Sustained Release of Catechin from Gelatin and Its Effect on Bone Formation in Critical Sized Defects in Rat Calvaria

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Abstract: The potential of plant-derived polyphenols in bone tissue engineering has not been fully realized owing to difficulties in maintaining their stability in affected parts. Catechins, such as epigallocatechin gallate (EGCG), are not fully utilized in bone regenerative medicine. Here, we demonstrated that chemical and non-chemical modifications of gelatin with EGCG resulted in distinct bone-forming abilities in critical-sized bone defects (9 mm) in rat calvaria. We prepared two EGCG-containing gelatin sponges: vacuum-heated gelatin sponges modified chemically with EGCG (AC-vhEGCG-GS) and vacuum-heated gelatin sponges with EGCG (no chemical modification; NC-vhEGCG-GS). Both sponges were characterized using scanning electron microscopy and degradability and EGCG-retention tests. The bone-forming ability of the sponges was estimated using micro-computed tomography and hematoxylin-eosin staining; the quality of newly formed bone (collagen maturation) was determined using picrosirius red staining and polarized microscopy. Both sponges had a spongy and soft texture with macropores ranging 50–150 µm with negligible differences in degradability. The NC-vhEGCG-GSs released all their EGCG content within 1 h, whereas AC-vhEGCG-GSs retained 75% of the EGCG for up to 24 h. In addition, AC-vhEGCG-GSs resulted in a significantly greater bone formation than NC-vhEGCG-GSs 4 w after implantation, with negligible differences in collagen maturation. These results suggest that the chemical modification of gelatin with EGCG might be a promising strategy to fully utilize the pharmacological effects of EGCG for natural polymer-based sponges. Moreover, the release rate of EGCG from gelatin is a promising screening parameter affecting the function of EGCG in vivo.

Key words: Bone formation, EGCG, Catechin, Gelatin

Introduction

The loss of bone tissue attributed to cancer, trauma, or infections significantly disrupts the patients’ quality of life.1,2 The functionalization of natural polymers is considered a seminal way to provide ideal materials applicable for bone diseases in advanced bone regenerative medicine.3 Denatured collagen, gelatin, derived from various animal species, such as fish, porcine, and bovine species, are utilized in the medical and pharmacological fields and in food industry for various purposes.4-9 The protein is widely applied in cell culture, bone substitute materials, and as a drug carrier because of its useful properties, such as high biocompatibility, degradability, and low cost.4-6 However, its clinical application, especially as a scaffold for bone regeneration, remains limited, possibly owing to its low bone-forming ability.10

Epigallocatechin gallate (EGCG), the most abundant bioactive polyphenolic compound in green tea, is a known health-promoting ingredient with antioxidant, antibacterial, anti-inflammatory, and anti-cancer properties.11 The molecule potentially has wide-ranging pharmacological effects on cardiac,12 inflammatory,13 oncological14,15 and psychiatric diseases.16 Thus, this polyphenol is thought to be a promising substance with applicability as a drug in nutritional supplements and biomaterials17-19.

The controlled release of therapeutic agents seems to be the most successful strategy for modulating various host reactions and diseases.20 Consequently, numerous delivery systems have been developed to provide localized release of these therapeutic agents in biomedical engineering.21 For EGCG, various techniques have been developed to retain or deliver EGCG in the affected areas; these techniques include chemical bonding to polymers,22 layer by layer technique, hydrophobic and/or hydrogen bonding,23,24 EGCG-coating associated with Na+ mediated physical self-assembly25 and oxidative polymerization26.
capsulation in drug carriers. Although our understanding of the application of this molecule in bone regenerative therapy remains limited when compared with that in other medical fields, we previously found that gelatin sponges chemically modified with EGCG using an aqueous chemical synthesis method (hereafter designated as AC-EGCG-GS) induced superior bone formation in critical-sized bone defects (4.2 mm of diameter) in mouse calvaria. More recently, we showed that the bone-forming ability of vacuum-heated AC-EGCG-GS (AC-vhEGCG-GS) was greater than that of vacuum-heated gelatin sponges (without EGCG) in critical-sized bone defects (9 mm of diameter) of rat calvaria. Alteration of the quantity of EGCG and gelatin in AC-vhEGCG-GSs affected the bone formation results. Although both AC-vhEGCG-GSs and vacuum-heated gelatin sponges successfully served as scaffolds for cells, the combination of AC-vhEGCG-GS with multipotent progenitor cells (dedifferentiated fat cells or adipose-derived stem cells) formed more bone in the congenital bone defects of rat jaws than the combination of vacuum-heated gelatin sponge with the above cells. Additionally, the solution containing the EGCG-modified gelatin impaired RANKL-induced osteoclastogenesis and delayed tooth movement by hindering oxidation. These results suggest that the chemical modification of gelatin with EGCG modulates bone formation and resorption. However, further investigation is essential to fully understand the effects of the synthesis process for functionalizing gelatin with EGCG, especially in terms of EGCG-release properties of the sponge and the effects on the formation and quality of newly formed bone.

