Osteoblastic Differentiation of Rabbit Mesenchymal Stem Cells Loaded in A Carrier System of Pluronic F127 and Interpore

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Background: Ideally, bone tissue engineering products should have the ability of osteoconduction and osteoinduction. According to the tissue engineering principle, mesenchymal stem cells (MSCs) combined with an appropriate scaffold can be used as a bone substitute for bone defects. Here we used Interpore as a scaffold loaded with MSCs mixed in hydrogel (Pluronic F127). In order to demonstrate the osteogenic ability of MSCs in the hydrogel, cell/hydrogel scaffold constructs were cultured in an induction medium to elicit an osteoblastic response.

Methods: MSCs aspirated from rabbit bone marrow were cultured in induction medium. MSCs were then loaded into scaffold Interpore with the aid of hydrogel (Pluronic F127). After culture for 7 and 14 days, osteoblastic differentiation ability was tested using Alizarin Red S stain, reverse transcription polymerase chain reaction (RT-PCR), measurement of calcium and alkaline phosphatase levels, and scanning electron microscopy (SEM).

Results: Calcium and alkaline phosphatase levels both increased after 7 and 14 days incubation. Alizarin Red S staining revealed MSCs could survive and differentiate to osteoblasts in the cell/hydrogel scaffold. RT-PCR showed mRNA expression of osteopontin and Core binding factor alpha 1 (Cbfa1). SEM revealed growth of osteoblast-like cells on ceramic pores.

Conclusions: Osteoconductive bone substitute (Interpore) has been used clinically for a long time. This study showed that MSCs could be held on Interpore with the aid of hydrogel (Pluronic F127) and that they could differentiate to osteoblasts.

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Key words: mesenchymal stem cell, bone substitute.

Skeletal defects are currently treated with autogenous grafts,[1] allogeneic grafts[2] or synthetic bone graft substitutes.[3-6] Autografting remains the treatment of choice but limitations, such as inadequate donor volume and donor site morbidity, contribute to a need for alternatives.[2] Allografts present similar scenarios and are further complicated with the potential for pathogenic transmission.[7] Advances in tissue-engineering technology utilizing synthetic bone graft substitutes offer the possibility of improvement in the repair of bone defects.[8]

As bone graft substitutes, biomaterials have pro-

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vided an anchor for the attachment and differentiation of endogenous migratory precursor cells.[9,10] The ability to characterize the interaction of target cells with modified biomaterial is an important tool for monitoring improvements in material technology. Therefore, bone cell/material interactions should be evaluated in a cell culture system in order to characterize the cellular response of osteogenic cells to the biomaterials involved.

Materials and gels based on poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) triblock copolymers (PEO-PPO-PEO) have been widely studied for pharmaceutical and biomedical applications, as reviewed by a few groups of investigators.[11-13] These polymers, which are known by the trade name Pluronics or the generic name poloxamers, are water-soluble, have low toxicity and certain molecular weights that have been approved by the Food and Drug Administration (FDA) for use in the human body. Pluronic F127, a copolymer formed with 70% polyethylene oxide and 30% polypropylene oxide, forms a reversible thermosensitive hydrogel that exists in a liquid state at cold temperatures and transforms into a thick gel at warm temperatures.[20] With greater viscosity, Pluronic F127 becomes a three-dimensional environment and gradually becomes bioabsorbable. Pluronic F127 is nontoxic, biocompatible and bioabsorbable,[20] and can thus be used as an injectable biomaterial.[21,22]

Ideally, bone substitutes should possess the ability of osteoconduction and osteoinduction but it is also extremely important to have enough bone cells.[23] It has been shown that some bone marrow cells have the ability to differentiate into bone, cartilage, muscle and tendon.[24,25] These cells are referred to as bone marrow mesenchymal stem cells (MSCs). The ideal MSCs-loaded scaffold should provide both osteoinductive stimulants and osteoconductive properties. According to the tissue engineering principle, MSCs combined with an appropriate scaffold can be used as an alternative for autogenous bone grafting.[26]

We used a commercial clinical calcium phosphate product, absorbable porous ceramics, Interpore (Pro-Osteon 500R, Interpore Cross International Inc, Irvine, California, USA) as the porous scaffold. A number of investigators have shown that osteoconductive materials alone do not increase fusion rate when compared with autogenous bone grafts.[27,28] In this study, MSCs were first encapsulated in Pluronic F127. In order to demonstrate the ability of MSCs to differentiate in the hydrogel, cell/hydrogel scaffolds were cultured in the presence of induction medium to elicit an osteoblastic response.

