

Research Article

Controlled Release of Granulocyte Colony-Stimulating Factor Enhances Osteoconductive and Biodegradable Properties of Beta-Tricalcium Phosphate in a Rat Calvarial Defect Model

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Autologous bone grafts remain the gold standard for the treatment of congenital craniofacial disorders; however, there are potential problems including donor site morbidity and limitations to the amount of bone that can be harvested. Recent studies suggest that granulocyte colony-stimulating factor (G-CSF) promotes fracture healing or osteogenesis. The purpose of the present study was to investigate whether topically applied G-CSF can stimulate the osteoconductive properties of beta-tricalcium phosphate (β -TCP) in a rat calvarial defect model. A total of 27 calvarial defects 5 mm in diameter were randomly divided into nine groups, which were treated with various combinations of a β -TCP disc and G-CSF in solution form or controlled release system using gelatin hydrogel. Histologic and histomorphometric analyses were performed at eight weeks postoperatively. The controlled release of low-dose (1 μ g and 5 μ g) G-CSF significantly enhanced new bone formation when combined with a β -TCP disc. Moreover, administration of 5 μ g G-CSF using a controlled release system significantly promoted the biodegradable properties of β -TCP. In conclusion, the controlled release of 5 μ g G-CSF significantly enhanced the osteoconductive and biodegradable properties of β -TCP. The combination of G-CSF slow-release and β -TCP is a novel and promising approach for treating pediatric craniofacial bone defects.

1. Introduction

Autologous bone grafts remain the gold standard for the treatment of congenital craniofacial bone disorders, such as alveolar cleft [1–8]. However, autologous bone grafts have potential problems, which include donor site morbidity and limitations to the amount of bone that can be harvested [9–13]. Porous beta-tricalcium phosphate (β -TCP), which is now commercially available, is known for its osteoconductive and biodegradable properties. However, its use as a replacement for autologous bone grafts remains controversial [14–18].

According to recent studies, various growth factors exhibit osteogenic properties [8, 19–23], such as bone morphogenetic protein 2 (BMP-2) [24–36], basic fibroblast growth factor (b-FGF) [37–42], platelet derived growth factor (PDGF) [43–46], transforming growth factor-beta 1 (TGF- β 1) [47–50], and vascular endothelial growth factor (VEGF) [51–54]. In general, growth factors administered in solution form are readily diffused or degraded *in vivo* [26, 50, 55, 56]. Thus, their enhanced and prolonged bioactivity at the target site is necessary to reduce bolus dosage, especially in pediatric patients. In addition, growth factors must be administered in combination with carrier materials.

TABLE 1: List of the experimental groups.

β -TCP	Types of G-CSF administration	Rat grouping (A-I)/ G-CSF dose ($\mu\text{g}/\text{defect}$)			
		0	1	5	20
+	Solution form	A	—	—	—
+	Solution form	—	B	—	—
+	Solution form	—	—	C	—
+	Solution form	—	—	—	D
+	Controlled release using gelatin hydrogel	E	—	—	—
+	Controlled release using gelatin hydrogel	—	F	—	—
+	Controlled release using gelatin hydrogel	—	—	G	—
+	Controlled release using gelatin hydrogel	—	—	—	H
None	Solution form	I	—	—	—

Recent studies suggest that granulocyte colony-stimulating factor (G-CSF) promotes fracture healing or osteogenesis [57–60]. Because G-CSF is an essential drug most frequently used to treat neutropenia secondary to chemotherapy, it is widely administered not only to adults but also to pediatric patients [61–69]. Accordingly, its biosafety is well established through extensive use in clinical contexts compared to other growth factors.

Commercially available β -TCP (Superpore, PENTAX, Tokyo, Japan) was used in the present study as an osteoconductive scaffold and space-maintaining material. To investigate the bone regenerative properties of G-CSF, topical supplementation either in solution or in sustained release form with a gelatin hydrogel system was performed. The purpose of this study was to investigate whether G-CSF with or without a controlled release system stimulates bone regeneration in combination with β -TCP using a rat calvarial defect model [70–73].

