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# Application of Perfusion Culture System Improves *in Vitro* and *in Vivo* Osteogenesis of Bone Marrow-Derived Osteoblastic Cells in Porous Ceramic Materials

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## ABSTRACT

**Composites of bone marrow-derived osteoblasts (BMOs) and porous ceramics have been widely used as a bone graft model for bone tissue engineering. Perfusion culture has potential utility for many cell types in three-dimensional (3D) culture. Our hypothesis was that perfusion of medium would increase the cell viability and biosynthetic activity of BMOs in porous ceramic materials, which would be revealed by increased levels of alkaline phosphate (ALP) activity and osteocalcin (OCN) and enhanced bone formation *in vivo*. For testing *in vitro*, BMO/β-tricalcium phosphate composites were cultured in a perfusion container (Minucells and Minutissue, Bad Abbach, Germany) with fresh medium delivered at a rate of 2 mL/h by a peristaltic pump. The ALP activity and OCN content of composites were measured at the end of 1, 2, 3, and 4 weeks of subculture. For testing *in vivo*, after subculturing for 2 weeks, the composites were subcutaneously implanted into syngeneic rats. These implants were harvested 4 or 8 weeks later. The samples then underwent a biochemical analysis of ALP activity and OCN content and were observed by light microscopy. The levels of ALP activity and OCN in the composites were significantly higher in the perfusion group than in the control group ($p < 0.01$), both *in vitro* and *in vivo*. Histomorphometric analysis of the hematoxylin- and eosin-stained sections revealed a higher average ratio of bone to pore in BMO/β-TCP composites of the perfusion group after implantation: 47.64 ± 6.16 for the perfusion group and 26.22 ± 4.84 for control at 4 weeks ($n = 6$, $p < 0.01$); 67.97 ± 3.58 for the perfusion group and 47.39 ± 4.10 for control at 8 weeks ($n = 6$, $p < 0.05$). These results show that the application of a perfusion culture system during the subculture of BMOs in a porous ceramic scaffold is beneficial to their osteogenesis. After differentiation culture *in vitro* with the perfusion culture system, the activity of the osteoblastic cells and the consequent bone formation *in vivo* were significantly enhanced. These results suggest that the perfusion culture system is a valuable and convenient tool for applications in tissue engineering, especially in the generation of artificial bone tissue.**

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## INTRODUCTION

IT IS STILL A GREAT CHALLENGE to repair miscellaneous tissue defects in clinical practice because suitable autologous tissue is available to only a limited extent and allografts are still under the shadow of potential infections, let alone risky immunological problems. Therefore, artificial tissues obtained by tissue engineering are important alternatives. Although tissue engineering is yet to overcome all these problems at the present time, it has attracted much attention in various fields.[1–8] As experiments have shown, cells cultured in dishes lose many of their typical characteristics as a result of dedifferentiation, although their numbers multiply enormously.[9,10] Concerning bone tissue engineering, many investigations have illustrated that osteoblasts cultured in a two-dimensional pattern with static medium could proliferate efficiently with the synthesis of bone-specific matrix proteins, while multilayered cultures could significantly accelerate osteoblast functions including matrix synthesis.[11–13] Numerous experiments have shown that the proper biomaterial as a cell framework is necessary for cells to develop their typical differentiated phenotypes. That is why three-dimensional (3D) models have been widely adopted for tissue engineering to obtain satisfying artificial bone grafts. A key question in 3D culture remains whether composites of cells and biomaterial can be kept in culture while maintaining their specific phenotype and functions. To achieve this, it is important to mimic the physiological nutrient and metabolic environment as closely as possible. Conventional culture methods with static medium do not satisfy these requirements any longer. A perfusion culture system is able to guarantee the continuous exchange of medium and constant removal of metabolic waste, and has been used for a variety of cells and tissues.[14–18] Perfusion enhanced the viability and function of murine osteosarcoma cells and murine bone marrow stromal cells and increased matrix synthesis by chondrocytes in 3D collagen sponges.[19–21] Perfusion cultures also gave higher productivities and a consistent product quality, and allowed steady state operations and better cell physiological control.[22] Direct perfusion of cultured constructs was supposed to reduce diffusional distances for mass transport; improve control of oxygen, pH, nutrients, and metabolites in the cell microenvironment; and thereby increase the thickness and spatial uniformity of engineered cardiac muscle.[23] Composite skin grafts were engineered by means of placing artificial skin seeded with keratinocytes in a perfusion culture system, resulting in improved cell growth and identical wound adherence and complete healing by a multilayered, keratinizing human epidermis.[24] Using a perfusion culture system, hepatocytes could be dynamically seeded onto biodegradable polymers and survive with a high rate of albumin synthesis.[25] All these results suggest that perfusion culture may also be beneficial for bone tissue engineering.

