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Effects of recombinant human bone morphogenic protein-2 and human bone marrow-derived stromal cells on *in vivo* bone regeneration of chitosan–poly(ethylene oxide) hydrogel

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Abstract: *In vivo* bone regeneration of chitosan–poly(ethylene oxide) (PEO) hydrogel in rat carlvarial defects was evaluated by using both human bone marrow-derived stromal cells (hMSCs) and recombinant human bone marrow protein-2 (rhBMP-2) for 4 and 8 weeks. *In situ* chitosan–PEO hydrogel was fabricated by mixing the precursor solutions of both chitosan-acrylate and PEO-thiol. Fabrication of the injectable hydrogels was modulated from within a minute to hours by controlling the temperature and pHs of the precursor solution. Gel swellings were dependent on the conditions of pHs and temperatures of the precursor solutions, showing higher gel swelling in basic water than in either acidic or neutral water. The compression strengths and *in vitro* degradation of hydrogels were also evaluated by controlling the concentrations of both precursor solutions and lysozyme, respectively, by referencing to the morphology of the control hydrogel with no enzyme added. Hydrogels showed sustained release of rhodamine-B over time. After implantation of the injectable hydrogels in rat calvarial defects for 4 and 8 weeks, *in vivo* bone regenerations were compared with by evaluating the degrees of new bone formations with Soft X-ray, microcomputed tomography, and histological stainings of hematoxylin and eosine Y and Masson's trichrome. Degrees of *in vivo* bone regeneration were controlled by encapsulating in advance either hMSCs, rhBMP-2, or both in the precursor solutions of the hydrogel. The defect implanted with hydrogel only showed higher amount of bone tissue regeneration than that of the control defect site. The defect sites with hydrogel containing both hMSCs and rhBMP-2 demonstrated highest amount of bone tissue regeneration among the samples. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 892–901, 2013.

Key Words: chitosan, hydrogel, bone regeneration, bone morphogenic protein-2, human bone marrow-derived stromal cells

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INTRODUCTION

Repairs of bone defects with irregular shapes are important challenges in dental and orthopedic societies.[1–4] Biological signals, cells, and biodegradable scaffolds are considered as three essential factors in tissue engineering, which could be recombinant human bone morphogenetic protein-2 (BMP-2), stem cells, and hydrogels among others in bone regeneration. Among the bioactive growth factors, such as various BMPs, platelet-derived growth factors, and other growth factors, BMP-2 has shown promising therapeutic potentials such as promotion of bone regeneration when delivered locally through a carrier.[5–8] Human bone marrow-derived stromal cells (hMSCs) have also demonstrated their differentiations into various cell types of mesenchymal lineages, such as osteoblasts and chondrocytes, thus being used in bone and cartilage tissue engineering.[5,6] To provide effective biological activity of growth factors and stem cells in local bone defects with various shapes, it is of importance to develop a sustained carrier system with both BMP-2 and hMSCs that allow local delivery to the bone defect sites with therapeutically effectiveness.[5–7] Furthermore, the carrier system should be effective, biocompatible, and biodegradable.

Among the carrier polymers, chitosan, a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine, has been

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attractive to these ends for its combined properties of biocompatibility, biodegradability, minimal toxicity, and promotion of tissue regeneration.[9–14] It has been used in various forms such as carriers for cell delivery and therapeutics, as well as polymeric scaffolds in either porous or films for its applications in bone tissue engineering because of its promotion of growth and mineral rich matrix deposition by osteoblasts in culture.[15–17] Chitosan demonstrated its biodegradation and biocompatibility with minimal local inflammation and osteoconduction through porous structures.[18] Furthermore, chitosan showed its ability to bind certain growth factors and also to release the bound ones in controlled rates due to its cationic nature, which is important in bone tissue engineering. The ability of growth factor binding of chitosan was reported by the introductions of both BMP-2 and a covalently linked imidazole group among various growth factors.[19,20] BMP-2 has been known to have abilities of stimulation of differentiation of hMSCs along the osteogenic lineage and enhancement of the functions of differentiated osteoblasts.[21]

The ability to produce tissue engineering bones with an injectable chitosan hydrogel would provide tremendous potential as a minimally invasive way to repair craniofacial defects that offers several important advantages over implanted scaffolds such as easy handling and possibility of its applications in irregular defects.[20] Furthermore, injectable chitosan hydrogel combined with both BMP-2 and hMSCs might synergistically enhance the performance of bone tissue regeneration in irregular defect sites. This injectable chitosan hydrogel will have further usefulness in terms of its fabrication, biodegradation, and applications in local delivery systems for bone tissue engineering with minimal damages to bioactive growth factors, live cells, and other bioactive elements. Subsequent to our previous reports on the fabrication of an *in situ* forming chitosan–poly(ethylene oxide) (PEO) hydrogel,[7,13,22] we here report the *in vivo* evaluation results of the *in situ* chitosan–PEO hydrogel containing both recombinant human bone marrow protein-2 (rhBMP-2) and hMSCs in rat calvarial defects by controlling their addition.

MATERIALS AND METHODS

Materials

Chitosan (5–10 kDa) was purchased from Kitto Life (Korea), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), triethanolamine, and 2-carboxyethyl acrylate (CEA) were purchased from Sigma–Aldrich, (St. Louis, MO). PEO with six thiols was purchased from Sun Bio (Orinda, CA). Carrier-free rhBMP-2 was purchased from R&D Systems (Minneapolis, MN), and fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were purchased from Gibco BRL (Carlsbad, CA). Low- and high-glucose Dulbecco's Modified Eagle's Medium (DMEM) was purchased from JBI Co. (Daegu, Korea) and Gibco BRL (Carlsbad, CA), respectively.

Synthesis of a chitosan–PEO hydrogel

Synthesis of chitosan–PEO hydrogel was previously described in detail in our research group.[12,13,22] In brief, after grafting chitosan with CEA through EDC chemistry (chitosan-acrylate), chitosan–PEO hydrogel was obtained via Michael type addition reaction by mixing separate 10% precursor solutions of chitosan-acrylate and PEO-thiols.

