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Novel Cell Seeding System Into a Porous Scaffold Using a Modified Low-Pressure Method to Enhance Cell Seeding Efficiency and Bone Formation

Ichiro Torigoe,* Shinichi Sotome,*† Akio Tsuchiya,*‡ Toshitaka Yoshii,* Makoto Takahashi,* Shigenori Kawabata,* and Kenichi Shinomiya*‡§¶

*Section of Orthopaedic and Spinal Surgery, Graduate School, Tokyo Medical and Dental University, Tokyo, 113-8519, Japan
†Section of Regenerative Therapeutics for Spine and Spinal Cord, Graduate School, Tokyo Medical and Dental University, Tokyo, 113-8519, Japan
‡The 21st Century Center of Excellence (COE) Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Tokyo, 101-0062, Japan
§Hard Tissue Genome Research Center, Tokyo Medical and Dental University, Tokyo, 101-0062, Japan
¶Core to Core Program for Advanced Bone and Joint Science, Tokyo Medical and Dental University, Tokyo, 101-0062, Japan

The efficient seeding of cells into porous scaffolds is important in bone tissue engineering techniques. To enhance efficiency, we modified the previously reported cell seeding techniques using low-pressure conditions. In this study, the effects of low pressure on bone marrow-derived stromal cells (BMSCs) of rats and the usefulness of the modified technique were assessed. There was no significant difference found in the proliferative and osteogenic capabilities among various low-pressure (50–760 mmHg, 1–10 min) conditions. To analyze the efficacies of the cell seeding techniques, BMSCs suspended in the plasma of rats were seeded into porous β-tricalcium phosphate (β-TCP) blocks by the following three procedures: 1) spontaneous penetration of cell suspension under atmospheric pressure (SP); 2) spontaneous penetration and subsequent low pressure treatment (SPSL), the conventional technique; and 3) spontaneous penetration under low pressure conditions (SPUL), the modified technique. Subsequently, these BMSCs/β-TCP composites were used for the analysis of cell seeding efficiency or in vivo bone formation capability. Both the number of BMSCs seeded into β-TCP blocks and the amount of bone formation of the SPUL group were significantly higher than those of the other groups. The SPUL method with a simple technique permits high cell seeding efficiency and is useful for bone tissue engineering using BMSCs and porous scaffolds.

Key words: Tissue engineering; Bone formation; Porous scaffolds; Bone marrow-derived stromal cells (BMSCs); Cell seeding method; Low pressure

INTRODUCTION

In the orthopedic fields, autologous bone grafts are commonly used for spinal fusion and repair of large bone defects caused by tumor, trauma, or infection. However, the utility of these grafts is complicated by the limited quantities of autologous bone and the invasive nature of harvesting autografts and its associated complications (1,3). To solve these problems, tissue engineering techniques for bone regeneration have been developed that combine cells such as bone marrow-derived stromal cells (BMSCs), which have osteogenic capacity when cultured under appropriate conditions (8,13,18), with porous scaffolds (4,17). There are many reports demonstrating the bone formation capability of BMSCs/porous ceramic composites even at extraskeletal sites (7,14,15,25).

To develop more efficient bone regeneration using cells and porous scaffolds, it is essential to introduce an adequate number of cells into porous scaffolds. In order to achieve this, a suspension with a higher concentration of cells or a more efficient cell seeding method can be used. However, it is well known that the osteogenic capacity of BMSCs decreases with proliferation (20,23,26), and furthermore, obtaining a large number of BMSCs is costly and requires long culture periods. The importance of cell seeding efficiency has been discussed in other tissue engineering fields as well, including cartilage

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Address correspondence to Shinichi Sotome, M.D., Ph.D., Section of Regenerative Therapeutics for Spine and Spinal Cord, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan. Tel: +81-3-5803-5272; Fax: +81-3-5803-5272; E-mail: sotome.orth@tmd.ac.jp

(22,27), heart (6), vessels (21), and liver (30). Therefore, developing an efficient method of seeding BMSCs into porous scaffolds will be a major step in tissue engineering techniques for bone regeneration. Stable and enhanced tissue regeneration, including bone formation, will be obtained by virtue of efficient seeding of cells into porous scaffolds.

Because seeding with simple droplets of cell suspension onto porous scaffolds is not efficient, other improved cell seeding methods have been reported. These seeding methods can be roughly classified as "perfusion," "rotational," "low pressure," and "centrifugation" methods (10). Perfusion methods using bioreactors are used for cell seeding into porous scaffolds in many laboratories (2,19,31), and modified methods with techniques such as an oscillating perfusion system have also been developed (29). Rotational methods seed cells by rotating the cell suspension with a stirrer (5). Application of low pressure is comparatively pervasive. Dong et al. and Wang et al. reported the enhancement of bone formation by seeding cells into porous ceramic scaffolds using a vacuum desiccator (9,28). However, most of these methods require special devices, thus complicating these procedures. In order for a cell seeding method to be clinically applicable, it must use simple techniques and devices and be able to be completed in a short time and in an efficient manner.

To satisfy both the cell seeding efficiency and the availability in techniques and devices, we devised a novel method in which only a small glass chamber, two syringes, and a connector with a valve are needed. In principle, this method is similar to the conventional method using a low-pressure condition, in which low pressure is applied to remove the air remaining in the pores after soaking porous scaffolds in a cell suspension (9,28). In our method, to remove air and penetrate cell suspension more efficiently than the conventional methods, porous scaffolds are soaked in a cell suspension under a low-pressure condition created by aspiration prior to soaking. An efficient seeding can be expected with efficient air removal and penetration of cell suspension. In this study, we examined the influence of low pressure on rat BMSCs and the usefulness of the modified method for bone tissue engineering.

