



Effect of porous titanium coated with IGF-1 and TGF- β_1 loaded gelatin microsphere on function of MG63 cells

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Abstract: Porous titanium with porosity of 60% was prepared by metal injection molding (MIM), and coated with gelatin sustained-release microspheres which were made by improved emulsified cold condensation method. The effects of porous titanium coated with insulin-like growth factor-1 (IGF-1) and transforming growth factor- β_1 (TGF- β_1) gelatin microspheres on the function of MG63 cells were evaluated in vitro. The results show that porous titanium coated with gelatin sustained-release microspheres has no cytotoxicity. The IGF-1 and TGF- β_1 loading concentrations are positively correlative with the proliferation and differentiation of MG63 after co-culturing with the concentrations of IGF-1 and TGF- β_1 gelatin microspheres in the range of 0.1–10 ng/mg and 0.25–2.5 ng/mg, respectively. The MG63 cells exhibit the best proliferation and differentiation with the IGF-1 and TGF- β_1 loading concentrations of 10 ng/mg and 2.5 ng/mg, respectively. The joint application of IGF-1 and TGF- β_1 group, which promote adhesion, proliferation and differentiation of MG63 cells, is superior to a single application group.

Key words: porous titanium; gelatin microsphere; insulin-like growth factor-1; transforming growth factor- β_1 ; MG63 cell

1 Introduction

Implant denture has become a mainstream method for repairing missing teeth during last decade. However, most implants for clinical use are fully dense. The disadvantages of the dense structure need to be improved, such as lack of space to allow bone tissue to grow in, long healing stage, poor early stability, weak bonding strength. In consideration of the factors above, researchers concentrate on developing a new kind of implant with porous structure, which is beneficial to permitting bone tissue ingrowth and can act as a scaffold to adhere with bioactive coatings. As far as the mechanical properties are concerned, the porous titanium matches better with bone tissue than the dense one [1]. The modification of the implant surface is necessary to accelerate the osseointegration, achieve early weight bearing and finally boost the possibility of successful implant denture. One strategy is to prepare bioactive coating on the implant surface, hoping that it can be helpful in promoting interaction between an implant and

the surrounding environment, activating the signal channel for osteogenesis.

Osseointegration is quite a complicated process under the regulation of various growth factors. The previous studies have proved that the growth factors can stimulate the differentiation of mesenchymal stem cells into osteogenic cells, as well as increase the activity of alkaline phosphatase and the expression of osteopontin mRNA [2,3]. It is reported that the growth factors adsorbed on the surface of bone substitute can induce mesenchymal cells to differentiate into bone cells and promote the synthesis of collagen, which is both time and dose dependent [4–8]. Whether the individual or combined application of relevant growth factors on the implant surface can promote the peri-implant bone formation is to be investigated. Among the relevant factors, insulin-like growth factor-1 (IGF-1) and transforming growth factors- β_1 (TGF- β_1) have attracted the most attention. IGF-1 is secreted by osteoblasts and can facilitate the recruitment, proliferation and differentiation of osteoblasts in return. Moreover, it contributes to the synthesis of type I collagen and

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protects bone matrix from degeneration. TGF- β_1 acts on osteoblasts and plays a significant role in bone and cartilage regeneration [9,10]. Nevertheless, little is known about the best exposed time and the optimal concentrations of IGF-1 and TGF- β_1 , and the effect on osteogenesis while applying IGF-1 and TGF- β_1 in combination is not clear either. Growth factors can escape and degenerate easily in partial drug delivery, which accounts for the short half-life. Excessive input may cause toxicity and carcinogenesis. To overcome these deficiencies, a proper application of sustained release system is needed. Our research group has developed gelatin microspheres with sustained growth factor release by the improved emulsified cold condensation method. The drug release kinetics in vitro show that the releasing time is up to 14 d. By the internal gelatin coating method, the coating can be formulated spreading the surface of micropores in porous implant without blocking the pores [11].

The focus of this study is to develop a sustained IGF-1 and TGF- β_1 release system with optimal concentration using gelatin microspheres. Thus, the influence and possible mechanism of porous titanium coated with IGF-1 and TGF- β_1 loaded gelatin microspheres individually or in combination on proliferation, differentiation and adhesion of MG63 cells are investigated.

2 Experimental

2.1 Preparation of experimental material

2.1.1 Preparation of porous titanium

The experimental porous titanium with pore diameters of 50–300 μm and porosity of 60% was prepared using the metal injection molding technology [12]. The structure of porous titanium was interconnected. The porous titanium was ultrasonically cleaned in acetone, ethanol and deionized water for 10 min, respectively, and then dried at 60 $^{\circ}\text{C}$ in a clean oven. The specimens were sterilized at high temperature and high pressure for the following preparation.

2.1.2 Preparation of growth factor gelatin sustained-release microspheres

Gelatin sustained-release microspheres with diameters of 10–40 μm were prepared through improved emulsified cold condensation method [11]. The growth factor in gelatin sustained-release microspheres released 93% after 14 d. According to Ref. [13], each 1 mg gelatin microsphere was added with 5 μL of TGF- β_1 solution with concentrations of 5000, 500, 50 and 5 ng/mL and IGF-1 solution with concentrations of 20000, 2000, 200, 20 and 2 ng/mL, respectively,

swelling 24 h under the condition of 4 $^{\circ}\text{C}$ with the pH value of 7.4. Then the gelatin microspheres were centrifuged at 1200–1500 r/min for 15 min, cleaned with steaming water three times, dried below 4 $^{\circ}\text{C}$ and sterilized with cobalt-60 radiation.

2.1.3 Preparation of porous titanium coated with growth factor gelatin sustained-release microspheres

The porous titanium was soaked in 5% (mass fraction) gelatin solution with the negative pressure treatment for 10 min and dried at 50 $^{\circ}\text{C}$ for 24 h. 20 mg/mL IGF-1 and TGF- β_1 gelatin sustained-release microspheres were dissolved in ethanol to coat on the surface of porous titanium and dried at 4 $^{\circ}\text{C}$ for 6 h. Subsequently, the above specimens were immersed in 2.5% (mass fraction) glutaraldehyde solution for 30 min, cleaned with steaming water three times, dried below 4 $^{\circ}\text{C}$ and sterilized with cobalt-60 radiation.