Evaluation of gelation behaviors

Behaviors of the formations of chitosan–PEO hydrogel were evaluated by using vial tilting method. Measurement of gelation times by the vial tilting method was processed by tilting the mixture of precursor solutions in a 1-mL conical vial over time. Gelation time was regarded at the point, when there was no flow for more than 1 min after repeatedly inverting 100 μL mixture of the precursor solutions in the vial.

Swelling behavior

Swelling of 10% chitosan–PEO hydrogel was measured in water at pHs of 4, 7.4, and 10 or in PBS at 37°C for up to 24 h. After measuring the initial weight of the prefabricated hydrogel with a microbalance, swelling of the chitosan–PEO hydrogel was determined by comparing the weights of the hydrogel sample before and after immersing it in water. Adherent water was removed by blotting the wet chitosan–PEO hydrogels with a piece of kimwipe paper before weighing them on a microbalance. The percentage of hydrogel swelling was calculated by using the following equation:

$$\text{Swelling percent } (\%) = [(W_s - W_i)/W_i] \times 100 \quad (1)$$

where W_s and W_i are the weights of the chitosan–PEO hydrogels at time t and at gel forming time, respectively.

Release experiment

Release of rhodamine B, a model drug, from the chitosan–PEO hydrogel was analyzed *in vitro*. After loading 0.05% (w/v) rhodamine B in the precursor solutions of 150 μL chitosan–PEO hydrogel, the amount of rhodamine B released from 10% chitosan–PEO hydrogel was measured at 37°C for 24 h by utilizing the ELISA microplate reader (Tecan, Switzerland) after by collection of 100 μL sample from the buffer solution reservoir. The volume of used 100 μL buffer solution was compensated after each collection of samples. The amount of the rhodamine B released was calculated by using the following equation:

$$\text{Cumulative percent of rhodamine B released } (\%) = R_o + (R_t/R_a) \times 100 \quad (2)$$

where R_o and R_t are the amount of rhodamine B released from the chitosan–PEO hydrogel at initial time $t = 0$ and time t, respectively, and R_a is the total amount of rhodamine B initially loaded in the precursor solution of chitosan–PEO hydrogel.

Compression strength

Compression strength of the formed chitosan–PEO hydrogel was measured by a texture analyzer by using a MT-LQ material tester (Stable Micro Systems, UK) equipped with a 5-kg load cell. Cell loading on both prefabricated 5% and 10%

chitosan–PEO hydrogel with a size of 0.5 cm height × 1 cm diameter was performed at a rate of 2 m/s at room temperature ($n = 3$).

Enzymatic degradation

In vitro degradation of 10% chitosan–PEO hydrogel was evaluated by addition of lysozyme (0.5 and 1.0 mg). After full swelling of 200 μL chitosan–PEO hydrogel to equilibrium in 3 mL PBS for 24 h, its weight (W_s) was measured at every other day with a microbalance ($n = 3$). Either 0.5 or 1.0 mg lysozyme in 10 μL buffer solution was sprayed over the hydrogel at every 3 day for 24 days. Weight percentage of the remained hydrogel at time t was calculated by using the following equation:

$$\text{Weight of chitosan–PEO hydrogel}(\%) = (W_t/W_s) \times 100 \quad (3)$$

W_t, weight of the chitosan–PEO hydrogel treated with lysozyme at time t; W_s, weight of the initial swollen chitosan–PEO hydrogel.

Preparation and culture of both hMSCs

Bone marrow aspirate was obtained from the iliac crest of one donor. The harvesting of bone marrow was performed on the donor who gave consent, and this study was approved by the local ethics committee (IRB at Seoul National University Dental Hospital, Nr: CRI05008) according to the legal regulations for human tissue and organs in Korea. hMSCs were cultured according to the protocol described by Caterson et al.[23] The marrow suspension was collected in a syringe containing 6000 U/mL heparin and was mixed with PBS solution in the same volume ratio and centrifuged at 2500 rpm for 10 min. After aspiration of the upper PBS layer, the marrow suspension was layered on Ficoll-paque (1:5 ratio) and centrifuged at 2580 rpm for 30 min. The nucleated cells concentrated at the interface were collected and washed with PBS. Adherent cells were plated at a density of 2×10^6 cells/100 mm culture plate and cultured in an expansion medium containing low-glucose DMEM, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% heat inactivated FBS (HIFBS) under a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every 3 or 4 days. Cells were passaged when they reached 70% confluence, and fifth passage cells were used for the present experiments.

In vivo bone regeneration in rat calvarial defect model

Prehydrogel solutions were prepared for calvarial implantation in the following five groups ($n = 5$ for each groups): (1) control without hydrogel, (2) hydrogel only, (3) hydrogel with 1×10^6 hMSCs/construct, (4) hydrogel with 1 μg rhBMP-2/construct (10 μg rhBMP-2/mL), and (5) hydrogel with both 1 μg rhBMP-2/construct (10 μg rhBMP-2/mL) and 1×10^6 hMSCs /construct. The samples were implanted for 4 and 8 weeks in the calvarial defects.

Each mixed hydrogel composite in a final volume of 100 μL was loaded onto one well of a 24-well culture dish in cell culture facility. The samples were incubated for 30 min at 37°C for cross-linking. Two microliters of 10% DMEM was added into the hydrogel-containing well and allowed to swell for overnight at 37°C in a humidified chamber. The swollen hydrogel disk fitted into the cranial bone defects ($d = 8$ mm).

The swollen hydrogel disk was applied to critical sized calvarial bone defects of rats cared according to the protocols of the Seoul National University Animal Cares. Male Sprague Dawley albino rats, age 8 weeks, were used for the experiments. All operations were performed under general anesthesia by intraperitoneal injection of ketamine hydrochloride (40 mg/kg, Ketaras, Yuhan Corp., Korea) mixed with xylazine (Rumpens Bayer Korea, Korea, 10 mg/kg). After disinfection of calvarial skin with 10% betadine (Potadines, Sam-Il Pharm., Korea) and subcutaneous injection of 2% lidocaine containing 1:100,000 epinephrine (Lidocaine HCL Injs. Yuhan Corp., Korea) at the calvaria, an incision was made along the sagittal suture. The periosteum was elevated, and critical-sized calvarial bone defect with a dimension of 8 mm in diameter and 1 mm in depth was created with a trephine bur without dura perforation. The defect area was either left empty in the control group, or was filled with each hydrogel. New bone formation at the defect site was analyzed at both 4 and 8 weeks after regular healing process using microcomputed tomography (Micro-CT) reconstruction and histological analysis.

