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Original Article

Mechanical behavior and cytocompatibility of calcium alginateor PVA/DCPP scaffolds

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Abstract: Objective: The present study aimed to evaluate the mechanical performance of calcium alginate or PVA/DCPP scaffolds and their compatibility with the tooth germ cells in jaw bone defect repair. Methods: We prepared a Calcium alginate or PVA (polyvinyl alcohol)/DCPP scaffolds and observed, the ability of resistance to dissolution, setting time and mechanical property. Tooth germ cells from Sprague Dawley (SD) rats were cultured and then co-cultured with two types of scaffolds, respectively. Result: In the test for resistance to dissolution, 3%, 4%, 5% and 7% calcium alginate/DCPP scaffolds and 15%, 20% and 25% PVA/DCCP scaffolds did not show obvious deformation. The calcium alginate/DCPP scaffolds had shorter setting time. PVA/DCCP scaffolds had higher shear strength which was proportional to the concentration of PVA. By scanning of electron microscopy, two scaffolds presented with inner and surface morphology similar to porous volcanic rock. The calcium alginate/DCCP scaffolds exhibited better compatibility with tooth germ cells from SD rats. Conclusion: Calcium alginate/DCCP scaffolds can be used for tissue-engineered bone repair or tissue-engineered denture repair after improvement of mechanical performance.

Keywords: Calcium alginate, polyvinyl alcohol, DCCP, tooth germ cells

Introduction

The incidence of jaw bone defect is rising in recent years due to trauma, tumors and periodontal diseases. Since jaw bone defect may be combined with dentition defect, conventional repair techniques such as oral implants and fixed or removable denture can hardly achieve the desired effect. Jaw bone defect not only affects the patients' life quality, but also brings challenges to practitioners in oral implant, maxillofacial surgery and periodontics [1, 2]. At present, the common materials used for jaw bone defect repair are autogenous bones [3], allogenic bones, heterogenous bones or artificial materials. However, these materials have shared problems including poor mechanical performance, low hardness and toughness, slow degradation, potential risk of rejection or mismatch with the growth rate of bone tissues [4]. It is necessary to develop new bone graft materials that are fit for jaw bone defect repair.

Complex morphology of jaw bone defect and high mechanical load borne by the implants are the main reasons for difficulties in jaw bone defect repair. Sodium alginate (SA) possesses the advantages of good biocompatibility, gelling property, degradability and film forming property [5, 6]. SA compounded with hydroxyapatite usually has enhanced mechanical performance. Polyvinyl alcohol (PVA) is a water-soluble polymer formed by the hydrolysis of polyvinyl acetate and alcohol. It has high water solubility, excellent film forming property, adhesion, strength and toughness as well as good biocompatibility and plasticity [7, 8]. Double crystal ceramic powder (DCCP) is composed of hydroxyapatite and a small amount of β-tricalcium phosphate and presents with the reticulated porous structure observed in bone tissues. DCCP can be used as a scaffold for guiding bone growth with high biocompatibility and excellent performance [9-11]. Thus DCCP scaffolds reinforced by SA or PVA may serve as the candidate materials for jaw bone defect

repair due to their high mechanical performance and biocompatibility.

Materials and methods

Materials and equipments

The experiment was carried out from June 2013 to October 2014 in Infection and Immunity Laboratory at Scientific Research Center of the First Affiliated Hospital of Xinjiang Medical University. The experimental animals were 6 juvenile SD rats (clean grade, 8-10 g). Thereagents used during this experiment includes SA (Sigma, USA), PVA (Guangdong Guanghua Chemical Factory Co., Ltd., China), calcium chloride (Sigma, USA), and CCK-8 kit (BestBio, China). The equipments utilized in the experiment include Electronic Universal Testing Machine WDW-20E (Jinan Fangchen Instrument & Equipment Co., Ltd.), bone crusher (multi-functional rocking crusher, XY-400A, Xiaobao), standard sample screener (200 mesh, Guangliang Sifter Plant, China), muffle furnace (AX-4-10, Tianjin Aixin Medical Equipment Co., Ltd.), SEM (Joel jsm63901v, Japan), and ZEIZZ microscope (Carl zeiss, Germany).

