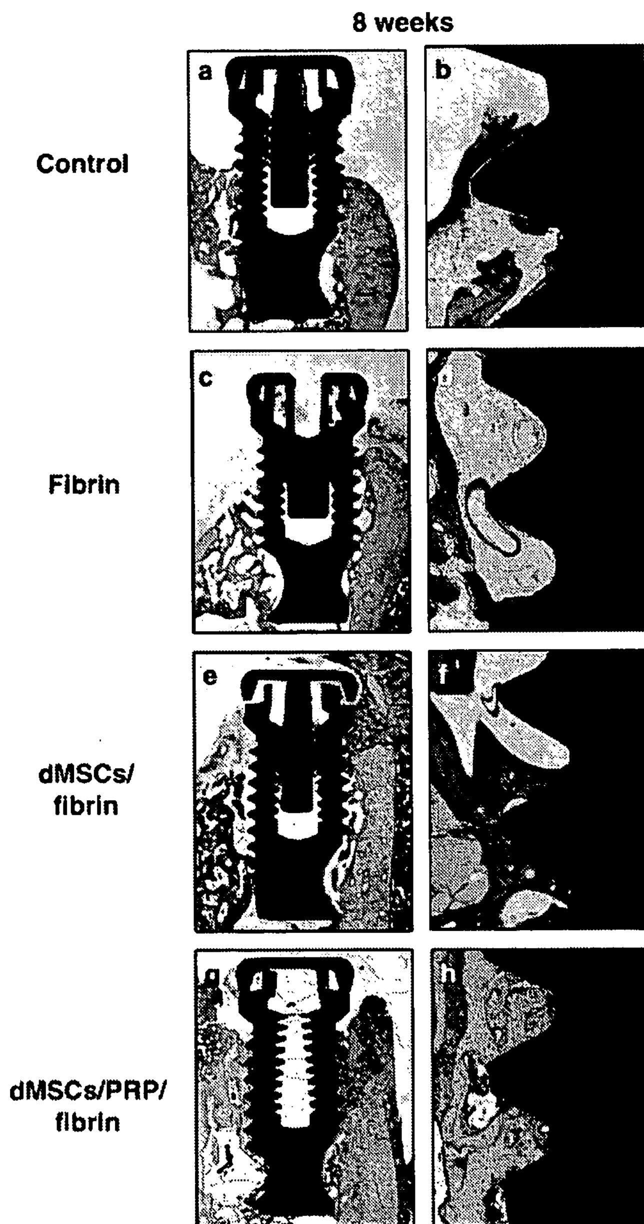

Fig. 5. Photographs of the histology sections seen by light microscopy at 8 weeks. Non-decalcified ground sections surface stained with toluidine blue. Original magnification, × 12.5for (a), (c), (e), (g) and × 250 for (b), (d), (f), (h). (a) In the control group, the regenerated bone was not sufficient for dental implants (lower magnification). (b) The control group (higher magnification). (c) Fibrin group (lower magnification). Parts of the threads on the buccal aspect were covered by soft tissue. (d) Fibrin group (higher magnification). (e) Dog mesenchymal stem cells (dMSCs)/fibrin group (lower magnification). The buccal wall was gradually regenerated. (f) dMSCs/fibrin group (higher magnification) (g) dMSCs/platelet-rich plasma (PRP)/fibrin group (lower magnification). The regenerated buccal bone was as wide as the lingual cortex. (h) The dMSCs/PRP/fibrin group (higher magnification).

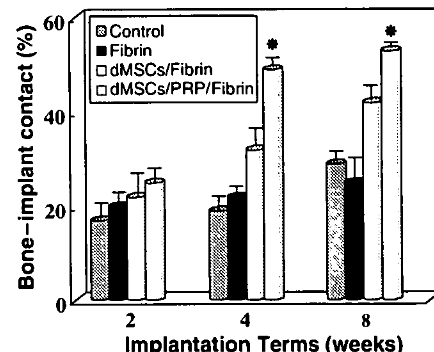

Fig. 6. Histomorphometrical evaluation. Comparison of the mean percentage of bone–implant contact among the graft materials. Measurements were made on all threads on the buccal and lingual aspects of the implants (see Materials and Methods and Fig. 1). A significant difference was seen between the dog mesenchymal stem cells (dMSCs)/platelet-rich plasma (PRP)/fibrin group and the other groups at 4 and 8 weeks.

since it has proven biocompatibility, biodegradability and capacity to bind to cells (Keller et al. 1985). Fibrin-stabilizing factor XIII found in fibrin glue favors the migration of undifferentiated mesenchymal cells on its highly cross-linked glue structure, and enhances the proliferation of these cells. In later studies, fibrin was used in combination with growth factors because it appeared to fulfill some of the requirements for a carrier for growth factors, i.e., the stimulation of angiogenesis and the maintenance of growth factors at the wound site and the prevention of soft tissue prolapse (Matras 1985; Cheng et al. 1998). In this study, as the correlation between growth factor (PRP) and fibrin had not been previously reported, at first we examined ALP activity, which is often used as a parameter of early osteoblast differentiation and is considered the single most accurate marker of bone formation (Allen 2003), *in vitro* to find the optimum mixing ratio between PRP and fibrin with MSCs (Table 1). The activity was low for ratios of 0% and 20%, and very little osteogenic ability was observed. At a PRP ratio of 40% or more, ALP activity gradually increased and was found to be high 9 days after induction. Based on these results, we attempted to determine the optimum mixing ratio at which PRP and fibrin would favorably affect cells in this *in vivo* study.

We also evaluated the performance of a dMSCs/PRP/fibrin admixture (tissue-engineered bone) in one-stage alveolar augmentation with simultaneous implant placement using a GBR membrane. GBR allows for bone augmentation, which makes it possible to prepare sites for implants. Histological proof of its efficacy and predictable clinical outcomes has also been obtained in animal and human studies (Buser et al. 1990; Hämmercle & Karring 1998). However, it has also been reported that little regenerated bone is present after bacterial contamination of the membrane and upon premature membrane removal (Buser et al. 1990, 1994; Kohal et al. 1998). Therefore, we did not permit membrane exposure in order to avoid the negative effects of the membrane.

In this study, the BIC of dMSCs/PRP/fibrin was 25, 49 and 53 at 2, 4 and 8

weeks, and that for dMSCs/fibrin was 22, 32 and 42. At 4 and 8 weeks, a significant difference between dMSCs/PRP/fibrin and other materials (fibrin, dMSCs/fibrin) was found (Fig. 6). The macro findings showed that dMSCs/PRP/fibrin induced sufficient bone regeneration and that the dental implant thread was not exposed (Fig. 2). In support of the BIC and macro findings, the histological findings showed that dMSCs/PRP/fibrin induced excellent bone formation from early stages compared with that in the other groups (Figs 3–5). Thus, these results indicate that sufficient bone regeneration was caused by dMSCs/PRP/fibrin and that simultaneous implantation using dMSCs/PRP/fibrin (tissue-engineered bone) is possible. Furthermore, growth factors are believed to be an important parameter in tissue engineering. Our findings suggest that PRP might promote bone regeneration. In previous studies, we investigated the influence of PRP in the tissue-engineered bone on the effects of the two-step procedure, and the results showed that newly regenerated bone formed by dMSCs/PRP perfomed better than that without PRP, suggesting a positive influence of MSCs combined with PRP (Yamada et al. 2004b). As PRP contains an autologous source of platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Lynch et al. 1991; Marx et al. 1998; Richard et al. 2001), it might also be useful in promoting bone regeneration around dental implants in a simultaneous technique. In addition, this type of tissue-engineered bone, which is a coagulated mass, might be suitable as a bone graft matrix of the simultaneous implant placement with PRP because it is easy to manipulate it around the dental implant finely. These results also suggest that increasing the growth factor concentration by the application of PRP in the wound improves soft tissue repair and bone regeneration. This study implicates that the acceleration of bone formation by PRP may be clinically helpful for the augmentation of alveolar defects at early stages.

In summary, these results indicate that it may be possible to achieve the osseointegration of implants placed simultaneously with injectable tissue-engineered bone grafts and that such grafts may be useful for shortening the period of implant treatment and improving the treatment outcome.

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要旨

本研究は、インプラント一期的埋入と同時に骨増生術のための移植材料として、組織工学的手法を用いて作製した注入型培養骨による効果を評価した。本研究にはハイブリッド成犬１２匹を用いた。下顎歯牙抜去後１ヵ月の治癒期間の後、直径１０ｍｍのトレフィン・バーを用いて下顎両側に骨欠損を形成した。腸骨より骨髄穿刺にて犬の間葉系幹細胞（ｄＭＳＣｓ）を採取し、埋入前４週間培養した。インプラント埋入と同時に以下の移植材料を填入した；(i) フィブリン、(ii)ｄＭＳＣｓとフィブリン（dMSCs/フィブリン）(iii)ｄＭＳＣｓと多血小板血漿(PRP)及びフィブリン(dMSCs/PRP/フィブリン)、(iv)対照(欠損のみ)。埋入後２、４、８週間後に屠殺し、移植部を組織学的及び組織形態学的分析によって評価した。インプラントとの界面は、それぞれ様々な骨－インプラント接触率（ＢＩＣ）を示した。このＢＩＣは２、４、８週間後に各々、対照群が１７、１９、２９％、フィブリン群が２０、２２、２５％、dMSCs/フィブリン群が２２、３２、４２％、さらにdMSCs/PRP/フィブリン群は２５、４９及び５３％であった。本研究では組織工学的手法によって作製した注入型培養骨をインプラント一期的埋入に応用した場合、この培養骨は歯牙インプラント周囲に予知性の高い骨再生を施す十分な能力を有する事が示唆された。

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Osteogenic potential of injectable tissue-engineered bone: A comparison among autogenous bone, bone substitute (Bio-oss®), platelet-rich plasma, and tissue-engineered bone with respect to their mechanical properties and histological findings

