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Seeding of mesenchymal stem cells into inner part of interconnected porous biodegradable scaffold by a new method with a filter paper

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An appropriate physical support provided by scaffolds creates a supportive environment that directs proliferation and differentiation of stem cells. However, it is difficult to homogenously inoculate stem cells into the inner part of scaffolds at high cell densities. In this study, mesenchymal stem cells were seeded into a hydroxyapatite/poly (D, L-lactic-co-glycolic acid) (HAP/PLGA) scaffold that had enough mechanical strength and porous 3-D structure. With an aid of a filter paper placed under the bottom of a HAP/PLGA block, the cells suspended in a culture medium flowed from the top to the bottom through interconnected pores in the scaffold, and distributed almost homogenously, as compared to cell distribution near the surface of the block by the conventional method using centrifugation or reduced pressure. This simple method with a filter paper may be useful in preparation of cell-scaffold complexes for tissue engineering.

Keywords: Seeding method, Scaffold, Apatite, Mesenchymal stem cell, PLGA

INTRODUCTION

Tissue engineering presents a promising approach to bone repair; for example, it induces bone formation around dental implants, in fracture gaps or within osteoporotic bone[1-3]. The advantages of this approach include *in vitro* cell expansion. Initially, a small number of cells can be used after expansion in culture to treat multiple patients with a large bone defect.

Bioactive ceramics have been used for bone tissue engineering. However, their clinical applications have been limited because of their brittleness, difficulty of shaping[4], and a slow degradation rate in the case of hydroxyapatite (HAP)[5]. Increasing interest has currently been focused on polymer/ceramic composite materials as bone substitutes[6-11], because these materials may have advantages over polymer and ceramic scaffolds. For example, the porous HAP/poly (D, L-lactic acid) (PDLLA) is neither too hard compared to bone nor too brittle compared to porous scaffolds made of calcium phosphates, and thus it can be cut using scissors or a scalpel to make shapes to fit any bone defect[12]. The combination of calcium phosphate ceramics and biodegradable polymer would allow for better malleability as well as reduce crush powder caused by the chipping from materials. In addition, the biodegradation rate of the material can be controlled by selecting a biodegradable polymer with suitable composition.

Various synthetic materials, including poly (lactic acid) (PLA), PLGA, polyglycolic acid (PGA), polyanhydrides, polyimides and polyphosphazenes, have been developed and used as scaffolds for cell transplantation[13-15]. Several techniques, including solvent-casting/particulate leaching[16], phase separation[17], fiber extrusion and fabric forming processing[18] and gas foaming have been used to fabricate the polymers into porous matrices[19]. However, interconnected pores made by these methods are less than 100 μm, and it is difficult to seed cells in large scaffolds homogenously or at high densities. To solve these problems, the present study used highly porous HAP/PLGA blocks prepared by compression of powders and subsequent melting of adjacent powder surfaces.

Several techniques, including the surface seeding[20] and injection seeding methods[21], have been used to seed cells into porous scaffolds. Sometimes cells are dynamically seeded using spinner flasks[22] and bioreactors[23,24]. In addition, cells are seeded by the perfusion method[25]. Dynamic seeding is effective and cell proliferation might be improved by physical agitation[26]. However, these approaches require special equipment and it takes a long time for cell seeding.

In this study, we developed a novel method of cell seeding. Cells in suspension at high densities were seeded on the top of a HAP/PLGA block scaffold, which was placed on a filter paper. With aid of the filter paper, cells flowed from the top to the bottom through interconnected pores in the scaffold, and distributed almost homogenously. We compared this method with

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the static seeding, centrifugation seeding and vacuum seeding methods, using human mesenchymal stem cells (hMSC).

