CLINICAL ORAL IMPLANTS RESEARCH

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Regeneration of rabbit calvarial defects using biphasic calcium phosphate and a strontium hydroxyapatite-containing collagen membrane

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Abstract

Objectives: Biphasic calcium phosphate (BCP) composed of 10% hydroxyapatite (HA) and 90% beta-tricalcium phosphate has been developed. Recently, a strontium hydroxyapatite-containing collagen membrane (Sr) was shown to stimulate early bone formation in rat calvarial defects at 4 weeks postoperatively, as compared with a cross-linked collagen membrane, for guided bone regeneration (GBR). The objective of this study was to evaluate these novel biomaterials for GBR in relation to a non-cross-linked collagen membrane (BG) and deproteinized bovine bone mineral (BO).

Materials and methods: Twenty New Zealand rabbits were used in this study. Four defects of 7 mm in diameter were created in each rabbit, and three of the defects were treated with BG/BO, Sr/BO, and Sr/BCP. Ten rabbits were sacrificed at 12 and 24 weeks, respectively. Histological and histomorphometric analyses were conducted. Volumetric densities of mineralized new bone (MNB), bone marrow (BM), residual grafting material (RG), and non-mineralized connective tissue (NCT) were determined for each group.

Results: After 12 weeks, Sr/BCP yielded more MNB than BG/BO and Sr/BO with no significant difference among the three groups. After 24 weeks, however, Sr/BCP demonstrated significantly more MNB than BG/BO and Sr/BO. Both after 12 and 24 weeks, Sr/BCP showed significantly less RG than BG/BO and Sr/BO. There was a significant increase in MNB in Sr/BCP from 12 to 24 weeks.

Conclusions: In defects grafted with BCP, more MNB was formed while less RG remained, than in defects grafted with BO. The Sr membrane was as effective as BG when comparing Sr/BO with BG/BO.

Guided bone regeneration (GBR) is a well-documented and routinely used method for regeneration of osseous defects prior to or simultaneous with implant placement. In the GBR technique, a barrier membrane is placed over an osseous defect to exclude epithelial and connective tissue proliferation into the defect. This allows for the creation and maintenance of a space where osteogenic cells derived from the host bone can repopulate and subsequently enhance bone regeneration (Dahlin et al. 1988; Hämmerle & Jung 2003).

Non-cross-linked resorbable collagen membranes are widely used due to their several advantages including biocompatiblity (Schlegel et al. 1997; Rothamel et al. 2012), hemostatic ability (Schwarz et al. 2008; Wang et al. 2012), chemostasis for gingival fibroblasts and osteoblasts (Locci et al. 1997; Alpar et al. 2000), and semi-permeability enabling nutrient transfer (Wang et al. 2012). Recently, Hao et al. (2015) explored the use of a strontium hydroxyapatite-containing collagen membrane (Sr) for bone regeneration of rat calvarial defects at 4 and 8 weeks postoperatively. It was demonstrated that the Sr membrane stimulated bone regeneration and maturation in the early phase of healing (week 4), compared with a cross-linked collagen membrane. Although there was no significant difference in the amount of newly formed bone between the Sr membrane and the cross-linked collagen membrane at 8 weeks, the later phase of healing with the

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Sr membrane has yet to be investigated and compared with the currently accepted gold standard, non-cross-linked collagen membrane (BG, Bio-Gide®; Geistlich Pharma AG, Wolhusen, Switzerland).

Deproteinized bovine bone mineral (DBBM) has been shown to have excellent biocompatibility and osteoconductivity (Berglundh & Lindhe 1997; Tapety et al. 2004; Orsini et al. 2007). Recent clinical studies, where DBBM and a non-cross-linked collagen membrane were used for GBR simultaneously with implant placement, demonstrated favorable clinical, esthetic, and radiological outcomes after 6 years (Buser et al. 2013) and a high survival rate of 91.9% after 12–14 years (Jung et al. 2013). Thus, the combination of DBBM and a non-cross-linked collagen membrane is predictable and can be considered as the gold standard for GBR.

Synthetic calcium phosphates, including hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), and biphasic calcium phosphates (BCPs), have been shown to be biocompatible and osteoconductive, and they have been used for orthopedic and dental applications (LeGeros et al. 2003). HA is considered as slowly resorbable or non-resorbable, thus providing the stable volume, but it remains in the body in the long term (LeGeros et al. 2003). On the other hand, β-TCP resorbs at a faster rate but has greater bioactivity in that during dissolution, it releases calcium and phosphate ions that stimulate new bone formation (LeGeros et al. 2003). By combining HA and β-TCP with varying HA/β-TCP ratios, BCPs have the advantage of controlled resorbability and bioactivity (LeGeros et al. 2003). Recently, a BCP with the HA/β-TCP ratio of 10/90 (BCP) has been developed. This novel BCP has to be evaluated and compared with the currently accepted gold standard, DBBM (BO, Bio-Oss®; Geistlich Pharma AG), in an animal model before being used in clinical trials.

Therefore, the objective of this study was to evaluate the novel BCP and the Sr membrane for GBR in rabbit calvarial defects, in relation to BO and BG at 12 and 24 weeks postoperatively. Due to the limited number of defects that can be created in each rabbit, two combinations of a barrier membrane and a bone substitute, Sr/BCP and Sr/BO, were compared to BG/BO.

Material and methods

Animals

The study protocol was approved by the Committee on the Use of Live Animals for Teaching and Research, the University of Hong Kong (CULATR 3058-13). Twenty New Zealand rabbits (age 6 months, weight 3.5–4.0 kg) were used in the study. They were checked for their health status and maintained by a veterinarian at the Laboratory Animal Unit of the Faculty of Medicine, the University of Hong Kong. Ten rabbits were sacrificed at week 12 and week 24 after surgery, respectively (Fig. 1).