Thus, this study was designed to compare the two types of EGCG-modified gelatin sponges prepared by different synthesis methods (Table 1), and evaluate their effects on bone formation and quality.

Materials and Methods

Sample preparation and characterization

Type A gelatin from porcine skin (Cat# G2500, gel strength < 300 g bloom) was obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), and EGCG from Bio Verde Inc. (Kyoto, Japan), respectively. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinum chloride (DMT-MM) and N-methylmorpholine (NMM) were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. The AC-vhEGCG-GS was prepared according to previous methods. Briefly, 0.07 mg of EGCG, 100 mg of gelatin, 27.5 μl of NMM, and 69.2 mg DMT-MM were mixed in 5 ml of MilliQ water at 23°C, followed by vacuum-heating as with AC-vhEGCG-GSs.

Characterization of sponges

The macroscopic and field emission scanning electron microscopic (FE-SEM) images of both sponges were obtained using the PowerShot A495 (Canon Inc., Tokyo, Japan) and FE-SEM (S-4800; Hitachi, Ltd., Tokyo, Japan). The sponges (2 mg) were immersed in 300 μl of phosphate buffer saline (PBS) and shaken at 60 r/min at 37°C for 24 h in a Bioshaker (Taitec BR-40LF; Taitec Co., Ltd., Saitama, Japan). Subsequently, the protein concentration in the supernatant was assessed to estimate the degradability of the sponges using a protein assay BCA kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s instructions. The residual EGCG in the sponges after immersion in PBS was assessed using the methods reported previously with slight modification. Briefly, 5 mg of NC- and AC-vhEGCG-GSs was immersed in 1 ml of PBS, and the sample tubes were fixed on an overturn stirring holder (MTR-103; AS ONE Corporation, Osaka, Japan) and incubated at 37°C for 24 h in a constant-temperature incubator (Taitec BR-40LF; Taitec Co., Ltd.). Following incubation, aliquots of 100 μl of the supernatant in each tube were pooled and the concentration of EGCG was measured using direct enzyme-linked immunosorbent assay (ELISA) and a mouse monoclonal anti-catechin antibody (Cat# KYU-TM-M001, Cosmo Bio Inc., Tokyo, Japan). Optical density was measured at 450 nm using a SpectraMax M5 plate reader (Molecular Devices, LLC., CA, USA). In addition, the percentage of residual EGCG in the sponges was estimated using the following formula:

\[
\text{Residual EGCG (\%)} = 100 - \left(\frac{X}{Y}\right) \times 100,
\]

where X and Y refer to the released (mg) and initial mass (mg) of EGCG in each tube, respectively.
Animal experiments and morphometric analysis

The animal experiments in this study were conducted with the permission of and in accordance with the guidelines approved by the local ethics committee of Osaka Dental University (approved No. 18-02003). Sprague-Dawley rats (8 w old, male) were purchased from Shimizu Laboratory Supplies Co. (Kyoto, Japan). The bone-forming capability of the sponges was evaluated using a critical-sized rat calvarial defect model. The rats were anesthetized by intraperitoneal injection of a mixture of medetomidine hydrochloride (Domitor; Zenoaq, Fukushima, Japan), midazolam (Midazolam Sandoz; Sandoz K.K., Yamagata, Japan), and butorphanol tartrate (Vetorphale; Meiji Sika Parma Co., Ltd., Tokyo, Japan). Bone defects (9 mm) were prepared at the center of the calvaria using a trephine bar as reported previously. Ten sponges (each sponge: 3 × 2 mm; D × H) were implanted in three defected calvaria per group. Four weeks after implantation, the treated calvaria were harvested and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for the histological and morphometric analyses described below. For morphometric analysis of newly formed bone, bone defects were analyzed using micro-computed tomography analysis (μCT, SMX-130CT; Shimadzu Corporation, Kyoto, Japan); the scanning parameters were 90 μA and 55 kV at 512 × 512 pixels. TRI/3D bone software (Ratoc Co., Ltd., Tokyo, Japan) was used to reconstruct the 3-D morphology of the calvaria following morphometric analysis. The bone formation and quality in the defects were quantified using the following parameters: bone volume (BV)/total volume (TV) ratio; bone mineral content (BMC)/TV ratio; and BMC/BV ratio. Cylindrical phantoms containing hydroxyapatite (200–1550 mg/cm³) were utilized to calculate the BMC, which exhibited calcified bone tissue. Calvaria were resected and kept in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C until used for histological evaluation.

