Chem. Res. Chinese Universities, 2019, 35(3), 514—520 doi: 10.1007/s40242-019-9007-7

DOPA-IGF-1 Coated HA/PLGA Microspheres Promoting Proliferation and Osteoclastic Differentiation of Rabbit Bone Mesenchymal Stem Cells

LIU Guomin[1,5], LI Yalong[1,5], YANG Shuting[2], ZHAO Yi'an[3,5], LU Tiancheng[4,5], JIA Wenyuan[1,5], JI Xuan[3,5] and LUO Yungang[3,5*]

1. Department of Orthopedics, the Second Hospital of Jilin University, Changchun 130041, P. R. China;
2. Department of Prosthdontics, the Hospital of Stomatology, Jilin University, Changchun 130021, P. R. China;
3. Department of Stomatology, the Second Hospital of Jilin University, Changchun 130041, P. R. China;
4. Life Sciences College, Jilin Agricultural University, Changchun 130118, P. R. China;
5. Jilin Provincial Changbai Mountain Medicine Anti-tumor Engineering Center, the Second Hospital of Jilin University, Changchun, 130041, P. R. China

Abstract To explore the ability of dihydroxyphenylalanine-insulin-like growth factor-1(DOPA-IGF-1) coated hydroxyapatite/poly(lactic-co-glycolic acid)(HA/PLGA) microspheres to promote the proliferation and osteoclastic differentiation of rabbit bone mesenchymal stem cells(rBMSCs), HA/PLGA microspheres with different HA content (10%, 30%, 50%, mass fraction) were prepared by electrospinning method and HA/PLGA microspheres with 50% HA were coated with IGF-1 and DOPA-IGF-1, respectively. They were co-cultured with rBMSCs, respctively. Cell counting kit-8(CCK-8) detection, confocal laser scanning microscopy(CLSM), alkaline phosphatase(ALP) detection and osteogenesis related genes *COL1A1*, *Runx2* and bone morphogenetic protein-2(*BMP-2*) detection were conducted to detect the proliferation activity, cell morphology, differentiation ability and the expression level of osteogenesis-related genes of cells cultured on all microspheres groups. The results showed that rBMSCs proliferation increased in an HA content dependent manner, and cells proliferated more in the IGF-1 coated and DOPA-IGF-1 coated groups, in particular in DOPA-IGF-1 coated group, and the differences were more remarkable over time (P<0.05). HA/PLGA microspheres promoted the proliferation and osteogenic differentiation of rBMSCs, and DOPA-IGF-1 coating enhanced the proliferation and osteogenic differentiation of rBMSCs.

Keywords Biomaterial; Electrospinning; Biocompatibility; Hydrophilic polymer

1 Introduction

The use of the implant is conducive to solving the common bone diseases, such as fracture and bone defect. However, traditional metal implant has some disadvantages, such as the limited osseointegration, osteolysis, non-degradation and implant spondylolysis[1—3]. Bone tissue engineering is commonly used clinically to develop and manufacture biodegradable scaffolds for bone repair, such as chitosan, gelatin, polycaprolactone(PCL) and poly(lactic-co-glycolic acid)(PLGA)[4—7]. However, most of the scaffolds used in clinic have their limitations, such as poor hydrophilicity, unsatisfactory cell adhesion, and the geometrical shape of scaffolds that cannot fully meet the clinical demand.

Microsphere can be made into various geometric shapes of scaffolds by injection molding to meet the clinical demand through injection molding, accelerate the cell proliferation, promote the up-regulation of osteogenesis related gene, growth factor and protein, and be conducive to the bone repair[8—10]. Recently, the important direction of research on related bone repair is to consider the microspheres as bone tissue engineering scaffolds[11]. In order to enhance the combination of the microspheres scaffolds and bone to promote cells proliferation on the surface of microspheres scaffolds, the microspheres are usually made into the porous scaffolds to improve the absorption area of cells, growth factor, drug and biological adhesive[12—15].

The methods for the preparation of microspheres scaffolds mainly include emulsified solvent evaporation method, phase separation method, spray drying method and freeze-drying method[14—18]. But the complex process, long preparation time, and the potential toxicity of organic solvents often limit their application[13]. Electrospinning is an effective method to prepare microspheres carrier[19]. It is simpler and more convenient

*Corresponding author. Email: luoyg@jlu.edu.cn

Received January 8, 2019; accepted March 22, 2019.

Supported by the Special Health Project of Jilin Province, China(No.3D516D703429) and the Special Health Project of Department of Finance of Jilin Province, China (No.3D5177703429).

than the emulsion solvent evaporation method, phase separation method and other process operations, and the condition of microspheres manufacturing was mild and applicable to adding the bioactive substance.

PLGA is commonly used as scaffolds for bone tissue engineering because of its good biocompatibility and biodegradability, but its wide application is limited by its hydrophobicity, lack of cell recognition sites and bone induction ability. Nano-hydroxyapatite(HA) has excellent bone induction and bone binding ability. HA/PLGA composite microspheres have been used for bone tissue engineering[8,15,20—22].

