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Induced Multipotent Stem Cells Combining with HAP/TCP/CS Composite Scaffold Repairs Bone Defect

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Abstract: The treatment of bone defect is the multi-disciplinary clinical problem. Although, several types of bone graft materials are used for clinical study, numerous hurdles such as infection and immune rejection need to be resolved in essence. Various types of stem cells combining with biological material for repairing bone defect is a promising way. However, the poor resource of stem cells has restricted their clinical application. In earlier research, researchers had reported that transient *in vitro* epigenetic reprogramming of mouse skin fibroblasts into multipotent stem cells. In this study, researchers used the similar method to reprogram dog skin fibroblasts into multipotent stem cells. Further, reprogrammed cells were re-differentiated into osteogenesis-like cells *in vitro*. Especially, iMS cells combining with Hydroxyapatite/β-Tricalcium Phosphate/Chitosan (HAP/β-TCP/CS) Composite Scaffold could repair bone defect in dog. The results indicated that the iMS could be used as seed cell for repairing bone defect in clinical application.

Key words: Induced multipotent stem cells, scaffold, cell hybrid material, bone defect, repairin

INTRODUCTION

The repairing of large bone defect in trauma, infection, tumor, pathology not only becomes the focus on the clinical research but also represents a big challenge to surgical. The bone tissue repair is a complex regeneration engineering and has special demands for bone-repairing materials. Therefore, choosing suitable material is vitally important for the success of repairing bone defects (Burg *et al*., 2000). At present, bone graft materials have been successfully used to the clinical study including autologous bone, allograft bone and various synthetic bones.

However, there are still some obvious limitations for autologous bone graft in practical applications such as infection, pain, donor site hematoma as well as associated donor site morbidity (Kessler *et al*., 2005; Younger and Chapman, 1989). In addition, in most cases, the amount of autologous bone can not meet the requirements of bone graft. On the other hand, negative effects are sometimes inevitable in allograft bone such as disease transmission (such as HIV, hepatitis virus, etc.), immune rejection, ethics and religion (Buck *et al*., 1989; Simonds *et al*., 1992). Moreover, to reduce the antigen, the allogeneic bone need to be frozen and freeze-dried which will damage the biological properties of allograft bone.

With the development of modern medicine, autologous and allograft bone are gradually replaced by artificial bone graft (Du *et al*., 1998; Ajaal and Smith, 2009; Deie *et al*., 2008). Artificial bone materials as scaffold can keep the original structure at the site of bone defect and play a supportive role for bone transplanting. However, the artificial bone materials have poorer biocompatibility. Bone tissue engineering establishes a new field of promising regenerative medicine for repairing bone defect. It is important to choose good seed cells and scaffold materials. Various types of stem cells have been considered as potential seeds for bone tissue engineering. However, the poor resource of stem cells has restricted their clinical application. The group have reported that skin fibroblasts were reprogrammed into induced Multipotent Stem (iMS) cells by short-time exposure of fish egg extracts. No any virus carriers were involved in the inducing process. iMS cells were bale to be re-differentiated into various types of tissue cells under specific inducing conditions *in vivo* and *in vitro* such as osteoblasts, muscle and nerves (Zhu *et al*., 2010).

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Several studies have demonstrated that Hydroxyapatite/Beta-Tricalcium Phosphate (HAP/β-TCP) composite was a kind of useful biological scaffold in tissue engineering. However, because the physical properties of HAP and β-TCP are both crisp, two kinds of materials are only used at non-stress bearing area (Harada, 1989; Rao *et al*., 1997). How to enhance the toughness of HAP/β-TCP and lengthen service life are current questions. It is interesting to note that Chitosan (CS) can both enhance mechanical strength of composition scaffold and provide suitable structure for osteogenic differentiation (Kim *et al*., 2008). HAP/β-TCP/CS composition scaffold not only had good mechanical properties but also biocompatibility to promote repairing injury tissue.

In this study, researchers used the similar method to reprogram dog skin fibroblasts into multipotent stem cells. Further, reprogrammed cells were re-differentiated into osteogenesis-like cells *in vitro*. Especially, iMS cells combining with HAP/β-TCP/CS composite scaffold could repair bone defect in dog. Researchers established an animal model of repairing bone defects by iMS cells combining with HAP/β-TCP/CS composite scaffolds.