Histological observation

After the μCT scan, the fixed calvaria were decalcified, dehydrated, and embedded in paraffin. Thin sections with 5 μm of thickness were
prepared and stained with hematoxylin-eosin (H-E) to confirm the existence of newly formed bone and picrosirius red to detect the collagen maturity associated with bone quality. The stained sections were analyzed using the fluorescence microscope (BZ-9000; Keyence Corporation, Osaka, Japan) for H-E staining and polarized microscopy (Eclipse Ci POL, NIKON INSTECH Co., Ltd., Tokyo, Japan; Leica DFC290 HD, Leica Microsystems, Tokyo, Japan) for the picrosirius red staining.

Statistical analysis
The data were analyzed using a Student’s t-test in GraphPad Prism 7J (GraphPad Software, Inc., San Diego, CA, USA). The data were presented as mean ± standard deviation (SD).

Results
Characterization of sponges
Fig. 1 shows the macroscopic images of NC- and AC-vhEGCG-GSs; both sponges had a spongy and soft texture. Irregular macropores and a smooth surface could be observed in the FE-SEM images of both sponges (Fig. 2). The pore size of the sponges approximately ranged from 50 to 150 μm.

Degradation of sponges and EGCG release
The vacuum-heating technique is extensively used for crosslinking gelatin and collagen to improve durability and degradability. Degradability of materials is considered to be associated with the release of drugs. Consequently, we verified the degradability of both sponges in PBS, and there were negligible differences between both sponges for up to 24 h (Fig. 3A). However, NC-vhEGCG-GSs released almost 100% of...
Yoshitomo Honda et al.: EGCG and Bone Formation

the EGCG within 1 h, whereas AC-vhEGCG-GSs retained nearly 75% of EGCG for up to 24 h (Fig. 3B). These results suggest that both sponges possessed similar degradability, but different EGCG-release properties.

Bone formation in defects

To evaluate the bone-forming abilities of NC- and AC-vhEGCG-GSs, both sponges were implanted in the critically sized defects (9 mm) of rat calvaria for 4 w, and analyzed morphometrically (Figs. 4 and 5). Our previous study showed that in defects with no implantation (negative control) there was a small amount of newly formed bone in the defect for up to 4 w\(^ {18, 24} \). The defects treated with AC-vhEGCG-GSs showed a greater radio-opacity than those treated with NC-vhEGCG-GSs (Fig. 4). We confirmed that the radio-opacities corresponded to newly formed bone using H-E staining (Fig. 5). The bone defects were nearly completely covered with newly formed bone within 4 w after implanting the AC-vhEGCG-GSs in the critical-sized rat calvarial defects.

Newly formed bone quality

Bone quality attracts much attention in the field of bone biology\(^ {30} \), as it is likely to affect the clinical outcome of the bone regenerative therapy. Picrosirius red staining analyzed by polarized microscopy was used to assess the maturity of collagen\(^ {31} \). To estimate the bone quality of newly formed bone, we evaluated bone mineral density (BMC/BV) and the maturation of collagen in defects treated with the two types of sponges (Figs. 4 and 6). Despite obvious differences in the release rate of EGCG from both sponges and in the bone formation rate in the defects, there were negligible differences in BMC/BV and in the images observed after picrosirius red staining for up to 4 w. For the picrosirius red staining, the green (unmaturated collagen) and red and yellow (mature collagen) stains were similarly dispersed in defects treated with NC- or AC-vhEGCG-GSs.

Discussion

This study demonstrated that two different gelatin sponges, non-chemically or chemically modified with EGCG, exhibited distinct properties in terms of EGCG-retention capacity and bone formation, but not in bone quality. Our results indicated that the different processing method for modifying gelatin with EGCG completely diverges in bone-forming ability of the EGCG-containing gelatin in vivo.

The FE-SEM images showed that the pore size of NC- and AC-vhEGCG-GSs ranged from 50 to 150 μm (Fig. 2). The pore size of AC-vhEGCG-GSs was larger than that of NC-vhEGCG-GSs. Generally, the pore size is known to alter the bone-forming ability of materials\(^ {32} \), and the optimal pore size of calcium phosphate-based materials for bone regeneration is recognized to be over 100 μm\(^ {21} \). However, the adequate...
pore size of polymer-based sponges for bone regeneration remains unclear because the immersion of materials in body fluid causes swelling, thereby changing the pore size\(^2\). In fact, both NC- and AC-vhEGCG-GSs could induce bone formation in the center of defects (Fig. 4). Previous studies have reported that pores sizes below 50 μm in collagen sponges promote osteoconduction\(^{31,33}\). Given these results, although small pores of ca. 50 μm might be able to induce bone formation when using biodegradable polymers, the superior bone-forming ability of AC-vhEGCG-GSs in comparison to that of NC-vhEGCG-GSs might be partially due to the difference in pore size between the two sponges.