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Abstract: Recently, tissue engineering has become available as a regenerative treatment for bone defects. However, the evaluation of its success is limited to histological analysis, and its effects on mechanical hardness remain to be investigated. This study investigated mechanical strength in support of histological findings, specifically for tissue-engineered bone with mesenchymal stem cells (MSCs) and platelet-rich-plasma (PRP). Initially, teeth were extracted, and bone defects on both sides of the mandible were prepared with a trephine bar. The defects were implanted by using the following graft materials: 1) PRP, 2) PRP and dog MSCs (dMSCs), 3) autogenous bone (PCBM), 4) bone substitute (Bio-Oss®), and 5) control (defects only). After 2, 4, 8, and 12 weeks of implantation, the defects were histologically assessed to examine their mechanical properties. According to histological observations, the dMSCs/PRP groups had well-formed mature bone compared with the control (defects only), Bio-Oss®, and PRP groups. The Vickers hardness test values were 8 (control), 9 (PRP), not detected (Bio-Oss®), 11 (PCBM), and 17 (dMSCs/PRP) after 2 weeks. Therefore, tissue-engineered bone can be used for early stage bone regeneration from the viewpoint of histology and mechanical properties. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 73A: 63–72, 2005

Key words: tissue engineering; mechanical properties; Vickers hardness; mesenchymal stem cells (MSCs); platelet-rich plasma (PRP)

INTRODUCTION

Various methods have been developed for the restoration of bone defects during craniomaxillofacial reconstructive, plastic, and orthopedic surgery, but the augmentation and manipulation of biocompatible material still remain as difficult clinical problems with respect to soft and hard tissues. One of these materials is an anorganic porous bovine-derived bone mineral, Bio-Oss®, and particular attention has been paid to its clinical use. Furthermore, excised autogenous tissues, including fat, fascia, cartilage, and bone chips, are frequently used for bone regeneration. Preferred autogenous material is limited in supply, is associated with attendant donor-site morbidity, and is occasionally not suitable for the proposed reconstruction because of poor tissue quality or difficulty in shaping the graft. A previous approach to this problem focused on the development of various artificial materials that could be used instead of autogenous bone. Most of these artificial materials were not osteogenic or osteoinductive. In addition, synthetic prostheses may present increased susceptibility to infection, elevated incidences of extrusion, and uncertain long-term interactions with host's physiology.

To address many of the concerns in the field of solid organ transplants, Vacanti et al.[1–3] have described a new

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technology called tissue engineering, which involves the morphogenesis of new tissue by using constructs formed from isolated cells with biocompatible scaffolds and growth factors. Extending is a technique to osteoblasts, and formulating a system whereby cell-scaffold constructs could be delivered less invasively would greatly expand the applicability of tissue engineering to fields such as maxillo-craniofacial reconstruction and plastic or orthopedic surgery. Recently, a new technology, tissue engineering, also has begun to make use of graft materials, where tissue-engineered bone has been introduced and frequently used for bone regeneration.[4,5] Yoshikawa et al.[6] have reported that hydroxyapatite (HA) loaded with mesenchymal stem cells (MSCs) has osteogenic potential comparable with autogenous particulate cancellous bone and marrow (PCBM). Furthermore, Boo et al. have confirmed similar osteogenic potential between β-TCP (tricalciumphosphate), which is a biodegradable material, and HA. However, these delivery substances exhibit no good plasticity, and the cellular implantation procedure involving their use is complicated by problems associated with delivery vehicles. Hence, Yamada et al.[4,7–9] have reported that novel injectable tissue-engineered bone is plastic and exhibits jelly-like flexibility and that their implantation procedures involve minimal invasiveness. It is possible to graft tissue with a syringe if MSCs, which are multipotential cells, are used. MSCs and platelet-rich plasma (PRP) admixtures could provide a three-dimensional scaffold for the successful transplantation and engraftment of osteoblasts. However, these studies also reported the osteogenic ability of tissue-engineered bone. The evaluation of their results was limited to histological analysis or macroscopic findings, and few reports have focused on the mechanical properties of tissue produced by this technique.[10,11] Therefore, we evaluated the osteogenic potential of tissue-engineered bone used for clinical application and obtained positive results for mechanical properties assessed by histological observations.[9,12] It is also clinically worth investigating mechanical hardness to permit the application of this technology to dental implants, periodontitis, and tumor resection.

Here, we explore the potential of PRP scaffolding combined with MSCs to increase the rate of bone formation and to enhance bone regeneration by comparing it with currently available matrices, including autogenous bone (PCBM), bone substitute (Bio-Oss®), and PRP. With these results, we may be able to enhance the reliability of clinical applications of injectable tissue-engineered bone.

MATERIALS AND METHODS

Canine animal models

All animal experiments were performed in strict accordance with protocols approved by the Institutional Animal Care Committee. After a period of housing, five adult hybrid dogs with a mean age of 2 years were operated on under general anesthesia. The first molars, the premolars, and the second and third premolars in the mandible were extracted, and their healing was allowed for 1 month. Bone defects on both sides of the mandible were prepared with a 10-mm diameter trephine bar. The defects were implanted by using graft materials as follows: PRP; dog MSCs (dMSCs) and PRP; PCBM; a natural bovine bone mineral graft material (Bio-Oss®); and the control defect, which was left without implant. We then examined the resultant osteogenesis by histological analysis and mechanical property testing.

Cell isolation and cultivation

The dMSCs were isolated from dog iliac crest marrow aspirates (10 mL). Bone marrow cell isolation and expansion were performed according to the previously published methods.[13] Briefly, basal medium, low-glucose DMEM, and growth supplements (50 mL of mesenchymal cell growth supplement, 10 mL of 200 m*M* L-glutamine, and 0.5 mL of penicillin-streptomycin mixture containing 25 units of penicillin and 25 μg of streptomycin) were purchased from Cambrex® Inc. (Walkersville, MD). Three supplements used for inducing osteogenesis, Dexamethason (Dex), sodium β-glycerophosphate (β-GP), and L-ascorbic acid 2-phosphate (AsAP), were purchased from Sigma Chemical Co. (St. Louis, MO). Cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. We replated dMSCs at a density of 3.1×10^3 cells/cm^2 in the 0.2 mL/cm^2 control medium. Differentiated dMSCs were confirmed by detecting alkaline phosphatase activity using p-nitrophenylphosphatase as a substrate. In culture, dMSCs were trypsinized and used for implantation.

PRP, PRP gel preparation, and injection of dMSCs/PRP

Approximately 50 mL of whole blood were withdrawn from the canine peripheral blood into a centrifuge tube containing 10 mL of culture medium with 250 U/mL, preservative-free heparin. The blood was first centrifuged in a standard laboratory Himac CT centrifuge (Hitachi Koki, Hitachi) for 5 min at 1100 rpm. Subsequently, the yellow plasma (containing the buffy coat with platelets and leukocytes) was taken up into a neutral monovette with a long cannula. The second centrifugation at 2500 rpm for 10 min was performed to combine platelets into a single pellet, and the plasma supernatant, which was platelet-poor plasma (PPP) and contained relatively few cells, was removed. The resulting pellet of platelets, termed the buffy coat/plasma fraction (PRP), was resuspended in 5 mL of residual plasma and used for the platelet gel. Platelets for PRP and PPP were counted by using a Beckman Coulter GEN*S® and STKS-RE® (Beckman Coulter, USA). PRP was stored at room temperature in a conventional shaker until use. Bovine thrombin in powder form (10,000 units) was dissolved in 10 mL of 10% calcium chloride in a separate sterile cup. Subsequently, 3.5 mL of PRP, dMSCs (1.0×10^7 cells/mL), and 0.5 mL of air were aspirated into a 5-mL syringe and into a second

2.5-mL syringe; 500 μL of this thrombin/calcium chloride mixture were aspirated. The two syringes were connected with a "T" connector, and the plungers of the syringes were alternatively pushed and pulled, thus allowing the trapped air bubble to transverse the two syringes. Within 5–30 s, the contents assumed gel-like consistency because thrombin affected the polymerization of fibrin to produce an insoluble gel. The gel was injected into the bone defect field using a 16-gauge needle attached to a 5-mL syringe. Samples were analyzed at 2 (n = 8), 4 (n = 8), 8 (n = 8), and 12 (n = 8) weeks after injection.

Histological analysis and microhardness measurements

Each implantation site was excised with a 2-mm diameter trephine bar after 2, 4, 8, and 12 weeks of implantation, and the half-section was assessed by histology. Specimens were fixed in 10% buffered formalin, decalcified (K-CX; Falma Co., Tokyo), and stained with hematoxylin and eosin. The other section was also examined under a light microscope, and microhardness of the undecalcified specimen was determined by using a hardness tester (Akashi MVK-E, Tokyo, Japan) (Fig. 1). Disk-shaped specimens were molded, and the microhardness was quantified by applying a 100-gf load to a pyramid diamond point. The dimensions of five indentations produced on the surface of each sample were measured at the microscopic level, and their average was used to determine the Vickers hardness number (VHN). Specimens were analyzed by a pathologist who was blinded to the identity of each specimen and who also determined the presence or absence of bone formation. The primary and second author, who agreed with the above-mentioned pathologist on all cases, reviewed all of the sections.

Statistical analysis

Group means and standard deviations were calculated for each measured parameter. A difference in newly formed bone was compared by using two-tailed paired Student's t test between the control, PRP, PCBM, Bio-Oss®, and dMSCs/PRP. A $p < 0.05$ was considered statistically significant.