Biomaterials

Biomaterials used in this study are listed in Table 1. The following three membranes were used:

- Non-cross-linked porcine dermis-derived collagen membrane (BG)
- Heat cross-linked strontium hydroxyapatite-containing collagen membrane (Sr)
- Pericardium-derived collagen membrane (PC) (Institute Straumann AG, Basel, Switzerland)

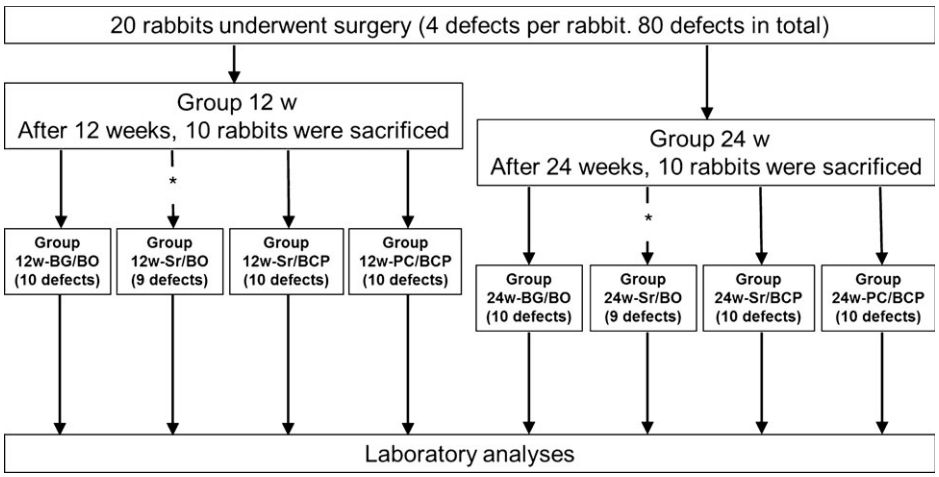

Fig. 1. Schematic illustration of the study design. *One defect in the Sr/BO group is left unfilled and uncovered to serve as a negative control at each healing period. Hence, the number of defects of Sr/BO is nine.

The following two bone substitutes were used:

- DBBM with a particle size of 250–1000 μm (BO)
- BCP composed of 10% HA and 90% β-TCP (BCP 10/90) with a particle size of 250–1000 μm (BCP) (Institute Straumann AG)

Preparation of strontium hydroxyapatite-containing membrane

Strontium hydroxyapatite (SrHA) membranes were prepared according to the method described by Hao et al. (2015). Briefly, strontium chloride ($SrCl_2$) and disodium hydrogen phosphate (Na_2HPO_4) were mixed in distilled water. The resulting precipitate, SrHA ($Sr_{10}[PO_4]_6[OH]_2$), was collected and mixed with a 5% (wt) gelatin solution to produce the compound with a 20 mg/ml SrHA concentration. The compound was dried at 40°C for 8 h and subsequently was allowed to swell in 4°C distilled water for 1 h. After that, the compound was put in a deep freezer at 70°C for 10 m and then freeze-dried overnight. The resulting membranes were cross-linked by heat at 160°C for 6 h.

Surgical protocol

The surgical protocol described by Jung et al. (2006) and Yip et al. (2015) was adopted. All surgical procedures were performed under general anesthesia. Before the operation, the animals received a subcutaneous injection of analgesic (Temgesic®, buprenorphine 0.05 mg/kg). They were anesthetized intramuscularly with Ketamine® (35 mg/kg), Acepromazine® (1 mg/kg), and Xylazine® (5 mg/kg). The dorsal part of the scalp covering the calvaria was shaved and disinfected with iodophor gauze. A midline dermo-periosteal incision was made in the area of the nasoincisal suture. Subsequently, a full thickness flap was elevated to expose the parietal bone. While the skin and periosteum were retracted, four defects were marked in each calvarium with a trephine drill (external diameter: 7 mm), under profuse saline irrigation. The bone in the defect outlined by the trephine drill was carefully removed with a piezoelectric surgery tip (Implant Centre 2; ACTEON Group, MERIGNAC Cedex, France) without damaging the dura mater and the lateral wall of the defect.

Guided bone regeneration

A total of 80 defects were created in 20 rabbits (Fig. 1). Four defects in each rabbit received different combinations of a barrier

Table 1. **Biomaterials used in this study**

Biomaterials	Abbreviation	Origin	Trade name	Manufacturer
Non-cross-linked collagen membrane	BG	Porcine dermis	Bio-Gide®	Geistlich Pharma AG, Wolhusen, Switzerland
Heat cross-linked strontium hydroxyapatite-containing collagen membrane	Sr	Animal (gelatin)	N.A.	N.A.
Experimental collagen membrane	PC	Pericardium of unknown animal	N.A.	Institute Straumann AG, Basel, Switzerland
Deproteinized bovine bone mineral	BO	Bovine	Bio-Oss®	Geistlich Pharma AG, Wolhusen, Switzerland
Biphasic calcium phosphate with the HA/β-TCP ratio of 10/90	BCP	Synthetic	N.A.	Institute Straumann AG, Basel, Switzerland

N.A., Not applicable.

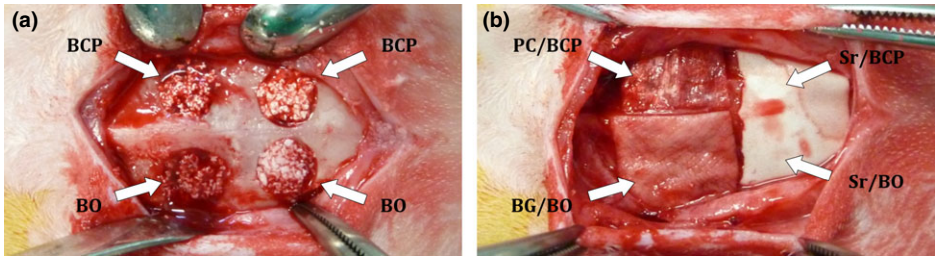

Fig. 2. The procedures of GBR. (a) The bone substitutes were placed in the defects. (b) The defects were covered with the membranes.

membrane and a bone substitute for GBR as follows (Fig. 2):

- Non-cross-linked collagen membrane and DBBM (BG/BO)
- Strontium hydroxyapatite-containing collagen membrane and DBBM (Sr/BO)
- Strontium hydroxyapatite-containing collagen membrane and BCP 10/90 (Sr/BCP)
- Pericardium-derived collagen membrane and BCP 10/90 (PC/BCP)

Thus, the four defects were grafted with either BO or BCP (Fig. 2a). The three membranes were carefully placed on the filled defects, according to the above combinations, in such a way that each membrane did not overlap the other; the membranes were then secured on the peripheral bone of the defect (Fig. 2b). For each observation period, one defect per 10 rabbits was left unfilled and uncovered, to serve as a negative control (Fig. 1). The periosteum was sutured with 4-0 vicryl (polyglactin 910) resorbable sutures (Coated VICRYL® Plus Antibacterial Suture; Ethicon, Edinburgh, UK). The skin was sutured with nylon monofilament sutures (ETHILON® Nylon Suture; Ethicon).

