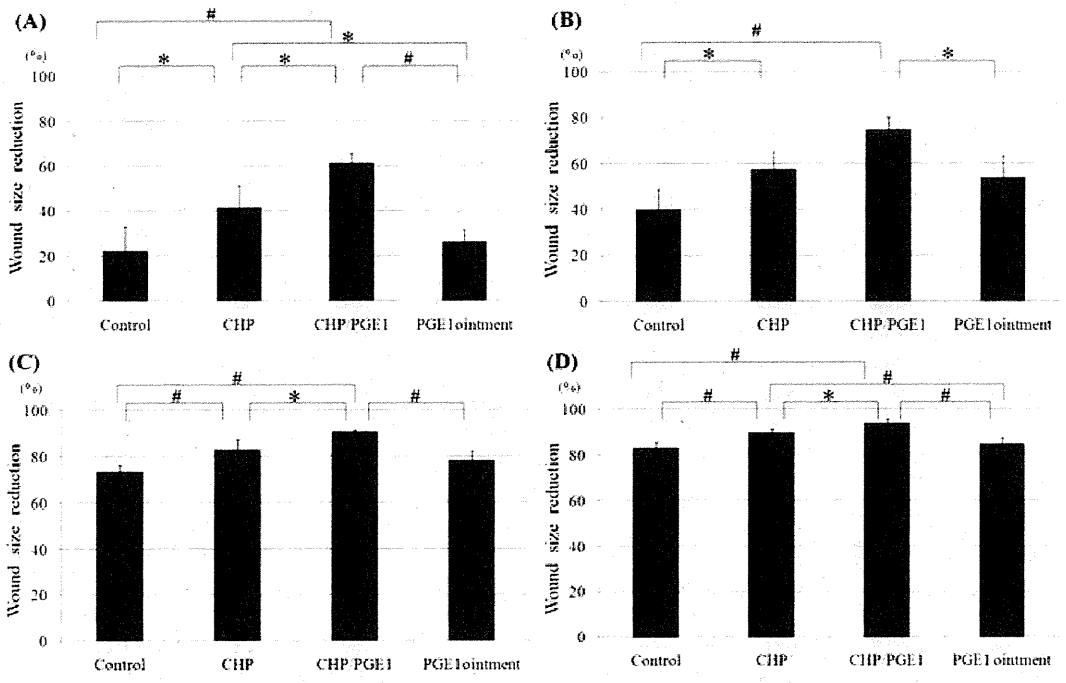

Figure 4. The rate of wound size reduction was calculated at (A) 5 days, (B) 7 days, (C) 10 days, and (D) 14 days. Data are presented as mean ± SEM. $^{\#}p < 0.01$, $^{*}p < 0.05$.

were a few inflammatory cells in the dermis. In the CHP/PGE1 group [Figure 7(C)], skin defect was completely regenerated and covered with new mature epithelium. Inflammatory cells were almost absent. In this group, the epidermal rete pegs were well developed and mature collagen was also present in the dermis. The skin appendage was regenerated. In the PGE1 ointment group [Figure 7(D)], the wound was not completely covered with epithelium and inflammatory cells were still seen in the dermis.

DISCUSSION

The ideal wound dressing should have the following properties: providing a moisturized wound-healing environment, being removable without additional trauma to the wound, protecting the wound from bacterial infection, controlling permeability of oxygen and carbon dioxide, being biocompatible, absorbing wound exudates, and finally promoting tissue reconstruction processes.[14] As wound dressing materials synthetic materials, such as polyurethane,[15–17] polyvinyl alcohol,[18]

polyhydroxyethylmethacrylate,[19] and copolymers,[20] as well as biological materials such as bovine collagen,[21–23] chitin,[24] and alginate[25–27] have been investigated. Compared with these materials, the uniqueness of CHP nanogels is to trap hydrophobic molecules including proteins and nucleic acids. Therefore, this material has been used as polymeric nanocarriers in cancer chemotherapy for protein delivery and for artificial vaccine.[6–8] We speculate that the character of CHP nanogels would also be ideal as a drug-carrier to stimulate wound healing. Interestingly, in this study CHP nanogels application alone accelerated the wound size reduction and epithelialization compared with not only untreatment (control) but also PGE1 ointment treatment, suggesting that CHP nanogels are effective for wound healing.

There would be several reasons for the favorable effects of CHP nanogels in wound healing. Our previous studies have demonstrated high biocompatibility of CHP nanogels,[9] which is one of the required properties for wound dressing. Since CHP nanogels contain more than 90% of water,[5] the water in the gels would be exchanged for wound exudates and keep the wound in wet condition continuously. Wound exudates, which are produced in the process of wound healing, keep the wound in a moist environment and smoothen the migration of the epidermal cells. Moreover, wound exudates contain various growth factors, such as epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor, which favorably stimulate wound healing. Thus, it is likely that CHP nanogels trap wound exudates together with various growth factors, which will gradually release from the gels, and that the gels also keep the wound in the moist environment.

PGE1 stimulates the proliferation and cytokine production of epidermal keratinocytes and dermal fibroblasts and it also dilates blood vessels improving peripheral circulation.[11] Thus, it has been expected that when PGE1 is

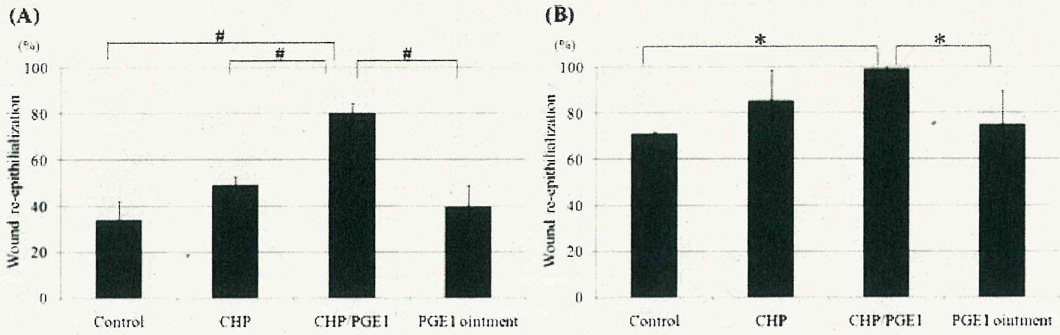

Figure 5. The rate of wound re-epithilialization was calculated at (A) 7 days and (B) 14 days. Data are presented as mean ± SEM. $^{\#}p < 0.01$, $^{*}p < 0.05$.

applied to the wound, PGE1 will directly act on keratinocytes, fibroblasts, and blood vessel cells consequently promoting wound healing. PGE1 ointment has been clinically used to treat skin ulcers and wounds; however, two time applications per day are recommended in the clinical use. In this study, when CHP/PGE1 was applied once to the wound after the wound preparation, the wound was completely covered with new epithelium and inflammatory cells were very few at 14 days. The healing of the wound was extremely well in CHP/PGE1 group compared with the other groups including PGE1 ointment group. The pharmacodynamic characteristics of PGE1 are its poor stability and very brief half-life of about 30 s because PGE1 is promptly oxidized and inactivated.[28] It is likely that CHP nanogels could trap PGE1 inside and protect PGE1 from oxidation and gradually release PGE1 for a prolonged period.

This study is the first report of the CHP nanogels application for wound healing. The present experimental results suggest that CHP nanogels in combination with PGE1 can promote wound healing, which confirms the efficiency of CHP nanogels-based drug delivery system. Using CHP nanogels as PGE1 carrier in the near future, we will be able to decrease the application frequency of PGE1 to the wounds, which is beneficial to both patients and nursing staff.

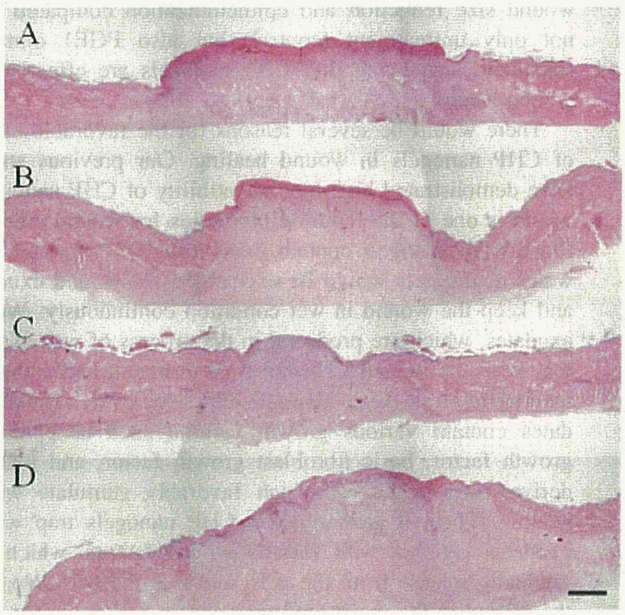

Figure 6. Histological examination of wound tissue stained with hematoxylin and eosin at 7 days: (A) control group; (B) CHP group; (C) CHP/PGE1 group; (D) PGE1 ointment group. Black line: 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

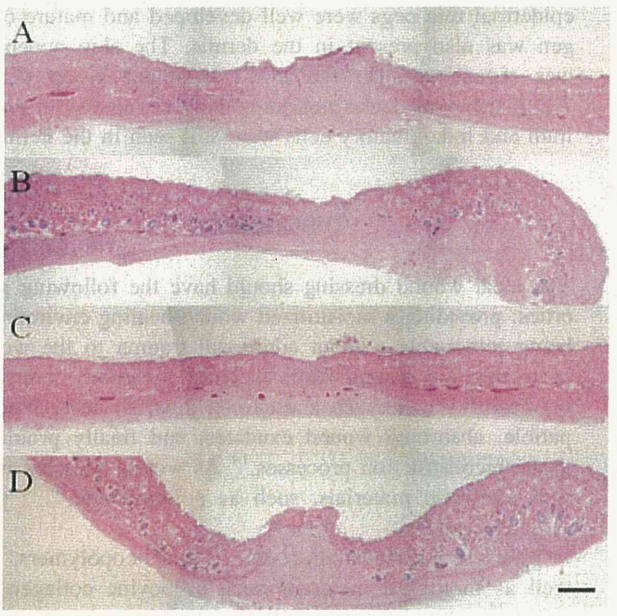

Figure 7. Histological examination of wound tissue stained with hematoxylin and eosin at 14 days: (A) control group; (B) CHP group; (C) CHP/PGE1 group; (D) PGE1 ointment group. Black line: 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSION

CHP-nanogels are effective for the controlled release of PGE1 and create a moist environment. CHP nanogels in combination with PGE1 can promote wound healing. The clinical application of CHP/PGE1 to full-thickness dermal defect would be promising.