Methods

Preparation of DCPP: The vertebrae were harvested from adult goat and the periosteum and cortical bone were removed, leaving only the cancellous bone, which was cut into blocks of about 0.5 × 0.5 × 0.5 cm. The bone blocks were repeatedly washed with running water until there was no more blood leached and then washed ultrasonically 3-5 times, 30 min for each time. Then the bones were treated with 5% sodium hydroxide solution and 15% hydrogen peroxide, dried at 70°C and calcinated at 800°C for 2 h to obtain the ceramic bovine bone. After the addition of proper amount of sodium pyrophosphate, the ceramic bovine bone was further calcinated at 800°C for 1 h and then left to cool to room temperature. The ceramic bovine bone was washed with distilled water ultrasonically for 15 min and dried using vacuum pump. After encapsulation, the ceramic bovine bone was disinfected by 60 Co (20 kGy) irradiation for 36 h. Finally, DCPP was obtained using bone crusher and passed through the 200-mesh sieve.

Preparation of calcium alginate/DCCP scaffold: SA powder of 0.3 g, 0.4 g, 0.5 g and 0.7 g was weighed respectively and mixed with 10 ml of ultra-pure water in a conical flask. The powder was heated in the 70°C water bath, mixed for 30 min and left to stand for 20 min. This process was repeated twice until the powder was completely dissolved, with the air bubbles removed by oscillation. Thus the 3%, 4%, 5% and 7% SA gel was obtained and mixed respectively with DCPP at the proportion of 1 ml for 0.1 g DCPP. Then the mixture was poured into the metal mold (cylindrical, diameter 9 mm, height 13 mm) and dried to obtain 3%, 4%, 5% and 7% SD/DCCP scaffolds. These scaffolds were immersed into 0.4 mol/L, 0.8 mol/L, 1.0 mol/L, 1.5 mol/L, 2.0 mol/L and 3.0 mol/L $Cacl_2$ solution for 48 h, respectively. After that, the scaffolds were washed with ultra-pure water for 3 times and preserved at -80°C for 24 h. Finally, the scaffolds were calcinated in the muffle furnace at 120°C for 20 min and left to cool to room temperature.

Preparation of PVA/DCCP scaffold: PVA powder of 1 g, 1.5 g, 2 g and 2.5 g was weighed respectively and mixed with 10 ml of ultra-pure water in the conical flask. The powder was heated in the 90°C water bath, mixed for 30 min and left to stand for 20 min. This process was repeated twice until the powder was completely dissolved, with the air bubbles removed by oscillation. Thus the 10%, 15%, 20% and 25% PVA gel was obtained and mixed respectively with DCPP at the proportion of 1 ml for 0.1 g DCPP. Then the PVA/DCCP scaffolds were prepared using the same method as with calcium alginate/DCCP scaffolds. Finally, the scaffolds were calcinated in the muffle furnace at 120°C for 20 min and left to cool to room temperature.

Detection of resistance to dissolution: The scaffold samples were immersed in 3 mL of ultra-pure water in the culture flask for 30 min, 1 h, 3 h, 24 h and 48 h, respectively. Dental explorer was used to test the softness of the surface of the scaffolds.

Dissolution and deformation of the scaffolds were classified into three grades: grade 1, no obvious dissolution or deformation of the surface of the scaffolds; grade 2, dissolution of the surface but not the inside of the scaffolds with no significant deformation; grade 3, dissolution of the surface and the inside of the scaffolds with severe deformation.

Testing for setting time: Six calcium alginate/DCCP scaffolds with calcium alginate concentration being 3%, 4%, 5% and 7% respectively were used for the testing, 24 scaffolds in total. Gillmore needles were used, including light needle (113.4 g, diameter 2.13 mm) and heavier needle (454.6 g, diameter 1.2 mm). The initial setting time (IT) and the final setting time (FT) were tested and recorded. The initial setting time was defined as the time of light needle leaving a mark on the surface of scaffolds, and the final setting time was the time of heavier needle leaving a mark.

Testing for shear strength: Four calcium alginate or PVA/DCCP scaffolds were prepared using the cylindrical mold described above and tested for shear strength using the electronic universal testing machine at the rate of 0.5 mm/min.

SEM: The scaffolds were made into round thin slices with the diameter of 9 mm and height of 1 cm. The scaffolds were dried with vacuum pump, and metal spraying was performed using an ion sputter. Then surface and inner morphology of the scaffolds as well as the average pore diameter were observed.