METHODS

Cell preparation

A three kg New Zealand rabbit was anesthetized by an intravenous injection of 5 ml of ketamine hydrochloride (Ketalar, Parke-Davis, Taiwan) and Rompum (Bayer, Leverkusen, Germany) mixture. Under sterile conditions, 10 ml of bone marrow aspirated from the iliac bone crest was collected into a syringe containing 6000 units of heparin to prevent clotting. The marrow sample was washed with Dulbecco's phosphate buffered saline (DPBS) and disaggregated, by passing it gently through a 21-gauge intravenous catheter and syringe, to create a single cell suspension. Cells were recovered after centrifugation at 600 g for 10 minutes. Up to 2×10^8 nucleated cells in 5 ml of DPBS were loaded onto 25 ml of Percoll cushion (Pharmacia Biotech, New Jersey, USA) of a density of 1.073 g/ml in a 50 ml conical tube. Cell separation was accomplished by centrifugation at 1100 g for 40 minutes at 20°C. The nucleated cells were collected from the interface, diluted with two volumes of DPBS and collected by centrifugation at 900 g. The cells were resuspended, counted and plated at 2×10^5 cells/cm^2 in T-75 flasks (Falcon Plastics, Bedford, MA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium - low glucose (DMEM-LG) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and antibiotics (mixture of 100 units/ml of penicillin and 100 ug/ml of streptomycin) (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 4 days of primary culture, the nonadherent cells were removed by changing the medium. The medium was changed every 3 days thereafter. MSCs grow as symmetrical colonies and were subcultured at 10 to 14 days. Subculture was carried out by treatment with 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA) for 5 minutes, rinsed from the substrate with serum-containing medium, collected by centrifugation at 800 g for 5 minutes and seeded into fresh flasks at 5000 to 6000 cells/cm^2. Cultures were incu-

bated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air until cell confluence.

Hydrogel and mesenchymal stem cells

Pluronic F127 was purchased from Sigma Chemical Corporation (Sigma, St. Louis, MO, USA). Pluronic F127 consists of approximately 70% ethylene oxide and 30% propylene oxide by weight. This material is soluble in water and enters a hydrogel state at room temperature.

The Pluronic F127 powder was weighed and slowly added to the culture medium at a low temperature. The mixture was prepared at 20% (wt/vol) under the room temperature above 25°C and sterilized by filtration through a 0.22 μm pore-size Millipore filter. The culture medium was divided into two groups: group I being the control group (DMED-LG containing 10% FBS) and group II being the experiment group (osteogenic medium). MSCs suspension was mixed at 4℃ with a 30% solution of Pluronic F127 at a cellular concentration of 2×10^6 just before use. The mixture of MSCs and polymer was injected into a 24-well plate, at room temperature; this mixture of MSCs and polymer became gel-like. In group I, each well had DMED-LG containing 10% FBS added; in group II, each well had osteogenic medium added. In order to promote osteogenic differentiation, MSCs/hydrogel were cultured in an osteogenic medium (consisting of DMEM-LG containing 10% FBS, antibiotics, 100 μM ascorbate-2-phosphate, 10^{-7} M dexamethasone and 10 mM β-glycerophosphate).

Evaluation of the osteogenic potential of MSCs/hydrogel

To determine the osteogenic differentiation potential of MSCs in hydrogel, four tests were performed on the cultures on day 7 and 14: Alizarin Red S staining, reverse transcription polymerase chain reaction (RT-PCR), alkaline phosphatase (ALP) activity measurements and calcium quantification.

Alizarin Red S staining

Alizarin Red staining was used for detecting calcium deposits. For osteogenesis studies, MSCs were cultured for 14 days with a composite made of Pluronic F127 and Interpore. The medium was then replaced with a calcification medium consisting of complete medium supplemented with 10^{-9} M dexamethasone, 20 mM β-glycerol phosphate and 50 ug/ml ascorbate-2-phosphate for additional 7 and 14 days. These cultures in dishes were stained with 0.5% Alizarin Red solution. Sections stained with Alizarin Red were then examined under a polarized light microscope.

RNA isolation and RT-PCR for detection of Core binding factor alpha 1 (Cbfa1) and osteopontin (OPN) mRNAs

After 14 days of various culture treatments, the alginate MSCs carriers were made soluble by incubation for 20 min at 4°C in a dissolving buffer composed of 55 mM sodium citrate, 30 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA) and 0.15 M sodium chloride at pH 6.8. After mild centrifugation, the cell pellets were washed twice with the dissolving buffer, collected by centrifugation and resuspended in DPBS. Extraction of RNA utilized TRIZOL reagent (Invitrogen, Life Technologies, California, USA). A spectrophotometer (Beckman Instruments GmbH, Munich, Germany) was used to quantify total RNA yield. The RNA samples were reverse transcribed using the ImProm-II reverse transcription system (Promega Corporation, Madison, WI, USA). Amplification by PCR of cDNA, utilizing a PCR Master Mix system (Promega Corporation, Madison, WI, USA), was performed with the following primers:

Cbfa1 (5'-CCGCACGACAACCGCACCAT-3' and 5'-CGCTCCGGCCCACAAATCTC-3');

OPN (5'-CCAAGTAAGTCCAACGAAAG-3' and 5'-ATGTCTGCTCCTGTAGTGG-3');

GAPDH (5'-GCCTGGTCACCAGGGCTGC-3' and 5'-TGCTAAGCAGTTGGTGGTGCA-3').

