



RESEARCH ARTICLE

THE EFFECTS OF DIFFERENT INFILL DENSITIES OF 3D PRINTED POLYAMIDE 12 COMPOSITE ON MC3T3-E1 ADHESION AND PROLIFERATION

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Abstract. Fused Deposition Modeling (FDM) three-dimensional printing (3D printing) technology has strong advantages in bone defect reconstruction due to its customizability, simple operation and relatively low cost. The hybrid β -tricalcium phosphate/zirconium oxide (β -TCP/ZrO₂) filled polyamide 12 (PA 12) filament feedstock is a new raw material that has been developed in recent years and is used to make FDM 3D-printed bone defect repair implants. Although previous studies have assessed thermal, mechanical and physical properties of 3D printed composites with in-house β -TCP/ZrO₂ filled PA 12 filament feedstock, the research on the biocompatibility of 3D printed PA 12 composite materials is still insufficient. Additionally, it has been proven that the pore size, porosity and crosslinking of 3D printed composites can affect cell growth and differentiation. The purpose of this article was to evaluate the biocompatibility of the 3D printed PA 12 composites through the use of MC3T3-E1 cells and try to explore the effects of the infill density of the 3D printed PA 12 composites on MC3T3-E1 proliferation. MC3T3-E1 cells were indirectly co-cultured with materials at 60%, 80% and 100% infill densities, respectively. Cell viability on day 1 and 3 was detected by Cell Counting Kit-8. The images of materials surface and cell adhesion on the composites were captured by Field Emission Scanning Electron Microscopy. The results showed that 3D printed PA 12 composite materials with 100%, 80% and 60% infill densities had no obvious cytotoxicity to MC3T3-E1. The 3D printed PA 12 composites had rough surfaces with fixed macropores and irregular micropores, making the composites conducive to cell adhesion. 3D printed PA 12 composites, especially the material with infill density of 80%, showed significant effects in promoting cell adhesion and proliferation.

Keywords: Adhesion, MC3T3-E1, PA 12 composite, proliferation, three-dimensional printing

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1. INTRODUCTION

Some patients may have craniofacial bone defects due to certain reasons, such as disease, trauma, surgery and some congenital factors. The defects destroy the integrity of the face and bring great psychological pressure to the patients. More importantly, some bone defects disrupt the continuity of the craniofacial bones, leaving internal tissues unprotected and even affecting the normal physiological functions. Studies [1-3] have shown that cranioplasty can effectively improve the cerebral hemodynamics and neurocognitive function while maintaining the appearance and relieving the anxiety of these patients. The repair materials for the bone defects mainly include autogenous bone, allogeneic bone and artificial bone. However, there are different limitations in the application of these materials, for example, limited resources, immune rejection, high cost, infection and ethical issues. Therefore, the development of implant materials has been a hot research topic. Three-dimensional (3D) printing technology provides innovative solutions for the treatment of complex bone defects through its advantages such as personalized customization, precise matching and promotion of bone regeneration.

3D printing is a high-speed imitation technology based on a computer-designed three-dimensional model. Biomaterials are bonded layer by layer to form simulated tissues or organs. 3D printing can be used to design implants that accurately match the bone defect site, especially the complex structures such as maxillofacial bone defects. In addition, the pore size, porosity and pore crosslinking can affect cell adhesion, viability and angiogenic and osteogenic differentiation [4-6]. The appropriate metrics of pore size, porosity and crosslinking vary by materials and applications [7,8]. 3D printing technology can control these parameters of the pores by adjusting the infill density.

Fused deposition modeling 3D printing (FDM 3DP) technology is the most used 3DP technology due to its simple operation, reliable dimensional accuracy and affordability [9]. Current established feedstock filaments for FDM are made from acrylonitrile butadiene styrene (ABS) and polylactic acid (PLA). Despite the advantages, insufficient mechanical properties and bioincompatible are the prominent disadvantages of current feedstock which hinder the application. The Polyamide 12 (PA 12) has excellent properties, for instance, high crystallinity, low melt viscosity, good fluidity, high surface hardness, easy processing and easy molding. Furthermore, the mechanical properties can be maintained after 3D printing. Additionally, hybrid ceramic fillers have been suggested to potentially improve the thermal stability and mechanical properties of materials. Study [10] has shown that PA 12 composite filament has been successfully compounded and is suitable for use with FDM. The PA 12 composite with fillers of zirconia (purity 99%) and hydroxyapatite (purity $\geq 90\%$) has good biocompatibility and machinability. Compared with hydroxyapatite, tricalcium phosphate (TCP) is easier to dissolve in the body. It seems that the hybrid β -tricalcium phosphate (β -TCP) enhances the thermal stability of the hybrid β -tricalcium phosphate/zirconium oxide (β -TCP/ZrO₂) filled PA 12 composite [11].