2. Materials and Methods

2.1. Study Design and Ethics. The present study was approved by the institutional committee of animal experiments at Hokkaido University (Institutional Animal Care and Use Committee Protocol number 12-0017). Fourteen Wistar rats (male, 13 weeks old; weight, 250–350 g) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). A total of 27 calvarial defects were randomly divided into nine treatment groups, with a total of three defects per treatment group. In solution-based treatment groups, defects were filled with a β -TCP disc containing normal saline alone (group A, control) or 1 (group B), 5 (group C), or 20 μg of G-CSF (group D). In controlled release groups, defects were filled with a β -TCP disk with an overlaid gelatin hydrogel sheet incorporating normal saline alone (group E) or, 1 (group F), 5 (group G), or 20 μg G-CSF (group H). The remaining defects were left empty to measure spontaneous healing (group I) (Table 1).

2.2. Preparation of β -TCP and Gelatin Hydrogel Incorporating G-CSF. Commercially available porous β -TCP blocks (Superpore) were kindly supplied by PENTAX (Tokyo,

Japan). Blocks were cut into discs 5 mm in diameter and 1 mm thick using a fine surgical saw and round bur.

Gelatin hydrogels were prepared by glutaraldehyde crosslinking of acidic gelatin as previously described [37]. Briefly, a mixed acidic gelatin-glutaraldehyde aqueous solution was cast into a polypropylene dish (80 \times 80 mm²) and maintained at 4°C for 12 hours. Hydrogel sheets were placed in a 100 mM glycine aqueous solution at 37°C. Discs were freeze-dried and sterilized with ethylene oxide gas. The water content of gelatin hydrogels (weight ratio of water present in hydrogel to wet hydrogel) was 95 wt%. Gelatin hydrogels were designed so that degradation would be complete in approximately two weeks under *in vivo* conditions [29, 39, 49, 74].

Hydrogel sheets were cut into discs 5 mm in diameter and 1 mm thick. Human recombinant G-CSF was kindly supplied by KYOWA KIRIN Co. (Tokyo, Japan). To prepare gelatin hydrogels incorporating G-CSF, 20 μL of normal saline solution containing 1, 5, or 20 μg G-CSF was dropped onto freeze-dried hydrogel discs and left at 4°C overnight. Similarly, 20 μL of G-CSF-free normal saline was dropped onto a freeze-dried hydrogel to obtain G-CSF empty hydrogels.

2.3. Surgical Procedures. Animals were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). Surgical areas were shaved and disinfected with povidone-iodine. Subsequently, a skin incision was made and subperiosteal dissection was performed under a surgical microscope to raise the periosteal flaps. A bone defect 5 mm in diameter was then prepared on each side lateral to the sagittal suture using a fine surgical bur under copious sterile saline irrigation. Defects were filled with bone substitutes according to the groups described above (Table 1 and Figure 1). Periosteal flaps were repositioned using a 4-0 nylon suture, and the skin was closed with a running 4-0 nylon suture. Finally, animals were euthanized by anesthetic overdose eight weeks after surgery.

2.4. Histological Processing. Specimens were prepared for decalcified sectioning by immersing them in 10% ethylenediaminetetraacetic acid (EDTA) for four weeks. Decalcified

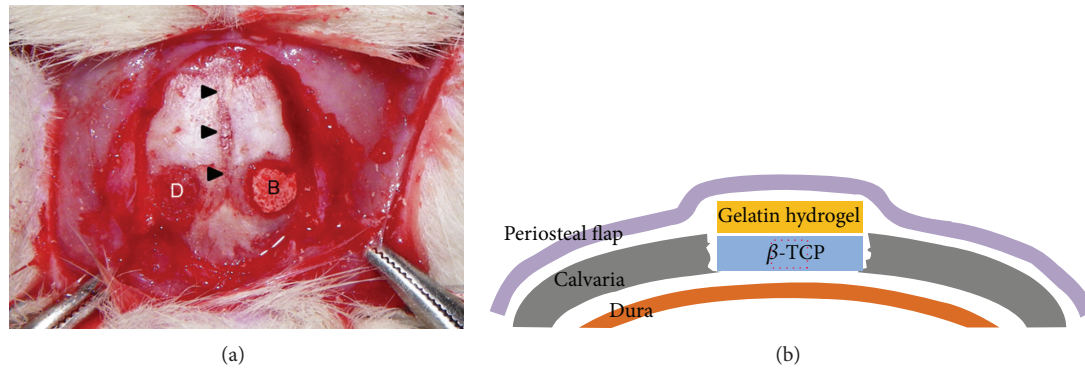


FIGURE 1: Surgical images. (a) Intraoperative view of rat calvaria. Two 5 mm diameter calvarial defects were created on each side of the sagittal suture (arrow heads). The defects were filled with a beta-tricalcium phosphate disc (B) or left untreated with exposed dura (D). (b) Schematic of a cross section of the calvarial defect. The dotted square in β -TCP indicates the most-central area where histomorphometric analysis was conducted.

specimens were dehydrated in ascending grades of ethanol and embedded in paraffin wax. Embedded samples were then sectioned into $3\ \mu\text{m}$ slices parallel to the sagittal suture across the center of each calvarial defect using a microtome (LEICA, SM2000R). Hematoxylin and eosin (HE) staining was used for histological analysis and aniline blue staining was used for histomorphometric analysis.

