

## Original Article

# Research of osteoblastic induced rat bone marrow mesenchymal stem cells cultured on $\beta$ -TCP/PLLA porous scaffold

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**Abstract:** Background: Ceramic and polymer composite scaffolds are widely used in tissue engineering for bone tissue regeneration. Composite of  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) and poly L-lactic acid (PLLA), due to its biocompatibility and biodegradability, is widely used in bioengineering. However, optimal ratio, porosity and pore size of this kind of scaffolds were not very clear yet. Materials and methods: We cultured osteoblastic induced rMSCs on  $\beta$ -TCP/PLLA scaffolds to investigate the optimum construction, which owned better properties for supporting cells growth, proliferation and differentiation. A total of 24 mice were divided into three groups: rMSCs +  $\beta$ -TCP/PLLA, osteoblastic rMSCs +  $\beta$ -TCP/PLLA and  $\beta$ -TCP/PLLA without cells. 8 mice were implanted with rMSCs +  $\beta$ -TCP/PLLA in the left thighs and  $\beta$ -TCP/PLLA without cells in the right thighs. 8 mice were implanted with osteoblastic rMSCs +  $\beta$ -TCP/PLLA in the left thighs and the same treatments in the right thighs as the above. After 8 and 12 weeks, the mice were sacrificed and implants with the surrounding tissues were harvested together. Paraffin sections were got and HE stain and Masson-Goldner stain were employed to observe the ectopic bone formation. Results: The scaffolds of  $\beta$ -TCP/PLLA = 2:1 significantly increased osteocalcin production of the cells. In addition, scaffolds with NaCl = 70 wt%, pore size 200~450  $\mu$ m showed better compatibility to these seeding cells. A significantly larger area of bone formation in the osteoblastic rMSCs and  $\beta$ -TCP/PLLA composite than that in rMSCs/scaffold and in the scaffold without cells in vivo. Conclusion: compounds of osteoblastic induced rMSCs and the scaffold with  $\beta$ -TCP/PLLA = 2:1, NaCl = 70 wt%, pore size = 200-450  $\mu$ m had good properties as a kind of bone substitute.

**Keywords:** Mesenchymal stem cells, osteoblastic induced, bone tissue engineering, poly-L-lactic acid (PLLA),  $\beta$ -tricalcium phosphate ( $\beta$ -TCP)

## Introduction

One type of produces for bone tissue engineering combines seed cells, growth factors and scaffolds. Currently, many composite scaffolds for bone tissue engineering were made to repair and reconstruct damaged bone tissue function. The composite scaffold should provide have favorable weight ratio of compound, porosity, pore size and mechanical properties. Ceramic and polymer composite scaffolds are widely used in tissue engineering for bone tissue regeneration [1]. As a absorbable ceramic material,  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) was suitably used in bone reconstruction implants for many years. However, these  $\beta$ -TCP implants

with low stress levels have a brittle fracture behavior in the clinical applications. Biodegradable polymers, such as poly L-lactic acid (PLLA), have a modulus of elasticity closer to that of natural cortical bone, and can retain sufficient mechanical strength for providing stabilization of bone implants [2, 3].

To overcome the shortcomings of these different materials, composites of  $\beta$ -TCP and PLLA, due to its biocompatibility and biodegradability, have been widely developed and used in bioengineering. However, optimal ratio, porosity and pore size of this kind of scaffolds were not very clear yet. In addition, the seed cell, especially the stem cell, is one of the critical factors

among those three components of bone tissue engineering. The ideal bio-active composite scaffold should have adequate bioactivity for stem cells attachment, proliferation, migration and differentiation. So, in this study, we cultured rat bone marrow mesenchymal stem cells (rMSCs) in vitro on  $\beta$ -TCP/PLLA scaffolds with different ratio, different pore size and different porosity. The aim is to investigate the optimum construction of  $\beta$ -TCP/PLLA porous scaffold for cells growth, proliferation and differentiation, and to explore the probability of osteogenesis of the composite in vivo.