Soft X-ray analysis

After harvesting the superficial aspect of the calvaria including the defect, their images were obtained by taking a radiogram with a cabinet-style Soft X-ray unit (CMB-2, Softex Co., Japan), which was operated at the conditions of 45 kVp, 3.5 mA, and 90 s exposure time. Digital images from the Soft X-ray were taken by means of a transmission and polarized light Axioskop Microscope, Olympus BX51 (Olympus Corp., Tokyo, Japan). Percentages of newly formed bone within the defects were calculated using computerized image analysis system, SPOT version 4.1 (Diagnostic instrument, Michigan). The new bone area was considered the white area of both ingrown bone from the defect margin and bone formation in the defect center. Total defect area was calculated as 50.27 mm^2 by using the SPOT program. The new bone areas of the defects were calculated, and their percentages (%) were presented as the area ratio of new bone versus total defect in all specimens of animals.

Micro-CT analysis

In one case of each five groups, Micro-CT scans were taken for the demonstration of three-dimensional new bone formation at the rat calvarial defects after 8 weeks using the SkyScan micro-focus X-ray system (SkyScanVR, Kontich Co., Belgium). The SkyScan micro-focus X-ray system was equipped with a micro-focus X-ray tube with a focal spot of 2 mm, producing a cone beam that was detected by a 12-bit cooled X-ray CCD camera of which fiber was optically coupled to scintillator. Although source voltage and current were set as 82 kV and 100 μA, respectively, the exposure

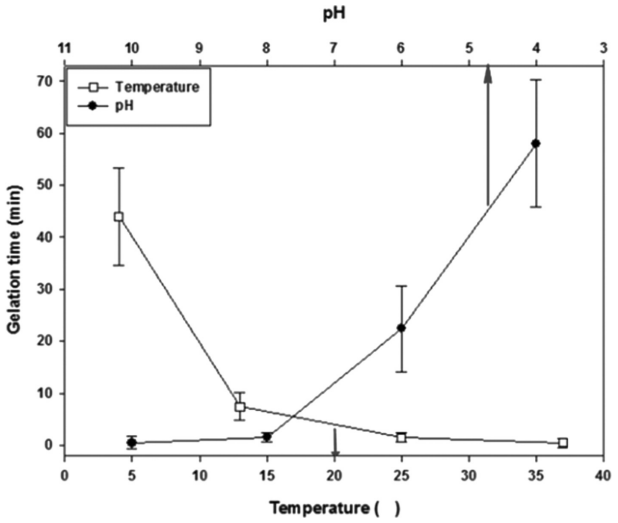

FIGURE 1. Gelation times of chitosan and PEO precursor solutions dependent on their temperature and pHs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

time was 3.4 s for optimized clearness. Three-dimensional images of the samples were obtained by using V works™ 4.0 personal.

Histochemical staining

After Micro-CT reconstruction, the operative fields at the calvaria were removed and decalcified through incubation in ethylene diamine *tetra*-acetic acid solution (7%, pH 7.0) for 3–4 days with replacement of the acid solution on day 2. The specimens were then dehydrated in 70% ethanol and embedded in paraffin. For histochemical staining, decalcified paraffin sections were cleaned with xylene for 10 min and then stained with both hematoxylin and eosine Y (H&E) and Masson's trichrome (MT) for the detection of cells and regenerated bone structures. Digital images of the stained sections were collected for the histological evaluation using a transmission and polarized light Axioskop microscope, Olympus BX51 (Olympus Corp., Tokyo, Japan), and the histomorphometric evaluation was not performed due to the tissue deformation during section procedures of the paraffin block.

Statistical analysis

All the data of Soft X-ray was reported as mean ± standard deviation. All the statistical analyses were performed with SPSS (ver. 18.0, SPSS, Chicago, IL). The comparisons between two groups were carried out using a *t*-test. Values were considered statistically significant when the *p*-value was <0.05.

RESULTS

Synthesis of chitosan–CEA

Chitosan–CEA has been synthesized by grafting CEA to chitosan via EDC chemistry. Detailed synthesis and chemical analysis of chitosan–CEA were reported in our previous report.[7] In brief, chemical grafting of CEA to chitosan (data not shown) was confirmed by observing the presence of new chemical signals from the three hydrogens from the unsaturated hydrocarbons at the positions of 6.5–5.8 ppm; O=C$\underline{H}$—C$\underline{H_2}$). The peaks at 2.0 ppm were considered the hydrogens in the acetyl methyl group of chitosan (—NHCOC$\underline{H_3}$). The degree of chitosan modification was estimated as 65% judged by comparing the hydrogen peak ratio between acetated methyl and the unsaturated hydrocarbons.

Fabrication of hydrogel

The chitosan–PEO hydrogel was spontaneously obtained via Michael type addition reaction by mixing the precursor solutions of chitosan-acrylate and PEO-thiol at a predefined molar ratio (1:1). Dependence of hydrogel formation on the temperatures and on the pHs of the precursor solutions was also analyzed by the tilting method (Fig. 1). The chitosan–PEO hydrogel was formed nearly instantaneously in mild basic conditions, that is, in pH 8.00 or 37°C in this experiment (Fig. 1). However, as the pH of the mixture solutions was changed from neutral to acidic and basic one, hydrogel formation was significantly delayed and expedited, respectively ($p = 0.00$ for all the cases). In specific, although the precursor solutions turned into a hydrogel in 28.8 s and 1.5 min at pH 10 and pH 8 in room temperature (25°C), respectively, it took 22.4 and 58 min for the same kind of precursor solutions at pH 6 and pH 4, respectively. In addition the temperature of the precursor solutions affected gel formation behaviors, that is, higher solution temperature induced quicker gel formation. In specific, although the precursor solutions at pH 8.0 turned into a gel in 0.5 and 1.5 min at 37°C and 24°C, respectively, gel formation of the precursor solution at 13°C was delayed to 7.4 min. F1