2.5. Histologic and Histomorphometric Analysis. Each specimen was examined under a light microscope and digital photographs were obtained for histological evaluation of a region corresponding to the center of the calvarial defect (Figure 1(b)). Images of HE staining were used for conventional histological analysis. High magnification images with aniline blue staining ($1.001\ \text{mm}^2$ or 1360×1024 pixels) of the most-central area of the defect were quantified to measure the percentage of newly formed bone and remaining bioceramics using imaging software (Adobe Photoshop CS5) [75]. All histomorphometric evaluations were conducted by a researcher blinded to the groupings.

2.6. Statistical Analysis. Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance (ANOVA). Data between groups were further analyzed using a Tukey-Kramer multiple comparisons test. $P < 0.05$ was considered statistically significant. Experimental results were expressed as mean \pm standard deviation (SD).

3. Results

3.1. Histological Findings. Figure 2 shows low magnification images of decalcified specimens stained with HE along the midline of each calvarial defect. No remaining gelatin hydrogel or surgical site infections were observed. In group A (control group), newly formed trabecular bone was observed focally but failed to occupy the entire defect. In groups B–D (solution-based treatment groups) and in group E (G-CSF-free gelatin hydrogel group), newly formed trabecular bone was observable but failed to fill the defect. In group F ($1\ \mu\text{g}$

G-CSF gelatin hydrogel group), newly formed bone tissue nearly bridged the calvarial gap, whereas residual β -TCP was also present. In group G ($5\ \mu\text{g}$ G-CSF gelatin hydrogel group), most of the defect was occupied with newly formed bone tissue; moreover, sparse residual β -TCP was observed. In contrast, group H ($20\ \mu\text{g}$ G-CSF gelatin hydrogel group) showed focal formation of new bone surrounded by fibrous connective tissue at the superficial area of the defect with the presence of remaining biomaterials. In group I (untreated defect group), the defect was filled with fibrous connective tissue with hardly any newly formed bone. Figure 3 shows higher magnification images of groups E (E') and G (G'). In group G, newly formed bone was observed immediately below the periosteal flap and multinuclear giant cells were detected around the newly formed bone. In contrast, in group E, the formation of fibrous tissue and blood vessels was significant compared with newly formed bone in the subperiosteal region.

3.2. Histomorphometric Evaluation. Figure 4 shows high magnification images of aniline blue staining in which matured bone tissue exhibits homogeneous dark blue and entrapped osteocytes. Residual β -TCP was observed as homogeneous white particles. Figure 5 shows the percentage of newly formed bone and remaining β -TCP per high-powered field. In groups A, B, C, D, E, and H, defects had a tendency to be occupied by more remaining β -TCP compared to newly formed bone tissue. In group A (control), the percentages of newly formed bone and remaining β -TCP were $20.77\% \pm 25.44\%$ and $35.01\% \pm 2.01\%$, respectively. In contrast, in groups F and G ($1\ \mu\text{g}$ and $5\ \mu\text{g}$ G-CSF gelatin hydrogel groups), the percentage of newly formed bone ($54.84\% \pm 9.46\%$ and $69.53\% \pm 5.35\%$ for groups F and G, resp.) conspicuously exceeded values of remaining β -TCP ($20.47\% \pm 2.89\%$ and $14.76\% \pm 7.36\%$ for groups F and G, resp.).