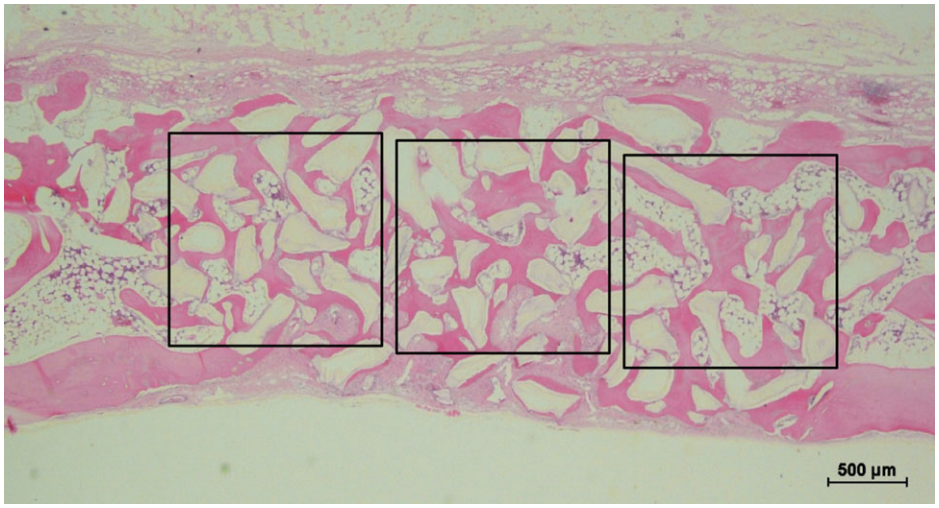

Fig. 3. Three areas used for histomorphometric analysis (original magnification ×50).

Postoperative care

An antibiotic (enrofloxacin 5–10 mg/kg, every 12 h for 3 days, subcutaneously) and analgesics (buprenorphine 0.05 mg/kg, every 8–12 h for 3 days, and thereafter, meloxicam 0.2 mg/kg every 24 h for 14 days, subcutaneously) were given postoperatively. The nylon monofilament sutures were removed 7 days after surgery. Their health status, weight, and food consumption were monitored and maintained until the time of sacrifice.

Sacrifice

After a healing period of 12 and 24 weeks, 10 rabbits were euthanized with an overdose of pentobarbital (150 mg/kg) at each time point (Fig. 1).

Preparation of histological specimens

The calvaria containing the entire grafted area were retrieved in blocks, perfused with 10% paraformaldehyde solution for immediate fixation, and subsequently decalcified with 12.5% ethylenediaminetetraacetic acid (EDTA) acid for 8 weeks. They were dehydrated in ethanol, embedded in paraffin, and sectioned in the frontal plane through the middle of the defects. Serial sections of 4 μm thickness were cut and then stained with hematoxylin–eosin (HE).

Histological and histomorphometric analyses

From each defect, one section was sampled for histological and histomorphometric analyses. For image acquisition, the defect was observed under a light microscope (Nikon® Eclipse VL100POL; Nikon, Tokyo, Japan) incorporated into a digital video camera (Nikon® Digital Sight DS-Ri1; Nikon). Digital images were evaluated using specialized software (NIS-Elements AR 3.00, Nikon® Laboratory Imaging software; Nikon).

Each defect was equally divided into three square areas: left, middle, and right (Fig. 3). The center part of each area was analyzed under higher magnification.

Histomorphometric measurements were performed by two trained and calibrated examiners, according to the methods described by Schroeder & Münzel-Pedrazzoli (1973). A lattice comprising 10 × 10 grid points was superimposed over the acquired image in the middle part of each area examined (Fig. 4).

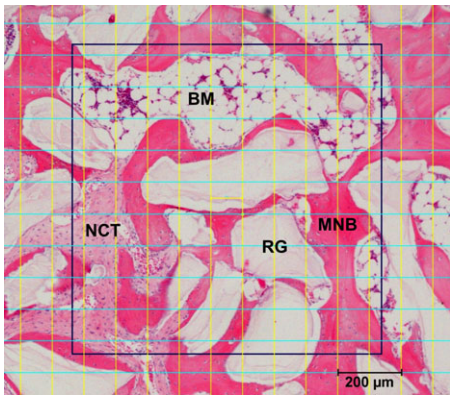

Fig. 4. Histomorphometric analysis was performed using 10 × 10 grid points within the navy square. MNB, mineralized new bone; BM, bone marrow; RG, residual grafting material; NCT, non-mineralized connective tissue (original magnification ×130).

The following tissue components were determined in each section:

- Mineralized new bone (MNB)
- Bone marrow (BM)
- Residual grafting material (RG)
- Non-mineralized connective tissue (NCT)

The volumetric density of different tissue components from the three square areas in each defect was averaged. Subsequently, mean volumetric density of each tissue component was calculated from the 10 animals for each group at the two healing periods (12 and 24 weeks).

Intra- and inter-examiner reliability was assessed by re-measurement of 20 slides 1 week after the first examination.

Statistical analysis

The data of BG/BO, Sr/BO, and Sr/BCP were analyzed to evaluate the clinical potential of BCP and the Sr membrane in relation to BO and BG. The results of PC/BCP will be reported in a separate report.