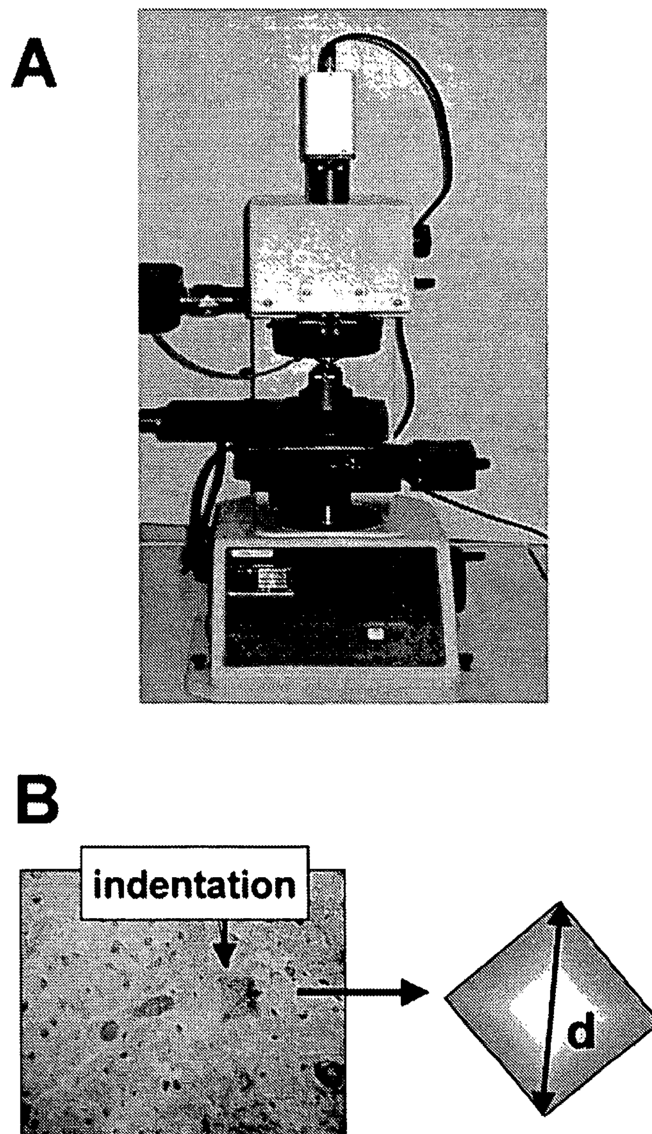

Figure 1. (A) Microhardness tester. (B) Sample surface indentation by a needle used to measure microhardness. The Vickers Hardness Number (VHN) was 0.1891 · P/d^2. P = applied load (kg); d = average diagonal length of an indentation (mm).

RESULTS

Establishment of a bone defect model in the dog mandible

Figure 2 shows the experimental design of 10-mm defects created in the dog mandible to obtain an environment hostile to bone regeneration. Subsequently, the bone-regenerating ability of the implants was evaluated histologically with a light microscope, and Vickers hardness test was conducted.

Macro finding observations for the implants

The PRP, dMSCs/PRP, PCBM and Bio-Oss® groups were implanted into 10-mm defects in the dog mandible. Macroscopic observations revealed that bone regeneration with dMSCs/PRP was greater than regeneration in the PCBM, PRP, Bio-Oss® groups and the control group (defect only), which was incomplete after 4 weeks. Furthermore, the control group exhibited only a shiny ap-

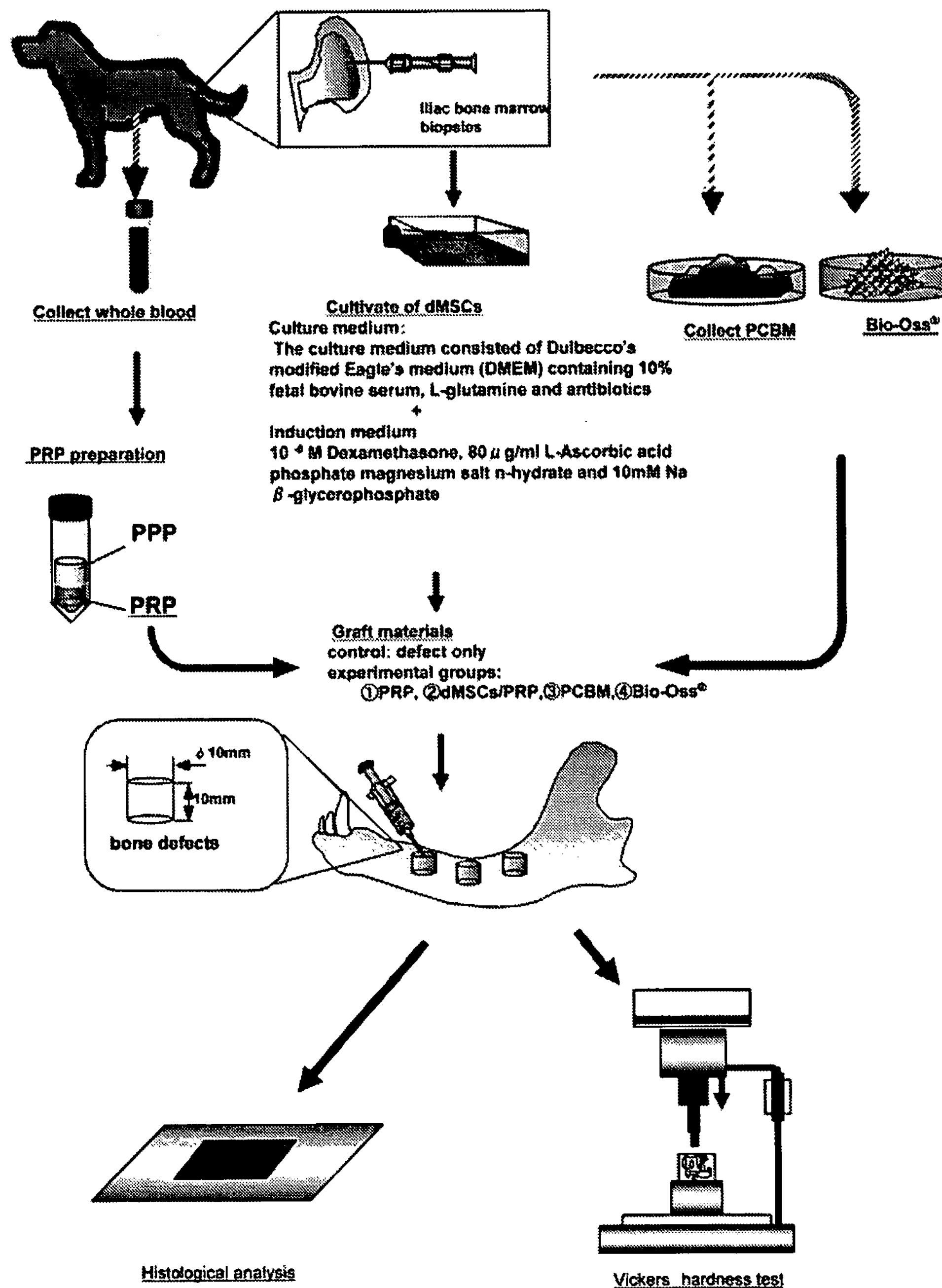

Figure 2. Schematic of the experimental protocol.

pearance and elastic consistency after 4 weeks. The dMSCs/PRP scaffold had almost completely disappeared without infection after implantation (Fig. 3).

Histological and light microscopic evaluation of the implants (PRP, dMSCs/PRP, PCBM, and Bio-Oss®) compared with the control *in vivo*

Implanted and nonimplanted control regions were collected after 2, 4, 8, and 12 weeks of implantation and were processed in decalcified and calcified forms for histology. For defects filled with PRP and the controls, cortical continuity was never restored, and the cavities were invaded by a vascular, fibrous tissue [Figs. 4(A–H) and 5(A–H)]. Furthermore, few new bone formations were seen after 4 weeks, and the Bio-Oss® group did not exhibit appreciable new bone formation up to the end of 12 weeks [Figs. 4(Q–T) and 5(Q–T)]. On the other hand, cavities filled with dMSCs/PRP resulted in new bone formation even after 2 weeks, which was manifested in a tubular pattern and by abundant vascularization after 4 weeks [Figs. 4(I,J) and 5(I,J)]. This pattern was indicative of a normal bone macrostructure with well-differentiated marrow cavity and cortices compared with cavities filled with

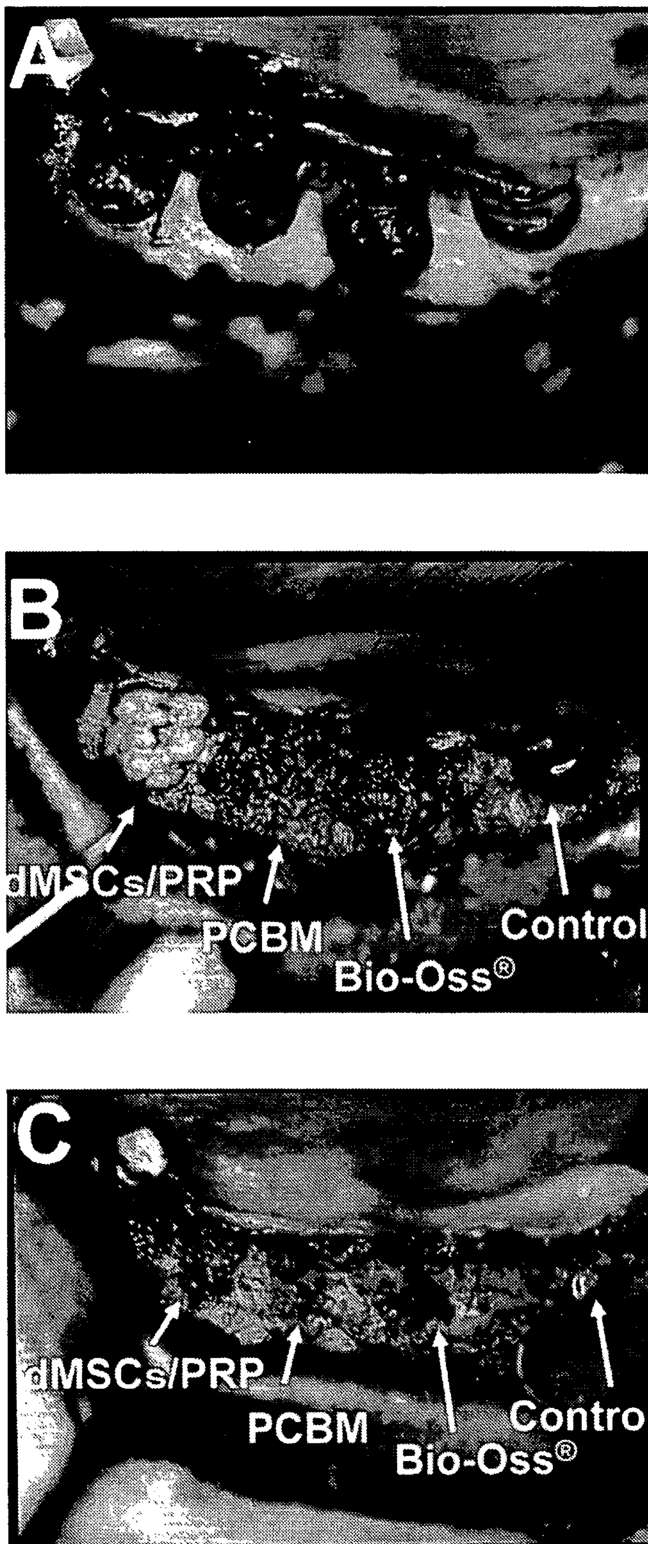

Figure 3. Macroscopic observations of bone regeneration. (A) Experimental design in the dog mandible. (B) Implanted materials in bone defects. (C) New bone regeneration for the dMSCs/PRP, PCBM, Bio-Oss®, and control groups after 4 weeks. Bone regeneration by dMSCs/PRP resulted in a natural marginal bone level, but the other materials were incomplete.