The combination of a porous ceramic material and bone marrow-derived osteoblastic (BMO) cells has been used for bone tissue engineering.[26–28] The challenge is how to increase bone formation in implants and so move forth steadily toward applications in clinical practice. Conventional culture systems with static medium may provide insufficient nutrition to support the survival and differentiation of BMO cells in composites in which the cell density is high and more active viability is expected. Optimal results could be achieved only when the culture conditions imitate closely those of the original tissues. Manipulation of the *in vitro* culture microenvironment has been a necessary approach in studies attempting to increase bone formation in tissue engineering. Conventional culture systems with static medium may provide insufficient nutrition to support the survival and differentiation of BMO cells in composites in which the cell density is high and more active viability is expected. Frequent medium exchange has shown certain benefits for bone marrow cells.[29] The perfusion of medium ensures fresh medium to cells and avoids the accumulation of harmful metabolic products, providing a constant microenvironment for a high degree of cellular differentiation and better tissue development. Our hypothesis was that perfusion of medium would increase the cell viability and biosynthetic activity of BMO cells in porous ceramic materials, which could be revealed by increased levels of alkaline phosphatase (ALP) activity and osteocalcin (OCN) and enhanced bone formation *in vivo*. In this study, we applied the perfusion system to the culture of composites of BMO/$\beta$-tricalcium phosphate ($\beta$-TCP) to investigate its effects on the biosynthetic activity of *in vitro* 3D-cultured osteoblasts and the effects on bone formation in the composites *in vivo*.

## MATERIALS AND METHODS

### *Porous ceramic material*

The porous ceramic blocks used in this study were made by Olympus Optical Co. (Tokyo, Japan). This kind of scaffold is composed of $\beta$-tricalcium phosphate [$Ca_3(PO_4)_2$]. The porosity of the $\beta$-TCP is 75%, with a pore size ranging from 100 to 400 $\mu$m. In this study, the dimensions of the $\beta$-TCP blocks were $5 \times 5 \times 5$ mm$^3$. Before being loaded with BMO cells, $\beta$-TCP blocks were sterilized in a dry heater at 180°C for 4 h.

### *Cell isolation and proliferation*

Japanese Government guidelines for the care and use of laboratory animals were strictly followed throughout the study.

Bone marrow of 7-week-old Fischer 344 male rats was obtained from their femurs aseptically, according to a method described previously.[30] After cutting both epiphyses of the femurs, the contents of the bone marrow cavity were harvested by flushing the cavity with minimal essential medium (MEM, with Earle's salt and glutamine; Nacalai Tesque, Kyoto, Japan) by rapid expulsion from 18- and 21-gauge needles. Single-cell suspensions were made by passing the marrow through 23-gauge needles three times, and then the cells were collected and resuspended in proliferation medium: MEM supplemented with 15% fetal bovine serum (Sigma-Aldrich, Irvine, UK), penicillin (100 units/mL), streptomycin (100 $\mu$g/mL), and amphotericin B (0.25 $\mu$g/mL) (GIBCO-BRL Life Technologies, Gaithersburg, MD). For cells from one femur, the suspension was adjusted to 15 mL of proliferation medium and then cultured in a 75-$cm^2$ culture flask (Falcon; BD Biosciences Discovery Labware, Franklin Lakes, NJ) at 37°C in a humidified 5% $CO_2$ incubator. The medium was changed after the first 24 h to remove unattached cells; the adherent cells left were mainly marrow stromal cells.[30] Hereafter, the medium was changed every other day.

### *Subculture into porous β-TCP*

When BMO cells became fully confluent after 10 days, they were detached with 0.1% trypsin and 20 million cells, suspended in 20 mL of differentiation medium, were used to seed $\beta$-TCP blocks. The differentiation medium consisted of proliferation medium supplemented with vitamin C phosphate (L-ascorbic acid phosphate magnesium salt *n*-hydrate, 50 $\mu$g/mL; Wako Pure Chemical Industries, Osaka, Japan), 10 mM sodium $\beta$-glycerophosphate (Merck Japan, Tokyo, Japan) and $10^{-8}$ M dexamethasone (Dex; Sigma). After being soaked and seeded in the above-described cell suspension and incubated for 2 h, composites were transferred one by one into a 24-well plate (Falcon) and kept cultured with differentiation medium at 37°C in a humidified $CO_2$ incubator for 2 days in order to ensure adhesion of BMO cells to $\beta$-TCP blocks. After subculturing for 2 days, all the composites were assigned randomly to either the control group or the perfusion group. Each BMO/$\beta$-TCP composite in the control group was transferred into 1 well of a new 24-well plate and cultured with 2mL of differentiation medium at 37°C in a humidified $CO_2$ incubator. The differentiation medium was replaced every other day and the culture period depended on the corresponding composites in the perfusion group.