The data were analyzed using IBM SPSS software version 20 (IBM Corporation, Armonk, NY, USA). The intra- and inter-examiner reliability was evaluated with intra-class correlation coefficient (ICC). Kolmogorov–Smirnov test was used to check the normal distribution of the data of each tissue component. When the normal distribution was verified, one-way analysis of variance (ANOVA) was used to assess the difference in the volumetric density of each tissue component between the three GBR groups at each healing period. When one-way ANOVA revealed a significant difference, Tukey's HSD test was used for multiple comparisons. When the data did not follow a normal distribution, Kruskal–Wallis test was used to assess the difference between the three groups. Additionally, to compare the volumetric density of each tissue component between the two healing periods (12 and 24 weeks) within the same GBR group, two-sample *t*-test was used. When the data did not follow a normal distribution, Mann–Whitney *U*-test was used. The level of significance was set at 0.05.

Results

Postoperative findings

All the rabbits recovered uneventfully without any sign of wound dehiscence, membrane exposure, or infection.

Descriptive histology

Negative control after 12 and 24 weeks

For each healing period, one defect per 10 rabbits served as a negative control where neither a barrier membrane nor a bone substitute was applied. After 12 weeks, the defect was occupied with newly formed bone (Fig. 5a). The entrance of the defect was bridged by trabecular bone. No volumetric shrinkage of the defect was observed.

In contrast, after 24 weeks, the defect presented a substantial volumetric shrinkage (Fig. 5b). New bone formation was noted from the margin toward the center of the defect; however, fibrous tissue was present in the defect and the bone bridging was incomplete.

Overview of GBR specimens after 12 and 24 weeks

Both at 12 and 24 weeks, one specimen of Sr/BCP was excluded, respectively, because the damage of the dura mater was suspected. In all the other specimens, the original volume of the defects was preserved regardless of the tissue components within the defects. In other words, BO or BCP particles of various size and configuration were distributed within the defects, and they were mainly surrounded by mineralized new bone and bone marrow, and occasionally by non-mineralized connective tissue.

After 12 weeks, in 9 of 10 BG/BO specimens, 9 of 9 Sr/BO specimens, and 9 of 9 Sr/BCP specimens, new bone formation reached up to the entrance of the defect, without any collapse of bony contour from the defect margin (Fig. 5c); occasionally, there was a minor depression at the center of the defect. In the remaining one BG/BO specimen, new bone formation was observed up to half to two-thirds of the defect height from the dura side, while the remaining area close to the periosteum was occupied by non-mineralized

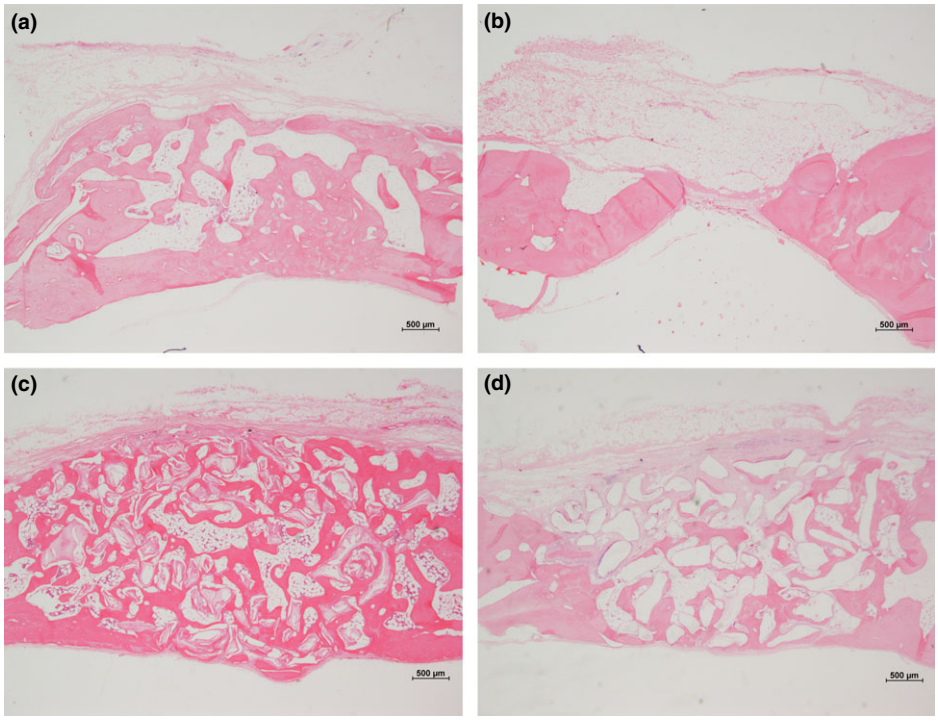

Fig. 5. Histological appearance of the negative control defect after 12 weeks (a) and 24 weeks (b), and complete bone regeneration (c: Sr/BO) and incomplete bone regeneration (d: BG/BO) of the defects after 12 weeks (original magnification ×50).

connective tissue (Fig. 5d). Despite the decreased amount of new bone formation in this specimen, the defect did not present a volumetric shrinkage due to the presence of BO particles encapsulated in the non-mineralized connective tissue.

After 24 weeks, in 9 of 10 specimens of BG/BO, 8 of 9 specimens of Sr/BO, and 9 of 9 specimens of Sr/BCP, new bone formation reached up to the entrance of the defect, without any collapse of bony contour from the defect margin or occasionally with minor depression of the bony contour. In the remaining two specimens, one BG/BO and the other Sr/BO, loose fibrous tissue and dense connective tissue occupied the center of the defect where BO particles were encapsulated by such non-mineralized connective tissues, and new bone formation was observed at the lateral aspects of the defect.

Defect surface of GBR specimens after 12 and 24 weeks

Fig. 6a,b shows the defect area directly below the periosteum of BG/BO and Sr/BO specimens after 12 weeks, respectively. No remarkable difference could be noted between the three GBR groups, both after 12 and 24 weeks. In the majority of the sites, new bone formation was observed at the surface of the grafted defect. Occasionally, BO was encapsulated in the non-mineralized connective tissue in the superficial layer of the defect. Both after 12 and 24 weeks, BG and Sr membranes could not be detected at the surface area of the defects. In general, no foreign body reactions were observed at the surface area of the defect in any GBR group.