Insulin-like growth factor-1(IGF-1) is widely used in the bone tissue engineering as the growth factor, which induces the bone regeneration. IGF-1 can up-regulate the expression of the alkaline phosphatase(ALP), osteocalcin and osteopontin, inhibit the formation of the osteoclasts, enhance the osteogenesis induced by bone morphogenetic protein(BMPs), stimulate the osteoblast differentiation of mesenchymal stem cells(MSCs) through activating the mammalian target of rapamycin(mTOR), and improve the bone micro-structure and bone mass[23—28]. IGF-1 is often loaded on the microspheres through grafting, coating or embedding, but the effect of IGF-1 on inducing cells proliferation is commonly affected because the combination of pure IGF-1 and microspheres is not stable.

Inspired by the adhesion mechanism of marine organism mussel, polydopamine(PDA) coating on the surface of scaffolds can improve the hydrophilicity of scaffolds, increase cell adhesion and enhance the biocompatibility of scaffolds[29,30]. The effective ingredient 3,4-dihydroxyphenylalanine(DOPA) of the mussel protein can be fused to IGF-1 by recombinant DNA technology, forming DOPA-IGF-1 compound, which can enhance the affinity of combination between IGF-1 and material[30].

In the current study, the electrospinning technology was used to prepare HA/PLGA microspheres, providing a technical reference for the construction of microspheres. Meanwhile, IGF-1 was immobilized on HA/PLGA microspheres by DOPA-IGF-1 to evaluate the biocompatibility and bone inducibility.

2 Experimental

2.1 Materials

New Zealand White rabbits[2 weeks, animal license number: SCXK(J) 2015-0005] were from Beijing Jinmuyang Laboratory Animal Cultivation Co., Ltd.(Beijing, China); PLGA[n(LA)/n(GA)=50/50, M_n=30000] was purchased from Changchun SinoBiomaterials Co., Ltd.(Changchun, China); IGF-1 and Dopa-IGF-1 were donated by Changchun Institute of Applied Chemistry, Chinese Academy of Sciences (Changchun, China); HA(20 nm, 99.9%) was purchased from Beijing DK Nano Technology Co., Ltd.(Beijing, China); *N*-methyl pyrrolidone(NMP, >99.0% purity) was purchased from Aladdin Biochem. Inc.(Shanghai, China); osteogenic induction culture medium was purchased from Cyagen Biosciences Inc.(Guangzhou, China); fetal bovine serum(FBS) was purchased from Gibco Company(Beijing, China); DMEM culture medium was purchased from Hyclon(Shanghai, China); cell counting kit-8(CCK-8) kit was purchased from Dojindo Molecular Technologies Inc.(Shanghai, China); ALP detection kit was purchased from Beyotime Biotech(Shanghai, China); reverse transcription kit and the quantitative real-time polymerase chain reaction(qRT-PCR) kit were purchased from GenStarBiosolutions(Beijing, China).

2.2 Extraction and Culture of Rabbit Bone Mesenchymal Stem Cells(rBMSCs)

The research had been approved by the Ethics Committee of the Second Hospital of Jilin University, Changchun, China(No.2018191). The rBMSCs were extracted according to the previously reported method[31]. They were cultured in a DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and placed in an incubator with 5% CO_2 at 37 °C and saturated humidity. The culture medium was changed for the first time after 72 h, and then every 48 h. For passage, cells were digested with 0.25% trypsin+0.02% EDTA at 37 °C for 3 min.

2.3 Preparation of HA/PLGA Microsphere

PLGA electrospinning solution of 10%(mass fraction) was prepared as follows: 1 g of PLGA was dissolved in 9 g of NMP solution, and fully dissolved after magnetic stirring for 12 h in a 70 °C water bath. The electrostatic generator(Fraser Anti-Static Techniques Ltd., Britain) was connected with the automatic propelling device. The receiving solution(60% ethanol solution, volume fracion) was placed below the shower nozzle, and the distance from the shower nozzle to the receiving solution level was 10 cm(d=10 cm). The prepared working solution was extracted with a 3 mL injector(d_{Inner}=8.66 mm) and then put in a propeller, 27G needle(d_{Inner}=0.15 mm) was adopted, the propelling speed was 0.2 mL/min, the receiving solution was accessed to the ZNCL-G intelligent automatic magnetic blender (Yuhua Instrument, Zhengzhou, China), and the microspheres were prepared by regulating voltage. Meanwhile, the receiving solution was stirred, so that the microspheres system was dispersed. After the shape of the microspheres in the receiver was stable, the microspheres were taken out, washed in 0.01 mol/L PBS solution three times and transferred to a vacuum drier for use. HA/PLGA mixed solutions in different proportions were prepared. Briefly, 1 g of PLGA was dissolved in 9 g of NMP, then 111, 429, 1000 mg of HA were added to the 10% PLGA solution, respectively, to form HA/PLGA microspheres with HA mass fraction to 10%, 30% and 50%, and HA/PLGA microspheres were prepared according to the above method. We divided the microspheres of different compositions into four groups: PLGA, 10%HA/PLGA, 30%HA/PLGA and 50%HA/PLGA, respectively.