2.2 In vitro analysis

Human osteoblast-like MG63 cells and mouse fibroblast cell line L929 were obtained from Cell Center of Xiangya School of Medicine, Central South University, Changsha, China. MG63 cells which were cultured in RPMI1640 (GibcoBRL Co.) were supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin at 37 $^{\circ}\text{C}$ in 5% CO_2 incubator. L929 cells were cultured under the same condition with MG63 cells. In this study, MG63 cells were used to evaluate the effect of different concentrations of IGF-1 and TGF- β_1 in gelatin microspheres on the behavior of cell proliferation and differentiation. L929 cells were used to evaluate the cytotoxicity of the sample with methylthiazolyldiphenyl- tetrazolium bromide (MTT) assay.

2.2.1 MTT assay

The samples of porous titanium coated with gelatin sustained-release microspheres (0.2 g/mL) were immersed in RPMI1640 media supplemented with 10% fetal bovine serum at 37 $^{\circ}\text{C}$ in 5% CO_2 incubator. After incubation for 72 h, the extraction medium was collected and saved in refrigerator at 4 $^{\circ}\text{C}$. L929 cells were seeded in 96-well plates at a density of 5000 cells per well and incubated for 24 h. The culture medium was replaced by 100% extraction in accordance with medium or 50% or 10% dilutions ($n=6$). The RPMI1640 medium with 10% phosphate buffer saline (FBS) acted as a negative control, and the culture medium containing 10 μL phenol acted as a positive control. After seeding for 3 d, the specimens were incubated with 10 μL MTT solution (5 mg/mL) at 37 $^{\circ}\text{C}$ for 4 h. Then, supernatant liquid was abandoned and 150 μL dimethyl sulfoxide was added to each well. The absorbance was measured using a microplate reader (Bio-Tek Co, USA) at 490 nm.

2.2.2 Optimizing concentrations of IGF-1 and TGF- β 1 in gelatin microspheres

1) Cell proliferation assay.

MG63 cells were seeded at a density of 5×10^4 cells/mL in 24-well plates. After incubated for 24 h, all the groups of gelatin microspheres were added to the corresponding wells. The cells were allowed to grow for 3, 7 and 14 d, respectively, then the culture media were removed to the eppendorf tubes. The MTT assay was used to assess the cell proliferation in accordance with the instructions. The absorbance was measured at 490 nm by using a microplate reader.

2) Cell differentiation assay.

The culture media were collected in eppendorf tubes at 3, 7 and 14 d. The alkaline phosphatase (ALP) activity of MG63 cells was assayed using an ALP kit in accordance with the protocol provided by the manufacture (NJJCBIO). The amount of ALP activity was calculated according to the standard curve.

3) Groups.

Each group was provided with three wells and 2 mg culture medium was added in it.

A: The concentrations of IGF-1 were 0.01, 0.1, 1, 10, 100 ng/mg, respectively.

B: The concentrations of TGF- β 1 were 0.025, 0.25, 2.5, 25.0 ng/mg, respectively.

C: The control group was gelatin microspheres without growth factor.

2.2.3 Effect of optimization of concentrations of IGF-1 and TGF- β 1 combined in gelatin microspheres on function of MG63 cells

The optimization of concentrations of IGF-1 and TGF- β 1 combined in gelatin microspheres was according to the above-mentioned methods for cell proliferation. Three groups were tested ($n=3$).

A: The optimal concentration of IGF-1 combined with TGF- β 1 gelatin microspheres with the concentrations of 0.025, 0.25, 2.5, 25.0 ng/mg, respectively.

B: The optimal concentration of TGF- β 1 combined with IGF-1 gelatin microspheres with the concentrations of 0.01, 0.1, 1, 10, 100.0 ng/mg, respectively.

C: The control group was gelatin microspheres without growth factor.

2.2.4 Comparison of individual use of IGF-1 or TGF- β 1 and combined use in gelatin microspheres on function of MG63 cells

The comparison of the optimized concentration of growth factor alone and combined in gelatin microspheres on the behavior of cell proliferation and differentiation was made with the same method as above. Four groups were tested ($n=4$).

A: The optimized concentration of IGF-1 gelatin

microspheres.

B: The optimized concentration of TGF- β 1 gelatin microspheres.

C: The optimized concentration of IGF-1 combined with TGF- β 1 gelatin microspheres.

D: The control group was gelatin microspheres without growth factor.

2.2.5 Effect of porous titanium coated with growth factor gelatin sustained-release microspheres on function of MG63 cells

1) Four groups were tested ($n=4$).

A: Porous titanium coated with the optimized concentration of IGF-1 gelatin sustained-release microspheres.

B: Porous titanium coated with the optimized concentration of TGF- β 1 gelatin sustained-release microspheres.

C: Porous titanium coated with the optimized concentration of IGF-1 combined with TGF- β 1 gelatin sustained-release microspheres.

D: The control group was porous titanium loaded on gelatin sustained-release microspheres without growth factor.

2) The cell proliferation assay was executed the same as above.

3) Cell differentiation assay.

The alkaline phosphatase activity was determined using the same method as above.

Osteocalcin assay: the culture media were collected in eppendorf tubes at 3, 7 and 14 d. The free osteocalcin released by MG63 cells was measured using a bone gla protein (BGP) enzyme-linked immunosorben assay (ELISA) kit. The assay was performed according to the directions provided by the manufacture (Purevalley Biotech, China). The radioactive count was measured by using a dosimeter and the amount of BGP activity was calculated referring to the standard curve.

4) Cell adhesion morphology assay.

MG63 cells were cultured with porous titanium samples for 7 or 14 d, respectively, the culture media were removed and the samples were washed three times with PBS to remove the excess media. The samples were fixated with 2.5% glutaraldehyde solution at 4 °C for 24 h and dehydrated in a graded series of ethanol. After air dried, the specimens were sputter-coated with gold, and the adhesion morphology of MG63 cells on the surface of porous titanium was examined using a scanning electron microscope (SEM) (JSM1230, Japan).

