

Original Article

Combined construction of injectable tissue-engineered bone with CPC and BMMSCs and its study of repair effect on rabbit bone defect model

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Abstract

Objective: to investigate the combined construction of injectable tissue-engineered bone with calcium phosphate bone cement composite (CPC) and bone marrow mesenchymal stem cells (BMMSCs). **Methods:** The proliferation activity of BMMSCs encapsulated was detected by CCK8 method on the 7th day after its self-coagulation by CPC. qRT-PCR was used to detect the expressions of mRNA. The microcapsules of BMMSCs combined with CPC were completely filled in the defect site in the experimental group, and the control group not filled. The two groups were sutured and routinely reared, double upper limb X-ray examination performed after operation. **Results:** Those of two groups were on the rise over time, which were higher at the 1st, 3rd, 5th and 7th days than those at the previous time points (all P<0.05). The relative expressions of ALP and CALCR at the 7th day were higher than those at the day in BMMSCs combined with the CPC group and BMMSCs group (all P<0.05). The relative expression of CALCR was significantly higher in BMMSCs combined with the CPC group than that in the BMMSCs group on the 7th day (P<0.05). **Conclusion:** With good cell activity and biological activity, the combined construction of the tissue-engineered bone with BMMSCs and CPC can be used as an ideal treatment material for bone tissue repair and connection.

Keywords: Bone Defect, Bone Marrow Mesenchymal Stem Cells, Bone Tissue Repair, Calcium Phosphate Bone Cement Composite, Tissue-Engineered Bone

Introduction

Bone defect, very common in clinical practice, especially in surgery, refers to bone loss due to trauma, infection, tumor resection, own bone diseases, and congenital malformation, resulting in the destruction of bone structure integrity^{1,2}. It can affect the normal function of bone tissue in the defect site, causing a series of secondary diseases. For example, long-term skull defect can cause the atrophy and cystic change of brain tissue, affecting the patient's intelligence and responsiveness, and the function of the local related

nervous system³. At present, the treatment methods for bone defects are mainly bone graft and gene therapy. Among them, bone graft, according to the different sources of it, can be divided into autologous bone graft, allogeneic bone graft, and tissue-engineered bone graft. The operation combined with gene therapy and physical therapy can accelerate the growth and healing of bone graft and bone in patients^{4,5}. However, large-area bone defect or comminuted fracture caused by strong external force is difficult to be treated by autologous or allogeneic bone graft, because grafting a large area of autologous bone from the patient itself will affect the function in the site of bone removal, thereby bringing severe complications and increasing the risk of canceration at the fracture site. The graft of large-area allogeneic bone will increase the risk of rejection reaction. These two large-area bone grafts have a higher risk of infection and a slower postoperative healing rate⁶⁻⁸.

The tissue-engineered bone, the most promising bone repair material in current studies, can simulate the natural process of human bone repair and regulate osteogenic precursor cells in the defect site to differentiate them

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into bone cells. It can also regulate the bracket and related bioactive substances so that the bone marrow microenvironment in the defect site is conducive to the formation and regeneration of bone^{9,10}. Compared with autologous or allogeneic bone graft, the tissue-engineered bone, making the bone graft source more accessible, reduces infection rate and carcinogenic rate, improving the safety of bone graft¹¹. Bone marrow mesenchymal stem cells (BMMSCs) that are fibroblast-like pluripotent stem cells in the bone marrow have great differentiation potential and weak immunogenicity. Therefore, they are ideal seed cells for the tissue-engineered bone¹². Injecting BMMSCs alone for bone repair is not effective because they are prone to lose, thereby affecting the effect. If a suitable vector can be used to immobilize them in the defect site, their residence time can be extended to play a role. Calcined hydroxyapatite as a bracket, traditional engineered bone guarantees strong mechanical property, but the repair effect is not ideal, and the doctor needs to trim them before graft. Calcium phosphate bone cement (CPC) has injectable property and in-situ plastic curing property. Hydroxyapatite (HA) formed after solidification has osteoinduction ability. Compared with traditional calcined hydroxyapatite, CPC not only guarantees strong mechanical property but also improves the plastic property of engineered bone^{13,14}. Therefore, in this paper, the feasibility of the combined construction of injectable tissue-engineered bone with CPC and BMMSCs and its application were investigated, to provide a safer and more effective method for constructing the tissue-engineered bone, so as to provide a theoretical basis for the improvement of bone graft surgery and improve the effective rate of operation and the prognosis of patients with bone defect.