### *Perfusion culture system*

A perfusion culture system, as shown in Fig. 1, was used in this study. After subculturing for 2 days, the BMO/$\beta$-TCP composites were transferred into a perfusion culture container (Minucells and Minutissue, Bad Abbach, Germany). The container was connected with the medium bottles by silicone tubes. The peristaltic pump was adjusted to deliver fresh medium at a rate of 2 mL/h. The whole system was put on an experimental table at room temperature, although the perfusion container was maintained at 37°C by a thermo plate. The medium used for perfusion culture was the same differentiation medium used for the control group, supplemented with 20 mM HEPES.

Each BMO/$\beta$-TCP composite in the perfusion group was transferred into the perfusion container, within which they were separated by support rings.

For the *in vitro* study, the culture of composites lasted for 1, 2, 3, or 4 weeks at most. For the *in vivo* study, the subculture lasted for 2 weeks before the composites were implanted in rats subcutaneously as described below.

### *Surgical procedures*

For the *in vivo* study, eight syngeneic 9-week-old male Fischer rats were anesthetized with a peritoneal injection (3.0 mg/100 g body weight) of chloral hydrate (Wako Pure Chemical Industries) shortly after superficially induced anesthesia by ether inhalation. On each side of the back of a rat, three small incisions were made and extended subcutaneously with forceps. Three pieces of BMO/$\beta$-TCP composites from the perfusion group were implanted subcutaneously into the three sites on the right side of the back and three counterparts from the control group into the left side. The wounds were then closed with 4-0 silk sutures.

### *Biochemical analysis*

The composites were harvested for biochemical analysis at 1, 2, 3, and 4 weeks *in vitro* or 4 and 8 weeks after implantation in rats. The alkaline phosphatase (ALP) activity and bone osteocalcin content of these composites were measured.[23] Briefly, implants were immediately crushed with a hammer, homogenized in 0.5 mL of 0.2% Nonidet P-40 containing 1 mM $MgCl_2$ with Physcotron (Micro-Tech Nition, Funahashi City, Chiba, Japan), and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was assayed for ALP activity, using *p*-nitrophenyl-phosphate as substrate. An aliquot (2.5 $\mu$l) of supernatant was added to 0.5 mL of 56 mM 2-amino-2-methyl-1,3-propanediol (pH 9.8) containing 10 mM *p*-nitrophenyl-phosphate with 1 mM $MgCl_2$, and the mixture was incubated at 37°C for 30 min. Then 0.5 mL of 0.2 N NaOH was added to the wells to stop the reaction before absorption at 405 nm was measured with a spectrophotometer. ALP activity was determined as millimoles of *p*-nitrophenyl released per implant after 30 min of incubation.

Osteocalcin was removed from the sediment after ex-

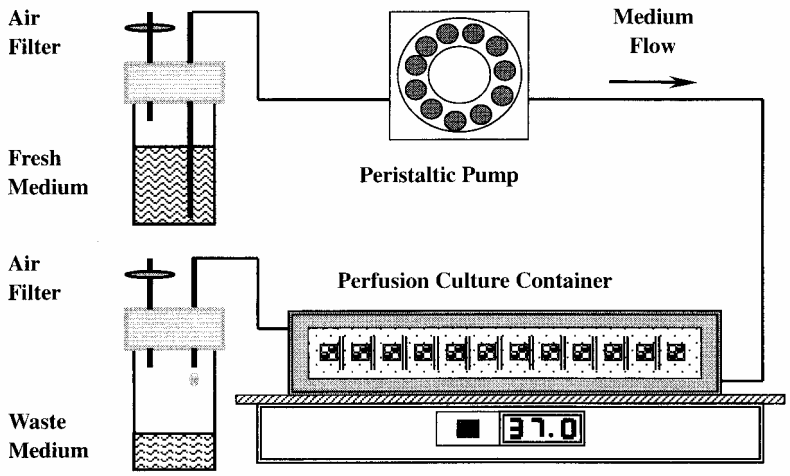


**FIG. 1.** Schematic diagram of the perfusion culture system. Block composites are held in a container through which fresh medium is delivered by a peristaltic pump at a rate of 2.0 mL/h. The container is maintained at 37°C by the thermo plate.