2.3 Statistical analysis

All data were expressed as mean \pm standard deviation. The statistical comparisons were carried out via one-way analysis of variance using SPSS 17.0

software (SPSS, USA). The differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Characterization of porous titanium coated with gelatin sustained-release microspheres

The representative SEM image of gelatin sustained-release microspheres is shown in Fig. 1(a). The microspheres have good dispersity, round shape and smooth surface. The particle size of the obtained microspheres is in the range of 5–40 μm and the average particle size is measured to be (20.33 ± 3.67) μm . As shown in Figs. 1(b) and (c), 20 mg/mL gelatin sustained-release microspheres are deposited in pore wall and small gap of porous titanium with no change of porous structure. Moreover, the porosity is still greater than 60% and the pore structure is not blocked.

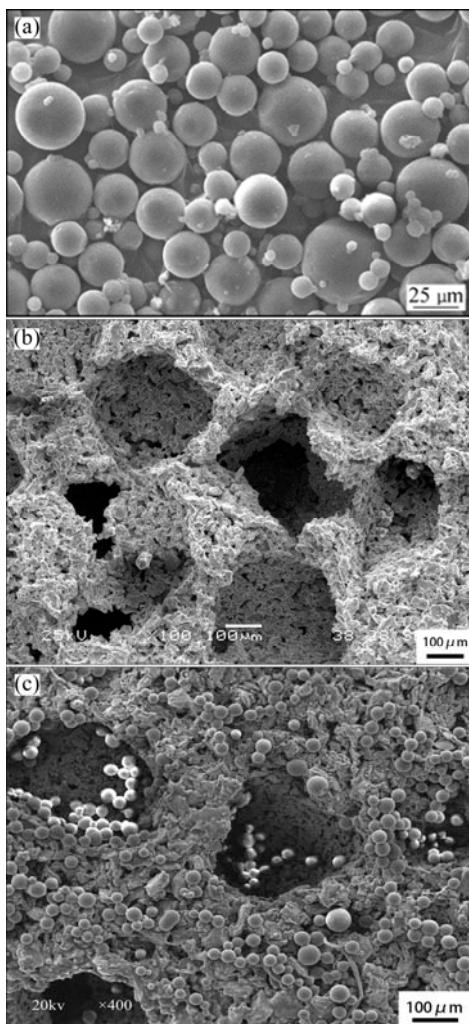


Fig. 1 SEM images of gelatin microspheres (a), porous titanium (b) and porous titanium coated with gelatin microspheres (c)

3.2 Cytotoxicity test

In this experiment, the cytotoxicity of the porous

titanium coated with gelatin sustained-release microspheres was examined by an MTT assay. L-929 cells were cultured in different extracts with concentrations of 100%, 50% and 10%. The optical density (D) value of positive control group was significantly lower than that of the experimental group, which reached a level of statistical significance ($P < 0.05$). The cell's relative growth rate (R) was calculated as follows: $R=D_{\text{test}}/D_{\text{negative}}\times 100\%$. The relative growth rates of the L-929 cells are shown in Table 1. It is shown that the relative growth rates of the cells in different extracts are almost similar to the negative control. There are significant differences between the relative growth rates of the cells in the extracts and those in the positive control after 3 d of culture ($P < 0.05$). According to the ISO 10993-5:1999 standard, the cytotoxicity of these extracts are grade 0–1, suggesting the non-cytotoxicity of porous titanium coated with gelatin sustained-release microspheres.

Table 1 Results of cytotoxicity tests of porous titanium coated with gelatin microspheres with L929 cells

Group	Concentration of extract	D	$R\%$	Cytotoxicity stage	$P^{[1]}$
Leaching liquor	100%	0.513 ± 0.094	97.34	1	<0.05
	50%	0.543 ± 0.219	103.03	0	<0.05
	10%	0.560 ± 0.020	106.26	0	<0.05
Negative liquor	–	0.527 ± 0.127	100.00	0	<0.05
Positive liquor	–	0.038 ± 0.014	7.2	4	–

[1] Compared with positive group

3.3 Effect of optimal of concentrations of IGF-1 and TGF- β 1 in gelatin microspheres on function of MG63 cells

The cell proliferation of MG63 on IGF-1 gelatin microspheres and TGF- β 1 gelatin microspheres with different concentrations were investigated with MTT assay after incubation for 3, 7 and 14 d, respectively. Figure 2(a) shows that the IGF-1 has effect on promoting the proliferation of MG63 cells which is positively correlative with the proliferation of MG63 cells in the range of 0.1 to 10 ng/mg. When the concentration of IGF-1 is 10 ng/mg, the IGF-1 gelatin microspheres have the most significant effect. Figure 2(b) shows that the cell proliferation increases gradually with increasing the concentration of TGF- β 1 in the range of 0.25 to 2.5 ng/mg. When the concentration of TGF- β 1 is above 2.5 ng/mg, the TGF- β 1 gelatin microspheres are not conducive to the proliferation of MG63 cells.

In order to evaluate the differentiation of MG63 cells under different concentrations of IGF-1 gelatin

microspheres and TGF- β 1 gelatin microspheres after culturing 3, 7 and 14 d, an ALP kit was used to estimate the differentiation. It can be seen from Fig. 2(c) that the effect on promoting the differentiation of MG63 cells alter with different concentrations of IGF-1 gelatin microspheres. When the concentration of IGF-1 is 10 ng/mg, the TGF- β 1 gelatin microspheres have better effect on promoting the differentiation of MG63 cells than other groups. Figure 2(d) shows that different concentrations of TGF- β 1 have different effects on the differentiation of MG63 cells, and the concentration of 2.5 ng/mg TGF- β 1 has a better effect on promoting the differentiation of MG63 cells than other groups.

3.4 Effect of optimal of concentrations of IGF-1 and TGF- β 1 combined in gelatin microspheres on function of MG63 cells

In order to optimize the combined concentrations of IGF-1 and TGF- β 1, the cell proliferation was evaluated with MTT assay. 10 ng/mg IGF-1 (A) combined with different concentrations of TGF- β 1 (B1: 0.025, B2: 0.25, B3: 2.5, B4: 25.0 ng/mg) and 2.5 ng/mg TGF- β 1 (B) combined with different concentrations of IGF-1 (A1: 0.01, A2: 0.1, A3: 1.0, A4: 10.0, A5: 100.0 ng/mg) were cultured with MG63 cells for 3 d, then the relative growth rate was calculated, respectively. Figure 3 shows

that the combination of IGF-1 and TGF- β 1 evidences a synergism of cell proliferation, particularly, 10 ng/mg IGF-1 combined with 2.5 ng/mg TGF- β 1 has better effect on promoting the cell proliferation than other concentration groups.