PCBM, which contained dead space due to transplanted PCBM resorption after 4 weeks [Figs. 4(M,N) and 5(M,N)]. After 8 weeks, we observed lamellar bone in the dMSCs/PRP group [Figs. 4(K) and 5(K)]. On the other hand, lamellar bone was still not observed in the PCBM group after 12 weeks [Figs. 4(P) and 5(P)]. The control group did not exhibit appreciable bone formation, and bone formation from natural bone was observed in the PRP group.

Histology is given in Figure 6. The control group exhibited soft tissue that was still present in the absence of bone bridging up to 8 weeks [Fig. 6(A–D)]. For the PRP group, there was fibrous tissue up to 4 weeks, and new bone and osteoid partly formed after 8 weeks [Fig. 6(E–H)]. The osteoblast lining was observed in the dMSCs/PRP group after 2 weeks, and bone formation was matured in a time-dependent manner [Fig. 6(I–L)]. In the PCBM group, osteoid was observed after 2 weeks, and bone that had formed from transplanted bone was observed even after 8 weeks [Fig. 6(M–P)]. In addition, the Bio-Oss® group could not produce well-calcified sections because of the presence of fibrous tissue and to the remains of the transplanted Bio-Oss® up to 8 weeks and could not initiate bone regeneration [Fig. 6(R)]. After 12 weeks, dMSCs/PRP and PCBM were almost completely replaced by new bone [Fig. 6(L,P)].

Microhardness test

Changes in each material's microhardness evaluated on the surface are summarized in Table I. The Vickers hardness test values (i.e., the newly formed bone area hardness values) after 2, 4, 8, and 12 weeks of implantation were 8, 16, 23, and 24 (control), 9, 15, 24, and 26 (PRP), Not Detected (ND), ND, ND, and 18 (Bio-Oss®), 11, 26, 28, and 30 (PCBM), and 17, 27, 31, and 34 (dMSCs/PRP), respectively (Table I). There were significant differences in newly formed bone hardness among the PCBM, PRP, Bio-Oss® groups, the control group, and the dMSCs/PRP group after 2 weeks ($p < 0.05$). In addition, hardness after 12 weeks was significantly greater in the dMSCs/PRP group than in the control, Bio-oss®, and PRP groups; however, no significant difference was noted among the PCBM, nature bone, and dMSCs/PRP groups ($p < 0.05$). In the tissue-engineered bone (dMSCs/PRP) group, hardness tended to increase during bone maturation at the early stage of implantation.

DISCUSSION

An ideal bone substitute should be biologically compatible, nonsupportive of local pathogens or

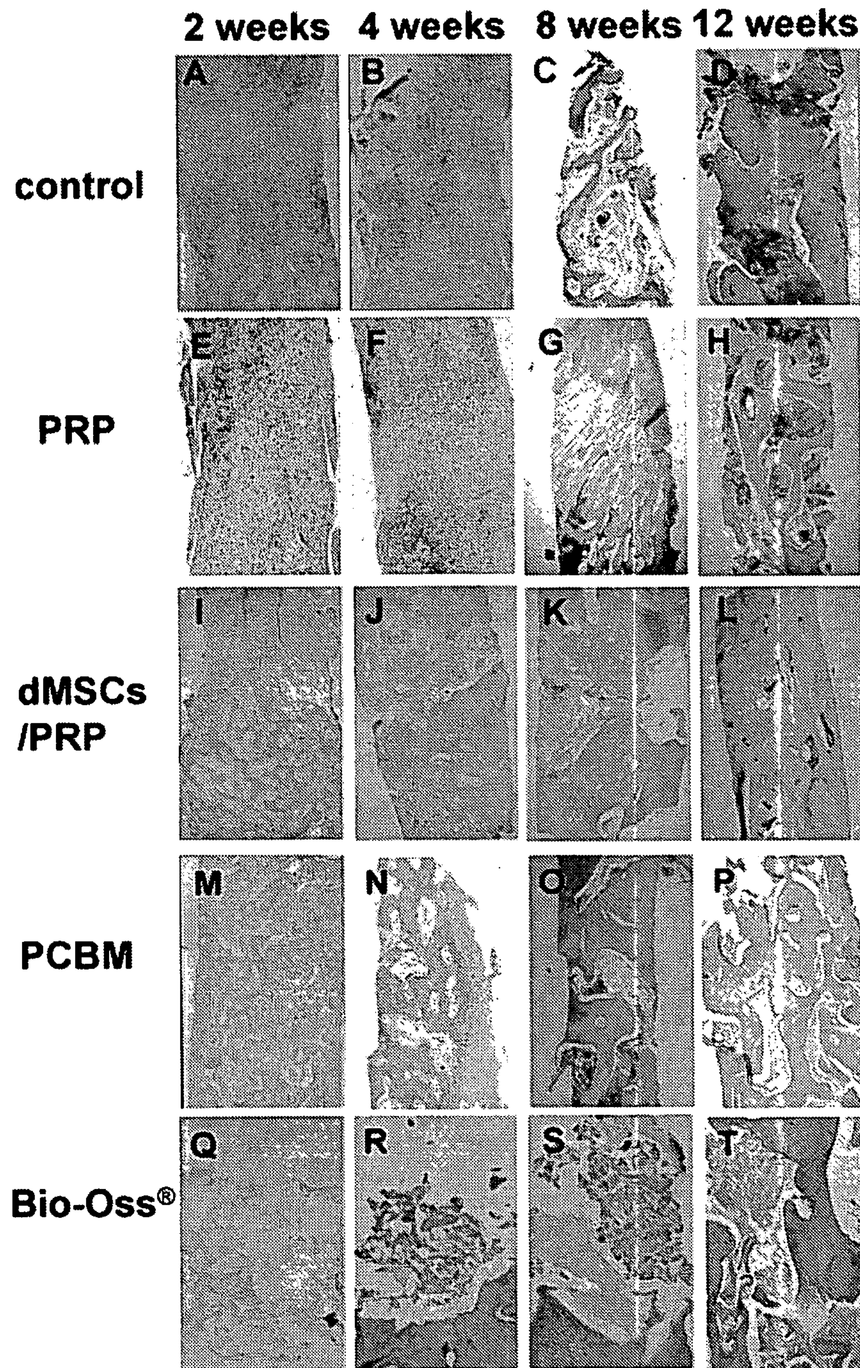

Figure 4. Histological evaluation of control (A–D), PRP (E–H), dMSCs/PRP (I–L), PCBM (M–P), and Bio-Oss® (Q–T) implantations for each week (lower magnification). Sections of representative implants are shown from their respective groups. The sections were stained by hematoxylin and eosin. Original magnification ×40 for all prints.

cross-infection, and osteogenic (i.e., an ability to facilitate bone cell ingrowth), and should match with the physical composition of natural bone trabeculae and provide scaffolding for new bone ingrowth. Furthermore, the substitute should be resorbable and osteotropic (i.e., bone formation enhanced by its chemical or structural characteristics), as well as microporous and easy to handle. Multiple methods have been used to conduct research in the bone restoration fields of craniomaxillofacial reconstructive, plastic, and orthopedic surgery. They involve a variety of materials and use techniques available to the surgeon for managing bone defects. At present, autogenous bone graft is still considered ideal because remodeling takes place without any immunological resistance, and graft material is advantageous in that it permits fast angiogenic ingrowth of vessels from transplanted or surrounding original bone.[14,15] Therefore, we examined the formation of autogenous bone, PCBM, as a positive control for these graft materials. According to histological