traction with 0.2% Nonidet P-40 by shaking in 5 mL of 20% formic acid for 10 days at 4°C. An aliquot (2 mL) of the formic acid extract was then applied to a Sephadex G-25 (PD-10) column. The protein fraction was collected, lyophilized, and prepared for measurement of intact rat osteocalcin as previously described.[24] All assays were performed with rabbit antiserum to rat osteocalcin and purified rat osteocalcin as a standard and tracer, respectively. We used a rat osteocalcin enzyme immunoassay (EIA) kit (BT-490; Biomedical Technologies, Stoughton, MA) and followed the manufacturer's protocol.

### *Statistical analysis*

Average values of ALP activity and osteocalcin content were expressed as the arithmetic mean ± SD. Analysis of data was performed by paired *t* test. Any difference was considered statistically significant when the *p* value was $<0.01$.

### *Histological analysis*

The implants were harvested for histological analysis 4 and 8 weeks after implantation. For each time point, composites from either group were obtained for light microscopic observation. Once they were removed from the subcutaneous sites on the back of rats, the composites were fixed in 10% neutral buffered formalin, decalcified with K-CX solution (Falma, Tokyo, Japan), embedded in histparaffin, and stained with hematoxylin and eosin. These specimens were observed by optical microscope (IX70; Olympus, Tokyo, Japan). The ratio of bone formed in $\beta$-TCP implants was determined by histomorphometric analysis of the sections. Briefly, the area of bone and pores in each chosen image was semiautomatically measured with the Image-Pro Plus system (MediaCybernetics, Silver Spring, MD). For each implant, four images from four sections were chosen for analysis. The average percentage of bone relative to the pores of each implant was then calculated. The difference among groups ($n = 6$) in terms of the percentage of bone to pores was analyzed by paired *t* test.

## RESULTS

### In vitro *study*

The experiments were repeated three times to confirm the reproducibility of the results. The activity of alkaline phosphatase was measured in subcultured BMO/$\beta$-TCP composites after 1, 2, 3, and 4 weeks of perfusion culture or conventional subculture. Figure 2 shows the levels of alkaline phosphatase activity of the composites in both groups. A similar tendency was observed in both groups, but much higher alkaline phosphatase activity was detected in the perfusion group at all four time points. In the perfusion group, the expression of alkaline phosphatase activity increased steeply toward its peak at 2 weeks (24.87 mM). From 1 week on, the ALP activity was 16.81 mM, about 2 times that of the control group (7.86 mM). At 3 weeks, the ALP activity decreased to some extent, but remained roughly three times that of the control group. At 4 weeks, the activity was still relatively high, at approximately three times the control level. Statistical analysis confirmed a significant difference between the perfusion group and control group ($n = 12$, $p < 0.01$).

Osteocalcin in composites could be detected from the first week (5.33 ng/mL) of perfusion culture *in vitro*. Figure 3 shows that the OCN content of composites in the perfusion group increased steadily at a higher level ($n =$

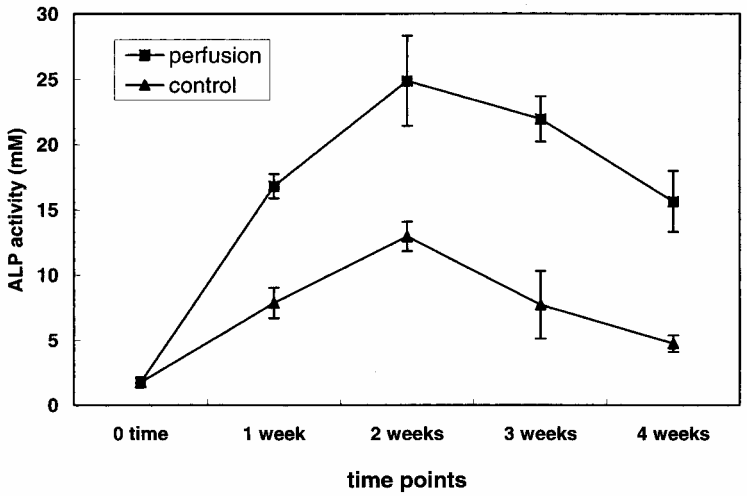


**FIG. 2.** Temporal changes in the alkaline phosphatase activity of *in vitro*-cultured composites. Significant differences exist between the perfusion and control group ($n = 12$, $p < 0.01$) by paired $t$ test. Details are described in Materials and Methods.