MATERIALS AND METHODS

Preparation of scaffolds

PLGA (LA:GA=75:25, Mw: 25 kDa) was dissolved in 1,4-dioxane (Wako, Osaka, Japan) to yield a solution of 8% w/v, and 2 specimens were prepared. One mixed with HAP powder (diameter 5–20 μm; HAP-200[27] Taihei Chemical, Osaka, Japan) (mixed HAP/PLGA mass ratio of 1:1) and one without HAP powder. 22 mL of this solution was loaded into Teflon molds (150×150 mm, height=25 mm) packed with 100 g of sodium chloride (Wako, Osaka, Japan). Following solvent evaporation, polymer sponges with entrapped salt particles (25 mm thick) were carefully removed from the molds. The salt was removed from the sponges by leaching in distilled water stirred with a magnetic bar for 48 h, and the sponges were crushed. The appropriate weight of obtained powders (HAP/PLGA powder=15 mg, PLGA powder=10 mg) was loaded into a stainless disk mold (diameter 5 mm; FTC, Chiba, Japan) and was packed into a thickness of 2 mm using rod-shaped press mold. The powder packed mold was heated at 70°C for 8 min in the oven, in order to melt the powder surface and consolidate powder. This mold was cooled at −30°C for 8 min in the freezer, and the configurated HAP/PLGA block or PLGA block was taken out from the stainless mold. The mechanical strength and pore structure can be regulated by processing parameters.

PLGA porous sponges were fabricated using the SCPL (solvent casting and particulate leaching) technique[16] which has been described previously. In this process, PLGA was dissolved in 1,4-dioxane (Wako, Osaka, Japan) to yield a solution of 10% w/v, and 2 specimens were prepared. One was mixed with HAP powder (mixed HAP/PLGA mass ratio of 1:1) and one without HAP powder. The mixed HAP/PLGA solution was loaded into Teflon cylinders (φ5×2 mm) packed with 0.4 g of sodium chloride, which had been sieved to a size between 250 and 500 μm. Following solvent evaporation, polymer sponges with entrapped salt particles were carefully removed from the molds. The salt was removed from the sponges by leaching in distilled water stirred with a magnetic bar for 48 h.

In the evaluation of scaffold characterization, we used the PLGA block and PLGA sponge produced using conditions that do not add HAP powder as a comparative control.

Scaffold characterization

The appearance of the scaffolds was examined using a scanning electron microscope (SEM; TM-3000, Hitachi High-Technologies, Tokyo, Japan). Samples were dehydrated in ascending grades of ethanol, dried, and mounted on an aluminum stub using a double-sided carbon tape. The specimens were coated with Au-Pd using a Magnetron Sputter Coater (MSP-1S, Vacuum Device Inc., Ibaraki, Japan) and examined with SEM at an acceleration voltage of 15 kV. Compression tests were performed with a mechanical tester (CR-500DX, Sun Scientific, Tokyo, Japan). The mean pore diameter was evaluated using the SEM images of cell-free scaffolds. Linear measurements from the most distant points of the pore openings were recorded (n=10/scaffolds)[28].

Cell culture

Bone marrow-derived hMSC were obtained from RIKEN CELLBANK (Age 59 males, 3rd passage culture), and maintained in monolayer cultures. The cells were seeded at 5×10^3 cells/cm^2 in 100 mm tissue culture dishes and maintained in 10 mL of alpha-MEM supplemented with 10% fetal bovine serum and 3 ng/mL fibroblast growth factor-2. Fibroblast growth factor-2 is essential for maintenance of proliferation activity and osteogenic potential of MSC in culture[29]. When cultures were approaching confluence, cells were harvested with 0.25% trypsin/EDTA. The cells were seeded at 5×10^3 cells/cm^2 in 100 mm tissue culture dishes and maintained in the above medium. hMSC obtained from the cultures at the 7th–9th passages were used for experiments. For fluorescent analysis, cells were harvested with 0.25% trypsin/EDTA and resuspended at 1×10^6 cells/mL with alpha-MEM. DiI fluorescent lipophilic tracer (Molecular Probes, Eugene, OR, USA) was added to the cell suspension at 5 μl/mL. After incubation for 20 min at 37°C in 5% CO_2/95% air, cells were centrifuged at 450 g for 5 min and washed twice with PBS.