A new 3D-printed implant material, β -TCP/ZrO₂ filled polyamide 12 composite, used for craniofacial reconstruction has been explored [10]. The resulting implants are expected to combine the advantages of PA 12 and hybrid ceramic. FDM 3DP can provide personalized customization for each patient or patient specific implant (PSI). Earlier studies [11-13] have evaluated and improved the properties of the PA 12 composite with different weight percentages of filler. When PA 12 composites with 30 wt%, 35 wt% and 40 wt% of filler, the activity of Human Periodontal Ligament Fibroblasts (hPDLF) cells was enhanced at material concentrations of 12.5, 25, 50 and 100 mg·mL⁻¹ [13]. With the increase of fillers, the thermal, mechanical and physical properties of PA 12 composites were enhanced [11].

3D printing technology allows the fabrication of complex solid and porous objects which could be realized via controlling the parameters such as infill density. However, the effects of the different architectures of 3D printed β -TCP/ZrO₂ filled PA 12 on cell attachment are yet to be assessed. The research focused on observing the macroscopic and microscopic characteristics of the surface of 3D printed materials with different infill densities, as well as the spreading and attachment of MC3T3-E1 cells on the materials.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Polyamide 12 (PA2200, EOS, Germany), β -TCP (21218, Sigma-Aldrich, USA) and ZrO_2 (230693, Sigma-Aldrich, USA) were purchased as raw materials for PA 12 composite materials. Synpor (Art. No. 08.510.140S, DePuy Synthes, USA) is used as a control.

Mouse Embryonic Osteoblasts Cells (iCell-m031, iCell Bioscience Inc., Shanghai, China) served as experimental cells. Minimum Essential Medium Alpha (10-022-CV, Corning®, USA), Fetal Bovine Serum (Gibco, USA) and Penicillin-Streptomycin Solution (Gibco, USA) were mixed at the ratio of 89:10:1 to prepare complete culture medium. Enhanced Cell Counting Kit-8, CCK8 (BL1055C, Biosharp®, China) was used to detect cell viability. 10% neutral formalin fixative solution was used as the fixative for Field Emission Scanning Electron Microscopy (FESEM).

2.2 Production of 3D Printed Samples

The fabrication method of 3D printed PA 12 specimens refers to Abdullah et al. [13]. As shown in Figure 1, the β -TCP/ ZrO_2 filled PA 12 filament feedstocks were printed into specimens (Dimensions= 1.0 cm $\times$ 1.0 cm $\times$ 0.3 cm) with infill densities of 60%, 80% and 100% by using a 3D printer (Ender-3, Creality, China). The 3D printed specimens were designed via the software Solidworks 2013 (Solidworks, USA). The 3D printing parameters are detailed in Table 1.