Swelling behaviors of chitosan–PEO hydrogel

After fabrication of 10% chitosan–PEO hydrogel, its physical properties were evaluated by measuring swelling behaviors under different pHs for 30 h (Fig. 2). The hydrogel swelled to 2.0–2.5 times and reached to an equilibrium F2

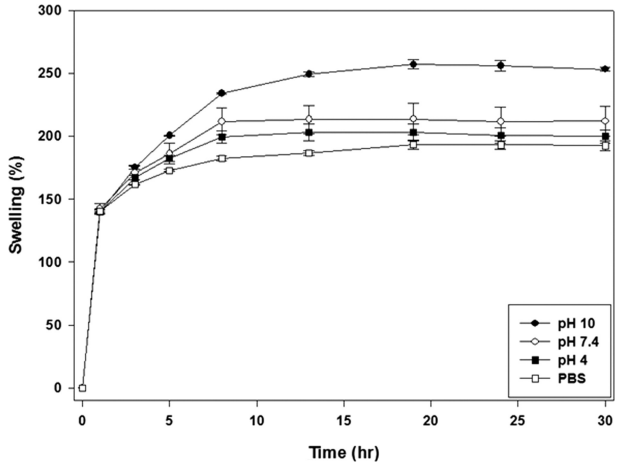

FIGURE 2. Swelling behaviors of 10% chitosan–PEO hydrogel in either PBS or water at different pHs over time.

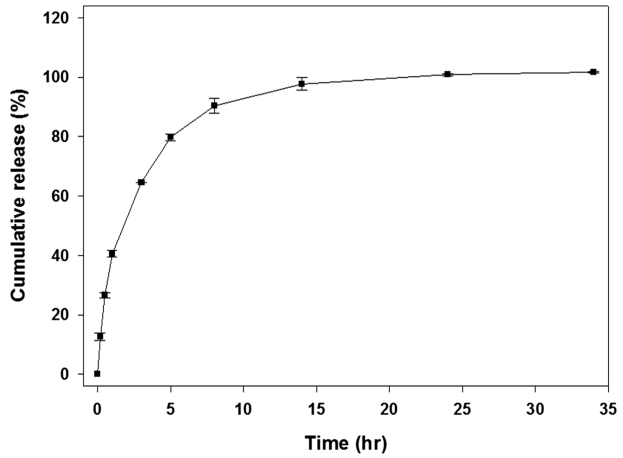

FIGURE 3. Release kinetics of rhodamine-B from 10% chitosan–PEO hydrogel in PBS (pH 7.4).

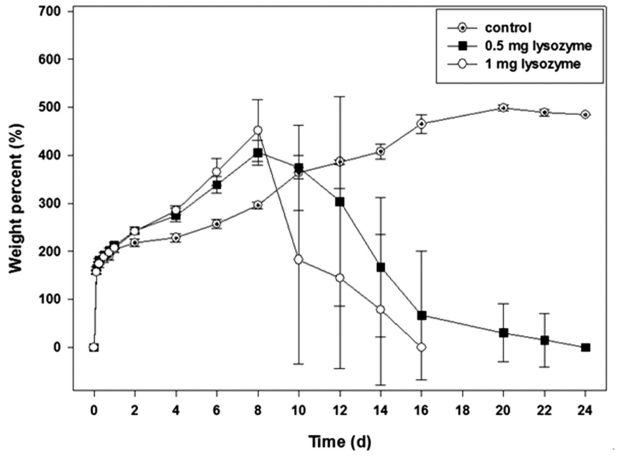

FIGURE 5. *In vitro* degradation of 10% chitosan–PEO hydrogels. Degradation of hydrogels was expedited by additions of 10 μL buffer solution containing either 0.5 or 1.0 mg lysozymes every 3 days.

approximately in 13–19 h in the mediums of both PBS and water depending on the conditions of pHs. The hydrogel in basic water showed significantly higher swellings of the hydrogels in either PBS or water at pHs of 4.0 and 7.4 ($p = 0.00$ for all the cases). In specific, the hydrogels swelled to 199% in PBS and to 204% and 210% in water at the conditions of pH 4 and 7.4, respectively, but the hydrogel swelled to 253% in water at pH 10.

Release kinetics of rhodamine B from chitosan–PEO hydrogel

The amount of rhodamine B released from the 10% chitosan–PEO hydrogel was *in vitro* measured for 24 h by comparing with its initial amount loaded (Fig. 3). It released approximately 80% and 98% in 5 and 14 h, respectively.

Compression strength of chitosan–PEO hydrogel

Compression strength of the chitosan–PEO hydrogel was dependent on the concentrations of the precursor solutions (Fig. 4). Although the 5% hydrogel had 17.5 Pa of compression strength, the 10% one did 39.8 Pa at their yield strengths, which is significantly different in statistic ($p = 0.01$).

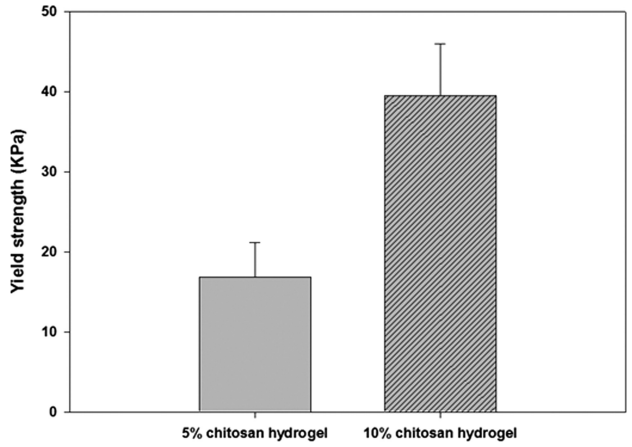

FIGURE 4. Compression strengths of chitosan–PEO hydrogels, where $p = 0.01$ in statistic.