3.5 Comparison of IGF-1, TGF- β 1, IGF-1 and TGF- β 1 combination in gelatin microspheres on function of MG63 cells

Figures 4 and 5(a) show that the TGF- β 1 (2.5 ng/mg) (Group A), IGF-1 (10 ng/mg) (Group B), combined application of TGF- β 1 (2.5 ng/mg) and IGF-1 (10 ng/mg) (Group C) and gelatin microspheres without growth factor (Group D) samples were co-cultured with MG63 for 3, 7, and 14 d, respectively, and different effects on the cell proliferation were observed from the four groups. IGF-1 has better effect on the cell proliferation than TGF- β 1. Furthermore, the joint functions of two growth factors groups on promoting the cell proliferation are superior to that of a single application group.

The ALP activity was determined to evaluate the effect of samples on the cell differentiation. MG63 cells and 4 groups of samples were co-cultured for 3, 7, 14 d and tested by alkaline phosphatase assay kit. The ALP activity test results (Fig. 5(b)) suggested that with

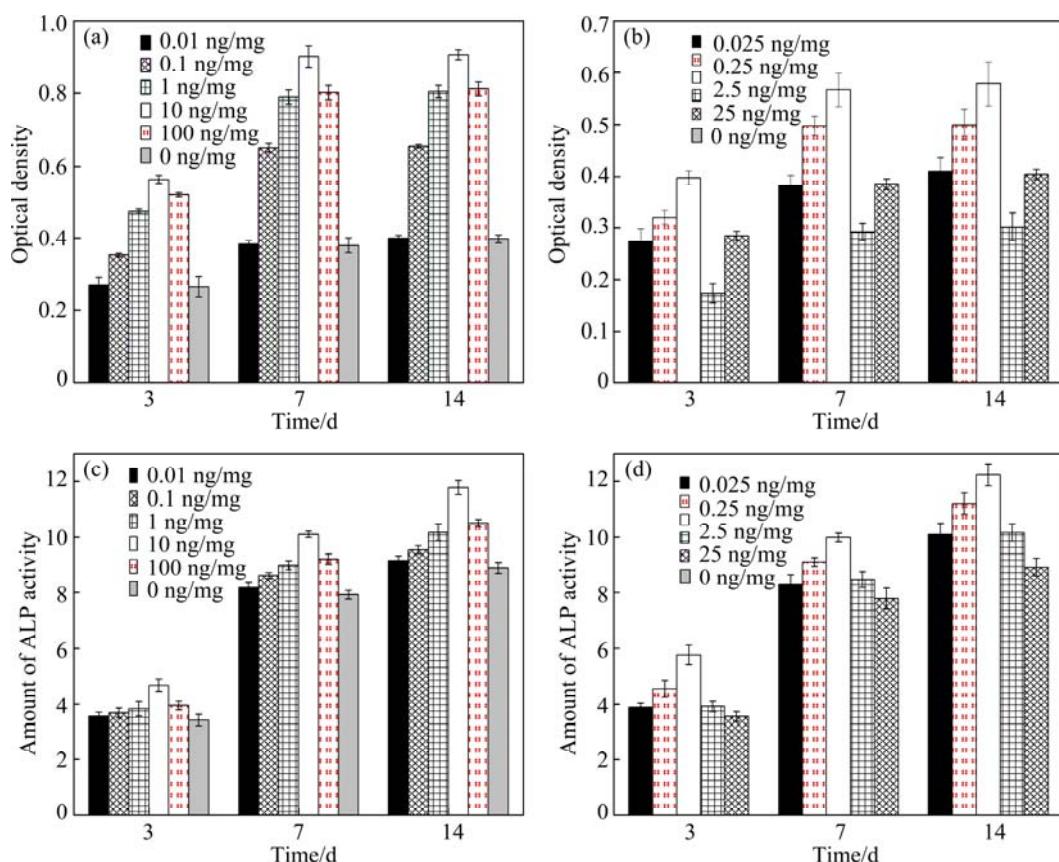


Fig. 2 Comparison of proliferation (a, b) and differentiation (c, d) of MG63 cells under different concentration of growth factors gelatin microspheres: (a, c) IGF-1; (b, d) TGF- β 1

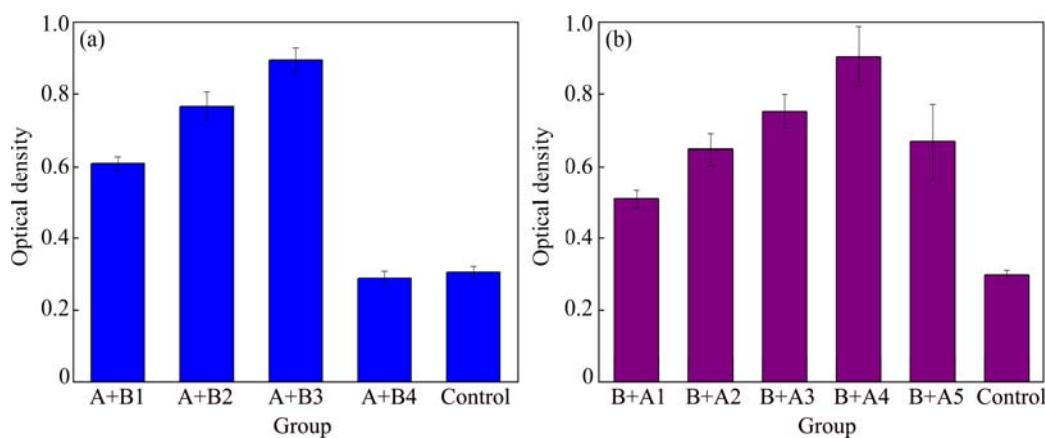


Fig. 3 Comparison of proliferation of MG63 cells under IGF-1 and TGF- β 1 combined in gelatin microspheres after culturing for 3 d: (a) IGF-1 combined with different concentrations of TGF- β 1; (b) TGF- β 1 combined with different concentrations of IGF-1