Amplification of Cbfa1 messages was performed for 30 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec after initial denaturation at 94°C for 2 min. Amplification of OPN messages was performed for 30 cycles at 94°C for 30 sec, 55°C for 2 min and 72°C for 2 min following initial denaturation at 94°C for 2 min. An aliquot of the reaction mixture was subjected to electrophoresis on 2% agarose gel. The PCR products were visualized by ethidium bromide staining and photographed. Band intensity was determined and normalized to the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Quantitative measurement of ALP activity

Since ALP is a cellular enzyme,[31] ALP activity was measured in living cultures. The culture medium was withdrawn and the MSCs carrier was washed twice with 10 mL of Tyrode's balanced salt solution. A 10 mL aliquot of ALP substrate buffer (50 mM glycine, 1 mM $MgCl_2$, pH 10.5), containing the soluble chromogenic ALP substrate (2.5 mM p-nitrophenyl phosphate), was added at room temperature. During incubation, cell-surface ALP converts p-nitrophenyl phosphate into p-nitrophenol, which then changes to a yellowish color. Twenty minutes after substrate addition, 1 mL of the buffer was removed from the culture and mixed with 1 mL of 1 N NaOH to stop the reaction. The absorbance of the mixture was read in triplicate on an ELISA MRX plate reader (Dynatech Labs, USA) at 405 nm. Enzyme activity was expressed as n mole p-nitrophenol/min/dish.

Calcium level quantification

MSCs carriers were rinsed twice with Tyrode's balanced salt solution and then poured in a 50 ml tube containing 10 ml of 0.5 N HCl. Calcium was extracted from the cells by shaking for 24 hours at 4°C. Cellular debris was centrifuged and calcium in the supernatant was measured quantitatively according to the manufacture's protocol described in the Sigma Kit #587. Absorbance of the samples was measured on the MRX multiplate reader (Dynatech Labs, USA) at 570 nm for 5-10 min after the addition of pertinent reagents. Total calcium was calculated from standard curves of solutions prepared in parallel with the experiments and expressed as μg Ca/dish.

Loading of interpore with MSCs/hydrogel

Absorbable Interpore (Pro Osteo 500) (Interpore International, Irvine, CA, USA) is a coralline hydroxyapatite derived from the goniopora coral by a hydrothermal exchange reaction with phosphate (pore volume 65%, mean pore diameter 600 um, interconnections 260 um). The cylinders were prefabricated.[36]

Interpore was first placed in a 24-well plate and then 1 ml of MSCs/hydrogel mixture was injected into each well along with the culture medium. The culture medium was renewed every 3 days. After 14 days, the Interpore with MSCs/hydrogel mixture was collected for Alizarin Red S staining and processed for scanning electron microscopy (SEM).

SEM

After MSCs/hydrogel mixture and MSCs/hydrogel loaded Interpore had been cultured for 14 days, these two constructs were fixed in 2.5% glutaraldehyde, dehydrated through a graded series of ethanol, vacuum dried, mounted onto aluminum stubs and sputter coated with gold. Samples were examined using an S-5000 SEM (Hitachi, Japan).

Statistical analysis

Categorical variables of different culture medium were evaluated by repeated measure analysis of variance (ANOVA) test. ANOVA was used to compare ALP activity (Table 1) and calcium (Table 2) difference with different culture medium. The level of significance was set at $p < 0.05$.

Table 1. Alkaline Phosphatase Activity

	Control Medium	Induction Medium
7 Days	$(5.94 \pm 0.395) \times 10^{-4}$	$(13.94 \pm 1.401) \times 10^{-4}$
14 Days	$(4.89 \pm 0.279) \times 10^{-4}$	$(6.78 \pm 0.278) \times 10^{-4}$
	n = 3, $p < 0.05$	

Enzyme activity is expressed as units of alkaline phosphatase activity.

Table 2. Calcium Level Quantification

	Control Medium	Induction Medium
7 Days	3.7 ± 0.30	15.0 ± 0.95
14 Days	21.9 ± 4.79	42.3 ± 2.56
	n = 3, $p < 0.05$	

Total calcium is expressed as μg Ca/dish.