### Materials and methods

#### *Synthesis and preparation of scaffolds*

The process which consists of solvent casting, compression molding and leaching stage had been used to fabricate scaffold of  $\beta$ -TCP/PLLA composite. Scaffolds were fabricated according to weight ratio of  $\beta$ -TCP/PLLA = 1:1, 1:2 and 2:1; the porosity of the composites ranges from 50~75% when the porogen NaCl added is 60 wt%, 70 wt% and 80 wt% respectively; the pore size ranges from 200 to 950  $\mu$ m by adding different crystal porogen NaCl.

#### *rMSCs culture and osteoblastic induced*

Bone marrow mesenchymal stem cells were isolated and cultured according to the method of Han [4]. Briefly, the Sprague-Dawley (SD) rats 3 months old were cervical dislocated and the femurs were aseptically excised. The proximal head of the femur was clipped off and the marrow was flushed with essential primary media (DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin) by a 20 gauge needle. The suspension was spun down at 1000 rpm for 10 min, the supernatant was aspirated, and the pellet was re-suspended in fresh primary media and added into Percoll separating medium (GIBCOBRL company). The monolayer cells were harvested and washed by essential primary media. The cells were cultured in flasks in an incubator at 37°C, 5% CO<sub>2</sub>, and 95% humidity. The medium was exchanged every 3 days and the cells were passaged when they were overgrowing in the flasks. Flow cytometry was used to assay the phenotype of rMSCs. To induce spontaneous differentiation into osteoblastic cells, we prepared a differentiation medium by addition of 50  $\mu$ g/ml ascorbic acid, 10 mM  $\beta$ -glycerophosphate and 100 nM

dexamethasone to the growth medium [5-7]. Then, we detected the proliferation and differentiation of osteoblastic rMSCs in different phases [8-12].

#### *Cell morphology and proliferation study*

When rMSCs were osteoblastic induced for 10 days, the cells were digested and seeded onto  $\phi$  = 10 mm  $\beta$ -TCP/PLLA scaffolds placed in 24 wells with  $1 \times 10^5$  cells per ml. Fluorescence microscope and scanning electron microscope (SEM) were used to investigate the cellular morphology and attachment. MTT assay was applied to detect the proliferation of the cells on the scaffolds.

#### *Alkaline phosphatase (ALP) activity and osteocalcin assay*

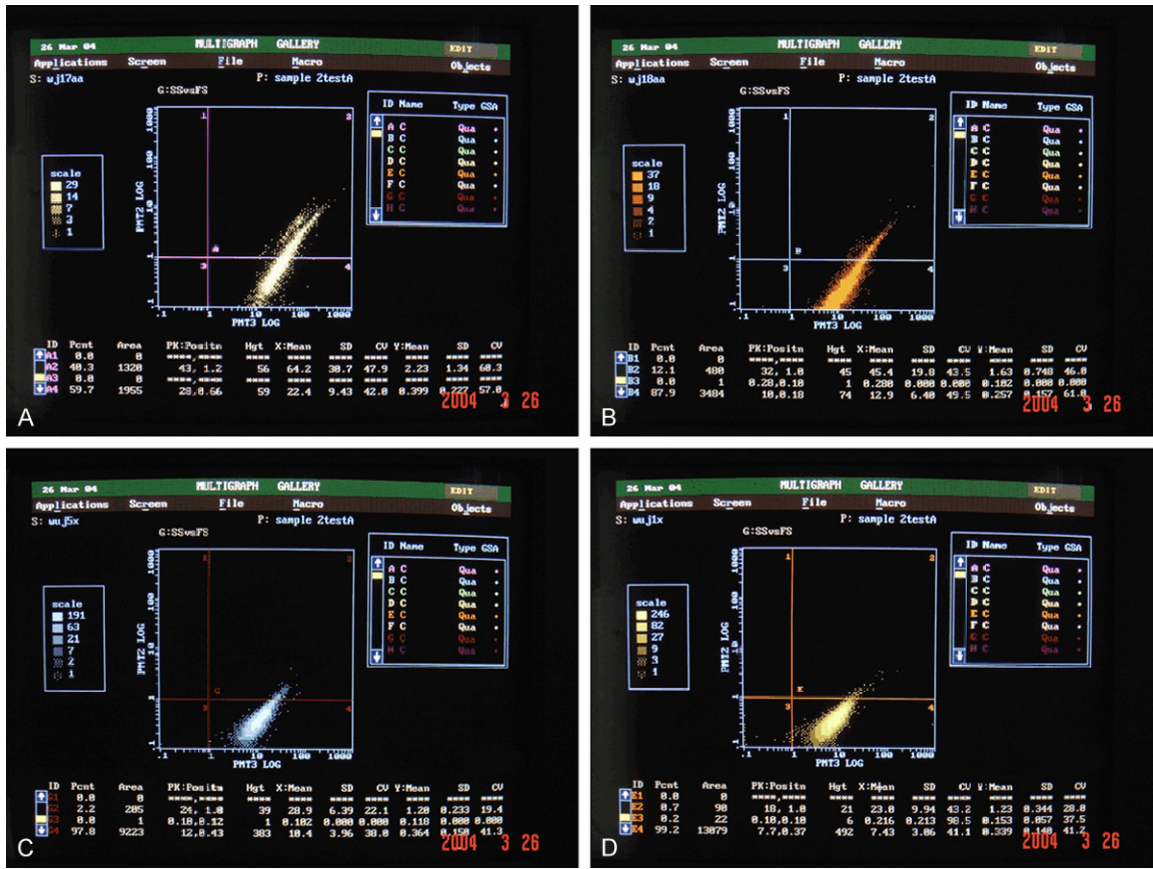
ALP activity was assessed by a commercial kit (IFCC Company); measuring optical density was at 405 nm. Results equaled to the amount of ALP dividing the amount of total protein. Osteoblastic rMSCs without materials were the control group.

