Three-dimensional Printed Scaffolds with Gelatin and Platelets Enhance *In vitro* Preosteoblast Growth Behavior and the Sustained-release Effect of Growth Factors

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Abstract

Background: Three-dimensional (3D) printing technology holds great promise for treating diseases or injuries that affect human bones with enhanced performance over traditional techniques. Different patterns of design can lead to various mechanical properties and biocompatibility to various degrees. However, there is still a long way to go before we can fully take advantage of 3D printing technologies.
Methods: This study tailored 3D printed scaffolds with gelatin and platelets to maximize bone regeneration. The scaffolds were designed with special internal porous structures that can allow bone tissue and large molecules to infiltrate better into the scaffolds. They were then treated with gelatin and platelets via thermo-crosslinking and freeze-drying, respectively. Vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β1 were measured at different time points after the scaffolds had been made. Cell proliferation and cytotoxicity were determined via cell counting kit-8 (CCK-8) assay.
Results: There was a massive boost in the level of VEGF and TGF-β1 released by the scaffolds with gelatin and platelets compared to that of scaffolds with only gelatin. After 21 days of culture, the CCK-8 cell counts of the control group and treated group were significantly higher than that of the blank group ($P < 0.05$). The cytotoxicity test also indicated the safety of the scaffolds.
Conclusions: Our experiments confirmed that the 3D printed scaffolds we had designed could provide a sustained-release effect for growth factors and improve the proliferation of preosteoblasts with little cytotoxicity *in vitro*. They may hold promise as bone graft substitute materials in the future.

Key words: Growth Factor; Platelet; Scaffold; Three-dimensional Print; Titanium

Introduction

The methodology of repairing osteochondral defects is a critical issue in orthopedic surgery. Bone scaffolds, which provide the benefit of avoiding unwanted immunological responses and eliminate the risk of acquiring infectious diseases from autografts and allografts, are widely used by orthopedic surgeons when repairing different types of bone defects.[1-4] The traditional methods of manufacturing scaffolds mainly focus on reshaping the structure of specific types of materials and give the scaffold some biomedical properties via processes such as leaching or soaking.[5-7] These methods can make scaffolds with higher porosity, but their size, shape, and interconnectivity are not easy to control, which may limit the prognosis in many aspects;[8] thus, getting satisfactory outcomes when treating bone defects using bone scaffolds is still very challenging.

The development of three-dimensional (3D) printing technology has dramatically changed scaffold designs

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in regenerative medicine.[9] Scaffold microstructures are able to regulate cell behaviors such as proliferation, differentiation, and apoptosis. With the recent rapid development of 3D printing technology, not only can we print 3D scaffolds with controllable inner microstructures but also we can have scaffolds composed with components in the extracellular matrix to deliver biomaterials. Platelets represent a type of specific source of growth factors and cytokines that are involved in wound healing and tissue repair.[10,11] Many platelet-derived factors play important roles in cell proliferation and differentiation including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, and insulin-like growth factors-1.

In this study, we designed a 3D printed scaffold with gelatin and platelets, examined the proliferation of preosteoblasts in the scaffolds using a cell counting kit-8 (CCK-8) assay and the growth factor release at various time points. Some of the PDGFs that were measured include TGF-β1 and vascular endothelial growth factor (VEGF), which play significant roles in wound healing and tissue regeneration. We also measured the cytotoxicity of the scaffold using CCK-8 assay.

METHODS

Preparation of platelets

Blood from healthy donors was collected into 3.8% (mass fraction) sodium citrate-containing tubes (Lankang Company, Jinan, China). Blood samples were centrifuged at 1500 ×g for 6 min, after which the samples were divided into three layers: a bottom layer composed of red blood cells, an intermediate layer composed of white blood cells, and a top player composed of plasma. All of the plasma layer and 3 mm of the intermediate layer were then centrifuged for another 6 min at 1000 ×g to obtain a two-part plasma: the upper three-quarters consisted of platelet-poor plasma and the lower quarter consisted of platelet-rich plasma (PRP).