Figure 6 shows the percentage of newly formed bone after statistical analysis. The values were significantly higher in groups F and G compared to the control group ($P < 0.01$). There was no significant difference between groups

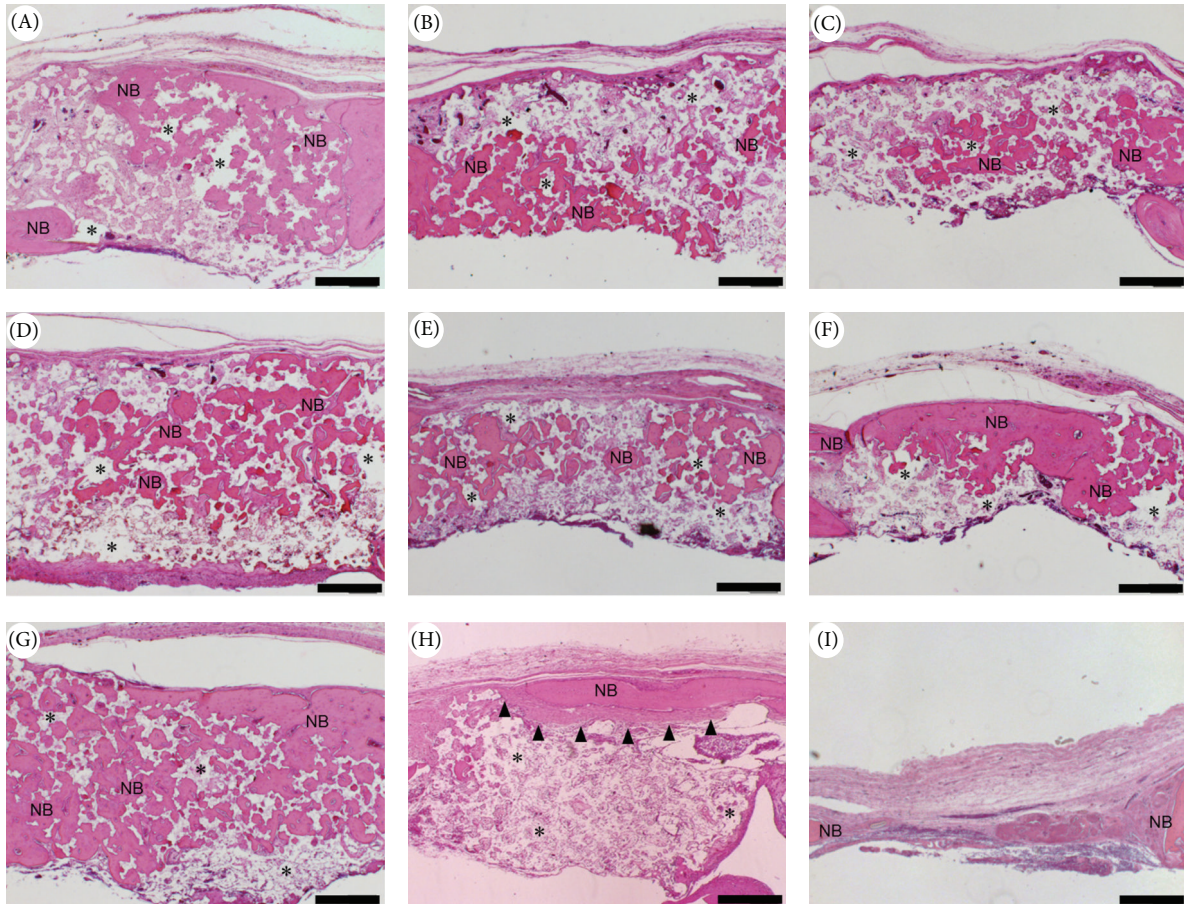


FIGURE 2: Low magnification images of hematoxylin and eosin (HE) staining. (a) β -TCP alone; (b) $1\ \mu\text{g}$ G-CSF in solution form; (c) $5\ \mu\text{g}$ G-CSF in solution form; (d) $20\ \mu\text{g}$ G-CSF in solution form; (e) free gelatin hydrogel; (f) $1\ \mu\text{g}$ G-CSF with gelatin hydrogel; (g) $5\ \mu\text{g}$ G-CSF with gelatin hydrogel; (h) $20\ \mu\text{g}$ G-CSF with gelatin hydrogel; and (i) untreated defect. Note the dense fibrous tissue surrounding focal bone formation in group H (arrow heads). Original magnification 40x; scale bar = $500\ \mu\text{m}$. NB: newly formed bone; * remaining β -TCP.

