CLINICAL ORAL IMPLANTS RESEARCH

*Yun-Young Chang**
*Jung-Seok Lee**
Min-Soo Kim
Seong-Ho Choi
Jung-Kiu Chai
Ui-Won Jung

Comparison of collagen membrane and bone substitute as a carrier for rhBMP-2 in lateral onlay graft

Authors' affiliations:
Yun-Young Chang, Jung-Seok Lee, Min-Soo Kim, Seong-Ho Choi, Jung-Kiu Chai, Ui-Won Jung, Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Yonsei University, Seoul, Korea

Corresponding author:
Ui-Won Jung
Department of Periodontology
Research Institute for Periodontal Regeneration
College of Dentistry
Yonsei University
50 Yonsei-ro, Seodaemun-gu, Seoul 120-752
Korea
Tel.: +82-2-2228-3185
Fax: +82-2-392-0398
e-mail: drjew@yuhs.ac

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Abstract

Objectives: To evaluate the bone regenerative effect of bioresorbable collagen membrane (CM) as a carrier for recombinant human bone morphogenetic protein-2 (rhBMP-2) when performing lateral onlay grafts using bovine hydroxyapatite incorporated with collagen matrix (BHC) in combination with CM in dogs.

Material and methods: A guided bone regeneration (GBR) was performed at the buccal aspect of edentulous maxillary alveolar ridges in dogs (n = 5): (1) BHC group, in which rhBMP-2-loaded BHC was covered by a CM, and (2) CM group, in which BHC was covered by an rhBMP-2-loaded CM. A histologic and histometric analysis was performed after 8 weeks of healing.

Results: Both the BHC and CM groups exhibited substantial newly formed bone (NB). More NB was found in the CM group than in the BHC group without statistical significance. Most of the NB was in direct contact with the residual bone substitute in the BHC group, whereas the projections and islands of NB were observed in the spaces between the residual bone substitute clusters in the CM group. The bone-to-residual bone substitute contact ratio was significantly lower in the CM group than in the BHC group (P = 0.043).

Conclusions: Within the limitations of this study, it can be concluded that rhBMP-2-loaded CM performed lateral onlay grafts as effectively as rhBMP-2-loaded BHC while showing less bone-residual bone substitute contact ratio in dogs. The loading of CMs with rhBMP-2 might therefore be a recommendable treatment option for facilitating lateral onlay graft combined with rhBMP-2.

Various treatment regimens have been used to overcome horizontal and vertical defects in atrophied alveolar ridges, and the guided bone regeneration (GBR) technique has resulted in successful bone regeneration in both preclinical and clinical studies (Hämmerle & Karring 1998; Hämmerle & Jung 2003; De Boever & De Boever 2005). However, several studies have shown that GBR exhibits very limited regenerative efficacy at defects that have limited healing sources, such as the vertically resorbed ridge (Caplanis et al. 1997; Wikesjö et al. 2004; Huang et al. 2008). The autogenous block bone graft may be considered the first choice for resolving such a challenging defect (McAllister & Haghighat 2007; Simion et al. 2007). However, while autogenous bone cannot be used routinely due to unavoidable problems such as donor-site morbidity, limited quantity of available bone, unpredictable resorption, and, most importantly, the need for a high level of surgical skill.

Tissue engineering technologies using growth factors have been rapidly developed for the regeneration of bone tissue, and much of the researches have focused on the use of recombinant human bone morphogenetic protein (rhBMP) to enhance the osteogenic potential of the bone substitute (Sigurdsson et al. 2001; Tatakis et al. 2002; Wikesjö et al. 2002). RhBMP-2 has received much attention for alveolar bone augmentation and has been extensively studied under various experimental conditions including onlay and vertical grafts (Wikesjö et al. 2002; Huang et al. 2008; Jung et al. 2008).

Recently, incorporation of bovine hydroxyapatite (BH) with collagen matrix (BHC) has used in several studies and showed improved manageability without dissipation of the graft particles during surgery (Jung et al. 2011). The BHC has a three-dimensional porous structure and is hydrophilic, which easily enables the absorption of fluid and could

*These authors equally contributed to this work.

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provide space for both vascular ingrowth and migration/proliferation of osteogenic progenitor cells.

The use of a bioresorbable collagen membrane (CM) as a barrier membrane in combination with grafting bone substitute as a scaffold is a well-documented GBR procedure (Hämmerle et al. 2002; Chiapasco & Zaniboni 2009). Previous studies combining GBR with rhBMP-2 have used conventionally bone substitute or absorbable collagen sponge as a carrier material for the rhBMP-2 (Jovanovic et al. 2007; Schwarz et al. 2008a, 2009). Bone substitute supports the CM and provides space for osteoinduction. However, the carrier should be absorbable or it will require additional processing, such as lyophilization, to enable rhBMP-2 loading (Jung et al. 2003).