MATERIALS AND METHODS

Preparation of fish oocyte extracts: Crucians were acquired from local fish production farms. Collecting fish oocytes under sterile condition were grinded using a mortar and pestle. The mixture was dissolved in an equal volume of 0.9% sodium chloride. Then, the mixture was centrifuged at a speed of 500×g for 10 min followed 1500×g for 10 min. Supernatants were collected and filtered using 0.22 mm filters. Protein levels of the extracts were detected as earlier described (Roobrouck *et al*., 2011; Lee *et al*., 2011). The extracts were diluted to 10 mg protein mL^{-1} using DMEM/F12 medium and stored at -80°C.

Cell reprogramming of dog skin fibroblasts *in vitro*: Exponentially growing fibroblasts were digested with trypsin-EDTA (Invitrogen, CA) and 4×10^5 cells were seeded in 6 well plates. Fibroblasts were directly reprogrammed into iMS cells by 72 h exposure of 2.4 mg mL^{-1} fish oocyte extracts without cell membrane permeabilization.

Immunohistochemical staining: Reprogrammed cells were fixed by 4% paraformaldehyde and washed with PBS three times for 5 min each. Cells were incubated with 0.3% Triton X-100 for 5 min. Endogenous peroxidase activity was blocked using 3% H_2O_2 for 5 min. The fixed cells were incubated with anti-dog-SSEA-4 (1:100) at 4°C for 12 h. Cells were incubated with Horseradish Peroxidase (HRP)-labeled (Gene Tech. Company Limited, Shanghai) at 37°C for 20 min. Cells were stained with DAB working solution according to instruction manual (MaiXin Bio-company, Fuzhou) and observed by optical microscope.

Flow cytometric analysis: Suspended cells (1×10^6) were fixed with 100 μL of 4% paraformaldehyde for 10-15 min. Cells were then incubated with 0.5% saponin solution (100 μL) at room temperature for 15 min. Cells were incubated with SSEA-4-FITC antibodies (LifeSpan BioSciences, USA) at room temperature for 30 min after washed twice with PBS/BSA buffer containing 0.5% saponin. Isotype controls were stained with an isotype-matched controls. Quadrant markers based on isotype controls and unstained cells were set. Data were acquired using FACSCalibur flow cytometer and analyzed with CELLQuest Software.

iMS cells differentiation into osteogenesis-like cells *in vitro*: The reprogrammed cells were differentiated into osteogenesis-like cells *in vitro* by modified StemPro® Osteogenesis Differentiation kit. After 14 days of cultivation, cells were fixed by 4% formaldehyde solution for 30 min. The fixed cells were stained with 2% Alizarin S Red S solution (pH 4.2) for 2-3 min after rinsed twice with distilled water.

Scaffold fabrication: Porous HAP/β-TCP scaffold was produced by foam impregnation method. The impregnation slurry was prepared with micro-HAP powder. The impregnated foams were fried from 700-1270°C. Chitosan was dissolved in acetic acid and poured on the surface of HAP/β-TCP foams. The acetic acid was removed with NaOH solution.

Degradation rate of scaffold *in vitro*: The HAP/β-TCP/CS scaffold immerged into PBS which was incubated with 5% CO_2 at 37°C. The PBS was changed twice a week. The degradation rate was analyzed as following the equation (Tomihata and Ikada, 1997):

$$\text{Degradation rate} = \frac{M_0 - M_1}{M_0} \times 100\%$$

Where:
M_0 = The original saturated wet weight of the scaffold
M_1 = The saturated wet weight after immersion

Dog Bone Defects Model: Dog was anesthetized with 3% pentobarbital sodium. Skin and subcutaneous tissues of surgical site were cut to expose the tibia and fibula. The

20 mm bone defect was made by stainless steel saw and the periosteum was removed together with bone. Then, scaffolds-cells complex was transplanted into the defect site. Ten healthy dogs were prepared from Kunming Animal Institute (Yunnan, China). All animal studies were approved by the Animal use Committee of the hospital.