MATERIALS AND METHODS

Culture of BMSCs

All experiments were performed in accordance with the guidelines of Tokyo Medical and Dental University for the care and use of laboratory animals.

BMSCs were obtained from the femurs of 7-week-old Fisher 344 male rats, according to the method described by Maniatopoulos et al. (13). Briefly, both ends of the femurs were cut and the bone marrow was flushed three times with Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., USA). The bone marrow was plated in T-75 culture flasks (BD Biosciences, USA) and cultured in DMEM, containing 10% fetal bovine serum (Sigma Chemical Co.) and 1% antibiotic-antimycotic supplement (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericine B; Invitrogen Co., USA) at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced twice a week thereafter. When adherent BMSCs in the cultured flasks became nearly confluent after about 10 days of culture, they were used for the following examinations.

Influence of Low Pressure for BMSCs

We first analyzed the proliferative and osteogenic capabilities of BMSCs exposed to low-pressure conditions to elucidate whether BMSCs were influenced by the low-pressure condition used in the following cell seeding method. Cultured BMSCs were harvested with 0.25% trypsin and 1 mM EDTA (Invitrogen Co.) and suspended in standard medium at a concentration of 2×10^6 cells/ml. This cell suspension was divided into five groups. Each cell suspension was infused into the glass chamber and exposed to control (760 mmHg) or various low-pressure conditions (50, 100, or 200 mmHg for 1 min, and 50 mmHg for 10 min) simulating the modified technique (SPUL) mentioned in the following section. The modified cell seeding technique can be completed within a few minutes, and cells are exposed to low pressure for less than 1 min. Therefore, we selected 1 min as the exposure time and compared it to 10 min to examine the influence of exposure time. Subsequently, the treated BMSCs suspensions were replated and used for analysis of proliferative capability, alkaline phosphatase (ALP) activity, and mineralization capability.

For the assessment of proliferative capability, each cell suspension was plated at a density of 2×10^3 cells/cm^2 in 12-well culture plates (BD Biosciences) and cultured in standard medium. These BMSCs were harvested at days 1, 5, and 10 with 500 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA) per well and sonicated to destruct the cell membrane. DNA concentrations of the samples were measured using PicoGreen® dsDNA Quantitation Reagent (Invitrogen Co.) according to the manufacturer's instructions. Total DNA content per well was represented as the number of the cultured BMSCs. For the assays of osteogenic capabilities, each cell suspension was seeded at a density of 1×10^4 cells/cm^2 in 12-well (ALP activity) or 6-well (mineralization capability) culture plates with standard medium. The standard medium was changed to an osteogenic medium after 24 h of plating. The osteogenic medium was composed of standard medium, 100 nM dexamethasone

(Sigma-Aldrich Co., USA), 50 μg/ml L-ascorbic acid phosphate magnesium salt *n*-hydrate (Wako, Japan) and 10 mM β-glycerophosphate (Sigma-Aldrich Co.). For the measurement of ALP activity, the cells were collected with 500 μl 0.2% Triton-X 100 (Sigma Chemical Co.) at days 0, 4, and 8 of osteogenic differentiation culture and sonicated. An aliquot (10 μl) of each sample was added into 100 μl of 10 mM *p*-nitrophenyl phosphatase hexahydrate containing 1 mM $MgCl_2$ and 56 mM 2-amino-2-methyl-1,3-propanediol in a 96-well plate and the mixture was incubated at 37°C for 30 min. By measurement of the absorption at 405 nm with a spectrophotometer, the concentration of *p*-nitrophenol product was confirmed. To normalize the concentration of *p*-nitrophenol, the DNA content of the sample was also measured using PicoGreen® dsDNA Quantitation Reagent. Mineralization capability was assayed by counting the number of mineralized nodules of each well using the von Kossa stain. At 3 weeks of the culture, medium was removed and cells were washed with 10 mM phosphate-buffered saline and fixed with 10% neutral buffered formalin for 10 min. After fixation, cells were washed with 0.1 mol/L cacodylic buffer (Wako), and then 3% silver nitrate solution (Wako) was added and irradiated with UV light for 1 h. Mineralization capability was represented by the number of mineralized nodules counted manually.

Three Procedures of Seeding Cells Into Porous Scaffolds

In this study, we compared three techniques including the modified method for seeding BMSCs into porous scaffolds. The porous materials used in the experiments were porous β-tricalcium phosphate (β-TCP) blocks, OSferion®, provided from Olympus Co. (Tokyo, Japan). The blocks were $5 \times 5 \times 5$ mm in dimension and 75% porous, with pore sizes ranging from 200 to 400 μm. The pores were interconnected by paths with diameters of 100–200 μm.

The three cell seeding techniques were as follows: 1) spontaneous penetration under normal pressure through the procedure (SP); 2) spontaneous penetration and subsequent low-pressure treatment to eliminate the remained air in pores (SPSL), the conventional vacuum technique; and 3) spontaneous penetration under low-pressure condition (SPUL), the modified technique. These techniques were carried out using a simple device shown in Figure 1. This device is composed of four major parts: an airtight glass chamber where porous scaffolds are placed, a stopper with a tube valve, a syringe to remove air in the chamber, and a syringe to infuse the cell suspension. Figure 2 is a scheme of each cell seeding technique using this device. In SP, spontaneous impregnation is used as the cell suspension is dropped into the pores. In SPSL, cell seeding efficiency is improved by bubbling out the residual air in the pores, which prevents liquid impregnation. This method is fundamentally the same as the previously reported technique using a vacuum desiccator (9,28). SPUL is expected to demonstrate a similar effect to SPSL but with higher efficiency. SPUL consists of the following four steps. In the first step, porous β-TCP blocks are placed into the glass chamber, and in the second step, the air in the chamber is removed by a syringe to create a vacuum. In the third step, the cell suspension is slowly infused into the chamber, maintaining the low-pressure condition, and the blocks are soaked in the suspension. In the final step, the valve is released so that the pressure in the chamber normalizes. At the penetration stage, the cell suspension is impregnated into the pores with less disturbance by surface tension, and at the subsequent vacuum breaking stage, the cell suspension is pushed into the pores without interference by air bubbles. The