Cell seeding

Prior to cell seeding, the HAP/PLGA blocks used were sterilized by γ irradiation. To prevent air bubble formation, each block was placed in 5 mL alpha-MEM in a 15 mL tube, which was placed in a vacuum desiccator for 2 min. Thereafter, the tube was incubated overnight at 37°C before cell seeding.

Flow seeding: Aliquots of 200 μL of the cell suspension (5×10^6 cells/mL, 1×10^6 cells/scaffold) were seeded onto the tops of the prewetted HAP/PLGA blocks placed on water absorption filter papers (30×30×5 mm) (Bio-RAD, Los Angeles, USA) (Fig. 1). A drop of cell suspension penetrated into the scaffold within 2 s. The penetration distance was determined by dividing the volume of the cell suspension by the area of the top side of the scaffold, and the flow rate was determined by dividing the penetration distance by the time required for the penetration of this medium into the scaffold. The flow rate of cell suspension was determined to be approximately 1.27 mm/s. The scaffolds were undisturbed in a CO_2 incubator for 1 h to allow the cells to attach to the scaffolds. Thereafter, the cell-scaffold complexes were incubated with 200 μL of alpha-MEM supplemented with 10% fetal bovine serum in 6-mm wells of a 96 multiwell plate for 12 h at 37°C in 5% CO_2/95% air.

Static seeding: The HAP/PLGA blocks were prewetted with alpha-MEM, and each block was placed in a 6-mm well of a 96 multiwell plate. 200 μL of cell

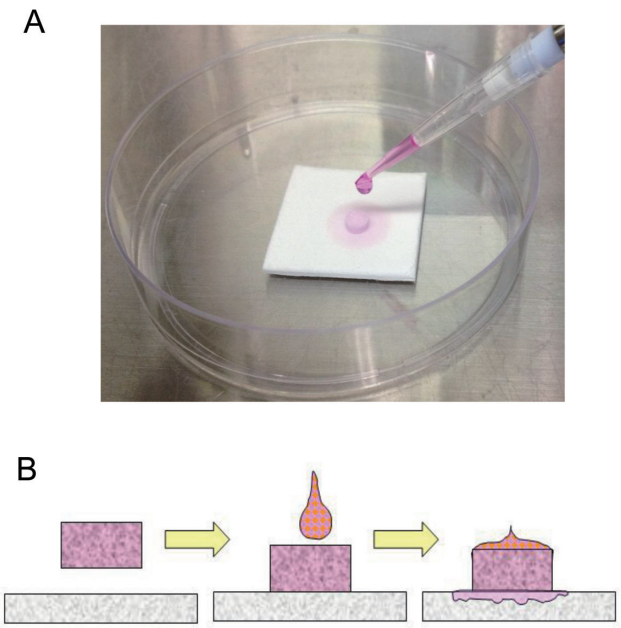

Fig. 1 A: A photograph showing cell seeding to a porous scaffold placed on a filter paper. B: Illustration of the cross-sectional view during cell seeding.

suspension (5×10^6 cells/mL, 1×10^6 cells/scaffold) was added in the 6-mm well, and incubated with the block for 12 h at 37°C in 5% CO_2/95% air.

Centrifugation seeding: The HAP/PLGA blocks were prewetted, and each block was placed in a 6-mm well of a 96 multiwell plate. Aliquots (200 μL) of the cell suspension (5×10^6 cells/mL, 1×10^6 cells/scaffold) were poured on the tops of the scaffolds and centrifuged (500 g) for 5 min using a microplate rotor (RT2S2, Hitachi Koki Co., Tokyo, Japan). After centrifugation, the cell-scaffold complexes were incubated for 12 h at 37°C in 5% CO_2/95% air.