2.4 HA/PLGA Microsphere Loads IGF-1 and Dopa-IGF-1

The surface of 50%HA/PLGA microspheres was modified by loading IGF-1 and Dopa-IGF-1, respectively. The steps were

as follows: the prepared HA/PLGA microspheres were immersed in 75%(volume fraction) ethyl alcohol for 30 min, and washed by PBS three times. Then HA/PLGA microspheres (50 mg) were soaked in a 10 ng/mL IGF-1 solution(10 mL) and a 10 ng/mL Dopa-IGF-1 solution(10 mL), respectively. Vitamin c(1.67 mg/mL) and 1 μL of tyrosinase were added, adjusting pH to 8.5, then the microspheres were incubated in an 5%(volume fracion) CO_2 incubator at 37 °C for 24 h. The microspheres were taken out and rinsed five times with sterile 1% PBS solution for use. We named the HA/PLGA microspheres with modified surface as 50%HA/PLGA/IGF-1, 50%HA/PLGA/DOPA-IGF-1, respectively.

2.5 Characterization

The general morphology of microspheres prepared under different electrostatic voltages was observed by a fluorescence inverted microscope with a digital photography system (IX71, Olympus Company, Japan). The prepared microspheres were metal sprayed, and the surface was observed by a scanning electron microscope(SEM, XL30 ESEM-FEG, Field Electron and Ion Company, America). FTIR-2000 Fourier transform infrared spectrometer(FTIR, Perkin Elmer, America) was used to analyze the chemical structure of the microspheres.

2.6 Cells Culture and Alizarin Red Dyeing of Calcification Nodule

Experiment was divided into six groups: 1 g of PLGA, 1 g of PLGA+111 mg of HA(10%HA/PLGA), 1 g of PLGA+143 mg of HA(30%HA/PLGA), 1 g of PLGA+1000 mg of HA(50%HA/PLGA), 1 g of PLGA+1000 mg of HA+IGF-1 (50%HA/PLHA/IGF-1) and 1 g of PLGA+1000 mg of HA+DOPA-IGF-1(50%HA/PLHA/DOPA-IGF-1). The third passaged cells were inoculated in a 24-well plate at 1.0×10^5 cells/mL, and added to the osteogenic induction culture medium for osteogenic induction, and the medium was refreshed every 48 h. After 14 d, alizarin red staining was performed to detect calcification nodules.

2.7 CCK-8 Assay

The microspheres(1 mg) were placed in a 96-well plate, disinfected with 75% ethanol for 30 min, washed with PBS 3 times, and incubated with 1 mL of DMEM overnight. The third passaged cells were inoculated at 2×10^4 cells/well. The plate was placed in a 5% CO_2 incubator at 37 °C, and the culture medium was refreshed every three days. On the 4th day and the 7th day, 100 μL of CCK-8 solution was added to each well, and incubated for 2 h. The absorbance at 450 nm was determined on a amicroplate reader(Varioskan Flash, Thermo, America). Each group was repeated five times.

2.8 Cell Morphology Observed by Confocal Laser Scanning Microscope

The cell/microsphere was taken out from a 96-well plate, and was washed twice in the PBS. It was fixed by 4% paraformaldehyde at room temperature for 20 min, fixed by 4% paraformaldehyde at 4 °C for 15 min, and washed three times in the PBS. The cells were incubated in the PBS including 0.25%(volumer fraction) Triton X-100 for 5 min, washed by PBS 2 times, and incubated by 1% BSA in dark for 20 min. After being washed by PBS, the cells were incubated by 1 μL/mL rhodamine-phalloidin at room temperature for 2 h. For nucleic labeling, microspheres were washed by PBS again, and dyed by 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The cell morphology and cytoskeletal structure were observed by a confocal laser scanning microscope(CLSM).

2.9 ALP Detection

According to the instruction of ALP kit, the ALP activity of rBMSCs was measured. Briefly, on the 4th and 7th day, RIPA lysate was used to lyse the cells, and centrifuged at 1000 r/min for 10 min. The supernatant(150 μL) was transferred to a 96-well plate, 50 μL of *p*-nitrophenyl phosphate (*p*-NPP) was added, and the mixture was incubated at 37 °C for 30 min. A reaction stop solution of 100 μL was added to each well to terminate the reaction, and the optical density at 405 nm was measured by a microplate reader. Meanwhile, the total protein content of each sample was calculated by a BCA protein assay kit. The measurement was in triplicate.

2.10 Osteogenesis Related Gene Detection

The mRNA expression of the osteogenesis related gene was quantitatively evaluated by qRT-PCR technology. On the 7th day, total RNA was extracted from BMSCs, and cDNA was obtained by reverse transcription. Two×RealStar Green Fast Mixture kit was used for qRT-PCR reaction on a 7500 Fast Real-Time PCR instrument(Applied Biosystems, America), and the expression levels of *Runx2*, *BMP-2*, *COL1A1* and *GAPDH* were quantified by measuring threshold cycles(Ct). *GAPDH* was used as internal reference. The reaction system was 20 μL, the reaction procedure included: pre-denature at 95 °C for 2 min, denature at 95 °C for 15 s, anneal at 60 °C for 30 s and extend at 72 °C for 30 s, 40 cycles. The melting curve was analyzed. The primer design in experiment is shown in Table 1. The measurement was in triplicate.