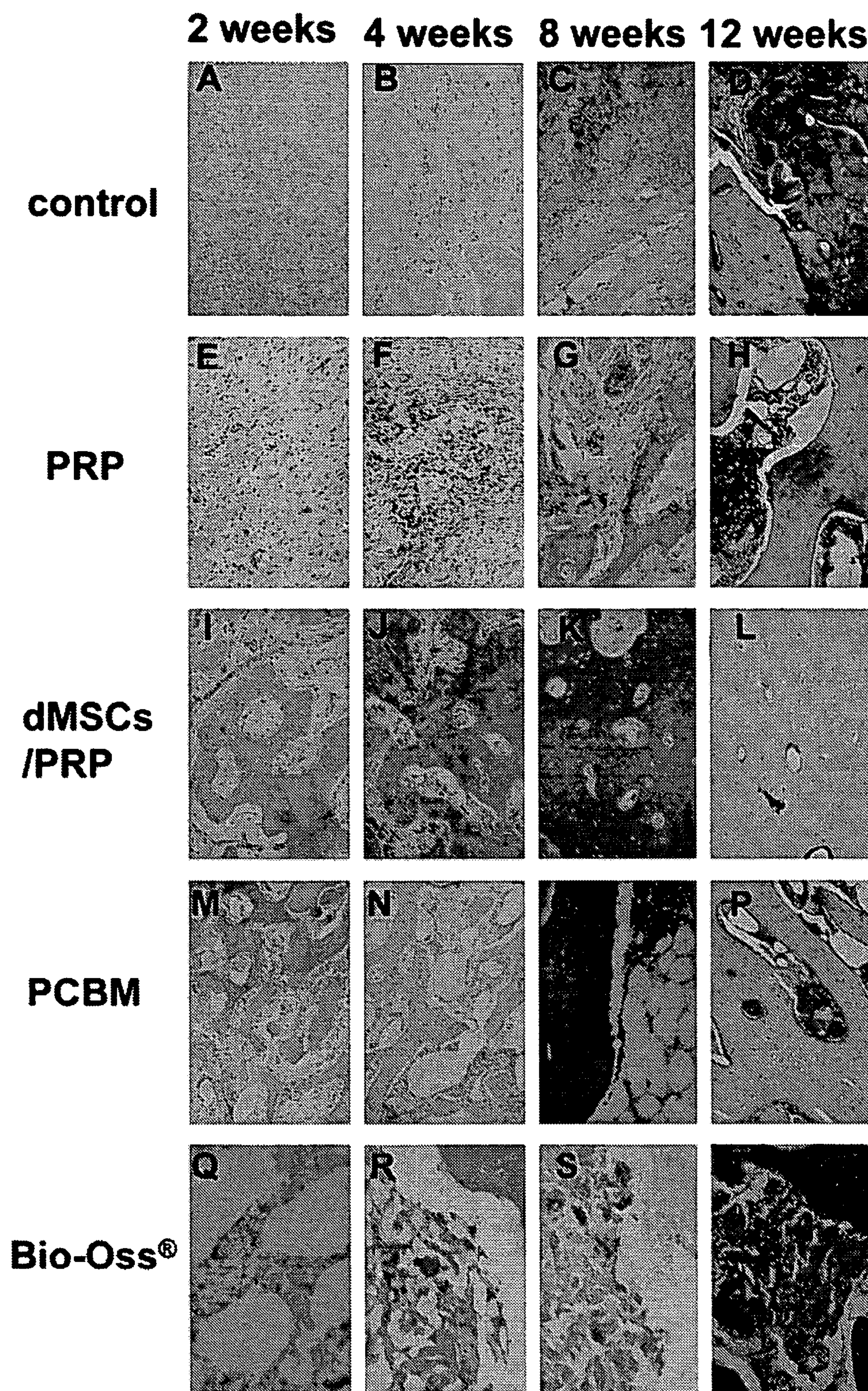

Figure 5. Histological evaluation of control (A–D), PRP (E–H), dMSCs/PRP (I–L), PCBM (M–P), and Bio-Oss® (Q–T) implantations for each week (higher magnification). Sections of representative implants are shown for each respective group. The sections were stained by hematoxylin and eosin. Original magnification ×200 for all prints.

observations, the PCBM group possessed dead space that was resorbed by implanted bone after 4 and 8 weeks, and newly formed bone gradually matured up to 12 weeks (Figs. 4 and 5). These results were identical to those seen by microscopic observation (Fig. 6). The Vickers hardness test values (i.e., the newly formed bone area's hardness values) in the PCBM group after 2, 4, 8, and 12 weeks were 11, 26, 28, and 30, respectively (Table I). On the other hand, the values in the dMSCs/PRP group after 2, 4, 8, and 12

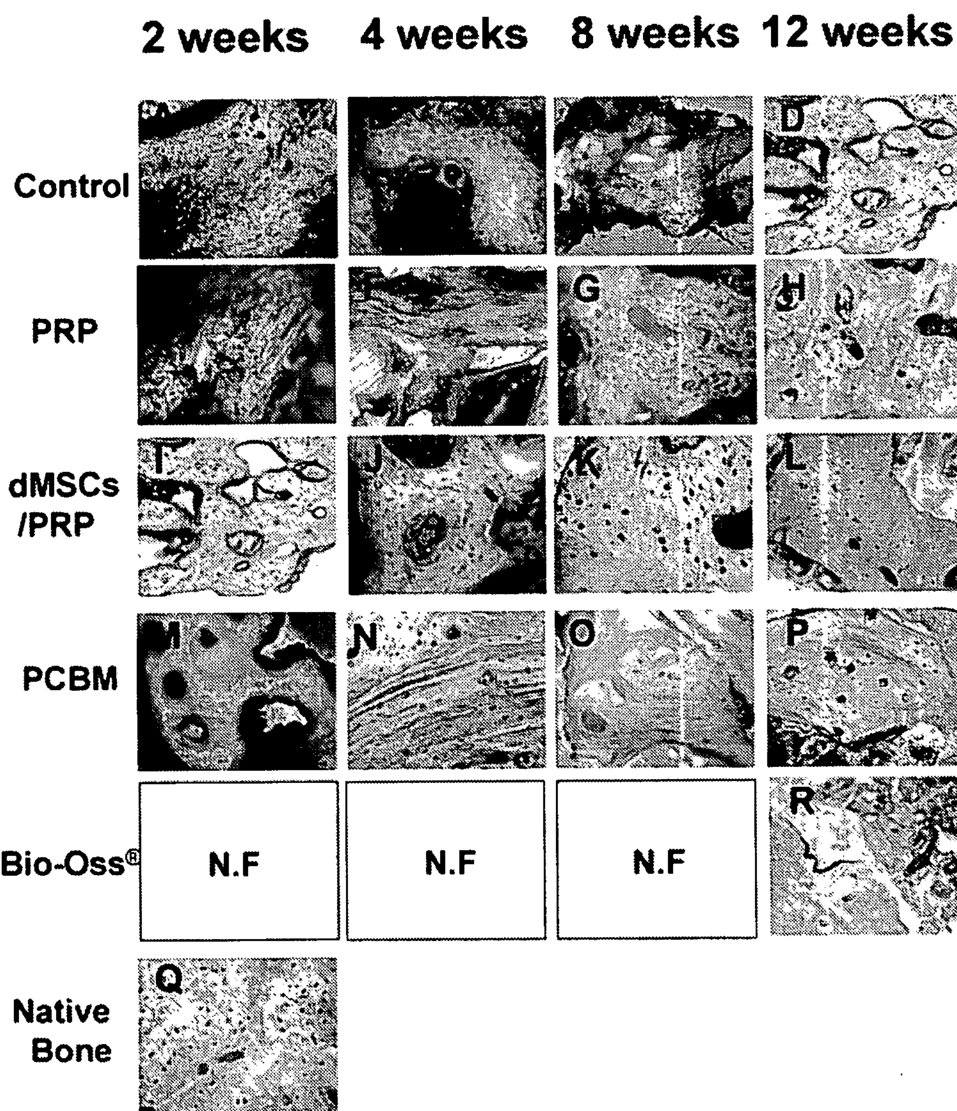

Figure 6. Light microscope evaluation of control (A–D), PRP (E–H), dMSCs/PRP (I–J), PCBM (M–P), and Bio-Oss® implantations for each week and native bone (Q) (higher magnification). Sections of representative implants are shown for each respective group. Original magnification ×200 for all prints.

weeks were 17, 27, 31, and 34, respectively. These values are equivalent and indicate better bone maturity than that in the control, PRP, and Bio-Oss® bone substitutes groups. Regarding mechanical hardness, furthermore, the dMSCs/PRP group also exhibited better bone formation than that in the PCBM group even at the early stage of implantation (2 weeks). In occlusal restoration with dental implants, our oral and maxillofacial surgeons must wait until the end of the healing period to allow good osseointegration, which requires 6 months for the maxilla and 3 months for the mandible.[16] However, these periods required for os-

TABLE I
Vickers Hardness Test

	2 weeks	4 weeks	8 weeks	12 weeks
Control	8.40 ± 1.50	16.10 ± 6.61	23.32 ± 2.53	24.20 ± 2.25
PRP	9.30 ± 1.64	15.40 ± 8.03	24.28 ± 2.94	26.30 ± 3.98
dMSCs/PRP	17.66 ± 2.60	27.42 ± 0.88	31.22 ± 3.27	34.46 ± 3.07
PCBM	11.24 ± 1.97	26.32 ± 2.94	28.44 ± 2.95	30.32 ± 2.62
Bio-Oss®	ND	ND	ND	18.24 ± 1.89

ND = not detected.
*$p < 0.05$.

teointegration constitute a burden for the patient. The better bone formation mentioned above suggests that bone formation with MSCs/PRP potentially shortens the healing period.

Recently, various bone substitutes have been used increasingly to simplify surgical procedures by eliminating the need for bone harvesting, which involves a potential risk of donor site morbidity as occurs with autogenous bone.[17] One such material is an anorganic porous bovine-derived bone mineral, Bio-Oss®. Bio-Oss® is useful as a graft material for sinus augmentation and guided bone regeneration (GBR) in dental and craniomaxillofacial surgery, but it is only slowly resorbed.[6,18,19] Therefore, we also examined its bone regeneration characteristics. In the present study, defects filled with Bio-Oss® displayed little bone formation up to 12 weeks (Figs. 4 and 5). In addition, light microscope observations showed that bone regeneration promoted by Bio-Oss® was not sufficient, and the same results were seen in the histological findings up to 12 weeks. The Vickers hardness test values of Bio-Oss® could not be measured until the end of 8 weeks because of insufficient bone regeneration and the presence of remnants and could be measured only at 12 weeks (Table I). Several studies have shown that this material possesses important properties (e.g., biocompatibility and osteoconductivity) when used as a scaffold for the ingrowth of host cells.[20–22] However, a bone substitute is gradually resorbed and replaced by vital bone. The biodegradable rate of Bio-Oss® is unknown, and most animal studies have indicated only slow bone substitution.[23-25] Bio-Oss® was hardly absorbed and did not promote sufficient bone formation in our study. A bone substitute should maintain its mechanical stability and volume during the initial healing phase and then be completely resorbed and replaced by newly formed bone.[26] From the view of resorption and mechanical stability, dMSCs/PRP is more suitable than the bone substitute.

