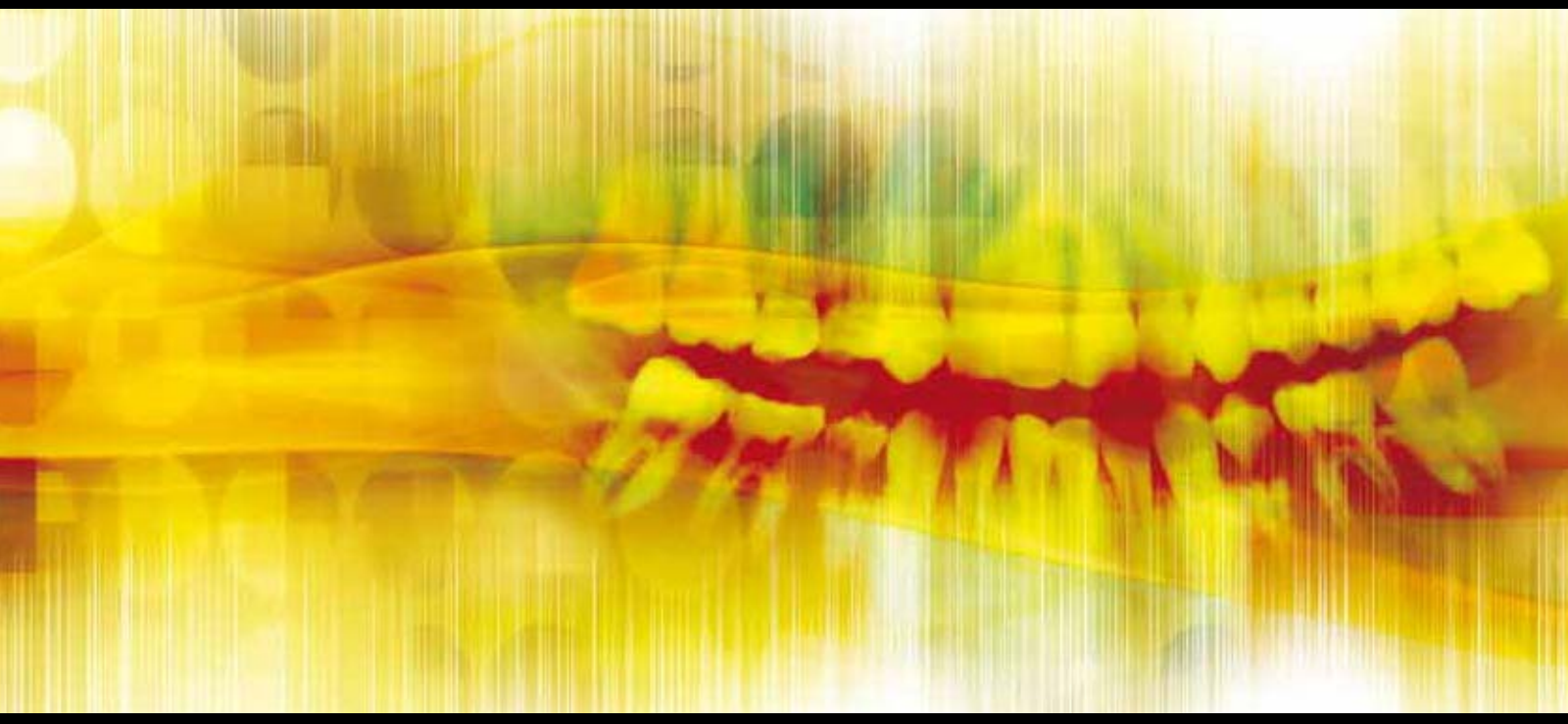
International Journal of Dentistry

Tissue Regeneration in Dentistry

Guest Editors: Kazuo Tanne, Petros Papagerakis, Gianpaolo Papaccio, Chiaki Kitamura, and Kotaro Tanimoto



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Review Article

Innovative Approaches to Regenerate Enamel and Dentin

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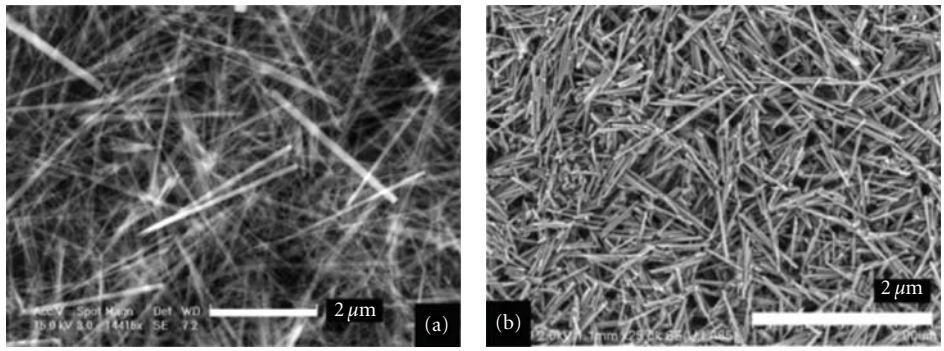
The process of tooth mineralization and the role of molecular control of cellular behavior during embryonic tooth development have attracted much attention the last few years. The knowledge gained from the research in these fields has improved the general understanding about the formation of dental tissues and the entire tooth and set the basis for teeth regeneration. Tissue engineering using scaffold and cell aggregate methods has been considered to produce bioengineered dental tissues, while dental stem/progenitor cells, which can differentiate into dental cell lineages, have been also introduced into the field of tooth mineralization and regeneration. Some of the main strategies for making enamel, dentin, and complex tooth-like structures are presented in this paper. However, there are still significant barriers that obstruct such strategies to move into the regular clinic practice, and these should be overcome in order to have the regenerative dentistry as the important mean that can treat the consequences of tooth-related diseases.

1. Introduction

Enamel is the outermost covering of vertebrate teeth and the hardest tissue in the vertebrate body. During tooth development, ectoderm-derived ameloblast cells create enamel by synthesizing a complex protein mixture into the extracellular space where the proteins self-assemble to form a matrix that patterns the hydroxyapatite [1] woven to form a tough, wear-resistant composite material [2]. The mature enamel composite contains almost no protein [3] and is a hard, crack-tolerant, and abrasion-resistant tissue [4]. During enamel biomineralization, the assembly of the protein matrix precedes mineral replacement. The dominant protein of mammalian enamel is amelogenin, a hydrophobic protein that self-assembles to form nanospheres that in turn influence the crystal habit and packing of the crystallites [5]. In contrast to the mesenchyme-controlled biomineralization of bone, which uses collagen and remodels both the organic and inorganic phases over a lifetime, enamel contains no collagen and does not remodel.

Mineralized dentin is synthesized by odontoblasts that line the centrally located dental pulp chamber and is deposited beneath the enamel and cementum [6]. Dentin, otherwise to the enamel, is soft flexible and able to absorb energy, and resists fracture. It is less mineralized than enamel, and it is a sort of sponge crossed by channels of one micron wide radically departing from the odontoblasts. These channels called "dentinal tubules," are occupied by a part of the odontoblasts whose cytoplasm body underlies the dentin-dental pulp interface. Dentinal fluids are also present in the tubules. Dentin is formed by mineralization of the dentin matrix mainly composed of collagen type I and some specific noncollagenous matrix proteins. The deposition of the dentin occurs over the life of the teeth. Sometimes in the immature dentin appear globules which are fusing during the maturation of the tissue [7]. Odontoblasts can be formed from dental pulp stem cells following a differentiation process induced by required signals [8]. It is also known that, in response to stimulation with recombinant BMPs, dental pulp cells differentiate into dentin-forming odontoblasts [9].



(a) (b)

Figure 1: SEM images of (a) fluorapatite nanorods prepared by direct precipitation from solution and (b) enamel crystals isolated from the maturation stage of rat incisor enamel [8]. (Reproduced with permission from the American Chemical Society.)

However, it is still unknown what is the required ideal combination of signals and the minimum set of cells, to engineer all the cellular components of a fully functional dental pulp, while the allegation that dental pulp stem cells may have the potential to differentiate into most cells of the dental pulp has not yet been strongly demonstrated in vivo.

Operative dentistry has been using regenerative processes to treat dental disease. The use of calcium hydroxide to stimulate reparative dentin is an example of therapeutic strategy. Tissue engineering enhances dentistry to move forward in the application of regeneration as important principle for the treatment of dental disease. It is based on fundamental approaches that involve the identification of appropriate cells, the development of conductive biomaterials, and an understanding of the morphogenic signals required to induce cells to regenerate the lost tissue. Extended research has started to emerge in the field of enamel and other dental tissue regeneration applying material-cell-based strategies. It is expected that strategies involving the use of tissue engineering, nanotechnology, and stem cells to have an increasing participation in clinical dentistry over the next 5–20 years [10]. There are major issues to overcome before such strategies be introduced into the clinic and used regularly to treat dental diseases. However, there is evidence that suggest tissue engineering as the main approach in the future of operative dentistry, for the development of new dental structures.

2. Making Enamel

Odontoblasts are found in the dental pulp of erupted teeth. In their absence, undifferentiated dental pulp cells or dental pulp stem cells can be differentiated into odontoblasts and restore the capability of the dental pulp to synthesize reparative dentin. However, ameloblasts which specialize in making enamel are not present in teeth with complete crown development. Consequently, an endogenous regeneration of enamel is not feasible, while the development of synthetic enamel and/or in situ cell-based approaches are being achieved by using the principles of tissue regeneration and nanotechnology.

2.1. Restoration: Synthetic Enamel Fabrication. Surfactants were used as reverse micelles or microemulsions to synthesize enamel, as they can mimic the biological action of enamel proteins [11]. The synthesized nanoscale structures may self-assemble into "one dimensional building blocks" leading to the development of hydroxyapatite nanorods similar to natural enamel crystals. The fabricated nanorods can potentially be applied as flowable restorative material for the restoration of lost enamel. Chen et al. [12] based on the biological processes involved in amelogenesis, combined with new approaches in nanotechnology, fabricated enamel prism-like structures consisted of fluorapatite nanorods (Figure 1(a)) precipitated directly from solution under controlled chemical conditions without the use of surfactants, proteins, or cells. The fabricated nanorods present similar characteristics to those of the natural enamel crystals isolated from rat incisor enamel, as it is confirmed from the scanning electron microscope (SEM) images in Figure 1(b).

Another enamel-based biomaterial having the added benefit of fluorapatite incorporated intrinsically into the composition was also observed. Particularly, amelogenin-driven apatite crystal growth, incorporating fluoride into the process, allowed the synthesis of elongated rod-like apatite crystals with dimensions similar to those observed in natural enamel [13]. Although the extended research for engineering advanced biomaterials, it is evidenced that none of the available material today can mimic all the physical, mechanical, and esthetic properties of enamel. This conclusion was an important parameter toward the establishment of cell-based strategies that could stimulate enamel regeneration.

