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Study on adhesion, proliferation and differentiation of osteoblasts promoted by new absorbable bioactive glass injection *in vitro*

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Abstract. – OBJECTIVE: The objective of this study was to evaluate adhesion, proliferation and differentiation of osteoblasts grown on absorbable bioactive glass-calcium phosphate cement injection (BG-CPC) materials *in vitro.*

MATERIALS AND METHODS: BG-CPC composite biomaterial samples were prepared *in vitro*, for culture with MC3T3-E1 rat osteoblasts. Cells were divided into CPC, BG and BG-CPC treated groups. After cultivation for 3d, cells were stained with rhodamine-phalloidin and 4',6-diamidino-2-phenylindole (DAPI) and observed by fluorescence microscopy for osteoblast morphology on the surface of biomaterials. At 24h, 48h and 72h, MTT assay was used to test adhesion and proliferation, and bicinchoninic acid assay (BCA) method was carried out to test ALP activity; ELISA was used to test bone morphogenetic protein (BMP) and TGF-β expression levels at day 3.

RESULTS: Compared with the other two groups, cells in the BG-CPC group had more attachments; the DAPI labelled nuclei were clearer and nuclear shape was more complete and full. Adhesion and proliferation, as well as alkaline phosphatase (ALP) activity of cells for all time points in the BG-CPC group were higher than those in the other two groups and differences were of statistically significant ($p<0.05$); BMP and TGF-β expression levels were higher than those in the other two groups and the differences were statistically significant ($p<0.05$).

CONCLUSIONS: *In vitro* use of new absorbable bioactive glass is able to promote adhesion, proliferation and differentiation of osteoblasts, which may be related to increased BMP and TGF-β expression.

Key Words:
New absorbable bioactive glass injection, Osteoblast, Adhesion, Proliferation, Differentiation, Bone morphogenetic protein, Transforming growth factor.

Introduction

With the wide application of orthopedic biomaterials, the requirements of these materials are becoming increasing important[1]. The biological material polymethyl methacrylate (PMMA) has disadvantages, in that it is not absorbable, with no functions of bone conduction or induction and it releases a significant amount of heat during the solidification process[2]. Calcium phosphate cement (CPC) can be shaped arbitrarily because it is self-setting and releases no heat as with PMMA. CPC has broad clinical application value due to features such as good biocompatibility, osteoconduction and being replaced by new bone after degradation[3]. Nevertheless, CPC degrades slowly within the body[4]. Bioactive glass (BG) receives much attention due to good biological activity and biocompatibility[5], and as a result, it is formed by new absorbable bioactive glass injection (BG-CPC) as an injectable material combined with CPC and possesses the advantages of both, and it's predicted for better application field[6]. Osteoblasts are the most important cell during the process of bone remodeling and also one of the most active cells during formation of material-implant-bone interface[7]. We performed *in vitro* experiments to address adhesion, proliferation and differentiation of osteoblasts treated by BG-CPC, to provide a theoretical basis for future *in vivo* studies.

Materials and Methods

Preparation of biological materials

CPC and BG power (45S5 bioactive glass, NovaBone®, LLC, Alachua, USA) were mixed

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according to the weight ratio 8:2.Next, 1M dipotassium phosphate and 1M monopotassium phosphate solutions were added, and mixed based on P/L=2.0 ratio. After mixing for 1 min, the mixture was poured into plastic molds of 10 mm diameter and height 2 mm at 37°C. They were then ready for use after solidification in an incubator with 100% relative humidity for 24h (Figure 1).

Cell Culture

MC3T3-E1 rat osteoblasts were cultured in advanced minimal essential media (a-MEM) containing 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin and grown in an incubator set to 37°C and 5% CO_2. The media was changed every 3-5 days until cells reached the logarithmic phase of growth and were 80% confluent before being passaged. The cells were then trypsinized and cell suspensions were prepared. They were then placed in new culture flasks at a density of $2\text{-}5\times10^5$ cells/ml.

Experimental Grouping

Cells were grown on top of CPC, BG or BG-CPC. Osteoblasts were treated with the biological materials in 96 well plates, and there were 30 wells in each group. Cells were treated for 24h, 48h and 72h. There were therefore 10 wells for each time point for which data was averaged.

Observation of Cellular Morphology

Before seeding cells, the three discoid materials were placed into wells of a 96 well plate and we performed ^{60}Co disinfection. Cells were then seeded on the biological materials at a density of 5×10^4/well and cultured for 3 days in a 5% CO_2 incubator at 37°C. 3.7% formaldehyde solution was used to fix cells on the materials and then 0.2% Triton X-100 was added for 30 min to permeabilize cells. Actin cytoskeleton was stained with phalloidin and DAPI was used to stain nuclei. A laser scanning confocal microscope (LEICA TMLA, Wetzlar, Germany) was used to observe adhesive conditions on the materials.