Preparation of scaffolds

The scaffolds possess an internal porous structure designed in a computer aided design environment using the software Rhino 5.0 (Robert McNeel & Associates, USA). The internal pores were orderly arranged regular dodecahedrons within the scaffolds with φ = 1500 μm. The titanium skeletons that formed the internal porous structure and the external appearance of scaffolds were 100 μm in diameter.[12] The 3D porous titanium was printed based on laser sintering technology (Concept Laser Mlab, Germany) in two sizes: 5 mm diameter and 3 mm thickness for cultivating cells in 96-well plate and 8 mm diameter and 3 mm thickness for implanting *in vivo*, respectively. Three groups of scaffolds were prepared including clear scaffolds (blank group), scaffolds with gelatin (control group), and scaffolds with gelatin and platelets (treated group). To prepare the scaffold with gelatin, 1 g NaCl was added to 5 ml of gelatin solution (5%), and then the solution was perfused into a 3D printed scaffold and lyophilized well. The mixed solid was treated at 180°C for thermo-crosslinking. The NaCl was absterged using distilled water, and then freeze-dried for the second time. The sterilization process was treatment with ethylene oxide, and the scaffolds were prepared with gelatin. The treated groups were prepared by adding 20 μl of PRP to the scaffolds with gelatin then freeze-dried. The 3D printed scaffold with completely interconnected pores was prepared according to our design. The structure of the clear scaffold and scaffolds with gelatin and platelets was imaged as shown in Figure 1 via scanning electron microscopy. All scaffolds were molded at 3 mm height and 5 mm diameter, which fit the wells of the 96-well microplate (3559, 96WL, Corning, USA).

Cell culture and culture medium

Preosteoblasts (MC3T3-E1, Osteoblast cell line) were bought from the Chinese Academy of Medical Sciences. The cells were cultured in an alpha-minimum essential medium (α-MEM) supplemented with 5% fetal bovine serum, 2 mmol/L glutamine, and 100 μg/ml penicillin–streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO_2. First, 5000 cells were seeded on a 75 cm^2 culture flask with 8 ml medium. After the cells adhered to the flasks, the medium was removed and replaced by a new 8 ml medium. The medium was replaced every 3 days until the cells were filled in the flasks. Then, 1 ml 0.25% trypsin was added to the flask, which was lightly joggled. The flask was cultivated in an incubator for 2–3 min and 6 ml of a new medium was added to stop digestion. The whole mixture medium was removed to a 15 ml centrifuge tube so that it could be centrifuged for 5 min at 1000 ×g. The supernatant was dumped, and 5 ml of a new medium was added followed by mechanical isolation.

Growth factors release test

We set four groups including a control group and three types of scaffold groups in a 96-well microplate. Three types of scaffolds with 200 μl α-MEM were placed into the wells of the treated groups. The wells of the control group only contained α-MEM. The α-MEM was exchanged every 4 days. Each well in the 96-well microplate then received 100 μl of cell suspension (cell concentration: 2×10^5 cells/ml). The 96-well microplate was then incubated in a CO_2 incubator until we measured the cell viability and cytotoxicity.
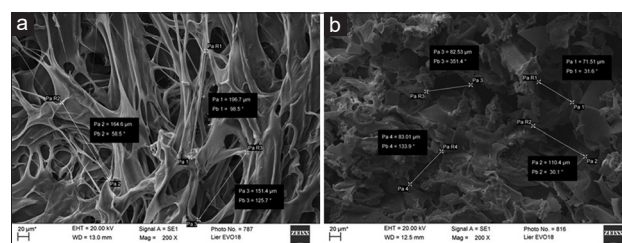
Figure 1: Scanning electron micrographs of the three-dimensional printed scaffolds. (a) The internal pores of the blank group (scaffolds with gelatin), diameters ranging from 150 to 220 μm. Bar = 20 μm. (b) The internal pores of the treated group (scaffolds with gelatin and platelet), diameters ranging from 70 to 200 μm. Bar = 20 μm.

The gelatin scaffolds group and the gelatin and platelet scaffolds group were compared for release levels of VEGF and TGF-β after 3, 6, 9, 12, 15, 18, and 21 days via enzyme-linked immunosorbent assay (ELISA) analysis. The levels of VEGF and TGF-β release were also measured via ELISA after the scaffolds were stored at 25°C for 3 months. Samples were treated at room temperature, and the concentrations of VEGF and TGF-β were measured using company kits (R&D Systems, Minneapolis, USA).