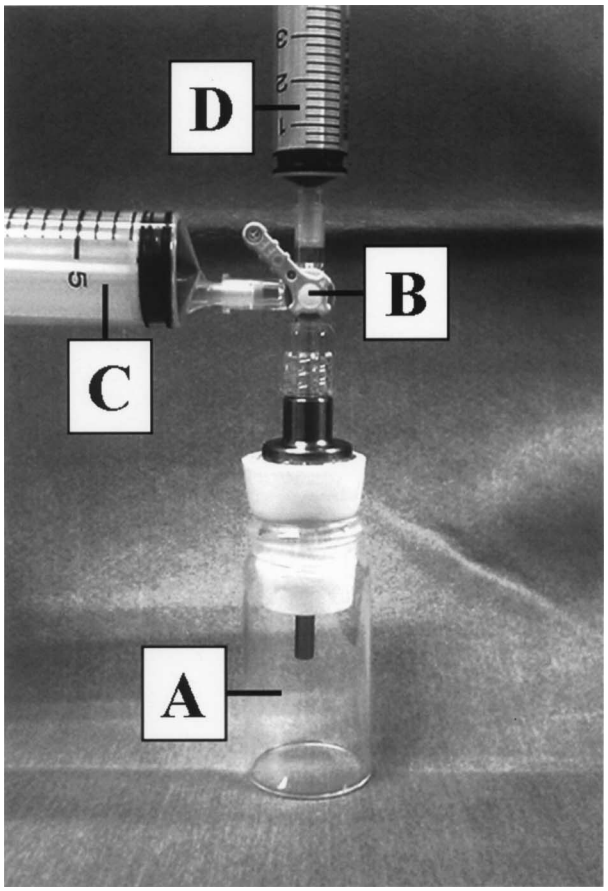

Figure 1. A novel device for seeding cell suspension into porous scaffolds is composed of four major parts: (A) an airtight glass chamber for porous β-TCP cubes, (B) a stopper with a tube valve, (C) a syringe to remove air in the chamber, and (D) a syringe to infuse the cell suspension.

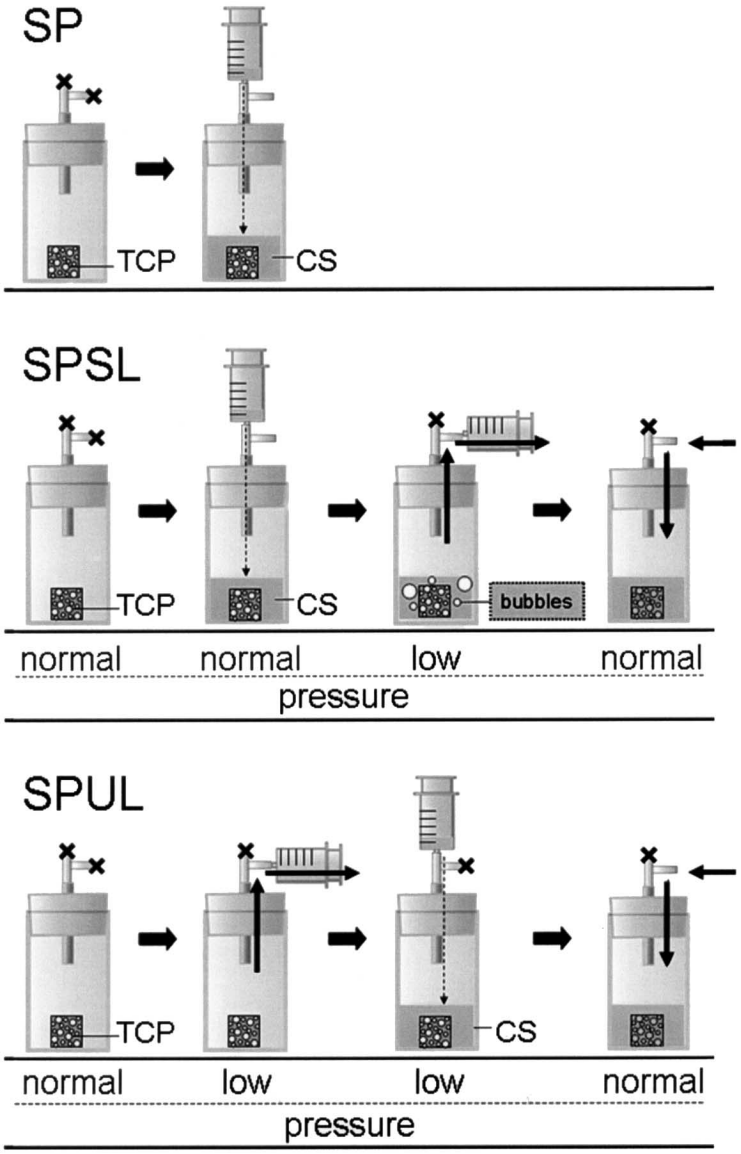

Figure 2. A schema of each cell seeding techniques using a novel device. Spontaneous penetration (SP): the porous β-TCP blocks in the chamber were only immersed in the cell suspension under normal pressure. Spontaneous penetration and subsequent low pressure (SPSL): after the porous β-TCP blocks were immersed in the cell suspension, low pressure was applied to remove residual air in the pores. Spontaneous penetration under low pressure (SPUL): under low-pressure conditions, the cell suspension was infused into the glass chamber to immerse the blocks, and then the valve was released to regain the normal pressure condition. In SPSL and SPUL, low pressure was adjusted to 50 mmHg. TCP: porous β-tricalcium phosphate, CS: cell suspension.

difference between SPSL and SPUL is the order of applying low pressure, and the low pressure was adjusted to 50 mmHg in both the methods.