Proliferation and cytotoxicity assay of rat tooth germ cells: Two juvenile SD rats aged four days were sacrificed by cervical dislocation. The jaw bones were dissected using the ophthalmic scissors under the upright microscope. The tooth germ was harvested, placed in the 25 mm culture dish and cut into pieces using the ophthalmic scissors. The cells were digested with 0.25% trypsin and then centrifuged to remove the trypsin. The cells were resuspended in complete culture medium (DMEM containing low glucose, 10% FBS, 1% penicillin-streptomycin solution) and inoculated to a 25 cm^2 culture flask. The culture medium was replaced every 3 days, and cell passage was performed every 7 days. The tooth germ cells of the third generation were used for subsequent experiment.

Preparation of leaching liquor of scaffold: According to the standard S010993-1 (surface area of the sample/volume of the leaching agent =3 cm^2/ml), 8 scaffolds were placed into the complete culture medium described above to prepare the leaching liquor, namely, 3%, 4%, 5% and 7% calcium alginate/DCCP scaffolds, and 10%, 15%, 20% and 25% PVA/DCCP scaffolds. The scaffolds were treated in the 37°C water bath for 72 h, then filtered and disinfected and preserved at 4°C.

Proliferation and cytotoxicity assay of rat tooth germ cells: Tooth germ cells of the third generation were made into 5×10^5/ml cell suspension and inoculated to a 96-well pate at 100 μl per well. The cells were then cultured at 37°C in a 5% CO_2 incubator so that the cells grew to form a single layer covering the base of the plate. The culture medium was discarded at 24 h after initial culture. For the blank group and the control group, 100 ul of complete culture medium was added, and for the experimental groups 100 ul of the leaching liquor of 8 different scaffolds was added, with 5 replicate wells for each group. At 1 d, 3 d and 5 d of culture, the cell growth was observed under the inverted microscope. Into each well 10 ul of CCK8 reagent was added to culture the cells at 37°C for 3 h. Absorbance (OD) was detected using the microplate reader at 450 nm, and relative growth rate (RGR) was calculated as by the following formula: RGR=(OD value of experimental group-OD value of blank group)/(OD value of negative control group-OD value of blank group) × 100%. Cytotoxicity was evaluated based on the value of RGR: grade 0, RGR≥100%; grade 1, RGR=75%-99%; grade 2, RGR=50%-74%; grade 3, RGR=25%-49%; grade 4, RGR=1%-24%; grade 5, RGR=0.

Statistical analysis

SPSS 14.0 was used for statistical analysis. ANOVA was carried out for the measurements in each group and at each time point. $P<0.05$ indicated significant difference.

Results

Resistance to dissolution

The 10% PVA/DCCP composite scaffold showed severe dissolution and deformation from 0.5 h to 48 h of treatment, and the dissolution and deformation belonged to grade 3. The scaffold was no longer suitable for the deposition of tooth germ cells at later stage. In comparison, the 3%, 4%, 5% and 7% calcium alginate/DCCP scaffolds and 15%, 20% and 25% PVA/DCCP scaffolds showed no significant dissolution and deformation. They were classified as grade 1 in

Table 1. Setting time of calcium alginate or PVA/DCCP scaffolds (min, Mean ± SD)

Group		Initial setting time	Final setting time	*F*	*P*
SA	3%	67.00±12.13	75.00±13.97	5.7	0.005
	4%	70.00±19.80	76.67±20.84		
	5%	94.67±23.75*	103.33±24.08*		
	7%	102.67±15.00$^{*,\#}$	110.00±15.07$^{*,\#}$		
PVA	10%	57.67±11.13	66.67±10.93	5.426	0.007
	15%	88.17±35.54^{a,***}	99.33±35.39^{a,***}		
	20%	93.83±33.19$^{a,b,\#\#}$	107.00±33.81$^{a,b,\#\#}$		
	25%	112.67±24.67^{a,b,c,**}	125.67±25.78^{a,b,c,**}		

Note: ANOVA was used to compare the difference among each concentration groups. LSD test was used to compare the difference between each two groups. *compared to 3% group, P<0.01; $^{\#}$compared to 5% group, P<0.01; ^{a}compared to 10% group, P<0.01; ^{b}compared to 15% group, P<0.01; ^{c}compared to 20% group, P<0.01; **compared to 7% group, P<0.01; $^{\#\#}$compared to 5% group, P<0.01; ***compared to 4% group, P<0.01.