Cell counting kit-8 scaffold proliferation and cytotoxicity test

CCK-8 kit (Boster, Wuhan, China) was used in the experiment. The time point chosen to evaluate cell proliferation was 21 days. Two wells in each group were measured simultaneously and averaged, and then 10 μl of CCK-8 was added to each well of the 96-well microplate. The wells were placed in a CO_2 incubator for 2 h to react. The absorbance was measured at 450 nm with a microplate reader (Thermo Scientific, Multiskan GO, USA). The time points chosen to evaluate the cytotoxicity were 1, 3, 5, and 7 days. Two wells in each group were measured simultaneously and averaged, then 10 μl of CCK-8 was added to each well of the 96-well microplate. The wells were placed in a CO_2 incubator for 2 h to react. The absorbance at 450 nm was measured with a microplate reader (Thermo Scientific).

Statistical analysis

Data are shown as mean ± standard deviation (SD). All data were analyzed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA) by Student's single-sample t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the complex scaffolds

As shown in Figure 1, the gelatin microscaffolds were successfully created in pores of the 3D printed porous titanium. We calculated that 100 gelatin pores existed in microscaffolds and found that their diameter was in the range of 100–300 μm. After freeze-drying the PRP, the microscaffold could also stay in the titanium pores because of the expansibility of gelatin when absorbing water. The scaffold with platelets became smaller than before because the platelets adhered to the gelatin.

Vascular endothelial growth factor and transforming growth factor-β1 sustained-release

ELISA was used to measure the VEGF and TGF-β1 released over 21 days, with results as shown in Figure 2. It could be determined that there was a triggered release after the first 3 days and the growth factors released would be low and plain at later days, which presented a better sustained release function. All the treated groups showed significantly higher release of VEGF and TGF-β1 than that in the control group ($P < 0.05$).

After the scaffolds were stored at 25°C for 3 months, VEGF and TGF-β1 were measured for another 21 days, with results as shown in Figure 3. The releasing curve not only seemed irregular compared to fresh scaffolds but also proved that the release function was sustained after 3 months of storage at room temperature. At 3, 9, 12, 15, 18, and 21 days' time points, treated group exhibited significantly higher than that in the control group ($P < 0.05$).

Cell proliferation

We chose three wells in each group randomly three times and measured the optical density at 450 nm to determine the number of viable cells [Figure 4]. The number of living cells was determined via CCK-8 assay. After 21 days of culture, the CCK-8 cell count of the control and treated groups was significantly higher than that of the blank group ($P = 0.019$ and 0.024, respectively). However, there was no significant difference ($P = 0.364$) between the control group and the treated group after 21 days of culture.