Collagen membrane could be considered as a carrier for rhBMP-2 if it stably covers the augmented volume while allowing the continuous release of rhBMP-2. Covering of the bone substitute with an rhBMP-2-loaded CM may enhance the osteogenic potential of the bone substitute, as it is in direct contact with the periosteum of the overlying mucogingival flap. It has been reported that the periosteum is highly osteogenic and rich in the necessary mesenchymal cells that can differentiate into osteoblastic cells (Zhu et al. 2006; Hayashi et al. 2008; Cho et al. 2011). Furthermore, it has been documented that a certain type of CM permits early transmembranous anastomosis between the reflected mucogingival flap and the inferior tissue (Schwarz et al. 2006, 2009). Therefore, it is anticipated that new bone induced by an rhBMP-2-loaded CM may form from the upper part of the onlay graft and subsequently surround the bone substitute located inside the onlay graft with native bone. The bone substitute will ultimately be surrounded by multiple osteogenic sources and thus undergo gradual osteogenesis. In other words, the osteoinductive potential of rhBMP-2-loaded CMs placed over a bone substitute will alter the osteogenic environment of onlay grafts in self-contained defects, such as in the sinus cavity, and influence the regenerative capacity of the bone substitute. In addition, previous studies have demonstrated that the addition of rhBMP-2 clinically enhances soft-tissue healing compared with the control condition (i.e., no addition of rhBMP-2) (Wikesjö et al. 2003a; Misch 2011). RhBMP-2-loaded CM that is in direct contact with the overlying mucoperiosteal flap could also exert a positive effect on the soft-tissue healing during GBR.

Based on these considerations, we conducted augmentation procedure using CM as a carrier for rhBMP-2 for comparison with a conventional bone substitute carrier in lateral onlay defect. Therefore, the aim of this study was to evaluate the bone regenerative effect of CM as a carrier for rhBMP-2 when performing lateral onlay graft using BHC in combination with CM in dogs.

Materials and methods

Animals

Five male mongrel dogs (Gukje, Pocheon, Korea) aged 20–24 months and weighing approximately 15 kg were used. All animals had a full, healthy permanent dentition and were allowed a period of adaptation of at least 1 week before the surgical procedure. They were kept in separate cages under standard laboratory conditions (an ambient temperature 22°C), provided with ad libitum access to water and a pelleted laboratory diet with an exception of 2 weeks postsurgery when they were fed a canned soft dog food diet. The animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea (no. 09-007).

RhBMP-2 constructs

A 2 mg of rhBMP-2 (Cowellmedi, Busan, Korea) was reconstituted with 1.34 ml of sterile water and further diluted with 2.68 ml of buffer to produce a stock solution with an rhBMP-2 concentration of 0.5 mg/ml. For experimental constructs, BHC (BioOss collagen®: width, 8 mm; thickness, 4 mm; height, 5 mm; Geistlich Biomaterials, Wolhusen, Switzerland) was formed into a rectangular shape of dimensions 4 × 5 × 4 mm (length × width × height), and the CM (BioGide®, 25 × 25-mm squares; Geistlich Biomaterials) was cut into 12.5 × 12.5-mm squares. The rhBMP-2 solution (0.2 ml of the 0.5 mg/ml stock) was withdrawn using a sterile 1-ml syringe and uniformly dispensed over the entire surface of both the BHC and CM on a sterile dish. Following a 15-min loading time at room temperature, the rhBMP-2-loaded BHC or CM was applied to the experimental site (according to the experimental group to which the site had been assigned).

Study design

Two experimental groups were defined according to the application protocol of rhBMP-2 as follows:

- BHC group: rhBMP-2-loaded BHC was applied and then covered with CM.
- CM group: BHC was applied to the surgical site and then covered with rhBMP-2-loaded CM.

Each animal ($n = 5$) received both surgical treatments (BHC group and CM group) at the same side of maxillary alveolar bone. The animals were allowed a healing period of 8 weeks.

Surgical protocol

All surgical procedures were performed by an experienced surgeon (YYC), under general anesthesia induced by an intravenous injection of atropine (0.05 mg/kg; Kwangmyung Pharmaceutical, Seoul, Korea) and an intramuscular injection with a combination of xylazine (2 mg/kg; Rompun, Bayer Korea, Seoul, Korea) and ketamine hydrochloride (10 mg/kg; Ketalar, Yuhan, Seoul Korea), followed by maintenance via inhalation anesthesia (Gerolan, Choongwae Pharmaceutical, Seoul, Korea). Vital sign monitoring was accompanied during the surgery. Routine local infiltrative anesthesia (2% lidocaine HCl with epinephrine 1 : 100,000, Kwangmyung Pharmaceutical) was used at the surgical sites. Oral prophylaxis including scaling and plaque control was performed 1 week before the surgical procedure.

The left maxillary first, second, and third premolar teeth were carefully extracted to create an edentulous alveolar ridge. The extraction sites were allowed to heal for 2 months. A separate experiment that did not form part of this study was performed on the right upper jaw (Jung et al. 2013). After the healing period, complete extraction socket healing was confirmed under general anesthesia (as described previously), and a midcrestal incision was made from the first molar to the canine. Two vertical incisions were then made at the mesial aspect of the first molar and the distal aspect of the canine on the left upper jaw quadrant. The mucoperiosteal flaps were increased buccally and the experimental site was completely exposed (Fig. 1a).