Methods

Materials and reagents

BMMSCs and iCell primary mesenchymal stem cell culture medium (Shanghai Saibaikang Biotechnology Co., Ltd.); tetracalcium phosphate (TTCP, Beijing Kangpuhuiwei Technology Co., Ltd.); calcium hydrogen phosphate (DCPA, Beijing Kangpuhuiwei Technology Co., Ltd.); TransScript Green Two-Step qRT-PCR SuperMix (TransGen Biotech Co. Ltd.); 2% sodium alginate hydrogel (Guangzhou Yikang New Material Technology Co., Ltd.).

Culture and amplification of BMMSCs

BMMSCs were cultured in a 37°C and 5% CO₂ incubator with iCell primary mesenchymal stem cell culture medium that contains primary mesenchymal stem cell basal culture medium, 0.5% primary mesenchymal stem cell culture additive and 5% fetal bovine serum. When cells grew adhering to 80% of the wall, 0.05% trypsin was added at a ratio of 1:2 for digestion and passage. The digestion was performed when the second generation cells grew to 100% to prepare cell suspension, with cell concentration adjusted to 2.5×10⁶/mL.

Construction of hydrogel BMMSCs

2% sodium alginate hydrogel and BMMSCs were mixed at a ratio of 1 mL: 1 mol. Then calcium chloride solution was dropped through a No.15 syringe needle to form a BMMSCs hydrogel microcapsule with a diameter of about 2 mm.

Preparation of CPC bracket

TTCP and DCPA were stirred, ground, and sieved to form TTCP and DCPA particles with a diameter of 1-80 μm and 0.4-3 μm, respectively. The refined TTCP and DCPA particles were stirred, mixed, and dried at ratio of 1:1 (molar ratio) to obtain CPC powder. Deionized water dissolution was added at a ratio of 1:3 (mass ratio) and quickly poured into a stainless steel model with a diameter of 12 mm and a height of 2 mm. They were placed together in a 37°C incubator for 4 hours of solidification, immersed in a water bath at 37°C for 24 hours, and dried. Then, they were sterilized for 12 hours and degassed for 72 hours in an ethylene oxide sterilizer for use.

CCK8 method for detection of proliferation after BMMSCs combined with CPC

CCK8 proliferation experiments were performed on the 7th day after the self-coagulation of BMMSCs by CPC. BMMSCs hydrogel microcapsule with 50 μl/well was seeded in a 96-well plate, and 0.1 g of CPC dough (CPC powder: ultrapure water=2 g: 1 mL) and 150 μL of iCell primary mesenchymal stem cell culture medium added as BMMSCs combined with CPC group. Altogether 50 μL of BMMSCs cell suspension and 150 μL of iCell primary mesenchymal stem cell culture medium were added to the BMMSCs group, and only equal amounts of icell culture medium added to the blank group. For each group of 3 duplicate wells, the 96-well plate was placed in a 37°C and 5% CO₂ incubator, incubated for 24 hours. CCK8 detection was performed in BMMSCs combined with the CPC group together with the control group after inoculation and adhering to the wall (Od) and after the 1st, 3rd, 5th, and 7th days of inoculation removing the CPC dough from BMMSCs combined with CPC group, respectively. The culture medium was discarded, washed twice with PBS, and then continued to incubate at 37°C and 5% CO₂ for 2 hours after 10 μL/well of CCK8 added. A450 was determined with a microplate reader. After the A450 of BMMSCs combined with the CPC group minus that of the blank group, the proliferation curve was plotted, as shown in Figure 1.

qRT-PCR detection of expressions of alkaline phosphatase (ALP) mRNA and calcitonin receptor (CALCR) mRNA after BMMSCs combined with CPC