12, $p < 0.01$) than that of the control group, which was also positively correlated with time.

## In vivo *study*

*Biological findings.* The alkaline phosphatase activity of implants was measured 4 and 8 weeks after the composites were implanted into subcutaneous sites in rats after perfusion culture or conventional subculture. Figure 4 shows the level of activity of the implants in both groups. In the perfusion group, the expression of alkaline phosphatase activity remained higher (26.38 mM) than the control level 4 weeks after implantation. Although it dropped considerably by 8 weeks (13.22 mM), the absolute value was not too low, and still higher than in the control group. The overall difference in ALP activity between the two groups was significant ($n = 6$, $p < 0.01$).

After implantation, the bone osteocalcin content continued increasing with time. As Fig. 5 shows, the OCN content of the implants in the perfusion group was 97.89 ng/mL at 4 weeks and 117.82 ng/mL at 8 weeks, much higher ($n = 6$, $p < 0.01$) than that of the control group at both 4 and 8 weeks after implantation subcutaneously.

*Histological findings.* At 4 weeks postimplantation, the decalcified sections with hematoxylin and eosin (H&E) staining showed primary bone formation in many pores of the composites in the perfusion group. Bone tissue

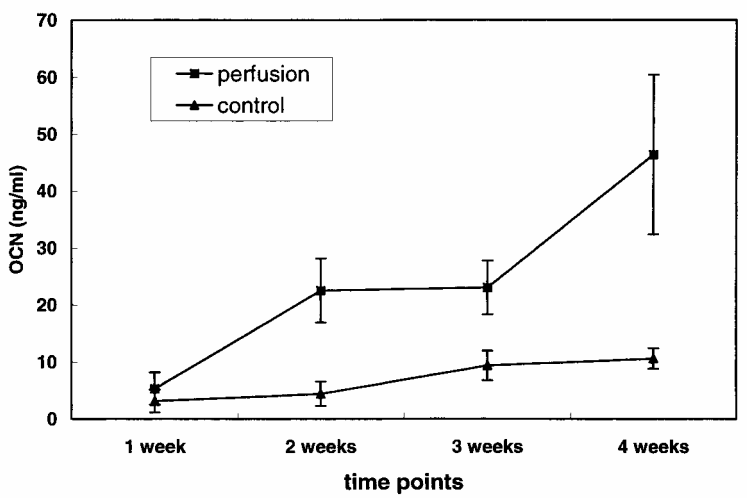


**FIG. 3.** Temporal changes in the bone osteocalcin content of *in vitro*-cultured composites. Details are described in Materials and Methods.

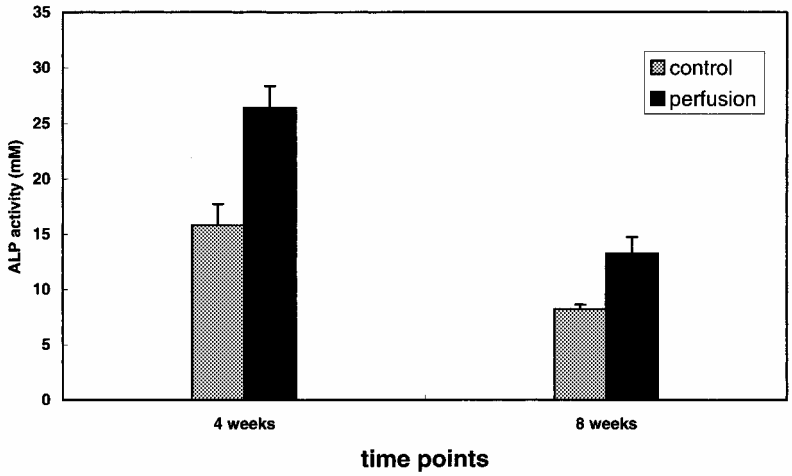


**FIG. 4.** Temporal changes in alkaline phosphatase activity of composites postimplantation. Significant differences exist between the perfusion and control group ($n = 6$, $p < 0.01$) by paired $t$ test. Details are described in Materials and Methods.

originated along the surfaces of the pores in $\beta$-TCP, stacked gradually toward the hollow centers, as shown in Fig. 6. The average percentage of bone to pores was $47.64 \pm 6.16$. In the pore regions, there were newly formed vessels among supporting tissues. Few small round cells, which were regarded as a sign of an inflammatory reaction, were seen in the regions. In the meantime, lineups of cuboidal cells representing active osteoblasts were observed along the bone-forming surfaces. By 8 weeks after implantation, new bone and osteocytes were dotted along the layers of the lamina bone. Regenerated bone marrow areas were also observed in association with the newly formed bone inside some porous regions. Bone was forming extensively to connect and fuse with the lamina bone in adjoining pores, while less fibrous tissue was left. The percentage of bone to pores was $67.97 \pm 3.58$. In the control group, gradual bone formation was also observed. The average percentage of bone to pores was $26.22 \pm 4.84$ at 4 weeks and $47.39 \pm 4.10$ at 8 weeks, which was lower than that of the perfusion group. The difference between the perfusion group and control was significant by paired $t$ test ($n = 6$, $p < 0.01$ at 4 weeks and $p < 0.05$ at 8 weeks), as shown in Fig. 7.