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RESEARCH ARTICLE

Exploitation of a novel polysaccharide nanogel cross-linking membrane for guided bone regeneration (GBR)

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Abstract

Cholesterol-bearing pullulan (CHP) nanogel is a synthetic degradable biomaterial for drug delivery with high biocompatibility. Guided bone regeneration (GBR) is a bone augmentation technique in which a membrane is used to create and keep a secluded regenerative space. The purpose of the present study was to evaluate the effects of the novel CHP nanogel membrane in GBR. Thirty-six adult Wistar rats were used and bilaterally symmetrical full-thickness parietal bone defects of 5 mm diameter were created with a bone trephine burr. Each defect was covered with the collagen membrane or the CHP nanogel membrane or untreated without any membrane. The animals were sacrificed at 2, 4 and 8 weeks and analysed radiologically and histologically. Furthermore, after incubating human serum with CHP nanogel or collagen, the amount of PDGF in the serum was measured using ELISA. New bone formation in terms of bone volume was higher in the nanogel group than in the control or collagen groups at 2 and 4 weeks. At 8 weeks, both membrane groups showed higher bone volumes than the control group. Notably, the newly-formed bone in the bone defect in the nanogel group was uniform and histologically indistinguishable from the original bone, whereas in the collagen group the new bone showed an irregular structure that was completely different from the original bone. After incubating with CHP nanogel, the amount of PDGF in the serum decreased significantly. CHP nanogel GBR membrane favourably stimulated bone regeneration, in which a unique characteristic of CHP nanogel, the storage of endogenous growth factors, was likely implicated. Copyright © 2011 John Wiley & Sons, Ltd.

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Keywords **guided bone regeneration; biomaterials; bone regeneration; acryloyl group-modified cholesterol-bearing pullulan (CHPOA); morphometric analysis; radiology; membrane; animal experiments**

1. Introduction

Dental rehabilitation of totally or partially edentulous patients with dental implants has become a routine treatment modality in recent decades, with reliable long-term results (Albrektsson *et al.*, 1981, 1986; van Steenberghe, 1989; van Steenberghe *et al.*, 1990; Lindquist *et al.*, 1996; Buser *et al.*, 1997; Arvidson *et al.*, 1998; Lekholm *et al.*, 1999; Brocard *et al.*, 2000; Leonhardt *et al.*, 2002). However, local conditions of the alveolar ridge, bone volume and bone quality, affect the long-term prognosis. Guided tissue regeneration (GTR) (Nyman *et al.*, 1982, 1990) was originally developed for the treatment of periodontal defects and then the same concept was later applied to bone regeneration, which is called guided bone regeneration (GBR) (Dahlin *et al.*, 1989). Vertical and horizontal bone augmentation with GBR has been

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applied to improve alveolar ridge deformities (Buser *et al.*, 1993; Simion *et al.*, 1996; Urban *et al.*, 2009). In GBR procedures, the barrier membrane keeps the bone regeneration space and it also prevents the invasion of fibrous connective tissue, resulting in new bone formation under the membrane.

Several different membranes, including non-resorbable and resorbable membranes, have been developed and clinically used in GBR. Resorbable membranes have been generally preferred because the second surgery for its removal was unnecessary, rendering the procedure less invasive to patients. Biomaterials such as collagen or copolylactic/glycolic acid (PLGA) have been developed as degradable GBR membranes. Since collagen is an animal-derived material, the risk of unknown infection is undeniable and an unfavourable immune response occurs in some patients (Charriere *et al.*, 1989; Keefe *et al.*, 1992; Lynn *et al.*, 2004).

Although PLGA is a completely synthetic material, it will gradually produce acids, inducing inflammatory responses. These disadvantages are not clearly noticed clinically; however, developing more biocompatible synthetic GBR membrane is beneficial.

Hydrophobized polysaccharide, such as cholesterol-bearing pullulan (CHP), is a unique material for drug delivery systems (DDSs) (Akiyoshi *et al.*, 1998, 1999, 2002, Akiyoshi, 2006). Cholesterol-bearing pullulan (CHP) self-aggregates to form a monodisperse and stable hydrogel nanoparticle, in which the domains of the associated cholesterol groups of CHP provide cross-linking points in a non-covalent manner (Figure 1). The size and density of the hydrogel nanoparticle can be controlled by changing the degree of substitution of the cholesterol groups of CHP. During that substitution process, it can incorporate growth factors and act as a molecular chaperone. We have reported that delivering prostaglandin E1 with CHP nanogel stimulates wound healing in rats (Kobayashi *et al.*, 2009). In this previous study it was surprising to find that CHP nanogel alone enhanced wound healing. Therefore, the barrier membrane containing CHP nanogel may have great potential as a GBR membrane. The purpose of the present study was to evaluate the effectiveness of the novel bioabsorbable CHP nanogel cross-linking membrane as a GBR membrane.

2. Materials and methods

2.1. Collagen membrane

A bioabsorbable membrane made of collagen (Koken Tissue Guide®, Japan) was used. This material was combined with bovine collagen derived from dermis tissue and bovine insoluble collagen derived from tendon (9:1). It was freeze-dried and cross-linked with the addition of hexamethylenediisocyanate (HMDIC). The collagen membranes were cut into circles with a diameter of approximately 6 mm.

2.2. Nanogel cross-linking membrane

CHP was synthesized as reported previously (Akiyoshi *et al.*, 1996). Acryloyl group-modified cholesterol-bearing pullulan (CHPOA) nanogel was synthesized by the reaction of 2-(acryloyloxy)-ethyl isocyanate (AOI) and CHP. CHPOA nanogel solution (40.5 μl, 26.7 mg/ml) in Dulbecco's phosphate-buffered saline (PBS; pH 7.4) and 5.4 μl DMSO were mixed and kept at 4 °C for 24 h. This CHPOA nanogel solution and 8.1.μl solution of PEG-SH in PBS (481.8 mg/ml) were mixed at the ratio of thiol groups to acryloyl groups, which was 1:1. Then 13 μl of the mixture was placed between two glass slides coated with Parafilm® for 4 h at 37 °C under humidified conditions, to form a membrane-shaped hydrogel. The diameter and thickness of the nanogel membrane was 6 and 0.4 mm, respectively. The prepared membrane was applied to bone defects of experimental animals within 24 h.

2.3. Animal experiments

The animal experiments in the present study were approved by the Committee of Animal Experiments, Tokyo Medical and Dental University.

2.4. Surgical procedures

Thirty-six adult male Wistar rats, 16 weeks old, were used and divided into three groups. Prior to the experiment, the overall health of each rat was monitored for 2 weeks. The rats were kept in a standard cage (Tokiwa, Japan) in an experimental animal room at 22 ± 3 °C at 40–60% humidity and 1 atm, on 6:00–20:00 light, fed a standard laboratory diet (CE-2 CLEA Japan Inc.) and given sterilized water. The animals were anaesthetized with a combination of ketamine (40 mg/kg)–xylazine (5 mg/kg). In addition, approximately 0.4 ml local anaesthesia with lidocaine–HCl containing epinephrine 1/80 000 (2% Xylocain, Astra Japan Ltd, Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan) was injected at the surgical site. A cutaneous flap was created by making a mid-sagittal incision through the skin, which was raised from the forehead. The periosteum was incised and elevated to expose the calvarial bone on both sides of the midline. Two symmetrical, full-thickness bone defects with outer diameter of 5 mm were created with a bone trephine burr (Tele Components Co., Germany) under continuous saline irrigation. The defects were covered with CHP nanogel cross-linking membrane or collagen membrane or without any membrane. The animals were sacrificed under chloroform anaesthesia at 2, 4 and 8 weeks after the surgery and analysed radiologically and histologically.

2.6. Radiographic evaluation

The calvariae were dissected out and fixed in neutral 10% formalin and then analysed using micro-CT (μCT; InspeXio, Shimadzu Science East Corp., Tokyo, Japan) to measure the bone volume in the defect area.