2.2. Regeneration: Cell-Based Strategies. It has been suggested that extracellular matrix proteins such as fibronectin [14], laminin [15], and ameloblastin [16] not only function as a mechanical scaffold for cell attachment and survival but also provide a microenvironment for guiding cell growth and differentiating on. Considering this suggestion Huang et al. used an in vitro cell and organ culture system, to study the effect of artificial bioactive nanostructures on ameloblasts with the long-term goal of developing cell-based

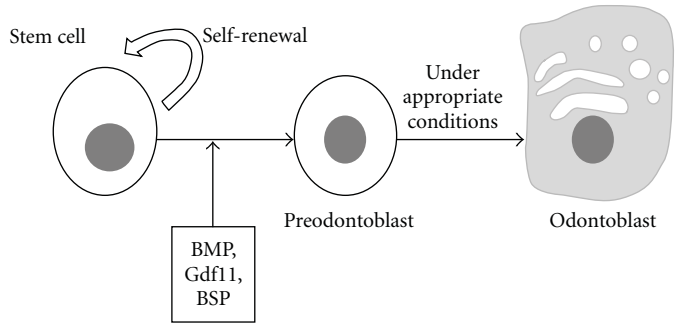


Figure 2: Differentiation of stem cell induced by appropriation signals such BMPs, Gdf11, or BSP into preodontoblast which can differentiate into odontoblast which can finally regenerate dentin.

strategies for tooth regeneration. Particularly, a branched peptide amphiphile molecules containing the peptide motif Arg-Gly-Asp or "RGD" (abbreviated BRGD-PA), known to self-assemble in physiologic environments into nanofibers network, was used in order to mimic the extracellular matrix that surrounds the ameloblasts. Ameloblast-like cells (line LS8) and primary enamel organ epithelial (EOE) cells were cultured within BRGD-PA hydrogels and formed focal multilayered structures that accumulated minerals [17]. BRGD-PA was also injected into the enamel organ epithelia of mouse embryonic incisors. At the site of injection, it was observed EOE cell proliferation with differentiation into ameloblasts as evidenced by the expression of enamel-specific proteins [17]. Moreover, it was shown the nanofibers within the forming extracellular matrix, in contact with the EOE cells engaged in enamel formation and regeneration. Finally it was concluded that BRGD-PA nanofibers present with enamel proteins participate in integrin-mediated cell binding to the matrix with delivery of instructive signals for enamel formation [17].

3. Making Dentin

A crosstalk that involves signals of diffusible molecules from the epithelium induces odontoblasts to synthesize extracellular matrix proteins required for dentin formation [18]. There is a big research in the field of the different inducers of dentin mineralization. The demineralized dentin powder, likewise the demineralized bone powder, observed to have also the capability to induce mineralization when applied directly to areas of pulp exposure [19, 20]. Specific functions of dentin seem to contain bone morphogenetic protein (BMP) activity, which induces reparative dentin formation, leading to the potentially use of BMPs in dentin regeneration [16, 20, 21].

Moreover the use of recombinant human proteins combined with collagen-based matrixes was applied to induce dentin regeneration. It was observed the induction of reparative dentin at the sites of pulp exposure within a period of 2 to 4 months [22, 23]. The general mechanism of this process is based on the fact that reparative dentin is formed where the stimulating agents were placed in direct contact with the dental pulp. This consideration was strengthened as it was observed a proportional dependence of the area of the induced reparative dentin with the amount of the applied BMP-7, which could eventually allow the predetermination of dentin's amount [24]. However the induction of reparative dentin was not successful in the case of inflamed dental pulps, which was assigned to insufficient amount of active recombinant protein due to its relative short half-life and to the faster degradation rates of the protein in the presence of the inflamed pulp [25].

The capability to induce reparative dentin was also found to growth/differentiation factor 11 (Gdf11) with a direct delivery to pulp cells applying a gene transfer strategy [26]. Additionally, bone sialoprotein (BSP) was observed to stimulate the differentiation of dental pulp cells into cells that can secrete extracellular matrix which is further mineralized into reparative dentin, presenting different morphological characteristics compared to the respective induced by BMP proteins [27]. This observation enhances the consideration that one day based on the patient's needs it will be possible to have the capability to select the ideal type of biological inducer for the desired reparative dentin.

In addition, the side population fraction of human dental pulp cells and the periodontal tissue stem cells derived from human-extracted teeth observed to partially regenerate dentin and periodontal tissue by cell transplantation into defects [28], suggesting that the transplantation of stem cells for partial tissue repair using autologous dental tissue stem/progenitor cells is possible when appropriate signals coexist, as it is schematically presented in Figure 2. These cells are thought to be already committed to dental cell lineages as they are able to form dental tissues without epithelial-mesenchymal interactions. In addition to specific cells and signaling molecules, the importance of scaffolds in guiding dentin regeneration has also been evaluated [29].

4. Current Research in Jointed Dentin-Enamel Regeneration

Tissue engineering using scaffold and cell aggregate methods has been also suggested to produce bioengineered complex dentin-enamel regeneration from dissociated cells. Shinmura et al. [30] investigated the capability of epithelial cell rests of Malassez (ERM) to regenerate dental tissues

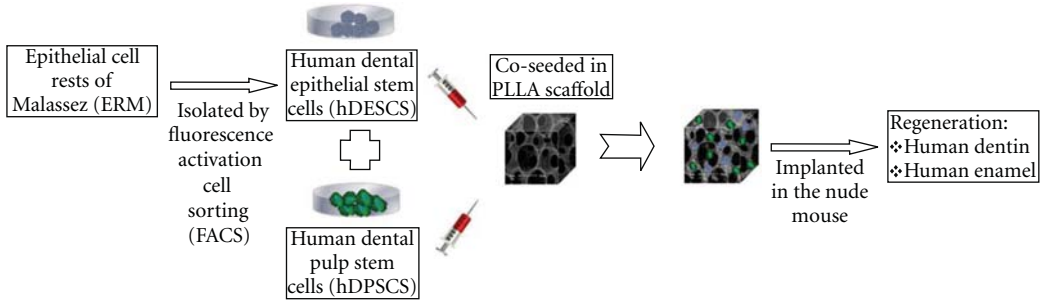


Figure 3: Layout of a cell-based strategy for the development of complex-like mineralized tissue by the co-seeding of hDESC and hDPSC.

by transplanting subcultured ERM seeded onto scaffolds into the omentum of athymic rats. Particularly, in combination with dental pulp cells at the crown formation stage, ERM was coseeded into collagen sponge scaffolds. After 8 weeks transplantation, enamel-dentin complex-like structures were recognized in the implants, as enamel-like tissue and the stellate reticulum-like structures were observed to some degree, while the tall columnar ameloblast-like cells were aligned with the surface of the enamel-like tissues. Similar results were observed in our lab with dental epithelial stem populations isolated by fluorescence activation cell sorting (FACS) using previously discovered epithelial stem cell markers [31] and subcultured under serum-free and xenon-free conditions. As it is illustrated in Figure 3, the collected human dental epithelial stem cells (hDESCs) can generate mineralized tissue in vivo when coseeded on PLLA scaffolds with human dental pulp stem cells (hDPSCs) and implanted subsequently in the nude mouse. After 10 weeks postimplantation mineralization is seen in the implants. Furthermore, complex dental tissues regeneration was investigated with different types of reassociations between epithelial and mesenchymal tissues and/or cells from mouse embryos which were cultured in vitro before in vivo implantation. In vitro the reassociated tissues developed and resulted in jointed dental structures that exhibited normal epithelial histogenesis and allowed the functional differentiation of odontoblasts and ameloblasts. After implantation, the reassociations formed roots and periodontal ligament, the latter connected to developing bone [32].

5. Conclusions: Future Trends

Regeneration of tooth parts is a complex attempt [33]. The treatment of tooth with inflamed pulp is considered as a main difficult challenge. A potential solution could be the application of appropriate advanced biological systems with therapeutic agents able to control the inflammatory response while inducing mineralization. An additional important challenge is the development of suitable carriers which can house all the necessary factors for the treatment and regeneration of lost/diseased tooth parts, while they should present biocompatibility, physicochemical, and mechanical properties compatible to their application in restorative dentistry. These new fabricated carriers should be able to create well-sealed restorations, preventing microleakages and subsequent contamination of the exposure pulp before the mineralization. The use of composites of synthetic or natural 3D scaffolds with bioactive antibacterial materials seeded with specific dental tissue stem cells could be a potential innovated system fulfilling all these significant requirements. Consequently, extended interdisciplinary research and effective collaboration between basic scientists and clinicians could potentially lead this field to the final goal of regeneration tooth parts or eventually the entire tooth.

Acknowledgment

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Research Article

Effect of Vitronectin Bound to Insulin-Like Growth Factor-I and Insulin-Like Growth Factor Binding Protein-3 on Porcine Enamel Organ-Derived Epithelial Cells

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The aim of this paper was to determine whether the interaction between IGF, IGFBP, and VN modulates the functions of porcine EOE cells. Enamel organs from 6-month-old porcine third molars were dissociated into single epithelial cells and subcultured on culture dishes pretreated with VN, IGF-I, and IGFBP-3 (IGF-IGFBP-VN complex). The subcultured EOE cells retained their capacity for ameloblast-related gene expression, as shown by semiquantitative reverse transcription-polymerase chain reaction. Amelogenin expression was detected in the subcultured EOE cells by immunostaining. The subcultured EOE cells were then seeded onto collagen sponge scaffolds in combination with fresh dental mesenchymal cells and transplanted into athymic rats. After 4 weeks, enamel-dentin-like complex structures were present in the implanted constructs. These results show that EOE cells cultured on IGF-IGFBP-VN complex differentiated into ameloblasts-like cells that were able to secrete amelogenin proteins and form enamel-like tissues *in vivo*. Functional assays demonstrated that the IGF/IGFBP/VN complex significantly enhanced porcine EOE cell proliferation and tissue forming capacity for enamel. This is the first study to demonstrate a functional role of the IGF-IGFBP-VN complex in EOE cells. This application of the subculturing technique provides a foundation for further tooth-tissue engineering and for improving our understanding of ameloblast biology.

1. Introduction

A frequent dental disease is dental caries which is a specific infectious disease that results in localized dissolution and destruction of the calcified enamel and dentin in teeth. However, enamel cannot regenerate by itself, because the layer of ameloblasts that forms the enamel degenerates after the tooth crown is completed. Thus dentistry has formulated artificial materials that mimic the hardness of enamel to repair enamel loss, but this may not be the most appropriate therapy. Therefore, the development of a novel approach to engineer natural enamel to repair enamel loss is strongly desired.