## DISCUSSION

Alkaline phosphatase is an ectoenzyme, produced by osteoblasts, that is likely to be involved in the degradation of inorganic pyrophosphate to provide a sufficient local concentration of phosphate or inorganic pyrophosphate for mineralization to proceed. Therefore, ALP is a useful marker for osteoblast activity. Osteocalcin (OCN), also known as bone Gla protein, is a highly conserved noncollagenous protein that contains three $\gamma$-carboxy-

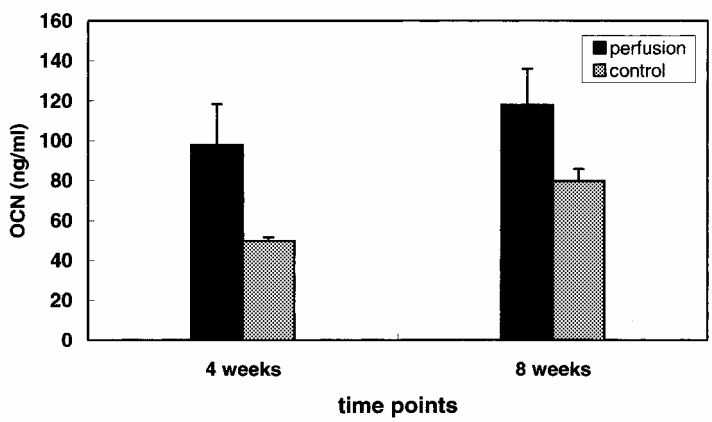


**FIG. 5.** Temporal changes in bone osteocalcin content of composites after a period of implantation. Details are described in Materials and Methods.