Six intramarrow perforations were made with a #330 carbide bur at two sites under copious sterile saline irrigation. RhBMP-2-loaded BHC was applied at the first site and then covered with an untreated CM (BHC group; Fig. 1b), while untreated BHC was applied and then covered with rhBMP-2-loaded CM at the second site (CM group; Fig. 1b). The CMs at each site (i.e., treated and untreated) were fixed by pin on the mesiodistal side of the CM (Membrane Pin, Dentium, Seoul, Korea). Periosteal releasing

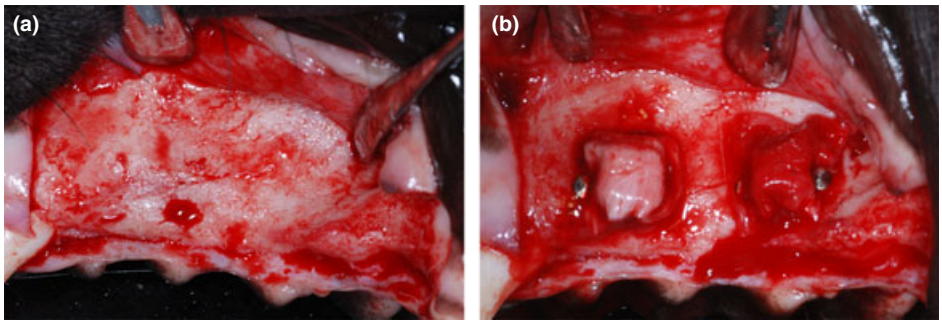

Fig. 1. (a) Clinical photograph of an experimental site after tooth extraction. (b) The rhBMP-2 loaded BHC was covered with untreated CM on the left side (BHC group), while untreated BHC was covered with an rhBMP-2-loaded CM on the right side (CM group).

incisions were made at the base of the flaps to obtain primary wound closure with tension-free adaptation. The buccal flaps were then sutured with 40 resorbable nylon (Monosyn 4.0 Glyconate Monofilament; B. Braun, Tuttlingen, Germany).

All of the animals were given antibiotics intramuscularly and fed a soft diet for 14 days postsurgery. Daily topical application of chlorhexidine was performed until suture removal, which took place 7–10 days postsurgery. The animals were killed after 8 weeks by an intravenous injection of concentrated sodium pentobarbital. The experimental sites, including soft tissues, were removed and then fixed in 10% buffered formalin for 10 days. The surgical procedures including tooth extraction began from September 2009, and the final animal was euthanized on June 2010.

Histologic and histometric analyses

Resected specimens were decalcified in 5% formic acid for 10 days. The experimental site of each specimen was cut apicocoronally, and one of the sections was embedded in paraffin. The central-most section of the experimental site was chosen for histologic and histometric analysis. Cross-sectioned specimens were further cut to a final thickness of 5 μm in the apicocoronal direction and stained with hematoxylin and eosin (HE) and Masson's trichrome (MTC). Each section was observed with the aid of a light microscope (Olympus Research System Microscope BX51; Olympus, Tokyo, Japan) equipped with a camera.

Histometric analysis was performed using an automated image analysis computer program (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA) by a single experienced investigator (JSL) who was masked as to surgical procedure and the experimental group to which the specimens belonged. For calibrating intraexaminer errors, above-mentioned investigator (JSL) performed histomorphometry twice with 2 weeks interval, using 10 sections. The following histometric parameters were measured as a primary variable (Fig. 2):

- Augmented area (AA) and height (AH) were determined by measuring the area and vertical height, respectively, between the inferior border of the CM and the native bone.
- Newly formed bone (NB) area (NBA) and height (NBH) were determined by measuring the area and vertical height, respectively, of NB between the inferior border of the CM and the native bone.
- Ratio of the components (%) were compared by calculating the proportions of NB, RB, and fibrovascular connective tissue (FV) within the AA.

To analyze differential healing process according to the loading site for rhBMP, another parameter was measured histomorphometrically, as a secondary variable as follows:

- Bone-to-residual bone substitute contact ratio (BRC; %) was measured as a proportion of residual bone substitute (RB) in direct contact with bone for entire circumferences of RB.

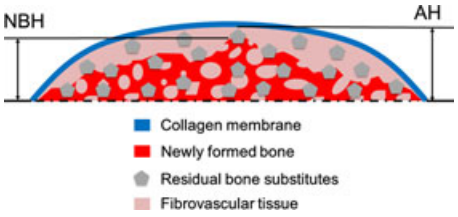

Fig. 2. Schematic drawing illustrating histometric landmarks. The augmented area (AA) was demarcated by the native bone (black dotted line) and the inferior border of the CM (blue line). The areas of newly formed bone (NBA), residual bone substitute (RB), and fibrovascular connective tissue (FV) were measured within the AA. Augmented height (AH) was measured according to the vertical distance between the native bone and the highest point of the AA. NB height (NBH) was measured according to the vertical distance between the native bone and the highest point of NB.

Statistical analysis

Mean and standard deviation values of each histometric parameter were calculated for each experimental group. The significance of differences between the groups was determined using the Wilcoxon signed-rank test. The level of statistical significance was set at $P < 0.05$. The Hodges–Lehman tests were used for calculating confidence intervals of median difference between two experimental groups in all measured parameters.

Results

Clinical observations

All of the animals healed uneventfully, and neither of the experimental sites (i.e., receiving either rhBMP-2-loaded BHC or rhBMP-2-loaded CM) in any of the animals exhibited wound dehiscence or signs of infection. Two specimens from experimental sites were obtained in each animal, and a total of 10 specimens (five specimens per group) were investigated for histomorphometric analysis.

Histologic analysis

Dome-shaped AAs were observed in both groups within the space circumscribed by the CM and native bone, well integrated into the native bone. The AAs were composed of NB, RB, and FV which included newly formed vascular tissue, fatty bone marrow, and dense connective tissue. The NB projected from the recipient bone bed into the AA and was present beneath the CM. The NB was composed of woven bone and mature lamellar bone with Haversian systems. Most of the RBs were located at the central portion of the grafted site.