In vitro degradation of chitosan–PEO hydrogel by enzyme

In vitro degradation of chitosan–PEO hydrogel was evaluated by measuring their weight changes during spraying of lysozymes every other day. The control hydrogel with no lysozyme-added swelled in PBS to 498% in maximum at nearly 20 days, and then started to degrade, probably due to hydrolysis. But when the 200 μL hydrogel was treated with 10 μL buffer solution with either 0.5 or 1.0 mg lysozymes, the hydrogel swelled rapidly and then degraded by lysozymes, which were regularly added on the hydrogel (Fig. 5). Different degrees of hydrogel swelling were observed between the lysozyme-added groups, that is, addition of higher amount of lysozyme induced quicker gel swelling. Even though maximum gel swelling was observed at day 8 for both lysozyme-added hydrogels, more and quicker gel swelling was observed in the hydrogel with higher amount of lysozyme added. In specific, the hydrogel swelled to 405% at day 8 for the 0.5 mg lysozyme-added one, but to 451% for the 1.0 mg lysozyme-added one. Dramatic difference in their weights was, however, observed from day 10. Although the 0.5 mg lysozyme-treated hydrogel showed slower weight loss, the 1.0 mg lysozyme-treated one demonstrated drastic reduction of its weight. In specific, the former showed maximally 451% swelling of hydrogel in its weight but the latter demonstrated 183% swelling at day 10. The 0.5 mg lysozyme-treated hydrogel showed reduction of its weight from 405% to 374%, 304%, 167%, 67%, and 30% at 8, 10, 12, 14, 16, and 20 days, respectively. In contrast, the 1.0 mg lysozyme-treated hydrogel demonstrated dramatic reduction of its weight from 451% to 183%, 143%, and 78% at day 8, 10, 12, and 14, respectively. The 10 μL buffer solution with either 0.5 or 1.0 mg lysozymes completely degraded the chitosan–PEO hydrogels in 16 and 24 days, respectively.

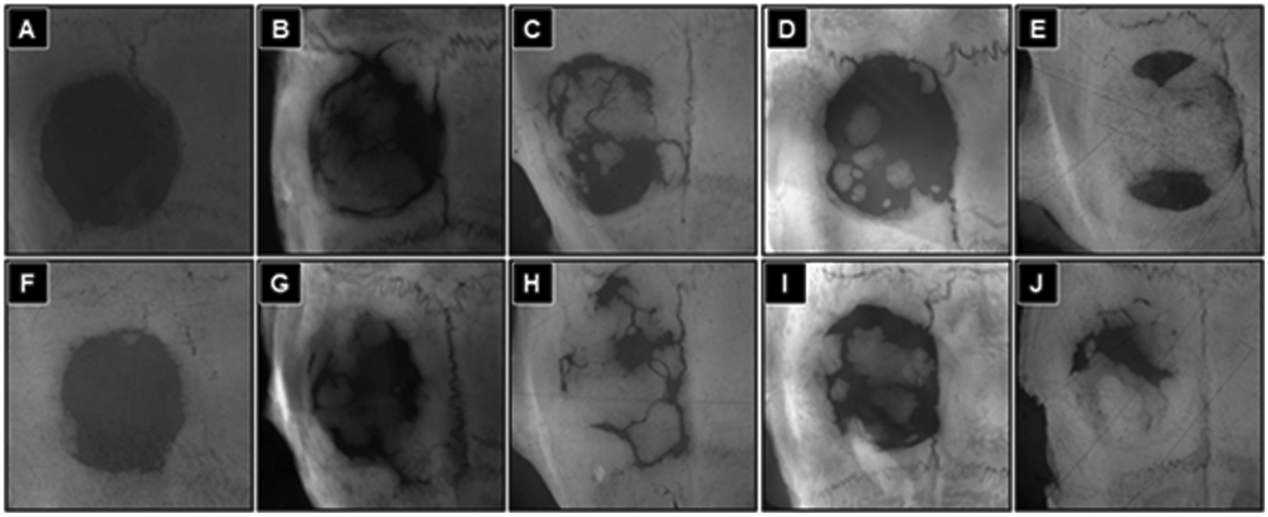

FIGURE 6. Soft X-ray images of the rat calvarial defects after 10% chitosan–PEO hydrogel implants for 4 and 8 weeks [(A and F) control; (B and G) chitosan–PEO hydrogel only; (C and H) chitosan–PEO hydrogel with hMSCs; (D and I) chitosan–PEO hydrogel with rhBMP-2; (E and J) chitosan–PEO hydrogel with both hMSCs and rhBMP-2; (A–E) 4 weeks; (F–J) 8 weeks]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In vivo bone regeneration in rat calvarial defects

In vivo bone regeneration by 10% chitosan–PEO hydrogel was evaluated with Soft X-ray and Micro-CT as well as histological staining in a rat calvarial defect model by addition of either rhBMP-2 or hMSCs, or both. *In vivo* new bone formation in Soft X-ray images was in two-dimensional evaluated with Soft X-ray images, by measuring the white areas of the defect sites on the X-ray images. On the Soft X-ray images, the white area presents the new bone area, whereas the black area of the defect sites indicates no bone formation. Clear differences in new bone formation were also observed between the control and experiment groups. The control without hydrogel implanted showed that new bone formation was not observed as indicated by the black area at 4 weeks but enhanced improvement of new bone formation was observed at 8 weeks [Fig. 6(A,F)]. Higher degree of new bone formation was clearly observed when hydrogel was implanted for 4 and 8 weeks [Fig. 6(B,G)]. The degrees of new bone formation between the four experiment groups were also different depending on the presences of hydrogel, cells, and/or growth factors. The hydrogels with cells and/or rhBMP-2 [Fig. 6(C–E) for 4 week implants; and Fig. 6(H–J) for 8 week implants] generated clearly higher degrees of new bone regeneration, that is, larger white areas, than those of control defect site with either no hydrogel [Fig. 6(A,F)] or hydrogel only [Fig. 6(B,G)].