At 7 d and 14 d, we collected the media from the wells and used the method of radioactive immunoassay (RIA) to test the osteocalcin. Two days prior to this assaying, 10<sup>-8</sup> M 1, 25 (OH) 2-VD3 was added to the media [13-15].

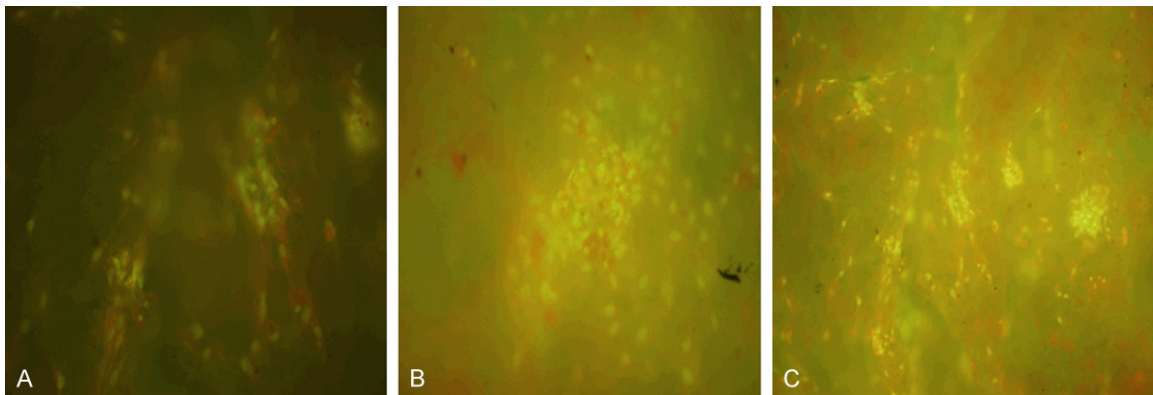
#### *Ectopic bone formation by implantation and degradation test in vivo*

All the animal experiments were approved by the Animal Research and Care Committee of the China. The mice were divided into three groups: rMSCs +  $\beta$ -TCP/PLLA, osteoblastic rMSCs +  $\beta$ -TCP/PLLA and  $\beta$ -TCP/PLLA without cells. All the disk scaffolds seeded with  $8 \times 10^5$  cells [16]. 24 Male nude mice (5 weeks old) were anesthetized and placed in a prone position. The bilateral thighs were accessed via a lateral approach and intramuscular pouches were created. 8 nude mice were implanted with rMSCs +  $\beta$ -TCP/PLLA in the left thighs and  $\beta$ -TCP/PLLA without cells in the right thighs. 8 nude mice were implanted with osteoblastic rMSCs +  $\beta$ -TCP/PLLA in the left thighs and the same treatments in the right thighs as the above. After 8 and 12 weeks, the mice were sacrificed and implants with the surrounding tissues were harvested together. Paraffin sections were got and HE stain and Masson-

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**Figure 1.** Rat bone marrow mesenchymal stem cells were CD44<sup>+</sup> and CD45<sup>-</sup> by FCM. A: rMSCsP0; B: rMSCsP1; C: rMSCsP3; D: rMSCsP5.