Within the defect after 12 weeks

In all the GBR groups, graft particles of various size and configuration were identified (Fig. 7a–c). They were mainly surrounded by mineralized new bone, and to a lesser extent, by bone marrow and non-mineralized connective tissue. The histological images of BG/BO and Sr/BO were similar after 12 weeks. Frequently, a blank space was observed between BO particles and the surrounding bone (Fig. 7a,b); this was considered to be the artifact due to the histological preparation (Berglundh & Lindhe 1997). On the other hand, in Sr/BCP specimens, such artifacts could not be observed, suggesting more intimate contact between BCP particles and the newly formed bone (Fig. 7c). Furthermore, new bone formation was also observed within BCP particles. No inflammatory cells were present around BO and BCP particles.

Within the defect after 24 weeks

The histological images of BG/BO and Sr/BO were similar after 24 weeks (Fig. 7d,e). The blank space between BO particles and the

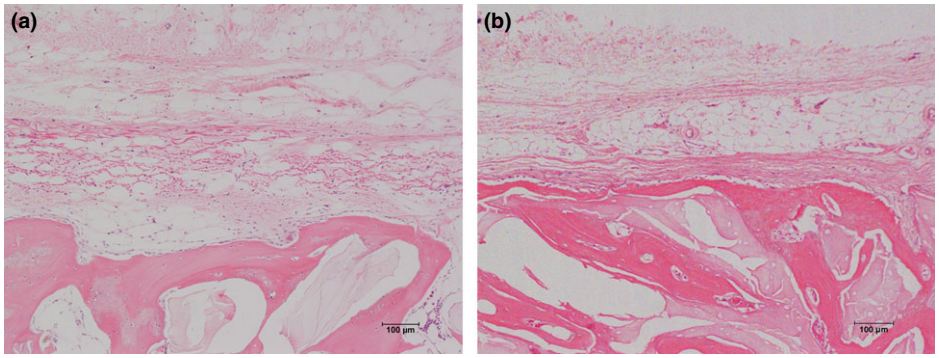

Fig. 6. Histological appearance of the defect area directly below the periosteum after 12 weeks (a: BG/BO; b: Sr/BO) (original magnification ×260).

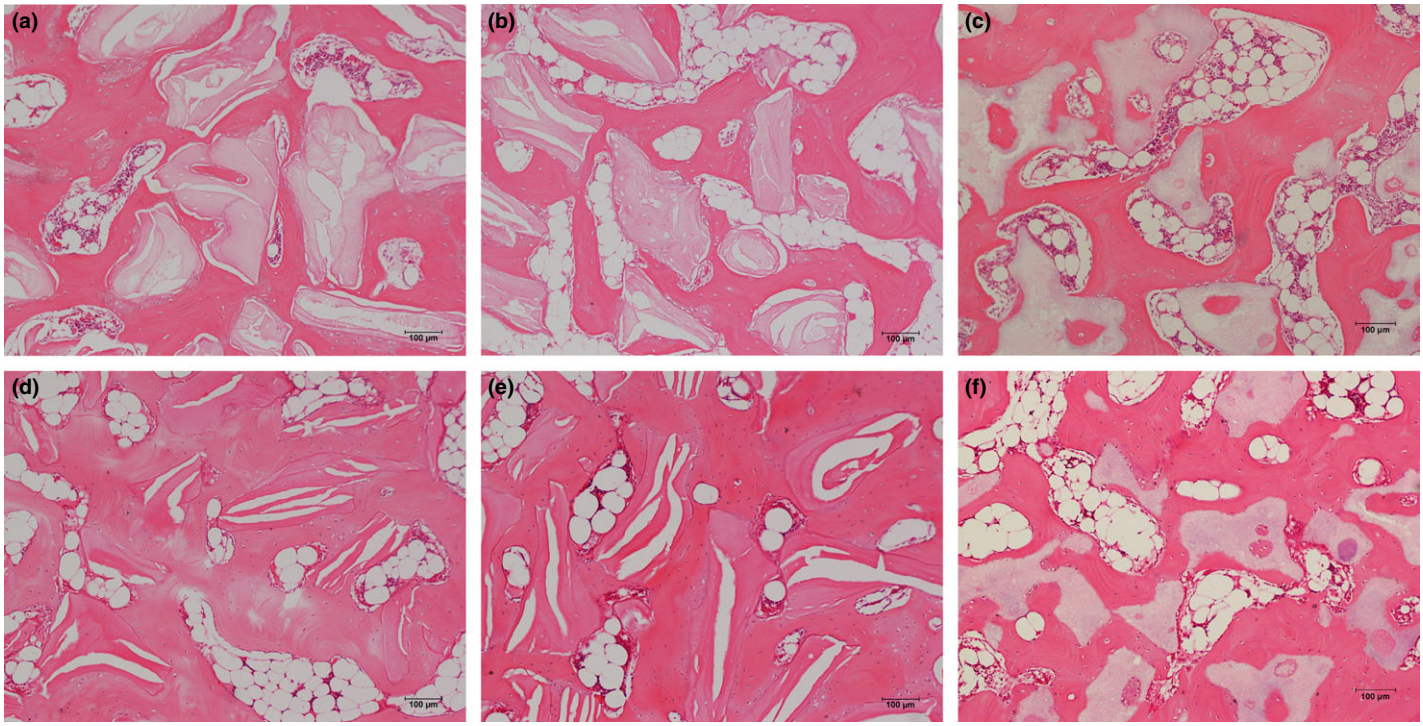

Fig. 7. Histological appearance of specimens at 12 weeks (a: BG/BO; b: Sr/BO; and c: Sr/BCP) and at 24 weeks (d: BG/BO; e: Sr/BO; and f: Sr/BCP) (original magnification ×260).

surrounding bone appeared to be less, if present, as compared with the 12-week specimens, indicating that the BO particles became better integrated into the surrounding bone. For the Sr/BCP specimens, the size of BCP particles appeared to be smaller and more fragmented, compared to the 12-week specimens (Fig. 7f). New bone formation could be observed within BCP particles.