In the BHC group, the cortical bone layer of the recipient bed was resorbed and the bone marrow space was in communication with the AA (Fig. 3a). The RB was in direct contact with the NB (Fig. 3b). The intact CM containing many capillaries was clearly observed at 8 weeks of healing (Fig. 3c). Multinucleated osteoclast-like cells were found at the resorption lacunae on the surface of the NB and RB (Fig. 3d,e).

The overall histologic features in the CM group were similar to those of the BHC group (Fig. 4a). However, the configuration of the NB differed slightly between the two groups. Finger- or column-like projections of NB that were directed toward or lying alongside the

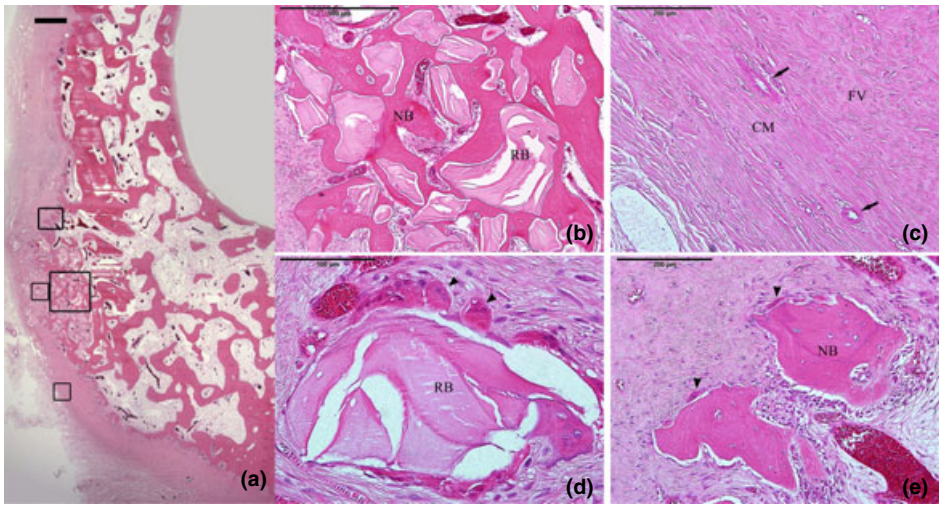

Fig. 3. (a) Low-magnification view of the histologic presentation in the BHC group (HE staining; scale bar = 1 mm). (b) The NB was in direct contact with the RB (HE staining; scale bar = 500 μm). (c) The intact CM containing many capillaries could be clearly observed (HE staining; scale bar = 200 μm). (d, e) Osteoclast-like cells (arrowhead) were observed on the surface of the NB and RB [HE staining; scale bar = 100 μm (d) and 200 μm (e)]. (BHC, bovine hydroxyapatite incorporated with collagen matrix; CM, collagen membrane; FV, fibrovascular connective tissue; NB, newly formed bone; RB, residual bone substitute).

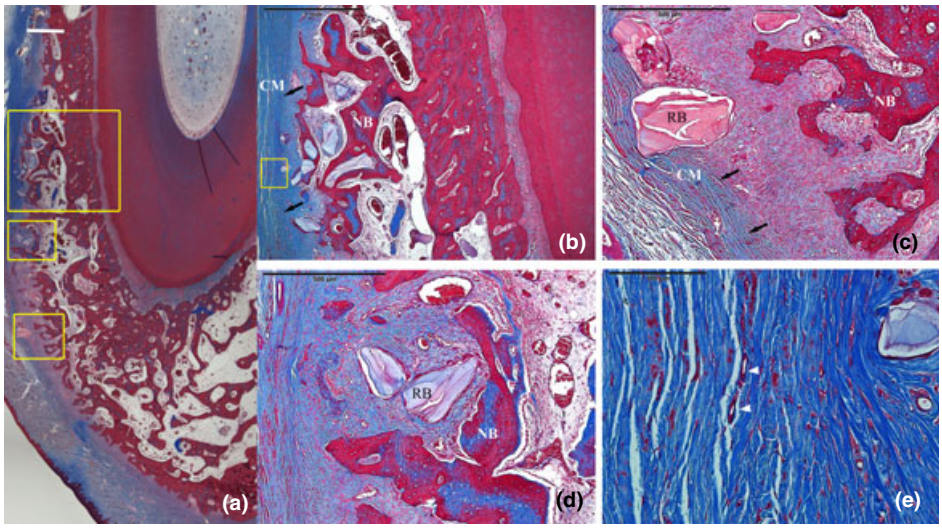

Fig. 4. (a) Low-magnification views of the histologic presentation in the CM group (MTC staining; scale bar = 1 mm). (b) and (c) Finger- or column-like projections of NB were observed underneath the CM (arrow). The NB was directed toward or lying alongside the CM [MTC staining; scale bar = 1 mm (b) and 500 μm (c)]. (d) NB was not integrated with the RB, instead existing as islands of bone within the FV (MTC staining; scale bar = 500 μm). (e) Newly formed vascular tissues (arrowhead) were observed passing through the CM (MTC staining; scale bar = 100 μm). (CM, collagen membrane; MTC, Masson's trichrome; NB, newly formed bone; RB, residual bone substitute).

rhBMP-2-loaded CM were commonly observed underneath the CM in the CM group, and the NB seemed to be closer to the CM than in the BHC group (Fig. 4b,c), with newly formed vascular tissues penetrating the rhBMP-2-loaded CM (Fig. 4e). While most of the RB was in direct contact with the NB in the BHC group, it was clustered into large or small areas and embedded within FV in the CM group. The projections and islands of NB were frequently observed in the spaces between the RB clusters in most specimens of the CM group (Figs 4d and 5).