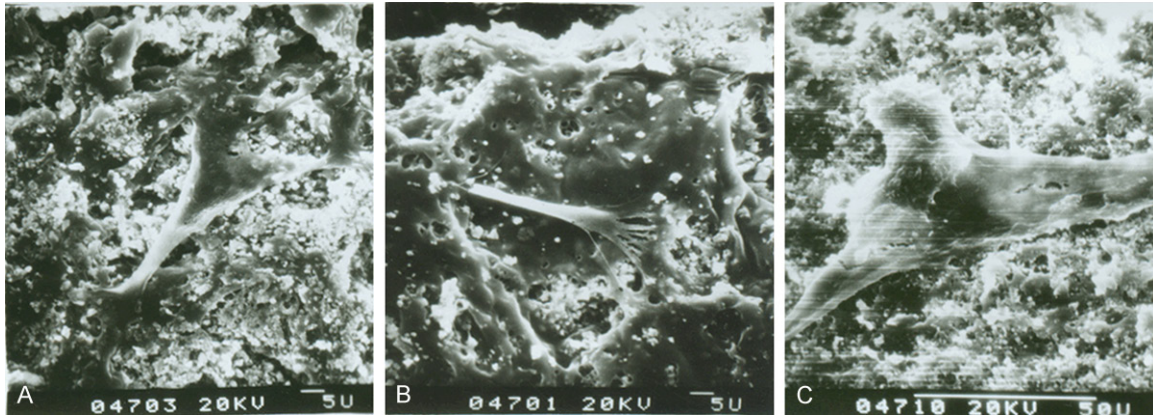
**Figure 2.** Fluorescence photomicrographs of osteoblastic rMSCs cocultured with  $\beta$ -TCP/PLLA of different ratio. 20 $\times$ . A:  $\beta$ -TCP/PLLA = 1:1; B:  $\beta$ -TCP/PLLA = 1:2; C:  $\beta$ -TCP/PLLA = 2:1.

Goldner stain were employed to observe the ectopic bone formation.

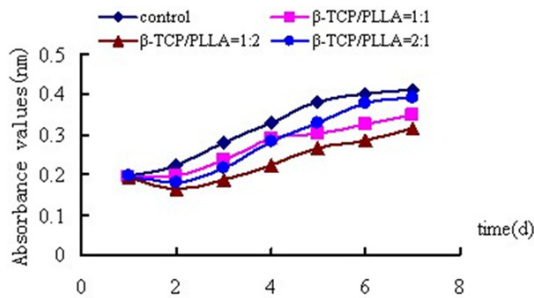
## Statistical analysis

The software SPSS 16.0 was used to do statistical analysis. The metering data was repre-

sented by mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). The comparison among multiple groups was analyzed with one-way ANOVA while the comparison between two groups was detected with LSD method, with  $P < 0.05$  considered statistically significant.



**Figure 3.** SEM of osteogenic rMSCs seeding on  $\beta$ -TCP/PLLA scaffolds for 4 d. A:  $\beta$ -TCP/PLLA = 1:1; B:  $\beta$ -TCP/PLLA = 1:2; C:  $\beta$ -TCP/PLLA = 2:1



**Figure 4.** Absorbance values of osteogenic rMSCs cultured on scaffolds with different ratio (Porosity = 60%, d = 100-400  $\mu$ m).