Table 1 Sequences of primers in the qRT-PCR

Gene	Primer sequence
Runx2	F: 5′-tcaggcatgtccctcggtat-3′, R:5′-tggcaggtaggtatggtagtgg-3′
BMP-2	F: 5′-cgtgaggattagcaggtctttg-3′, R:5′-aggcgtttccgctgtttg-3′
COL1A1	F: 5′-aattcggcttcgacgtttg-3′, R:5′-gggttgtttgtcgtctgtttct-3′
GAPDH	F: 5′-gagcaccagaggaggacga-3′, R:5′-tgggatggaaactgtgaagag-3′

2.11 Statistical Analysis

The SPSS20.0 software was used for statistical analysis (SPSS Inc., Chicago, USA). The proliferation of the rBMSCs were expressed by mean±SD. The difference of the cell proliferation, osteogenic activity and gene expression quantity between the experimental groups and control groups was analyzed by independent sample Student-*t* test. $P<0.05$ showed the statistically significant difference.

3 Results

3.1 HA/PLGA Microsphere Characterization

The general morphologies of the prepared microspheres were observed under the inverted microscope to explore the influence of different electrostatic voltages on the morphology of microspheres[Fig.1(A)].

When the electrostatic voltage was 4.5—5.0 kV(U=4.5—5.0 kV), the morphology of the microcarrier was stable and spherical(d=150—300 μm at 5.0 kV), the edge of the microspheres was smooth, and the size and shape of the microspheres were uniform. There was no difference between different HA/PLGA microspheres. When the voltage was more than 5.0 kV or less than 4.5 kV, the morphology of the microspheres was irregular, and the fluctuation amplitude of particle size was increased(Table 2).

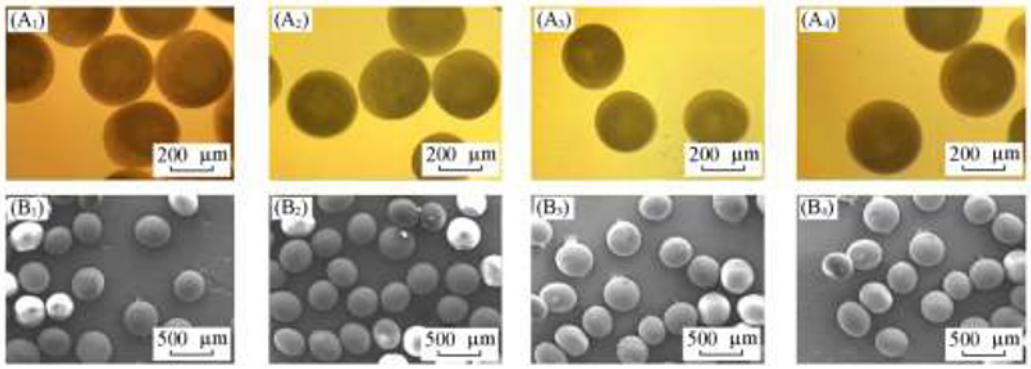

Fig.1 Inverted microscope maps(A_1—A_4) and SEM images(B_1—B_4) of PLGA(A_1, B_1), 10%HA/PLGA(A_2, B_2), 30%HA/PLGA(A_3, B_3) and 50%HA/PLGA(A_4, B_4)

Table 2 Influence of different voltages on the morphology of 30%HA/PLGA*

U/kV	d/μm	Microsphere appearance
3.5	150—500	Irregular sphere or hemisphere
4.0	150—350	Irregular sphere or hemisphere
4.5	200—300	Sphere
5.0	150—300	Sphere
5.5	100—300	Irregular sphere/hemisphere
6.0	100—250	Irregular sphere/hemisphere

In order to observe the surface morphology and the fine structure of the microspheres surface, SEM was used to characterize the microspheres. The particle sizes of the microspheres prepared by 4.5—5.0 kV were uniform, and they were round or sphere without sharp edges, adhesion or aggregation between the spheres. Compared with the pure PLGA microspheres, the surface of HA/PLGA microspheres was rough because the microspheres contained HA[Fig.1(B)].

FTIR spectra of pure PLGA microspheres and HA/PLGA microspheres with different HA concentrations prepared by electrospinning technology are shown in Fig.2. The characteristic absorption peaks of PLGA were located at 2990 and 2940 cm^{-1}(—CH_3), 1753 cm^{-1}(C═O), 1183 and 1083 cm^{-1}(C—O). HA characteristic absorption peak was observed at 500—600 cm^{-1}(P—O), and it clearly indicated that HA was cooperated in the microspheres.