Intra- and inter-examiner reliability for histomorphometric analysis

The intra-class correlation coefficients (ICC) to determine the intra- and inter-examiner reliability were >0.95 for each tissue component. This meant that the histomorphometric measurements were highly reproducible.

Histomorphometric analyses

Comparison among the groups at 12 weeks

The mean percentage of each tissue component in the three GBR groups after 12 weeks and the summary of the statistical analysis are summarized in Table 2 and Fig. 8. Sr/BCP demonstrated the greatest amount of mineralized new bone (40.93%), followed by Sr/BO (36.18%) and BG/BO (35.07%). However, one-way ANOVA revealed no significant difference in the mean percentages of mineralized new bone among the groups ($P = 0.106$).

Regarding the bone marrow, one-way ANOVA supplemented with Tukey's HSD test showed significantly higher amount in Sr/BCP (33.19%) than in Sr/BO (21.07%) and BG/BO (16.83%) ($P = 0.042$ and $P = 0.004$, respectively). Also, the mean percentage of residual grafting material in Sr/BCP (24.37%) was significantly lower than that in BG/BO (42.83%) and Sr/BO (37.78%) ($P < 0.001$, respectively).

The mean percentage of non-mineralized connective tissue in each group was about 1–5%. The data did not follow a normal distribution, and Kruskal–Wallis test revealed no significant difference between the three groups ($P = 0.153$).

Comparison among the groups at 24 weeks

The mean percentage of each tissue in the three GBR groups after 24 weeks and the summary of the statistical analysis are summarized in Table 3 and Fig. 9. Sr/BCP demonstrated significantly higher mean percentage of mineralized new bone (50.15%) than Sr/BO (40.67%) and BG/BO (39.20%) ($P = 0.013$ and $P = 0.003$, respectively).

Sr/BCP had greater amount of bone marrow (27.07%) than BG/BO (19.17%) and Sr/BO (19.81%). However, no significant difference was observed among the three groups ($P = 0.251$).

The mean percentage of residual grafting material in Sr/BCP (20.81%) was significantly lower than that in BG/BO (38.40%) and Sr/BO (35.59%) ($P < 0.001$, respectively).

The mean percentage of non-mineralized connective tissue in each group was about 2–4%. The data did not follow a normal distribution, and Kruskal–Wallis test revealed no significant difference between the three groups ($P = 0.685$).

Intra-group comparison between 12 and 24 weeks

The mean percentage of each tissue component, comparing the 12-week and 24-week groups within the same GBR group, is summarized in Fig. 10. In general, the proportion

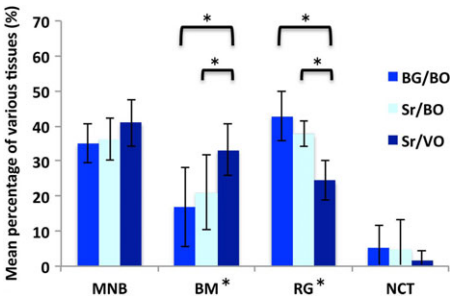

Fig 8. Mean percentages of analyzed tissue components in BG/BO, Sr/BO, and Sr/BCP groups after 12 weeks. *Statistically significant ($P < 0.05$).

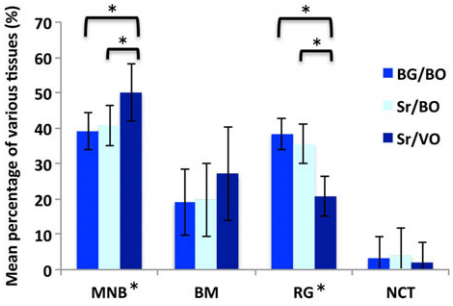

Fig 9. Mean percentages of analyzed tissue components in BG/BO, Sr/BO, and Sr/BCP groups after 24 weeks. *Statistically significant ($P < 0.05$).

Table 2. **Percentages of analyzed tissue components in each group after 12 weeks (mean ± SD) using one-way ANOVA with multiple comparisons using Tukey's HSD test**

	BG/BO ($n = 10$) (%)	Sr/BO ($n = 9$) (%)	Sr/BCP ($n = 9$) (%)	*P*-value	Significant multiple comparisons with *P*-value
Mineralized new bone (MNB)	35.07 ± 5.53	36.18 ± 5.89	40.93 ± 6.70	0.106	
Bone marrow (BM)	16.83 ± 11.26	21.07 ± 10.73	33.19 ± 7.27	0.005	Sr/BCP > BG/BO, $P = 0.004$; Sr/BCP > Sr/BO, $P = 0.042$
Residual graft (RG)	42.83 ± 7.01	37.78 ± 3.60	24.37 ± 5.63	<0.001	Sr/BCP < BG/BO, $P < 0.001$; Sr/BCP < Sr/BO, $P < 0.001$
Non-mineralized connective tissue (NCT)	5.27 ± 6.20	4.96 ± 8.20	1.52 ± 2.71	0.153*	

*Kruskal–Wallis test (due to non-normality).

Table 3. **Percentages of analyzed tissue components in each group after 24 weeks (mean ± SD) using one-way ANOVA with multiple comparisons using Tukey's HSD test**

	BG/BO ($n = 10$) (%)	Sr/BO ($n = 9$) (%)	Sr/BCP ($n = 9$) (%)	*P*-value	Significant multiple comparisons with *P*-value
Mineralized new bone (MNB)	39.20 ± 5.29	40.67 ± 5.79	50.15 ± 8.17	0.002	Sr/BCP > BG/BO, $P = 0.003$; Sr/BCP > Sr/BO, $P = 0.013$
Bone marrow (BM)	19.17 ± 9.43	19.81 ± 10.38	27.07 ± 13.09	0.251	
Residual graft (RG)	38.40 ± 4.50	35.59 ± 5.54	20.81 ± 5.62	<0.001	Sr/BCP < BG/BO, $P < 0.001$; Sr/BCP < Sr/BO, $P < 0.001$
Non-mineralized connective tissue (NCT)	3.23 ± 6.17	3.93 ± 8.00	1.96 ± 5.77	0.685*	

*Kruskal–Wallis test (due to non-normality).