RESULTS

Scaffold characterization: The microstructure and macrostructure of the 3D porous scaffolds were performed using Scanning Electron Microscope (SEM). The results showed that there was a rough surface, high ratio of porosity and irregular macroporosity in HAP/β-TCP composite scaffold (Fig. 1a and b). 3D interconnected porous were formed by the process of heating organic foaming agent. When Chitosan (CS) was integrated into HAP/β-TCP composite scaffold, the result showed that the surface of HAP/β-TCP composite scaffold was covered with chitosan. Mostly, the distribution of pore diameter of HAP/β-TCP/CS was from 50-100 μm (Fig. 1c). Figure 1d was the amplification of the red arrow area in Fig. 1c. Although, the SEM images showed that the macroporosity were covered by the formed membrance by chitosan, the structure remained opening. Therefore, the formed membrane by chitosan did not affect the porosity of the scaffolds.

Pore diameter analysis of scaffold: The distribution of pore diameter of HAP/β-TCP composite scaffold was from 50-400 μm but mostly distributed between 100 and 300 μm (Fig. 2a). When CS covered the surface of HAP/β-TCP composite scaffold, the distribution of pore diameter was from 50-100 μm that exactly meet with the size of stem cells (Fig. 2b). Pore diameter of HAP/β-TCP/CS composite scaffold was suitable for the proliferation and adhesion of stem cells.

Degradation rate of composite scaffolds *in vitro*: The degradation of composite scaffolds mainly included two stages. The first stage was that CS was completely degraded and HAP was slowly partly degraded within 9 weeks. The second stage was that β-TCP was slowly degraded within ten to twelve weeks. The degradation rate of HAP/β-TCP/CS composite scaffold was obviously faster than that of HAP/β-TCP that mainly was due to high degradation rate of CS. The total degradation rate of HAP/β-TCP/CS scaffold *in vitro* was about 54% at the 12th week (Fig. 3).

Expression of stem cell marker genes: The dog skin fibroblasts were reprogrammed into iMS cells by short-time exposure of fish oocyte extracts. Immunohistochemical staining was performed to detect

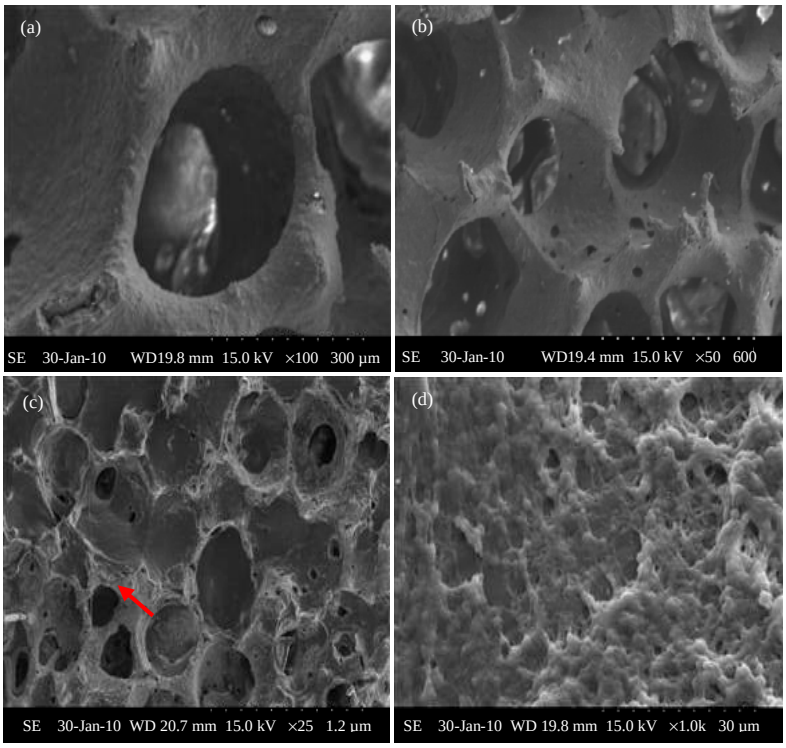

Fig. 1: Structure of composite scaffolds

expression of Nanog proteins related to cell pluripotency. Cells were stained with Nanog antibody. The results showed that Nanog was not expressed in untreated skin fibroblasts as control (Fig. 4a). In contrast, stem cell markers Nanog was activated in reprogrammed cells (Fig. 4b).