Quantification of Cells Introduced Into Porous Scaffolds

Cultured BMSCs were harvested and suspended in the plasma of Fisher 344 male rats at a concentration of 2×10^6 cells/ml. According to our previous report, which demonstrated that the bone formation of rat BMSCs/β-TCP composites was enhanced by using blood plasma to introduce the BMSCs into the scaffolds (24), rat blood plasma was used to prepare a cell suspension instead of culture medium. Kruyt et al. also reported the usefulness of blood plasma (11). Plasma was prepared by centrifugation ($2380 \times g$, 10 min) of the

whole blood of the Fisher 344 male rats containing 10% citrate phosphate dextrose as an anticoagulant. Immediately after mixing the cell suspension with 2% calcium chloride at a volume ratio of 1:7 to form a fibrin gel, the cell suspension was seeded into porous β-TCP cubes using the three techniques. For each cell seeding method, four porous blocks were used. After gelation of the cell suspension caused by fibrinogen contained in plasma was completed, these blocks were fixed with 10% neutral buffered formalin for 1 week, and the samples were embedded in methylmethacrylate (Wako). Each sample was sectioned at 5 μm thickness and five sections at equal intervals were made from one sample. After staining with toluidine blue, BMSCs in all pores of each whole sectional area were manually counted. The efficiency of seeding BMSCs was defined as the average number of BMSCs per sectional area.

In Vivo Bone Formation Capability

We examined whether the cell seeding procedures influenced in vivo bone formation capability of the BMSCs. After the primary culture became nearly confluent, the BMSCs were cultured in the osteogenic medium for 4 days. These BMSCs were detached and suspended in the plasma of Fisher 344 male rats at a concentration of 2×10^6 cells/ml and seeded into porous β-TCP blocks by the three cell seeding methods. Five blocks were used for each method and each time point. When the gelation of the cell suspension was completed, the BMSCs/porous β-TCP composites were implanted subcutaneously into the backs of 7-week-old Fisher 344 male rats. These implants were harvested at 3 and 6 weeks after implantation and fixed with 10% neutral buffered formalin. Then these implants were decalcified with K-CX solution (Falma, Japan), dehydrated, and embedded in paraffin and sectioned at 5 μm thickness. Seven sections at equal intervals were made from one implant and stained with hematoxylin and eosin. By using an image-editing software (Photoshop®, Adobe Systems Incorporated, USA) and an image analysis software (Scion Image®, Scion corporation, USA), the area of bone formation was manually selected and measured (Fig. 3). The bone formation area was defined as the ratio of newly formed bone per whole sectional area including pores and β-TCP. The bone formation area of one implant was an average of seven sections.

Statistics

Average values were expressed as the mean ± SD. When significant differences were detected by one-way ANOVA, post hoc pairwise comparisons were performed using the Tukey-Kramer test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Proliferative and Osteogenic Capabilities of BMSCs After Exposure to Various Pressure Conditions

Proliferative capability was defined as the DNA content, measured at 1, 5, and 10 days after exposure to various pressure conditions. The DNA content of each group increased according to the duration of culture, and there was no significant difference among the groups at any time point (Fig. 4A). Osteogenic capability was analyzed by both ALP activity and mineralization capability. ALP activity had peak at day 4 and decreased at day 8, and there was also no significant difference among the groups at any time point (Fig. 4B). Figure 4C shows the cultured BMSCs stained with von Kossa solution and all groups seemed to be similarly stained. Mineralization capability was quantified as the number of mineralized nodules. By quantitative analysis, the number of mineralized nodules in 200 mmHg for 1 min and 50 mmHg for 10 min appeared to be smaller than other groups. However, there was no statistically significant difference in the mineralization capabilities among the groups treated with various low-pressure conditions, including the control (Fig. 4D). From these results, it was clarified that the low-pressure conditions tested in this experiment did not influence the proliferative and osteogenic capabilities of BMSCs [i.e., the modified method (SPUL) would not influence BMSCs seeded into porous scaffolds].

Efficiency of Seeding BMSCs

Because the in vitro studies showed that various low-pressure conditions had no effect on BMSCs, the pressure level in low-pressure seeding techniques (SPSL and SPUL) was adjusted to 50 mmHg, and we analyzed the difference in cell seeding efficiency among the three cell seeding methods. Histological sections were made of the β-TCP blocks immediately after the introduction of cells. Most of the BMSCs in the blocks were seen inside the pores retained by fibrin networks and did not attach to the β-TCP scaffolds directly (Fig. 5A). The BMSCs seeded by SPUL existed inside almost all the pores of the whole sectional area while there were some pores without BMSCs in the other cell seeding techniques. For quantitative analysis, the number of the BMSCs in the pores of whole sectional areas was counted manually. Compared to the SP group, cell seeding efficiencies were 1.8-fold higher in the SPSL group and threefold higher in the SPUL group. SPUL (seeding under low pressure) showed the highest cell seeding efficiency among the three techniques (Fig. 5B).

