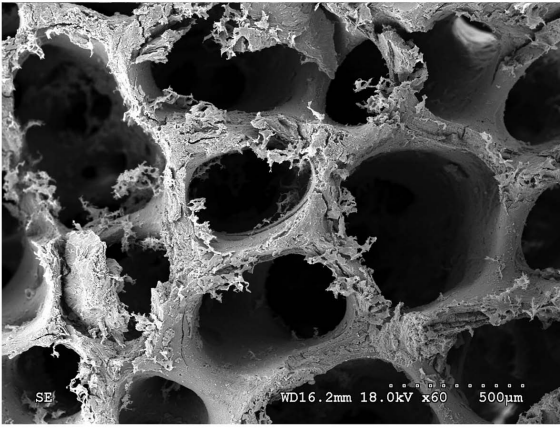


Figure 1. Scanning electron microscopy shows high levels of sheet protein crystallization on the surface of the partially deproteinized biologic bone modified by fibronectin.

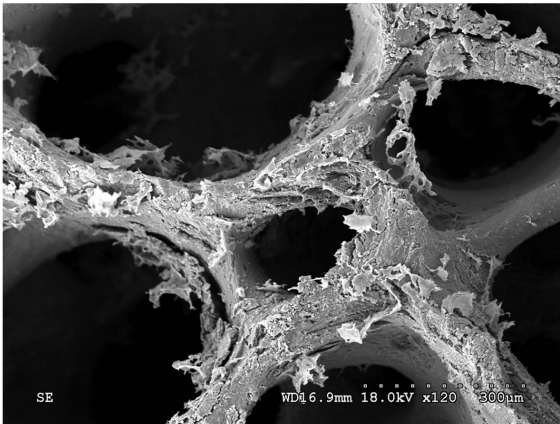


Figure 2. Scanning electron microscopy shows proteins on the surface of the partially deproteinized biologic bone modified by fibronectin. The proteins on the surface were uneven, as it was unstably attached to the scaffold materials.



Figure 3. Appearance of vascular endothelial cells isolated from umbilical cord blood after culturing for 6 weeks. Magnification, x40.

a polygonal-like morphology (Fig. 3). The primary ADSCs showed a slender fusiform shape (Fig. 4). The third-generation ADSCs were observed to be spindle-shaped, with a fusiform

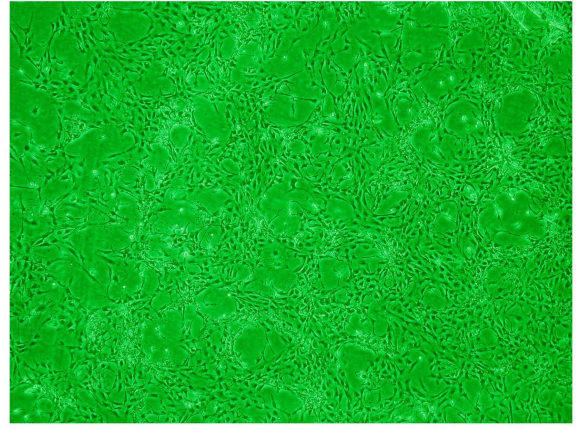


Figure 4. Primary adipose-derived stem cells isolated from adipose tissue. Magnification, x40.

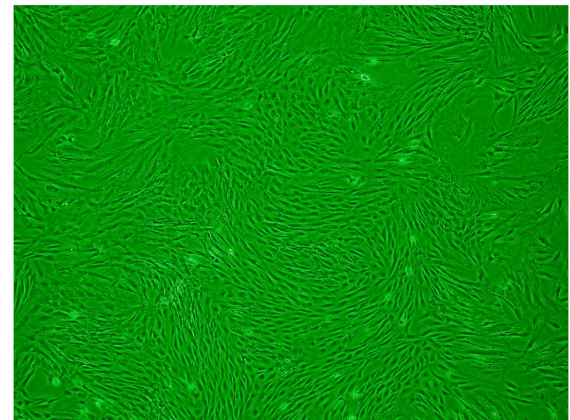


Figure 5. Third generation adipose-derived stem cells. Magnification, x40.

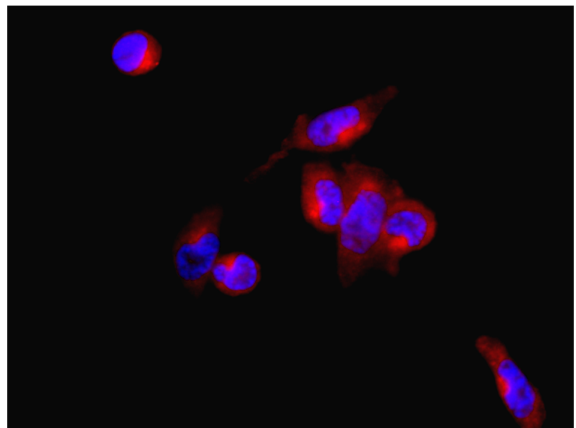


Figure 6. Von Willebrand factor immunofluorescent staining of vascular endothelial cells. Magnification, x630.

morphology, and the cells were arranged in a spiral shape without cell overlap (Fig. 5).