Scaffold cytotoxicity test

The number of the living cells was determined by the CCK-8 assay to detect cytotoxicity [Figure 5]. At the 3 days' time point of culturing, the value of the CCK-8 cell count of the control and treated groups was obviously higher than that of the blank group ($P < 0.05$). At 5 day of culture, the value of the CCK-8 cell count of the control group remained above
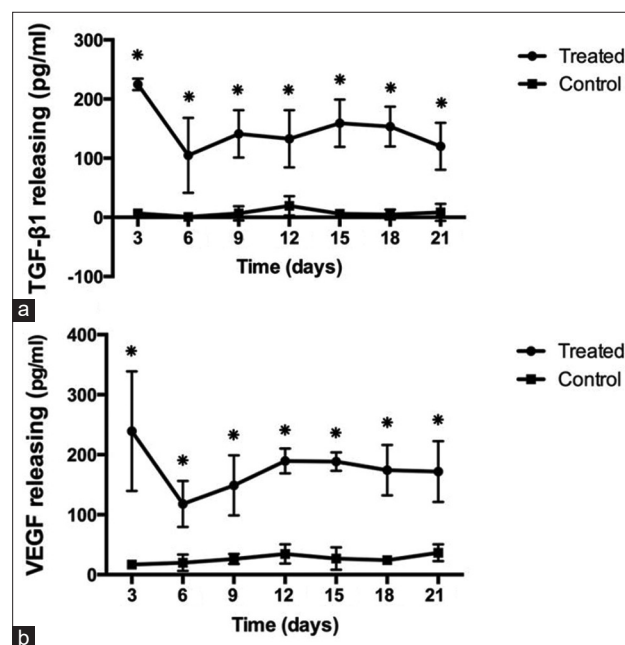
Figure 2: Transforming growth factor-β1 and vascular endothelial growth factor releasing in the supernatant of MC3T3-E1 cultured with scaffolds of treated group was measured by enzyme-linked immunosorbent assay. (a) TGF-β1 releasing in the supernatant of MC3T3-E1 cultured with scaffolds of treated group after the scaffolds were made. (b) VEGF releasing in the supernatant of MC3T3-E1 cultured with scaffolds of treated group after the scaffolds were made. Data are shown as mean ± standard deviation, $n = 3$. *$P < 0.05$ versus control. TGF-β1: Transforming growth factor-β1; VEGF: Vascular endothelial growth factor.
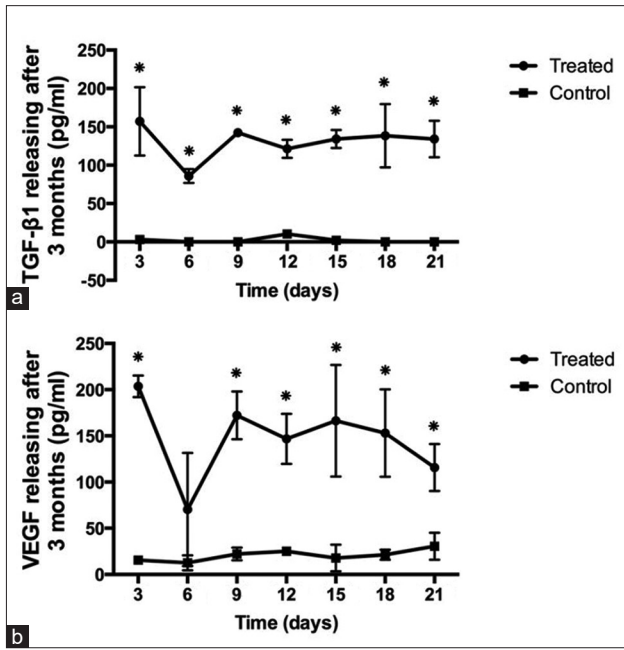
Figure 3: Transforming growth factor-β1 and vascular endothelial growth factor releasing in the supernatant of MC3T3-E1 cultured with scaffolds of treated group was measured by enzyme-linked immunosorbent assay. (a) TGF-β1 releasing in the supernatant of MC3T3-E1 cultured with scaffolds of treated group after the scaffolds were stored at 25°C for 3 months. (b) VEGF releasing in the supernatant of MC3T3-E1 cultured with scaffolds of treated group after the scaffolds were stored at 25°C for 3 months. Data are shown as mean ± standard deviation, $n = 3$. *$P < 0.05$ versus control group. TGF-β1: Transforming growth factor-β1; VEGF: Vascular endothelial growth factor.
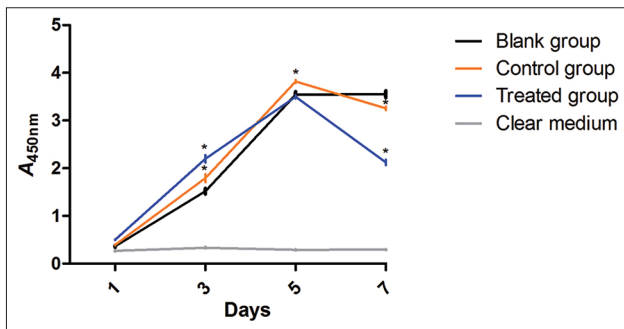

Figure 5: Cytotoxicity assay of the MC3T3-E1 at different time points using cell counting kit-8 assay. Data are shown as mean ± standard deviation, $n = 3$. *$P < 0.05$ versus blank group.

the level of the blank group ($P < 0.05$), with the treated group almost equal to the blank group. However, at 7 d of culture, CCK-8 cell count of the control and treated groups was lower than that of the blank group ($P < 0.05$).

DISCUSSION

As modern medicine advances, many varied methodologies are being explored to treat severe bone diseases. However, curing bone defects it remains very challenging, not only because of the complex stratified architecture of bone itself
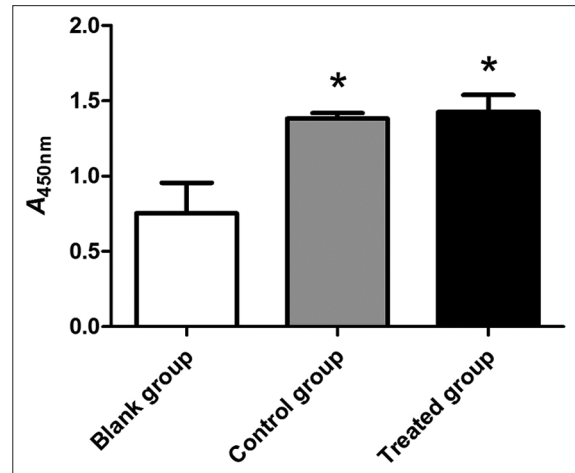
Figure 4: Cell proliferation assay of the MC3T3-E1 using cell counting kit-8 assay after a 21-day culture. *$P < 0.05$ versus the blank group. Data are shown as mean ± standard deviation, $n = 3$.

but also due to changes in the microenvironment including cytokines and blood supplies.[13-15] Our work is a combination of 3D printing technology that gives the scaffold an accurate structure, and biological treatments with platelets to enhance the osteogenesis effect, thus helping patients with bone defects obtain a better prognosis.