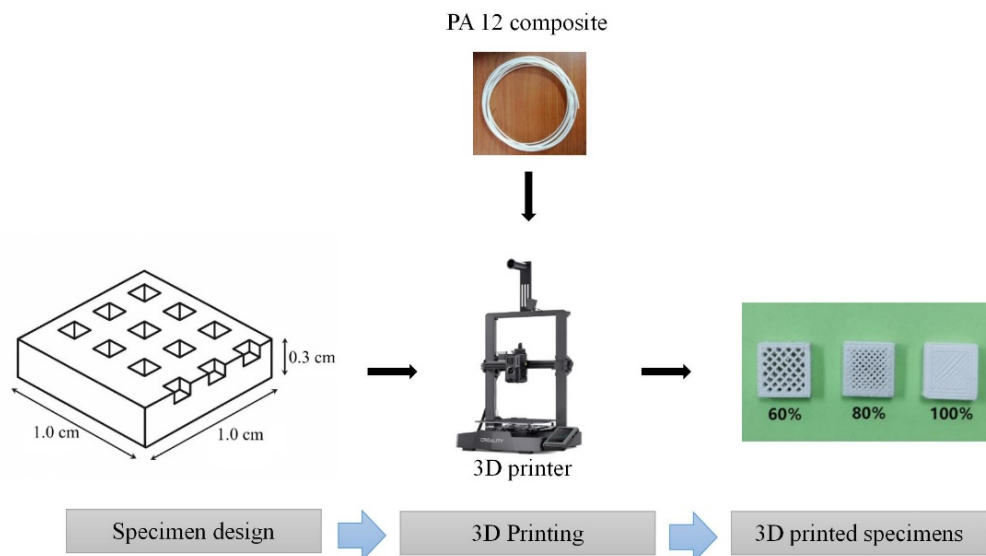


Figure 1: The production process of 3D printed specimens

Table 1: Setting of 3D printer

Item	Setting
Resolution	Standard
Infill density	60%, 80% and 100%
Number of shells	3-5 walls
Layer Height	0.2 mm
Extruding speed	50 mm/s
Travelling speed	25 mm/s
Nozzle temperature	245 °C
Build plate temperature	60 °C

2.3 Preparation of Soaking Extraction Solution of 3D Printed Samples

The method of preparing soaking extract refers to existing research [14]. After washed and autoclaved, 3D printed specimens (Dimensions= 1.0 cm × 1.0 cm × 0.3 cm) with different infill densities (60%, 80% and 100%) were soaked in complete culture medium at a rate of one piece per milliliter, respectively. The containers were sealed and placed in a 37 °C incubator for 72 hours. Then the soaking extract of samples with different infill densities was collected and stored at 4 °C, respectively.

2.4 Cell Culture

MC3T3-E1 cells were cultured mainly according to the experimental manual provided by the supplier. Cells were placed in a 5% CO₂ incubator and cultured at 37 °C. Complete medium was replaced every two days. When the cell confluence reached 80%, subculture or cell cryopreservation was performed. The eighth passage cells were used for this study.

2.5 Enhanced Cell Counting Kit-8

The indirect co-culture method was used in this study rather than the direct co-culture method. The 3D printed PA 12 specimen with pore structure is opaque, therefore, the cell growth status on the surface of the material cannot be directly monitored in real time under an inverted microscope in the direct co-culture method. Moreover, the static cell-seeding method in the study cannot guarantee the uniform distribution of cells on materials with pore structure and enough contact between cells and material surface, which may affect the results of CCK8. It should be emphasized that the use of soaking extract in the indirect co-culture method can further enhance the effects of material dissociates on cell proliferation.

Adding MC3T3-E1 cell suspension (Concentration=5×10⁴ mL⁻¹) into 96-well culture plates, 100 μL per well. After incubation for 24 hours under original conditions, the culture medium was replaced with different soaking extracts or new complete medium. Three parallel wells were set for each extraction solution. Then, the culture plates were placed in the incubator for further culture. After 1 day, a 96-well culture plate was taken out. The media in the plate were replaced by a mixture of CCK-8 buffer and complete culture medium, 100 μL per well. Then the plate was placed back into the incubator and incubated in the dark. Three and a half hours later, OD value was measured with a microplate reader at 450 nm. After culturing the cells for another 2 days, another 96-well culture plate was taken out and the OD value was measured according to the previous method. Cell viability was calculated by the following formula,

$$\text{Cell viability (\%)} = \frac{\text{OD}_{\text{extract}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{medium}} - \text{OD}_{\text{blank}}} \times 100\% \quad (1)$$

2.6 Field Emission Scanning Electron Microscopy

3D printed PA 12 specimens with infill densities of 60% and 80% were scanned under a scanning electron microscope (Quanta 450 FEG, Fei, USA). The accelerating voltage (kV) used in FESEM was 5 kV. Three samples (Dimensions= 0.5 cm × 0.5 cm × 0.3 cm) were randomly selected from each infill density. After being autoclaved, the materials were placed one by one into a 96-well culture plate. Then complete culture medium was added into the well, 100 μL per well. 24 hours after the culture plate was placed in the incubator, the original culture medium was discarded. Cells were seeded onto each sample as a cell suspension (Concentration= 5×10⁴ mL⁻¹), 100 μL per well. The plate was then placed into the incubator. After 24 hours, culture medium was discarded. Then samples were fixed, dehydrated and dried. After gold spraying, the sample surface and cell adhesion were observed by FESEM at magnifications of 500× to 10,000×.