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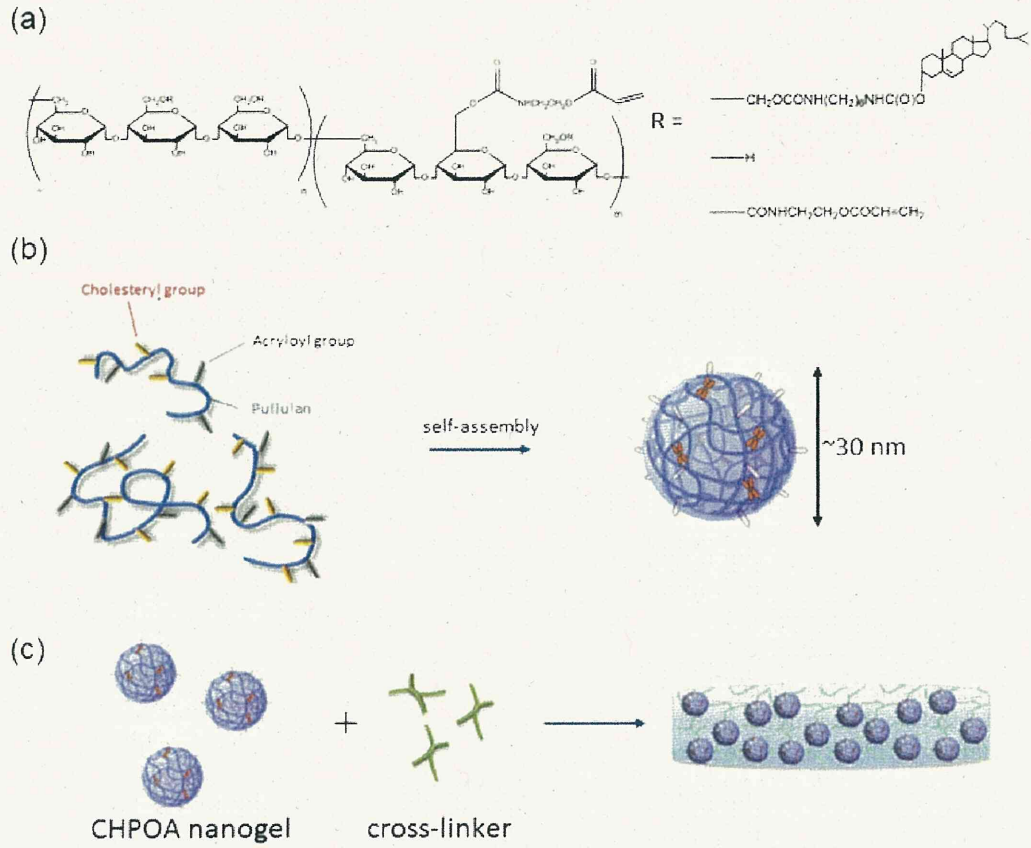

Figure 1. Scheme of acryloyl group-modified cholesterol-bearing pullulan (CHPOA). (A) Chemical structure of CHPOA nanogel. (B) Schematic illustration of CHPOA. (C) Schematic illustration of CHPOA nanogel cross-linking hydrogel (CHPOA–PEGSH)

2.7. Histological analysis

After radiographic analysis the calvariae were decalcified in 5% formic acid for 2 weeks. The specimens were dehydrated in ascending grades of ethanol, embedded in paraffin and sectioned 5 μm thick in the sagittal direction with a microtome. The sections were stained with haematoxylin and eosin (H&E).

2.8. PDGF concentration after incubating serum with materials, ELISA

Whole blood (50 ml) was obtained from the arm vein of a healthy, non-smoker male donor aged 34 years. After being stored overnight in a glass tube at room temperature, serum was prepared. The serum was incubated in three different combinations: serum alone, serum with collagen membrane and serum with nanogel membrane. The diameter of both membranes was 6 mm. These combinations were prepared in Eppendorf-type 1.5 ml vials and incubated for 3 h at room temperature. Then, PDGF-BB concentration in the serum was measured using an ELISA kit (Human PDGF-BB ELISA Kit, Ray Bio®, Ray Biotech Inc., Norcross, GA, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

Data, apart from the ELISA result, were first analysed by one-way ANOVA. When this analysis suggested a significant difference between groups, the data were further analysed by Tukey *post hoc* multiple comparison tests, using SPSS software (v 11.5, SPSS, Chicago, IL, USA). For ELISA assay, statistical evaluation was performed with Student's *t*-tests (SPSS v 11.5). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Radiographical images

Post-operative soft tissue healing was similar in all three groups without any membrane exposure in both membrane groups. The radiographical images of all the groups at 2, 4, and 8 weeks are presented in Figure 2. In the control group, newly formed bone could be seen only at the surrounding edge of the defect. In the collagen membrane group, part of the membrane appeared to be calcified, whereas in the CHP nanogel membrane group newly formed bone could be observed in almost the entire defected area. The surface of new bone in the CHP

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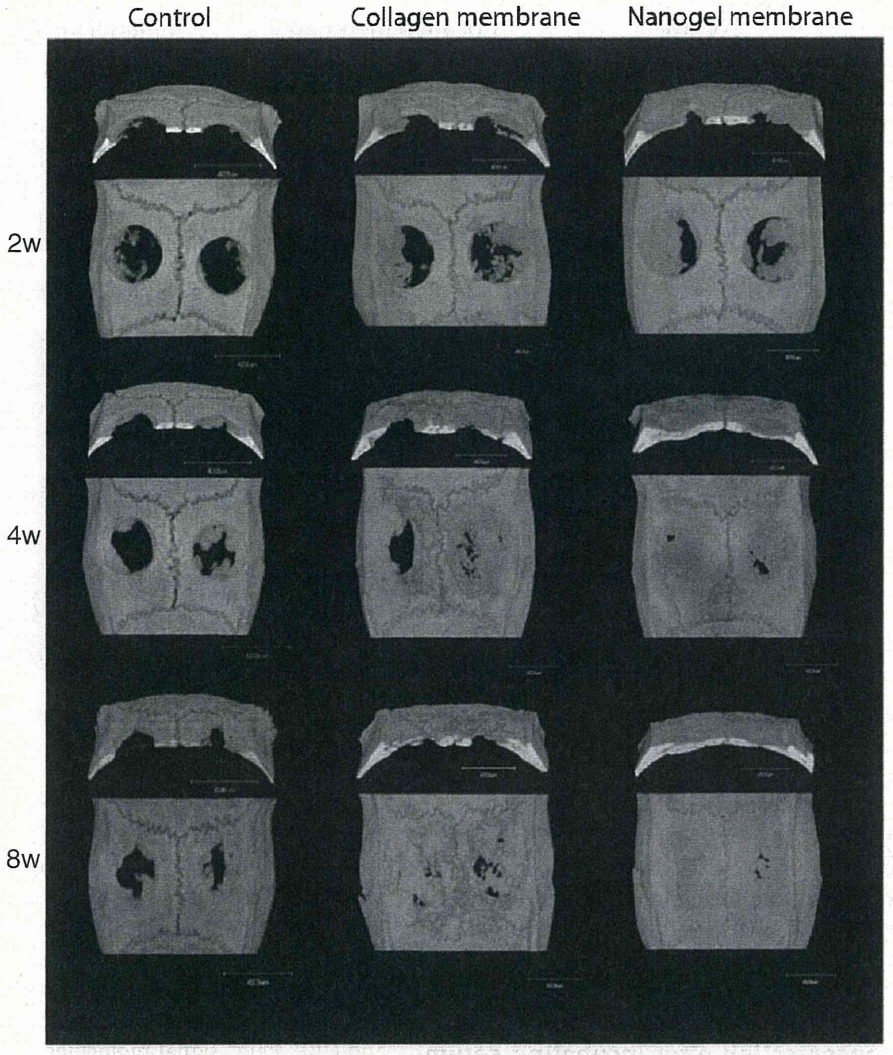

Figure 2. Cross-sectional micro-CT images of the bone defects at 2, 4 and 8 weeks

nanogel group was smooth; however, that in the collagen group was irregular. Volume of the newly formed bone is shown in Figure 3. New bone volume in the defect area in CHP nanogel group was highest at 2 and 4 weeks. At 8 weeks there was no statistical difference of newly-formed bone volume between the collagen and CHP nanogel groups. Newly-formed bone volume in the control group was lowest at the three time points.

