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Osteogenic effect of fluvastatin combined with biodegradable gelatin-hydrogel

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The aim was to investigate the local osteogenic effect of fluvastatin incorporated into a biodegradable gelatin-hydrogel (GH) scaffold. The GH scaffolds were prepared through crosslinking by ultraviolet irradiation followed by freeze-drying. Two circular defects were surgically created on fifteen-week-old male rats calvaria. All defects of each rat were randomly filled with two of three treatments, specifically: fluvastatin incorporated into a GH disk (Flu-GH), distilled water incorporated into a GH disk, and no treatment. New bone formation was quantitatively analyzed after 7, 14, and 28 days using a micro-computed tomography (micro-CT) system, and histologically observed. Evaluation by micro-CT revealed a significant difference in new bone formation among the three kinds of defect. A highly osteogenic effect was observed in the Flu-GH group. The results showed that the fluvastatin incorporated into a biodegradable GH scaffold promoted osteogenesis in rat calvarial bone, indicating its potential for bone regeneration.

Keywords: Fluvastatin, Scaffold, Osteogenic

INTRODUCTION

Statins are small, stable lactones unsusceptible to proteolytic degradation, and are competitive inhibitors of 3-hydroxy-2-methyl-glutaryl coenzyme A (HMG CoA) reductase¹. Statins are widely used to lower cholesterol levels, which is an important factor in the treatment of hyperlipidemia and arteriosclerosis. Recently, another *in vivo* effect of statins has also attracted attention, with a number of studies finding that statins promote differentiation of osteoblasts produced by stimulation with bone morphogenetic protein-2 (BMP-2)²⁻⁷, indicating their potential in the development of new osteogenic drugs. Statins are expected as a new treatment modality for bone diseases instead of BMP-2, because they can be easily synthesized and be made 16,000-times cheaper to produce than BMP-2². However, a major hurdle in the clinical application of statins is finding an appropriate method of local administration. When administered by injection their effect has been reported to be lost within 5 days⁷, making this modality impractical for clinical application in bone healing. Therefore, various methods of local administration, or drug delivery system (DDS), have been explored, and include the use of collagen gel, polyethylene glycol, and polylactide-co-glycolide⁸⁻¹⁰. One such carrier, gelatin-hydrogel, is biodegradable and already in use in various types of food and drug. New bone formation was observed around fluvastatin incorporated into gelatin-hydrogel¹¹. It has been suggested, however, that degradation of the remaining hydrogel sponge hinders bone healing. In the present study, a different approach toward the design of the gelatin-hydrogel scaffold was taken, making it biodegradable.

The overall objective of this study was to investigate the local osteogenic effect of fluvastatin incorporated into

a biodegradable gelatin-hydrogel scaffold.

MATERIALS AND METHODS

Preparation of gelatin-hydrogel disk incorporating fluvastatin

Fluvastatin sodium salt (Toronto Research Chemicals, Ontario, Canada) as a water soluble statin was used in this study. Alkaline gelatin with an isoelectric point of 7.0–9.0 was obtained from Nitta Gelatin Co., Ltd (Osaka, Japan). An aqueous solution (2 mL) of 5 wt% gelatin was placed in a polystyrene dish (44×44 mm²) and crosslinked by ultraviolet radiation for 120 min. After crosslinking, the hydrogel was placed in an electric freezer at –30 for 4 h. The frozen hydrogel was then lyophilized for 1 day in order to obtain a completely dry sheet of gelatin-hydrogel. Discs of 3.5 mm in diameter were then punched out of the resulting sheet. Fluvastatin (4.3 mg) was dissolved in 10 mL of distilled water (1 mM, pH of the solution was 6.0). The discs were placed in the fluvastatin solution for 1 day in order to bind electrostatically between alkaline gelatin and acidic statin. All experimental procedures were conducted under sterile conditions.

Animal experiment

Fifteen-week-old, male, Sprague-Dawley rats ($n=10$) obtained from Sankyo Laboratory (Tokyo, Japan) were used in the experiment. The rats were allowed food and water ad libitum and maintained on a 12-h light/dark cycle (lights on from 8:00 to 20:00) at 23±1°C with 60±10% humidity during the experiment. Two rats each were euthanized with an overdose of ether anesthesia on both days 14 and 28 after surgery. All animal experiments in this study were conducted in accordance with the Tokyo Dental College Guidelines for Animal Experimentation (Approval date: 6/1/2011; Approval

number: 235101). All rats were anesthetized by intraperitoneal injection (60 mg/kg body weight) of sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). A linear sagittal skin incision was then made in the skull with a #15 surgical blade. Full thickness epidermal, hypodermal, and periosteal flaps were then elevated, creating 2 full-thickness bone defects of 3.8 mm in diameter in the dorsal area of the calvarial bone. A 3.8-mm trephine bur was used to create the defects under constant irrigation with normal saline solution to prevent overheating of the bone edges. The animals were randomly allocated to 3 groups, with one of 3 treatments being applied in each defect as follows: Group 1 ($n=4$): one defect was filled with fluvastatin incorporated into a gelatin-hydrogel disk (Flu-GH), while the other was left untreated (control); Group 2 ($n=3$): one defect was filled with distilled water incorporated into a gelatin-hydrogel disk (DW-GH), while the other was treated with Flu-GH; Group 3 ($n=3$): one defect was treated with DW-GH, while the other was left untreated. In all rats, the periosteal flap was closed by suturing with 4-0 silk sutures.