Vacuum seeding: 200 μL of the cell suspension (5×10^6 cells/mL, 1×10^6 cells/scaffold) was added into a 15 mL polypropylene tube (Becton Dickinson) containing a dried HAP/PLGA block that was not prewetted. The tubes were put immediately into a vacuum desiccator. Low pressure was applied for 2 min. Thereafter, the cell-scaffold complexes in centrifuge tubes were cultured in a humidified incubator at 37°C in 5% CO_2/95% air for 12 h.

Fluorescent microscopy and SEM

The cell-scaffold complexes obtained by the above methods were fixed in 10% formaldehyde and cut in half at the middle. The sections at the middle were mounted on a slide and observed using a fluorescent microscopy (BX51-FL, Olympus, Tokyo, Japan). In addition, the samples were dehydrated in ascending grades of ethanol, dried, and mounted on an aluminum stub using a double-sided carbon tape. The specimens were coated with Au-Pd using a magnetron sputter coater (Vacuum Device Inc., Ibaraki, Japan) and examined with SEM (TM-3000, Hitachi High-Technologies, Tokyo, Japan) at an acceleration voltage of 15 kV.

DNA and calculation of cell number

The cell-scaffold complexes were homogenized in 1 mL of 350 mM 2-Amino-2-methyl-1-propanol, 1 mM $MgSO_4$, 2 mM $ZnSO_4$, 2 mM EDTA (pH 10.4) for 15 s at 4°C by a high intensity ultrasonic processor (40W×15 s, VCX130, SONICS, Newtown, USA). DNA content in each sample was determined using a DNA kit (PicoGreen, Molecular Probe, Chicago, USA). Fluorescence was measured in a spectrofluorophotometer (Spectramax M2, Molecular Devices, Sunnyvale, USA) at excitation and emission wavelengths 480 nm and 520 nm, respectively. In some studies, DNA contents of 2.5×10^5, 5×10^5, and 1×10^6 hMSC were determined. Using this standard curve, we estimated the number of cells in the cell scaffold complexes.

Statistical analysis

Results are presented as the mean and the standard deviation (SD) in all experiments. Student's *t*-test was performed to determine the significance of the results between selected groups.

RESULTS

Characterization of scaffolds

The HAP/PLGA and PLGA porous blocks showed highly porous and open-pore structures compared with HAP/PLGA and PLGA porous sponge fabricated by a standard solvent casting and particulate leaching technique (Fig. 2). The range of pore size was 11–320 μm (Table 1), and 70% or more was from 110 to 220 μm. Therefore, the pore size of the blocks was almost uniform throughout the matrix (Fig. 2A). The average pore sizes of the HAP/PLGA and PLGA blocks were 169 and 185 μm, respectively, which was larger than that of the HAP/PLGA and PLGA sponges. The average porosities of the scaffolds were calculated with the density of HAP and PLGA, respectively (Table 1). The average compressive strength of the HAP/PLGA and PLGA blocks was 0.33 and 0.35 MPa, respectively, which was much greater than that of the HAP/PLGA and PLGA sponges (Fig. 2C). HAP powders exposed to the surfaces of the HAP/PLGA sponge and block were observed in high magnification images of SEM (Fig. 2B).

Seeding of cells into HAP/PLGA blocks by various methods

hMSC were seeded into HAP/PLGA blocks using various methods, and the distribution of the cells in the scaffolds were examined 12 h after cell seeding. Cross sections were prepared to examine the distribution of seeded cells inside the HAP/PLGA scaffold. The cells penetrated into the inner part of the scaffold by the flow seeding method with a filter paper (Fig. 3A e.), but not by the other methods (Fig. 3A b, c, and d). Cell densities and the distribution profile of DiI-labeled hMSC in the scaffold