We calculated the degrees of new bone formation by measuring the white area obtained by Soft X-ray (Fig. 7). The control group demonstrated only 19% and 27% bone growth during 4 and 8 weeks, respectively. Enhanced increase in new bone formation was observed for the hydrogel-implanted sites. For the defect sites implanted with the chitosan–PEO hydrogel only, 38% and 45% new bone formation were observed during 4 and 8 weeks, respectively, which were higher areas of new bones than those of the control groups. Three experimental groups with hMSCs and/or rhBMP-2 also showed higher degrees of new bone formation compared with those of the control group, and the added hMSCs and rhBMP-2 affected differently on the degrees of new bone formation. In specific, the degrees of new bone formations by the hydrogels with hMSCs were measured as 41% and 36%, respectively, which were similar to those of the group with hydrogel only when implanted for 4 and 8 weeks. The hydrogel with rhBMP-2 added demonstrated a little higher degrees of new bone formations, 44% and 51%, during 4 and 8 week implants, respectively, than those of the hydrogel with hMSCs. When compared with the hydrogel only, higher amount of new bone formation was observed for the hydrogel with both hMSCs and rhBMP-2, which showed 45% and 71% new bone formation during 4 and 8 week implants, respectively.

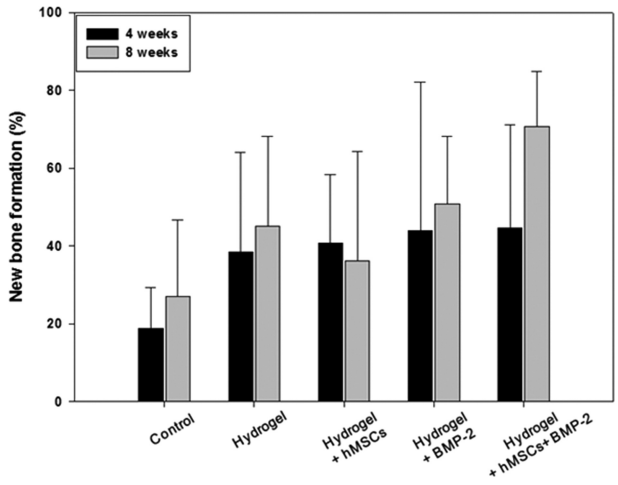

FIGURE 7. Degrees of new bone formation of the rat calvarial defects after 10% chitosan–PEO hydrogel implants for 4 and 8 weeks measured by Soft X-ray. Statistical analyses of hydrogel samples were not significantly different over the control.

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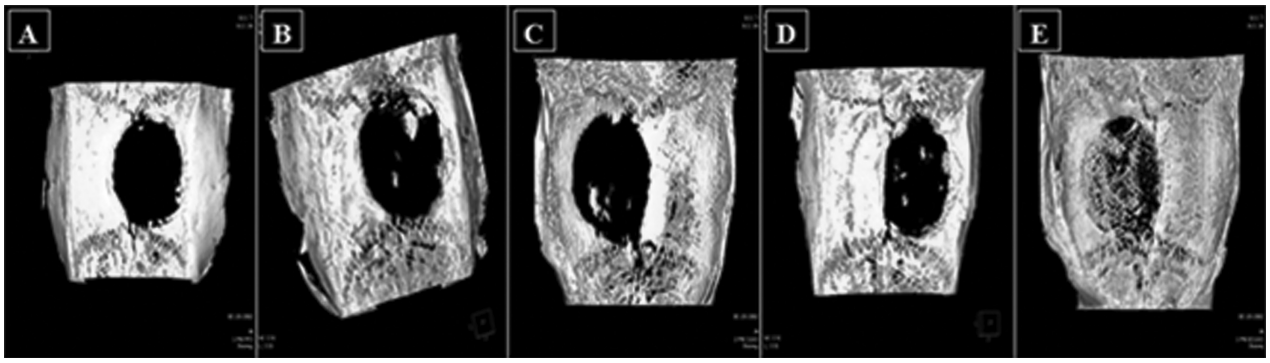

FIGURE 8. Micro-CT images (8 weeks) of new bone formation in rat calvarial defects (one case in each group) after 10% chitosan–PEO hydrogel implants for 8 weeks, where control (A), chitosan–PEO hydrogel (B), chitosan–PEO hydrogels with hMSCs (C), with rhBMP-2 (D), and with both hMSCs and rhBMP-2 (E). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

New bone formation in the defect sites were three-dimensionally demonstrated by Micro-CT in one sample of each groups after 8 weeks (Fig. 8). All the hydrogel samples induced higher degrees of new bone formation than that of the control without hydrogel [Fig. 8(A)]. The defects with hydrogel only [Fig. 8(B)] induced higher amount of new bone formation than those of both the control site without hydrogel [Fig. 8(A)] but showed similar extent of new bone formation to those of the hydrogels with either hMSCs or rhBMP-2 [Fig. 8(C,D)]. As we described in the previous sessions of the Soft X-ray, the Micro-CT results also showed that the hydrogel with both hMSCs and rhBMP-2 [Fig. 8(E)]