In Vivo Bone Formation Capability

To evaluate whether the efficiency of seeding BMSCs influenced the quantity of bone formation, the bone for-

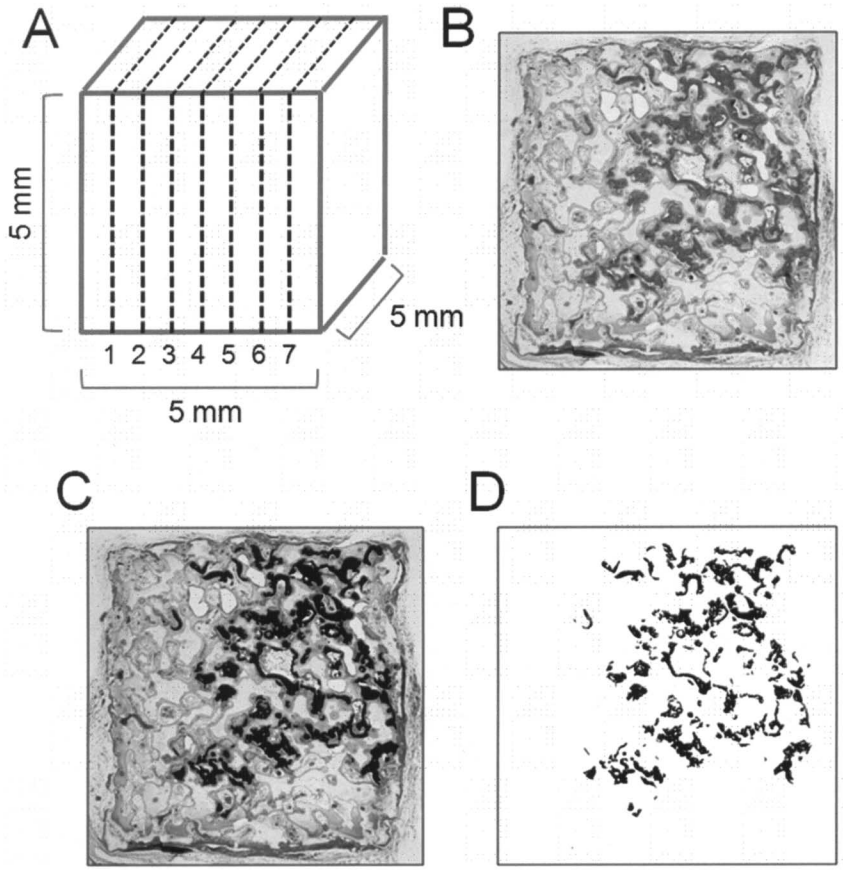

Figure 3. The method of quantitative analysis of newly formed bone. (A) Seven sections at equal intervals were made from one implant. (B) Each section was stained with hematoxylin and eosin. (C) The area of bone formation was manually painted with black color using Photoshop® image-editing software. (D) Only the area of the black color of each section was extracted using the software and measured using Scion Image® image analysis software. The bone formation area was defined as the ratio of newly formed bone per whole sectional area including pores and β-TCP. The bone formation area of one implant is an average of seven sections.

mation capabilities of the implants, which were combined with BMSCs by the three methods, were examined by an ectopic bone formation model. At postoperative week 3, the implants prepared using SPUL had more bone formation than the other methods, although it was localized in the peripheral area. Additionally, the formed bone of the SPUL group was thicker than the others (data not shown). At postoperative week 6, differences in bone distribution became more prominent than those of week 3. Figure 6 shows a representative section at the central part of the extracted implants. Bone formation in the implants by SPUL spread over almost the entire implant whereas the distribution of bone formation in implants by SP and SPSL was localized. In the high-magnification views, there was no significant difference in local bone thickness among the three groups. However, absorption and replacement to newly formed bone of β-TCP seems to be more advanced and, further, bone marrow appeared only in the implants of the SPUL group (Fig. 6). The quantitative analysis demonstrates more bone formation in the SPUL group, although there was no significant difference statistically at 3 weeks after implantation. Consistent with the result of the cell seeding efficiency experiment, a significant amount of bone formation was seen at postoperative week 6 in the SPUL group compared with the other two groups (Fig. 7).

DISCUSSION

In this study, we demonstrated the role of SPUL with a simple device for tissue engineered bone regeneration using BMSCs. Though, in principle, it was predictable that SPUL would be more efficient than SPSL at seeding cells into porous scaffolds, it was unclear whether the low-pressure conditions applied in this procedure would influence cell viability and differentiation capability. Previously, we reported that SPSL using a vacuum des-