The 3D printed scaffold appears gray, bright, and clean. Using a scanning electron microscope, the scaffold exhibits a 3D spatial structure. Under ×200 magnification, the pores are equally distributed and connected, with a diameter of 1500 μm, and are visible within the scaffold. The trabecula between the pores is bumpy. The micropore structure is clearly visible at ×200 magnification, with diameters in the range of 100–300 μm. It has been reported that when the pore diameter of a porous structure is 15–40 μm, fibrous tissue would grow into the interior of the material.[16,17] When the diameter of pores is 40–100 μm, the growth of noncalcified bone tissue improves. When the diameter of the pores exceeds 150 μm, the bone tissue can infiltrate completely into the pore structure of the scaffold, potentially improving the osteogenesis effect. The 3D geometric structure and interconnected pores of our scaffold offer a quite large internal surface area and a 3D structure, in which preosteoblasts are able to absorb more large molecules, promote cell adhesion, proliferation, and osteogenic capabilities.[18,19]

During the design process, we treated the scaffold with platelets for the sake of utilizing its cytokines secretion to enhance the regeneration of the defected area. Platelets attract considerable attention for their bioactivation of scaffolds due to their simplicity, safety, and cost-effectiveness of autologous blood preparation. We tested the VEGF and TGF-β1 release for 21 days for different times at which the platelets were made immediately and stored for 3 months at 25°C [Figures 2 and 3] to address whether the control group and treated group had different effects on cytokines

control-release. From these results, it was indicated that the scaffolds with platelets adhered on the microscaffold could release growth factors effectively and be restored for a long time via this special preparation method.

In the study, preosteoblasts cultured *in vitro* were seeded onto a 96-well microplate containing an α-MEM. The cells proliferated further and numerous cells were measured at day 21 via CCK-8 assay, the cell count of the control group and treated groups was significantly higher than that of the blank group. However, there was no significant difference between the control group and the treated group. This result demonstrated that the gelatin and platelets in the 3D porous structure of the scaffold could also promote cytokine secretion and the infiltration of nutrients and metabolites in the medium, which enhanced the 3D printed scaffolds' biocompatibility. Therefore, this kind of scaffold may be used as a bone graft substitute material.

The cytotoxicity test is one of the most important tests in biological safety evaluation.[20] The method we used to evaluate the viability was CCK-8 assay, which calculated the relative cell proliferation rate based on 450 nm light absorption indirectly, to assess the degree of cytotoxicity. A higher cell proliferation rate was associated with the lower toxicity of different types of scaffolds. In this study, detection based on cytotoxicity test standards *in vitro* showed that preosteoblast proliferation increased rapidly in the early stage (days 1–5) and then the velocity decreased; we can see the results in Figure 5. In three groups with scaffolds and one group without a scaffold, preosteoblasts maintained remarkably good proliferation in the experimental group. However, the preosteoblasts proliferation seems poor in the treated group compared to the other two groups with scaffolds at day 7 because the cells became confluent on the well surface. This finding is in agreement with previous studies that PRP at low concentration in a culture medium has positive effects on cell growth, while high concentrations of PRP suppress viability and proliferation.[21] Therefore, the results indicate that the scaffolds we designed are not toxic to preosteoblast cells and may be safe to implant in animals.

In conclusion, the results of this study confirm that our 3D printed scaffolds provided good tissue compatibility and improved the adhesion and proliferation of preosteoblasts. Furthermore, the platelets we treated also showed a terrific sustained release effect for VEGF and TGF-β1, even after being stored for 3 months. Therefore, a 3D printed scaffold with gelatin and platelets is a suitable implant for cell proliferation and may be a good bone graft substitute material for bone defects. In the present study, 3D scaffolds were only observed *in vitro* for 3 months; therefore, a longer observation period and *in vivo* experiments will be performed in future studies.

Acknowledgments

Conflicts of interest

There are no conflicts of interest.

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