3.2. Histological images

Histological images are presented in Figure 4. Corresponding to the radiographical images, new bone formation in CHP nanogel was prominent at 2 and 4 weeks compared with the other two groups. Notably, newly-formed bone in the CHP nanogel group was mature bone containing less connective tissue (Figure 4c, f, i, l),

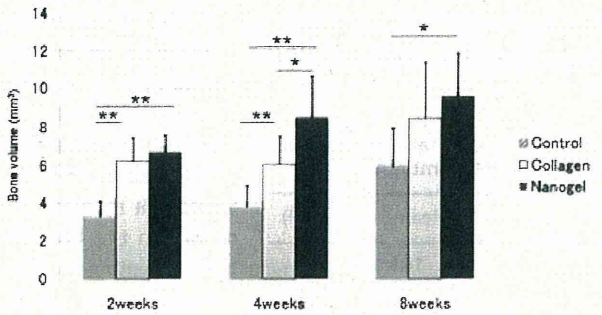

Figure 3. Bone volume in the defects at 2, 4 and 8 weeks. The data were obtained with micro-CT analysis. $*p < 0.05$, $**p < 0.01$

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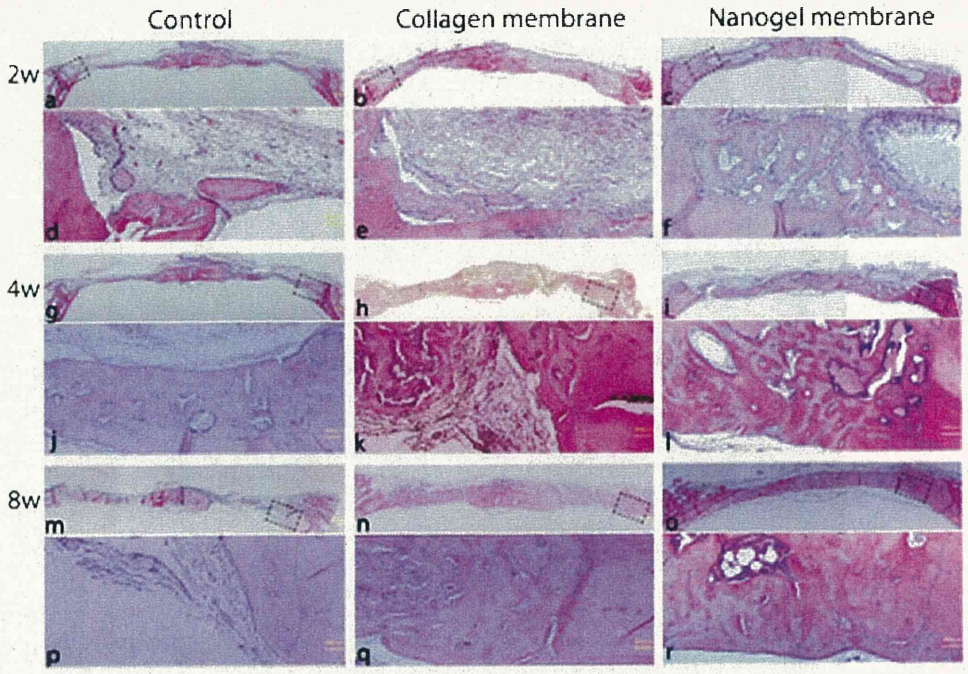

Figure 4. Histological images at 2, 4 and 8 weeks. High-magnification images of the parts of low-magnified images are presented; H&E staining

whereas in the collagen group less mature irregular bone was observed (Figure 4b, e, h, k). In addition, in the CHP nanogel group new bone formation occurred under the membrane, while it was observed both inside and under the membrane in the collagen group.

3.3. PDGF concentration after incubating serum with materials, ELISA

After incubating serum with the membranes, PDGF-BB concentration in the serum was measured. PDGF-BB concentration in the serum was the lowest when the serum was incubated with CHP nanogel membrane (Table 1), suggesting that CHP nanogel membrane trapped PDGF-BB in the material.

4. Discussion

The principle of GBR was originally explored for bone augmentation of the alveolar process in conjunction with oral implant therapy (Dahlin *et al.*, 1988; Buser *et al.*, 1993; Simion *et al.*, 1996; Urban *et al.*, 2009). In GTR and GBR, a membrane is used to keep regenerative space, preventing the invasion of the unfavourable surrounding tissue. The concept of tissue engineering is to provide the three key players to the regenerative site: cells, signal molecules and scaffolds; it is obvious that in both GTR and GBR, cells, signal molecules and scaffolds are endogenous. The progenitor cells are derived from the tissue facing the regenerative site, not from the tissue separated by a membrane. Signal molecules from activated platelets, such as PDGF and TGF-β, initially work for proliferation and differentiation of osteogenic cells. As scaffolds, fibrin initially plays important roles in regeneration. Collagen and other extracellular matrices are produced by the cells in the regenerative space and they work as scaffolds. Thus, GTR and GBR are characterized as encouraging endogenous regenerative ability by providing a space for regeneration.

In the present study, a bone defect model of rat calvaria was used to evaluate two types of GBR membrane. Although bone regeneration in the internal bone defect is different from external bone augmentation in GBR, the potential of the material for GBR membrane could be evaluated in the internal bone defect model of the present study. The present bone defect of 5 mm diameter did not heal spontaneously at 8 weeks when the defect was left without a membrane. The proliferation of undesired soft tissues in the bone defect interrupts the proliferation of bone forming cells from the periphery of the defect (Dahlin *et al.*, 1988; Kostopoulos *et al.*, 1994; Hämmerle *et al.*, 1995). Any material with some degree of biocompatibility can work as GTR or GBR membrane. Methyl

Table 1. PDGF concentration in the serum after incubating with collagen membrane or CHP nanogel membrane

	Mean ± SD (pg/ml)	
Serum	164.0 ± 5.0	*
Serum + collagen	163.3 ± 4.7	*
Serum + nanogel	151.9 ± 6.7	

*$p < 0.05$, significantly different from one another.

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cellulose was initially used as a GTR membrane. Polytetrafluoroethylene (PTFE), polyglycolic and polylactic acids (PLGA) and collagen have been clinically applied as GBR membranes (Bunyaratavej and Wang, 2001). Furthermore, other materials, such as chitin/chitosan and alginate, have also been investigated as GBR membranes (Eun-Jung Lee *et al.*, 2009). Our previous studies demonstrated the high biocompatibility of CHP nanogel (Kobayashi *et al.*, 2009), which is one of the required properties for wound dressing and also for GBR membranes. Compared with the other materials, the uniqueness of CHP nanogel is to trap hydrophobic and hydrophilic molecules inside. Since CHP nanogel contains > 90% of water, the water in the gels would be exchanged for tissue exudates. After incubating in serum for the ELISA assay, the colour of the nanogel changed into yellow, similar to the colour of the serum. PDGF is synthesized mainly by megakaryocytes and is stored in the α-granules of platelets. When platelets are activated, PDGF is released. *In vitro*, PDGF-AA and PDGF-BB enhance the proliferation of multiple types of bone cells, including both osteoblast and osteoclast lineages (Hadjidakis and Androulakis, 2006; Zhang *et al.*, 1998). Although long-term exposure to PDGF reduces alkaline phosphatase activity and mineralization (Hsieh and Graves, 1998), application of PDGF stimulates regeneration of periodontal tissue and bone (Nevins *et al.*, 2009).

In our previous study, CHP nanogel alone stimulated wound healing in rats (Kobayashi *et al.*, 2009) and we speculated that CHP nanogel stored endogenous growth factors in wound exudates. In the present study, the decrease in the amount of PDGF-BB in the serum was small but significant after incubating the serum with CHP nanogel membrane. The result of the ELISA assay indicated that CHP nanogel membrane has an ability to store PDGF-BB. It is also possible that CHP nanogel membrane would store not only PDGF-BB but also other growth factors produced at the regenerative site. In addition to PDGF-BB, TGF-β, VEGF and FGF are also produced in and around the bone defects. It is likely that after storing growth factors at the regenerative site, CHP nanogel membrane would gradually release these growth factors during membrane degradation; in other words, it is reasonable to conclude that CHP nanogel membrane works as signal molecule attractant and reservoir at the regenerative site.

In the present study, both collagen and CHP nanogel membrane stimulated bone regeneration compared to the control, in which no membrane was applied. The amount of newly-formed bone at the early time point and the quality of the bone in the CHP nanogel group were superior to that of the collagen group. The character of the CHP nanogel membrane, storing and releasing endogenous growth factors, could partly explain this difference. In the histological images, bone was formed under the membrane in the CHP nanogel group, whereas it was formed both inside and under the membrane in the collagen group. In the μCT images we also observed the irregular surface of the regenerated bone facing the membrane in the collagen group. It is plausible that the collagen membrane used in the present study worked not only as a barrier membrane of GBR but also as a scaffold for bone regeneration.

CHP nanogel consists of pullulan and cholestesterol. Pullulan is a polysaccharide industrially prepared from starch and its medical application has been approved. CHP nanogel has already been used clinically for delivering insulin (Akiyoshi *et al.*, 1998), interleukin 12 (Shimizu et al., 2008) and cancer antigen (Kageyama *et al.*, 2008); in these studies, CHP nanogel did not exert any adverse effect. Therefore, CHP nanogel is an extremely safe material. The uniqueness of CHP nanogel is that it can incorporate growth factors and acts as a molecular chaperone. Thus, CHP nanogel is an ideal material for delivering growth factors. We have delivered BMP2 with CHP nanogel to the parietal bone of mice and observed new bone formation (Hayashi *et al.*, 2009). In addition to growth factors, CHP nanogel can incorporate small biologically active molecules. Using CHP nanogel we have also delivered prostaglandin E agonist and prostaglandin E1 to bone and skin wounds, respectively, and reported the stimulation of bone and skin regeneration (Kobayashi *et al.*, 2009; Kamolratanakul *et al.*, 2011). The present study demonstrated that CHP nanogel membrane alone stimulated bone regeneration; however, delivering biologically active molecules, such as BMP and prostaglandin E1, with CHP nanogel would be effective and promising in bone regeneration (Hayashi *et al.*, 2009). Although CHP nanogel membrane is promising as a GBR membrane, we have to solve the following two points before applying this membrane in GBR clinically. First, the membrane should be stable for a long time at room temperature. In the present study, CHP nanogel membrane was prepared within 24 h before the surgical application. It would be possible to make CHP nanogel membrane durable for a long storage period, because we observed that dried CHP nanogel membrane was also effective in the same bone defect model (authors' unpublished data). Second, enhancement of mechanical strength of the membrane is absolutely required. The present CHP nanongel membrane is strong enough to cover over a relatively small bone defect; however, it is obviously too weak to be applied for vertical or horizontal bone augmentation. Combining CHP nanogel membrane with biodegradable polymer would solve this problem.