**Table 1.** ALP activity of osteogenic rMSCs on  $\beta$ -TCP/PLLA composite with different weight percent of porogen-NaCl ( $\beta$ -TCP/PLLA = 2:1, d = 200-450  $\mu$ m) ( $\bar{x} \pm s$ , n=8)

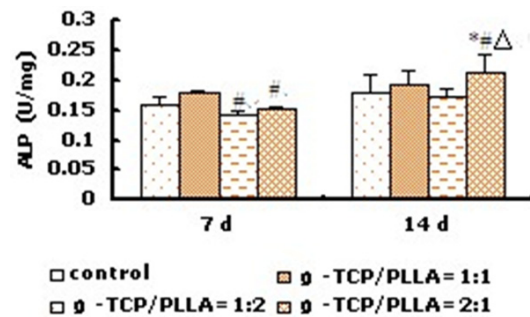
Groups	ALP (U/mg) 7 d	ALP (U/mg) 14 d
60 wt%	0.13 $\pm$ 0.01*	0.20 $\pm$ 0.03
70 wt%	0.15 $\pm$ 0.00	0.21 $\pm$ 0.03
80 wt%	0.10 $\pm$ 0.01**,#	0.13 $\pm$ 0.01*,#

\*P < 0.05, \*\*P < 0.01 vs NaCl = 70 wt%; #P < 0.05, ##P < 0.01 vs NaCl = 60 wt%.

## Results

### Study in vitro

Through observing the cells morphology and assaying the cells surface antigen by flow cytometry (FCM), we found that the cells were CD44 positive and CD45 negative (Figure 1), which were some characters of bone marrow mesenchymal stem cells [17-21].



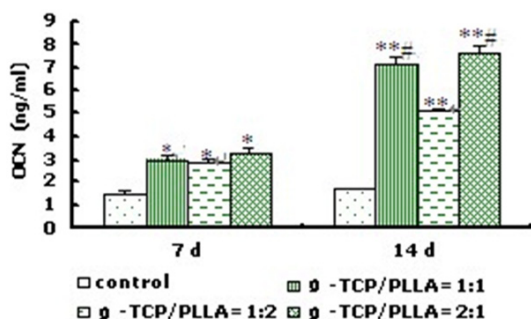
**Figure 5.** ALP activity of osteogenic rMSCs on  $\beta$ -TCP/PLLA composite with different ratio (Porosity = 60%, d = 100-400  $\mu$ m) (n=8). \*P < 0.05 vs corresponding control group;  $^{\Delta}$ P < 0.01 vs  $\beta$ -TCP/PLLA = 1:2; #P < 0.05 vs  $\beta$ -TCP/PLLA = 1:1.

**Table 2.** ALP activity of osteogenic rMSCs on  $\beta$ -TCP/PLLA composite with different pore size ( $\beta$ -TCP/PLLA = 2:1, porosity = 70%) ( $\bar{x} \pm s$ , n = 8)

Groups	ALP (U/mg) 7 d	ALP (U/mg) 14 d
d=200-450 $\mu$ m	0.15 $\pm$ 0.00	0.21 $\pm$ 0.03
d=450-900 $\mu$ m	0.15 $\pm$ 0.01	0.18 $\pm$ 0.01

Fluorescence microscope and SEM showed that osteoblastic rMSCs could attach and proliferate on the scaffolds with different ratio, different porosity and different pore size (Figures 2, 3). However, MTT assay showed that there were more osteoblastic cells grew on  $\beta$ -TCP/PLLA =2:1 after the 5th day (Figure 4; Table 1). On the other hand, the cell density increased significantly (P < 0.01) on  $\beta$ -TCP/PLLA = 2:1 compared with other ratio scaffolds (Figure 5; Table 2).

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**Figure 6.** OCN production of osteogenic rMSCs on  $\beta$ -TCP/PLLA composite with different ratio (Porosity = 60%, d = 100-400  $\mu$ m) (n=8). \* $P < 0.05$ , \*\* $P < 0.01$  vs control group; # $P < 0.05$  vs  $\beta$ -TCP/PLLA = 1:2.

**Table 3.** OCN production of osteogenic rMSCs on  $\beta$ -TCP/PLLA composite with different porosity ( $\beta$ -TCP/PLLA = 2:1, d = 200-450  $\mu$ m) ( $\bar{x} \pm s$ , n=8)

Groups	OCN (ng/ml) 7 d	OCN (ng/ml) 14 d
60 wt%	2.79 $\pm$ 0.11	6.21 $\pm$ 0.11*
70 wt%	3.26 $\pm$ 0.29	7.60 $\pm$ 0.32
80 wt%	2.47 $\pm$ 0.10*	4.88 $\pm$ 0.67*

\* $P < 0.05$  vs NaCl = 70 wt%.