Recently, tissue-engineered bone, such as MSCs/PRP used for regeneration, has been applied increasingly.[4–9] Tissue-engineered bone composed of MSCs/PRP provides better bone formation, suggesting that MSCs exhibit a positive effect when combined with PRP. In our study, the Vickers hardness test values after 2, 4, 8, and 12 weeks in the dMSCs/PRP group were 1.9, 1.8, 1.3, and 1.3 times higher than those in the PRP group, respectively. PRP matrices alone might not have an ability for bone formation, but the PRP scaffold for MSCs might encourage MSCs adhesion, proliferation, and differentiation, thus eliciting bone formation.[14] The implanted scaffold may become vascularized, because osteogenesis requires a well-developed vascular supply.[15] PRP contains an autologous source of platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β). These reinforcing growth factors introduced through the application of PRP to the wound improve soft tissue repair and bone regeneration. Ideally, the scaffold should be resorbed at a rate commensurate with new bone formation within a few weeks. Therefore, the use of tissue-engineered bone may provide conditions suitable for obtaining more rapid and effective bone regeneration attributable to PRP.

Light microscopy is greatly advantageous in distinguishing mineralized bone from osteoid. Because our samples were undecalcified, bone construction was clearly observable. In this study, dMSCs/PRP and PCBM were observed in mineralized bone structures and lamellar bone at the early stage of implantation in dMSCs/PRP. However, PRP or the control did not exhibit satisfactory bone formation after 2, 4, and 8 weeks, although PRP was observed in the osteoid after 8 weeks. We believe that these light microscopic observations are correlated with the hardness test. For the hardness test, the dMSCs/PRP group exhibited greater bone maturation than that in the PCBM group at the early stage of implantation. Other materials were not more suitable because bone maturation occurred slowly. These results were identical to those seen by histological observations. Thus, light microscopic observations reflect the microhardness tendency.

The present findings suggest that MSCs/PRP possesses excellent osteogenic characteristics and may serve for repairing of bone defects. In the future, this procedure of tissue-engineered bone grafting could be used instead of the procedures that use autogenous bone and could be used for the reconstruction of segmental bone defects after tumor dissection, trauma, dental implant, or periodontitis. In addition, the new bone formed by this procedure may provide excellent environments for other bony areas.

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Distraction Osteogenesis Assisted by Tissue Engineering in an Irradiated Mandible: A Case Report

Hideharu Hibi, DDS, PhD[1]/Yoichi Yamada, DDS, PhD[2]/Hideaki Kagami, DDS, PhD[3]/Minoru Ueda, DDS, PhD[4]

Distraction osteogenesis (DO) can provide predictable bone regeneration without grafting procedures but requires long treatment time and forms less bone transverse to the direction of distraction. To promote 3-dimensional bone formation and shorten the consolidation period, tissue-engineered osteogenic material (injectable bone) was applied in a patient who was being treated with vertical DO with an osteocutaneous fibular flap to reconstruct the mandible. The material, which comprised autologous mesenchymal stem cells culture-expanded then induced to be osteogenic in character and platelet-rich plasma (PRP) activated with thrombin and calcium chloride, was infiltrated into the distracted tissue at the end of distraction and injected into a space created labially with a titanium mesh at implant placement. The infiltration contributed to full consolidation of the regenerate for 3 months, and the injection thickened the regenerated ridge and bridged a gap between the native mandible and distracted fibula. The reconstructed mandible was expanded from 10 mm to 25 mm in height despite a lacerated and opened labial periosteum in the distracted area. Six implants 18 mm in length were placed and subsequently achieved osseointegration. The cutaneous flap covering the implants was trimmed, and the palatal mucosa was transplanted to the regenerated ridge for vestibuloplasty. These raw surfaces were covered with PRP; within 3 weeks, they had attained an epithelium. The implants have supported a fixed prosthesis with adequate surrounding bone and attached mucosa. DO was assisted by tissue engineering and became effective in restoring the compromised mandible. INT J ORAL MAXILLOFAC IMPLANTS 2006;21:141–147

Key words: *distraction osteogenesis, injectable bone, platelet-rich plasma, stem cells, tissue engineering*

Distraction osteogenesis (DO) has become a widely accepted technique for reconstructing bone defects in the maxillofacial region. This technique provides predictable bone formation without grafting procedures but requires a long healing time which includes latent, lengthening, and consolidation periods. To promote bone formation and shorten the consolidation period, some attempts at applying hyperbaric oxygenation or electrical, ultrasonic, or chemical stimulation have been made.[1] Several recent studies have shown that injecting cells with osteogenic potential into distracted callus enhances its consolidation.[2–5]

The present authors have recently reported on a tissue-engineered osteogenic material called "injectable bone," which comprises culture-expanded mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP).[6] Not only animal studies but also clinical trials have demonstrated that this material can effectively regenerate osseous tissue. It was therefore decided to apply the material to DO and present this case of the reconstruction of a mandible with damaged healing potential.

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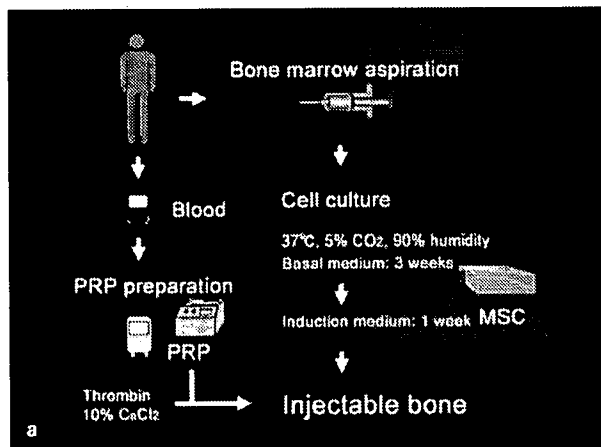

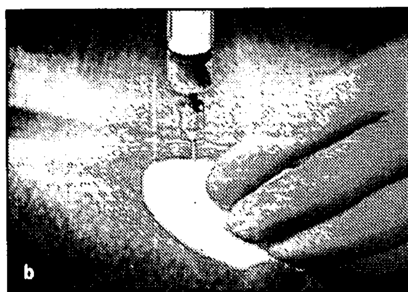

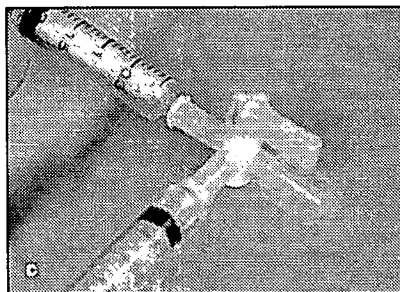

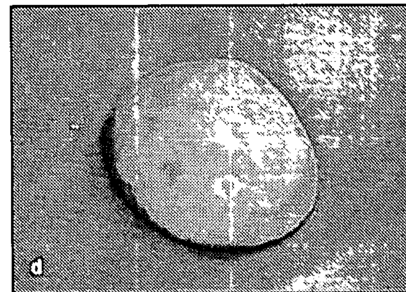

Fig 1a Preparation of MSCs, PRP, and injectable bone.

Fig 1b Aspiration of bone marrow.

Fig 1c Mixing materials of injectable bone. One syringe contains air, calcium chloride, and human thrombin; the other contains PRP and MSCs.

Fig 1d Injectable bone. Mixture keeps gel form for about 20 seconds.

MATERIALS AND METHODS

The MSCs and PRP are prepared as described previously.[6] The MSCs are isolated from iliac marrow aspirates, expanded in culture media for 3 weeks, and differentiated in supplemented osteogenesis induction media for another week. The PRP is isolated from autologous blood using density gradient centrifugation and a selective collection technique (Figs 1a and 1b). A 3-way stopcock connects 2 syringes; one contains 1 mL of air, 1 mL of 10% calcium chloride, and 1,000 units of human thrombin; the other contains 6 mL of PRP and all of the induced MSCs. This formula is standard except for the MSCs; the amount of those varies according to need. With the stopcock open, the contents of the 2 syringes are completely mixed for 5 seconds. The injectable bone mixture then maintains its gel form for about 20 seconds (Figs 1c and 1d).

CASE REPORT

A 54-year-old male patient was referred to the authors' hospital for rehabilitation of his reconstructed edentulous mandible. Two years earlier, the patient had undergone a segmental resection and immediate reconstruction of the mandible in conjunction with the oral floor resultant from squamous cell carcinoma, following chemotherapy and irradiation of 60 Gy. The reconstruction consisted of a 9-cm vascularized fibular graft osteotomized into 3 segments and fixed with 8 miniplates for the mandible and its cutaneous flap for the oral floor (Figs 2a and 2b). Computed tomograms demonstrated that the grafted fibula had remodeled into a bi-angled body of 1 cm in height and width (Fig 2c).

Vertical DO was planned in the area between the right mental foramen and the left reconstructed segment to allow dental implant placement. From the submandibular approach through the previous scar line under general anesthesia, complete osteotomies were performed with a sagittal saw following the removal of 6 plates and screws. A transport segment, which was 7 cm long, 5 mm high, and attached by a pedicle to the lingual periosteum, was created in the reconstructed mandible with the fibula. A distraction device (TRACK 1.5; Gebruder Martin, Tuttlingen, Germany) was adjusted and fixed with microscrews (Fig 3a). In closing the wound in layers, the periosteum labial to the horizontal osteotomy line mostly became lacerated and opened because of simultaneous removal of the previous osteosyntheses on this line. After a latent period of 7 days, the distractor was activated at a rate of 0.5 mm twice per day for 15 days (Fig 3b).