5. Conclusion

The present results indicate that novel CHP nanogel crosslinking membrane would be potentially effective as a GBR membrane.

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Clinical Device-Related Article

Evaluation of a biodegradable novel periosteal distractor

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Abstract: We developed a new device composed of a thin biodegradable mesh (poly-L-lactide/hydroxyapatite composite) for distracting periosteum. The purpose of this study is to evaluate the effect of using this device as a periosteal distractor.
Materials and Methods: Eight Japanese male rabbits were divided into two groups according to time of sacrifice. The calvarial periosteum was elevated and one side of a biodegradable mesh was fixed to the bone surface with two titanium screws. Seven days after the surgery, an elevating screw was inserted into the other side of the mesh. Then, the calvarial periosteum was elevated at maximum 0.5 mm every 12 h for 5 days. The device was designed to distract the periosteum at different rates along its entire surface. At 4 and 6 weeks of the consolidation, the animals were sacrificed and newly formed bone was histologically and radiographically evaluated.
Results: The new device simplified periosteal distraction and reduced its invasiveness. Moreover, it successfully induced new bone formation from two sources; the periosteum and the underlying basal bone. Histomorphometric analysis of the distracted space showed that there is a relation between the rate of distraction and the amount of newly formed bone. We suggest that the optimal speed range for periosteal distraction in rabbit calvarial model could be less than 0.33 mm/day.
Conclusions: The new device is slim, biodegradable and the procedure is simple. Thus, periosteal distraction with this device is potential for vertical and horizontal ridge augmentation in oral cavity. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 100B: 882–889, 2012.

Key Words: periosteal distraction, bone regeneration, biodegradable material, dental implants

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INTRODUCTION

Oral rehabilitation with endosseous dental implants is effective and predictable; however, osseous defect is a frequent obstacle for this treatment. To achieve satisfactory functional and aesthetic requirements, ridge augmentation is required before implant installation.[1] There are several augmentation techniques: bone graft, guided bone regeneration, and alveolar distraction.[2] However, these augmentation options have some disadvantages. Although autogenous bone graft is still a gold standard for bone augmentation, donor side morbidity, and limitation of harvestable bone are problems in this technique.[1] Although autogenous bone substitutes are used; however, they are not effective enough compared with autogenous bone because of their material and chemical characteristics.[2] Although augmenting the bone with guided bone regeneration is effective, this technique is usually limited to undersized and regularly shaped defects and membrane exposure is a common problem.[3] Distraction osteogenesis is applicable to challenging case in which large augmentation is required. This technique is advantageous especially in vertical bone augmentation; however, it needs osteotomy and two surgeries.[4]

The osteogenic potential of the periosteum is well established.[5] Progressively uplifting the periosteum and expanding the interface between the bone surface and periosteum has resulted in the formation of new bone either with cortical perforation[6] or without.[1] Although Schmidt et al. were the first to demonstrate a device for periosteal distraction, they suggested the further modification of the periosteal distraction device to eliminate the dislodging action of the distracted tissues on the distracting end.[1] Estrada et al. reported device instability, displacement and perforation of soft tissue. In these previous studies, the devices were applied in the rabbit forehead or in the dog oral cavity and loss of the devices commonly occurred.[7] Casap et al. also reported difficulty of device placement thus the selection of the experimental animal was restrained by adequate size and facial anatomy.[8] Their device was similar to the one designed by Schmidt et al.[1] It is a metallic u-shaped device of 15 mm height with a distraction rod passing through it

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and the distraction plate was only 6 mm length.[9] This device was fixed to the body of the mandible and extended to the outside, which adversely affected the animal feeding habit. Although this technique seems to be promising, improvement of periosteal distraction device is strongly required. First, the device should be compact for less interfering of oral functions. Second, it should not harm mucosa or periosteum and, finally, it should be easily activated.

Studies of periosteal distraction with different devices and with different protocols have been reported; however, optimal activation conditions have not yet been clarified.[8] In previous periosteal distraction studies, the distraction rates varied from 0.2–0.5 mm/day, distraction period ranged from 8–32 days and latency period ranged from 5–10 days, whereas consolidation period ranged from 7 to 56 days.[1,2,6–10]

Poly-L-lactide is a synthetic polymer that has been extensively studied as a biodegradable material for medical devices. This material has the advantage of complete hydrolysis into its components and finally absorbed totally inside the body.[11] A slim biodegradable mesh, which consists of poly-L-lactate and hydroxyapatite (u-HA) fine particles, has been approved by FDA in 2007 for the repair of fracture and fixation of bone fragments in maxillofacial region. Some clinical studies reported using this composite in fractures of maxillofacial and other regions.[12–16] u-HA fine particles in this mesh are bioactive and totally bioresorbable inorganic component being responsible for osteoconductivity and faster dissolution of this composite when compared with that without u-HA particles.[11] We developed a simple device using this biodegradable mesh for periosteal distraction. The purpose of this study is to evaluate the effect of gradually distracting the periosteum using this biodegradable device.

MATERIALS AND METHODS

Japanese male white rabbits, weighing from 2.5 to 3 kg, were used and were divided into two groups, four rabbits in each group, according to the time of sacrifice. The experimental protocol was approved by the Committee of Animal Experiments in Tokyo Medical and Dental University.

Device description

The device consisted of composite materials of bioactive, bioresorbable unsintered HA (u-HA; Ca/P = 1.69 (mol.ratio), carbonate ion = 3.8 mol %, having fine particles (average size 3–5 μm) of 40% weight combined with poly-L-lactide (PLLA), which has been reinforced using a unique compression forging process. The u-HA fine particles are uniformly distributed throughout the composite materials. (Super FIXSORB MX, TAKIRON, Osaka, Japan) The distraction device has three components [Figure 1(A,B)].

i. Rectangular shape bioresorbable mesh (20 × 10 × 0.5 mm^3), in which three holes were prepared: two holes for fixation screws and 1 serrated hole for distraction screw [Figure 1(A)].
ii. Two titanium fixation mini screws (3 mm in length, 1 mm in diameter) [Figure 1(B)].
iii. Titanium elevating screw (5 mm in length, 2 mm in diameter) [Figure 1(B)].

Surgical procedures

Animals were anesthetized preoperatively with an intramuscular injection of ketamine (50 mg/kg Ketalar, Sankyo, Tokyo, Japan) and thiopental sodium (25 mg/kg Rabonal, Tanabe, Tokyo, Japan). In addition, 1.8 mL of a local anesthetic (2% xylocaine/epinephrine 1:80,000, Dentsply Sankin, Tokyo, Japan) was injected into the surgical sites before the start of surgery.

All operations were performed under aseptic condition. The forehead of the animal was shaved and disinfected with tincture of 1% iodine solution. A U-shaped skin and subperiosteal incision was done over the calvarial bone. The skin and periosteal flap were carefully raised to expose the bone surface and, then, the periosteum was retracted away from the operative site. Under irrigation with saline, 2 or 3 perforations were made with a round bur of number 4 to expose the modularly cavity in the external cortical plate of the occipital bone. The mesh was first placed over the perforated area and then fixed to the bone surface from one end by means of the two mini screws [Figure 2(A)]. The periosteum was then positioned back in its place and stabilized by suturing. [Figure 2(B)]. Finally, the skin flaps were sutured with 3–0 silk [Figure 2(C)].

Device activation

After 1 week a soft tissue incision of 2 mm length was done over the screw place of the mesh. The elevating screw was threaded through the mesh to raise it. The rotating screw perforated the covering soft tissue and was resting on the external cortical layer of the calvarial bone. Rotating the screw 180° cause the titanium mesh to be elevated by 0.5 mm. A rate of 0.5 mm distraction was applied twice a day for 5 days [Figure 2(D)].

During the observation period, all rabbits were given water and a standard rabbit feed *ad libitum*. Rabbits were sacrificed after 4 and 6 weeks consolidation period with a lethal dose of thiopental sodium. The entire cranial bone was removed and fixed for 14 days in neutral 10% formalin.

Microcomputed tomography (micro-CT) analysis

After fixation, specimens were scanned using a high resolution micro CT imaging system (SMX-90CT, Shimadzu, Kyoto, Japan) continuously in increments of 60 μm. The bone images were extracted by processing the gray scale images using a median filter to remove noise and a fixed threshold to extract the mineralized bone phase. Following phantom calibration of the images, scanned images were analyzed with three-dimensional 3D image analysis software (TRI/3D-BON; Rotac system engineering, Tokyo Japan). 10 Micro CT serial longitudinal images were obtained for each specimen (1 image/mm). The distracted area in each image was divided equally into three segments by the imaginary lines L1, L2, L3, and L4 [Figure 3(A)].