Histometric analysis

The results of the histometric analysis are summarized in Tables 1 and 2. The mean values of AA and NBA were slightly higher in the CM group than in the BHC group; however, the difference between the two groups was not statistically significant. The AH and NBH also appeared to be greater in the CM group than in the BHC group; however, the difference did not reach statistical significance. Each histologic component that occupied AAs showed similar distribution ratio between both groups (Fig. 6). The percentage of NBH of the original BHC height did not differ significantly between the BHC group (41.8%) and the CM group (46.8%). The BRC ratio was significantly lower for the CM group than for the BHC group (43.4 ± 17.7 and 10.1 ± 5.4, respectively; $P = 0.043$; Table 1).

Discussion

This study compared effects of CM and BHC as a carrier for rhBMP-2 on bone regeneration in GBR at lateral onlay graft model. Most of the previous studies on growth factors and their clinical use have focused on the biomaterials for the scaffold as a carrier of the growth factors. However, there may be several difficulties when using the conventional carrier system and growth factors in certain clinical cases, especially for non-contained defects. It is not easy to manage and apply rhBMP-2-loaded bone substitute to non-contained defects without dissipation. In addition, a certain amount of rhBMP-2-loaded bone substitute could be lost throughout surgical process, which will adversely influence the outcome of GBR. Therefore, this study was based on the hypothesis that loading of rhBMP-2 at another GBR component (CM) instead of bone substitutes might result in comparable bone regeneration to the conventional loading at the bone substitutes while minimizing the above-mentioned issues.

The present study used the onlay graft model, which is a challenging defect model, to compare the differential effects of rhBMP-2 according to selective loading site, rather than a well-defined inlay defect model in which the healing source is provided by the surrounding walls of the defect. The present experimental model mimicked horizontal augmentation in the narrow alveolar bone ridge. Even though the vertical augmentation model has been used more frequently as a challenging defect model, horizontal onlay graft can be used more frequently in clinical situations, such as dehiscence or fenestration defect on narrow alveolar ridge. In addition, volumetric stability of regenerated tissues in experimental sites can be increased, because compressive force to augmentation by primary intention can be decreased in the horizontal augmentation model compared with the vertical one.

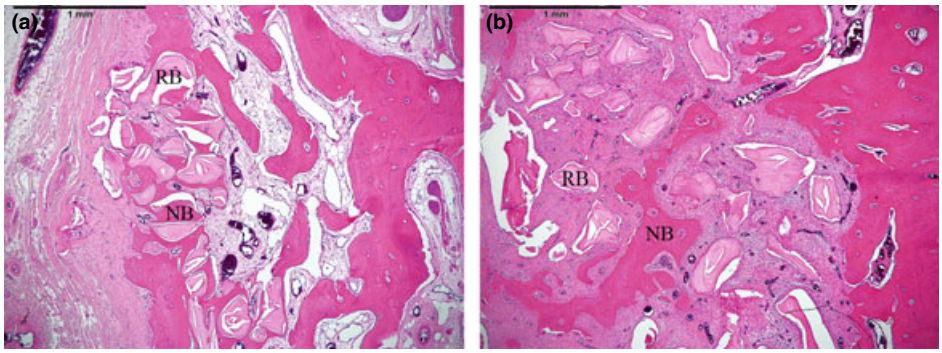

Fig. 5. High-magnification views of the histologic presentation in other specimens of the BHC groups (a) and CM (b). (a) Most of the RBs were in direct contact with the NB (HE staining; scale bar = 1 mm). (b) Numerous clusters of RB particles were embedded within the FV. NB that was not in direct contact with the RB was observed in the spaces between the RB clusters (HE staining; scale bar = 1 mm). (BHC, bovine hydroxyapatite incorporated with collagen matrix; CM, collagen membrane; FV, fibrovascular connective tissue; NB, newly formed bone; RB, residual bone substitute).

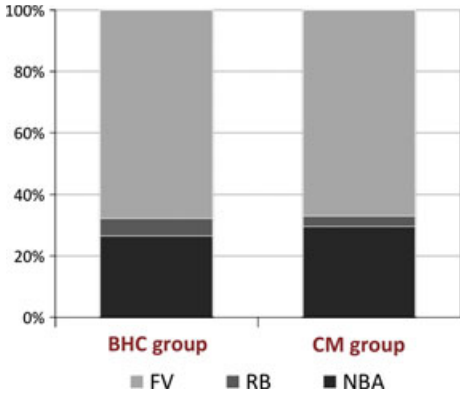

Fig. 6. Proportions of individual composition in augmented area by histomorphometric analysis (NBA, newly formed bone; RB, residual bone substitute; FV, fibrovascular connective tissue).

Table 1. **Results of histomorphometric measurements (mean ± standard deviation) and confidence interval**

			95% confidence interval		
	BHC group	CM group	Lower	Upper	Estimate
AA (mm^2)	20.21 ± 10.24	25.95 ± 12.71	−2.240	23.050	2.685
AH (mm)	2.17 ± 0.76	2.27 ± 0.94	−1.070	0.850	0.240
NBH (mm)	1.67 ± 0.65	1.87 ± 0.66	−0.290	0.710	0.210
BRC (%)	43.4 ± 17.7	10.1 ± 5.4*	−55.500	−12.200	−33.850

AA, augmented area; AH, augmented height; NBH, newly formed bone height; BRC, Bone-residual bone substitute contact ratio.
*Statistically significant difference compared with BHC group (P = 0.043).