2.7 Statistical Analysis

Each experiment was repeated three times independently. Data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Statistical analysis was performed using the software GraphPad Prism 10. Analysis of variance was used for comparison between samples, and $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Surface Structure of 3D Printed Specimens

As shown in Figure 1, the pore size of 3D printed specimen decreases with the increase of infill density. On the contrary, the number of pores increases. Observed by FESEM, the surface of 3D printed specimens with infill densities of 60% and 80% was rough and had macropores and irregular micropores (Figure 2). It means that the 3D printed material was conducive to cell adhesion. Meanwhile, pore structure facilitates the transfer of oxygen and nutrients to cells and avoids the accumulation of metabolites.

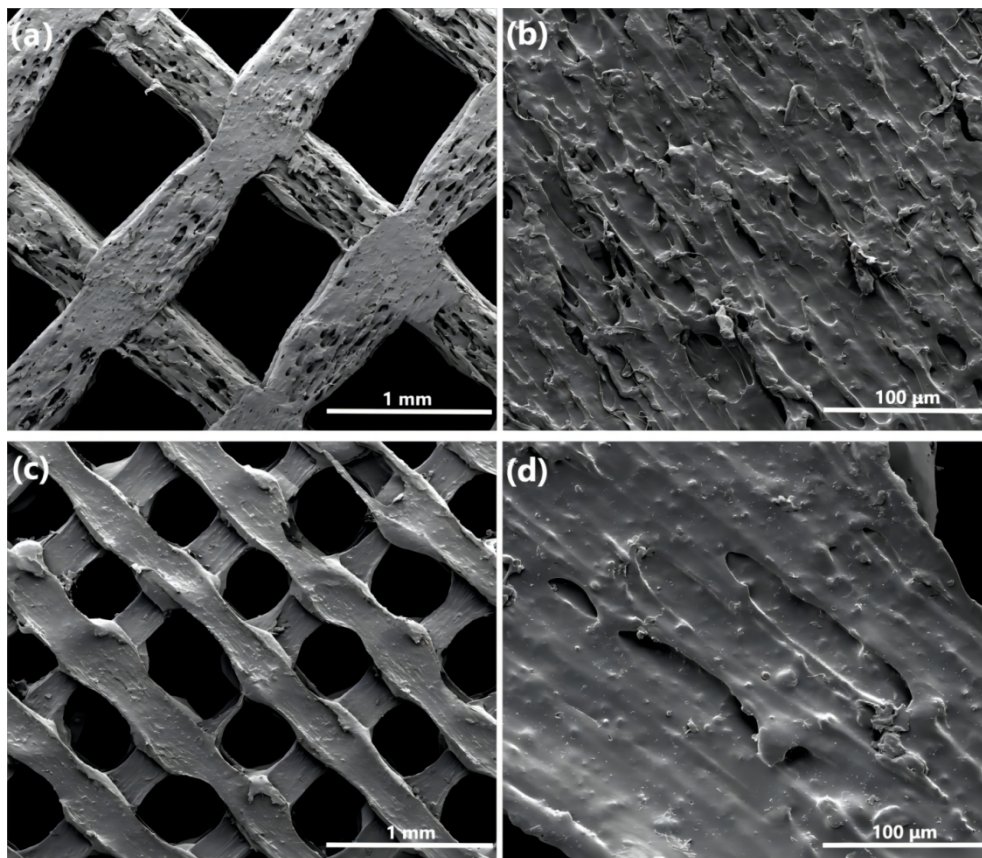


Figure 2: Surface characterization of 3D printed PA 12 composites using FESEM analysis: (a) PA 12 composite with 60% infill density (b) PA 12 composite with 60% infill density (c) PA 12 composite with 80% infill density and (b) PA 12 composite with 80% infill density

It should be noted that the roughness of 3D printed PA 12 specimens was not completely consistent, even if the specimens were printed in the same way. The roughness of the samples may be different due to the principle of FDM 3DP technology. For instance, the roughness of the specimen may differ significantly between the X-axis and the Y-axis. In addition, the aggregation of fillers may cause nozzle clogging during the print process, requiring occasional stops and adjustments. The process could

be improved. Temperature can be set above the melting temperature of PA 12, up to 245 °C, to ensure that the PA composite completely melts and flows without clogging at the nozzle.