In each segment the area occupied by new bone (BA) and the total distracted area (DA) were measured using image analysis software (Image j, 1.43 Hz, NIH, Bethesda, MD). Images were automatically corrected for brightness and contrast, then were converted into 8-bit gray scale before measurement.[17]

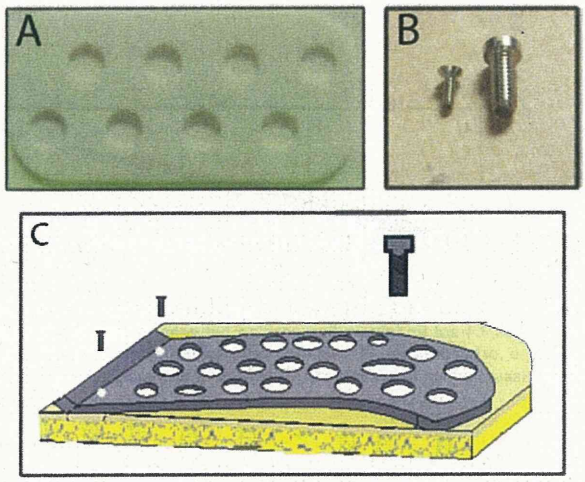

FIGURE 1. Image of PLLA/HA distraction device (A).Image of Distraction and fixation screws (B). Illustration showing the device and Position of fixation and elevation screws (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The percentage of the newly formed bone per distracted volume (BV/DV) in each segment was calculated. A table of (BV/DV) means for all segments in the eight animals representing the two groups was formed including the mean and SD of each segment. (Table I)

Heights attained by the periosteum at the end of activation at L2 and L3 axis were calculated by rules of right angled triangle [Figure 3(A)] and consequently elevation rates at these points were calculated (Table II).

Statistical analysis was performed with SPSS statistical package. Descriptive statistics included mean and standard deviation as well as one way analysis of variance test to compare the ratio of newly formed bone volume per distracted volume in the three segments between the two groups. The level of significance was set to 95%.

Histological processing

Following fixation calvarial bone was dehydrated in ascending grades of ethanol, and then embedded in polyester resin (Rigolac- 70F, Rigolac-2004, Nisshin EM Co., Tokyo, Japan). The distraction devices were kept in place then sections were cut (Exakt, Mesmer, Ost Einbeck, Germany) and ground to a thickness of about 100 μm. The sections were finally stained with 0.1% toluidine blue. Histological observation was performed under a light microscope.

RESULTS

In all animals normal dietary habit was resumed immediately after cessation of general anesthesia effect. No infectious symptom was detected during the entire period of the experiment. In two animals slightly more inflammation was observed compared with the other animals after the first surgery; however, it diminished before activation of the distraction. All devices remained rigidly fixed to the calvarium during the experiment. They were totally concealed under the soft tissue during activation and until time of sacrifice.

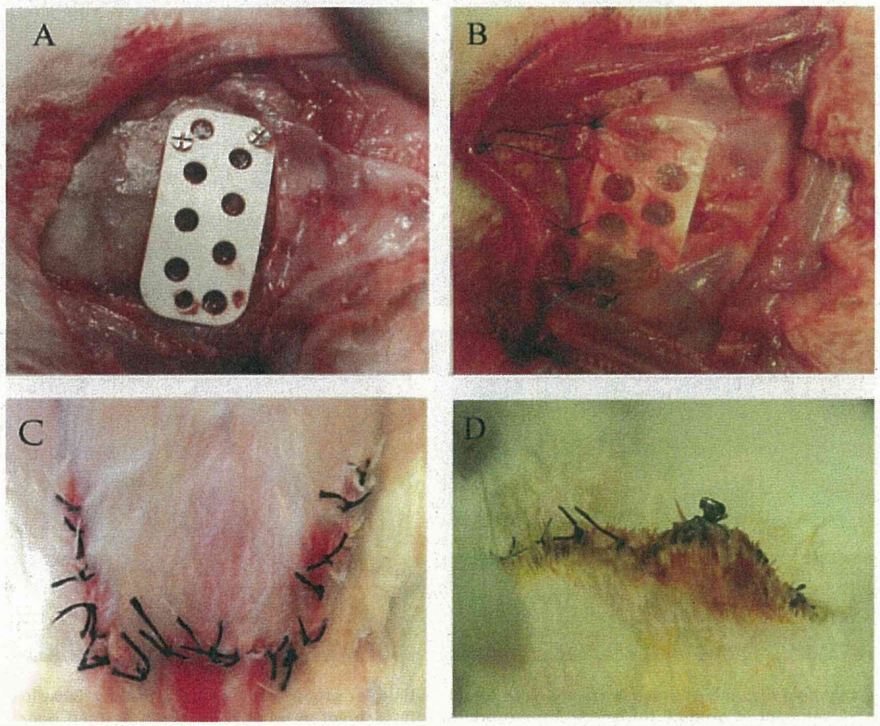

FIGURE 2. Image of Periosteal distractor fixed on calvarium bone (A). Distractor covered by periosteum (B). Flab closure (C). Device activation (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

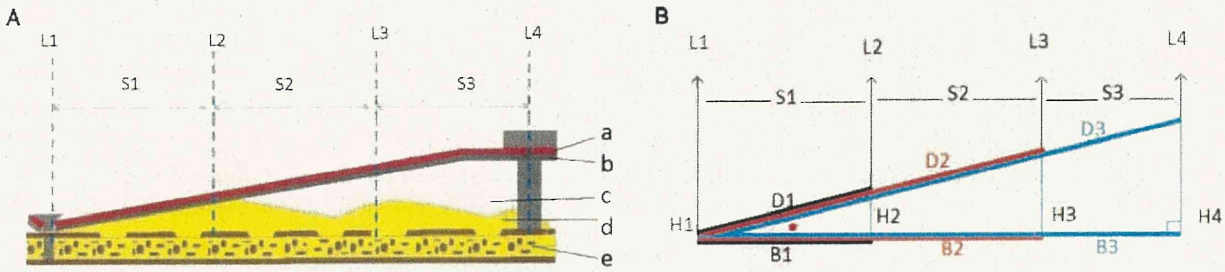

FIGURE 3. Illustration showing a transverse section showing the 3 distracted segments,S1, distracted segment close to the fixed end.S2,middle distracted segment .S3, distracted segment close to the movable end. L1, L2, L3, L4 are imaginary line passing between the S1, S2, and S3 segments. a, periosteum; b, device. c connective tissue. d, newly formed bone. e ,original bone. (A) Geometrical Illustration of the device and distracted site after full activation (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

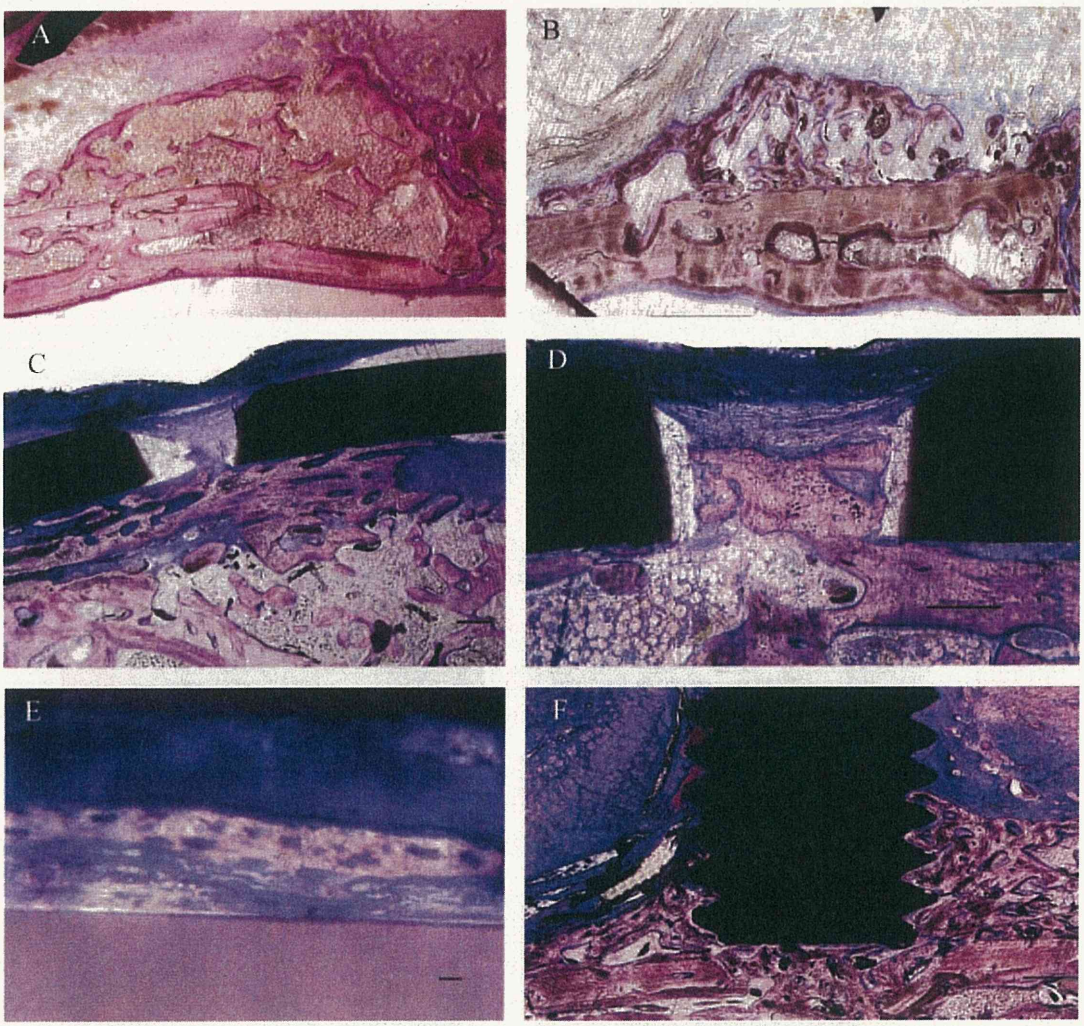

FIGURE 4. Representative histological images stained with toluidine blue for group 1 and group 2 Scale = 300μ in A,B,C,D,F 10 μ in E. Newly formed bone in S2 segment in group1. (A) Newly formed bone in S2 segment in group2. (B) Newly formed bone in S1 segment in group 2 showing more dense trabeculae than those in S2 segment. (C) Extended bone trabeculae through device perforations and contact with periosteum. (D) New bone trabeculae over the device and under the periosteum. (E) New bone trabeculae in S3 segment creeping over the apical 2 mm of the distraction screw (F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I. Showing Percent of Volume of Newly Formed Bone per Distracted Volume in Each Segment in Group 1 and 2