The growth of enamel is a highly complex process that is tightly regulated through a number of control mechanisms. Numerous growth factors involved in enamel development have been shown to interact with components of the extracellular matrix (ECM). Growth factors can regulate proliferation, determination, and differentiation of enamel-lineage cell phenotypes. The interaction between the ECM and growth factors is believed to be an important modulator of enamel development. However, many of the mechanisms behind these interactions remain unclear.

The insulin-like growth factor (IGF) family consists of two growth factors, IGF-I and IGF-II, which are mitogenic peptide growth factors. They are involved in a diverse

range of biological functions including development [1], cell proliferation and differentiation [2, 3], and DNA synthesis [4, 5], as well as insulin-like effects including an involvement in fat metabolism [1]. The IGF family is tightly regulated by two IGF receptors (IGF-IR and IGF-IIR), six IGF binding proteins (IGFBP-1 to IGFBP-6), and multiple IGFBP proteases [1, 6, 7]. Both IGF-I and IGF-II and their corresponding receptors are expressed throughout amelogenesis in rat incisors [8]. The IGF family is associated with the secretion of enamel-related proteins in rodent teeth [9], and furthermore, IGF-I stimulates cell proliferation in Hertwig's epithelial root sheath in the mouse molar [10].

Vitronectin (VN) is a multifunctional 75 kDa glycoprotein that is highly abundant in the blood and numerous tissues and forms a major component of the ECM [11]. VN plays an important role in diverse cellular processes, including cell migration, cell attachment, cell spreading, and hemostasis, that are mediated via α_v integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors which recognize an Arg-Gly-Asp [RGD] sequence) adjacent to the protein's N-terminus [12–15]. VN is also involved in the immune defense system through its interaction with the terminal complex of complement [16]. It has been suggested that VN binds to growth factors including epidermal growth factor and fibroblast growth factor [17, 18], hepatocyte growth factor, IGF-II [19], and IGF-I (via IGFBPs) [20]. The functional significance of these interactions has been confirmed through observation of *in vitro* cellular responses in culture plates pretreated with VN and IGF [21, 22], and the ability of VN to modify IGF action in smooth muscle cells has also been demonstrated [12]. Subsequent studies have revealed that IGFBP-3, enhances IGF-I binding to VN by forming a heterotrimeric complex comprising of IGF-1, IGFBP-3 and VN (IGF-IGFBP-VN) [20, 23, 24] and the complex results in enhanced functional responses [20, 21]. However, there have been no reports of this IGF-IGFBP-VN complex in the enamel-lineage cells, the enamel organ-derived epithelial (EOE) cells.

This study therefore examined whether the approach of prebinding IGF and IGFBP to VN-coated culture dishes would be effective in culturing EOE cells in order to produce enamel by tissue engineering methods. This strategy was adopted in an attempt to more accurately reflect the *in vivo* cellular environment (rather than an effect of IGF and IGFBP in solution) in which growth factors "captured" by ECM proteins participate in coordinated matricellular signaling [25]. The environment significantly enhanced cell proliferation and maintained the phenotype of the primary EOE cells. In addition, EOE cells cultured on IGF-IGFBP-VN-coated dishes, in combination with primary dental pulp cells, were capable of growing new enamel-like tissues. This study is one of the first studies to demonstrate the critical role of IGF-I, IGFBP-3, and VN on EOE cells.

2. Materials and Methods

2.1. Isolation and Subculture of Porcine EOE Cells. EOE cells were prepared as previously described [26, 27]. Briefly, impacted third molars were harvested from the mandibles of 6-month-old pigs. After hard tissues were disconnected from the tooth, the enamel organ was separated from the dental pulp by treatment with dispase II (Goudou Syuzei, Tokyo, Japan) and then mechanically isolated. Minced enamel organ was treated with collagenase (Wako, Osaka, Japan) in Hank's balanced salt solution (Invitrogen, Life Technologies, NY). The released cells were passed through a 70 μm cell strainer (Becton Dickinson & Co., Franklin Lakes, NJ) and were then cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen) under 10% CO_2 in air for 7 days.

A mixed cell population of EOE cells and dental follicle cells were observed in primary culture. Then, to isolate the epithelial cells from the mesenchymal cells, the medium was replaced with LHC-9 medium (Biofluids, Bethesda, MD) without FBS after the cell populations reached confluence [26]. The cells were cultured under 10% CO_2 for 2 weeks, during which time most of the contaminating dental follicle cells disappeared, leaving only morphologically identifiable epithelial cells. The epithelial cells were trypsinized and inoculated onto the specified culture dishes (1×10^5 cells/cm^2). Complete minimum essential medium based on α-MEM (Invitrogen) supplemented with 5% FBS, insulin (5.0 μg/mL), transferrin (5 μg/mL), triiodothyronine (2×10^{-10} M), cholera toxin (1×10^{-10} M), hydrocortisone (0.5 μg/mL), epidermal growth factor (0.1 μg/mL), penicillin (1000 U/mL), streptomycin (1 mg/mL), and amphotericin B (2.5 μg/mL) was applied to the subsequent subcultures. The cultured cells were observed by phase-contrast microscopy on indicated days.

2.2. Prebinding of IGF-I and IGFBP-3 to VN (IGF-IGFBP-VN). To examine the effect of IGF-IGFBP-VN on EOE cells, culture plates were pretreated overnight with VN (150 ng/cm^2; Tissue Therapies, Brisbane, Australia) prior to the addition of growth factors including IGF-I (50 ng/cm^2; Tissue Therapies) and IGFBP-3 (150 ng/cm^2; Tissue Therapies), according to the protocol [19, 28], and each treatment was usually performed overnight at 4°C. All reagents were prepared in serum-free medium. For comparison, either polystyrene (PS) or collagen- type- I- (Col-I-) coated dishes (Becton Dickinson & Co.) were used to evaluate cell growth and differentiation.

2.3. Measurement of Cell Growth. The growth of 1st and 2nd passage EOE cells on IGF-IGFBP-VN-coated dishes was examined in comparison with PS, and Col-I dishes. Subcultured EOE cells were plated at a density of 5×10^3 cells/mL into 6-well IGF-IGFBP-VN, PS and Col-I culture plates. The EOE cells in each well were counted using a WST-8 kit (Cell-counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). The counting technique employed a tetrazolium salt that produced a highly water-soluble formazan dye. After 1 hour of incubation with the reagent according to the manufacturer's instructions, relative cell numbers were determined by measuring the absorbance of light at a wavelength of 450 nm on days 1, 10, and 25 (Model 650 Microplate reader; Bio-Rad Laboratories, Hercules, CA). The experiment on cell growth was performed in triplicate.

Statistical analysis was performed using Mann-whitney U test with Bonferroni's correction. Data are presented as the mean ± standard deviation for three separate experiments. A significant difference ($P < 0.05$) between paired conditions is indicated on Figures by an asterisk.

2.4. RNA Preparation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. Total cellular RNA was purified from primary cells in nonserum culture medium for 10 days and subcultured cells at 10 days after first passage from three samples, using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using Superscript III RNase H- (Invitrogen). Synthesized cDNA served as a template for subsequent polymerase chain reaction (PCR) amplification. PCR primers for amelogenin, ameloblastin, enamelin, matrix-metalloprotease- (MMP-) 20, collagen type I, IGF-I, and IGF-I receptor are listed in Table 1. Amplification was performed in a PCR Thermal Cycler SP (Takara, Tokyo, Japan) for 25–35 cycles according to the following reaction profile: 95°C for 30 s, 45–60°C for 30 s, and 72°C for 30 s. Porcine β-actin primer was used as an internal standard.

2.5. Immunocytochemistry. We tested whether the EOE cells at second passages differentiated into ameloblast-lineage cells in the IGF-IGFBP-VN or Col-I dishes. EOE cells, grown for 10 days on coverslips, were fixed in 4% paraformaldehyde for 10 min at room temperature and then treated with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 5 min to render them permeable. The cells were then incubated with the 4% horse serum diluted in 0.01 MPBS. After blocking, the cells were incubated for 60 min in affinity-purified rabbit anti-pig amelogenin polyclonal antibody (1 : 100 dilution; a gift from Dr. J. P. Simmer, University of Michigan, Ann Arbor, MI) as an ameloblast marker. FITC-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH) was then applied for 60 min at room temperature. The stained cells were sealed with Vectashield mounting medium containing DPAI (Dojindo) diluted 1 : 2000. Nonimmune rabbit serum was used to replace primary antibody as a fluorescence control.

2.6. Preparation of Collagen Sponge Scaffolds. Based on our previous results, collagen sponges were selected as scaffolds for our *in vivo* study (product number: CL025-PH56f/FD90H48-02F26; a gift from NIPRO Corporation, Osaka, Japan). The performance of collagen sponge has been shown to be superior to that of polyglycolic acid fiber mesh [29]. Briefly, scaffolds approximately 10 mm in diameter and 2 mm in thickness were prepared from a 2.5% aqueous solution of collagen extracted from porcine skin. They contained 75% (dry weight) type I atelocollagen and 25% type III atelocollagen and were frozen at −40°C and vacuum-dried to produce a porous matrix (pore volume fraction, 97.5%).

2.7. Enamel-Tissue Engineering Using a Combination of EOE Progenitor Cells and Dental Pulp Cells In Vivo. All experiments involving the use of animals were approved by the Institutional Animal Care and Use Committees of the Institute of Medical Science at the University of Tokyo, Japan.

Previously, we established a method to generate enamel based on a cell-scaffold construct followed by transplantation *in vivo*. To determine whether EOE cells subcultured using IGF-IGFBP-VN culture dishes have the potential to generate enamel tissues, we used our transplantation experiment. Primary dental pulp cells were obtained from impacted third molar teeth in the mandibles of 6-month-old pigs. After hard tissues were discarded from the tooth, only dental pulp was disconnected from the enamel organ by treatment with dispase II (Goudou Syuzei, Tokyo, Japan) and then the pulp core in the center of the dental pulp was mechanically isolated to prevent contamination of the dental follicle. Minced pulp core was treated with collagenase (Wako) in Hank's balanced salt solution (Invitrogen) for 30 min at 37°C. The released cells were passed through a 70 μm cell strainer (Becton Dickinson & Co.).