The expressions of ALP and CALCR were detected by qRT-PCR in BMMSCs combined with the CPC group together with the BMMSCs group after inoculation and adhering to the wall (Od) and after the 7th day removing the CPC dough

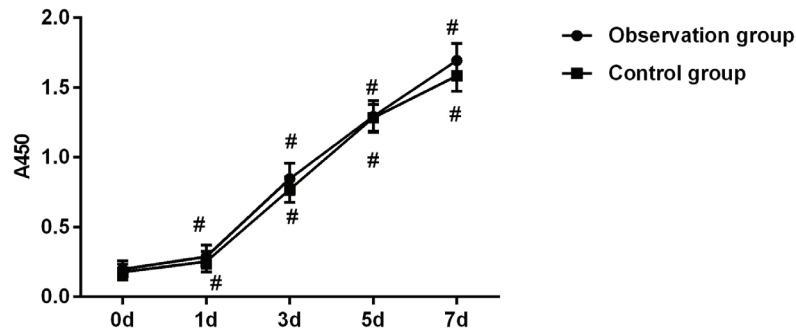


Figure 1. CCK8 method for detection of proliferation after BMMSCs microcapsule combined with CPC. A450 values in BMMSCs combined with CPC group were not different from those in BMMSCs group at the day, the 1st, 3rd, 5th, and 7th days (all $P > 0.05$). Those of two groups were on the rise over time, which were higher at the 1st, 3rd, 5th and 7th days than those at the previous time points (all $P < 0.05$). Note: # indicates that in the same group, compared with the previous time point, $P < 0.05$.

Table 1. Primer sequences.

Gene	Upstream primer	Downstream primer
U6	5'-CTCGCTTCGGCAGCAC-3'	5'-AACGCTTCACGAATTTGCGT-3'
ALP	5'-CCCCGTGGCAACTCTA-3'	5'-GCGGCAGACTTTGGTTT-3'
CALCR	5'-GAGGTCCAGACCACCGTAA-3'	5'-AAGGGGATGATCTCGGCAC-3'

from BMMSCs combined with CPC group, respectively. Trizol method was used for the extraction of total RNA, and UV spectrophotometer for the detection and extraction of RNA concentration and purity, with A260/A280 between 1.8 and 2.0 as the standard. The reverse transcription and amplification of total RNA were performed in strict accordance with the TransScript Green Two-Step qRT-PCR SuperMix kit instruction. The reverse transcription system was: 1 μ g of total RNA, 4 μ L of 5xTransScript Green All-in-One SuperMix for qPCR, 1 μ L of gDNA Remover, and RNase-free Water added to 20 μ L, after mixed, incubated at 42°C for 15 min, and at 85°C for 5 s. The amplification system was: 2 μ L of cDNA, 0.4 μ L of each of the upstream/downstream primers (10 μ M), 10 μ L of 2xTranscript Tip Green qPCR SuperMix, and Nuclease-free Water added to 20 μ L. The amplification was performed using a two-step method: at 94°C for 30 s, at 94°C for 5 s, and at 60°C for 30 s, for a total of 40 cycles. U6 was used as an internal reference. The primer sequences of U6, ALP and CALCR (synthesized by Shanghai Shenggong Bioengineering Co., Ltd.) are shown in Table 1. The results were processed by $2^{-\Delta\Delta CT}$ method.

Repair effect of combined construction of tissue-engineered bone with CPC and BMMSCs on rabbit radius defect

Altogether 18 healthy SPF-class 6-month-old New Zealand white rabbits (provided by Jinan Ono

Bioengineering Co., Ltd.) were selected, with a weight of (2.1 \pm 0.76) kg, 9 in the experimental group and 9 in control group. After the rabbit was anesthetized with 10% chloral hydrate (1 mL/kg), the forearm of the rabbit was incised and exfoliated to the periosteum of the radius, and about 1 cm of the bone taken to construct the model of rabbit radius defect. The microcapsules of BMMSCs combined with CPC were completely filled in the defect site in the experimental group, and the control group not filled. The two groups were sutured. They were fed with basal feed, free water intake and diet, alternating light for 12 hours. One week later, antibiotics were injected into muscles to prevent infection. Double upper limb X-ray examination was performed on the day, 4th, 8th and 12th weeks after operation.