Table 2. Shear strength of calcium alginate or PVA/DCCP scaffolds.

		N	Mean ± SD	*F*	*P*
Shear force	3%	6	83.95±27.95	30.3	0.000
	4%	6	208.85±223.78		
	5%	6	215±246.32		
	7%	6	134.55±92.92		
Compressive force	3%	6	227.43±67.43	30.8	0.000
	4%	6	348.78±49.49		
	5%	6	690.89±289.84		
	7%	6	427.98±231.43		

terms of dissolution and deformation, so they were qualified as scaffolds.

Setting time

As the concentration of SA and PVA increased, both initial setting time and final setting time were prolonged. Compared with 15%, 20% and 25% PVA/DCCP scaffolds, the 4%, 5% and 7% calcium alginate/DCCP scaffolds took less time to achieve final setting (**Table 1**).

Shear strength

The diameter of the electron probe was 1 mm for 4 samples. As the concentration of SA increased, the shear strength of 3%, 4% and 5% calcium alginate/DCCP scaffolds also increased. But when SA concentration was increased to 7%, the shear strength of 7% calcium alginate/DCCP scaffold decreased, probably due to the addition of excess SA. Thus, the shear strength of the calcium alginate/DCCP scaffold cannot increase all the way with the increasing of SA concentration, but only within a certain range of SA concentration.

As the concentration of PVA increased, the shear strength of 10%, 15%, 20% and 25% PVA/DCCP scaffolds also increased. According to the experimental results, the concentration of PVA was proportional to the shear strength of PVA/DCCP scaffold within the concentration range tested (**Table 2**).

SEM

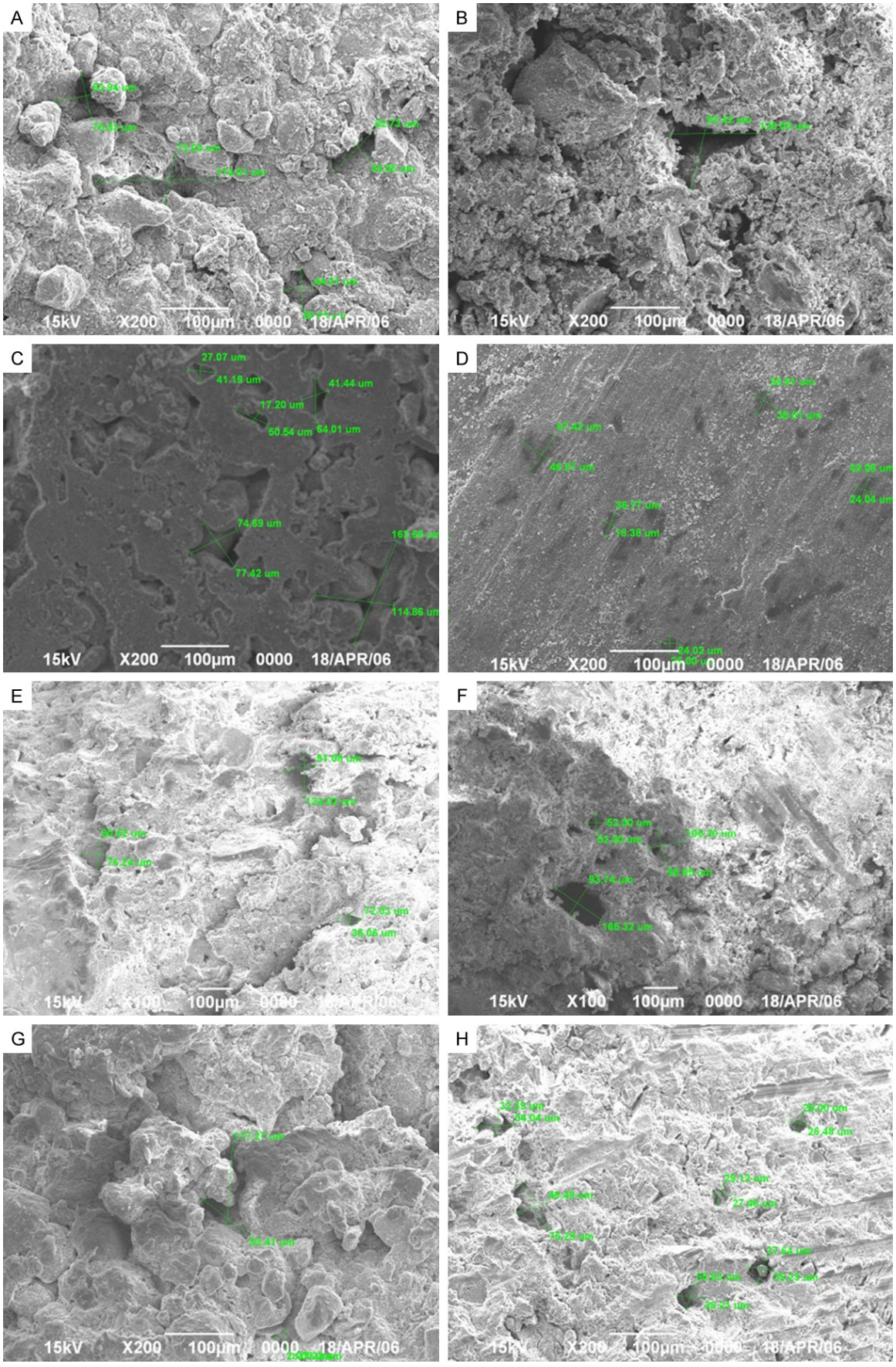
It was observed under the SEM that the surface and inner morphology of calcium alginate/DCCP scaffolds was similar to porous volcanic rock. The pores were of irregular shapes and varying sizes, about 18.38-174.01 μm. The surface and inner morphology of the PVA/DCCP scaffolds was similar to that of the calcium alginate/DCCP scaffolds, with pore size of about 11.18-241.63 μm (**Figure 1**).