Immunofluorescent staining. Immunofluorescent staining of VECs showed that the cells were polygon-shaped and positive for vWF, which is a glycoprotein and a useful marker

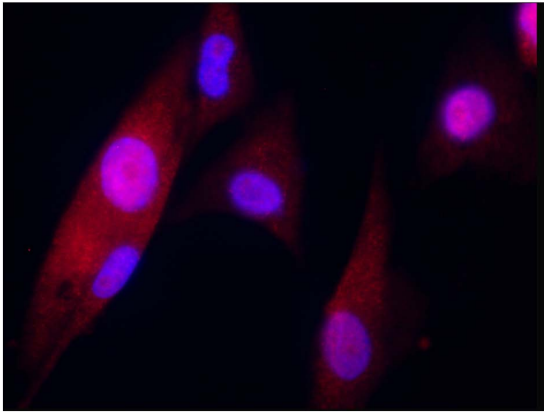


Figure 7. Immunofluorescent staining for CD90 in adipose-derived stem cells. Magnification, x630.

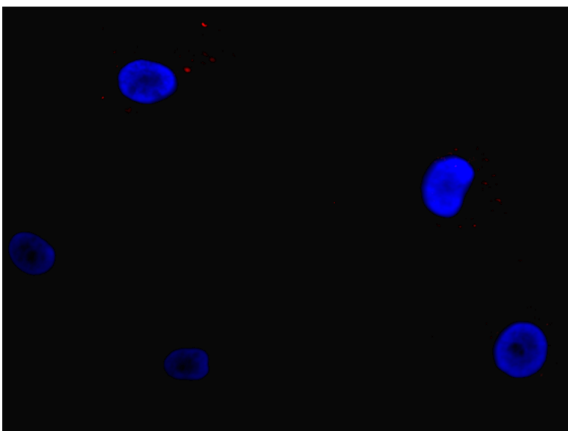


Figure 8. Immunofluorescent staining of the negative control group. Magnification, x630.

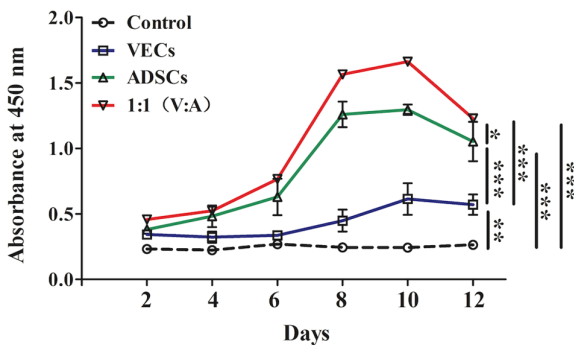


Figure 9. Cell viability in each group. ADSCs, adipose-derived stem cells; VECs, vascular endothelial cells; 1:1 (ADSCs: VECs) and control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

for ECs (26) (Fig. 6). ADSCs showed a long, spindle-shape morphology and were positive for CD90, which is an established stem cell marker in the hematopoietic system (27) (Fig. 7). In the control group, cells were not positive for vWF or CD90 (Fig. 8).

Cell viability. Cell viability was measured in all groups, as shown in Fig. 9. In all groups, except the control group, cell

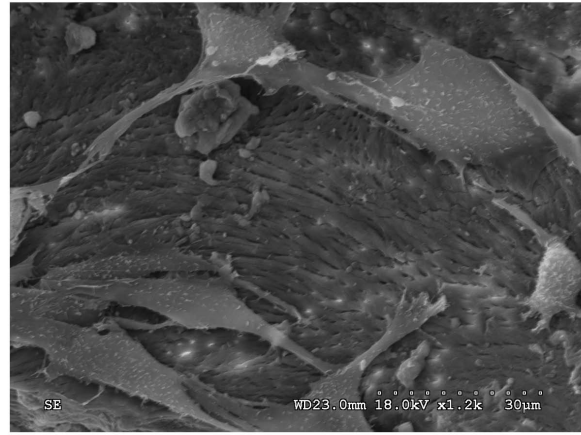


Figure 10. Scanning electron microscopy showing ADSCs on PDPBB. ADSCs, which were fusiform in shape with an extended pseudopod, were adhered to the micropores on the surface of PDPBB. PDPBB, partially deproteinized biologic bone; ADSCs, adipose-derived stem cells.