The injectable bone was applied to the distracted tissue at the end of the DO. The MSCs were derived from 10-mL iliac marrow aspirates and expanded in culture to the number of 5×10^7 cells. After induction, they

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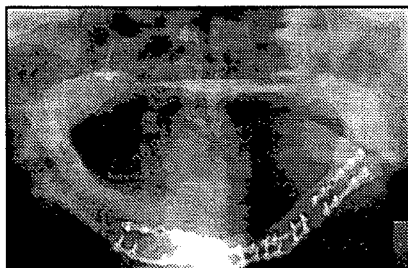
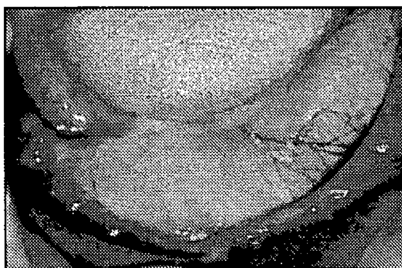




Figs 2a and 2b Reconstructed mandible and oral floor with vascularized osteocutaneous fibular flap.

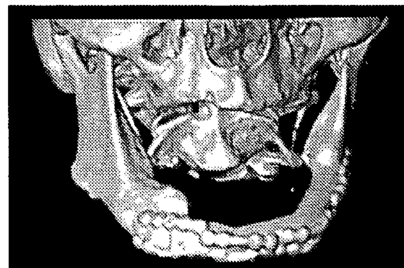


Fig 2c Grafted fibula remodeling into a bi-angled body of 1 cm in height and width.

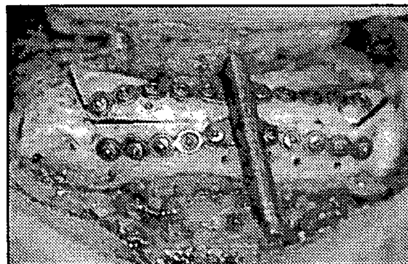


Fig 3a Distraction device. The periosteum lacerated and opened due to simultaneous removal of the previous osteosynthetic plates and screws.

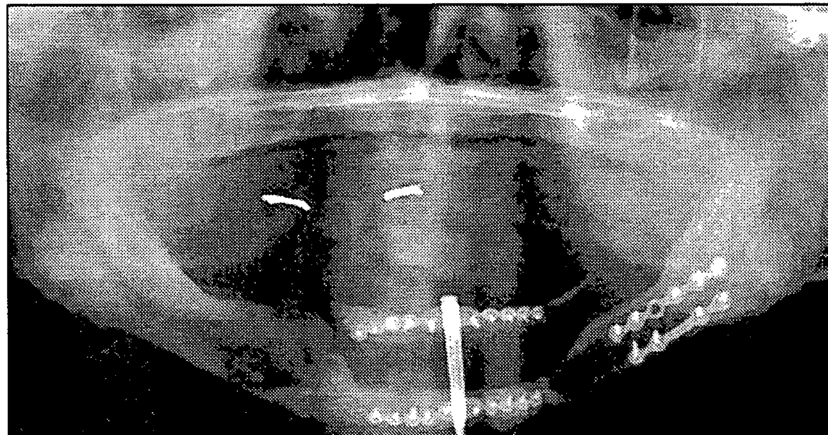
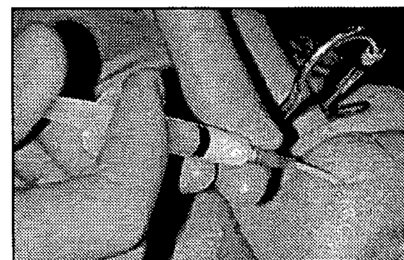


Fig 3b Immediately after distraction. Transport segment was repositioned 15 mm superiorly.



Figs 4a and 4b Application of injectable bone to distracted tissue with fluoroscopic guide.

expressed high alkaline phosphatase activity in assay. Twenty milliliters of PRP were isolated from 200 mL of blood; this PRP contained 1.6×10^9 platelets/mL, a concentration 8.3 times stronger than that of the original whole blood. With a C-arm fluoroscope for guidance, while the patient was under intravenous sedation, a 18-gauge needle was placed into the distraction gap (Fig 4a). The 3 mL of injectable bone was prepared and infiltrated for 15 seconds (Fig 4b). The needle was left in place for an additional minute to allow the gel to increase in viscosity and to prevent the injected material from leaking out of the puncture. No complications were observed during the injection, and the subsequent course was uneventful.

A series of monthly panoramic radiographs showed that radiopacity in the distraction gap had begun to appear at 1 month. After 2 to 3 months, during which the transport segment resorbed marginally (Fig 5a), the area became wholly radiopaque. Computed tomograms at 3 months revealed that newly formed bone in the distraction gap had

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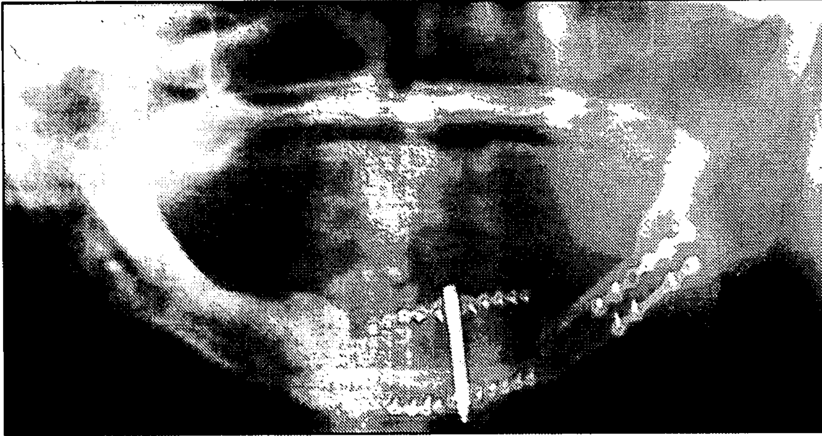

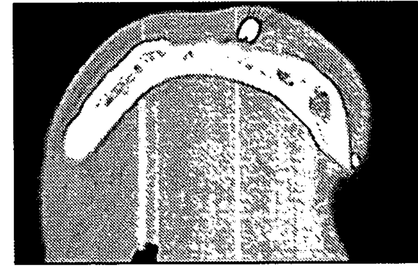

Figs 5a and 5b Three months after distraction. Radiopacity in the distraction gap. Newly formed bone in the distraction gap appeared unclear at the labial aspect but clear on the lingual cortical surface. The area in between, which had a relatively even density, was higher in terms of Hounsfield units than the neighboring cancellous area.

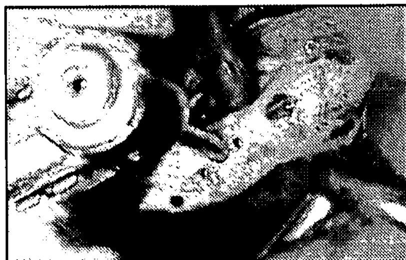

Fig 6a Biopsy sample removal with a trephine bur.

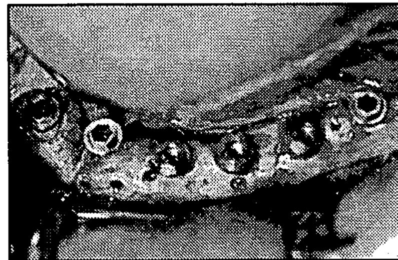

Fig 6b Shortage of marginal bone around the 2 implants furthest to the right.

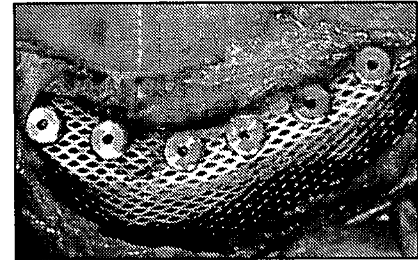

Fig 6c Marginal and labial space created with a titanium mesh.

Fig 6d Filling space with injectable bone.

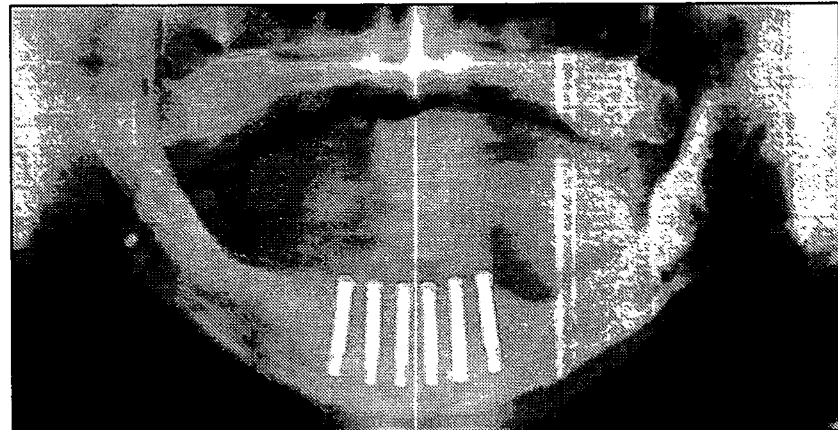

Fig 6e Radiograph obtained immediately after implant placement.

unclear labial surfaces but clear lingual cortical surfaces. The area in between, which was relatively even with respect to density, scored higher in Hounsfield units than the cancellous bone areas in the neighboring mandibular and fibular bone (Fig 5b).

The distraction device was removed and 6 titanium screw-type implants, 3.75 mm in diameter and 18 mm in length (Brånemark System, Nobel Biocare, Göteborg, Sweden), were placed under general anesthesia. During the preparation tissue specimens were taken with a trephine (Fig 6a). The implant furthest to the right was in native mandible, while the other 5 were in distracted bone. All implants required a torque of 40 Ncm for placement and achieved primary stability. The 2 implants furthest to the right had a shortage of surrounding marginal bone because of a gap in the bone between them (Fig 6b). A 0.1-mm-thick titanium mesh (Micromesh, Stryker, Kalamazoo, MI) was fixed to the platforms of the implants with cover screws, and additional space was created marginally and labially (Fig 6c). This space was filled with 3 mL of injectable bone prepared in the manner already described with 6×10^7 induced MSCs and PRP containing 3.6×10^9 platelets (Fig 6d).