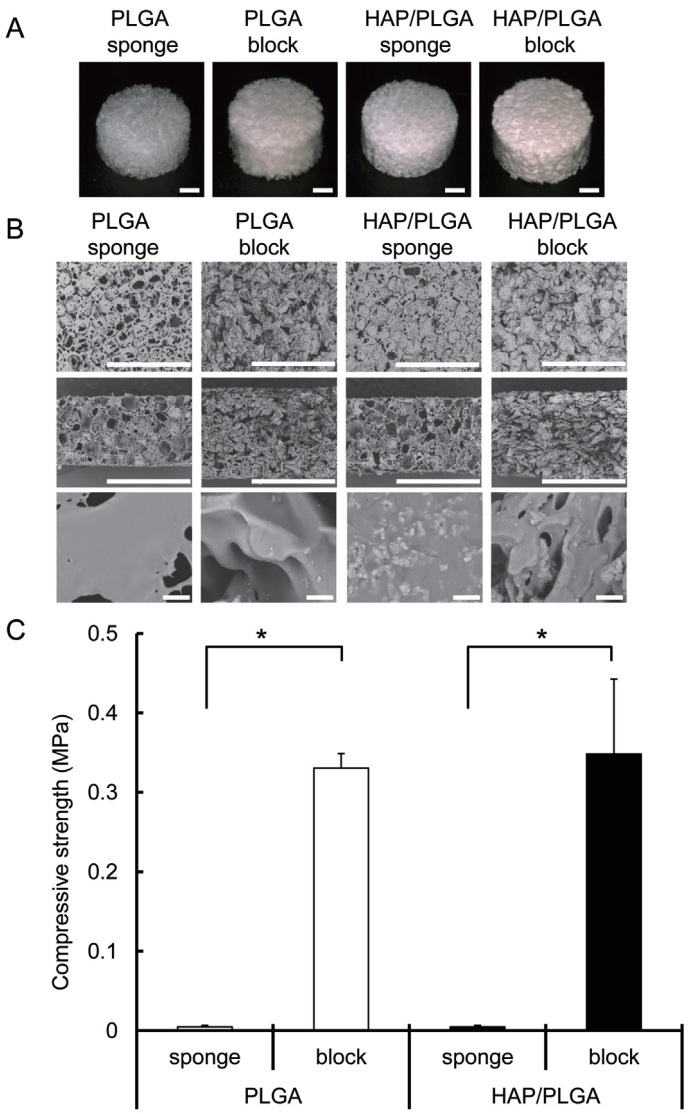

Fig. 2 A: Macroscopic images of the PLGA block, HAP/PLGA block, PLGA sponge and HAP/PLGA sponge. Scale bars, 1 mm. B: Surface (upper, low magnification; lower, high magnification) and cross-sectional (middle, low magnification) scanning electron micrographs of the PLGA block, HAP/PLGA block, PLGA sponge and HAP/PLGA sponge. Scale bars: upper and middle, 2 mm; lower, 10 μm. C: Compressive strength of the PLGA sponge, PLGA block, HAP/PLGA sponge and HAP/PLGA block. Asterisks represent significant difference (p<0.001) between the sponge and block.

Table 1 Physical characteristics of the PLGA sponge, PLGA block, HAP/PLGA sponge and HAP/PLGA block

Property	PLGA sponge	PLGA block	HAP/PLGA sponge	HAP/PLGA block
Diameter (mm)	4.90 (0.03)	4.94 (0.04)	5.01 (0.03)	5.08 (0.05)
Thickness (mm)	1.66 (0.07)	2.00 (0.03)	1.80 (0.06)	2.02 (0.02)
Volume (mL)	0.031 (0.001)	0.038 (0.001)	0.036 (0.001)	0.041 (0.001)
Porosity (%)	95.8	80.7	95.9	80.9
Avg. pore diam. (μm)	78	185	78	169
Pore size range (μm)	3–282	25–473	3–276	11–320