The results of flow cytometric analysis showed that the expression of pluripotent cell marker SSEA-4 protein increased in the treated skin fibroblasts with fish oocyte extracts (Fig. 4d). In contrast, the expression of SSEA-4 showed negative in untreated the skin fibroblasts (Fig. 4c).

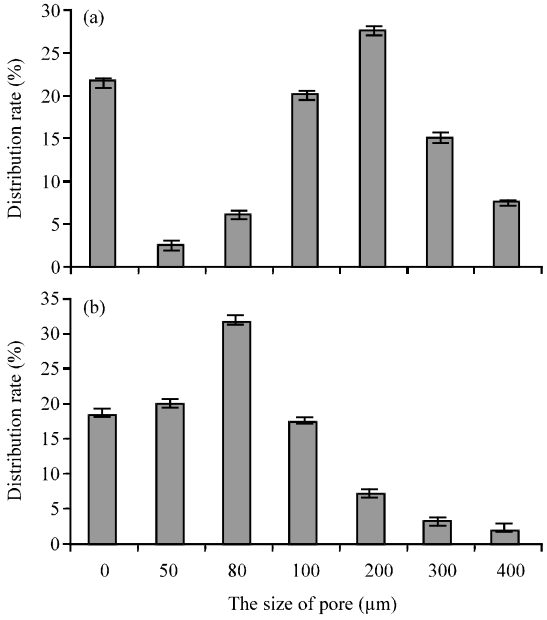

Fig. 2(a, b): Pore diameter distribution of scaffolds

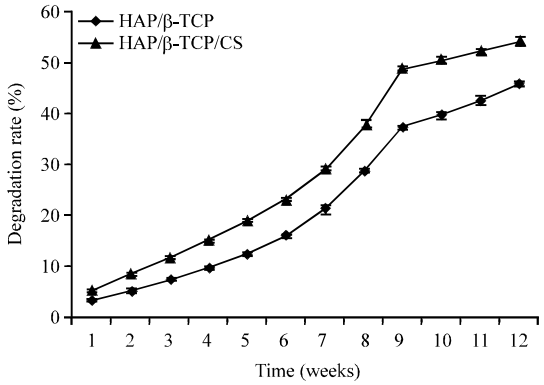

Fig. 3: Degradation rate of composite scaffold *in vitro*

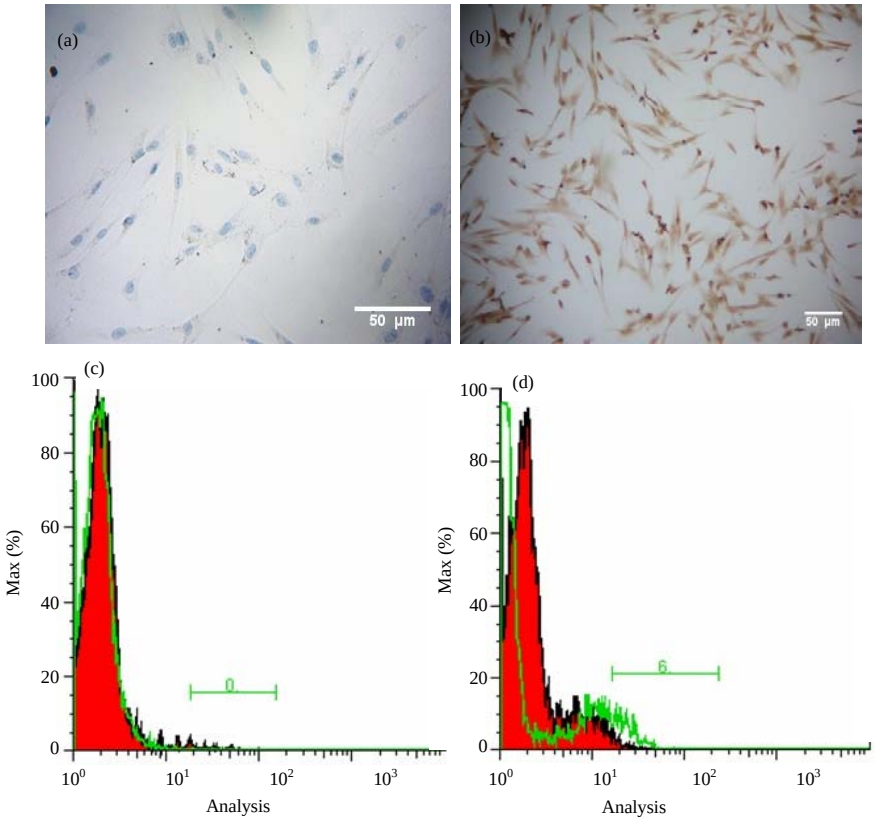

Fig. 4(a-d): Immunohistochemical staining and flow cytometric analysis

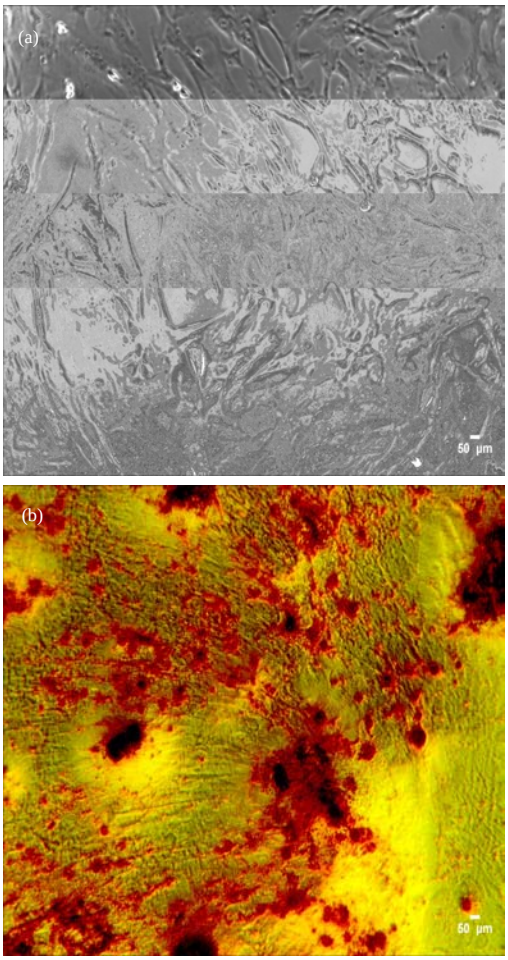

Fig. 5(a, b): iMS cells differentiated into osteogenesis-like cells

Differentiation of dog iMS cells into osteogenesis-like cells: The iMS cells were continuously treated for 14 days by StemPro® Osteogenesis Differentiation kit. The result of Alizarin Red S specific staining showed that iMS cells were differentiated into osteogenesis-like cells (Fig. 5b). Dog fibroblasts were not stained as negative control (Fig. 5a).

Cytotoxicity of scaffold against cells: Cytotoxicity of scaffold against cells was detected by cells co-cultured with leaching solution of HAP/β-TCP/CS. The results showed that the leaching solution nearly did not affect cell growth and cell proliferation rate was between 93 and 99% at a concentration of 0.25~3 mg mL^{-1} (Fig. 6).

The cell morphology was normal (Fig. 7). According to toxicity grading standards, non-toxic of scaffold against cells was evaluated at a concentration of 0.25~3 mg mL^{-1}.