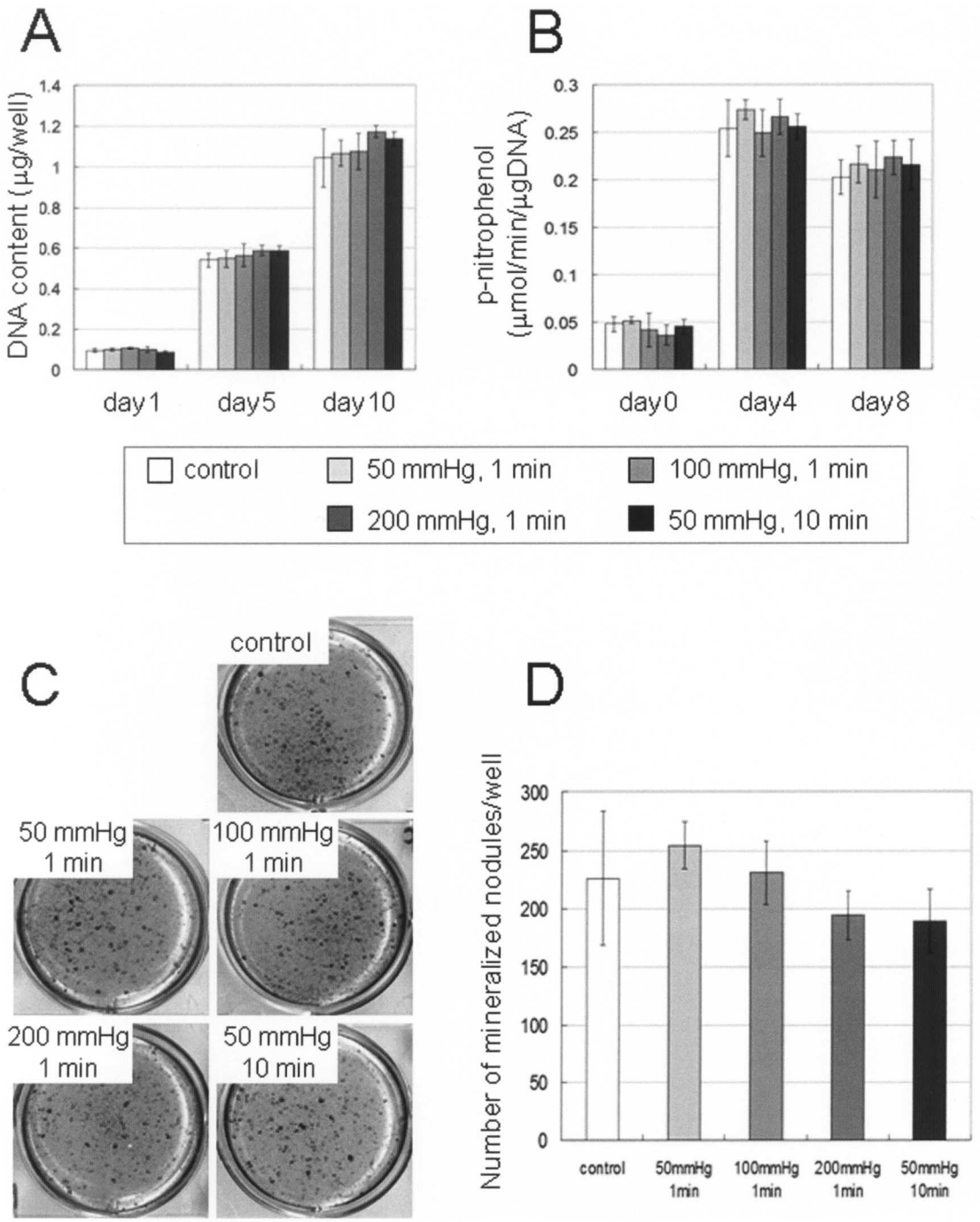

Figure 4. Proliferative and osteogenic capabilities of BMSCs treated with various low-pressure conditions: (A) Proliferative capability expressed as DNA content, (B) ALP activity, (C) the photographs of BMSCs stained with von Kossa solution, and (D) number of the mineralized nodules. There was no significant difference between normal and various low-pressure-treated groups in all proliferative and osteogenic capability assays. The data are expressed as mean ± SD.