Table 2. **Histomorphometric measurements of individual compositions in augmented area (mean ± standard deviation in mm^2) and confidence interval**

			95% confidence interval		
	BHC group	CM group	Lower	Upper	Estimate
NBA	5.32 ± 1.97	7.77 ± 3.26	0.797	4.081	2.415
RB	1.17 ± 1.43	0.85 ± 1.12	−0.819	0.172	−0.324
FV	13.68 ± 6.97	17.43 ± 9.49	−5.123	19.888	1.641

NBA, newly formed bone; RB, residual bone substitute; FV, fibrovascular connective tissue.

Both types of carriers used in the present study were based on porcine collagen, in which the previous studies demonstrated burst release in the initial phase and gradual release in the late phase on loading of rhBMP-2 (Hollinger et al. 1998; Friess et al. 1999). Hänseler et al. (2012) also reported similar results when rhBMP-2 was incorporated with BH, in which two different release profiles were recorded in the initial and late phases. Approximately16 ± 7% rhBMP-2 was detected in rhBMP-2-soaked BH after 15 days. The biological activity of rhBMP-2 tested in the cell culture medium BH exhibited high activity (25 ± 8% activity of the positive control) after 15 days. Although further study is needed for the exact release profile of BHC, we could expect similar release profiles from both biomaterials; CM and BHC. Therefore, this study used the same collagen-based barrier membrane (CM) and bone substitutes (BHC) to focus on the evaluation of loading site of rhBMP-2 among GBR components. However, the use of collagen-based bone substitutes (BHC) resulted in the decrease in augmented volume and height at 8 weeks after surgery compared with the original implanted volume. This may be caused by lack of volumetric stability due to resorption of collagen composition in BHC at the initial healing phase.

In the present results, both the BHC and CM groups exhibited substantial NB that was well integrated with native bone. The NBA and NBH appeared to be slightly better in the CM group than in the BHC group, but the difference was not statistically significant. The percentage of NBA within the AA also appeared to be greater in the CM group than in the BHC group, but again the difference was not significant. The percentage of NBA obtained in the CM group (29.5%) was comparable with that obtained in previous studies in a dehiscence defect augmented with rhBMP-2-coated BH (37%) (Jung et al. 2003) and in an onlay defect augmented with 10 μg/ml rhBMP-2-loaded bone substitute (26.3 ± 8.5%) (Jung et al. 2008). The NBH of 1.87 ± 0.66 mm (46.8%) obtained in our CM group is consistent with that of 1.89 ± 0.55 mm (47.3%) found in a recent evaluation of vertical augmentation using rhBMP-2-loaded BHC in rabbit calvaria (Kim et al. 2010). Therefore, it was confirmed that the application protocol of loading CM with rhBMP-2 could be an effective treatment option for lateral onlay grafting.

Collagen membranes are convenient for soaking up rhBMP-2 and are easily manipulated at surgical sites in the clinical context (Jung et al. 2003). In the present context, the use of CM as a carrier for rhBMP-2 could simplify surgical procedures while simultaneously obtaining comparable NB. Furthermore, it would help to reduce the amount of rhBMP-2 required to load particulate or block-type bone substitutes. Minimizing the dose of rhBMP-2 would provide both cost and safety benefits.

The necessity of an osteoinductive agent is being increasingly emphasized in defects with a restricted healing source. In our previous study (Jung et al. 2013), we evaluated lateral onlay grafts treated using BHC with/without CM using the same protocol and conditions as in the present study and demonstrated that NB was observed only at the experimental sites that were covered with a CM. Both sites of the BHC and CM groups in the present study exhibited significantly

greater NB than those that received the same biomaterials without rhBMP-2 in the previous study (3.26 ± 1.04 mm^2). These findings are highly consistent with those of Wikesjö et al. (2003c, 2004), who reported that rhBMP-2 increased the regenerative efficacy of GBR at onlay grafts.

The present results also showed peculiar characteristics in bone regeneration according to the loading protocol. Most of the RB particles were in direct contact with the NB in BHC group, which was consistent with a previous report that rhBMP-2-coated BH enhanced the NB-to-graft-materials contact ratio (Jung et al. 2003). Whereas, the RB particles that were not loaded with rhBMP-2 in CM group did not show increased BRC, instead being clustered and embedded within the dense FV. Finger- or column-like projections of NB were observed in the spaces between the clusters of RB particles, communicating with each other or the native bone. These are similar results to the previous studies investigating surgical implantation of BHC with no rhBMP-2 loading in one-wall intrabony defects (Stavropoulos & Wikesjö 2010; Jung et al. 2011) or using the same experimental protocol in the same model but without loading of rhBMP-2 (Jung et al. 2013). The histometric analysis indicated that the BRC ratio was significantly lower in the CM group than in the BHC group. However, NB appeared to be directed toward or lying alongside the rhBMP-2-loaded CM. RhBMP-2 loaded at CM might enhance chemotactic and mitogenic potential from periosteum-like connective tissue and promote new bone formation around CM. Schwarz et al. (2008b) demonstrated that enhanced bone formation was observed just below the barrier membrane covering a dehiscence defect. They attributed this formation of peripheral bone underneath the barrier membrane to the early transmembranous angiogenesis of the barrier membrane, permitting the migration of preosteoblastic cells from the periosteum to the submembranous tissue via newly formed vascular tissues (Schwarz et al. 2008b, 2009). In the present results, the rhBMP-2-loaded CMs persisted after 8 weeks of healing and prevented ingrowth of the epithelium and the outer connective tissue (Moses et al. 2008). Thus, enhanced transmembranous angiogenesis by the rhBMP-2 and the barrier function of the CM may have affected the histologic morphogenesis of NB and RB.