The cell-seeding technique involving the combination of high densities of subcultured EOE cells with high densities of primary dental pulp cells has been described previously [30]. A high density of primary dental pulp cells (30 μL of 5.0×10^6 cells/mL) was first placed on top of the collagen sponges and incubated for 1 h. Subsequently, a high density of EOE cells at day 10 of cultivation (30 μL of 5×10^6 cells/mL) after 1 passage was seeded directly on top of the dental pulp cells. The subcultured EOE cells were allowed to adhere onto the dental pulp cells for an additional hour. In the control group, oral keratinocytes obtained from the oral mucosa of 6-month-old pig mandibular jaws were subcultured and seeded at high density on the top of the dental pulp cells after the primary dental pulp cells had been seeded onto the collagen sponges ($n = 3$). The scaffolds with cells were then transplanted into the omentum of immunodeficient rats aged 5–7 weeks (F344/n Jcl-rnu, Nihoncrea, Japan) ($n = 3$). The omentum was sutured to prevent movement of the test and control implants [31–33]. The implants were allowed to develop for 4 weeks after which time they were fixed in Bouin's solution and demineralized in 30% EDTA and then embedded in paraffin. 5 μm sections were cut and stained with hematoxylin and eosin for histological analysis.

Since epithelium and pulp tissues are tightly connected, there is a high chance of contamination. This was monitored by observation with phase contrast microscopy and RT-PCR using the cultivation of dental pulp cells. This confirmation has been used in our previous studies [34, 35].

2.8. Immunohistochemistry. Immunohistochemical analyses were performed on paraffin-embedded tissue sections with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame) using affinity-purified rabbit anti-pig amelogenin polyclonal antibody (1 : 2000 dilution, gift from Dr Simmer, University of Michigan, USA) as the primary antibody. Standard procedures [36] were modified as described in detail by Chen et al. [37].

Table 1: Sequence of primer pairs used for semiquantitative RT-PCR.

Gene		Sequence	Annealing temperature (°C)	Amplicon (bp)	Accession number or reference
Amelogenin	Forward	5-CCTGCCTTTTGGGAGCA	50	328	NM213800
	Reverse	5-TGGTGGTGTTGGGTTGGA			
Ameloblastin	Forward	5-ATTCCCAACCTGGCAAGAGG	55	380	NM214037
	Reverse	5-AGCGCTTTTAATGCCTTTGC			
Enamelin	Forward	5-TGAGGAGATGATGCGCTATG	45	315	NM214241
	Reverse	5-TGAGGTGTCTGGGTTTCCTC			
Enamelysin	Forward	5-ATACGTGCAGCGAATAGATGC	45	290	NM213905
	Reverse	5-CTATTTAGCAACCAATCCAGG			
Collagen type I	Forward	5-GATCCTGCTGACGTGGCCAT	55	212	AY350905
	Reverse	5-ACTCGTGCAGCCGTCGTAGA			
IGF-I	Forward	5-GACGCTCTTCAGTTCGTGTG	50	348	NM214256
	Reverse	5-ACTCGTGCAGAGCAAAGGAT			
IGF-IR	Forward	5-ACTGTATGGTGGCCGAAGAC	50	391	NM214172
	Reverse	5-ATCTCGTCCTTGATGCTGCT			
beta-actin	Forward	5-TCGACCACAGGGTAGGTTTC	45	497	AF017079
	Reverse	5-CCCCAGCATCAAAGGTAGAA			

3. Results

3.1. Effect of IGF-IGFBP-VN on EOE Cell Growth. EOE cells were easily obtained from explant culture, but the culture was a mixed population (Figure 1(a)). After replacing the serum-containing medium with nonserum medium, the dental follicle cells disappeared and only the EOE cells were left. The EOE cells showed the cobblestone morphology that is typical of epithelial cells (Figure 1(b)). However the EOE cells did not grow in the nonserum medium, so selected EOE cells were then plated onto the IGF-IGFBP-VN-coated dishes (passage 1) and after four days of subculture, several small colonies of EOE cells appeared (Figure 1(c)). The colonies increased with time and became confluent after 25 days of cultivation and were then subcultured as passage 2 (Figure 1(d)). Both groups of passaged cells showed the typical polygonal-shaped epithelial morphology. EOE cells were also cultured onto PS (Figure 1(d)) and Col-I-coated dishes (passage 1) (Figure 1(e)); however, the EOE cells displayed an extended morphology. In addition, EOE cells could not be grown on either PS or Col-I dishes after the second passage.

We compared the rate of cell proliferation of EOE cells that were cultured on the IGF-IGFBP-VN, Col-I, and PS culture dishes at passage 1 (Figure 2). We found that EOE cells grew at similar rates on the IGF-IGFBP-VN and Col-I culture dishes until day 10 of cultivation, but the cells exhibited a much lower proliferation rate at day 25 of cultivation when cultured on the Col-I dishes. There was a significant difference ($P < 0.05$) at day 25 between the growth rates in these different culture conditions. Interestingly, EOE cells did not grow on the PS dishes.

3.2. Differentiation of EOE Cells. We studied whether the EOE cells would be able to differentiate into ameloblasts. RT-PCR was used to examine the expression of various ameloblast-related genes (Table 1) in the EOE cells. The EOE cells, grown in LHC-9 as primary culture cells, expressed mRNA for amelogenin, ameloblastin, enamelin, MMP-20, IGF-I, IGF-I receptor (IGF-IR), and collagen type I. After subculture at passage 1, expression of amelogenin and MMP-20 was not detected in the PS culture though some expression of ameloblastin and enamelin was detected (Figure 3). The expression pattern of the ameloblast-related genes of the EOE cells in the Col-I culture was similar to that of the IGF-IGFBP-VN culture. Interestingly, mRNA of amelogenin in the IGF-IGFBP-VN culture was more highly expressed than that in the Col-I culture. In addition, expression of IGF-I and IGF-IR mRNA was higher in the IGF-IGFBP-VN culture than that in the Col-I culture. Collagen type I gene was not detected in any of the cultures at passage 1 (Figure 3).

Using immunocytochemistry, we next examined the protein expression of amelogenin to determine whether the EOE cells were differentiated into ameloblasts. Amelogenin expression was detected in the EOE cells in both the Col-I (Figures 4(a) and 4(b)) and IGF-IGFBP-VN cultures (Figures 4(c), 4(d), and 4(e)) after 14 days cultivation at passage 1. The degree of expression in the IGF-IGFBP-VN culture was higher than that of the Col-I culture. There was no expression of amelogenin in the EOE cells under PS culture conditions (data not shown).

3.3. Histology of the Tissue-Engineered Enamel-Dentin Complexes. We examined the enamel-forming capability of the EOE cells by transplanting seeded collagen sponges into the omentum of athymic rats. These *in vivo* experiments were performed 3 times for a transplantation period and obtained consistent data at the time period examined.

At four weeks after transplantation, the implants from the scaffolds seeded with both cultured EOE cells and fresh dental pulp cells revealed hard tissue formation (Figure 5(a)). At this time, the scaffolds were already

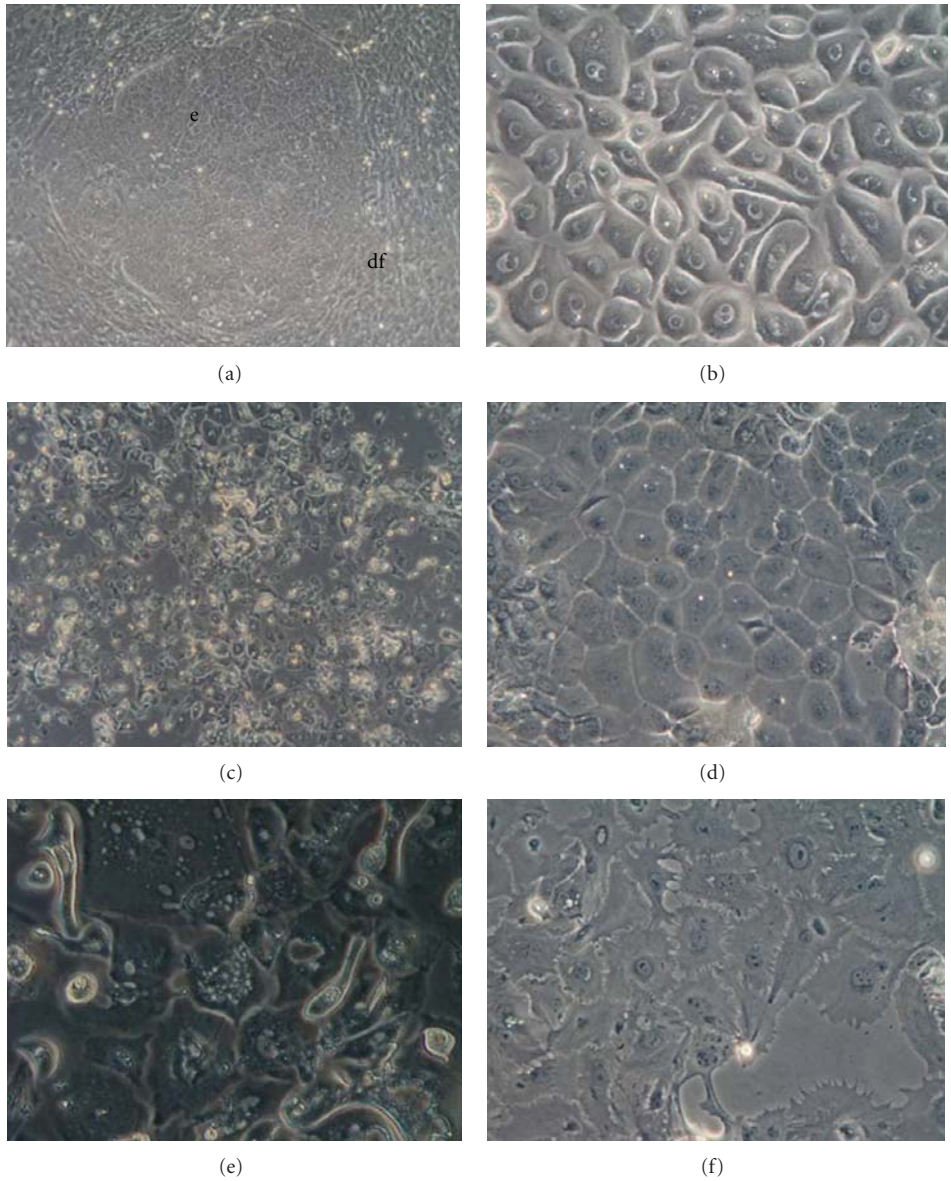

Figure 1: Phase-contrast micrographs. (a) Mixed culture of enamel organ-derived epithelial (EOE) cells and dental follicle cells after 1 week of culture. The epithelial cells (e) have formed an island within the strongly proliferating dental follicle cells (df). (b) EOE cells in LHC-9 medium. After replacing the serum-medium with LHC-9, only EOE cells survived in the nonserum medium. The colony of EOE cells showed a cobblestone appearance associated with typical epithelial morphology. (c) Subcultured EOE cells (passage 1) grown on the insulin-like growth factor-I/insulin-like growth factor binding protein-3/vitronectin complex after 10 days of cultivation. (d) EOE cells cultured on insulin-like growth factor-I/insulin-like growth factor binding protein-3/vitronectin (IGF-IGFBP-VN) complex. After 1 passaged, EOE cells had the same characteristic morphology. After 25 days of cultivation, the colony became confluent. The EOE cells had the same characteristic morphology. (e) EOE cells cultured on collagen type I-coated dishes. The cells showed a vague outline and expanded morphology. (f) EOE cells cultured on PS. The EOE cells displayed on extended morphology. Scale bars: 100 μm (a), 50 μm (b–d) length.