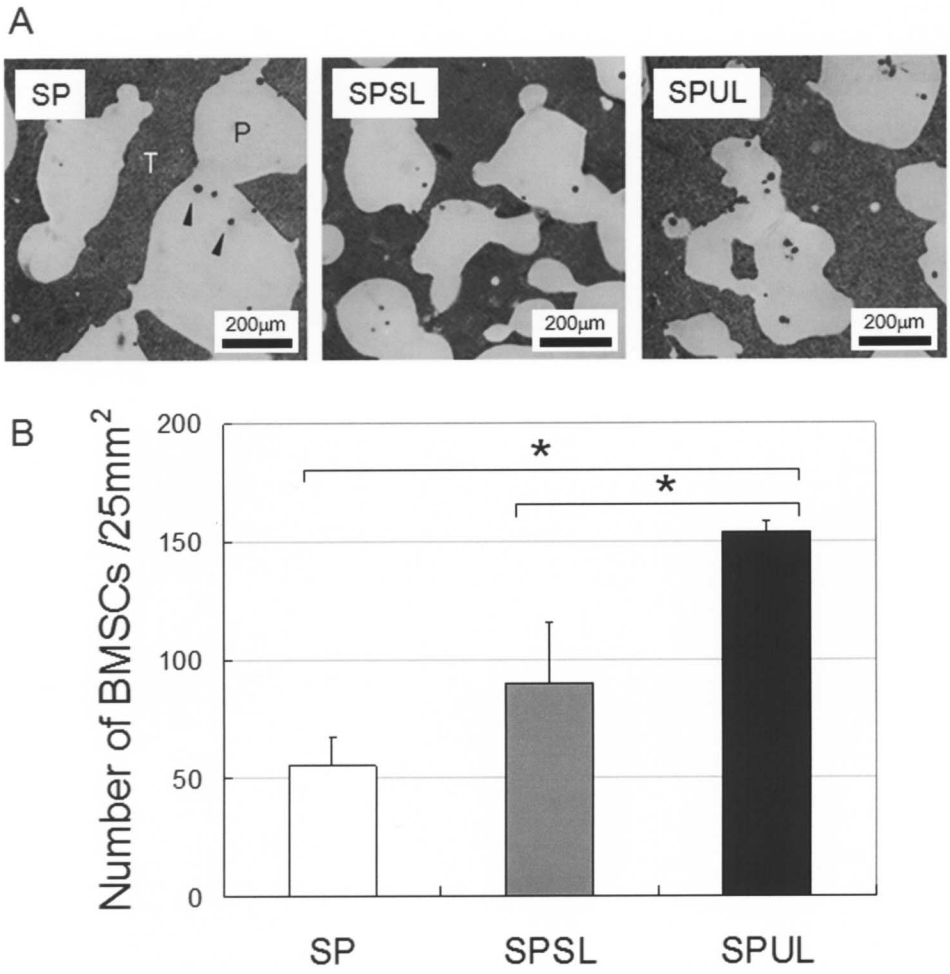

Figure 5. (A) Toluidine blue-stained sections of β-TCP blocks combined with BMSCs by the three techniques: SP, SPSL, and SPUL. The BMSCs seeded by SPUL existed inside almost all the pores of the whole sectional area while there were some pores without BMSCs in SP and SPSL. Arrowheads indicate seeded BMSCs retained inside the pores of β-TCP by fibrin networks. T: β-TCP, P: pores. Original magnification: 40×, scale bar: 200 μm. (B) Quantitative analysis of BMSCs number seeded into the pores of β-TCP blocks. A significantly large number of BMSCs could be seeded in the pores of β-TCP blocks with SPUL compared to the other two methods. The data are expressed as mean ± SD (*$p < 0.05$).

iccator (100 mmHg for 100 s) facilitated bone formation in porous scaffolds (28), and Dong et al. also reported that SPSL increased ALP activity and osteocalcin content in BMSCs/hydroxyappatite (HA) composites at 4 and 8 weeks after implantation (9). However, the results of these reports were due to both increased cell seeding efficiency and the effects of low-pressure conditions on the abilities of BMSCs. There are few reports that examined the relationship simply between low pressure and cells (22). This study examined the sole influence of low pressure conditions on viability of BMSCs and osteogenic capability in vitro. The results indicated that there was no significant difference in proliferative and differentiation capabilities among the BMSC groups exposed to each low-pressure condition, although it seemed that some groups decreased in mineralization capability. Based on these results, we confirmed that acute and short exposure to low pressure would not affect the capabilities of BMSCs, at least within the ranges tested in this study, and then we examined SPUL for cell seeding efficiency and in vivo bone formation.

To further analyze SPUL, we compared the cell numbers in the BMSCs/porous β-TCP composites prepared by each cell seeding method and bone formation quantitatively. The scaffolds used in these studies were porous β-TCP blocks, which have excellent osteoconductivity, biodegradability, biocompatibility, and rich interpore connections (12,16,25). SPUL resulted in higher number