Figure 11. Scanning electron microscopy showing VECs on PDPBB. Fewer VECs, which showed a polygonal-like shape, adhered on the surface of PDPBB. VECs, vascular endothelial cells; PDPBB, partially deproteinized biologic bone.

viability in each group gradually increased and peaked on the 10th day. The 1:1 (VECs:ADSCs) co-cultured cells exhibited the highest cell viability, followed by the ADSCs group. After the 10th day, the absorbance gradually decreased. The viability of the control group remained unchanged throughout. It was revealed that there was a significant effect of treatment on cell viability ($F_{(3,20)}=1282.6$; $P < 0.001$), as well as a significant interaction between treatment and time. Pairwise comparisons revealed that all groups were significantly different from each other ($P < 0.001$), with the highest cell viability in the 1:1 co-culture group and the lowest in the control group (Table II).

SEM images showing cells adhered to the surface of PDPBBs. On the 10th day, ADSCs that adhered to the surface of the PDPBB were spindle-shaped and protruded into the micropores on the surface of the PDPBB (Fig. 10), whereas only a small number of VECs adhered, which were polygonal in shape (Fig. 11). In the co-culture cell group, a large number of cells were observed to adhere to the PDPBB in a nest-like distribution, and the cells had accumulated to form a cell

Table II. Results of data analysis of each cell culture group on partially deproteinized biologic bone.

Group	Absorbance at 450 nm, mean ± SD					
	2nd day	4th day	6th day	8th day	10th day	12th day
VECs	0.34±0.02	0.32±0.04	0.34±0.02	0.45±0.05	0.61±0.12	0.57±0.07
ADSCs	0.38±0.01	0.48±0.08	0.63±0.14	1.26±0.10	1.36±0.04	1.05±0.05
1:1	0.46±0.01	0.52±0.13	0.77±0.03	1.57±0.02	1.66±0.01	1.23±0.10
Control	0.25±0.01	0.22±0.13	0.27±0.03	0.24±0.02	0.24±0.01	0.26±0.10

N=6/group. ADSCs, adipose-derived stem cells; VECs, vascular endothelial cells.

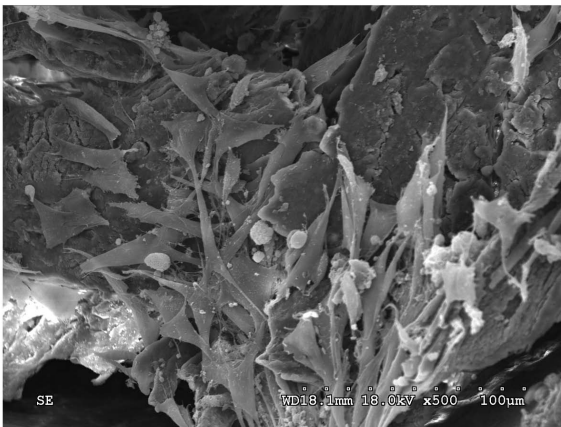


Figure 12. Scanning electron microscopy showing the co-culture of VECs and ADSCs on PDPBB. There were a large number of cells in the co-cultured cells group attached to the PDPBB scaffold, exhibiting a nest-like distribution. The cells accumulated to form clusters and morphologically appeared to be a mixed population of both polygonal (VECs) and spindle-shaped (ADSCs) cells. VECs, vascular endothelial cells; ADSCs, adipose-derived stem cells; PDPBB, partially deproteinized biologic bone.

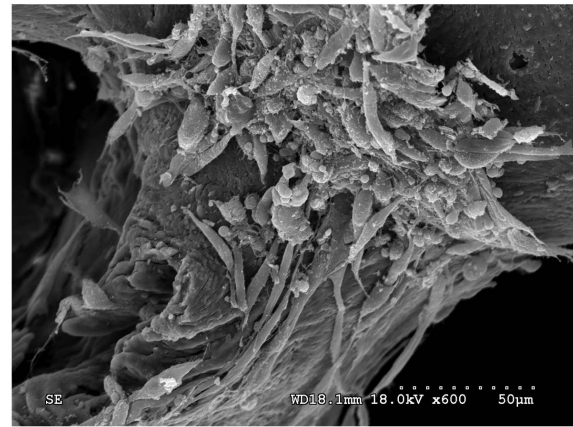


Figure 13. Scanning electron microscopy showing cell boundaries in the co-culture of vascular endothelial and adipose-derived stem cells. Cell boundaries in the co-cultured cells group were unclear as there were a large number of granular materials of different sizes.

cluster. The cell morphology was diverse, with a mixed population of both polygonal and spindle cells (Fig. 12). In Fig. 13, a large number of granular materials of different sizes can be observed; furthermore, it was shown that the cells grew along the trabecular bone to form a cell layer (Fig. 14).