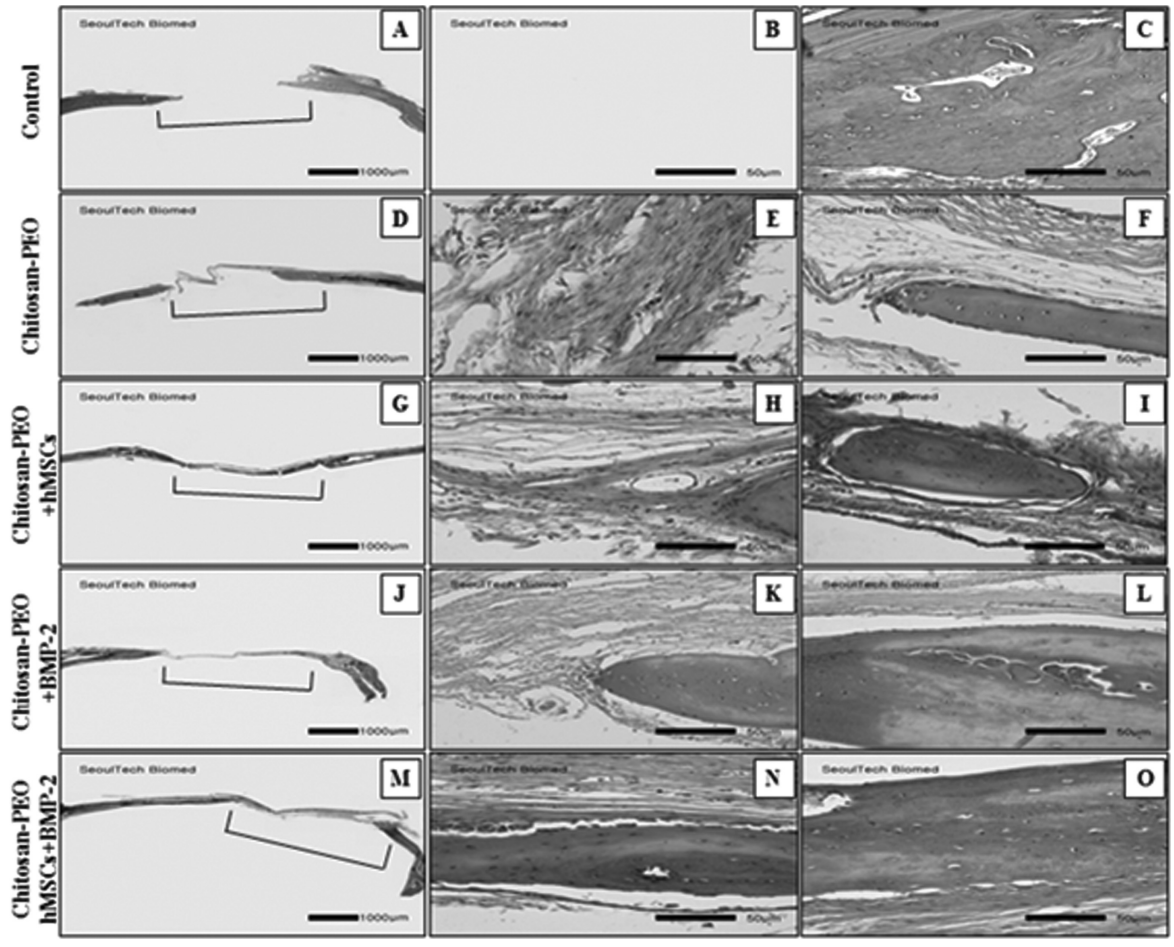

FIGURE 9. H&E stainings of rat calvarial defects after 4 and 8 week implants of 10% chitosan–PEO hydrogel samples, where C is the control calvaria with no defects (×400). Control defects (A and B); the defect sites implanted without (A and B) and with (D–F) hydrogel; the defects with hydrogels containing either hMSCs (G–I) or rhBMP-2 (J–L), containing both hMSCs and rhBMP-2 (M–O) [(A, D, G, J, and M) 8 weeks; ×12.5], [(E, H, K, and N) 4 weeks; ×400), and [(C, F, I, L, and O) 8 weeks; ×400]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

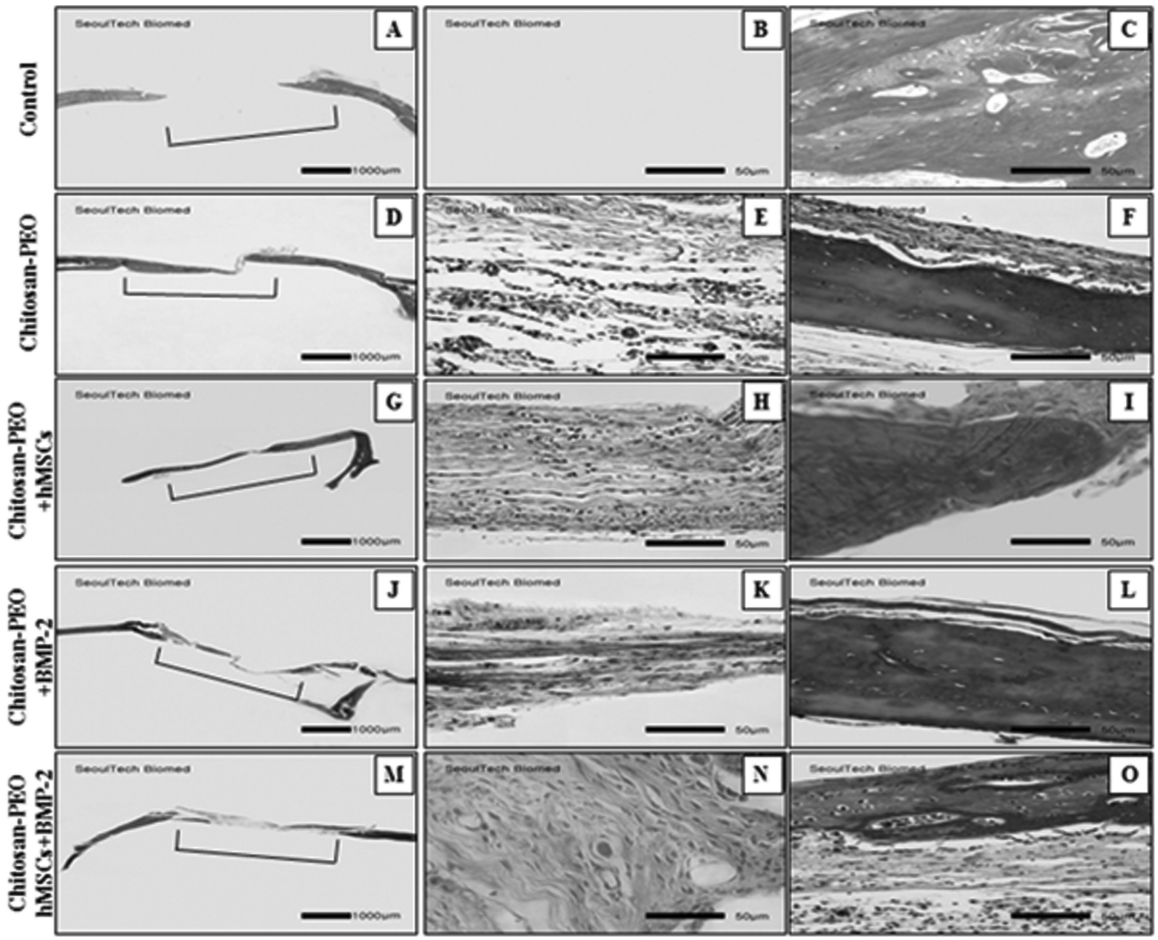

FIGURE 10. MT stainings of rat calvarial defects after 4 and 8 weeks implantation of 10% chitosan–PEO hydrogel samples. All the legends are the same as those of Figure 9. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

induced the highest degree of new bone formation among the used conditions.