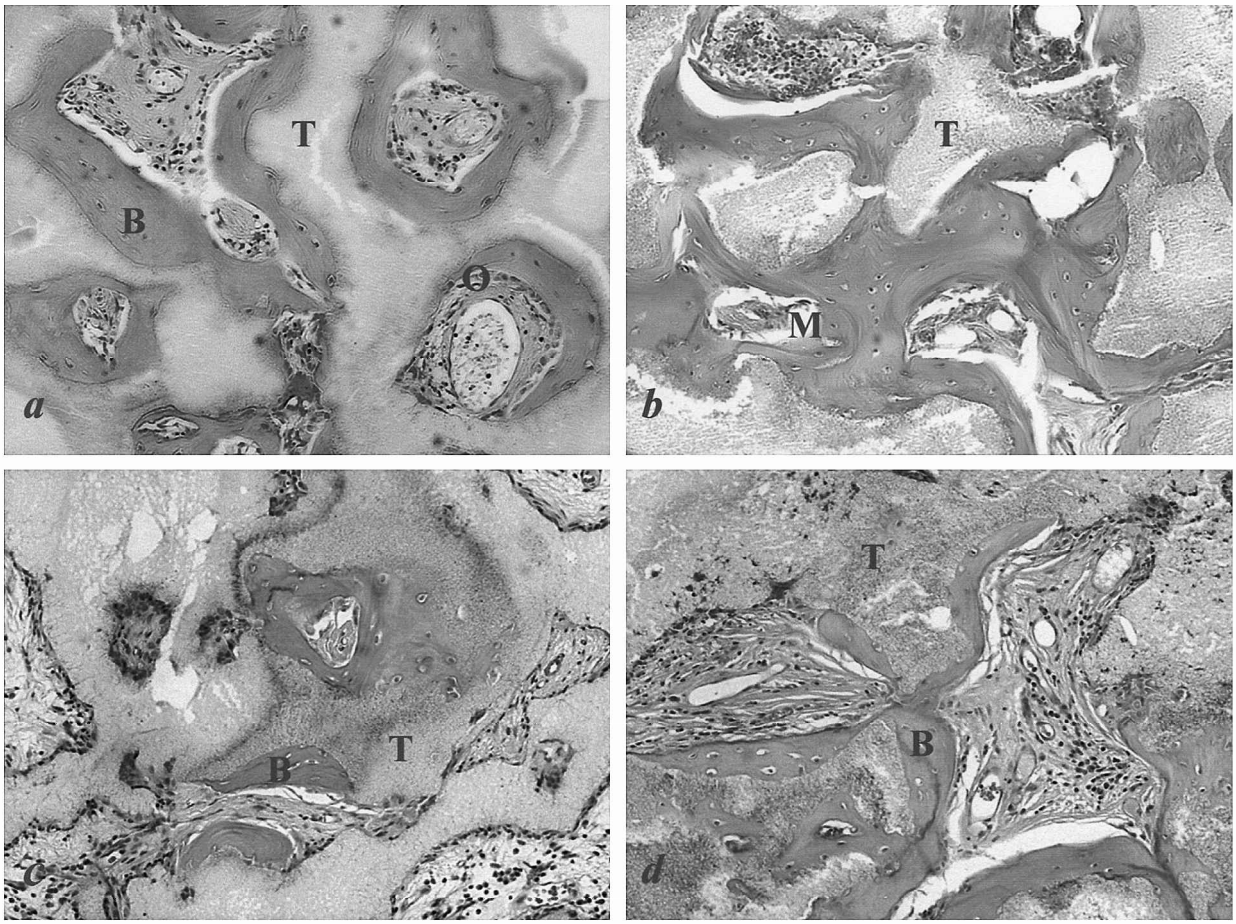


**FIG. 6.** Sections of implants in the perfusion group 4 (**a**) and 8 (**b**) weeks after implantation, and of their counterpart implants from the control group (*c* and **d**). B, bone formed; T, ghost of $\beta$-TCP after decalcification; O, active osteoblasts; M, rebuilt marrow cavity. Hematoxylin and eosin stain. Original magnification, ×100.

glutamic acid residues that allow it to bind calcium. Although the function of OCN is not quite clear, it is deemed that only osteoblasts or cells with the characteristics of osteoblasts produce osteocalcin. OCN is already known to play an important role in the process of ossification for bone formation. Like alkaline phosphatase, osteocalcin is also being used as a marker of osteoblast activity.[25] In our study, like in other reports,[31–35] ALP activity strengthened rapidly and peaked at about 2 weeks, while the temporal changes in OCN content increased steadily with time, which was well in line with the course of bone formation in the composites.

In the connective tissue network of the bone marrow, there is a heterogeneous population of mesenchymal progenitor cells (bone marrow stromal cells, BMSCs), capable of differentiating into several skeletal tissue lineages.[36,37] When cultured *in vivo*, BMSCs are able to regenerate connective tissues like bone, cartilage, muscle, and tendon, hence they are widely used in 3D cultures with biomaterials to obtain tissue-engineering bone grafts, although the factors involved in modulating the differentiation of cells of the osteoblast lineage have not yet been fully identified. In our study, BMSCs were used for differentiation into osteoblasts and forming of bone. In this study, the levels of both ALP and OCN, the markers of osteoblastic cells, were significantly increased during culture and postimplantation; meanwhile, in the H&E sections, spindle-like active osteoblasts were observed near the bone-forming area, which demonstrated that BMSCs were transferred successfully and suggests they are appropriate for bone tissue engineering.

It is now recognized that tissue engineering must be done in three principal steps: the proliferation of cells, the seeding of cells into a suitable scaffold, and the maintenance of the differentiated phenotype of the engineered tissues.[17] A biocompatible porous scaffold plays an important role in seeding the cells and serving as a template for tissue regeneration. The scaffolding materials should be biocompatible, biodegradable, and osteoinductive to accept the attachment and migration of osteoblasts. Because