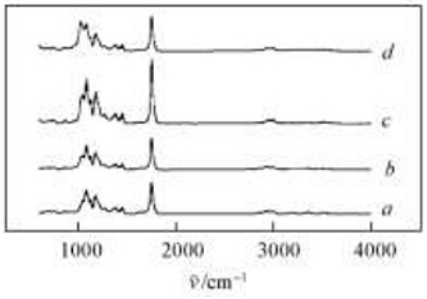

Fig.2 FTIR spectra of PLGA(*a*), 10%HA/PLGA(*b*), 30%HA/PLGA(*c*) and 50%HA/PLGA(*d*)

3.2 BMSCs' Morphological Observation and Alizarin Red Dyeing

The third passaged rBMSCs had the highest viability. Alizarin red staining was used to observe whether there was calcium deposition in cultured rabbit BMSCs[Fig.3(A)]. When the cells were cultured to the 14th day, they were dyed with Alizarin red, and the calcification nodule dyed with red in the cells matrix can be seen in Fig.3(B).

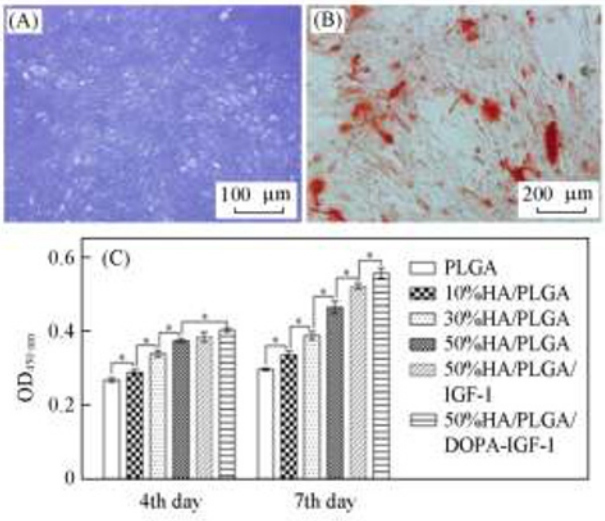

Fig.3 Morphologys of rBMSCs cells incubated on the surface of microspheres(A), Alizarin red staining result(B) and cytotoxicity of microspheres on the 4th day and the 7th day by CCK-8 assay(C, *P<0.05)

3.3 rBMSC Proliferation on Microsphere

The adhesion and proliferation of cells were limited to the surface property of the microspheres. The cell proliferation was determined by CCK-8 on the 4th day and the 7th day[Fig.3(C)]. For different types of HA/PLGA microspheres, the cell proliferation was in an HA content dependent manner. The cell proliferation ability of HA/PLGA microspheres coated with DOPA-IGF-1 and IGF-1 was stronger than that of the uncoating HA/PLGA microspheres, and the highest ability was in the DOPA-IGF-1 coating group. With time extended, the proliferation activity of the 7th day was higher than that of the 4th day ($P<0.05$) .

3.4 LSCM Observation of Microsphere/Cell

As shown in Fig.4, the blue fluorescence displays the DAPI stained nucleus on the 7th day, which was used to evaluate the quantity of rBMSCs on microsphere surface. The green fluorescence was the actin microfilament dyed by FITC, which was used to evaluate the structure and function of the cytoskeleton. The rBMSCs grew well and evenly adhered on the HA/PLGA microspheres surface. For the microspheres with different HA concentrations, HA/PLGA microsphere surface with a higher HA concentration carried more cells, the microspheres coated with DOPA-IGF-1 and IGF-1 showed more cells and cytoskeletons than the uncoating microspheres, and the DOPA-IGF-1 coating group had the largest number of cells(Fig.4).

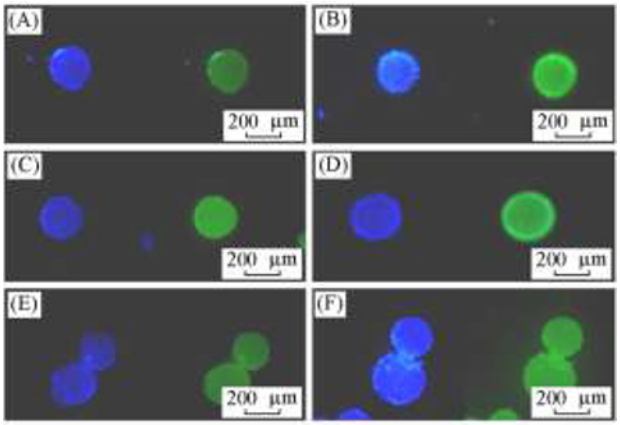

Fig.4 CLSM observation results of PLGA(A), 10%HA/PLGA(B), 30%HA/PLGA(C), 50%HA/PLGA(D), 50%HA/PLGA/IGF-1(E) and 50%HA/PLGA/DOPA-IGF-1(F)

The blue fluorescence was DAPI stained nucleus and the green fluorescence was the actin microfilament dyed by FITC.

3.5 ALP Activity

ALP activity is commonly used as the marker of osteoblastic differentiation at early stage, so the bone inducibility of the microspheres can be evaluated through the determination of ALP activity. ALP activity was determined by ALP kit on the 4th day and the 7th day, and the detection result was consistent with that of CCK-8 assay(Fig.5).