Cell proliferation-cytotoxicity assay

Cultured rat tooth germ cells were used as shown in **Figure 2A**. According to the result of cell proliferation-cytotoxicity assay, the 3%, 5% and 7% calcium alginate/DCCP scaffolds had poor compatibility with the tooth germ cells, and the cytotoxicity was of grade 2-3. The cytotoxicity was not enhanced with time. The cytotoxicity of 20% and 25% PVA/DCCP scaffolds was of grade 3-4, and the cytotoxicity was not enhanced with time either (**Figure 2**).

Discussion

To achieve a good repair effect for jaw bone defect, the bone implants should have high plasticity and mechanical performance. DCPP reinforced by SA or PVA may serve as an ideal material for the repair of jaw bone defect. We tested for the resistance to dissolution, setting time, shear strength, SEM characteristics and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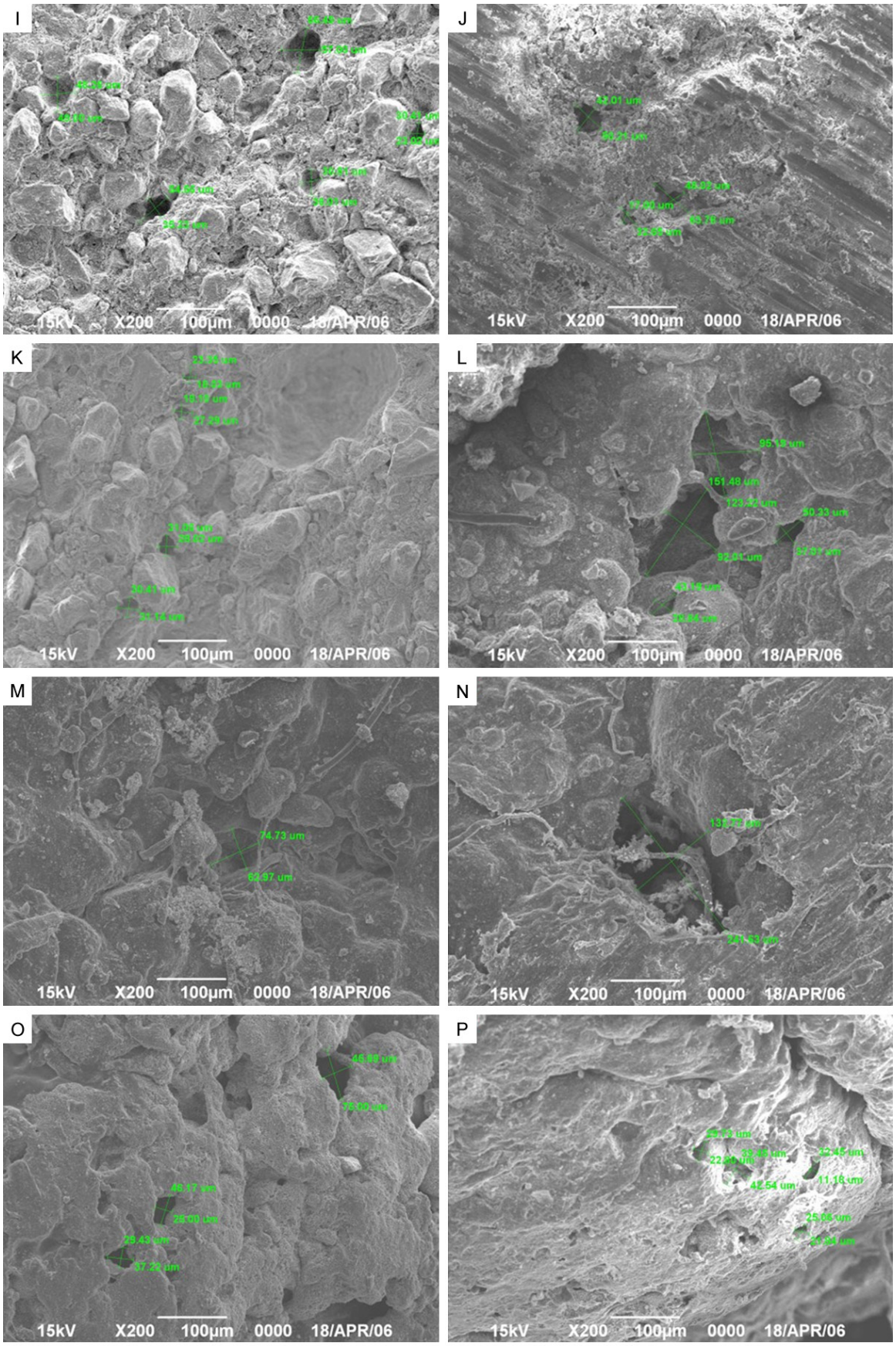