RESULTS

Alizarin Red S staining

Using Alizarin Red S staining, calcium deposits were detected in MSCs/hydrogel culture and MSCs/hydrogel loaded Interpore. Figure 1A shows MSCs/hydrogel cultured in induction medium for 14 days and Figure 2A shows MSCs/hydrogel mixture loaded in Interpore and cultured in induction medium for 14 days. Figure 1B revealed MSCs/hydrogel cultured in control medium for 14 days and Figure 2B revealed MSCs/hydrogel mixture loaded in Interpore and cultured in control medium for 14

days. As can be seen, after 14 days in vitro culture, mineralization in the MSCs/hydrogel and MSCs/hydrogel mixture loaded in Interpore were clearly demonstrated in both induction medium and control medium; MSCs encapsulated in Pluronic F127 could be induced to an osteoblastic phenotype and were able to mineralize.

RT-PCR

To determine gene expression of Cbfa1 and OPN mRNA in MSCs/hydrogel, RT-PCR was carried out with primers specific for Cbfa1 and OPN. Figure 3 shows the results. Two bands, Cbfa1 (an osteoblast specific transcription factor essential for developing a mature osteoblast phenotype) and OPN (found during early osteoblastic differentiation), were found in the group cultured in the presence of induction medium. The presence of Cbfa1 and OPN mRNA bands indicates osteogenesis of MSCs.

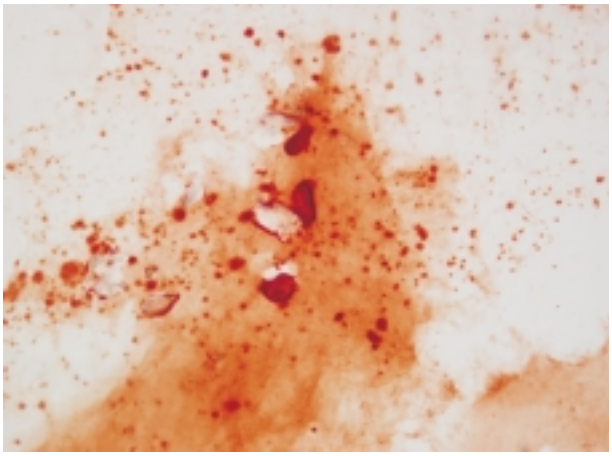

Fig. 1A Mesenchymal stem cell cultured with Pluronic F127 in induction medium. Red color indicates calcium deposition. (Alizarin Red S staining)

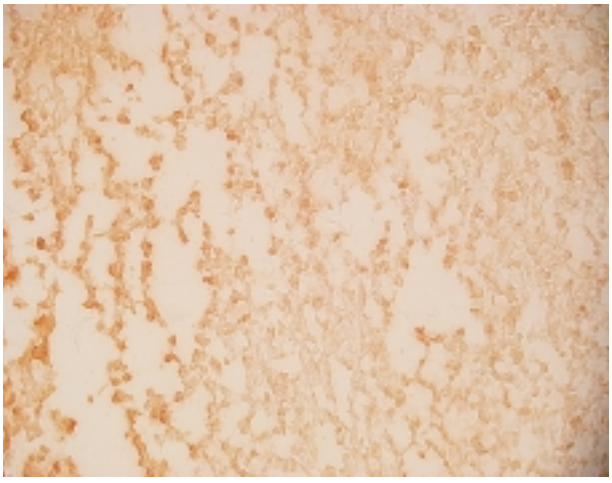

Fig. 1B Mesenchymal stem cell cultured with Pluronic F127 in control medium. (Alizarin Red S staining)

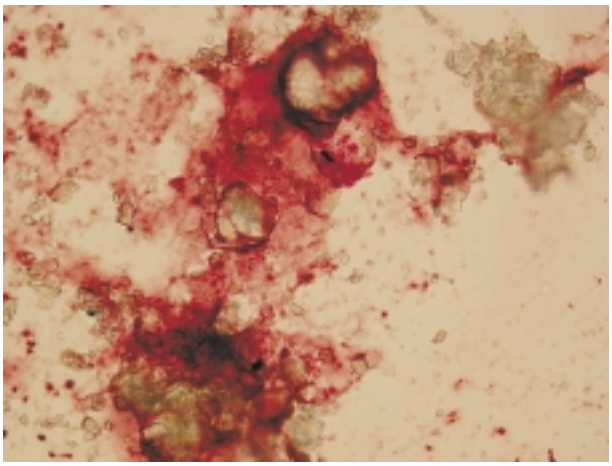

Fig. 2A Mesenchymal stem cell cultured with Pluronic F127 and Interpore in induction medium. Red color indicates calcium deposition. (Alizarin Red S staining)