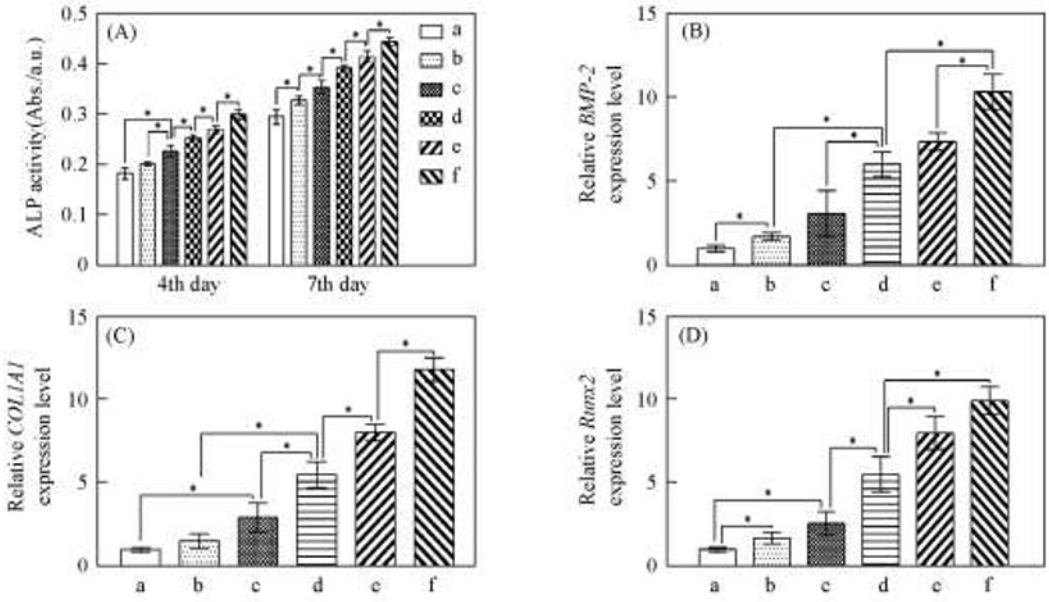

Fig.5 ALP detection results of rBMSCs on the 4th and the 7th day(A), *BMP-2* detection(B), osteogenesis related genes *COL1A1*(C), and *Runx2*(D) expression detected by qRT-PCR on the 4th day

a—f in (A)—(D) corresponded to PLGA, 10%HA/PLGA, 30%HA/PLGA, 50%HA/PLGA, 50%HA/PLGA/IGF-1 and 50%HA/PLGA/DOPA-IGF-1, respectively.

3.6 qRT-PCR Detecting Osteogenesis Related Gene Expression

The expression of osteogenesis marker *COL1A1*, *Runx2* and *BMP-2* in rBMSCs cultured on microspheres for 7 d was quantitatively analyzed by qRT-PCR. The bone gene expression of 10%HA/PLGA microspheres was not significantly increased compared with the PLGA/cell group($P>0.05$), and then the expression level of the osteogenic genes was significantly increased in an HA content dependent manner($P<0.05$).

The *COL1A1*, *Runx2* and *BMP-2* expression level of 50%HA/PLGA microspheres group was about 5.5-, 5.5- and 6.0-fold that of the PLGA group, respectively, demonstrating that HA promoted rBMSCs osteogenesis differentiation. The expression level of osteogenesis gene of rBMSCs in the 50%HA/PLGA/DOPA-IGF-1 group and the 50%HA/PLGA/IGF-1 group was higher than those in the uncoating group. The expression level of *COL1A1*, *Runx2* and *BMP-2* of 50%HA/PLGA/DOPA-IGF-1 group was the highest, about 11.8-, 9.9- and 10.3-fold that of the PLGA group. The expression level of *COL1A1*, *Runx2* and *BMP-2* of 50%HA/PLGA/IGF-1 group was 8.0-, 8.0- and 7.3-fold that of the PLGA group, respectively(Fig.5).

4 Discussion

It has become one of the hotspots in the field of bone tissue engineering to induce and differentiate mesenchymal stem cells(MSCs) for repairing the bone defect by compositing HA and degradable PLGA to make microspheres scaffolds[32,33]. HA/PLGA microspheres were prepared by electrospinning technology in the current research to optimize the preparation process. It was simpler and more convenient than the emulsion solvent evaporation method, phase separation method and other process operations. The condition of microspheres manufacturing was mild, and applicable to adding the bioactive substance. In the current study, the HA/PLGA microspheres prepared were characterized as uniform particle size, non-adhesion, uniform morphology and smooth surface under the conditions of d=10 cm, speed=0.2 mL/h and U=4.5—5.0 kV. FTIR showed characteristic absorption peaks of PLGA and HA, indicating that HA was incorporated in microspheres.