degraded and not visible in the implants. The developmental stage of amelogenesis was recognized in one implant at this stage. Enamel organ-like structures and enamel-dentin complex-like structures were recognized in the implants from the scaffolds seeded with both cultured EOE cells and fresh dental papilla cells by histological analysis (Figures 5(b) and 5(c)). In hematoxylin-eosin stained sections, at high magnification, enamel-like tissue was easily found inside the dentin-like tissue generated in the implants (Figure 5(d)). At higher magnification, the width of the dentin-like tissue was approximately 50 μm. The tall columnar ameloblast-like cells were aligned with the surface of the thin enamel-like tissues. The nuclei of the ameloblasts were localized at the edge of the cells most distal to the epithelial cells. At this stage, dentin tubules were clearly identified in the enamel-dentin-like complex (Figure 5(e)).

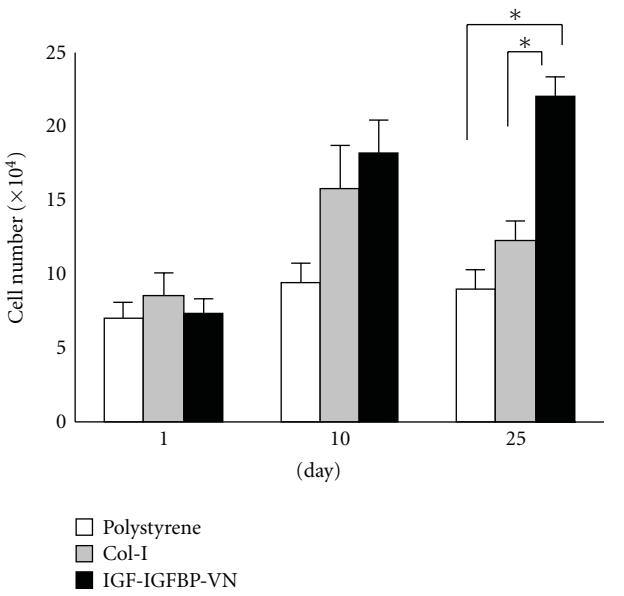

Figure 2: Comparison of the cell proliferation of enamel organ-derived epithelial (EOE) cells on polystyrene, collagen type I, and insulin-like growth factor-I/insulin-like growth factor binding protein-3/vitronectin (IGF-IGFBP-VN) complex at passage 1. The number of cells was counted on days 1, 10, and 25. The growth of EOE cells on IGF-IGFBP-VN complex was faster than that of EOE cells on Col-I. The EOE cells did not grow on PS. Asterisks indicate a significant difference ($P < 0.05$) between paired conditions.

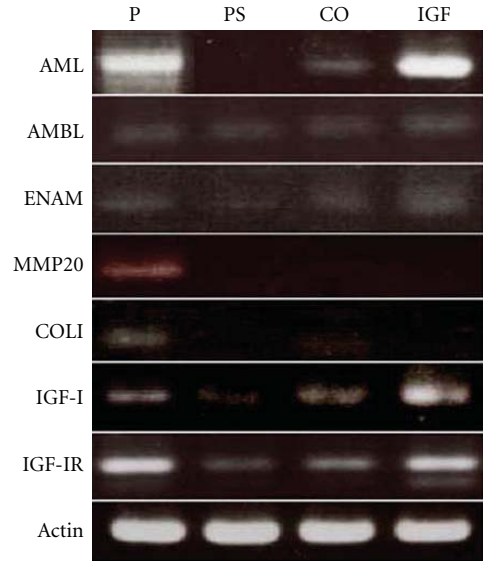

Figure 3: Semiquantitative reverse transcription-polymerase chain reaction analysis of enamel organ-derived epithelial (EOE) cells cultured on insulin-like growth factor-I/insulin-like growth factor binding protein-3/vitronectin complex (IGF) in comparison with the mixed cells from the primary culture (P), and EOE cells on polystyrene (PS) and collagen- type-I-(Co)- coated dishes. Amelogenin (AML), ameloblastin (AMBL), enamelin (ENAM), matrix metalloprotease-20 (enamelysin, MMP20), collagen I (COLI), insulin-like growth factor-I (IGF-I), insulin-like growth factor I receptor (IGF-IR), and beta-actin (Actin) in cultured EOE cells were examined. Primary cells (P) expressed all the examined genes. Cultured EOE cells at passage 1 on PS expressed ameloblastin and enamelin. The expression pattern in the IGF culture was similar to that of the CO culture. Both IGF-I and IGF-IR were more strongly expressed in IGF compared to CO.

Immunohistochemistry was used to examine the distribution of amelogenin in the implants at 4 weeks after transplantation. Amelogenin expression was present in the enamel-like tissue and ameloblast-like cells, while the ameloblast-like cells without enamel formation stained negative for amelogenin (Figure 5(f)).

4. Discussion

This study reports a new *in vitro* culture technique for EOE cells using a complex of IGF-I, IGFBP-3, and VN. We have demonstrated the potential use of this IGF-IGFBP-VN complex in the manufacture of bioengineered enamel through the transplantation assay of EOE cells, but some interaction with dental mesenchymal cells is required for enamel generation. Four weeks after transplantation, enamel was produced on the surface of the dentin after dentin formation. During the enamel formation process, the presence of components of the enamel organ, such as the cells resembling stellate reticulum, inner enamel epithelium, and ameloblasts, appeared to mimic the course of normal enamel development. On the other hand, after the propagation of EOE cells *in vitro*, EOE cells were differentiated into preameloblasts or ameloblasts associated with ameloblast-related gene and protein expressions. The characterization of stem/progenitor cells includes the capacity for both self-renewal and differentiation. Based on our *in vitro* and *in vivo* research, we have demonstrated that cultured EOE cells using IGF-IGFBP-VN include the cells with stem/progenitor characteristics. However, further analysis is needed to examine the precisely developmental stage of EOE cells.

Our previous approach solved the main obstacle to propagation of EOE cells *in vitro* by using a feeder layer of cells [27]. Cultured EOE cells, under a feeder layer, in combination with dental papilla cells formed enamel-dentin complexes in our *in vivo* experiments. As a control, we examined the potential of oral keratinocytes to form enamel by using the same methods, but there was no enamel formation in the implants *in vivo*. This was the first report of enamel-tissue engineering using cultured EOE cells. Although the approach of using a feeder layer has proven to be a major advance [38, 39], the method is quite complicated and there is the possibility that the cells in the feeder layer may turn cancerous because immortalized 3T3-J2 cells are used as the feeder layer [39]. Thus, a new approach to propagate EOE cells without feeder cells was desirable. Interestingly, the period required for enamel generation by our approach using IGF-IGFBP-VN was similar to that of using a feeder layer [34]. Since the source of the dental pulp cells for combination with the scaffold is the same [34], these results suggest that the potential of this new technique

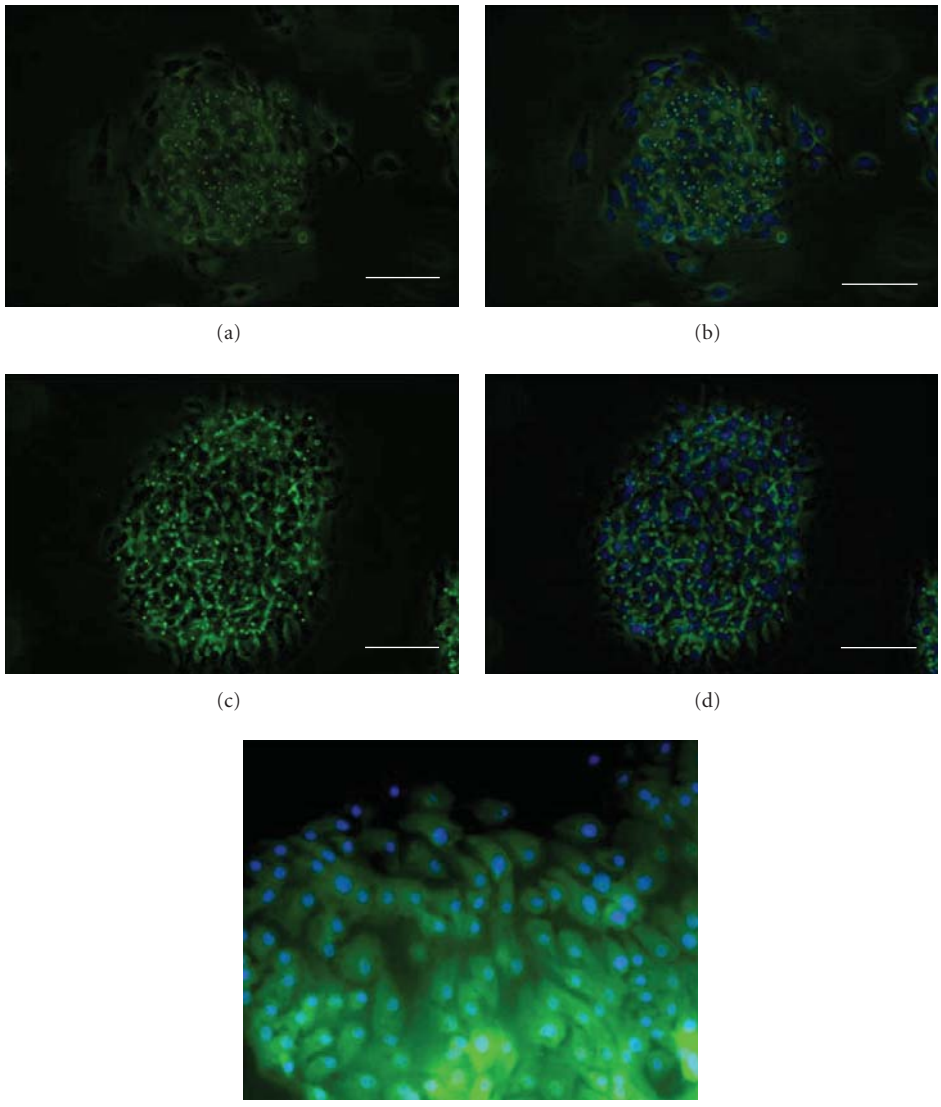

Figure 4: Immunofluorescence analysis in enamel organ-derived epithelial (EOE) cells. (a) Immunofluorescence showed that EOE cells were positive for amelogenin in the collagen type I-coated dishes. (b) Merged image to (a). Amelogenin staining in combination with DAPI to stain DNA (blue). (c) EOE cells were positive for amelogenin by immunofluorescence in the insulin-like growth factor-I/insulin-like growth factor binding protein-3/vitronectin complex-coated dishes. The staining of amelogenin in the EOE cells was more intense than that in the collagen type I-coated dishes. (d) Merged image to (c). Amelogenin staining in combination with DAPI to stain DNA (blue). Scale bars: 50 μm length (a–d). (e) The high magnification view of (d). EOE cells cultured on in the insulin-like growth factor-I/insulin-like growth factor binding protein-3/vitronectin complex-coated dishes expressed strongly amelogenin.

using the IGF-IGFBP-VN complex is similar to that of the technique using a feeder layer of cells.