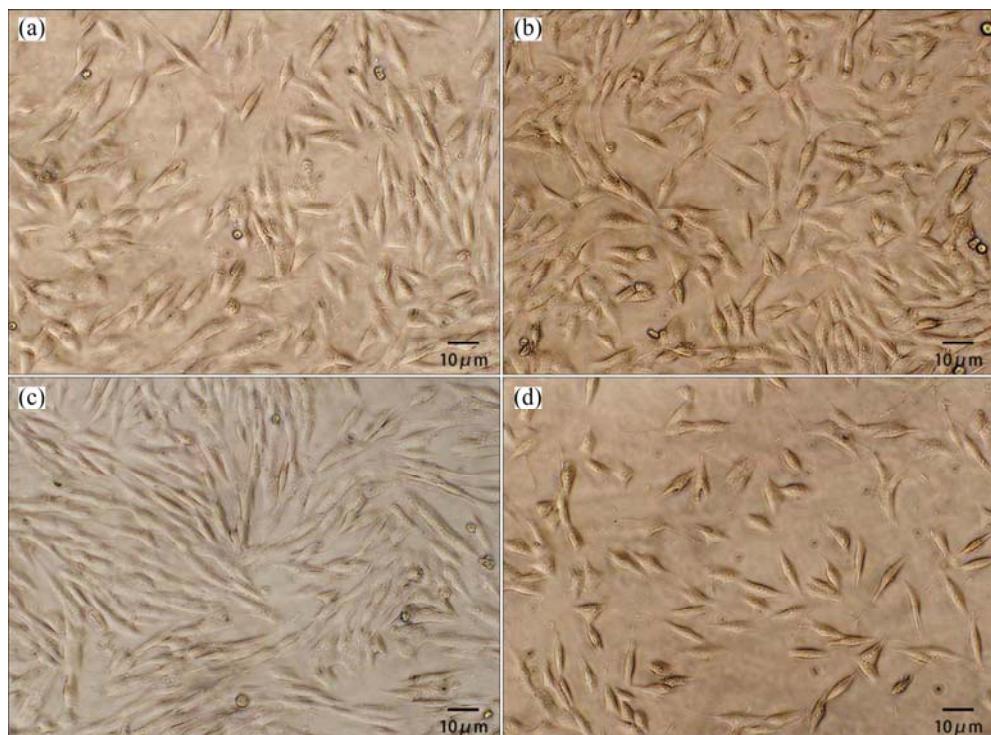


Fig. 4 Growth of MG63 cells in different growth factor groups after culturing for 7 d: (a) TGF- β 1 group; (b) IGF-1 group; (c) Combined group; (d) Control group

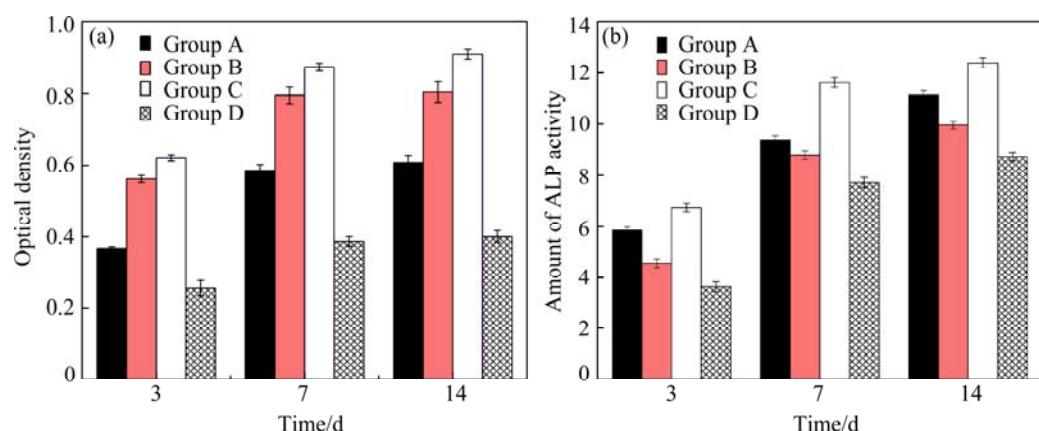


Fig. 5 Comparison of proliferation (a) and differentiation (b) of MG63 cells in different growth factors gelatin microspheres groups

increasing the culture time, the ALP activity of all groups increases. Among the four groups, TGF- β 1 has better effect on the cell differentiation than IGF-1, the joint functions of two growth factors group on promoting the cell differentiation are superior to a single application group.

3.6 Effects of porous titanium coated with growth factor gelatin sustained-release microspheres on function of MG63 cells

The porous titanium was coated with the growth factor gelatin sustained-release microspheres to prepare Groups A, B, C and D of samples. Four groups of samples were co-cultured with MG63 cells for 3, 7, 14 d and the cell proliferation was measured with MTT assay. Figure 6(a) shows a comparison of viable cell densities for different samples after 3, 7 and 14 d. The results indicate that different groups have different quantities of cell proliferation at the same time point. Along with increasing the incubation time, the quantity of cells increases. Moreover, the amount of cell proliferation is shown as Group C > Group B > Group A > Group D. That is to say, IGF-1 group has better effect on the cell proliferation than TGF- β 1 group, and the joint functions of two growth factors groups on promoting the cell proliferation are superior to IGF-1 group or TGF- β 1 group. There are statistically significant differences between each two groups ($P<0.05$).

The activities of ALP and BGP were detected using an alkaline phosphatase assay kit and a BGP ELISA kit, respectively after cultivating for 3, 7 and 14 d. As shown in Figs. 6(b) and (c), with increasing the co-culture time, the activity of all groups increases in this order: Group C > Group A > Group B > Group D. The coating with growth factors has the effect on promoting the differentiation of MG63 cells, the joint functions of IGF-1 and TGF- β 1 on promoting the cell differentiation are superior to a single factor. TGF- β 1 group has better effect on the cell differentiation than IGF-1 group, and there is statistically significant difference between the two groups ($P<0.05$).