BMSCs are not abundant enough and only account for 0.001%—0.01% of the karyocyte[34]. Thus, for the reparative treatment of the bone defect, the BMSCs should be amplified on biological scaffolds *in vitro*, and then transplanted to the bone defect part for proliferating and differentiating to repair the bone defect[35]. In the current study, the third passaged rBMSCs and microspheres were cultured together, and the Alizarin red dyeing results indicated that the extracted and cultured rBMSCs had the ability of adherence and osteogenesis. CCK-8 detection result showed that the proliferation activity of cells in all microspheres groups was obviously enhanced in an HA content dependent manner on the 4th day and the 7th day. It may be related to the role of HA in promoting the proliferation of rBMSCs, and it is more conducive to the adherence and proliferation of cells because the roughness of the microspheres surface is also increased with the increase of HA content.

IGF-1 can adjust the gene expression and cells matrix composition in the cartilage layer around the bone defect, and promote the osteogenic induction differentiation of the BMSCs and bone repair[36,37]. It can effectively improve the combining capacity of IGF-1 and materials through DOPA-IGF-1 prepared by recombinant DNA technology[38]. In order to improve the osteogenic differentiation ability of HA/PLGA microspheres, we modified the surface of HA/PLGA microspheres by either loading IGF-1 or DOPA-IGF-1.

The activity to promote proliferation for the DOPA-IGF-1 group and IGF-1 group was stronger than that for the HA/PLGA microspheres group, in particular DOPA-IGF-1 group. This result suggested that IGF-1 played the role in promoting the adhesion and proliferation of rBMSCs. We speculated that the adhesion efficiency of the DOPA-IGF-1 on the microspheres surface was higher than that of IGF-1, and consequently its ability to promote cell proliferation was more obviously. It has been reported that the PDA can immobilize small molecules, such as growth factor or nano-HA on the material surface to enhance the ability of cell proliferation and differentiation[15,29,30], but there are few studies on the proliferation and differentiation ability of the BMSCs induced by DOPA-IGF-1 prepared by recombinant DNA technology. In addition, the detection result of ALP was consistent with that of CCK-8 assay, and it supported our deduction with CSLM observation result.

Meanwhile, we also detected the expression level of osteogenesis-related genes to explore the differences in the ability of microspheres to promote osteogenic differentiation of rBMSCs. After co-culture for 7 days, *COL1A1*, *Runx2* and *BMP-2* genes of 10%HA/PLGA group were not increased obviously($P>0.05$) compared with the PLGA group, this result may be related to the low content of HA. While, the other HA/PLGA groups showed higher expression state and ascending trend, in which the expression level of *COL1A1*, *Runx2* and *BMP-2* of 50%HA/PLGA/DOPA-IGF-1 group was the highest, that of 50%HA/PLGA/IGF-1 group ranked the second. These results showed that IGF-1 and HA can collaboratively promote the proliferation and differentiation of rBMSCs. Meanwhile, the ability to promote the bone differentiation for the 50%HA/PLGA/DOPA-IGF-1 group was the best, suggesting the deduction that the adhesion efficiency of DOPA-IGF-1 on material surface was higher than that of only IGF-1, and thus the ability to promote osteogenesis differentiation for the 50%HA/PLGA/DOPA-IGF-1 group was stronger.

All results showed that the microspheres prepared in the current research had good biocompatibility and bioactivity. But because no *in vivo* study was carried out in the current study, the long-term influences of DOPA-IGF-1, such as teratogenicity and carcinogenicity were not clear. The development and application of this new material construct needs to be explored *in vivo*, which would be the focus of our next study.

References

[1] Kalsbeek J. H., van Walsum A. D. P., Vroemen J., Janzing H. M. J., Winkelhorst J. T., Bertelink B. P., Roerdink W. H., *Bone Joint J*., **2018**, *100-B*(4), 443

[2] Pura J. A., Bobyn J. D., Tanzer M., *Clin. Orthop. Relat. Res.*, **2016**, *474*(5), 1224

[3] King V., Swart A., Winder M. J., *J. Clin. Neurosci*., **2016**, *32*, 91

[4] Nguyen T. H., Lee B. T., *Sci. Technol. Adv. Mater*., **2012**, *13*(3), 035002

[5] Nguyen T. H., Lee B. T., *J. Mater. Sci.: Mater. M*., **2010**, *21*(6), 1969

[6] Ullah S., Zainol I., Idrus R. H., *Int. J. Biol. Macromol.*, **2017**, *104*, 1020

[7] Agarwal T., Narayan R., Maji S., Behera S., Kulanthaivel S., Maiti T. K., Banerjee I., Pal K., Giri S., *Int. J. Biol. Macromol*., **2016**, *93*, 1499

[8] Shen H., Hu X. X., Yang F., Bei J. Z., Wang S. G., *Acta Biomaterialia*., **2010**, *6*(2), 455

[9] Chung H. J., Kim I. K., Kim T. G., Park T. G., *Tissue Eng. Pt. A*., **2008**, *14*(5), 607

[10] Cebatariuniene A., Jarmalaviciute A., Tunaitis V., Puriene A., Venalis A., Pivoriunas A., *J. Cranio. Maxill. Surg*., **2017**, *45*(6), 845