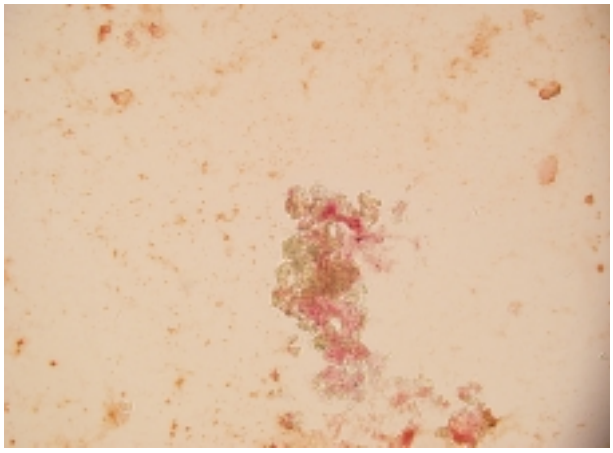

Fig. 2B Mesenchymal stem cell cultured with Pluronic F127 and Interpore in control medium. (Alizarin Red S staining)

ALP measurements and calcium level quantification

In our study, ALP activity measured in the control medium was 0.594 (0.549, 0.610, 0.623) and 0.489 (0.458, 0.512, 0.497) n mole p-nitrophenol/min/dish after 7 days and 14 days, respectively. ALP activity measured in the induction

medium revealed 1.394 (1.414, 1.523, 1.245) and 0.678 (0.710, 0.660, 0.664) n mole p-nitrophenol/min/dish after 7 days and 14 days' culture, respectively (Table 1). A standard solution of *p*-nitrophenol (Sigma, St. Louis, MO, USA) was used for assay of released *p*-nitrophenol. One unit of activity corresponds to the release of 1 umol *p*-nitrophenol/min.[16] Calcium level quantification was measured as 3.7 (3.4, 3.7, 4.0) and 21.9 (16.4, 24.1, 25.2) μg Ca/dish after 7 days and 14 days' culture in control medium, respectively. Calcium level quantification was measured as 15.0 (14.2, 14.7, 16.1) and 42.3 (42, 45, 39.9) μg Ca/dish after 7 days and 14 days' culture in induction medium, respectively (Table 2). The data showed that in the induction medium, MSCs/hydrogel mixture could transform MSCs to osteoblastic phenotype more efficiently than in the control medium ($p < 0.05$).

SEM

In the inductive medium, by SEM, cells on MSCs/hydrogel loaded Interpore appeared to be polygonal osteoblast/osteocyte-like cells. Figure 4 shows that cells on MSCs/hydrogel mixture cultured in Interpore can grow onto the pores of Interpore.

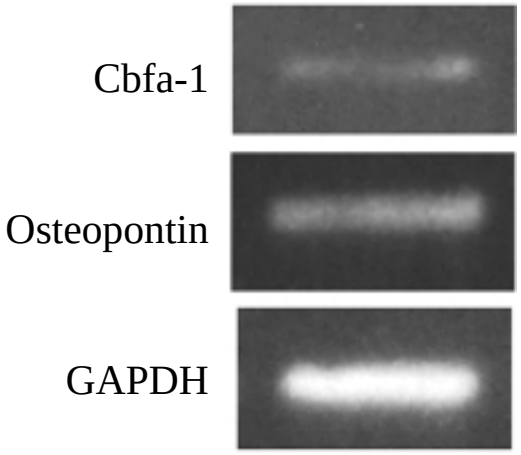

Fig. 3 Total RNA was isolated from MSCs/hydrogel mixture. The primers were specific for Core binding factor alpha 1 (Cbfa1) and osteopontin (OPN). Cbfa1 and OPN were semi-quantified by reverse transcription polymerase chain reaction in the group cultured in the presence of induction medium.

DISCUSSION

Previous in vitro investigations have been suitable for determining some of the parameters encompassing the cell interactions with bone graft substitutes.[14,15] Techniques have included morphologic assessment of mineralized tissue[31] and osteoclastic attachment.[17] A common theme for most of these investigations has been how to monitor the state of osteogenic differentiation.[14,18,19]

Substitutes for autogenous bone grafts have been studied for many years. Materials with osteoconductive properties have been tested but the results of these studies have shown that osteoconductive materials do not meet a high rate of fusion without induction media in the animal model. The addition of osteoinductive substances, particularly bone morphogenetic proteins (BMPs), led to a significantly higher rate of fusion than was seen with osteoconductive materials alone. We hypothesized that mesenchymal stem cells could provide adequate osteoinductive material for the carrier.