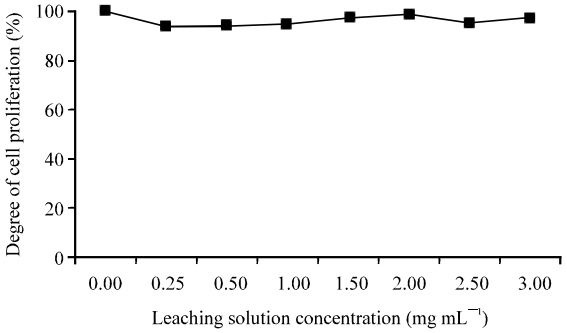

Fig. 6: Effection of HAP/β-TCP/CS leaching solution on iMS cell proliferation

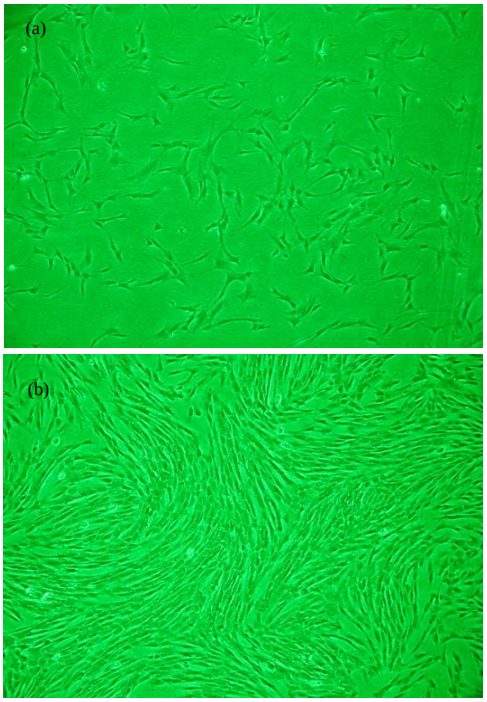

Fig. 7(a, b): Effection of HAP/β-TCP/CS leaching solution on iMS cell growth

Cells-scffolds hybrid material: About 1×10^5 iMS cells were co-cultured with scaffolds in 6 well plates. The results showed that a small number of cells adhered the surface of scaffolds at the third day (Fig. 8a and b). The cell pseudopods were seen and extended toward the pores. As time increasing, more and more cells attached the surface of composite scaffolds. Cells were nearly covered the surface of composite scaffolds at the 12 days (Fig. 8c and d).

Bone regeneration: The cells-scaffolds hybrid material was transplanted into the fibula defect area of dog after