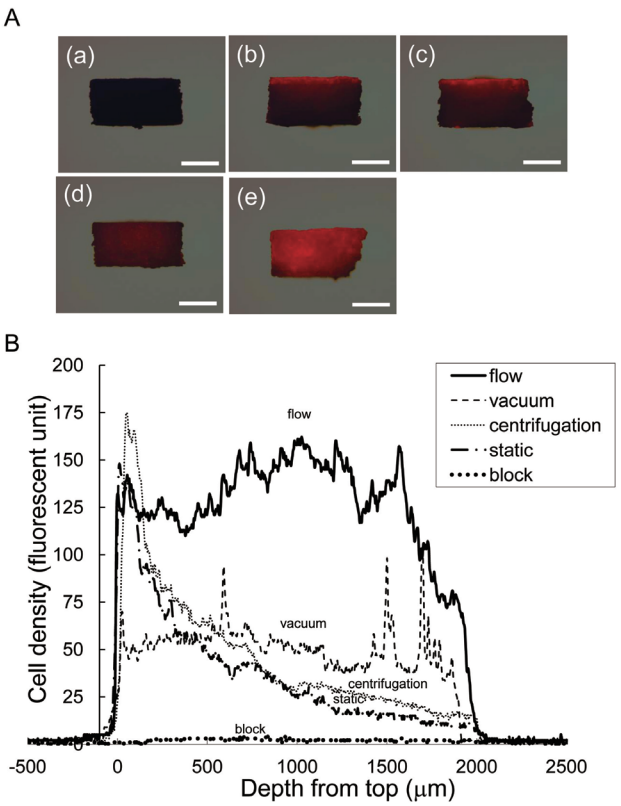

Fig. 3 A: Cross sections of HAP/PLGA blocks seeded with DiI-labeled hMSC using the different cell seeding methods: a) scaffold alone without cells, b) static seeding, c) centrifugation seeding, d) vacuum seeding, e) seeding with a filter paper. Scale bars, 2 mm. B: Cell density and distribution profiles from the fluorescent microscopic images in A. Image processing and analysis were performed using the public domain NIH Image program (developed at the US National Institutes of Health). Seeding with a filter paper gave uniform cell distribution at a high density. The static and centrifugation methods gave uneven cell distribution. The vacuum method gave uniform cell distribution at a low density.

were analyzed by using the fluorescent microscopic images and the public domain NIH Image program. To subtract background fluorescence, we used cell-free scaffolds incubated for 12 h. As shown in Fig. 3B, the flow seeding method with a filter paper allowed uniform distribution of DiI-labeled cells at high densities in the scaffold, whereas cells were located near the top of the scaffold by the centrifugation and the static methods. The vacuum method allowed uniform distribution, but the cell density in the scaffold was lower than it was in the flow seeding method.

Figure 4 shows SEM images of hMSC adhered on the top, middle and bottom of HAP/PLGA blocks by the static, centrifugation, vacuum and flow seeding methods. On the top of HAP/PLGA blocks, 4–14 cells were observed in a picture, irrespective of the methods. In the middle and bottom of the blocks, no cells were found by the static and centrifugation methods, and only a few cells were present by the vacuum method. In contrast, about 13 cells were present in both the middle and bottom by the flow method. The cell number in HAP/PLGA blocks obtained by the flow method was higher than that obtained by the other methods ($p<0.001$) (Fig. 5). In these studies, we estimated cell numbers in the scaffolds using the standard curve for DNA as a function of the number of hMSC. The approximate cell numbers in the blocks were 499,689, 385,952, 123,230 and 923,669 for the static, centrifugation, vacuum and flow methods, respectively, utilizing 1,000,000 cells/block. Similar results were obtained in repeated studies, indicating high efficacy of the flow method.

DISCUSSION

Highly porous, open-pore matrices were formed by the compression of HAP/PLGA powders and the melting of powder surfaces by heat. The interconnected pore structure was more uniform than that of matrices fabricated with the standard solvent casting and particulate leaching technique. Furthermore, the HAP/PLGA blocks exhibited better mechanical properties than HAP/PLGA sponges fabricated with the standard technique. The HAP/PLGA blocks were maintained in culture without shrinkage for more than 5 weeks (data not shown).