Many kinds of hydrogel can be applied to enhance the adherence of cells.[29] In order to enhance the cells' adhesion into the inner pores, we used the hydrogel polymer (Pluronic F127) to help MSCs to grow on the pores of Interpore. These cells not only adhered to the external surface but also entered into the pores of the ceramics. Pluronic F127 meets the

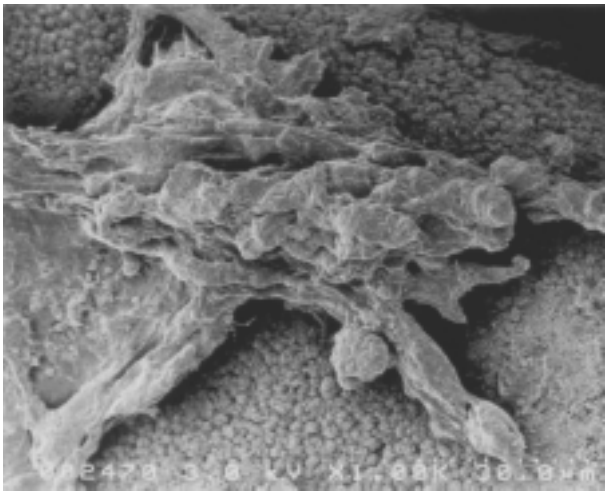

Fig. 4 Mesenchymal stem cells cultured with Pluronic F127 loaded in Interpore showing the cells can grow into the ceramics. (Scanning electron microscope)

following three criteria for being an excellent hydrogel: good tolerability, biodegradability, non-toxicity. The material dissolves slowly, is cleared by renal and biliary excretion,[30] is soluble in water and becomes a hydrogel at room temperature in concentrations above 20%.[20] This thermally reversible swelling of the polymer solution also undergoes a transition from a soluble to an insoluble state when the temperature is raised, making it useful in pharmaceutical compounding. The material can be drawn into a syringe for accurate dose measurement when it is cold. The non-toxic properties and stability of Pluronic F127 aqueous solutions make them suitable for use as injectable vehicles.[32,33] Pluronic F127 gel has already been used in the treatment of burns[34] and the topical application of drugs in cancer treatment.[35] It could increase cell numbers with the ceramic and may be more effective in promoting MSCs to form new bone.

Alizarin Red S staining for permanent cytologic preparations is a valuable test since it can be complementary to compensated polarized light microscopic examination for detecting calcium crystals. Alizarin Red S staining has the greatest sensitivity for the detection of calcium pyrophosphate crystals because crystals are stained regardless of how weakly or strongly birefringent they may be.[37] In this study, mineralization could be seen in both MSCs/hydrogel and MSCs/hydrogel loaded scaffold after Alizarin Red S staining.

The enhancement of osteoblastic differentiation observed in our experiments is further supported by the findings of increased ALP and calcium levels as a result of being incubated with induction medium. This means that even though there is no induction medium, the aspirated bone marrow cells contain osteoblastic promoting potential. In the presence of the induction medium, ALP and calcium levels markedly increased at day 7 and 14, respectively.

OPN is a major non-collagenous protein synthesized by differentiated osteoblasts and deposited into the mineralizing matrix.[40] Cbfa1 has been identified as an essential factor for osteoblastic differentiation and bone disappears in Cbfa1 knock-out mice.[41] In this study, increased amounts of transcripts for Cbfa1 and OPN were expressed after incubation for 7 days in the presence of induction medium, suggesting osteoblastic differentiation in the MSCs/hydrogel mixture.

A critical variable in cell-based techniques is the number of cells implanted. A larger volume of bone marrow results in a better union rate, remodeling and bone formation.[38] The fusion rate of implantation of 100 million cells is better than 1 million cells.[39] In our study, the number of MSCs was approximately 8 million/dish. The different number of MSCs can be estimated in our future study to verify the clinical result of bone fusion.

The results of this study demonstrated that porous ceramics (Interpore) loaded with injectable mesenchymal stem cells in the presence of induction medium can induce MSCs to differentiate to osteoblasts successfully. This approach may provide an alternative to grafting in bone fusion.

In this study, our results show that MSCs can be held on Interpore with the aid of hydrogel (Pluronic F127) and differentiate to osteoblasts, indicating that this type of bone tissue engineering products has the ability of osteoconduction and osteoinduction in vitro.