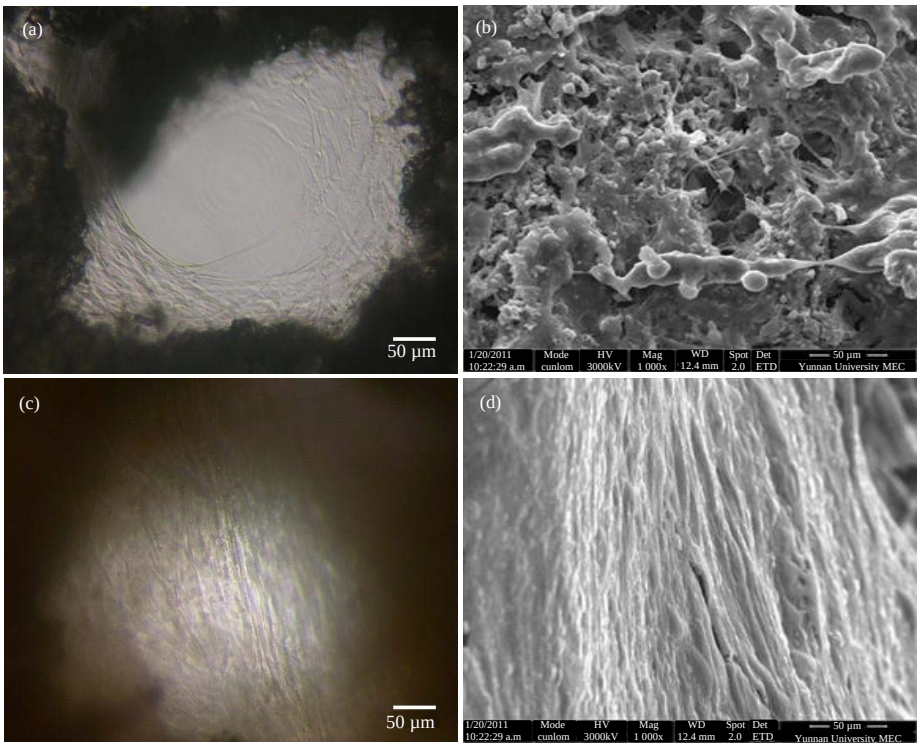

Fig. 8(a, b): Morphology of iMS cell-scaffold hybrid material

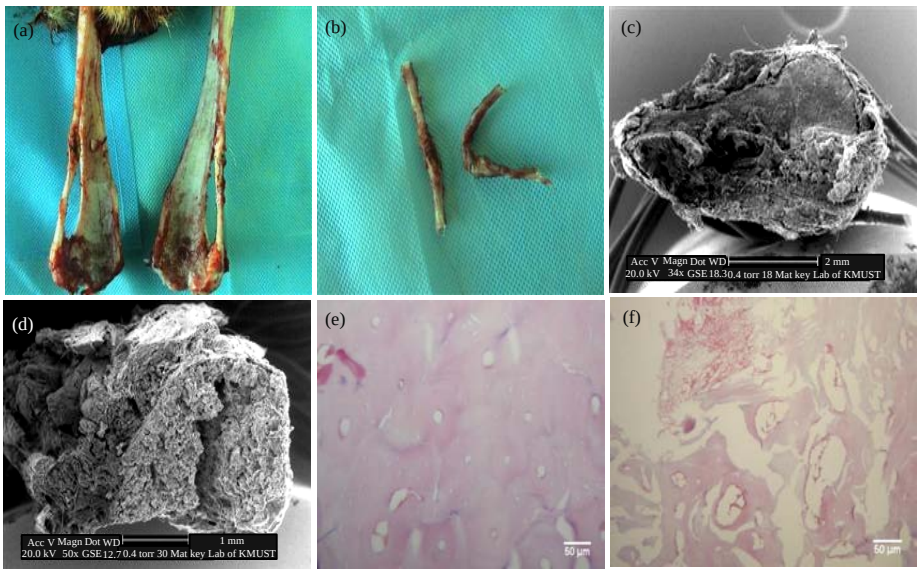

Fig. 9(a-f): iMS cells combining with HAP/β-TCP/CS composite scaffold repaired bone defect

the iMS co-cultured with HAP/TCP/CS composite scaffolds for 20 days. Only transplanting HAP/TCP/CS composite scaffold was as control. After 6 months of transplantation, researchers found that bone defects of the experimental group and control group were both repaired (Fig. 9a). However, the repairing area of experimental group was stronger than that of control group (Fig. 9b) that indicated iMS cell was a key factor for repairing bone. The SEM result showed that the scaffold was almost completely biodegraded (Fig. 9c and d). The

structure of forming bone tissue had nearly no difference with natural bone tissue. In contrast, the control group did not form new bone (Fig. 9e and f).

DISCUSSION

Bone marrow mesenchymal stem cells can be obtained from all parts of the body. It is an idea seed cell of tissue engineering whereas the amount of bone marrow mononuclear cells only accounted for 0.001-0.01%. Meanwhile, the quantity of the cells will reduce as age increasing and body physically fading.

Induced Pluripotent Stem Cells (iPSCs) can differentiate into various tissue cells under specific inducing conditions *in vivo* and *in vitro* such as osteoblasts, chondrocytes, adipocytes ligaments, nerves, liver, heart, endothelial and so on (Yu *et al*., 2007). However, because of the intervention of virus carriers, the iPS cells have troubles with toxic side effects and low transfection efficiency. To avoid the above problem in the previous studies, the mouse skin fibroblasts were able to be reprogrammed into multipotent stem cells which had potential of differentiating into bone cells *in vitro* and *in vivo* (Zhu *et al*., 2010).

Using the same method, dog skin fibroblasts were reprogrammed into multipotent stem cells by short-time exposure of fish oocyte extracts. Then, iMS cells re-differentiated into osteogenesis-like cells *in vitro*. iMS cells co-cultured with HAP/TCP/CS composite scaffolds could form cell hybrid material. The SEM showed that iMS cells finely adhered to the surface of scaffold and grew toward the pores. After transplanting cell hybrid material into the dog's large bone defects area, the defect could be finely repaired with completely biodegradable of scaffold. Only transplanting HAP/β0-TCP/CS composite scaffold into the site of large bone defect that could not be finely repaired. All these experiment results indicated that iMS cells could be seen as an ideal seed cell for repairing bone defect in clinical application.

CONCLUSION

In this study, the dog skin fibroblasts were able to be reprogrammed into iMS cells and iMS cells were able to be re-differentiated into osteogenesis cells *in vitro*. iMS cells co-cultured with HAP/TCP/CS formed cell-scaffold hybrid material *in vitro*. The results showed that iMS cells adhered to the surface of composite scaffold and grew toward the pores. The result of animal model showed that cell hybrid material not only could repair the dog's the fibula defect area at 6 months after transplantation but also HAP/TCP/CS composite scaffold can be completely biodegradable after transplantation. Based on these findings, researchers conclude that iMS cell is a better type of seed cells for bone tissue engineering to regeneration of bone defect.

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