Homogenous spatial distribution of hMSC at high cell densities within the 3-D scaffold is important in constructing tissue substitutes. By evenly distributing cells in scaffolds, problems of excess or deficient cell numbers in different parts of the same scaffold can be avoided. Uneven cell distribution would limit surface area for cell growth. Furthermore, the high cell density region may suffer from nutrient deficiency[30]. The spatial distribution of cells in scaffolds should influence the kinetics of cell proliferation and extracellular matrix deposition. In this study, the novel cell seeding method with a filter paper allowed uniform distribution of hMSC in the scaffold. In contrast, the centrifugation and static methods limited the distribution of hMSC near the upper surface of scaffold, while the vacuum method showed a lower seeding density throughout the scaffold. In the case of the centrifugation method, centrifugal force acts on cells without the flow of a medium. Therefore cells may be collected in the upper side of a scaffold. On the other hand, the flow method causes the flow of cells and a medium, and the flow may carry cells into the inner part of a scaffold more smoothly. In the case of the static method, cells adhere on the surface of the 6 mm well besides the top of the scaffold during the incubation for 12 h, which decreases the efficiency of cell seeding, although this method has been used in studies on tissue engineering.

The fluorescent microscopy was useful in evaluation of cell distribution profiles in HAP/PLGA blocks after