Data analysis and processing

SPSS19.0 statistical package (Asia Analytics Formerly SPSS China) was used to analyze and process data. Measurement data were expressed as $\bar{x} \pm s$. The t-test was used for the comparison between two groups, paired t-test for the comparison of two-time points in the same group, repeated measures analysis of variance for the comparison of multiple time points. Count data were expressed as ratio, and χ^2 test and analysis was used. The level of significance is $P < 0.05$.

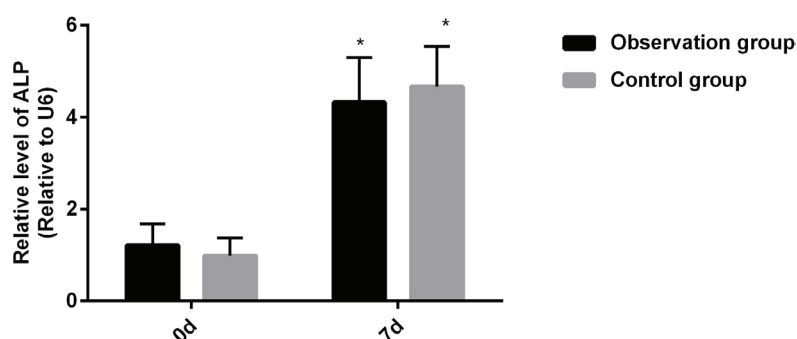


Figure 2. qRT-PCR detection of expression of ALP mRNA. The relative expressions of ALP mRNA at the 7th day were higher than those at the day in BMMSCs combined with the CPC group and BMMSCs group ($P < 0.05$). Those of ALP in BMMSCs combined with the CPC group were not significantly different from those in the BMMSCs group at the day and the 7th day ($P > 0.05$). Note: * indicates that in the same group, compared with the day, $P < 0.05$.

Table 2. CCK8 method for detection of A450 values at different time points after BMMSCs microcapsule combined with CPC.

	0 d	1 d	3 d	5 d	7 d	F	P
Observation group	0.198±0.061	0.287±0.083*	0.846±0.112*	1.292±0.114*	1.692±0.124*	359.300	0.000
Control group	0.178±0.058	0.252±0.076*	0.769±0.091*	1.283±0.096*	1.582±0.108*	450.000	0.000
t	0.713	0.933	1.601	00.181	2.007		
P	0.486	0.365	0.130	0.859	0.062		

Note: # indicates that in the same group, compared to the previous time point, $P < 0.05$.

Results

CCK8 method for detection of proliferation after BMMSCs microcapsule combined with CPC

A450 values in BMMSCs combined with the CPC group were not different from those in BMMSCs group at the day, the 1st, 3rd, 5th and 7th days (all $P > 0.05$). Those of two groups were on the rise over time, which were higher at the 1st, 3rd, 5th and 7th days than those at the previous time points (all $P < 0.05$). See Table 2 and Figure 1.

qRT-PCR detection of expression of ALP mRNA

The relative expressions of ALP in BMMSCs combined with CPC group at the day and the 7th day were (1.212±0.466) and (4.323±0.972), respectively, and those of ALP in BMMSCs group were (0.987±0.392) and (4.667±0.872), respectively. Those of ALP at the 7th day were higher than those at the day in BMMSCs combined with CPC group and BMMSCs group ($P < 0.05$). Those of ALP in BMMSCs combined with the CPC group were not significantly different from those in the BMMSCs group at the day and the 7th day ($P > 0.05$). See Figure 2.

qRT-PCR detection of expression of CALCR mRNA

The relative expressions of CALCR in BMMSCs combined with CPC group at the day and the 7th day were (0.872±0.041)

and (3.231±0.029), respectively, and those of CALCR in BMMSCs group were (0.913±0.036) and (2.891±0.392), respectively. Those of CALCR at the 7th day were higher than those at the day in BMMSCs combined with the CPC group and control group ($P < 0.05$). The relative expression of CALCR in BMMSCs combined with the CPC group was not significantly different from that in the BMMSCs group at the day ($P > 0.05$). The relative expression of CALCR in BMMSCs combined with the CPC group was significantly higher than that in the BMMSCs group on the 7th day ($P < 0.05$). See Figure 3.