How does the IGF-IGFBP-VN complex work in the EOE cells? Although our results indicated that IGF-I and IGF receptor expression was observed in the EOE cells in all culture conditions, interestingly, the EOE cells in the IGF-IGFBP-VN culture dishes highly expressed IGF-I and the IGF-I receptor, which indicated that the activity could be increased by autocrine or paracrine signals. Little is known about the effects of VN on EOE cells although an increasing number of functions are being discovered for VN. The molecule is best known for its actions on cell attachment and spreading [16, 40]. In a study of epithelial cells, it was demonstrated that VN has an effect on the metabolic activity of cultured corneal-limbal epithelial cells [23] and skin keratinocytes [28]. It is therefore not surprising that the cellular activity of EOE cells was enhanced by VN.

It is well known that IGF-I is a potent mitogen involved in normal growth and development [1] and influences cell division and differentiation [41, 42]. IGFs are also recognized as having important roles in tooth development; addition of exogenous IGF-I to molar teeth maintained in culture

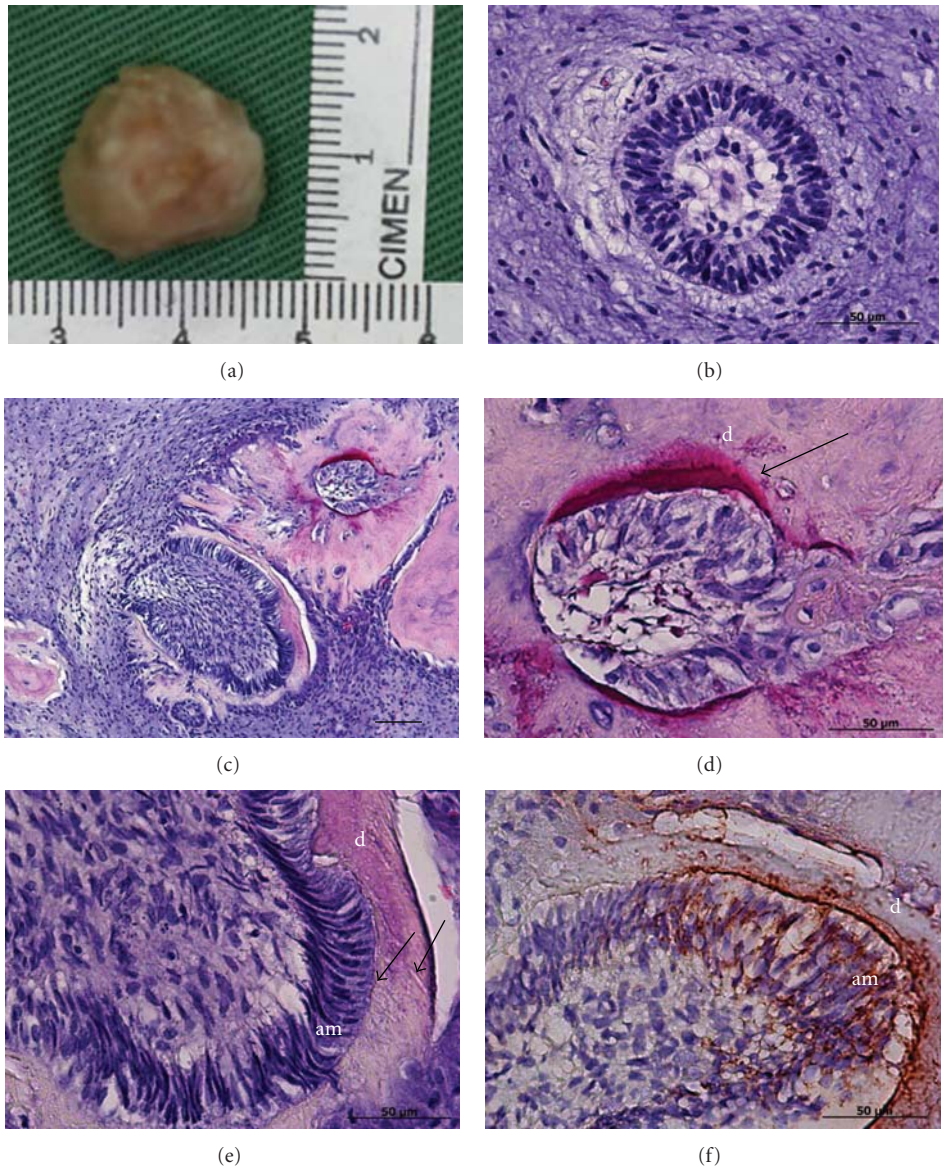

Figure 5: The implants at four weeks after transplantation. (a) Round-shaped implants were produced by the constructs formed between cultured enamel organ-derived epithelial (EOE) cells and dental pulp cells in a scaffold. (b) An enamel organ-like structure associated with an epithelial cell cluster was seen in the implant. Columnar cells were organized in a circle and dental pulp cells embraced the enamel organ. (c) Enamel-like tissue formation on the surface of the dentin, stained with hematoxylin-eosin. (d) High magnification of (c), showing that enamel-like tissue (black arrow) stained with eosin was strongly displayed in the implant. (e) High magnification of (c), showing that tall columnar ameloblast-like cells (am) were aligned perpendicular to the dentin-like tissues (d). (f) High magnification of (c), showing that amelogenin expression was strongly identified in the enamel-like tissue adjacent to the dentin-like tissues and ameloblast-like cells. Scale bars: 50 μm (b, d–f), 200 μm (c) length.

results in an increase in tooth volume [43]. In organ cultures of mouse molars, exogenous administration of IGF-I increases the synthesis of amelogenin, ameloblastin, and enamelin [44]. These results suggest that IGF-I promotes differentiation and development of ameloblasts. The IGF-I receptor has also been identified in the enamel organ across amelogenesis [8]. Moreover, IGF-I appears to be effective in promoting proliferation of Hertwig's epithelial root sheath [10]. Thus, IGF and its receptors are involved in the growth of the epithelium as the tooth develops, including the stages of crown and root formation. In our study, the expression of IGF-I was dramatically increased in EOE cells in culture with the IGF-IGFBP-VN complex. The significance of the IGF-IGFBP-VN complex has been confirmed through observation of other cellular responses in culture plates pretreated with VN, IGF-I, and IGFBP-3 [21, 22]. This is the first study to demonstrate that the IGF-IGFBP-VN complex can enhance the proliferation activity

associated with the maintenance of the phenotype of EOE cells. Although IGF-IGFBP-VN plays critical functions in the cell cycle, the exact mechanism by which IGF-IGFBP-VN facilitates cell proliferation remains unclear. Recently, there is accumulating evidence for direct cooperation between the IGF-I receptor and α_v-integrins as the signaling pathways between these receptors are clearly interconnected [45, 46]. Furthermore, IGF-I-IGFBP-VN complexes induce synergistic increases in intracellular signal transduction, in particular, an increased and sustained activation of the phosphatidylinositol 3-kinase/AKT pathway [25]. Through the IGF-I receptor, IGF-I can activate multiple signaling pathways, including the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways in tumor cells [47]. Facilitation of the cell cycles may accelerate tissue development, however further studies will be required to clarify this issue.

To develop a protocol for enamel-tissue engineering, an appropriate culture method is required to obtain a sufficient number of stem/progenitor cells. The present study revealed that the IGF-IGFBP-VN complex provides a viable alternative to the feeder layer technique. Nevertheless, there is room for refinement of this technology, including extended serial propagation studies, the use of alternative markers of differentiated ameloblasts and formed enamel, and further optimization of the IGF-IGFBP-VN complex formulation to increase the formation of enamel.

Acknowledgments

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Research Article

Bone Regeneration in Artificial Jaw Cleft by Use of Carbonated Hydroxyapatite Particles and Mesenchymal Stem Cells Derived from Iliac Bone

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Objectives of the Study. Cleft lip and palate (CLP) is a prevalent congenital anomaly in the orofacial region. Autogenous iliac bone grafting has been frequently employed for the closure of bone defects at the jaw cleft site. Since the related surgical procedures are quite invasive for patients, it is of great importance to develop a new less invasive technique. The aim of this study was to examine bone regeneration with mesenchyme stem cells (MSCs) for the treatment of bone defect in artificially created jaw cleft in dogs. *Materials and Methods*. A bone defect was prepared bilaterally in the upper incisor regions of beagle dogs. MSCs derived from iliac bone marrow were cultured and transplanted with carbonated hydroxyapatite (CAP) particles into the bone defect area. The bone regeneration was evaluated by standardized occlusal X-ray examination and histological observation. *Results*. Six months after the transplantation, perfect closure of the jaw cleft was achieved on the experimental side. The X-ray and histological examination revealed that the regenerated bone on the experimental side was almost equivalent to the original bone adjoining the jaw cleft. *Conclusion*. It was suggested that the application of MSCs with CAP particles can become a new treatment modality for bone regeneration for CLP patients.

1. Introduction

Cleft lip and palate (CLP) is a prevalent congenital anomaly in the orofacial region characterized by a jaw cleft due to failure of the palatal shelves to fuse properly. CLP is caused by various genetic and environmental factors, and the incidence rate of CLP is 0.19% in Japan [1]. Generally, CLP patients receive labiaplasty and initial palatoplasty at the infant stage as the initial treatment, and speech therapy is also needed for the recovery of speech function. In addition, discontinuity of the upper dental arch due to the jaw cleft frequently causes malocclusion, and orthodontic treatment is conducted on most CLP patients for acquisition of stable occlusion.