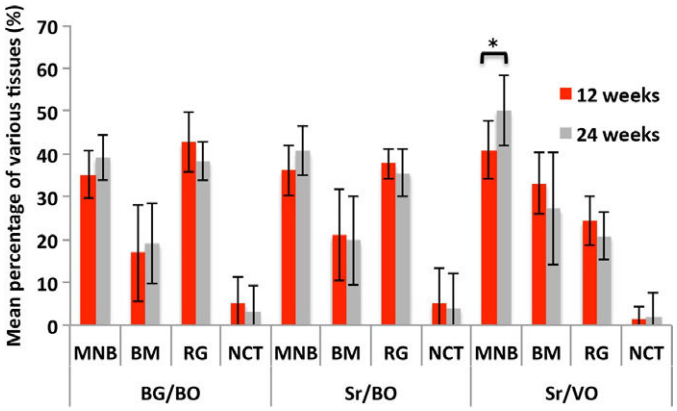

Fig. 10. Intra-group comparison of the mean percentages of the analyzed tissues between 12 and 24 weeks. *Statistically significant ($P = 0.019$).

of mineralized new bone increased, whereas that of residual graft decreased in each group.

Both in BG/BO and Sr/BO, there were no significant differences in the mean percentage of any tissue component from 12 to 24 weeks ($P > 0.05$). In Sr/BCP, mineralized new bone significantly increased from 12 (40.93%) to 24 weeks (50.15%) ($P = 0.019$). There were no significant differences in the mean percentages of the other tissue components in Sr/BCP ($P > 0.05$). In all the GBR groups, the data of non-mineralized connective tissue did not follow a normal distribution, and Mann–Whitney *U*-tests revealed no significant difference from 12 to 24 weeks ($P > 0.05$).

Discussion

The present study demonstrated that in defects grafted with BCP (Sr/BCP), greater amount of mineralized new bone was formed while smaller amount of residual grafting material was noted, compared with defects grafted with BO (Sr/BO and BG/BO). In addition, the Sr membrane was as effective as BG when comparing Sr/BO with BG/BO.

A calvarial defect heals by means of intramembranous ossification, thus resembling the healing of the maxillofacial bone (Schmitz & Hollinger 1986). The rabbit calvaria was large enough to create four defects, which enabled multiple comparisons of the biomaterials tested (Jung et al. 2006; Yip et al. 2015). In the present study, four combinations of a barrier membrane and a bone substitute were applied in each rabbit. The results of PC/BCP are not of our primary interest and will be reported elsewhere. Thus, the three combinations, namely BG/BO, Sr/BO, and Sr/BCP, were compared with regard to the defect healing. Due to the lack of the BG/BCP group, the effect of each biomaterial cannot be directly compared. Yet, the results of the three combinations provide useful information to evaluate the performance of BCP and Sr, by comparing Sr/BO and Sr/BCP in relation to BG/BO.

One defect served as a negative control, where neither a bone grafting material nor a barrier membrane was placed, for each healing period. In the 12-week specimen, the defect was filled with mineralized new bone and bone marrow without a volumetric shrinkage, which was unexpected but may be explained by the concept of "critical size defects." A critical size defect is defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal (Schmitz & Hollinger 1986). For the rabbit calvaria, the critical size defect was reported to be 15 mm (Frame 1980). In the present study, the defect size was 7 mm in diameter, and the periosteum was repositioned and sutured with resorbable sutures after the GBR procedure. For defects smaller than the critical size, the periosteal sheath may act as a supporting scaffold above the defect, and also proper closure of the periosteum may contribute to the prevention of soft tissue ingrowth into the defect (Schmitz & Hollinger 1986). In addition, it was reported that the osteoprogenitor cells present in the inner osteogenic layer of the periosteum play an important role in osteogenesis (Reynders et al. 1999; Ueno et al. 2003). These may explain the bone regeneration in the defect without a volumetric shrinkage in the 12-week specimen. On the contrary, in the 24-week specimen, the defect presented a substantial volumetric shrinkage with the incomplete bony bridging. Thus, despite the limited number of the negative control defect, it could be postulated that bone regeneration in an unfilled defect without a barrier membrane did not occur predictably. This is reinforced by the fact that all the GBR-treated sites, adding 12- and 24-week specimens, preserved the original volume of the defects, and also 53 of 56 (95%) GBR-treated defects demonstrated new bone formation reaching up to the entrance of the defect. Thus, the combinations of the membrane and the bone substitute used in the present study predictably recovered the original contour of the defects and successfully regenerated bone in the defects. This is in agreement with the findings of a previous study that the combination of BG and BO maintained the volume of the original defect in rat calvaria (Donos et al. 2004). In the present study, adding 12- and 24-week specimens, 18 of 20 BG/BO specimens (90%), 17 of 18 Sr/BO specimens (94%), and 18 of 18 Sr/BCP specimens (100%) successfully regenerated bone in the defects. Thus, the Sr membrane was as effective as BG for GBR, when used in combination with BO or BCP.

For histomorphometric analyses, at 12 weeks, greater amount of MNB was observed in Sr/BCP (40.93%) than in Sr/BO (36.18%) and BG/BO (35.17%), although the difference between the three groups was not significant. At 24 weeks, however, Sr/BCP showed significantly greater amount of MNB (50.15%) than Sr/BO (40.67%) and BG/BO (39.20%). With regard to RG, both after 12 and 24 weeks, the percentage of RG in Sr/BCP was significantly smaller than that of BG/BO and Sr/BO. These observations can be explained by the findings of a previous study by Jensen et al. (2009). They compared the amount of MNB and RG in defects grafted with either autograft, DBBM (BO), or BCP with HA/β-TCP ratio of 80/20, 60/40, or 20/80 (BCP 80/20, 60/40, or 20/80) in the mandible of minipigs. Autograft yielded significantly more MNB and less RG than BO at week 4, 13, and 26. In contrast, comparing autograft with BCP 20/80, there were no significant differences in the amounts of MNB and RG at week 4, 13, 26, and 52. They suggested that the amount of MNB and degraded bone substitute was inversely proportional to HA/β-TCP ratios of BCPs and concluded that autograft and BCP 20/80 had a higher resorption rate. In the present study, the HA/β-TCP ratio of BCP was further reduced to 10/90; thus, it can be expected to perform similarly to autograft. This explains more MNB and less RG in Sr/BCP than in Sr/BO and BG/BO both at 12 and 24 weeks. In Sr/BCP, there was no significant difference in the amount of RG from 12 to 24 weeks, which is