Repair effect of combined construction of tissue-engineered bone with CPC and BMMSCs on rabbit radius defect

The results of the X-ray examination on the day after operation showed that there was 1 cm of bone defect in the middle part of the rabbit radius of two groups. The results of the X-ray examination at the 4th week after operation showed that in the observation group, the density of the defect area gradually increased, and the creep replacement occurred. The tissue-engineered bone was gradually connected with the surrounding bone tissue. In the control group, the bone density of the defect area decreased, with poor creep replacement. The tissue in the defect area was not connected. The results of the X-ray examination at the 8th week after operation showed that in the observation group, the creep replacement gradually increased, and the tissue-engineered bone was gradually integrated with the surrounding bone

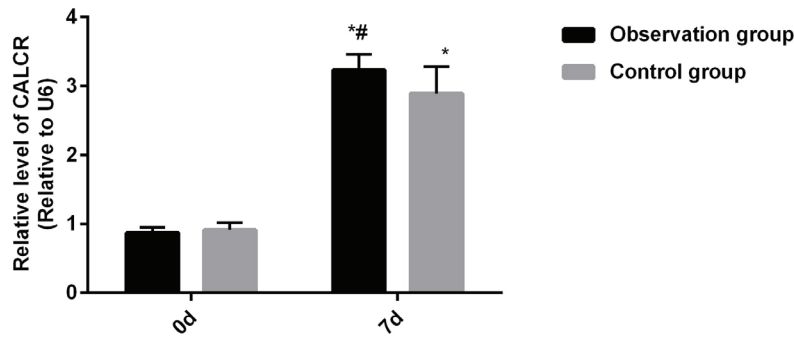


Figure 3. qRT-PCR detection of expression of CALCR mRNA. The relative expressions of CALCR at the 7th day were higher than those at the day in BMMSCs combined with the CPC group and BMMSCs group ($P < 0.05$). The relative expression of CALCR in BMMSCs combined with the CPC group was not significantly different from that in the BMMSCs group at the day ($P > 0.05$). The relative expression of CALCR in BMMSCs combined with the CPC group was significantly higher than that in the BMMSCs group on the 7th day ($P < 0.05$). Note: * indicates that in the same group, compared with the day, $P < 0.05$. # indicates that at the same time point, compared with the control group, $P < 0.05$.

tissue. In the control group, the gap between the defect area and the surrounding normal tissue was clearly seen, and no connection and integration were found. The results of the X-ray examination at the 12th week after operation showed that in the observation group, the density of the defect area was closed to that of the surrounding bone tissue. The gap between the defect area and the surrounding normal tissue gradually became blurred or even disappeared. The tissue-engineered bone was completely connected and integrated with the surrounding bone tissue. In the control group, the density of the defect area was lower than that of the surrounding bone tissue, and the gap between the defect area and the surrounding bone tissue still existed. No complete connection and integration were found in the defect area.

Discussion

With the aging of the population, the incidence of fracture and musculoskeletal disorders increases year by year, so does the treatment cost of bone defect¹⁵. Although autologous bone graft has long been considered as the first choice for operative treatment, infection, limited bone source and other problems still exist¹⁶. The simulation of the natural process of bone repair by the tissue-engineered bone, with CPC as the bracket and BMMSCs as seed cells, can solve many problems in autologous or allogeneic bone graft¹⁷. Therefore, in this paper, the combined construction of the injectable tissue-engineered bone with CPC and BMMSCs and its application study were investigated, the provide a safer and more effective bone repair material for the operative treatment of bone graft, to improve the effective rate and prognosis of patients with bone defect.