[11] Lee Y. S., Lim K. S., Oh J. E., Yoon A. R., Joo W. S., Kim H. S., Yun C. O., Kim S., *J. Control. Release*, **2015**, *205*, 128

[12] Sivandzade F., Mashayekhan S., *J. Biomat. Sci. Polym. E*., **2018**, *29*(6), 683

[13] Kuterbekov M., Machillot P., Lhuissier P., Picart C., Jonas A. M., Glinel K., *Acta Biomater*., **2018**, *75*, 300

[14] Yan S., Xia P., Xu S., Zhang K., Li G., Cui L., Yin J., *ACS Appl. Mater. Interfaces*, **2018**, *10*(19), 16270

[15] Gao T., Zhang N., Wang Z., Wang Y., Liu Y., Ito Y., Zhang P., *Macromol. Biosci*., **2015**, *15*(8), 1070

[16] Wang H., Li P., Zhang L., Zhang J., Ling X., Ran H., *China Journal of Chinese Materia Medica*, **2009**, *34*(23), 3021

[17] Singh S. K., Vuddanda P. R., Singh S., Srivastava A. K., *Biomed. Research International*, **2013**, *2013*, 909045

[18] Li C., Qian Y., Zhao S., Yin Y., Li J., *Mater. Sci. Eng. C, Mater. Biol. Appl*., **2016**, *64*, 43

[19] Malik S. A., Ng W. H., Bowen J., Tang J., Gomez A., Kenyon A. J., Day R. M., *J. Colloid Interf. Sci*., **2016**, *467*, 220

[20] Fu C., Yang X., Tan S., Song L., *Sci. Rep*., **2017**, *7*(1), 12549

[21] Li Y., Zhang Z., Zhang Z., *Cell Tissues Organs*, **2018**, *205*(1), 20

[22] Mao Z., Li Y., Yang Y., Fang Z., Chen X., Wang Y., Kang J., Qu X., Yuan W., Dai K., Yue B., *Front Pharmacol*., **2018**, *9*, 368

[23] Poudel S. B., Bhattarai G., Kook S. H., Shin Y. J., Kwon T. H., Lee S. Y., Lee J. C., *Growth HormIgf Res*., **2017**, *36,* 1

[24] Rico-Llanos G. A., Becerra J., Visser R., *J. Biomed. Mater. Res. A*., **2017**, *105*(7), 1867

[25] Reible B., Schmidmaier G., Moghaddam A., Westhauser F., *Int. J. Mol. Sci.*, **2018**, *19*(6), 1674

[26] Shu X. L., Feng J., Feng J., Huang X. M., Li L. Q., Shi Q. S., *J. Biomater. Appl*., **2017**, *32*(5), 547

[27] Xian L., Wu X., Pang L., Lou M., Rosen C. J., Qiu T., Crane J., Frassica F., Zhang L., Rodriguez J. P., Xiaofeng J., Shoshana Y., Shouhong X., Argiris E., Mei W., Xu C., *Nat. Med*., **2012**, *18*(7), 1095

[28] Hawkins-Carranza F. G., Munoz-Calvo M. T., Martos-Moreno G. A., Allo-Miguel G., Del Rio L., Pozo J., Chowen J. A., Perez-Jurado L. A., Argente J., *Horm. Res. Paediat*., **2018**, *89*(3), 200

[29] Liu C., Li Y., Wang J., Liu C., Liu W., Jian X., *Molecules*, **2018**, *23*(7), 1643

[30] Cho H. J., Perikamana S. K. M., Lee J. H., Lee J., Lee K. M., Shin C. S., Shin H., *Acs. Appl. Mater. Inter*., **2014**, *6*(14), 11225

[31] Wang R., Xu B., Xu H. G., *Cell Physiol. Biochem*., **2017**, *41*(1), 213

[32] Shi P. J., Wang Q., Yu C. P., Fan F. J., Liu M., Tu M. L., Lu W. H., Du M., *Colloids Surf. B Biointerfaces*, **2017**, *155*, 477

[33] Zhou P., Wu J., Xia Y., Yuan Y., Zhang H., Xu S., Lin K., *Int. J. Nanomedicine*, **2018**, *13*, 4083

[34] Mostafa N. Z., Uludag H., Varkey M., Dederich D. N., Doschak M. R., El-Bialy T. H., *Open Dent. J*., **2011**, *5*, 139

[35] Zhang G., Na Z., Ren B., Zhao X., Liu W., *Int. J. Clin. Exp. Med*., **2015**, *8*(8), 12172

[36] Reible B., Schmidmaier G., Moghaddam A.,Westhauser F., *Int. J. Mol. Sci*., **2018**, *19*(6), 1674

[37] Zhang Z., Li L., Yang W., Cao Y., Shi Y., Li X., Zhang Q., *Osteoarthritis Cartilage*, **2017**, *25*(2), 309

[38] Zhang C., Miyatake H., Wang Y., Inaba T., Wang Y., Zhang P. B., Ito Y., *Angew. Chem. Int. Edit*., **2016**, *55*(38), 11447