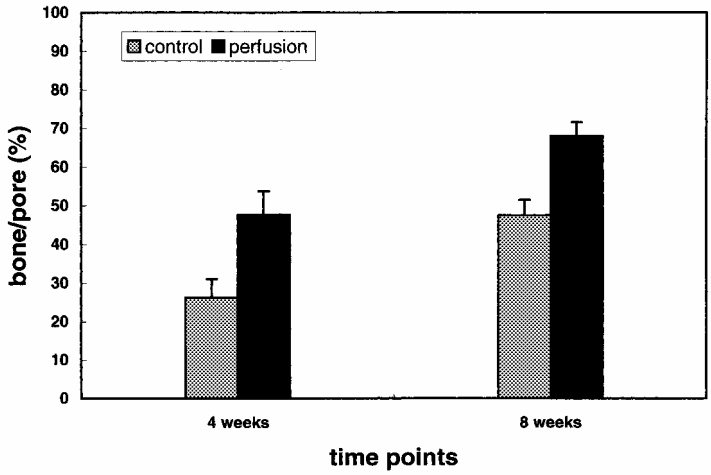


**FIG. 7.** The average ratio of bone to pore in composites after implantation. For composites of the perfusion group, it was 47.64 ± 6.16 at 4 weeks an 67.97 ± 3.58 at 8 weeks; for the control group, it was 26.22 ± 4.84 at 4 weeks and 47.39 ± 4.10 at 8 weeks. The difference between the perfusion group and control was significant at 4 weeks ($n = 6$ $p < 0.01$) and 8 weeks ($n = 6$, $p < 0.05$).

of its good biocompatibility and characteristics of absorption, $\beta$-TCP has been used as a bone substitute or scaffold for tissue engineering. As a scaffold, $\beta$-TCP not only provides a favorable environment for osteoblast attachment and growth, but also acts as nucleation sites for the deposition of calcium and phosphate ions and the formation of apatite crystas so that an apatite layer is easily formed and bone formation improved.[38] When $\beta$-TCP was implanted into bone defects, bone formed directly on the material in an osteoconductive manner and $\beta$-TCP was absorbed gradually, maintaining direct contact with the bone.[39–41] In our study, we also observed a direct connection between newly formed bone and $\beta$-TCP surfaces of the pores without any fibrous tissue in between. This implies that the formation of solid bone could be expected in a subcutaneous model when the composites are implanted into bone to repair defects during clinical application.

Although it is not so difficult to obtain bone in porous ceramics, especially with a combination of BMO cells and ceramics, a pending problem is the discrepant and often limited bone formation in artificial bone grafts.[31,34,41–44] It is necessary to optimize various aspects of tissue engineering so as to obtain more reliable and more extensive bone formation. Of all these aspects, culture conditions are perhaps the most important.

In bone tissue engineering, a popular model is to combine an adequate three-dimensional porous scaffold with osteoprogenitor cells to achieve bone formation in the composites *in vitro* and *in vivo*. The cell density in the 3D structures is high and the degradation of some resorbable materials may release harmful substrate around cells, so that the physiological system cannot be sufficiently mimicked by the stagnant environment in conventional culture dishes. A perfusion system was designed to meet such demands to optimize the culture conditions for artificial tissues.

The benefits of the perfusion culture system could be summarized[45] as follows: under perfusion conditions, tissue receives constant nutrition and paracrine factors remain at a physiological level, while harmful metabolic products do not accumulate inside the artificial tissue. Because the media in perfusion cultures are continuously pumped through thin, gas-permeable silicone tubes, which allows a continuous and optimal exchange of gases, the $O_2$ partial pressure in the culture medium is considerably higher than that in a conventional culture plate, and the desired pH value can be constantly stabilized for the entire culture period under a laboratory air atmosphere with a certain concentration of HEPES without injection of gas into the system, which provides the convenience of enabling the system to be kept on a laboratory table instead of in a $CO_2$ incubator. Besides, during growth and differentiation, metabolically active cells produce lactate and secrete it into the medium, which would harm the cells themselves by causing a pH shift in the case of the accumulation of lactate to a nonphysiological concentration. Although in most 2D cultures the lactate concentration can be neglected, this is not the case in 3D structures because of the high cell density.[46] By perfusion culture, the culture medium is not recycled but permanently renewed, and the level of lactate is kept at a physiological concentration so that damage to the cells is prevented. In our study, probably for all the abovecited reasons, a higher level of activity of osteoblasts and consequently more bone in the composites of the perfusion group were observed. This finding of enhanced cell

viability and biosynthetic activity by perfusion culture is consistent with other investigations.[19,21] All these results showed that perfusion conditions are also beneficial for the generation of autologous artificial grafts for skeletal tissue repair.

In summary, this study demonstrated that a relatively large artificial bone graft ($5 \times 5 \times 5$ mm$^3$) could be generated by culturing composites of BMSCs and porous ceramic ($\beta$-TCP) blocks before implantation into subcutaneous sites in rats. The markers of ALP and OCN show that the application of a perfusion culture system during the subculture of BMO cells in a porous ceramic scaffold is beneficial to osteogenesis. After differentiation culture *in vitro* with the perfusion culture system, the activity of the osteoblastic cells and the consequent bone formation *in vivo* are significantly enhanced. These results suggest that the perfusion culture system is a valuable and convenient tool for applications in tissue engineering, especially in the generation of artificial bone tissue.

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