Surface morphology

Inner morphology

Figure 1. The surface and inner morphology of calcium alginate/DCCP scaffolds under SEM. A. Surface morphology of 3% calcium alginate/DCCP scaffold (× 200); B. Inner morphology of 3% calcium alginate/DCCP scaffold (× 200); C. Surface morphology of 4% calcium alginate/DCCP scaffold (× 200); D. Inner morphology of 4% calcium alginate/DCCP scaffold (× 200); E. Surface morphology of 5% calcium alginate/DCCP scaffold (× 200); F. Inner morphology of 5% calcium alginate/DCCP scaffold (× 200); G: Surface morphology of 7% calcium alginate/DCCP scaffold (× 200); H. Inner morphology of 7% calcium alginate/DCCP scaffold (× 200); I. Surface morphology of 10% PVA/DCCP scaffold (× 200); J. Inner morphology of 10% PVA/DCCP scaffold (× 200); K. Surface morphology of 15% PVA/DCCP scaffold (× 200); L. Inner morphology of 15% PVA/DCCP scaffold (× 200); M. Surface morphology of 20% PVA/DCCP scaffold (× 200); N. Inner morphology of 20% PVA/DCCP scaffold (× 200); O. Surface morphology of 25% PVA/DCCP scaffold (× 200); P. Inner morphology of 25% PVA/DCCP scaffold (× 200).