3.2 Cell Cytotoxicity and Proliferation Assay

Mouse embryo osteoblast precursor (MC3T3-E1) cells are fibroblast-like cells that grow adherently. It has been demonstrated that MC3T3-E1 can differentiate into osteoblasts and can be used to study osteoblast differentiation in vitro [15]. The porous material, SynPor, was used as a control. SynPor has been used in craniofacial reconstruction due to its biocompatibility, however, it needs to be prepared by trimmed and shaped beforehand. The procedure will prolong the operation time. Furthermore, its mechanical strength may not be sufficient for high-load areas such as the mandibular weight-bearing area.

CCK8 kit was used to detect the cytotoxicity and proliferation effects of 3D printed PA 12 composite. As shown in Figure 3, after 1 day of indirect co-culture, the extract solution of Synpor or 3D printed PA 12 specimens with different infill densities had no significant effects on the activity of MC3T3-E1 cells compared with complete culture medium. However, after 3 days of indirect co-culture, the proliferation of MC3T3-E1 cultured in the extract solution of Synpor and 3D printed PA 12 composite with 60% infill density was significantly lower than that of cells in complete culture medium. On the contrary, 3D printed PA 12 composite with 80% infill density and 100% infill density showed obvious promoting effects on cell growth. There was no significant difference between the 3D printed PA 12 specimens with 100% infill density and 80% infill density. Regardless of which extract was used, the cell viability was not less than 80% after 1 and 3 days (Figure 3). This indicates that 3D printed PA 12 composite did not have significant cytotoxicity to MC3T3-E1 cells.

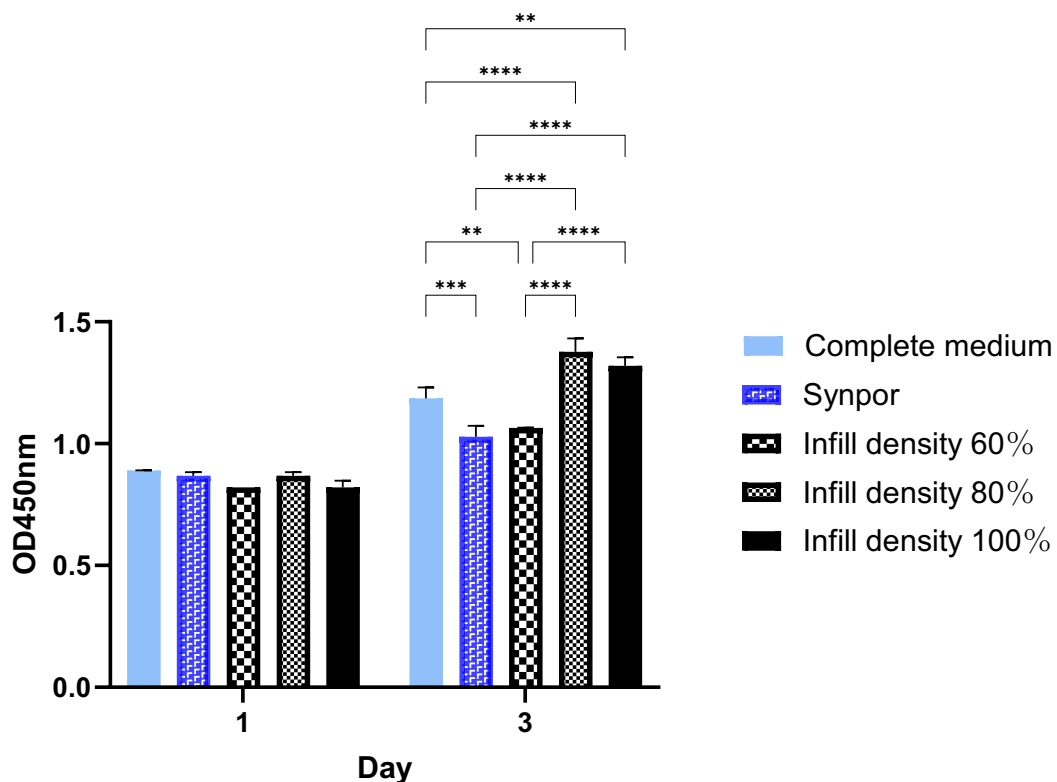


Figure 3: OD450 of ME3T3-E1 cells cultured in complete medium and extracts of Synpor and 3D printed specimens with infill densities of 60%, 80% and 100%. $p < 0.05$, the difference is statistically significant. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

The indirect co-culture method was used in the research. The effects of PA 12 composite materials on cells were mainly achieved through the dissolution of the materials in the soaking extraction solution. Polar molecular (-CO-NH-) structure in polyamide chains that mimics the structure of collagen [16], is an essential factor that promotes the osteoblast. Besides, it has been speculated that hybrid ceramics dissolve into the culture medium and promote cell growth [14]. This research enhances findings from the previous study [14] that the 3D printed samples affect cell proliferation.