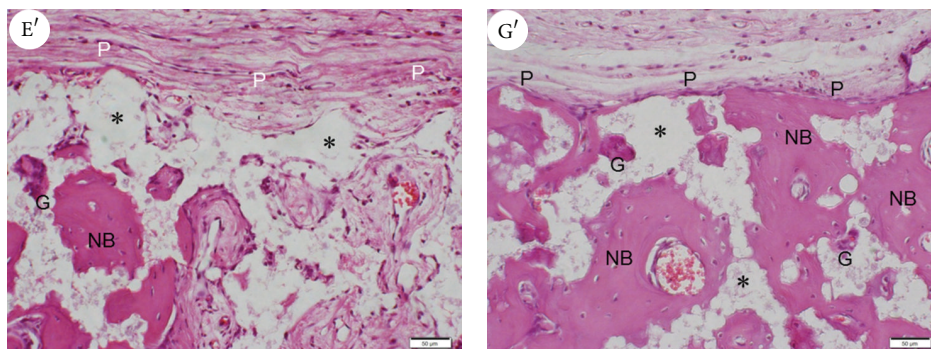


FIGURE 3: High magnification images of hematoxylin and eosin staining (E') group E and (G') group G. Note the obvious fibrous tissue and vessel formation in group E. In group G, newly formed bone is observed immediately below the periosteal layer and multinuclear giant cells are present. Original magnification 200x; scale bar = $50\ \mu\text{m}$. G: multinuclear giant cell; NB: newly formed bone; P: periosteal flap; * remaining β -TCP.

A (control) and I (empty defect). Values corresponding to groups B, C, D, E, and H showed no significant difference compared to that of group A.

Figure 7 shows the percentage of remaining β -TCP, which was used to evaluate biodegradability *in vivo*. There was

no significant difference between groups A, B, C, D, E, F, and H. In contrast, only in group G ($5\ \mu\text{g}$ G-CSF gelatin hydrogel group) the percentage was significantly lower compared to group A ($14.76\% \pm 7.36\%$ versus $33.53\% \pm 0.80\%$, $P < 0.05$). This result indicated a prominent enhancement of