Although the present study is a controlled animal experimental study, there were some limitations including small number of experimental subjects and exclusion of a control group without rhBMP-2. These were for decreasing unnecessary sacrifice of experimental animals, by using the same and controlled experimental conditions (both groups in the same area of the same animal and similar age/weight of animals) for non-inferiority test. In addition, results from our previous study (Jung et al. 2013) were employed in this study to avoid repeated experiments and overuse of animals. Therefore, despite these ethical issues, further studies including a large-scale experiment using another extensive augmentation model are needed to confirm the present results.

Within the limitations of the present study, it can be concluded that rhBMP-2 loaded CM performed lateral onlay grafts as effectively as rhBMP-2-loaded BHC while showing less bone-residual bone substitute contact ratio in dogs. The loading of CMs with rhBMP-2 might therefore be a recommendable treatment option for facilitating lateral onlay graft combined with rhBMP-2.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References

Caplanis, N., Sigurdsson, T.J., Rohrer, M.D. & Wikesjo, U.M. (1997) Effect of allogeneic, freeze-dried, demineralized bone matrix on guided bone regeneration in supra-alveolar peri-implant defects in dogs. *International Journal of Oral and Maxillofacial Implants* **12**: 634–642.

Chiapasco, M. & Zaniboni, M. (2009) Clinical outcomes of GBR procedures to correct peri-implant dehiscences and fenestrations: a systematic review. *Clinical Oral Implants Research* **20**: 113–123.

Cho, E.H., Park, J.C., Cha, J.K., Kim, Y.T., Jung, U.W., Kim, C.S., Choi, S.H. & Kim, C.K. (2011) Dimensional change of the healed periosteum on surgically created defects. *Journal of Periodontal & Implant Science* **41**: 176–184.

De Boever, A.L. & De Boever, J.A. (2005) Guided bone regeneration around non-submerged implants in narrow alveolar ridges: a prospective long-term clinical study. *Clinical Oral Implants Research* **16**: 549–556.

Friess, W., Uludag, H., Foskett, S., Biron, R. & Sargeant, C. (1999) Characterization of absorbable collagen sponges as recombinant human bone morphogenetic protein-2 carriers. *International Journal of Pharmaceutics* **185**: 51–60.

Hämmerle, C.H. & Jung, R.E. (2003) Bone augmentation by means of barrier membranes. *Periodontology 2000* **33**: 36–53.

Hämmerle, C.H., Jung, R.E. & Feloutzis, A. (2002) A systematic review of the survival of implants in bone sites augmented with barrier membranes (guided bone regeneration) in partially edentulous patients. *Journal of Clinical Periodontology* **29**: 226–231; discussion 232–223.

Hämmerle, C.H. & Karring, T. (1998) Guided bone regeneration at oral implant sites. *Periodontology 2000* **17**: 151–175.

Hänseler, P., Jung, U.W., Jung, R.E., Choi, K.H., Cho, K.S., Hämmerle, C.H. & Weber, F.E. (2012) Analysis of hydrolyzable polyethylene glycol hydrogels and deproteinized bone mineral as delivery systems for glycosylated and non-glycosylated bone morphogenetic protein-2. *Acta Biomaterialia* **8**: 116–123.

Hayashi, O., Katsube, Y., Hirose, M., Ohgushi, H. & Ito, H. (2008) Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. *Calcified Tissue International* **82**: 238–247.

Hollinger, J.O., Uludag, H. & Winn, S.R. (1998) Sustained release emphasizing recombinant human bone morphogenetic protein-2. *Advanced Drug Delivery Reviews* **31**: 303–318.

Huang, Y.H., Polimeni, G., Qahash, M. & Wikesjo, U.M. (2008) Bone morphogenetic proteins and osseointegration: current knowledge – future possibilities. *Periodontology 2000* **47**: 206–223.

Jovanovic, S.A., Hunt, D.R., Bernard, G.W., Spiekermann, H., Wozney, J.M. & Wikesjo, U.M. (2007) Bone reconstruction following implantation of rhBMP-2 and guided bone regeneration in canine alveolar ridge defects. *Clinical Oral Implants Research* **18**: 224–230.

Jung, R.E., Glauser, R., Scharer, P., Hämmerle, C.H., Sailer, H.F. & Weber, F.E. (2003) Effect of rhBMP-2 on guided bone regeneration in humans. *Clinical Oral Implants Research* **14**: 556–568.

Jung, U.W., Lee, J.S., Lee, G., Lee, I.K., Hwang, J.W., Kim, M.S., Choi, S.H. & Chai, J.K. (2013) Role of collagen membrane in lateral onlay grafting with bovine hydroxyapatite incorporated with collagen matrix in dogs. *Journal of Periodontal & Implant Science* **43**: 64–71.