Osteocalcin (OCN) production assay showed that all the composite could promote cell osteocalcin production which manifested that the compound had good osteoinductive. In detail, cells on  $\beta$ -TCP/PLLA = 1:2 produced less osteocalcin than that of  $\beta$ -TCP/PLLA = 1:1 and  $\beta$ -TCP/PLLA = 2:1 ( $P < 0.05$ ) (Figure 6). The cells on scaffold with NaCl = 70 wt% could produce more OCN than that with NaCl = 80 wt% (Table 3) and the cells on scaffold with the pore size = 200-450  $\mu$ m could produce more OCN than that with 450-900  $\mu$ m (Table 4).

### Study in vivo

Before the designated time points after surgery, all the animals had no inflammation. The scanning electron microscope (Figure 7) and histological study (Figure 8) found that at 8 weeks and 12 weeks post-implantation, there were few cells existed in the scaffolds which had not been seeded by the cells. In addition, there were some fibrous connective tissue surrounded the scaffolds. In rMSCs plus  $\beta$ -TCP/PLLA group, there were some cell interconnections forming honeycomb appearance. On the brink of the scaffolds, a great quantity cells aggregated and there were some zones of

**Table 4.** OCN production of osteogenic rMSCs on  $\beta$ -TCP/PLLA composite with different pore size ( $\beta$ -TCP/PLLA = 2:1, porosity = 70%) ( $\bar{x} \pm s$ , n = 8)

Groups	OCN (ng/ml) 7 d	OCN (ng/ml) 14 d
d=200-450 $\mu$ m	3.3 $\pm$ 0.29	7.6 $\pm$ 0.32
d=450-900 $\mu$ m	3.1 $\pm$ 0.16	6.6 $\pm$ 0.30*

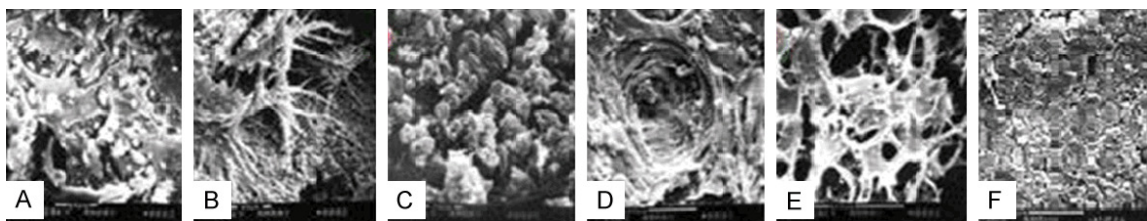
\* $P < 0.05$  vs d = 200-450  $\mu$ m.

necrosis. There were a few osteoblastic cells growing along with the pore. Few micro-capillary could be seen and the material was degraded slowly in this group. In the osteoblastic induced rMSCs +  $\beta$ -TCP/PLLA group, a layer of osseous precursor cells on the surface of the constructs, some cuboidal-shaped active osteoblasts were seen in the pores. Also, plenty of blood vessels were seen in the pores without fibrous tissue. At 12 weeks, some multinuclear cells and blood vessels were found and the material of the scaffold was degraded quickly in this group. Masson-Goldner staining showed collagenous structures on the surface of the constructs, which supported bone formation progressively.

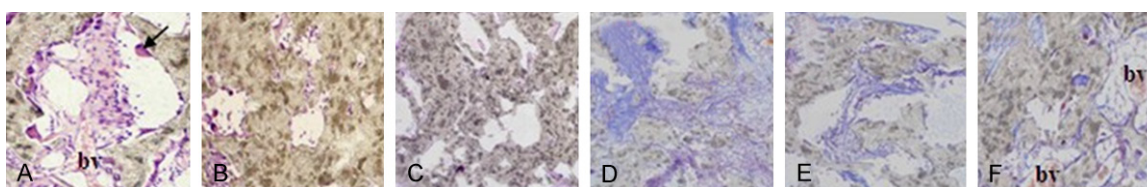
### Discussion

Ideal bio-active compound requires a number of factors: cells that can undergo differentiation to form osteoblasts; biological factors that control the growth and differentiation of these cells; and osteoconductive bioresorbable scaffolding matrices that promote cellular attachment, migration, and proliferation. Composite of  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) and poly L-lactic acid (PLLA) is widely used in bioengineering. However, optimal ratio, porosity and pore size of this kind of scaffolds were not very clear yet. In this study, we cultured osteoblastic induced rat bone marrow mesenchymal stem cells (rMSCs) on  $\beta$ -TCP/PLLA scaffolds to investigate the optimum construction, which owned better properties for supporting cells growth, proliferation and differentiation.