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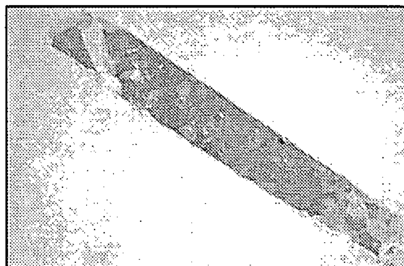
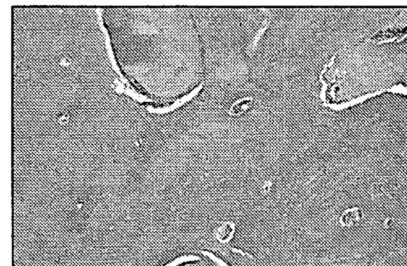

Fig 7a Decalcified section of specimen (hematoxylin and eosin; original magnification ×1.25).

Fig 7b Remodeling lamellar bone with abundant osteocytes in lacunae (hematoxylin and eosin; original magnification ×10).

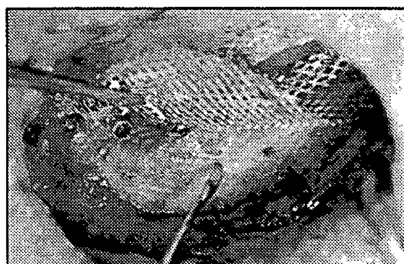
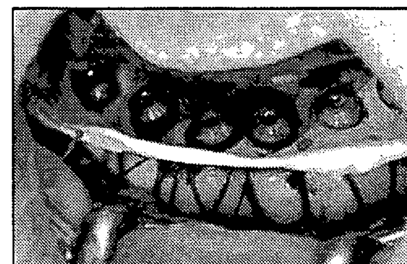

Fig 8a Regenerated hard tissue covered with periosteumlike membrane under a titanium mesh.

Fig 8b Vestibuloplasty of regenerated ridge. Transplanted palatal mucosa and dressing sheet.

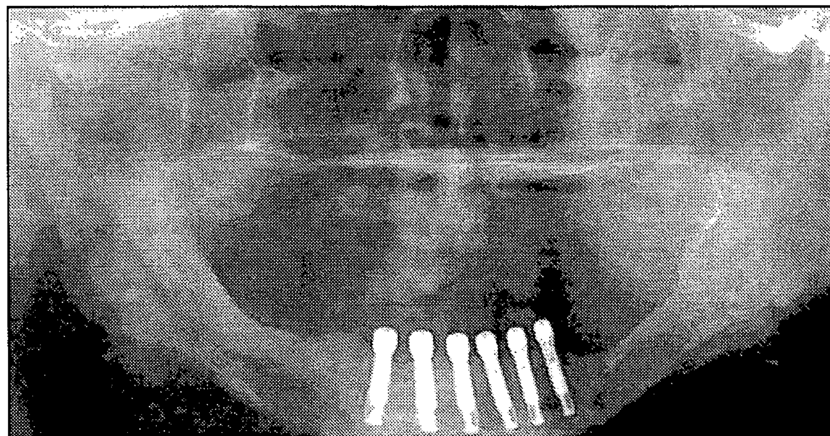

Fig 8c Radiograph obtained immediately after uncovering the implants.

The postoperative course was uneventful (Fig 6e). A decalcified section of the histologic specimen showed remodeling lamellar bone with abundant osteocytes in lacunae in the distracted zone (Figs 7a and 7b).

Three months after implant placement, the implants were uncovered, and the mesh was removed under general anesthesia. All implants had achieved osseointegration, and healing abutments were connected. Under the mesh regenerated hard tissue covered with the periosteumlike membrane was seen (Fig 8a). On this membrane at the labial and lingual sides of the regenerated ridge, palatal mucosa was transplanted for vestibuloplasty with the uncovered cutaneous flap defatted and positioned lingually and apically. The PRP activated with human thrombin and calcium chloride were applied to the raw surfaces in the palate and the mandibular ridge. These were covered with a temporary prosthesis and a lyophilized and irradiated porcine skin (Alloask, Taiho Pharmaceutical, Tokyo, Japan) for 5 days (Figs 8b and 8c).

Three weeks after the uncovering surgery, the donor sites in the palate fully epithelized and a marginal attached mucosa formed around the implants, which were connected to multiunit abutments (Fig 9a). A maxillary complete denture and a mandibular implant-supported prosthesis were placed and have functioned for a year without problem (Figs 9b and 9c).

DISCUSSION

A vascularized fibular flap is often selected for mandibular reconstruction because it offers adequate length of bone and pedicle, constant geometry, and low donor site morbidity. However, to follow the mandibular arch, the fibula requires multiple

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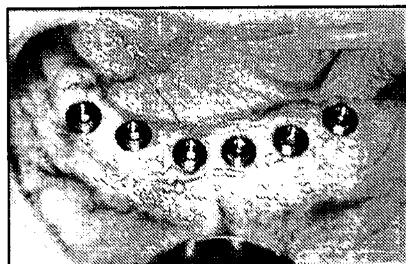

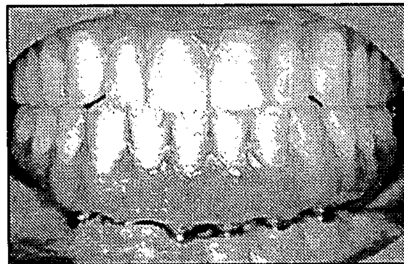

Fig 9a View of the implants 3 weeks after uncovering.

Fig 9b Prosthesis in place.

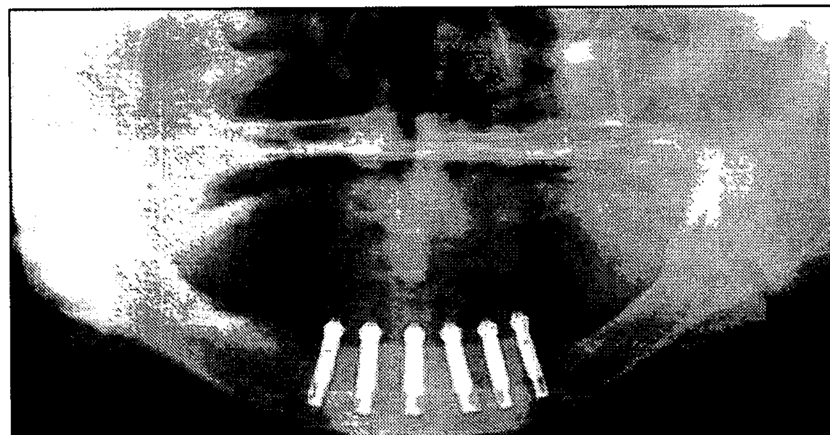

Fig 9c Radiograph obtained 1 year after seating the prosthesis.

osteotomies, which interrupt the medullary vessel and thereby vascular supply since the entire flap depends on the periosteum.[7] The fibular periosteum still supplies the external two thirds of the cortex after revascularization, while its internal third and the medulla have a reduced vascular supply.[8] Preservation of periosteal attachment is therefore considered a critical factor in DO, even if grafted fibular segments have healed and united. Several authors have reported on successful cases of vertical DO of the fibula grafted to reconstruct the mandible.[7,9] These cases were less complex than the present case, which included a patient with older age, a higher dose of irradiation, a larger transport segment, a longer distance of distraction, and damage to the labial periosteum resultant to simultaneous removal of osteosynthetic plates and screws. These conditions should reflect upon the partial resorption of the superior transport segment. Despite the reflection, the present case demonstrated new bone formation. Not only was the new bone formation less complicated on the labial side of the regenerate, it was also better quality inside, as observed radiographically and histologically, without a longer consolidation period. These favorable results might be attributed to the material injected into the distracted tissue.

Tissue engineering combines 3 key elements: cells, signaling molecules, and scaffolds.[10] For cells, the MSCs were applied; for signaling molecules, there were the growth and transforming factors in the PRP; and for scaffolding, there was the fibrin network of the PRP gel for the injectable bone.[6] In applying injectable bone to DO, they regarded the fibrous tissues in the distracted zone as the scaffold. Several animal studies have shown that the injections of cells with osteogenic potential into distraction gaps enhanced new bone formation with respect to volume and strength and that this enhancement led to shortening of the consolidation period.[2–5] The timing of the cell injections was further investigated; it appeared to have no effect on experimental outcome.[4] In this case the 15-mm distraction was considered relatively short, and the injection was administered at the end of the distraction because that is when the number of cells in the distraction gap with osteogenic potenial is the lowest. The injected cells could work before their gradual recruitment via vessel. Growth factors which alpha granules of the platelets secrete can activate cells, including MSCs and osteoblasts, through their membrane receptors.[11]

Partial resorption of the transport segment, which left the gap between its neighboring bone, was recovered with the injectable bone. Its gel form allowed the contained cells to contact surface microarchitecture of implants placed simultaneously. For space making with a relatively large shield, a titanium mesh was considered superior to polytetrafluoroethylene membranes because they restrict new vascularity.[12] The lack of blood supply might limit bone regeneration with the injectable bone to a certain amount. DO has few limitations regarding distraction length but requires longer treatment time

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than grafting. These innovative methods in combination can allow more effective bone regeneration for adequate implant placement.

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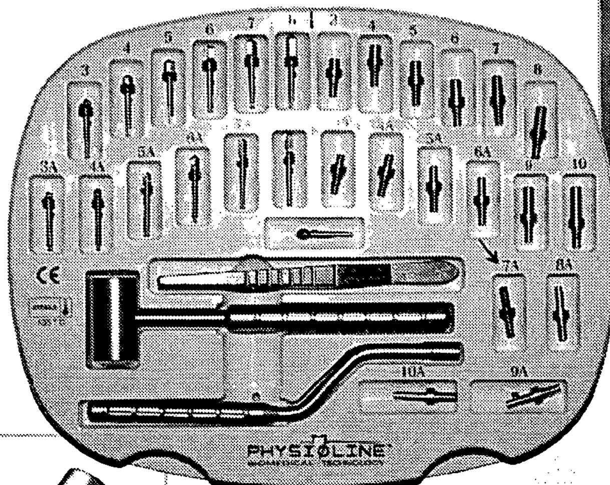

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