In 1972, Boyne and Sands [2] reported that smooth eruption of the canine to the bone transplant area was induced and excellent dental arch form was obtained by autogenous iliac bone grafting before canine eruption. Since then, autogenous iliac bone grafting has been frequently employed for the closure of bone defects at the jaw cleft site to establish well-balanced occlusion [3–5].

However, the related surgical procedures to collect transplant material from iliac bone are quite invasive, causing large stress for the patients. Therefore, artificial transplant materials for bone regeneration instead of iliac bone, such as hydroxyapatite (HAP) and β-tricalcium phosphate (β-TCP),

have been suggested [6, 7]. To date, general clinical application of these materials alone has been limited because of the difficulty in induction of canine eruption and tooth movement to the transplant area [6, 7]. Therefore, it is of great importance to develop a new less invasive technique.

Tissue engineering with stem cell transplantation is an expected candidate to achieve osteogenesis in bone defects with less surgical invasion for ilium extraction. Recently, stem cells have been applied to regenerative medicine, even for internal organs such as blood vessels, nerves, and heart [8–10]. For bone regeneration, mesenchyme stem cells (MSCs) have been expected to become useful transplant material. MSCs account for 0.001~0.01% of the subcellular component in bone marrow, having the potential to differentiate into multiple mesenchyme lineages such as chondrocytes, adipocytes, and osteoblasts by appropriate biological stimuli [11]. Sufficient volume of bone marrow for separation of MSCs can be aspirated using a bone marrow puncture needle. The pain score was significantly lower in CLP patients who underwent bone marrow puncture from iliac bone than in those who underwent conventional surgical separation of iliac bone marrow, suggesting that the bone regeneration using MSCs can relieve stress of patients [12]. However, there have been few studies on osteogenesis with MSCs in CLP patients [13, 14].

Three elements (cell, scaffold, and growth factor) are believed to be crucial for successful tissue regeneration. In the present study, we used carbonated hydroxyapatite (CAP) particles as a scaffold. CAP contains 3~5% carbonate ions by substitution in the HAP lattice structure and is the major mineral constituent of bone and teeth. Therefore, CAP scaffold has quite high biocompatibility with the body, suggesting a big advantage as a scaffold material.

In this study, we examined bone regeneration using MSC transplantation with CAP scaffold for artificial bone defect.

2. Materials and Methods

2.1. Preparation of Artificial Jaw Cleft in Beagle Dogs. Three-month-old female beagle dogs were used ($N = 3$). Permission for a series of experiments in this study was granted by the Animal Experiment Committee of Hiroshima University. Briefly, the upper third incisors on both sides were extracted under general anesthesia (Somnopentyl, Kyoritsu, Tokyo, Japan). The alveolar and palatal bones were ground by about 5 mm in width and 10 mm in length to create bilateral bone defects. The osteoepiphyses were epibolied by suturing mucosa. Antibiotic (Baytril, Bayer HealthCare, Tokyo, Japan) was used to prevent infection before and after the surgery. After a 1-month healing period, the artificial jaw cleft was evaluated by X-ray imaging to check whether or not the bone defect was filled with bone by spontaneous recovery.

2.2. Isolation of MSCs and the Culture. During the healing period, bone marrow MSCs were isolated from the iliac bone of each dog and cultured. The bone marrow was aspirated with a bone marrow puncture needle (Taiyu Medical Co., Tokyo, Japan) from the iliac bone of beagle dogs under general anesthesia with pentobarbital (Somnopentyl, Kyoritsu). MSCs were seeded at a density of 5×10^5 cells per 100 mm cell-culture dish (Corning, New York, NY, USA) in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 10% $NaHCO_3$, 0.7 μg/mL L-glutamine, and antibiotics, under 5% CO_2 atmosphere in a humidified incubator at 37°C. The medium was changed every other day, and the MSCs were subcultured until confluence. The second-passaged cells were used in all experiments.

2.3. Transplant Body. MSCs were cultured to 1×10^8 cells/well and detached from the culture plates *just before transplantation. Unsintered CAP particles (600–800 μm) containing about 10% carbonate ions were used as scaffold. One hundred and eighty mg CAP particles were mixed with MSCs to allow the cells to attach on the surface of CAP particles.* A transplant *consisting* of the same volume of CAP particles without MSCs was used as the control.

2.4. Transplantation of MSCs into Artificial Jaw Cleft. One month after the preparation of artificial jaw cleft, the artificial jaw cleft on the both sides was opened under general anesthesia with pentobarbital (Somnopentyl, Kyoritsu). MSCs with CAP particles were transplanted into the bone defect on the left (experimental side), whereas transplant *consisting* of CAP particles alone was filled on the right side cleft (control side). The bone defect filled with a transplant was covered with poly-lactic-co-glycolic-acid (PLGA) barrier membrane (GC membrane, GC Co., Tokyo, Japan) to prevent leakage of the transplant. Afterward, mucous membrane was sutured to intimacy. Antibiotic (Baytril, Bayer HealthCare) was used to prevent infection before and after the surgery.

2.5. Evaluation of Bone Regeneration by X-Ray Imaging. Bone regeneration was evaluated using standardized occlusal X-ray images and histological examination. All occlusal X-ray examinations in this study were standardized using a film holder to maintain positions among X-ray irradiator, object, and film (Figure 1(a)).

The radio-opacity of the artificial jaw cleft area was measured using *NIH-image* 1.59 software (National Institutes of Health, Bethesda, Washington DC, USA) on the standardized occlusal X-ray images (Figure 1(b)). *The signals of regenerated bone and CAP particles were evaluated by radio-opacity of the jaw cleft area.*

2.6. Evaluation of Bone Regeneration by Histological Staining. A small piece of the regenerated tissue was separated from the dogs under general anesthesia with pentobarbital (Somnopentyl, Kyoritsu) 3 and 6 months after the transplantation. The tissue specimens were immediately fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, decalcified with EDTA for 1 month, and embedded in paraffin. Tissue sections of 7 μm thickness were made and stained with hematoxylin and eosin (HE).

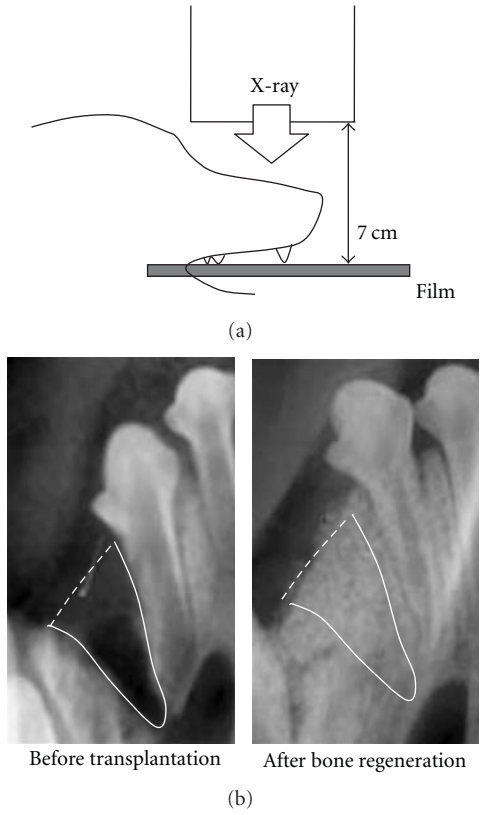

Figure 1: X-ray examination of bone regeneration (a) Schema of standardized occlusal X-ray examination. A film was held parallel to the upper dentition of a beagle dog, and X-rays were irradiated vertically to the film at 250 mV. The angle and distance between X-ray bulb and film were standardized using a locator. (b) The radio-opacity without signals of CAP particles in the artificial jaw cleft area was measured on standardized occlusal X-ray images.

The number of capillary vessels in the regenerated area was counted on the tissue sections using a phase contrast microscope (BZ8000, KEYENCE, Osaka, Japan).

2.7. Statistical Analysis. The transplantation of MSCs was performed using 3 dogs. Means and standard deviations (SD) were calculated from the data obtained and then subjected to Student's t-test using Graphpad Prism 4.0a software (Graphpad Software, Inc., San Diego, CA, USA) to examine significant differences in the means at the 1% and 5% levels of significance.

3. Results

3.1. Intraoral Findings before and after the Transplantation of MSCs to Artificial Jaw Cleft. Bilateral artificial jaw cleft was prepared on the dog maxilla (Figure 2(a), arrowhead). The artificial jaw cleft was opened after 3 months, and no spontaneous recovery of bone defect was shown. Thus, the CAP particles with or without MSCs were transplanted to the bone defects (Figure 2(c), arrowhead). After the transplantation, no inflammation was shown in the transplanted area. Six months after the transplantation, the shape of the alveolar ridge at the transplanted area was maintained on both experimental and control sides (Figure 2(d)).

3.2. Radiographic Findings before and after the Transplantation of MSCs to Artificial Jaw Cleft. No spontaneous recovery of bone defects was shown by X-ray examination 3 months after the preparation of bilateral artificial jaw cleft (Figures 3(a) and 3(b), arrowhead). Immediately after the transplantation, CAP particles were observed as opaque regions in images of the jaw cleft (Figure 3(c), arrowhead). Three months after the transplantation, radio-opacity of CAP particles was reduced on the control side compared with that on the experimental side (Figure 3(d), arrowhead), and decreased more substantially after 6 months (Figure 3(e), arrowhead). The radio-opacity on the experimental side after 6 months was increased whereas the number of CAP particles was decreased compared with those at 3 months.

The radio-opacity of CAP particles on the experimental side was significantly ($P < 0.05$) lower than that on the control side (Figure 3(f)). On the other hand, the radio-opacity of regenerated bone on the experimental side was significantly (3 months, $P < 0.05$; 6 months, $P < 0.01$) higher than that on the control side. These results *indicate* the digestion of CAP particles and calcification in the jaw cleft on the experimental side.

3.3. Histological Observation before and after the Transplantation of MSCs to Artificial Jaw Cleft. Three and *Six* months after the transplantation, the tissues of the transplanted area were separated and evaluated by histological observation.

Three months after the transplantation, a large *number* of CAP particles *remained* on the control side, whereas only a few CAP particles were found on the experimental side on the intraoral photographs (Figures 4(a) and 4(b)). In addition, the CAP particles had become smaller than the original particles (600–800 μm) on the experimental side. Histological observation revealed the presence of fibroblastic cells around CAP particles on the control side (Figures 4(c), 4(d), and 4(e)). On the other hand, no fibroblastic cells were shown around the CAP particles, and new bone formation was observed on the *experimental* side (Figures 4(f), 4(g), and 4(h)).