Observation of Cell Adhesion and Proliferation

Cells were seeded at a density of 5.0×10^4/well on disinfected material in 96 well plates and then 1ml culture medium was added. Cells were cultured for 4h at 37°C, 5% CO_2 and saturation humidity conditions. 100 µl MTT solution was added to each well at 24h, 48h and 72h and then allowed to incubate for another 4h. Culture medium was removed carefully and placed in new wells of a 96 well cell culture plate. 600 µl medium was then added to each well. Plates were placed on a rocking platform for 10 min and three 150 µl aliquots were removed from each well and transferred to another 96 well culture plate. Dimethyl sulfoxide (DMSO) was added to blank wells for the zero setting and then an ELISA survey meter (Multiskan FC, Thermo Scientific, Waltham, MA, USA) was used to test OD values at 490 nm. Data was averaged.

ALP Activity

Cells were seeded at a density of 2.0×10^4/well on disinfected material in 96 well plates and culture medium was removed after 24h, 48h and 72h. Cells were washed carefully with phosphate buffered saline (PBS) three times; 1% Triton-X100 solution was added to permeabilize osteoblasts and then were incubated at 4°C for 12h. Alkaline phosphatase (ALP) activity was determined using a specific assay kit (Nanjing Jiancheng, China). Light absorption of the solution at 405 nm was taken, and bicinchoninic acid assay (BCA) method was used to measure total protein content of cells in each well. Activity is expressed as the value normalized to total protein content and averaged.

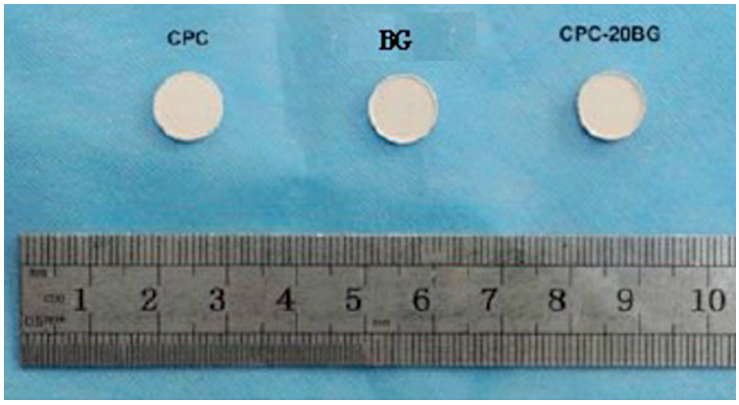

Figure 1. Preparation of the three different biological materials.

Table I. Comparison of adhesion and proliferation of cells (OD value).

	Adhesion			Proliferation		
Group	**24h**	**48h**	**72h**	**24h**	**48h**	**72h**
CPC group	0.029±0.003	0.036±0.004	0.055±0.006	0.153±0.033	0.294±0.036	0.365±0.039
BG group	0.044±0.005	0.063±0.007	0.085±0.008	0.247±0.042	0.365±0.045	0.486±0.047
BG-CPC group	0.063±0.008	0.082±0.006	0.124±0.005	0.365±0.022	0.557±0.024	0.849±0.026
F	5.624	5.967	6.325	5.934	6.235	6.658
p	0.027	0.023	0.015	0.023	0.015	0.007

PCT: procalcitonin; CRP: C reactive protein; LAC: blood lactic acid; *represents $p < 0.05$; x^a means Chi-square x^2.

Determination of BMP and TGF-β Expression levels

BMP-2 ELISA kit was from Ji'nan Aonuo Biological Engineering Co., Ltd and TGF-β ELISA kit was from Shanghai Jimian Shiye Co., Ltd and they were used according to manufacturer's instructions.

Statistical Analysis

SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was used for data input and analysis; measurement data is expressed as mean ± standard deviation ($\bar{x} \pm s$); comparison between groups was through single factor ANOVA analysis followed by Post Hoc test (LSD); enumeration data are expressed as cases or percentage; comparison between groups was through χ^2 testing; $p<0.05$ was taken as statistically significant.

Results

Comparison of cell morphology

Compared with the other two groups, cells grown on BG-CPC had more attachments and nuclei were clearer and more complete and full (Figure 2).

Comparison of Adhesion and Proliferation of cells

Adhesion and proliferation of cells at all time points in the BG-CPC group were higher than those in the other groups and the differences were statistically significant ($p<0.05$) (Table I).

Comparison of ALP Activity

ALP activities at all time points in the BG-CPC group were higher than those in the other two groups and the differences were statistically significant ($p<0.05$) (Figure 3).

Comparison of BMP and TGF-β Expression Levels

BMP and TGF-β expression levels in the BG-CPC group were higher than those in the other two groups and the differences were statistically significant ($p<0.05$) (Table II).