Histochemical staining

Degrees of new bone formation by hydrogels, hMSCs and rhBMP-2 were also histologically inspected by staining with both H&E and MT for overall new bone tissue formations and new bones with collagen tissues, respectively (Fig. 9). Although the normal bone without defects showed its typical histology [Fig. 9(C)], the control defects without hydrogel implanted showed negligence amount of new bone tissues at both low and high magnifications [Fig. 9(A,B)]. As we noted in the previous analyses of both Soft X-ray and Micro-CT, quantitatively higher degrees of new bone formation of the hydrogels [Fig. 9(D–P)] were observed than the control defect without hydrogel did [Fig. 9(A,B)]. The control with hydrogel only [Fig. 9(E)] seemed to induce new bone tissues similar to those regenerated by the hydrogels containing either hMSCs [Fig. 9(H)] or rhBMP-2 [Fig. 9(I)], when implanted for 4 weeks; but the hydrogel containing both hMSCs and rhBMP-2 [Fig. 9(N)] seemed to induce more new bone tissues, approaching to the morphology of the natural bone tissue [Fig. 9(C)]. Increased differences in the degrees of their new bone tissue regeneration were observed for the defects with hydrogels implanted for 8 weeks, compared with those of the defects with hydrogels implanted for 4 weeks. The hydrogels containing either hMSCs [Fig. 9(G,I)] or rhBMP-2 [Fig. 9(J,L)] and both hMSCs and rhBMP-2 [Fig. 9(M,O)] demonstrated connection of the defects by formation of new bone tissues. These results showed better new bone formation than those of the defects with hydrogel only implanted [Fig. 9(D,F)].

In MT staining (Fig. 10), all the defects implanted for 8 weeks showed different degrees of new bone tissue formation in each group quantitatively similar to those implanted for 4 weeks as described in Figure 9.

DISCUSSION AND CONCUSIONS

Injectable chitosan–PEO hydrogel has been evaluated in rat calvarial defects after both synthesis of chitosan-acrylate and fabrication of hydrogel. Spontaneous chitosan–PEO hydrogel was fabricated through Michael type addition reaction by simple mixing of precursor solutions of both chitosan-acrylate and PEO-thiols. Hydrogel gelation times were controlled by changing the temperatures from 5 to 37°C and pHs from acidic to neutral and basic conditions of the precursor solutions, ranging from a minute to 40 min. Basic condition and higher temperature induced quicker hydrogel

formation, and the formed hydrogel swelled more in basic water to 2.5 times of the fabricated hydrogel in weight. Higher compression strength of the hydrogel was controlled by higher concentrations of precursor solutions, and its mechanical stability and *in vitro* degradation were controlled by addition of lysozymes. Addition of higher concentrations of lysozymes on the hydrogel surface induced quicker *in vitro* biodegradation. The hydrogel showed excellent possibility of controlled release of bioactive molecules such as drugs, growth factors and cells, as tested by encapsulation of rhodamine-B, rhBMP-2, and hMSCs, respectively, in the hydrogels through mixing in the hydrophilic precursor solutions in advance. For controlled release of the bioactive rhBMP-2 and live stem cells to induce *in vivo* new bone formation, we encapsulated them in the precursor polymer solutions and then converted into a chemically cross-linked hydrogel. *In vivo* bone regenerations were evaluated by implanting the hydrogels in rat calvarial defects with a critical defect size with Soft X-ray, Micro-CT, and hisotological stainings with H&E and MT. The results showed that while the control defect without hydrogel added induced minimal amount of new bone tissue formation, the defect with hydrogels implanted did higher amount of new bone formation. The defects with hydrogels containing either hMSCs or rhBMP-2 showed quantitatively similar extents of new bone tissue formation to that incorporated with hydrogel only when observed with Soft X-ray, Micro-CT, and H&E staining. Even though the degrees of new bone formations were similar to between the hydrogels containing either hMSCs or rhBMP-2 when observed by H&E, clear difference in new bone tissue formation was observed on the defects with hydrogel containing both hMSCs and rhBMP-2, showing higher amount of collagen regenerations and similar bone tissue formation, when implanted for 8 weeks. From these results, the chitosan–PEO hydrogel was evaluated as useful in regeneration of new bone tissues by injecting the precursor solutions with bioactive agents such as rhBMP-2 and hMSCs in rat calvarial defects. Encapsulated rhBMP-2 and stem cells in the hydrogel might be released in a sustained manner as demonstrated by the tests of both rhodamine-B release and biodegradation by enzymes, leading to better *in vivo* induction of new bone tissues in rat calvarial defects. A 10-μg rhBMP-2/mL seemed to stimulate both hMSCs in the hydrogel and nearby host precursor cells *in vivo* in the hydrogel construct with both rhBMP-2 and hMSCs.

The fate of transplanted MSCs should play an important role in the regenerative procedure. The recent experiment for vasculogenesis showed that seeded hMSCs were no longer detectable in the immune-deficient mouse within a few days of implantation, differently from the expectation that the seeded cells differentiate into the mature vascular cells of the neo-vessel, whereas transplanted vessel scaffold with hMSCs transformed into living blood vessels over a 6-month time course.[24] After transplantation, scaffolds were initially repopulated by cells originated from mouse tissue, and hBMCs secreted cytokines for inflammatory process,[24] which will act as a stimulating factor for tissue regeneration. In this aspect, xenogenic hMSC used in this study might induce more accelerating response in the osteogenesis via immunogenic inflammatory process, compared with the use of autogenous MSC. On the contrary, the xenogenic treatment group displayed inferior osteogenesis compared with the autogenous MSC treatment group in the comparative study of osteogenesis between hMSC and autogenous MSC with rabbit calvarial defect model, where no local or systemic inflammatory response resulting from xenogenic transplantation was observed.[25] Therefore, our results should be carefully interpreted, because it may be different from cases with autogenous MSC.

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