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REFERENCES

1. Friedlaender GE. Bone grafts. The basic science rationale for clinical applications. J Bone Joint Surg Am 1987;69:786-90.
2. Mayer MH. Clinical perspectives on bone grafting: avoiding the complications of developmental malformations. In: Seyfer AE, Hollinger JO, eds. Bone repair and regeneration. Philadelphia: WB Saunders, 1994:365-76.
3. Harakas NK. Demineralized-bone-matrix- induced osteogenesis. Clin Orthop Relat Res 1984;188:239-51.
4. Matukas VJ, Clanton JT, Langford KH, Aronin PA. Hydroxylapatite: an adjunct to cranial bone grafting. J Neurosurg 1988;69:514-7.
5. Kadiyala S, Lo H, Leong K. Biodegradable polymers as synthetic bone grafts. In: Brighton CT, Friedlaender G, Lane JM, eds. Bone formation and repair. Rosement: American Academy of Orthopaedic Surgeons, 1994:316-24.
6. Hollinger JO, Mayer M, Buck D, Zegzula HD, Ron E, Smith J, Lin L, Wozney JM. Poly (alpha-hydroxy acid)

carrier for delivering recombinant human bone morphogenetic protein-2 for bone regeneration. J Control Release 1996;39:287-304.
7. Tomford WW. Transmission of disease through transplantation of musculoskeletal allografts. J Bone Joint Surg Am 1995;77:1742-54.
8. Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920-6.
9. Hollinger JO, Buck DC, Bruder S. Biology of bone healing: its impact on clinical therapy. In: Lynch RGS, Marx R, eds. Tissue Engineering: applications in maxillofacial surgery and periodontics. San Diego: Quintessence, 1998:17-53.
10. Winn SR, Schmitt JM, Buck D, Hu Y, Grainger D, Hollinger JO. Tissue engineered bone biomimetic to regenerate calvarial critical-sized defects in athymic rats. J Biomed Mater Res 1999;45:414-21.
11. Malsten M. Block copolymers in pharmaceutics. In: Alexandridis P, Lindman B, eds. Amphiphilic block copolymers: self-assembly and applications. New York: Elserier, 2000;139-46.
12. Moghimi SM, Hunter AC. Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. Trends Biotechnol 2000;18:412-20.
13. Kabanov AV, Alakhov VY. Micelles of amphiphilic block polymers as vehicles for drug delivery. In: Alexandridis P, Lindman B, eds. Amphiphilic block polymers. Self-assembly and applications. New York: Elsevier, 2000;347-76.
14. Puleo DA, Holleran LA, Doremus RH, Bizios R. Osteoblast responses to orthopedic implant materials in vitro. J Biomed Mater Res 1991;25:711-23.
15. Brook IM, Craig GT, Hatton PV, Jonck LM. Bone cell interactions with a granular glass-ionomer bone substitute material: in vivo and in vitro culture models. Biomaterials 1992;13:721-5.
16. Nakamura T, Gross M, Yamamuro T, Liao SK. Identification of a human osteosarcoma-associated glycoprotein with monoclonal antibodies: relationship with alkaline phosphatase. Biochem Cell Biol 1987;65:1091-100.
17. Sidqui M, Collin P, Vitte C, Forest N. Osteoblast adherence and resorption activity of isolated osteoclasts on calcium sulphate hemihydrate. Biomaterials 1995;16:1327-32.
18. Begley CT, Doherty MJ, Hankey DP, Wilson DJ. The culture of human osteoblasts upon bone graft substitutes. Bone 1993;14:661-6.
19. Elgendy HM, Norman ME, Keaton AR, Laurencin CT. Osteoblast-like cell (MC3T3-E1) proliferation on bioerodible polymers: an approach towards development of a bone-erodible polymer composite material. Biomaterials 1993;14:263-9.
20. Schmolka IR. Artificial skin. I. Preparation and properties of pluronic F-127 gels for treatment of burns. J Biomed Mater Res 1972;66:571-82.
21. Kamil SH, Eavey RD, Vacanti MP, Vacanti CA, Hartnick CJ. Tissue-engineered cartilage as a graft source for laryngotracheal reconstruction: a pig model. Arch Otolaryngol Head Neck Surg 2004;130:1048-51.
22. Cao YL, Lach E, Kim TH, Rodriguez A, Arevalo CA, Vacanti CA. Tissue-engineered nipple reconstruction. Plast Reconstr Surg 1998;102:2293-8.
23. Muschler GF, Matsukura Y, Nitto H, Boehm CA, Valdevit AD, Kambic H, Davros WJ, Easley KA, Powell KA. Selective retention of bone marrow-derived cells to enhance spinal fusion. Clin Orthop and Rel Res 2005;432:242-51.
24. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK. Douglas R. Mosca JD. Moorman MA. Simonetti DW. Craig S. Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
25. Krebsbach PH, Mankani MH, Satomura K, Kuznetsov SA, Robey P. Repair of craniotomy defects using bone marrow stromal cells. Transplantation 1998;66:1272-8.
26. Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin Orthop Relat Res 1998;355 Suppl:S247-56.
27. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effects of implants loaded with mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 1998;80:985-96.
28. Cinotti G, Patti AM, Vulcano A, Della Rocca C, Polveroni G., Giannicola G, Postacchini F. Experimental posterolateral spinal fusion with porous ceramics and mesenchymal stem cells. J Bone Joint Surg Br 2004;86:135-42.
29. Bensaid W, Triffitt JT, Blanchat C, Oudina K, Sedel L, Petite H. A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials 2003;24:2497-502.
30. Wang ZY, Stern IJ. Disposition in rats of a polyoxypropylene-polyoxyethylene copolymer used in plasma fractionation. Drug Metab Dispos 1975;3:536-42.
31. Yoshimine Y, Sumi M, Isobe R, Anan H, Maeda K. In vitro interaction between tetracalcium phosphate-based cement and calvarial osteogenic cells. Biomaterials 1996;17:2241-5.
32. Johnston TP, Miller SC. Toxicological evaluation of poloxamer vehicles for intramuscular use. J Parenter Sci Technol 1985;39:83-9.
33. Henry RL, Schmolka IR. Burn wound coverings and the use of poloxamer preparations. Crit Rev Biocompat 1989;5:207-20.
34. Nalbandian RM, Henry RL, Balko KW, Adams DV. Pluronic F-127 gel preparation as an artificial skin in the treatment of third-degree burns in pigs. J Biomed Mater Res 1987;21:1135-48.
35. Miyazaki S, Takeuchi S, Yokouchi C, Takada M. Pluronic F-127 gels is as a vehicle for topical administration of anticancer agents. Chem Pharm Bull (Tokyo)