Micro-computed tomography analysis

Quantitative image analysis of new bone formation was performed using the R-mCT[®]. Defects in the calvarial bone were scanned using the R-mCT[®] with an X-ray source of 85 kV/140 μ A at 0, 7, 14, and 28 days after surgery. After anesthesia with sodium pentobarbital, each rat was set on the object stage and imaging performed over a full 360-degree rotation with an exposure time of 17 s. An isotropic resolution of $50 \times 50 \times 50$ μ m voxel size was selected in order to display the microstructure of the bone. Analysis of new bone formation was performed in each bone defect. The TRI/3D-BON software (Ratoc System Engineering, Tokyo, Co. Ltd, Tokyo, Japan) was used to obtain a 3-dimensional (3-D) reconstruction from the obtained set of scans. Out of the entire 3-D data set, a cylindrical region of interest (ROI) with a diameter of 3.6 mm and height of 1.3 mm covering the entire thickness of the calvarial bone was selected for analysis. The ROI was placed where the original defect was located, as the margins were visually recognizable. Mask work was performed to binarize the 3-D data set in the ROI. Only 3-D images of new bone formation were displayed, and bone volume (BV, mm^3) was measured directly (Fig. 1).

Histological observation

Two rats each were randomly selected and euthanized on both days 14 and 28 after surgery. The calvarial bone area including the 2 defects was extirpated en bloc. The sample was fixed with 10% neutral buffered formalin solution for 1 week at room temperature. Subsequently, the specimens were dehydrated in an increasing series of ethanol concentrations, embedded in methyl methacrylate (WAKO, Japan) for 4 weeks, and polymerized according to the manufacturer's recommendations. After polymerization, non-decalcified sections (70 μ m) were prepared with a modified diamond

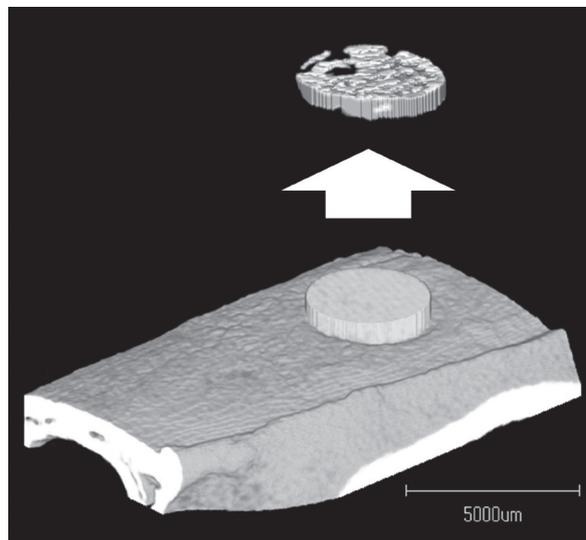


Fig. 1 3-D images of new bone formation at before and after mask work.

blade sawing microtome technique. The sections were stained with toluidine blue and then observed by light microscopy.

Statistical analysis

All data are expressed as the mean \pm standard deviation (SD). The data were analyzed using a statistical analysis software package (Excel Statistics 2006, SSRI, Japan). Significance between groups was estimated using a one-way analysis of variance and Fischer's protected least significance test. A p -value of less than 0.05 was considered statistically significant.

RESULTS

Micro-computed tomography analysis

Figure 2 shows representative micro-CT images of the three kinds of defect at days 14 and 28. New bone formation was clearly observed (Fig. 2 (a–f): white arrows).

Figure 3 (a–c) shows bone volumes in each animal at days 0, 7, 14, and 28. New bone formation was consistently observed in one animal during the period tested. Large differences were observed in bone formation between individuals (SD=1.582). However, the Flu-GH-treated areas in most of the animals showed more bone volume than the untreated side.