In studying in vitro, results found that the cells were CD44 positive and CD45 negative, which were some characters of bone marrow mesenchymal stem cells. The results suggested that the osteoblastic rMSCs came into proliferative phase, matrix synthesis phase and mineralization phase after 10, 14 and 20 days induced respectively. To evaluate the biocompatibility of



**Figure 7.** Scanning electron micrographic of Obi/scaffold, rMSCs/scaffold and scaffold without cells transplanted intramuscular of nude mice for 8 weeks and 12 weeks. Noted: Obi/scaffold (A: 8 weeks, 2000×) (D: 12 weeks, 600×); rMSCs/scaffold (B: 8 weeks, 2000×) (E: 12 weeks, 600×); scaffold without cells (C: 8 weeks, 2000×) (F: 12 weeks, 600×). nude mice for 8 weeks and 12 weeks. Noted: composite of osteogenic rMSCs and  $\beta$ -TCP/PLLA scaffold (A: 8 weeks, 200×, D: 12 weeks, 100×); composites of rMSCs and  $\beta$ -TCP/PLLA scaffold (B: 8 weeks, 200×, E: 12 weeks, 100×);  $\beta$ -TCP/PLLA scaffold (C: 8 weeks, 200×, F: 12 weeks, 100×), (ABC: HE stain; DEF: Masson stain), the arrow in A shows osteoclast and "bv" means blood vessels.



**Figure 8.** Histological section photographs of different groups transplanted intramuscular of nude mice for 8 weeks and 12 weeks. Noted: composite of osteogenic rMSCs and  $\beta$ -TCP/PLLA scaffold (A: 8 weeks, 200×, D: 12 weeks, 100×); composites of rMSCs and  $\beta$ -TCP/PLLA scaffold (B: 8 weeks, 200×, E: 12 weeks, 100×);  $\beta$ -TCP/PLLA scaffold (C: 8 weeks, 200×, F: 12 weeks, 100×), (ABC: HE stain; DEF: Masson stain), the arrow in A shows osteoclast and "bv" means blood vessels.

the scaffold with different technical parameter and to build new type bio-active scaffold, we chose passage 2 osteoblastic induced rMSCs as seeding cells in the latter study [10-12].

In studying in vivo, the scanning electron microscope and histological study found that at 8 weeks and 12 weeks post-implantation, there were few cells existed in the scaffolds which had not been seeded by the cells. In addition, there were some fibrous connective tissue surrounded the scaffolds. This phenomenon was in according with simple  $\beta$ -TCP or PLLA implantation [26-31]. In rMSCs plus  $\beta$ -TCP/PLLA group, there were some cell interconnections forming honeycomb appearance. In the osteoblastic induced rMSCs +  $\beta$ -TCP/PLLA group, At 12 weeks, some multinuclear cells and blood vessels were found and the material of the scaffold was degraded quickly in this group. Masson-Goldner staining showed collagenous structures on the surface of the constructs, which supported bone formation progressively.

The results showed that, 1. The osteoblastic rMSCs came into proliferation, matrix synthesis and mineralization phase respectively after 10, 14 and 20 days induction. 2. The scaffolds of

$\beta$ -TCP/PLLA = 2:1 could not only promote the cells proliferation, but also significantly increased osteocalcin production of the cells. In addition, scaffolds with NaCl = 70 wt%, pore size 200~450  $\mu$ m showed better compatibility to these seeding cells. 3. A significantly larger area of bone formation in the osteoblastic rMSCs and  $\beta$ -TCP/PLLA composite than that in rMSCs/scaffold and in the scaffold without cells in vivo. The results demonstrated that osteoblastic induced rMSCs and the scaffold with  $\beta$ -TCP/PLLA = 2:1, NaCl = 70 wt%, pore size = 200-450  $\mu$ m had good properties as a kind of bone substitute, which could form a bone-like tissue in vivo.

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#### Disclosure of conflict of interest

None.

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