1984;32:4204-8.
36. Jensen SS, Aaboe M, Pinholt EM, Hjorting-Hansen E, Melsen F, Ruyter IE. Tissue reaction and material characteristics of four bone substitutes. Int J Oral Maxillofac Implants 1996;11:55-66.
37. Lazcano O, Li CY, Pierre RV, O'Duffy JD, Beissner RS, Abell-Aleff PC. Clinical utility of the alizarin red S stain on permanent preparations to detect calcium-containing compounds in synovial fluid. Am J Clin Pathol 1993;99:90-6.
38. Werntz JR, Lane JM, Burstein AH, Justin R, Klein R, Tomin E. Qualitative and quantitative analysis of orthotopic bone regeneration by marrow. J Orthop Res 1996;14:85-93.
39. Minamide A, Yoshida M, Kawakami M. Yamasaki S. Kojima H, Hashizume H, Boden SD. The use of cultured bone marrow cells in type I collagen gel and porous hydroxyapatite for posterolateral lumbar spine fusion. Spine 2005;30:1134-8.
40. Kasugai S, Nagata T, Sodek J. Temporal studies on the tissue compartmentalization of bone sialoprotein (BSP), osteopontin (OPN), and SPARC protein during bone formation in vitro. J Cell Physiol 1992;152:467-77.
41. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997;89:747-54.

間葉幹細胞在人工代用骨複合物 (pluronic + interpore) 的骨生成能力評估

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背 景： 理想的人工代用骨必須有骨傳導與骨引導的能力。根據組織工程的原則，間葉幹細胞加入適當的人工代用骨，可以做爲自體骨移植的代用品。我們使用 interpore 做爲支架並加入包被在 pluronic F-127 中的間葉幹細胞，在引導生成的環境中，去測試骨生成能力。

方 法： 從兔子骨髓中抽取的間葉幹細胞在引導生成的環境中培養。然後我們將細胞在水膠 pluronic F-127 的協助下，加入支架 interpore 中。在培養 7 至 14 天後，使用 Alizarin Red S 染色，RT-PCR，鈣濃度，鹼性磷酸酶濃度和掃瞄電子顯微鏡測試骨生成能力。

結 果： 在 7 天與 14 天的培養後，鈣濃度，鹼性磷酸酶活性都增加。Alizarin Red S 染色則顯示間葉幹細胞能在細胞／水膠支架系統中存活並分化成骨細胞。RT-PCR 則顯示出 osteopontin，Cbfa-1。掃瞄電子顯微鏡展示出細胞長在孔洞上。

結 論： 具有骨傳導性的人工代用骨 -interpore，在臨床上使用已有一段時間。在這個研究中，間葉幹細胞可以經由 hydrogel-pluronic F-127 的協助，而附著在 interpore 上，並進而達成成骨母細胞的分化。

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關鍵字： 間葉幹細胞，人工代用骨。

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