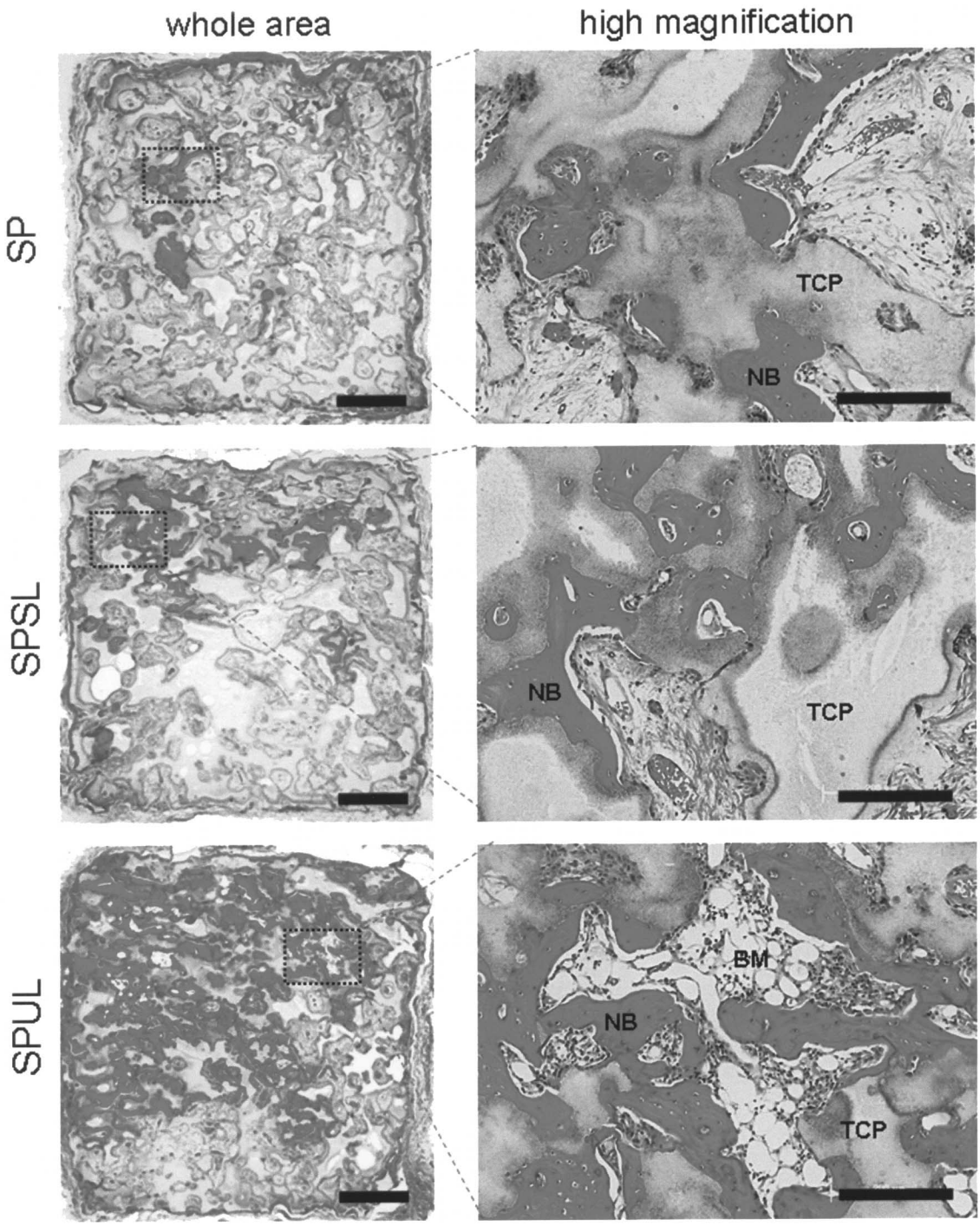

Figure 6. Hematoxylin and eosin staining of histological sections at 6 weeks after implantation. The amount of bone formation of the SPUL group was significantly larger than the other groups. In the high-magnification views, there was no clear difference in local bone thickness among the three groups. However, absorption and replacement to newly formed bone of β-TCP seemed to be more advanced, and bone marrow appeared only in the SPUL group. TCP: β-TCP, NB: newly formed bone, BM: bone marrow. Original magnification: whole area 12.5×, high magnification 40×; scale bar: whole area 1 mm, high magnification 200 μm.

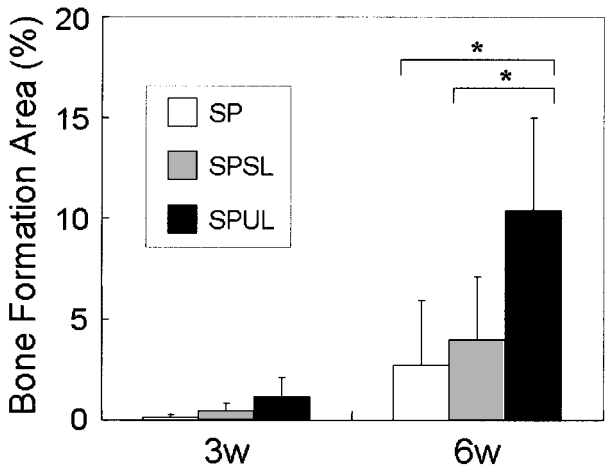

Figure 7. Quantitative analysis of bone formation area. Although there was no significant difference at 3 weeks, more bone formation was seen in the SPUL group. Consistent with the result of the cell seeding efficiency experiment, a statistically larger amount of bone formation was seen at 6 weeks in the SPUL group than in the other two groups. The data are expressed as mean ± SD ($*p < 0.05$).

of cells immediately after seeding than the other techniques. Furthermore, in BMSCs/β-TCP composites prepared by SPUL, the cell distributions were more uniform, and almost all the pores were filled with BMSCs, while BMSCs existed in partial pores in the other techniques. The bone formation analysis also demonstrated the advantages of SPUL. In this examination, BMSCs/β-TCP composites were implanted immediately after cell seeding without further cell culture. Therefore, the influence on seeding efficiency would be confirmed more accurately. Three weeks after implantation, there were no significant differences in the quantitative analysis of newly formed bone among the groups; however, the SPUL group showed more bone formation, and the formed bone tended to be thicker. At 6 weeks, the differences among the groups were larger and statistically significant. The quantity of bone formation of the SPUL group was more than two times that of the other groups. Because the differences among the three methods involved the cell seeding efficiency and distribution of the cells, this result suggests that the number and the distribution of the seeded cells influences bone tissue regeneration.

We think the mechanism for the superior efficiency of the SPUL group is as follows. SPUL and SPSL can be divided into three phases: "low pressurizing," "penetration," and "low-pressure release." In the low-pressurizing phase of SPUL, air in the pores, which disturbs the entry of cell suspension at the next penetration phase, can be efficiently removed by decreased pressure without interruption by the cell suspension. In the penetration phase, the cell suspension is injected into the low-pressure chamber. Therefore, the cell suspension penetrates efficiently into the pores because of less interruption by residual low pressurized air and lower surface tension. In the pressure release phase, the cell suspension is pushed into almost all the pores by normal air pressure. The first phase of SPSL is the penetration phase. In contrast to SPUL, penetration of cell suspension is partly interrupted by the normobaric air remaining in the pores. The next phase is the low-pressurizing phase. When the air is removed from the pores, the cell suspension has already penetrated some pores and disturbs the extrusion of air. Furthermore, a part of the cell suspension, which has already entered into the pores, is also extruded with the air. The last phase of SPSL is the low-pressure releasing phase as well as SPUL. However, at the start of this step, there is more residual air in the pores than in the SPUL method. Furthermore, it is inevitable that the cell suspension introduced into the pores contaminates the air bubbles attached around the porous block, but there was no air bubble formation during SPUL. Through the three phases, the efficiency of SPUL at every phase is superior to that of SPSL. In addition, the difference will be larger when using a cell suspension with higher viscosity because the interruptions by the cell suspension and the air that arises in SPSL are more potent.

There are three advantages of SPUL: 1) this method has high cell seeding efficiency, 2) the devices are simple and readily available, and 3) the technique is easy and completed within a few minutes. These advantages are very important for clinical usage of the tissue engineering technique. The glass chamber used in SPUL may be substituted with an injection syringe, which is inexpensive and available at most institutions. Furthermore, this method will also be applied for introduction of other liquids into porous materials not only in the tissue engineering fields but in other fields as well.

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