Discussion

The main components of injectable bioactive glass are SiO_2, CaO, P_2O_3 and Na_2O, which

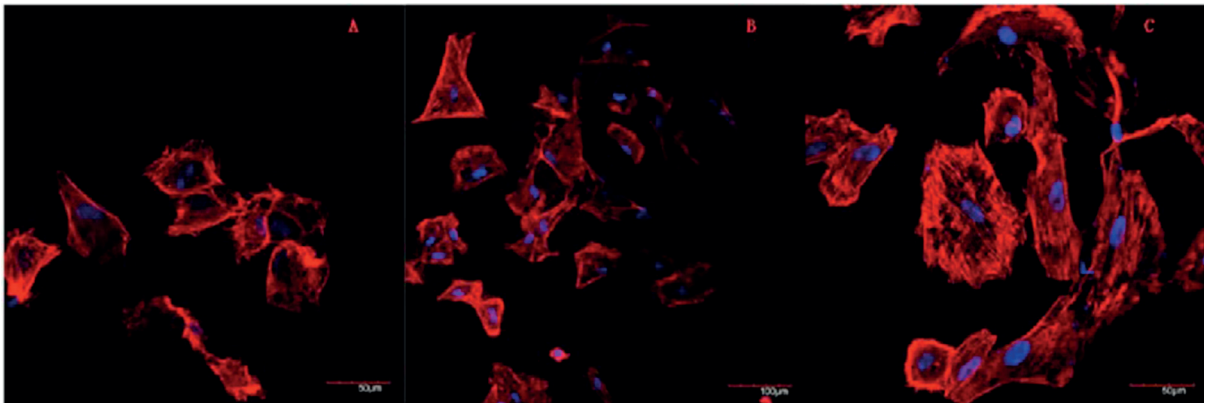

Figure 2. Cell morphology of the three different groups. A: CPC group; B: BG group; C: BG-CPC group.

Table II. Comparison of BMP and TGF-β expression levels.

Group	BMP-2 (pg/mL)	TGF-β (μg/L)
CPC group	43.5±10.2	84.7±23.4
BG group	51.3±12.6	92.8±32.5
BG-CPC group	72.6±18.7	123.5±45.7
F	5.217	5.632
p	0.027	0.024

is chemically similar to human bone and forms firm chemical bonds between the material interface and human bone tissue, and as a result, its joint strength provides high stability between transplant and articular surface. Furthermore, its conductivity is better than calcium phosphate bone cement[8]. It has been shown through *in vivo* experiments[9] that after the bioactive glass is planted within the human body, mature osteocytes can be found in HCA-collagen layers and it is mineralized. Bioactive glass has good biocompatibility and biological activity, as well as good bone conduction and has osteoinductive activity, which can cause a response inside and outside cells, between tissue and material[10]. Yuan et al[11] made 45S5 bioactive glass into porous cylinders and implanted them into muscle pouch of dogs and found that there were bone tissues formed within the transplant after 3 months through histological observation, which demonstrates that bioactive glass has osteoinductive activity. Kirk et al[12] used gelatin as ground substance and combined it with bioactive glass powder and allogeneic bone meal. This kind of biological material has good bone conduction and osteoinductive activity.

Bioactive materials have certain limitations when compared with human bone tissue. For example, bioactive glass contains silicon, which cannot be degraded within the human body and its metabolic mechanism is not yet clear; therefore, no matter how long bioactive glass is implanted in the body, it cannot be transferred so as to be similar to human bone tissue[13]. Calcium phosphate bone cement can be formed into any shape, and it has broad clinical application value due to features such as good biocompatibility, osteoconduction and being replaced by new bone after degradation. New absorbable bioactive glass injection possesses the above advantages and is predicted to have better applied space. Currently, there are fewer studies on this subject and it can be concluded from out study that compared with the other two groups, cells on the BG-CPC surface group formed more cell attachments and DAPI stained nuclei were clearer and nuclear shape was more complete and full. Adhesion and proliferation, as well as ALP activities of cells for all time points in the BG-CPC group were higher than those in the other two groups and the differences were statistically significant.

Cells are stimulated by the autocrine response of soluble ions released slowly from the surface of bioactive glass particles. Such stimulation includes promoting the secretion of bone induction molecules such as BMP, transforming growth factor (TGF-β), insulin-like growth factor, platelet-derived growth factor and fibroblast growth factors[14,15]. These factors can maintain high concentrations and continuous release both locally and in circulation, which effectively increases osteoblast activity, promotes ostosis and chondrogenesis and increases the success rate of the implant[16]. It can be concluded from this study that BMP and TGF-β levels in the BG-CPC group were higher than those in the other two groups and the differences were statistically significant.

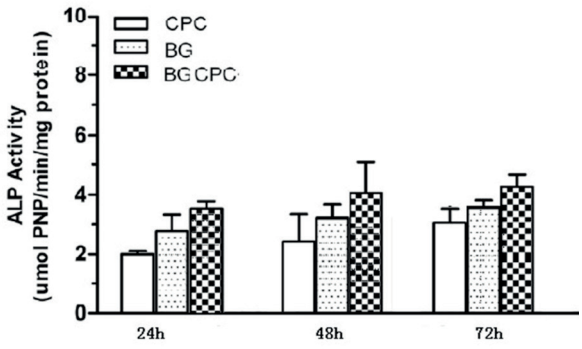

Figure 3. ALP activity of the three different groups.

Conclusions

Above all, new absorbable bioactive glass injection *in vitro* is able to promote adhesion, proliferation and differentiation of osteoblasts, which may be related to increased BMP and TGF-β expression, and this biological material has great potential for clinical application.

Conflict of Interest

The authors declare no conflicts of interest.

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