in agreement with the study by Yip et al. (2015). They used the same rabbit calvarial model, and the defect healing was evaluated using BO, BCP 10/90 and 60/40 at 12 and 24 weeks, postoperatively. BCP 10/90 tested had a particle size of 500–1000 μm, in contrast to BCP with a particle size of 250–1000 μm in the present study. Yet, there was no significant difference in the amount of RG in BCP 10/90 from 12 (34.83%) to 24 weeks (35.33%). These values are larger than the amounts of RG in Sr/BCP at 12 weeks (24.57%) and 24 weeks (20.81%), as demonstrated in the present study. In terms of MNB, there were no significant differences between BO and BCP 10/90 both at 12 and 24 weeks in the study by Yip et al., whereas in our study, Sr/BCP showed significantly more MNB than Sr/BO and BG/BO at 24 weeks. In the study by Yip et al., there was no significant difference in the amount of MNB in BCP 10/90 from 12 to 24 weeks, in contrast to BCP showing a significant increase from 12 to 24 weeks in the present study. The reasons for these inconsistencies are unclear but may be related to the difference in the particle size and other morphological properties between the two BCPs. A previous study found a difference in the resorption rate between three β-TCP bone substitutes with the same chemistry but with different macro- and micro-design, which may be due to their particle geometry, pore structures, porosity, and interconnectivity (Walsh et al. 2008).

In terms of BM, Sr/BCP yielded significantly greater amount (33.19%) than Sr/BO (21.07%) and BG/BO (16.83%) at 12 weeks. At 24 weeks, Sr/BCP (27.07%) yielded greater amount of BM than Sr/BO (19.81%) and BG/BO (19.17%) although the difference between the three groups was not significant. The amounts of NCT were small (about 1–5%) and comparable among the three groups both at 12 and 24 weeks. Bone marrow is a living soft tissue that contains important components such as vessels, osteoblasts, osteoclasts, and mesenchymal stem cells. A human histological study investigated the osteogenic and osteoclastic potential around β-TCP particles used for sinus augmentation after 6 months (Zerbo et al. 2005). It was shown that most soft tissue cells around β-TCP particles were osteogenic and that few tartrate-resistant acid phosphatase (TRAP)-positive cells were identified. The authors indicated that further bone deposition would occur in due time in the grafted area while β-TCP particles and soft tissue components are replaced by mineralized new bone. Thus, BCP with its high β-TCP content, together with the fact that Sr/BCP had greater amount of BM than Sr/BO and BG/BO, may be beneficial in regenerating more MNB in the long term than BO.

Dahlin et al. (2014) evaluated the bone regeneration in the mandibular defect of minipigs, using BO and BCP 10/90 with a particle size of 500–1000 μm. Interestingly, the amount of RG in BCP 10/90 was significantly higher than that in BO both at 3 and 8 weeks. Yet, BCP 10/90 showed significantly more MNB than BO both at 3 and 8 weeks; thus, the authors mentioned that BCP 10/90 demonstrated great potential. Previous animal studies showed that β-TCP was almost completely or completely resorbed after the short (8 weeks) or long healing period (24 months), respectively (Artzi et al. 2004; Jensen et al. 2007). As 90% β-TCP in BCP theoretically contributes to its high resorbability and bioactivity, it is of clear interest to investigate to what extent the inclusion of 10% HA affects the resorbability of BCP as well as the volume stability of the peri-implant defect grafted with BCP in the long term. Further studies are required.

Histologically, BO particles appeared to be better integrated to the newly formed bone after 24 weeks. This was based on the fact that the blank space (histological artifact), which was frequently observed between BO particles and MNB after 12 weeks, was observed less frequently after 24 weeks in both BG/BO and Sr/BO. This is in agreement with the findings of a previous human study where biopsies were obtained after sinus augmentation at two time points for comparison (Valentini et al. 2000). In contrast to BO, the blank space could not be observed between BCP particles and MNB both at 12 and 24 weeks, suggesting more intimate bone to graft contact in BCP than in BO. Furthermore, MNB was also observed within BCP particles. It was reported that in a BCP, the resorbable phase of β-TCP released calcium and phosphate ions while being dissolved. These ions became saturated in the local body fluid and consequently precipitated bone-like apatite crystals onto the stable phase of HA (LeGeros et al. 2003).

In the study by Hao et al. (2015) using rat calvaria, significantly greater amount of MNB and faster bone maturation were observed after 4 weeks with the Sr membrane than with a cross-linked collagen membrane. However, after 8 weeks, there was no significant difference in the amount of MNB between the Sr membrane and the cross-linked collagen membrane. It may be speculated that the beneficial effect of the Sr membrane diminished as it was degraded. This is in agreement with the results of the present study although the experimental conditions are not identical. In the present study, the Sr membrane was completely resorbed without any sign of foreign body reaction, and no significant differences in the amounts of the analyzed tissue components were found between BG/BO and Sr/BO both after 12 and 24 weeks. Yet, further studies are required to assess whether the Sr membrane has any clinical advantage over BG in terms of new bone formation and maturation in the early phase.

The results of the present animal study cannot be directly applicable to humans. In addition, the defect created was self-contained, whereas in real clinical situation, the morphology of the peri-implant defect would be more severe in terms of angiogenesis and osteogenesis. Furthermore, the surgically created acute-type defect has a tendency to heal spontaneously, due to its high osteogenic potential (Hanisch et al. 2003). Thus, the regenerative potential of the biomaterials used in the present study may be lower in real clinical situation. Nevertheless, this study could be useful to estimate the clinical performance of BCP and the Sr membrane.

In conclusion, in defects grafted with BCP, significantly more mineralized new bone was observed while significantly less grafting material remained, than in defects grafted with BO. The Sr membrane was as effective as BG in terms of defect healing when supported by the bone substitute.

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