The SEM observation reveals the MG63 cell attachment, growth and spreading on four different groups of samples' surfaces. Figure 7 shows that with increasing the incubation time, the amount of MG63 cells which adheres to the surface and pores of the samples increases. The surface morphologies of MG63 cells on four groups of samples after 7 d of culture are compared as follows. From Fig. 7(e), it can be seen that the cell numbers of Group C on the sample's surface increase much more than those of other groups, and the sample's surface is covered with cells which have become irregular shape with numerous filopodia extensions attached to the edge of pores. In addition,

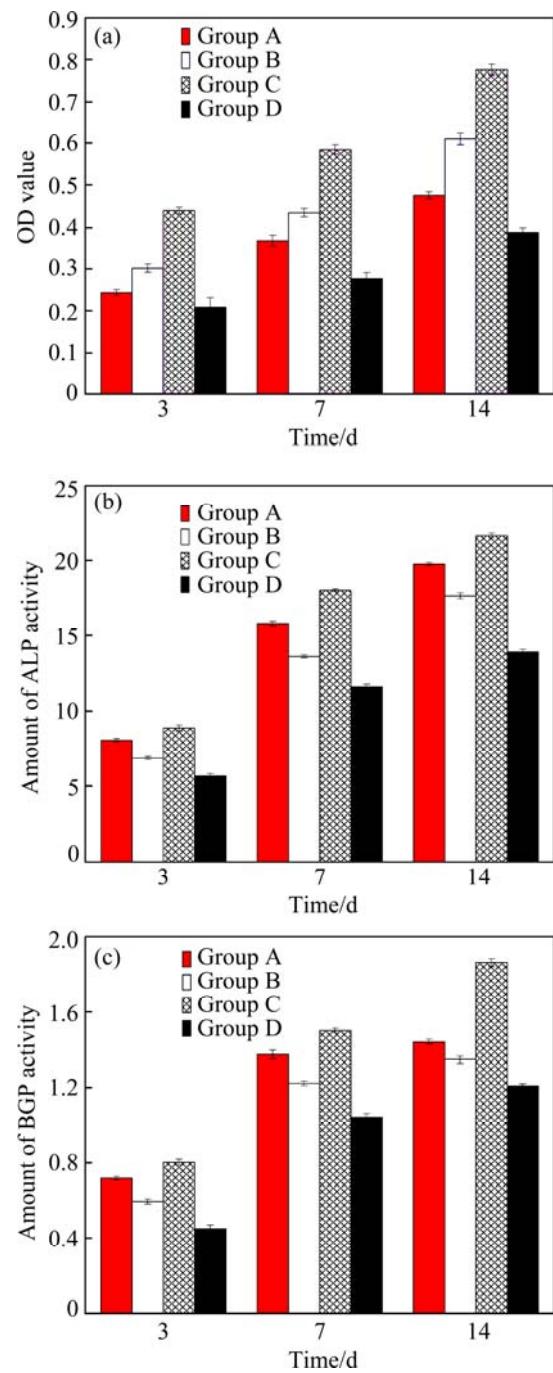


Fig. 6 Comparison of proliferation (a) and differentiation (b, c) of MG63 cells in different growth factors gelatin microspheres coated on porous titanium groups

some cells are found to migrate into the pores. MG63 cells which adhere to the samples of Group A and Group B well spread with its filopodia attached to the edge of pores (Figs. 7(a) and (c)). In contrast, relatively fewer MG63 cells are observed to adhere on the samples of Group D than that on other groups. The cells on the surface of Group D spread well and have diffused or under-developed filopodia, as shown in Fig. 7(g). After culturing for 14 d, the cells on the samples surface of Groups A, B and C become polygonal or irregular shape,