It should be emphasized that the effects of the porous structure of biomaterials on cell differentiation, 3D growth structure and the formation of extracellular micro-environment need to be valued in practical applications [4,5,17]. Therefore, the 3D printed PA 12 specimen with 80% infill density is preferred, although the specimens with 80% infill density and 100% infill density have stronger effects on promoting MC3T3-E1 cell growth than that with 60% infill density.

3.3 Cell Adhesion

The effect of 3D printed PA 12 specimen on cell proliferation varied with infill density (Figure 3). The 3D printed specimen with 100% infill density was a solid piece, while the 3D printed specimen with 60% infill density or 80% infill density had a simulated porous structure (Figure 1). The pore structure can increase the surface area, and promote nutrient and oxygen diffusion. Also, the pore structure is conducive to osteoblast growth and blood vessel growth. Therefore, the materials with 60% and 80% infill densities were selected in this study for further evaluation of cell adhesion by FESEM.

The adhesion of MC3T3-E1 cells on 3D printed materials with 60% and 80% infill densities was observed by FESEM. One day after cell inoculation, cell attachment was visible on both materials. The pseudopodia of the cells were extended and interconnected (Figure 4).

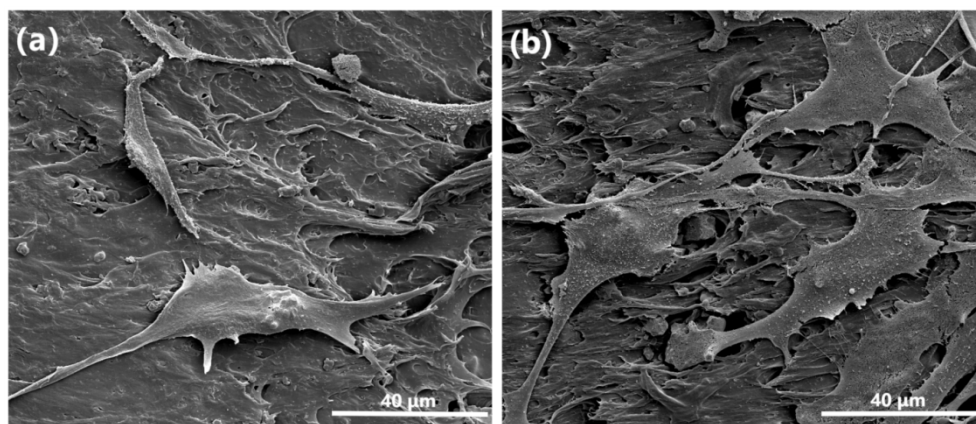


Figure 4: FESEM micrographs of cell attachment after cell seeding (24 hours): (a) PA 12 composite with 60% infill density and (b) PA 12 composite with 80% infill density

The pore structure of 3D printed PA 12 specimen is conducive to cell attachment and growth, but excessive pore size showed negative effects (Figure 3). The size and number of material pores can be controlled by adjusting the 3DP infill density, thereby optimizing cell adhesion and proliferation.

4. CONCLUSIONS

As the infill density increased (60% and 80%), the number of pores in the 3D printed β -TCP/ZrO₂ filled PA 12 composite gradually increased, and conversely, the size of the pores gradually became smaller. Cell viability analysis showed that the 3D printed PA 12 composite materials with 60%, 80% and 100% infill densities had no obvious cytotoxicity to MC3T3-E1, and the materials with 80%

and 100% infill densities significantly promoted cell proliferation. The images obtained by FESEM directly proved that the cells attached and spread on the composite materials with 60% and 80% infill densities, and the pseudopods stretched.

Although it has shown that the 3D printed PA 12 composites have good biocompatibility and growth promoting effects on MC3T3-E1 cells, the positive effects of the PA 12 composites on MC3T3-E1 osteogenic differentiation need to be further confirmed. These data can provide support for continued research on the osteoinductivity of 3D printed PA 12 composites.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no conflicts of interest to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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