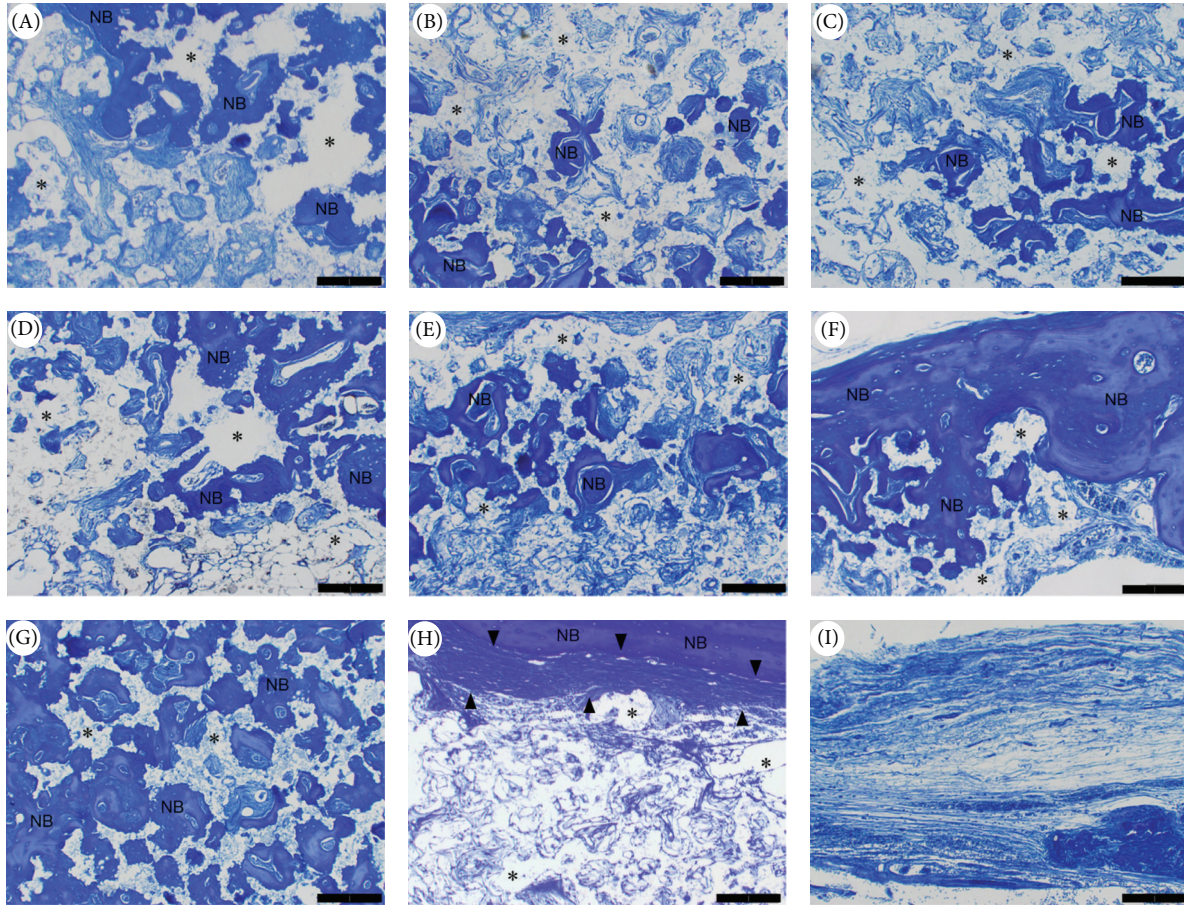


FIGURE 4: High magnification images of aniline blue staining. Capital letters A–I correspond to the treatment groups. Newly formed bone (NB) appears as a homogenous dark blue area, which includes osteocytes. The remaining β -TCP shows a homogenous white area (asterisk). Bony bridging was nearly complete in groups F and G. Less residual β -TCP was seen in group G. Original magnification 100x; scale bar = 200 μ m.

the biodegradable properties of β -TCP, which was further accelerated by 5 μ g G-CSF in sustained release form.

4. Discussion

In the present study, we demonstrated that the controlled release of low-dose (1 μ g and 5 μ g) G-CSF significantly enhanced bone regeneration when combined with a β -TCP disc. Moreover, administration of 5 μ g G-CSF using a controlled release system significantly promoted the biodegradable properties of β -TCP. According to our results, this tissue-engineering approach combining β -TCP and the sustained release of G-CSF is potentially feasible and promising for clinical use. To our knowledge, this is the first report which demonstrates the bone regeneration properties of G-CSF at membranous ossification sites. Because systemic administration of 5–10 μ g G-CSF/kg/day is commonly used for pediatric malignancies [64, 66–68], the results shown here indicate that notably low doses of G-CSF (1–5 μ g/defect/2 weeks) with controlled release can promote osteogenesis.

In this study, we used β -TCP as an osteoconductive scaffold and space-maintaining material [76]. In the present

study, the left untreated defect group showed thin connective tissue formation with minimal bone regeneration. Although the control group (β -TCP alone) showed a greater tendency for bone formation compared to the untreated defect group, there were no significant differences between the groups. Furthermore, the defect in the control group had more residual β -TCP than newly formed bone tissue. These results suggest that β -TCP alone implantation is not sufficient to fill the defect with regenerated bone in the craniofacial region. Some experimental studies have confirmed the osteoconductive properties of β -TCP, which were comparable to autologous bone grafts [15, 17, 70]. However, other groups have emphasized versatility by combining β -TCP with autologous bone fragments [16, 77–79], growth factors [45, 46, 80–84], simvastatin [71], or stem cells [18, 85, 86] in both experimental and clinical studies.

Interestingly, Ishida et al. reported that topical application of G-CSF had bone regenerative properties via neovascularization and osteogenesis [59]. That study revealed a significant increase in CD34⁺ cells—an endothelial and hematopoietic progenitor-enriched cell population—in capillaries corresponding to the bone defect site. These findings

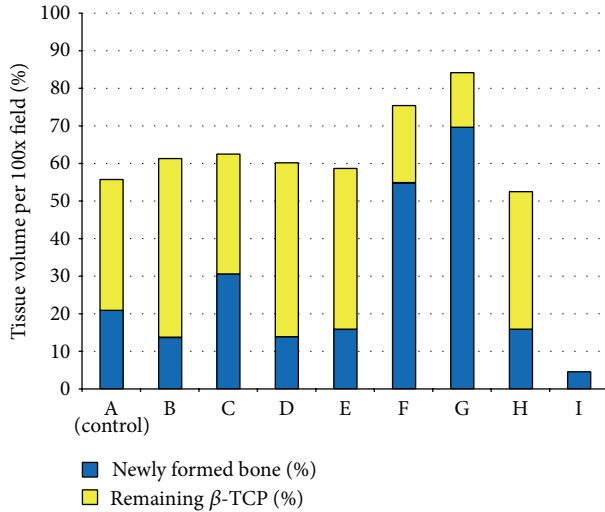


FIGURE 5: Percentage of newly formed bone and remaining β -TCP eight weeks after surgery. Values are shown as mean. Capital letters A–I correspond to treatment groups. Note that newly formed bone occupies more than 50% of the central area of the defect in groups F and G. In addition, the percentage of newly formed bone exceeds that of remaining β -TCP.