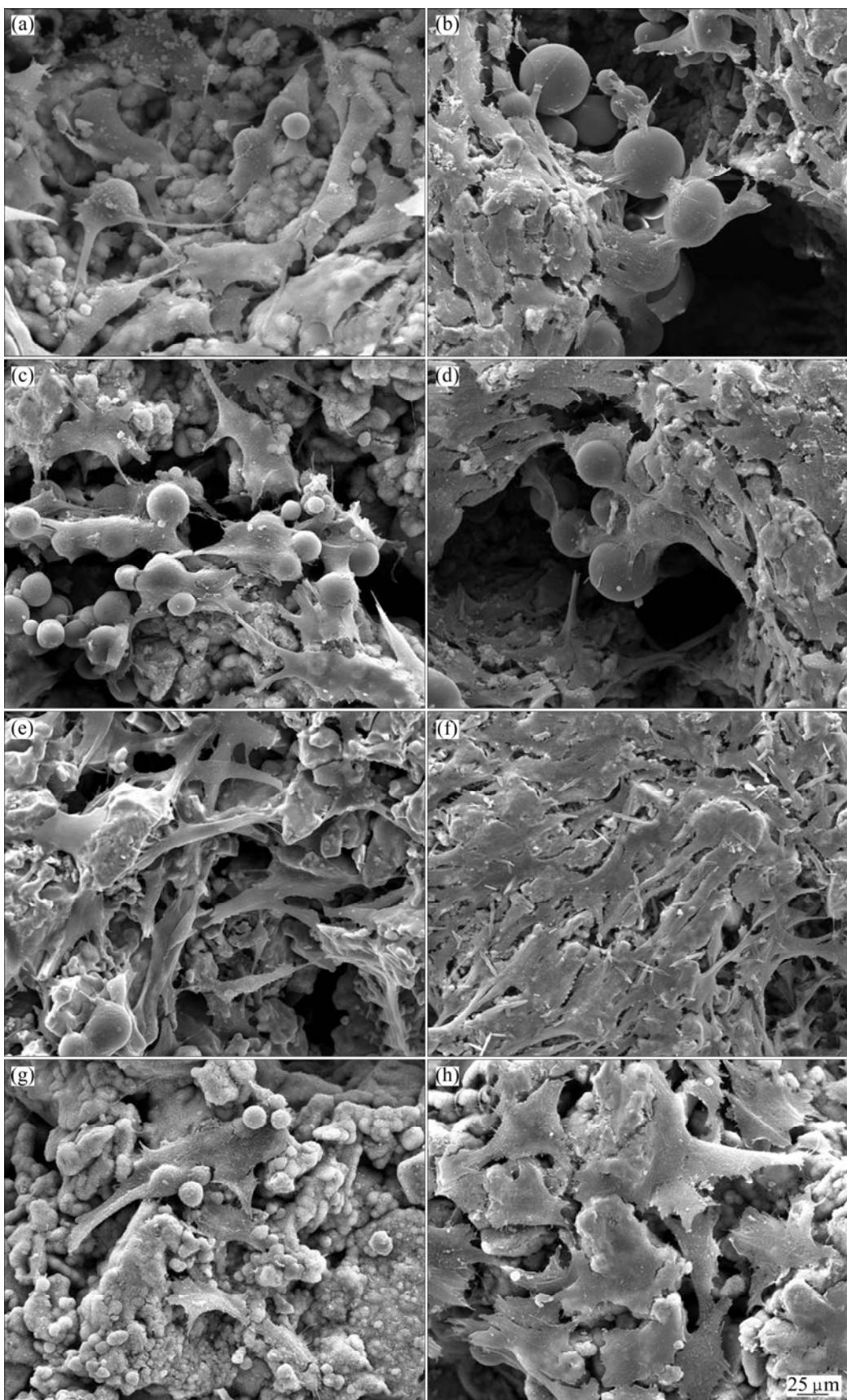


Fig. 7 Surface morphologies of MG63 cells cultured for 7 d (a, c, e, g) and 14 d (b, d, f, h) on different coated porous titanium samples: (a, b) Group A; (c, d) Group B; (e, f) Group C; (g, h) Group D

forming the cladding tile net-like or imbricate morphology covered on the pores and surface of samples, as shown in Figs. 7(b), (d) and (f). The adherent cells on the samples surface of Group D which depended on its filopodia form a complete cell layer (Fig. 7(h)). As shown in Figs. 8(a) and (b), the surface morphologies of MG63 cells on samples of Group C are netlike or imbricate in pores.

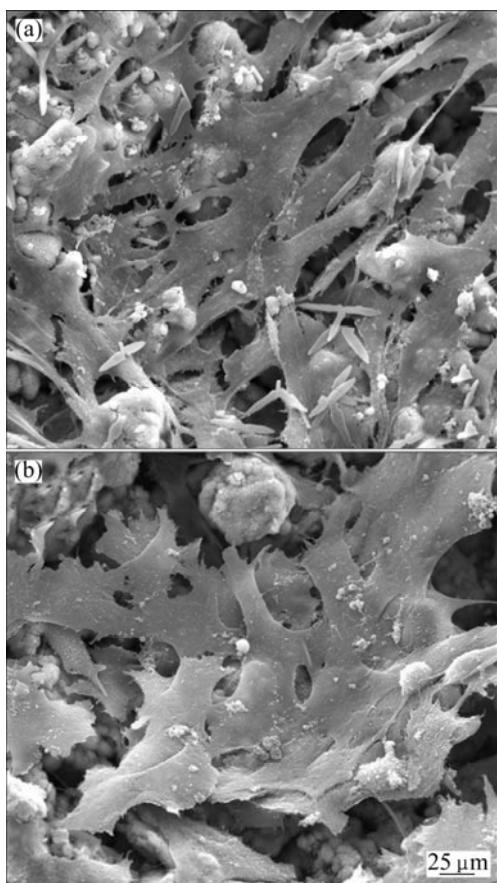


Fig. 8 Surface morphologies of MG63 cells cultured for 14 d on pores of porous titanium samples in Group C: (a) Netlike; (b) Imbricate

4 Discussion

The existing titanium implants are dense that can not provide a structure for bone ingrowth and achieve biological fixation. To enhance the biocompatibility, a number of coating methods have been applied on the implant surfaces, but the coatings are easily damaged and lost during implantation. In order to overcome the shortcoming, implant with porous structure on the surface, which can match the bone mechanical properties and provide supporting for the coatings, has been widely used. Because titanium is shortage of biological activity, the progress of osteointegration usually needs three to six months after implanted in bone. To improve the surface bioactivity and osteointegration after implant insertion

has become a hot topic in the research of new implant pattern.

The studies have suggested that growth factors, including mitogenic factor (such as IGF-1), enhancement factor of osteocyte activity (such as TGF- β_1) and osteoinductive factor (such as bone morphogenetic proteins (BMPs)) absorbed on the surface of bone substitute material, could promote the differentiation, matrix secretion and calcification of osteoblast [14]. IGF-1 and TGF- β_1 can increase osteoblast collagen protein and total protein synthesis, and enhance the expression of mRNA of type I collagen in osteoblast. BMP-2 is one of BMPs that can affect osteoblast directly, enhance the activity of alkaline phosphatase, and stimulate collagen synthesis. The application of one or several growth factors for bone-implant interface may enhance the activity of the osteoblasts, activate osteogenesis program instantaneously, and promote the formation of osteointegration. Growth factors have many deficiencies in the local application, including short biological half-lives, rapid spread and degradation under physiological conditions. What is worse, only when the concentration of growth factors reaches the threshold level can the corresponding response on cells be activated. In order to achieve controlled release of the growth factor in the local application, a slow release system is needed. The gelatin is easier to be identified by cell adhesion molecule with bioactive peptide [15]. In the previous studies, the gelatin microspheres were made by the improved emulsified cold condensation method, which made the releasing drugs time sustained up to 14 d in vitro. The sustained-release microspheres coating formed by infiltration method in pores of porous titanium cannot block the pore structure. This sustained-release system could make the application of growth factors in porous titanium implant possible. The concentration of TGF- β_1 has bidirectional effect on the osteoblast proliferation [16]. IGF-1 has a positive regulation effect on the proliferation activity of osteoblast, increasing the activity of ALP, and promoting the formation of bone and mineralization of bone matrix. In addition, the deficiency of IGF-1 in local tissues may cause bone loss [17,18]. In this study, sustained-release microsphere was used to enhance the level of growth factors in the local micro environment. Furthermore, the effects of porous titanium coated with IGF-1 and TGF- β_1 gelatin microspheres on the function of MG63 cells in vitro were evaluated.