Our \textit{in vitro} study showed that NC-vhEGCG-GSs released their EGCG content within 1 h (Fig. 3). However, our data is insufficient to conclude that the simple mixture of EGCG and gelatin was ineffective at retaining the polyphenol in the gelatin sponge. Other studies have addressed the efficacy of the non-chemical modification technique using EGCG in combination with other biomaterials, including synthetic polymers\(^5,22\), alpha-tricalcium phosphate\(^{36}\), and collagen\(^{21}\) for bone tissue engineering. Some studies showed that physical adsorption of EGCG on the other biomaterials without any chemical modification increased the capacity for bone formation\(^{1,21,34}\). Given these results, type A gelatin might enable the interaction, albeit weak, of EGCG with itself. The reason why the simple mixture of type A gelatin and EGCG was unable to yield a strong and stable interaction remains unclear, and further investigation is necessary to elucidate the underlying mechanisms driving EGCG and gelatin interactions. Nevertheless, our results may provide insights into the use of EGCG in natural polymers and biomaterials in regenerative medicine.

The biodegradability and durability of a scaffold potentially alters its bone-forming abilities\(^2\). Although we have not elucidated the precise biodegradability of NC- and AC-vhEGCG-GSs \textit{in vivo}, they had similar levels of degradability in PBS \textit{in vitro} (Fig. 3). Our previous study revealed that AC-vhEGCG-GSs and vacuum-heated gelatin sponges (without EGCG) showed similar levels of degradability, and were effective scaffolds for osteoblastic cells UMR106 \textit{in vitro}\(^{36}\). In view of bone formation by the NC-vhEGCG-GS, this sponge possibly works as a sufficient scaffold for the bone-forming cells \textit{in vivo} as with AC-vhEGCG-GSs and vacuum-heated gelatin sponges.

The bone-forming ability of AC-vhEGCG-GSs was significantly greater than that of NC-vhEGCG-GSs when an equal dose of EGCG was incorporated into NC- and AC-vhEGCG-GSs before implantation (Figs. 4 and 5). Meanwhile, the collagen maturity of newly formed bone was similar in the defects treated with both sponges for a period of at least 4 w (Fig. 6). Previous studies have reported that surgery alone (without any pathogens and foreign body reactions) could cause inflammation within hours and persist for up to 5 d\(^{37,38}\). Free radicals emerged at the bone fracture site, modulating bone metabolism\(^{39}\). Furthermore, chronic inflammation shows adverse effects for the bone healing\(^{40}\). Meanwhile, it is consensusal that EGCG is a strong antioxidant and anti-inflammatory agent\(^{41}\). Additionally, the presence of EGCG in biomaterials treated by coating or modification approaches alters the materials properties, including wettability, charge, and calcium deposition\(^{4,21}\). After the transplantation of sponges, the rapid release of EGCG from NC-vhEGCG-GSs might have caused the loss of the pharmacological effect of EGCG and changed the surface properties of the sponges over time. In contrast, the AC-vhEGCG-GS might have been able to retain these properties owing to the chemical modification, thereby resulting in the augmentation of its bone-forming ability \textit{in vivo}.

Thus, we found that NC- and AC-vhEGCG-GSs prepared under different synthesis conditions, but containing the same dose of EGCG, resulted in varying EGCG-retention properties and bone-forming abilities, but similar bone qualities. The stable retention of EGCG in AC-vhEGCG-GSs resulted in superior bone formation over that induced by NC-vhEGCG-GSs. There are various studies reporting the pharmacological effects of polyphenols, including EGCG, \textit{in vitro}. However, our data suggests that caution should be taken when transferring findings obtained \textit{in vitro} to \textit{in vivo} studies. For example, the release rate of the active ingredient from substrates and biomaterials might be a significant factor affecting the function of polyphenols \textit{in vivo}.

Acknowledgements

We are very grateful to Mr. Naoya Kawade (Institute of Dental Research, Osaka Dental University) for the technical support. This investigation was supported in part by Grant-in-Aid for Scientific Research (16K11667, and 18H02986) from the Japan Society for the Promotion of Science.

Conflicts of Interest

The authors declare no conflicts of interest associated with this manuscript.

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