Figure 4 shows the mean bone volumes at days 0, 7, 14, and 28. Significant differences in bone volume were observed among the 3 treatments at days 7, 14, and 28. At day 7 after surgery, bone volume in the Flu-GH-treated area was significantly greater than that in the untreated area ($p=0.0281$). Bone volume in the Flu-GH-treated area was significantly greater than that in the DW-GH and untreated groups at day 14 after surgery ($p=0.0236$, 0.016, respectively). At day 28, bone volume

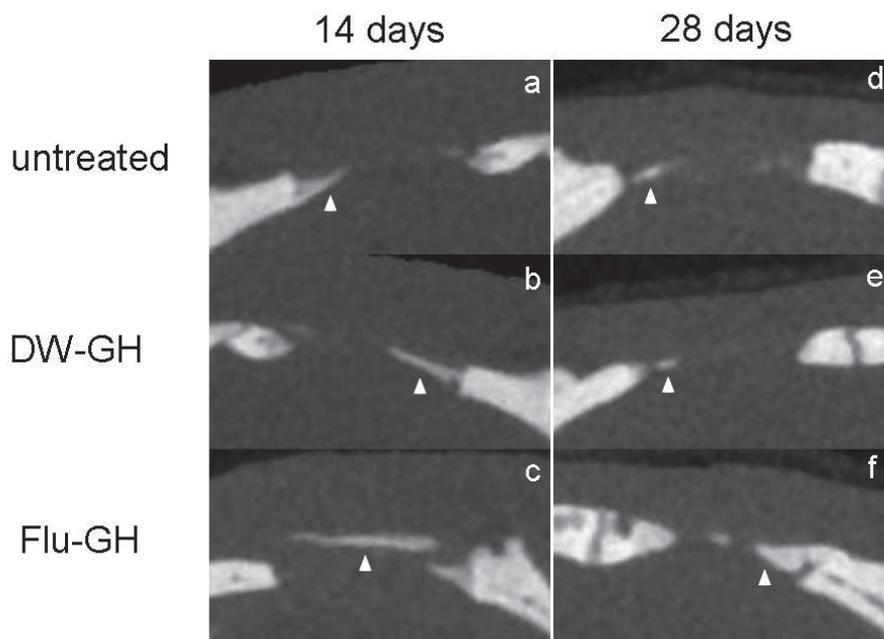


Fig. 2 Representative micro-CT images of the three kinds of defect at days 14 and 28. New bone formation was clearly observed (white arrows).

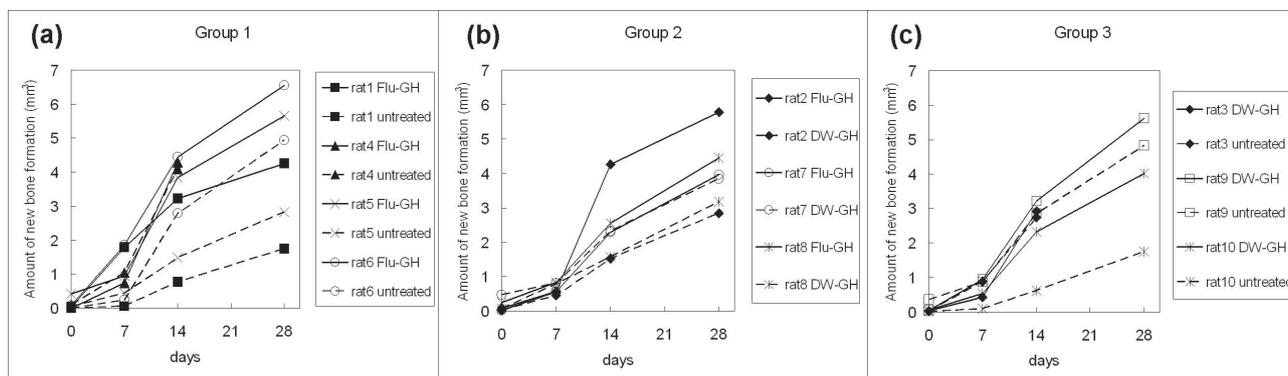


Fig. 3 Bone volume in each animal at days 0, 7, 14, and 28.

in the Flu-GH-treated area was significantly higher than that in the untreated area ($p=0.0097$). No significant differences were observed between the DW-GH-treated and untreated areas due to an especially large standard deviation in the untreated area at 28 days.

Histological observation

Figure 5 shows representative histological changes in the defect regions. New bone formation was clearly observed in the Flu-GH-treated area at days 14 and 28 (Fig. 5 (c), (f): red arrows). No clear differences were observed when the histological images were compared with the micro-CT images from which Fig. 2 was obtained. No degradation of the hydrogel disks was observed in the gelatin-hydrogel-treated area at 14 or 28 days.