	Group 1			Group 2		
Distracted Segment	S1	S2	S3	S1	S2	S3
R1	69.37	20.79	27.37	100	45.39	9.21
R2	84.28	19.6	15.27	94.21	73.76	18.89
R3	98.33	29.32	17.25	100	70.82	18.2
R4	96.84	47.81	6.51	85.89	82.79	22.89
Mean	87.205	29.38	16.6	95.025	68.19	17.2975
SD	13.45662	13.02696	8.56334	6.673672	16.03061	5.774492

R, rabbit; S, distracted segment.

Distraction screws were easily adjusted and remained attached to the device until the end of the experiment.

At 4 weeks of the consolidation period in transverse histological sections multiple dome shape bone outlined by thin bone trabeculae and scattered bone trabeculae within abundant adipose tissue were evident. A layer of connective tissue was covering the newly formed bone layer [Figure 4(A)]. At 6 weeks of the consolidation period transverse sections demonstrated the similar histological patterns; however, bone trabeculae were thicker and adipose tissue was less abundant [Figure 4(B)].

Newly formed bone trabeculae showed different patterns among the distraction segments at 4 and 6 weeks of the consolidation period. Bone trabeculae in S1 Segment at both time points showed more compact appearance with marked decrease in adipose tissue [Figure 4(C)]. In addition, trabeculae in the S1 segment were likely to extend outside the limits of the distraction segment through the holes of the plate and attach to proliferating periosteum [Figure 4(D)]. Bone trabeculae in S2 segment showed more loose appearance with thicker overlying connective tissue layer [Figure 4(A,B)]. In S1 and S2 segments at 6 weeks of consolidation period small bone trabeculae was observed between the periosteum and the mesh [Figure 4(E)]. S3 showed almost similar histological pattern to S2 with thicker connective tissue layer although minute bone trabeculae was not observed between the periosteum and the device. At both 4 and 6 weeks of consolidation S3 was distinguished by regeneration of promoted quality bone with scant adipose tissue over the serrations of the apical 2 mm of the distraction screw [Figure 4(F)]. Periosteum was characterized by numerous blood vessels and tended to extend down into the distraction area through the plate holes [Figure 4(D)].

At 4 and 6 weeks of consolidation micro-CT 3D image showed that the new bone is less radiopaque than the original basal bone. Connective tissue appeared as radiolucent area of considerable thickness in S3; however, it tapered off from S3 to S1 and disappeared in the S1 segment [Figure 5(A,B,C)].

Quantitative data showed that S1 segment at both time points contain the highest new bone volume per distracted segment volume compared with S2 and S3 segments. The percentage of new bone volume at 6 weeks was higher than the one at 4 weeks (Table I). There were statistical differences between S1 and S2 segments and between S1and S3 segments at 4 weeks. At 6 weeks there were significant differences between all segments [Figure 6(A,B)].

DISCUSSION

In this study, we demonstrated that our new device induced osteogenesis and distracted soft tissue successfully in a 6 weeks rabbit calvarial model. The results also showed that there is a relation between the rate of periosteal distraction and quality of formed bone. In addition, the device sensibly simplified the periosteal distraction procedure and reduced the invasiveness.

The success of our new device in inducing new bone and distracting the periosteum in the rabbit model was attributed to several key design features. First, the whole device except the part of the activating screw is entirely concealed under the periosteum and soft tissues, which reduced the dislodging effect to the distracted tissues. Second, the design of the new device kept the integrity of the periosteum because the device perforated the periosteum only at the site of the activation screw. These points are advantageous compared with the previous devices, which perforated the periosteum at more than one site. Indeed, periosteal integrity following its elevation is the principle factor for success of periosteal distraction.

Interestingly, the sloping design of the device enabled to apply different distraction speeds simultaneously depending on the distance from the fixation screws, all of which were 1 mm per day and less.

In the segment near to the fixed end (S1) newly formed bone underneath the device is characterized by relatively thick trabecular bone and less adipose tissue. Moreover, growth of periosteal bone over the mesh was usually confined in this segment. This may suggest that the use of slower speed during periosteal distraction is prudent to promote newly formed bone. It is likely that the slower periosteal distraction speed decreased the opportunity for soft tissue to occupy the created space and increased the possibility for osteoblasts to form bone. Furthermore, this study demonstrated that the periosteum over the mesh had

TABLE II. Showing Elevation Rates in S1, S2, and S3 Segments

Segment	S1	S2	S3
Elevation rate range (mm/day)	0–0.33	0.33–0.66	0.66–1

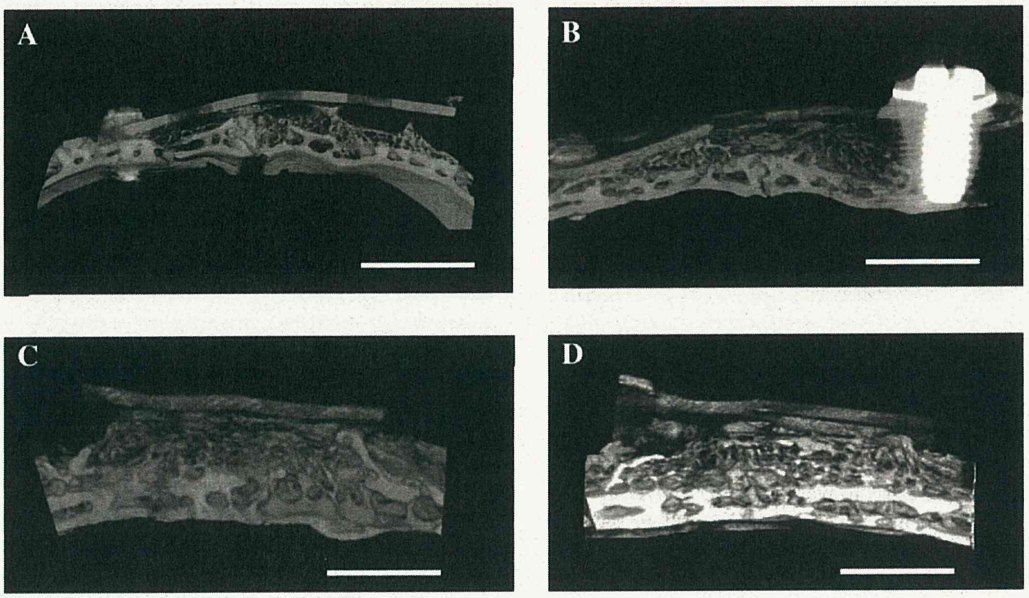

FIGURE 5. Micro CT images showing Transverse view of newly formed bone under the device in group1 (A) and in group2 (B). Micro CT images showing Crossectional view of newly formed bone under the device in group2 in S1 segment (C) and S2 segment (D). Scale bar = 5000 μ in A,B,C,D.

capacity to form minute bone when it received appropriate level of tension. However, the periosteum could not produce bone under the condition of excess tension level, which existed at the place away from the fixation screws in this study.

In this study at the area of the highest distraction speed, the segment 3, the less bone and more connective tissue were observed; however, the threads of the titanium distraction screw were covered with large amount of good quality of bone. Bone trabeculae crept on the titanium surface and covered the thread in which the distraction screw worked as a scaffold for bone regeneration. [Figure 4(f)]

In this study the histological finding demonstrated that newly formed bone originated mainly from the basal bone. It is very likely that progenitor cells of blood vessels and osteoblasts were provided from the basal bone, especially through the perforated bone holes. These cells proliferated and differentiated in the space, which was gradually produced by the elevating mesh. Nutrition to the regenerated area was also mainly provided from the basal bone. Furthermore, the distracted periosteum through the mesh holes was also an additional nourishment source for the new bone.

The speed of the regenerative space expansion by periosteum elevation should be optimum, thus osteoprogenitor cell supply, proliferation and differentiation of these cells and nutritional supply are able to catch up. The periosteal distraction is consistent to Elizarov's principle of tissue distraction in which the slow distraction speed for tissues is recommended.