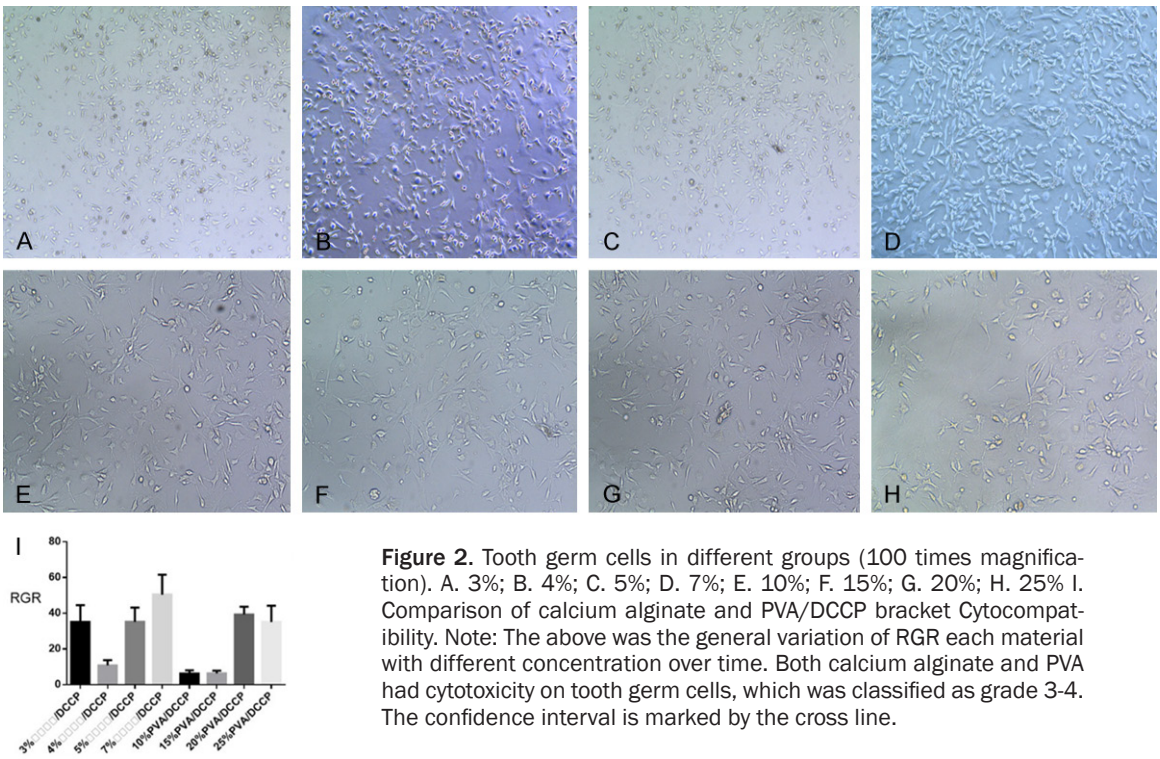

Figure 2. Tooth germ cells in different groups (100 times magnification). A. 3%; B. 4%; C. 5%; D. 7%; E. 10%; F. 15%; G. 20%; H. 25% I. Comparison of calcium alginate and PVA/DCCP bracket Cytocompatibility. Note: The above was the general variation of RGR each material with different concentration over time. Both calcium alginate and PVA had cytotoxicity on tooth germ cells, which was classified as grade 3-4. The confidence interval is marked by the cross line.

cell proliferative activity and evaluated the mechanical performance, microscopic morphology and biocompatibility of the calcium alginate or PVA/DCCP scaffolds. These were important parameters that indicated the feasibility of the scaffolds for jaw bone defect repair.

We found that 3%, 4%, 5% and 7% calcium alginate/DCCP scaffolds and 15%, 20% and 25% PVA/DCCP scaffolds had good resistance to dissolution in liquid. As the concentration of SA or PVA increased, both the initial setting time and the final setting time increased. With in the concentration range under testing, SA had shorter final setting time. In shear strength test, 3%, 4% and 5% calcium alginate/DCCP scaffolds had higher shear strength, and the shear strength increased with the increasing concentration of SA. This was consistent with the findings by Liu et al. [12]. Moreover, the shear strength increased obviously with the increase of PVA concentration. The shear strength of PVA/DCCP scaffolds was directly proportional to PVA concentration. Compared with calcium alginate/DCCP scaffolds, PVA/DCCP scaffolds had higher mechanical strength [13, 14].

As shown by SEM observation, both two scaffolds shared similar inner and surface morphology which resembled the porous volcanic rock with irregular pore shape and non-uniform pore size. These pores provided the space for the growth of cells. According to cell proliferation-cytotoxicity test, the calcium alginate/DCCP scaffolds had better biocompatibility [15, 16]. However, the cytotoxicity of the scaffolds was still of grade 2-3, which indicated the need to optimize the preparation process so as to improve the biocompatibility. The cytotoxicity

may arise from the calcination process and the use of calcium chloride to treat the calcium alginate/DCCP scaffolds. More studies are needed to reveal the origin of cytotoxicity.

To conclude, PVA/DCCP scaffolds have better mechanical performance, while calcium alginate/DCCP scaffolds have better biocompatibility. The preparation process can be optimized in the direction of integrating the advantages of SA and PVA so as to create more suitable scaffolds for jaw bone defect repair.

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Disclosure of conflict of interest

None.

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