To explore the combined effects and mechanism of different growth factors has become a hot spot in recent years. Recent studies have shown that the combination of platelet derived growth factor (PDGF) and IGF-1, vascular endothelial growth factor (VEGF) and BMP, BMP and TGF- β_1 , fibroblast growth factor (FGF) and

TGF- β_1 has synergistic effect [19–21]. This study finds out that the partial application of IGF-1 and TGF- β_1 has a close association with the concentration of growth factors for the proliferation and differentiation of MG63 cells. The concentration is positively correlated with the proliferation and differentiation of MG63 when the concentration of IGF-1 loaded gelatin microspheres ranges from 0.1 to 10 ng/mg, and the effect reaches the peak when the density is 10 ng/mg. Promoting the proliferation and differentiation has a positive relationship with the concentration when the concentration of TGF- β_1 loaded gelatin microspheres ranges from 0.25 to 2.5 ng/mg, and the peak reaches 2.5 ng/mg. When the density exceeds 2.5 ng/mg, the proliferation is restrained but the differentiation is facilitated. The effect of combined application of two growth factors at their best loaded density surpasses the individual application of IGF-1 or TGF- β_1 in the aspect of facilitating proliferation and differentiation of MG63 cells. One of the possible reasons why the effect of combined application of IGF-1 and TGF- β_1 is better than that of single application is that there is an interaction between the two growth factors which can increase the synthesis and release of endogenous growth factors of osteoblast. Studies have shown that TGF- β_1 could induce osteoblast to produce IGF-1 [22], the combined effect of them facilitated the biological effect of osteoblast. Another possibility is that the combination of two factors generates the conveying of correspondent signal conduction. Regulating and controlling function is enhanced due to the interaction of signal conduction. IGF-1 promotes the proliferation and differentiation of osteoblast mainly through PI3K/Akt pathway, TGF- β_1 promotes the phosphorylation of Akt quickly by Smad2/3 independent method, thereby activating PI3K/Akt pathway. They have interaction between the intracellular signaling pathways and could strengthen the biological effects of osteoblast. At the same time, IGF-1 activates the interactions between Akt or PI3K and Smad3, and prevents its phosphorylation and verification, and then interdicts the effect of TGF- β_1 induced cell apoptosis and growth inhibition [23]. The possible reason for that the effect of facilitating proliferation and differentiation of combined application of IGF-1 and TGF- β_1 is not as good as the sum effect when they are applied separately is related to the common receptor on the cell surface. When the number of cells reaches at one certain point, the two growth factors compete for the surface receptor, which results in the defective function of all the factors [24]. The synergy effect between different growth factors does not mean that the combined effect of single factors additions. Because the interaction adjustment mechanism between different growth factors exists, and thus makes the significance of joint application of

growth factors not only focus on increasing its functional intensity. The network control effect between growth factors is more important that makes its biological effects of osteoblast more perfect, conducive osteoblast proliferation and differentiation, and promotes the bone formation.

The cell adhered to the extracellular matrix is the initial phase of cell tissue. After cells adhere to the material interface properly, cell migration, proliferation and differentiation occur [25,26]. The surface chemical composition, rough surface structure, surface hydrophilicity, surface charge and surface energy can affect the adhesion of osteoblast. In this study, a porous titanium which has interconnected pores with a size of 50–300 μm was made, allowing cells to grow and to proliferate and differentiate. The obtained results for cell adhesion morphology show that the cell number and morphology of gelatin microspheres loaded with growth factor samples are better than those of uncoated samples. The coating, conducive to cell adhesion, can have three origins. Firstly, the gelatin containing RGD bioactive short-chain peptides could mediate cell adhesion [27]. Secondly, the coatings increase the surface area and surface energy of the sample which would benefit cell adhesion. Thirdly, the coating sustains the release of growth factors which could keep the effective concentration of growth factor around the sample. That is conducive to cell proliferation and differentiation, so the number of cells adherent to the sample surface will increase at the same period of time. In our study, the porous structure of titanium sample could provide good environment for cell adhesion, and the coating loading the growth factor on the gelatin microspheres could sustain the release of growth factors, that could promote cell adhesion, proliferation and aggregation on the sample surface. In the meantime, studies have shown that the high density of cells could induce cell differentiation rapidly, and the morphology change of cells could also promote cell differentiation [28–30].

5 Conclusions

1) The IGF-1 loading concentration is positively correlative with the proliferation and differentiation of MG63 in the range of 0.1–10 ng/mg. The MG63 cells exhibit the best proliferation and differentiation with the IGF-1 loading concentration of 10 ng/mg.

2) The TGF- β_1 loading concentration is positively correlative with the proliferation and differentiation of MG63 in the range of 0.25–2.5 ng/mg. The MG63 cells exhibit the best proliferation and differentiation with the TGF- β_1 loading concentration of 2.5 ng/mg.

3) IGF-1 has better effects on promoting cells' proliferation than TGF- β_1 , while TGF- β_1 has better

effect on promoting cells' differentiation than IGF-1, showing that there is a different effect on MG63 cells functions. The joint application of IGF-1 and TGF- β 1 group which promote adhesion, proliferation and differentiation of MG63 cells is superior to a single application group. The joint functions of IGF-1 and TGF- β 1 have a synergistic effect.

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载 IGF-1 与 TGF- β_1 明胶微球涂层的 多孔钛对 MG63 细胞功能的影响

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摘要: 用金属注射成形(MIM)技术制备孔隙度为 60%的多孔钛, 用改良冷凝聚合交联法制备明胶缓释微球并涂覆于多孔钛表面, 体外细胞评价胰岛素生长因子-1(IGF-1)、转化生长因子- β_1 (TGF- β_1)明胶缓释微球涂层多孔钛对 MG63 细胞功能的影响。结果表明: 明胶缓释微球涂层多孔钛无细胞毒性; 当 IGF-1、TGF- β_1 明胶缓释微球的载药浓度分别在 0.1~10 ng/mg 和 0.25~2.5 ng/mg 范围内时, 与 MG63 细胞共培养, IGF-1 和 TGF- β_1 的载药浓度与细胞的增殖和分化呈正相关; 当微球的载药浓度 IGF-1 为 10 ng/mg, TGF- β_1 为 2.5 ng/mg 时, MG63 细胞具有最优的增殖和分化; IGF-1 和 TGF- β_1 联合应用对 MG63 细胞的黏附、增殖与分化作用明显优于单一应用。

关键词: 多孔钛; 明胶微球; 胰岛素样生长因子-1; 转化生长因子- β_1 ; MG63 细胞

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