Sencimen et al. reported dominance of adipose tissue under the periosteum in the periosteal distraction.[9] This study clearly demonstrated that the quality of the newly formed bone depended on the distraction speed. Slowest speed produced bone of thicker trabeculae and less connective tissue and radiopacity closer to original bone. This newly formed bone can be sustained and matured if it receives appropriate level of mechanical stress. Periosteal

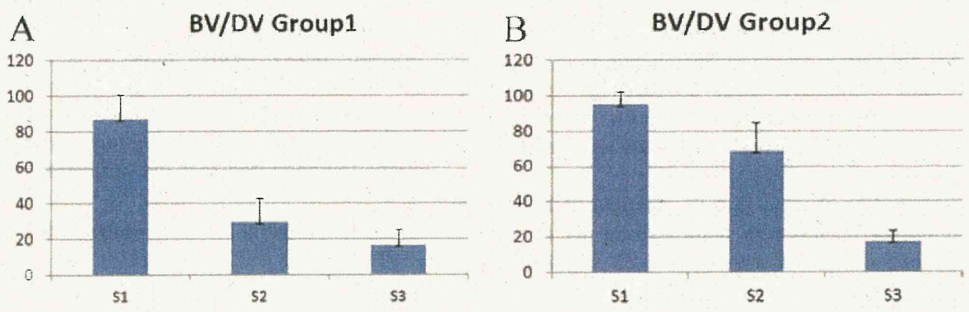

FIGURE 6. A, B: Graphic representation of the mean and standard deviation of the percent of bone volume /distracted volume (BV/DV) among segments in the 2 groups. $P < 0.01$ Scheffe test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

distraction followed by dental implant installation could be beneficial because the implants transmit mechanical stress to the newly formed bone. The important finding in this study was that the optimum speed range of periosteal distraction for bone augmentation was 330 μm per day and less, which decreased soft tissue formation. In bone distraction speed from 0.5 mm to 1.0 mm or sometimes more per day is clinically applied, which is based on previous animal and clinical studies.[10,18,19] In osteogenic distraction, cell and nutrition supplies originate from both ends of bone and from the surrounding periosteum, whereas in periosteal distraction those supplies are derived only from basal bone and overlying periosteum. Therefore, it seems to be reasonable that in osteogenic distraction normal distraction speed is more than that of periosteal distraction suggested in this study.

In addition to autologous bone graft and distraction osteogenesis, several bone augmentation modalities have been developed and clinically applied: applications of osteogenic cells[20,21] or signal molecules, such as BMP-2[22,23] or PDGF[24,25] combining with scaffold materials. Although these modalities are clinically effective and promising, vertical bone augmentation in oral cavity is still challenging except distraction osteogenesis because of difficulties in covering grafted bone or material with soft tissue.

Distraction osteogenesis makes vertical augmentation together with soft tissue expansion possible; however, bone distraction in the oral cavity is technically sensitive because of difficulties in segmenting bone and setting a bone distraction device in narrow space. In addition, a patient is uncomfortable when bone distraction device exists in the oral cavity.

Future intraoral application of this biodegradable device as a periosteal distraction could be less sensitive technically especially when it is set free from fixation screws. Moreover, as the accessible elevation screws can be easily removed before complete bone mineralization a second surgery will not be necessary.

Lethaus et al. have recently reported a new device for periosteal distraction demonstrating effectiveness of periosteal distraction in vertical bone augmentation.[26,27] This study further demonstrated effectiveness of periosteal distraction in vertical augmentation with a new biodegradable device. Conclusively, although improvement of a device is still required, periosteal distraction would be potentially effective in vertical bone augmentation in dental field.

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Induced Osteogenesis Using a New Periosteal Distractor

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Purpose: We developed a new device mainly composed of a titanium mesh to distract the periosteum. The purpose of this study is to evaluate induced osteogenesis by periosteal distraction with the new device.

Materials and Methods: We divided 12 Japanese male rabbits into 3 groups, with 4 rabbits in each. In all groups the calvarial periosteum was reflected, and 1 side of the titanium mesh was fixed to the bone surface with 2 micro-screws. In groups 1 and 2, an elevation screw was inserted into a serrated hole on the other side of the plate 7 days after surgery. Then the device was activated at a rate of 0.5 mm every 12 hours for 5 days. At 4 weeks of the consolidation period, group 1 was killed, followed by group 2 at 6 weeks. Group 3 (control) received no screws, and hence no activation was performed. In group 3, 2 animals were killed 4 weeks after titanium mesh insertion, followed by the other 2 animals at 6 weeks. The device was designed to simultaneously distract the periosteum at different rates along its inclined surface. Newly formed bone was histologically and radiographically evaluated.

Results: The new device effectively induced osteogenesis and successfully distracted the soft tissue after 6 weeks in a rabbit model.

Conclusions: The new device is slim, and the procedure is straightforward. Thus, periosteal distraction with this device can potentially be used for vertical and horizontal ridge augmentation in the oral cavity. In addition, the results suggest that connective tissue growth in the distraction site might be controlled by reducing the speed of periosteal distraction.

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Oral rehabilitation with endosseous dental implants is effective and predictable; however, implant placement, without augmentation procedures, may lead to an esthetically compromised situation.[1] The presence of an osseous defect is a frequent obstacle for this treatment. To achieve satisfactory functional and esthetic requirements, ridge augmentation is often required before implant installation.[1]

Several reconstruction procedures have been proposed to augment the alveolar ridge, including bone grafting, guided bone regeneration, and alveolar distraction.[2] However, the outcome of some of these augmentation options varies. Although autogenous bone graft is still a gold standard for bone augmentation, donor-side morbidity and limitation of harvestable bone are problems of this technique.[1] Instead of autogenous bone, bone substitutes are used; however, they are not effective enough compared with autogenous bone because of their material and chemical characteristics.[2] Though effective, the guided bone regeneration technique is usually limited to undersized and regularly shaped defects; moreover, membrane exposure is a common problem.[3]

Distraction osteogenesis is used in more extensive alveolar augmentation, especially vertical type; however, limitations of this technique have been reported. They have ranged from aggressive procedures[4] and frequent lingual inclination of the distracted segment[5,6] to some relapse of the initial bone gain before implant placement.

Schmidt et al[1] were the first authors to apply a device for periosteal distraction. By gradual periosteal lifting, this technique enlarges the interface between the original bone surface and the periosteum, inducing supraosseous neogenesis. They recommended ad-

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FIGURE 1. Titanium device.

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ditional improvement of the periosteal distraction device to eliminate the displacing effect of the distracted tissues on the device.[1] Subsequent periosteal distraction studies also reported device problems. Some studies reported device instability, displacement, and soft tissue dehiscence.[8,9] Casap et al[10] also reported difficulty in device placement; thus the selection of the experimental animal was restrained by adequate size and facial anatomy.

The periosteal tissue was proved highly osteogenic.[11] Contact with bone seems to be essential for its osteogenicity.[12-14] Periosteal distraction procedures involve separation of the periosteum from its underlying bone; however, some periosteal distraction studies showed periosteal bone formation.[15,16]

Though promising, periosteal distraction greatly requires enhancements to the device and the protocol regulating the device activation.[12] In previous periosteal distraction studies, distraction rates varied from 0.2 to 0.5 mm/d, the distraction period ranged from 8 to 32 days, and the latency period ranged from 1 to 10 days, whereas the consolidation period ranged from 7 to 60 days.[1,2,8,10,15-19]

We developed a compact titanium device for periosteal distraction that is activated in an inclined position. The purpose of this study is to evaluate the induced osteogenesis as a result of using this device.

Materials and Methods

We divided 12 Japanese male rabbits aged 6 weeks and weighing from 2.5 to 3 kg into 3 groups, with 4 rabbits in each. Two groups were experimental, and one group served as a control. The experimental protocol was approved by the Committee of Animal Experiments at Tokyo Medical and Dental University, Tokyo, Japan.

Device Description

The distraction device has 3 components (Figs 1-3): (*1*) titanium mesh (20 mm × 10 mm × 0.3 mm), in which 3 holes were prepared—2 holes for fixation screws and 1 serrated hole for distraction screw (Fig 1); (*2*) 2 titanium fixation mini-screws (3 mm in length and 1 mm in diameter) (Fig 2); and (*3*) a titanium elevation screw (5 mm in length and 2 mm in diameter) (Fig 2).

Surgical Procedures

Animals were anesthetized preoperatively with an intramuscular injection of ketamine (50-mg/kg Ketalar; Sankyo, Tokyo, Japan) and thiopental sodium (25-mg/kg Rabonal; Tanabe, Tokyo, Japan). In addition, 1.8 mL of a local anesthetic (2% Xylocaine/epinephrine 1:80,000; Dentsply Sankin, Tokyo, Japan) was injected into the surgical sites before the start of surgery.

All operations were performed under aseptic condition. In all groups the forehead of the animal was shaved and disinfected with tincture of 1% iodine solution. A U-shaped skin and subperiosteal

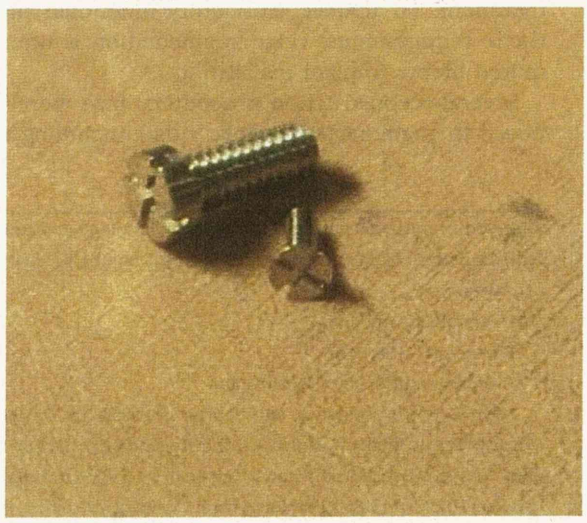

FIGURE 2. Distraction and fixation screws.

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