CCK8 method was used for the detection of proliferation activity after BMMSCs microcapsule combined with CPC. It was found that A450 values in BMMSCs combined with the CPC group were not different from those in BMMSCs group

at the day, the 1st, 3rd, 5th, and 7th days. Those of two groups were on the rise over time, which were higher at the 1st, 3rd, 5th and 7th days than those at the previous time points. This indicates that BMMSCs hydrogel combined with CPC coated with sodium alginate still have good cell proliferation activity. Both ALP and CALCR, osteogenic related genes, are osteogenic markers^{18,19}. ALP is a group of isoenzymes from tissues such as liver, bone and intestine. ALP3 type is derived from bone tissue. The dephosphorylation of ALP in osteoblasts produces phosphoric acid, which together with calcium-producing calcium phosphate is deposited on bone, promoting the formation of bone tissue²⁰. When binding to calcitonin, CALCR inhibits the formation of osteoclasts and bone absorption, thereby promoting bone formation. BMMSCs, belonging to progenitor cells, do not express ALP and CALCR, and mature osteoblasts express the former, but osteoclasts express the latter^{21,22}. The increased expressions of ALP and CALCR mean that BMMSCs are successfully differentiated into mature bone cells, forming bone tissue²³.

The relative expressions of ALP and CALCR at the 7th day were higher than those at the day in BMMSCs combined with the CPC group and BMMSCs group. Those of ALP and CALCR in BMMSCs combined with the CPC group were not significantly different from those in the BMMSCs group at the day. The relative expression of CALCR in BMMSCs combined with the CPC group was significantly higher than that in the BMMSCs group on the 7th day, but that of ALP was not different between the two groups. This indicates that BMMSCs hydrogel, combined with CPC, does not affect the ability of BMMSCs to differentiate into osteoblasts. The results of the X-ray examination on the day after operation showed that there was 1 cm of bone defect in the middle part of the rabbit radius of two groups, indicating that the model of rabbit radius defect was successfully constructed. The results of the X-ray examination at the 4th, 8th and 12th weeks after operation showed that in the

observation group, the density of the defect area gradually increased, close to that of surrounding normal bone tissue, and the creep replacement gradually became ideal. The gap between the bone defect and the surrounding normal tissue gradually became blurred or even disappeared. The tissue-engineered bone was gradually connected and integrated with the surrounding bone tissue. However, in the control group, the density of the defect area was always lower than that of the surrounding normal bone tissue, and the creep replacement was not ideal. No complete connection and integration were found in the defect area. It is indicated that the combined construction of the tissue-engineered bone with CPC and BMMSCs has a repair and connective effect, which is faster than that of the natural process of bone defect repair, on rabbit radius defect.

In the study of CPC materials, Xu²⁴ et al. have found that chitosan as the liquid phase of CPC, improving the processing performance of it and accelerating its degradation after grafting, has good heterotopic bone induction. Therefore, when CPC and BMMSCs are combined to construct the tissue-engineered bone, this experiment can be improved by replacing sodium alginate with chitosan. Having prepared a calcium phosphate composite bracket containing simvastatin PLGA microsphere (SIM-PLGA-CPC), Zhang²⁵ et al. have found that when transplanted onto rabbit femoral condyle, compared to conventional CPC materials, SIM-PLGA-CPC has good biocompatibility and higher bone formation efficiency. Therefore, another improvement to the tissue-engineered bone in this experiment can be performed. Simvastatin is introduced, and the superiority of these two improved methods in subsequent experiments is compared. Deng²⁶ has found that having a more obvious repair effect on bone defect, the porous CPC transfected with VEGF gene can increase the blood supply to the defect site. Therefore, the tissue-engineered bone in this study can be modified by transfecting CPC with VEGF gene.

In summary, with good cell activity and biological activity, the combined construction of the tissue-engineered bone with BMMSCs and CPC, having a good repair effect on the model of rabbit radius defect, can be used as an ideal treatment material for bone tissue repair and connection.

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Authors' contributions

GL and WS were responsible for the culture and amplification of BMMSCs. LZ and TW performed CCK8 assay. ZP, SS, and WX contributed to PCR. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of Deqing People's Hospital.

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