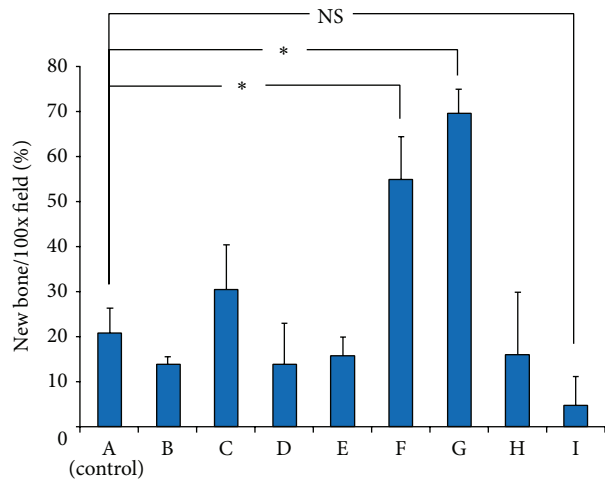


FIGURE 6: Percentage of newly formed bone in the high magnification field. Values are shown as mean \pm standard deviation (SD, $*P < 0.01$). The values are significantly higher in groups F and G (1 and 5 μ g G-CSF with gelatin hydrogel, resp.) compared to group A (β -TCP alone).

suggested that $CD34^+$ cells were important promoters of neovascularization. The study also showed that G-CSF was responsible for mobilizing osteoblasts to the bone defect site. In addition, recent studies demonstrated the promotion of fracture healing by $CD34^+$ cells [58, 87–89]. Kuroda et al. reported the first successful clinical case of a tibial nonunion treated with topically applied G-CSF-mobilized $CD34^+$ cells [60]. Some reports have shown that $CD34^+$ cells play an important role in releasing angiogenic factors, including vascular endothelial growth factor (VEGF), hepatocyte growth

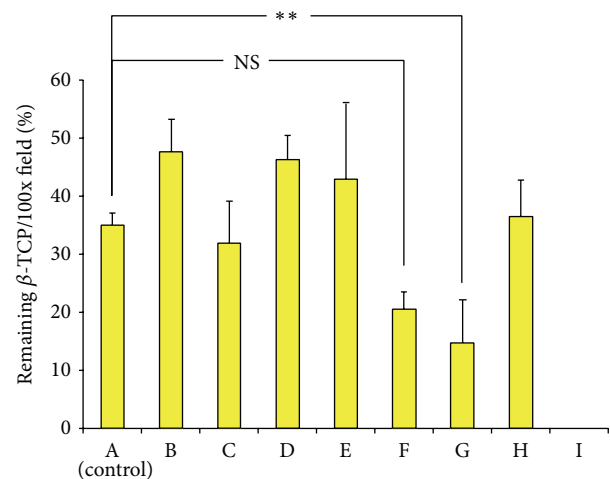


FIGURE 7: Percentage of remaining β -TCP in the high magnification field. Values are shown as mean \pm SD ($**P < 0.05$). The percentage in group G (5 μ g G-CSF with gelatin hydrogel) is significantly lower compared to group A. This result highlights the biodegradable properties of β -TCP.

factor (HGF), and fibroblast growth factor 2 (FGF2) [58, 90, 91]. Moreover, the differentiation capacity of $CD34^+$ cells into osteoblasts has been shown in previous reports [59, 92]. In the present study, the controlled release G-CSF groups showed more newly formed bone immediately below the periosteum compared to the other groups. On the other hand, Rojvani et al. reported that osteoprogenitor cells differentiate from the dura mater [71]. Presumably, the sustained release of G-CSF may stimulate periosteal cells along an osteogenic lineage, resulting in enhanced bone formation.