Jung, U.W., Lee, J.S., Park, W.Y., Cha, J.K., Hwang, J.W., Park, J.C., Kim, C.S., Cho, K.S., Chai, J.K. & Choi, S.H. (2011) Periodontal regenerative effect of a bovine hydroxyapatite/collagen block in one-wall intrabony defects in dogs: a histometric analysis. *Journal of Periodontal & Implant Science* **41**: 285–292.

Jung, R.E., Weber, F.E., Thoma, D.S., Ehrbar, M., Cochran, D.L. & Hämmerle, C.H. (2008) Bone morphogenetic protein-2 enhances bone formation when delivered by a synthetic matrix

containing hydroxyapatite/tricalciumphosphate. *Clinical Oral Implants Research* **19**: 188–195.

Kim, S.J., Shin, H.S. & Shin, S.W. (2010) Effect of bone block graft with rhBMP-2 on vertical bone augmentation. *International Journal of Oral and Maxillofacial Surgery* **39**: 883–888.

McAllister, B.S. & Haghighat, K. (2007) Bone augmentation techniques. *Journal of Periodontology* **78**: 377–396.

Misch, C.M. (2011) Bone augmentation of the atrophic posterior mandible for dental implants using rhBMP-2 and titanium mesh: clinical technique and early results. *The International Journal of Periodontics & Restorative Dentistry* **31**: 581–589.

Moses, O., Vitrial, D., Aboodi, G., Sculean, A., Tal, H., Kozlovsky, A., Artzi, Z., Weinreb, M. & Nemcovsky, C.E. (2008) Biodegradation of three different collagen membranes in the rat calvarium: a comparative study. *Journal of Periodontology* **79**: 905–911.

Schwarz, F., Ferrari, D., Sager, M., Herten, M., Hartig, B. & Becker, J. (2009) Guided bone regeneration using rhGDF-5- and rhBMP-2-coated natural bone mineral in rat calvarial defects. *Clinical Oral Implants Research* **20**: 1219–1230.

Schwarz, F., Rothamel, D., Herten, M., Ferrari, D., Sager, M. & Becker, J. (2008a) Lateral ridge augmentation using particulated or block bone substitutes biocoated with rhGDF-5 and rhBMP-2: an immunohistochemical study in dogs. *Clinical Oral Implants Research* **19**: 642–652.

Schwarz, F., Rothamel, D., Herten, M., Sager, M. & Becker, J. (2006) Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat. *Clinical Oral Implants Research* **17**: 403–409.

Schwarz, F., Rothamel, D., Herten, M., Wustefeld, M., Sager, M., Ferrari, D. & Becker, J. (2008b) Immunohistochemical characterization of guided bone regeneration at a dehiscence-type defect using different barrier membranes: an experimental study in dogs. *Clinical Oral Implants Research* **19**: 402–415.

Sigurdsson, T.J., Nguyen, S. & Wikesjö, U.M. (2001) Alveolar ridge augmentation with rhBMP-2 and bone-to-implant contact in induced bone. *The International Journal of Periodontics & Restorative Dentistry* **21**: 461–473.

Simion, M., Fontana, F., Rasperini, G. & Maiorana, C. (2007) Vertical ridge augmentation by expanded-polytetrafluoroethylene membrane and a combination of intraoral autogenous bone graft and deproteinized anorganic bovine bone (Bio Oss). *Clinical Oral Implants Research* **18**: 620–629.

Stavropoulos, A. & Wikesjö, U.M. (2010) Influence of defect dimensions on periodontal wound healing/regeneration in intrabony defects following implantation of a bovine bone biomaterial and provisions for guided tissue regeneration: an experimental study in the dog. *Journal of Clinical Periodontology* **37**: 534–543.

Tatakis, D.N., Koh, A., Jin, L., Wozney, J.M., Rohrer, M.D. & Wikesjö, U.M. (2002) Peri-implant bone regeneration using recombinant human bone morphogenetic protein-2 in a canine model: a dose-response study. *Journal of Periodontal Research* **37**: 93–100.

Wikesjö, U.M., Lim, W.H., Thomson, R.C., Cook, A.D., Wozney, J.M. & Hardwick, W.R. (2003a) Periodontal repair in dogs: evaluation of a bioabsorbable space-providing macroporous membrane with recombinant human bone morphogenetic protein-2. *Journal of Periodontology* **74**: 635–647.

Wikesjö, U.M., Qahash, M., Thomson, R.C., Cook, A.D., Rohrer, M.D., Wozney, J.M. & Hardwick, W.R. (2004) rhBMP-2 significantly enhances guided bone regeneration. *Clinical Oral Implants Research* **15**: 194–204.

Wikesjö, U.M., Sorensen, R.G., Kinoshita, A. & Wozney, J.M. (2002) RhBMP-2/alphaBSM induces significant vertical alveolar ridge augmentation and dental implant osseointegration. *Clinical Implant Dentistry and Related Research* **4**: 174–182.

Wikesjö, U.M., Xiropaidis, A.V., Thomson, R.C., Cook, A.D., Selvig, K.A. & Hardwick, W.R. (2003c) Periodontal repair in dogs: rhBMP-2 significantly enhances bone formation under provisions for guided tissue regeneration. *Journal of Clinical Periodontology* **30**: 705–714.

Zhu, S.J., Choi, B.H., Huh, J.Y., Jung, J.H., Kim, B.Y. & Lee, S.H. (2006) A comparative qualitative histological analysis of tissue-engineered bone using bone marrow mesenchymal stem cells, alveolar bone cells, and periosteal cells. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **101**: 164–169.