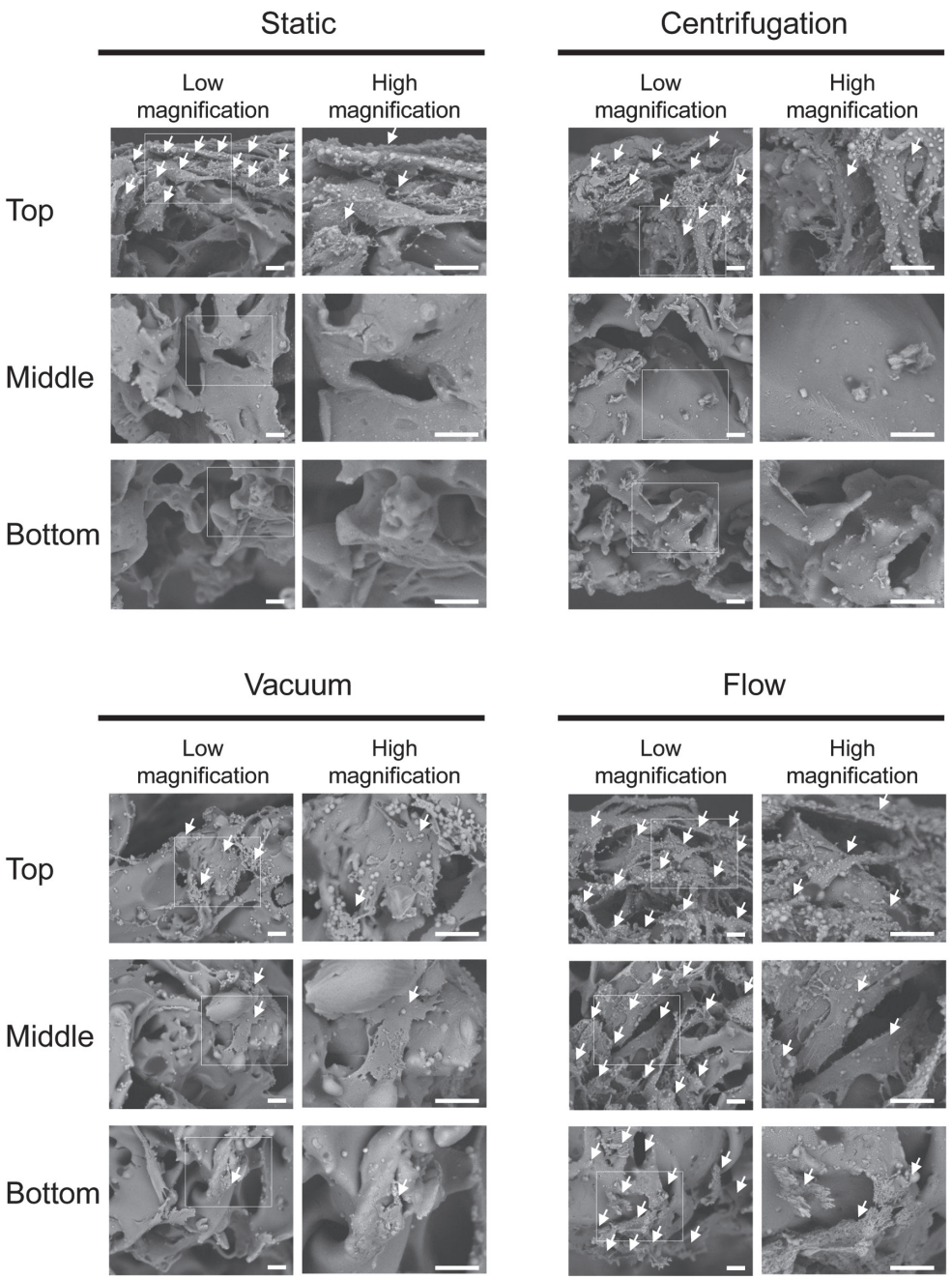

Fig. 4 SEM images of hMSC in different layers of HAP/PLGA scaffolds at 12 h. Scale bars, 10 μm. The arrows indicate adherent cells.

cell seeding by various methods. SEM also allowed evaluation of individual cell distribution in the blocks. Both studies showed even distribution of cells by the flow method and uneven distribution by the static and the centrifugation methods. In addition, DNA analysis quantitatively showed that the total cell number adhered in the blocks was higher by using the flow method than by the other methods (Fig. 5). These observations, taken together, proved that the flow method improves seeding efficiency and the uniform distribution of hMSC in porous scaffolds.

Using the flow method, a high seeding efficiency of up to 90% can be achieved for a short time (1 h, see Materials and Methods). In addition, the flow seeding method has the advantage of preventing loss of inoculated cells outside of the scaffold in a 6-mm well or centrifuge tube. Compared to other methods[22,31-38], the flow method is simple, and can be carried out in usual

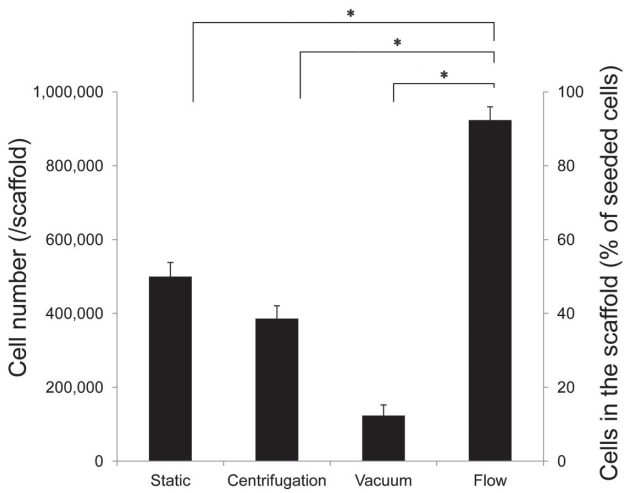

Fig. 5 The number of hMSC adhered in the HAP/PLGA blocks.
Suspensions containing 1×10^6 cells were applied by the four methods. DNA was determined using a DNA kit (PicoGreen, Molecular Probe, Chicago, USA). Cell numbers were calculated on a basis of DNA using the standard curve for DNA as a function of cell numbers. Asterisks represent significant difference ($p<0.001$) between the method with a filter paper and the other methods.

laboratories without special instruments. In addition, we believe that the flow method may be applied to other types of interconnected porous scaffolds.

In a separate study, the hMSC-HAP/PLGA block complex prepared by the flow method enhanced bone regeneration in experimental animals. We intend to report the effect of the hMSC-HAP/PLGA block complex on bone regeneration in a separate article in the near future.

CONCLUSION

The flow seeding method with a filter paper allowed uniform distribution of cells in the inner part of porous scaffolds within a short time. This new method will be useful in tissue engineering using hMSC and other cells.

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