In the present study, we used gelatin hydrogel as a sustained release carrier of G-CSF. Various growth factors have been shown to have bone regenerative properties, such as bone morphogenetic proteins (BMPs) [24–36], b-FGF [37–42, 93], PDGF [43–46], TGF- β 1 [47–50], and VEGF [51–54]. BMP-2 has the strongest osteoinductive activity in promoting ectopic bone regeneration [26, 29] and has been approved by the Food and Drug Administration for use in orthopedics and oral surgery [30, 34, 35]. In general, growth factors administered in solution form are easily diffused or degraded prior to achieving full bioactivity [26, 50, 55, 56]. Therefore, commercially available BMP-2 in combination with a collagen sponge kit must contain milligram amounts of the growth factor (1.5 mg/mL) [30, 34, 35]. Potential risk for local inflammatory responses should be taken into consideration after topical application [35]. In order to reduce bolus dosage, enhanced and prolonged bioactivity of growth factors at the targeting site is necessary. One of the practical ways to control the *in vivo* release of growth factors is to use gelatin hydrogel, in which the growth factor is physicochemically immobilized and subsequently released in proportion to hydrogel degradation [74, 94]. In the present study, the water content of gelatin hydrogels (weight ratio of water present in hydrogel to wet hydrogel) was 95 wt%. The hydrogels were designed so that degradation would be complete in approximately 2

weeks under *in vivo* conditions [29, 39, 49, 50, 74]. Gelatin is commercially available and its biosafety is well established through its long clinical use as a plasma expander and drug ingredient.

In the present study, the controlled release of 5 μg G-CSF (group G) significantly promoted the osteoconductive properties and biodegradability of β -TCP. Improved biodegradability compared to hydroxyapatite is a major characteristic of porous β -TCP [71, 95–97]. Biodegradability is generally thought to occur in harmony with bone remodeling, in which β -TCP allows tissue fluid dissolution and absorption by osteoclasts *in vivo* [72, 95]. Brouard et al. reported that G-CSF increased both osteoclast activity and bone resorption in the bone marrow, triggering an increase in the number of mesenchymal precursor cells in the bone marrow using a mouse model [98]. In another study, PDGF modified β -TCP resorption, although the underlying mechanism was not provided [46]. Some studies have shown that BMP-2 does not facilitate β -TCP resorption [70, 99]. In group G of the present study, multinuclear giant cells were observed around newly formed bone immediately below the periosteum. We can speculate from the results that the controlled release of 5 μg G-CSF may stimulate the mobilization and differentiation of mesenchymal precursor cells in the periosteum as well as osteoclast activation. In contrast, group H (20 μg G-CSF gelatin hydrogel group) showed less new bone formation and β -TCP resorption. This might be explained by the multidifferentiation potential of G-CSF-mobilized progenitor cells, which is consistent with previously published reports [92, 100–102]. Interestingly, Ishida et al. stated that topical application of 50 μg G-CSF did not induce bone regeneration according to preliminary data [59]. Moreover, some reports have shown that sustained release of G-CSF enhances tendon-bone integration with significantly more formation of Sharpey's fibers and microvessels [103]. These results led us to speculate that a prolonged high concentration of topical G-CSF drives progenitor cells toward fibrous tissue formation rather than osteogenesis. Therefore, sustaining relatively low concentrations of topical G-CSF can play an important role in inducing balanced bone regeneration and β -TCP resorption. Our findings suggest an optimal dose of 5 μg per defect for controlled release of G-CSF, which is consistent with previously published reports [59, 103].

There are several limitations in this study that must be noted. First, the study was designed using small animals and a limited number per experimental group. Second, although some reports accept the calvarial defect rat model [70–73], the decortication procedure may not fully reflect clinical situations of congenital craniofacial anomalies [104], since some evidence suggests that fractures mobilize CD34⁺ cells from the bone marrow into the peripheral blood [88, 89]. Third, we used histomorphometric analysis to characterize newly formed bone and biodegradation of β -TCP; however, we did not identify CD34⁺ cells or evaluate the activity of osteogenic cells at the bone defect site. Future studies should incorporate experimental models without decortication, larger animals, and immunohistochemical analysis.

In conclusion, controlled release of 5 μg G-CSF using a gelatin hydrogel system significantly enhances the osteoconductive and biodegradable properties of porous β -TCP. The present results indicate that the combination of G-CSF slow-release and β -TCP is feasible and promising for the treatment of congenital craniofacial bone defects.

Conflict of Interests

All authors declared that there is no conflict of interests to report regarding this work.

Acknowledgments

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