Discussion

The present preliminary study sought to evaluate the adhesion and proliferation of the co-culture system of VECs and ADSCs *in vitro* on PDPBB scaffolds, and to determine the optimal time period for maximum cell proliferation that could be used for transplanting cells during *in vivo* experiments. It was observed that the adhesion and cell viability of the co-cultured cells was higher, in terms of the total number of cells adhered to the PDPBB, as well as the morphologically formed cell layers as compared with those of the single cell types. Furthermore, it was found that the cell viability was the highest on the 10th day.

Previous studies have demonstrated that co-culturing VECs and ADSCs influences the differentiation of osteogenic cells and is a useful source for bone engineering (7,10,28). Therefore, in the present study, the same system was

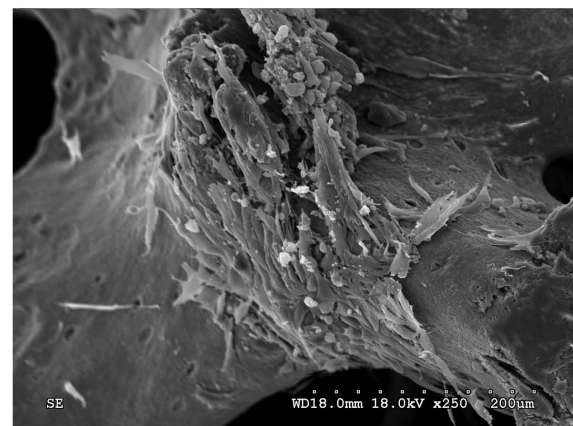


Figure 14. Scanning electron microscopy showing the co-culture system. Vascular endothelial and adipose-derived stem cells of the co-cultured cells group grew along the trabecular bone and formed a cell layer on the partially deproteinized biologic bone.

adopted to verify the cell viability and adhesion efficiency of VECs and ADSCs. VECs secrete factors such as BMP and VEGF (29,30); BMP-7 stimulates angiogenesis by increasing VEGF expression in ECs via direct and indirect mechanisms (31). ADSCs, when used in a co-culture, can increase bone regeneration, and show high levels of CD90 and CD105

expression. Additionally, ADSCs show high expression levels of stemness genes, including SOX2, octamer-binding transcription factor 4, NANOG and Kruppel-like factor 4, and they are able to differentiate into osteogenic, chondrogenic and adipogenic cells (32). The present study is consistent with a previous study that found that PDPBB seeded with a co-cultured system provided an ideal environment for cell growth and osteogenesis, along with cytocompatibility (16). Therefore, it is necessary to use a co-culture system instead of single cells for tissue engineering experiments. Furthermore, it was found that a 1:1 ratio of the cells yielded favorable results. Although the present study did not explore molecular pathways, an MTT assay revealed that the cell viability of the co-culture on the PDBPP scaffold was significantly higher than either of the individual cell types.

Accurate timing of the transplantation steps (implantation of scaffolds and transplantation of cells) is important to reach sufficient vascularization and a proper level of tissue ingrowth prior to transplantation. When cells are transplanted too early, premature cells with poor adherence grow, whereas when cells are transplanted too late, cell apoptosis increases (33). The adhesion and function of ECs on smooth muscle cells in a co-culture, with the addition of fibronectin, could be consistently maintained for up to 10 days, although there was no change in the growth rate of ECs (34). In the present study, it was observed that cell viability gradually increased over time and was the highest on the 10th day, so this is hypothesized to be the optimum length of time for the transplantation of tissue-engineered bone grafts to an *in vivo* system, though further experiments are needed.

Fibronectin mediates several cellular interactions with the extracellular matrix and plays important roles in cell adhesion, migration, growth and differentiation (35). Fibronectin, together with transforming growth factor- β 1, may affect bone formation, in part by regulating the survival of osteoblasts (36). Additionally, fibronectin coating on discs of hydroxyapatite or α -type alumina as scaffolds led to enhanced adhesion and the spreading of MC3T3-E1 osteoblastic cells (37). In the present study, it was shown that the modification of PDPB scaffold material using fibronectin promoted adhesion and viability of cells on the scaffold material, and also induced osteogenic differentiation in the co-culture, as evidenced by the SEM images.

The present study has certain limitations. It is preliminary, and requires further in-depth molecular and cellular experiments to validate the results. Nevertheless, this study provides an initial understanding of the benefit of co-culturing VECs and ADSCs in bone grafting experiments. Additionally, future studies are required to address molecular pathways involved and the behavior, safety and efficacy of the PDBPP scaffold in an *in vivo* system.

In conclusion, this study demonstrated that VECs and ADSCs as a co-culture with a PDBPP scaffold leads to increased adhesion and cell viability, which is the highest on the 10th day, thus indicating that until this time point, the cells can be maintained in a healthy condition.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GY, FW, YL, JH and DL analyzed and interpreted the experimental data, contributed equally in writing the manuscript and revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols of this study were approved by Kunming Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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