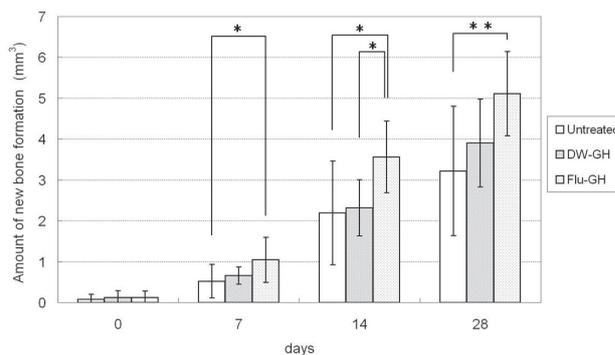


Fig. 4 New bone formation at days 0, 7, 14, and 28 postsurgically. Bone volume was measured 3-dimensionally with TRI/3D-BON software.

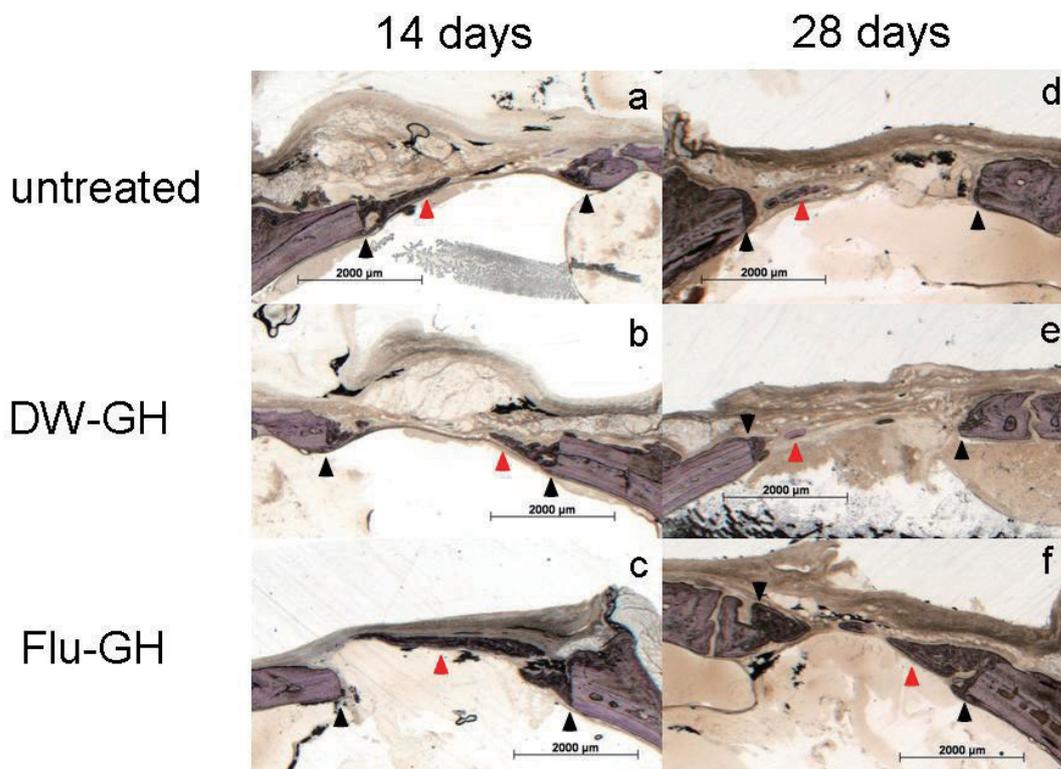


Fig. 5 Histological observation of bone defects at days 14 ((a), (b), (c)) and 28 ((d), (e), (f)). Black arrows indicate margin of bone defects. Red arrows indicate new bone formation.

DISCUSSION

In this study, quantitative image analysis and histological observation revealed that the significant increases in new bone formation and the critical osteogenic effect were observed in the Flu-GH-treated area in all animals. In Fig. 4, changes in bone volume with each treated area were observed. Significant increases of bone volume have been sustained for 14 days only in Flu-GH-treated area. This indicates that fluvastatin exerted a positive osteogenic effect and continuously released from the gelatin-hydrogel disk during the first half of the postsurgical observation period. Furthermore, Flu-GH-treated and DW-GH-treated areas showed similar increases of bone formation from 14 to 28 days. This result suggests that the osteogenic effect of gelatin-hydrogel without fluvastatin occurred in the second half of the postsurgical observation period. Moreover, a large standard deviation was only observed in defects which were left untreated. These results suggest that the gelatin-hydrogel did reduce individual differences. No degradation of the hydrogel disks was observed histologically. In an earlier study, we found that degradation of gelatin-hydrogel placed in a surgically created bone defect disturbed bone formation after 28 days¹¹. In this study, however, inhibition of

osteogenesis was prevented as the hydrogel was designed to be absorbed *in vivo* within 14 days, thus ameliorating the adverse effects of degradation. These results indicate the importance of designing a DDS that is bioresorbable within 14 days unless it is to be removed postsurgically.

In conclusion, the results showed that our new biodegradable Flu-GH delivery system promoted osteogenesis in rat calvarial bone, indicating the potential of this system in regenerative treatment.

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