Six months after the transplantation, the number of CAP particles on the control side was decreased but many particles still remained, whereas almost no CAP particles were observed on the experimental side (Figures 4(i) and 4(j)). Histological examination revealed that new bone formation was present locally in the transplanted area on the control side, but fibroblastic cells were still located around CAP particles (Figures 4(k), 4(l), and 4(m)). On the other hand, new bone formation was observed in almost the whole area on the experimental side, and the CAP particles had almost disappeared (Figures 4(n) and 4(o)).

The number of capillary *vessels* was significantly ($P < 0.01$) greater on the experimental side than on the control side after 3 and 6 months (Figure 5).

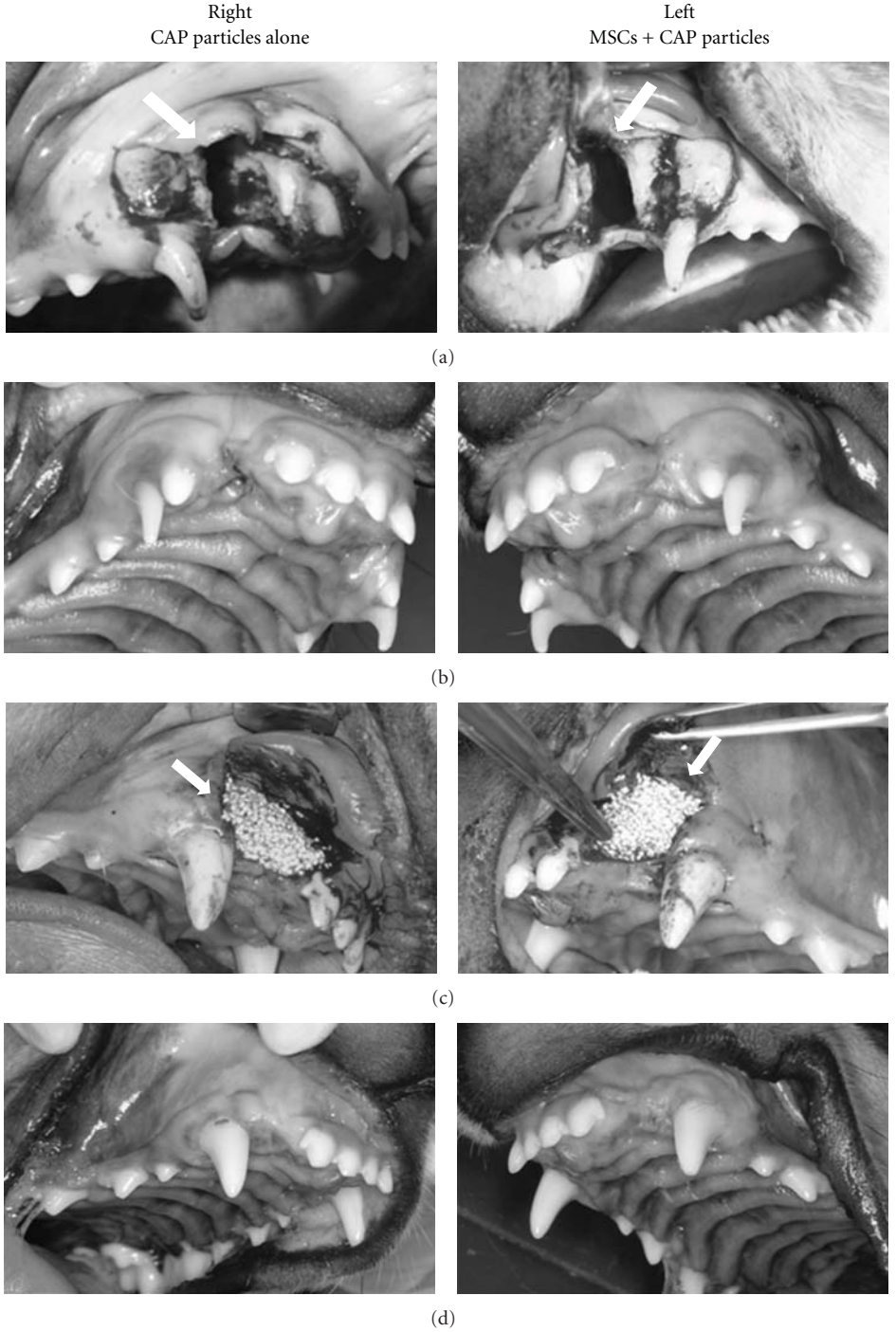

Figure 2: Intraoral findings before and after the transplantation of MSCs to artificial jaw cleft. Intraoral images (a) after preparation of bilateral artificial jaw cleft in maxilla (arrowhead, jaw cleft), (b) before and (c) after transplantation of MSCs with CAP particles to the jaw cleft (3 months after the preparation of artificial jaw cleft). The cleft on the right side (control side) was filled with CAP particles alone, whereas the cleft on the left side (experimental side) was filled with MSCs and CAP particles (arrowhead). (d) 6 months after the transplantation.

4. Discussion

In the present study, bone regeneration of artificial jaw cleft was demonstrated by the transplantation of MSCs with CAP particles. Radio-opacity of regenerated tissue on the experimental side was significantly higher than that on the control side, suggesting a contribution of MSCs to new bone formation. The CAP particles used in the present study *were* unsintered, and substituted for 3–5% carbonate ions in the HAP structure, leading to unstable

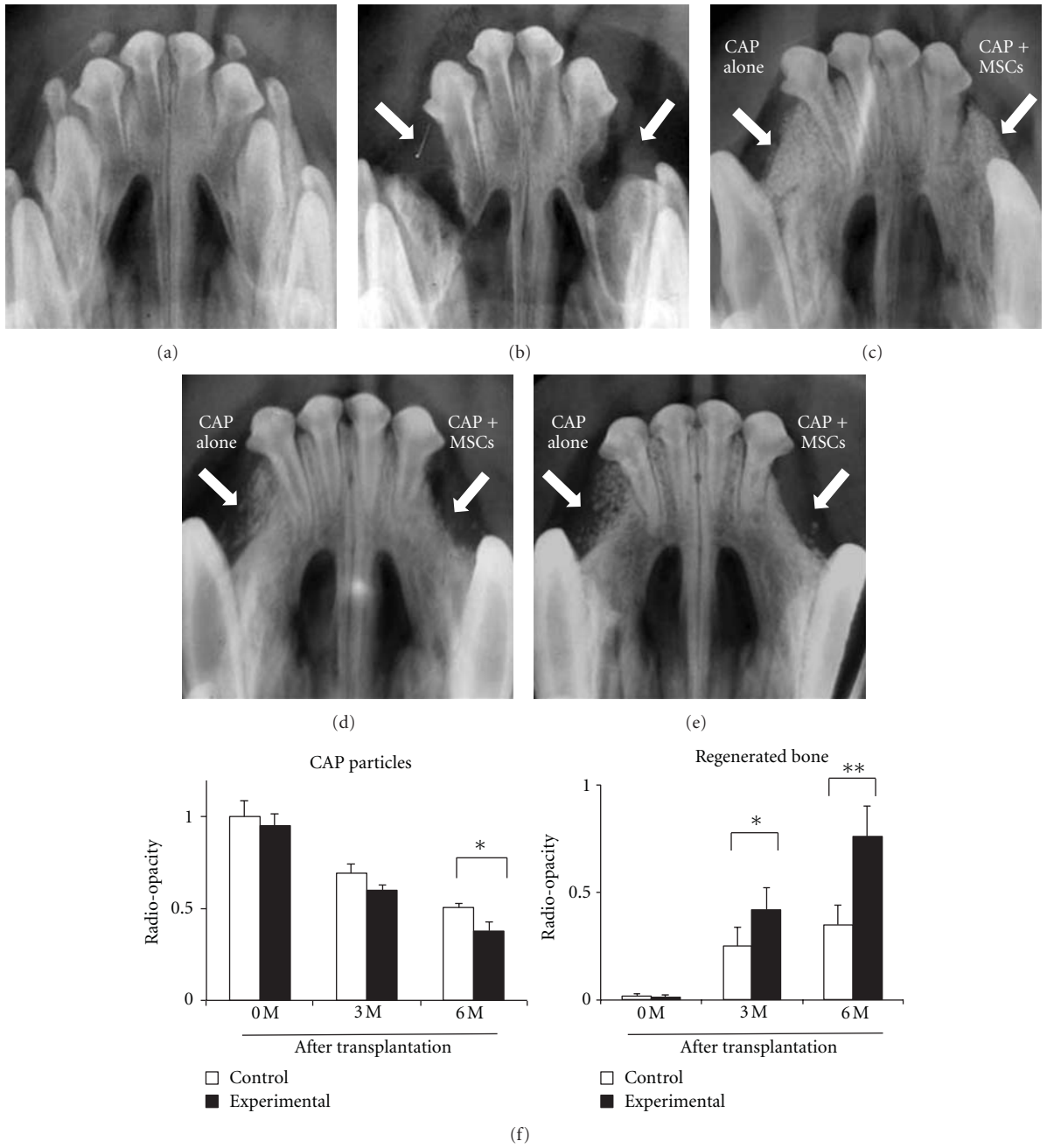

Figure 3: Radiographic findings before and after the transplantation of MSCs to artificial jaw cleft. Standardized occlusal X-ray images (a) after preparation of bilateral artificial jaw cleft in maxilla, (b) before and (c) after transplantation of MSCs with CAP particles to the jaw cleft (3 months after the preparation of artificial jaw cleft; arrowhead, jaw cleft). The cleft on the right side (control side) was filled with CAP particles alone, whereas the cleft on the left side (experimental side) was filled with MSCs and CAP particles, (d) 3 months and (e) 6 months after the transplantation (arrowhead, transplanted area). (f) The radio-opacity of the artificial jaw cleft area was measured on the standardized occlusal X-ray images after the transplantation to the artificial jaw cleft. *The signals of regenerated bone and CAP particles were evaluated by radio-opacity of the jaw cleft area.* $N = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

crystal structure compared with that of HAP. Since the solubility of pure HAP is quite low, it would take a long time to be digested and replaced *by* new bone if used for transplantation into bone defect. In a previous study [15], CAP transplanted above a tooth bud did not disturb tooth eruption, suggesting its high biocompatibility and solubility in the body. However, there have been no reports of bone regeneration in jaw cleft by the use of unsintered CAP scaffold. In our previous study, alveolar bone regeneration was achieved by transplantation of β-TCP in mouse [6]. However, the scaffold for CLP alveolar bone regeneration requires some special characteristics. *The transplantation of*

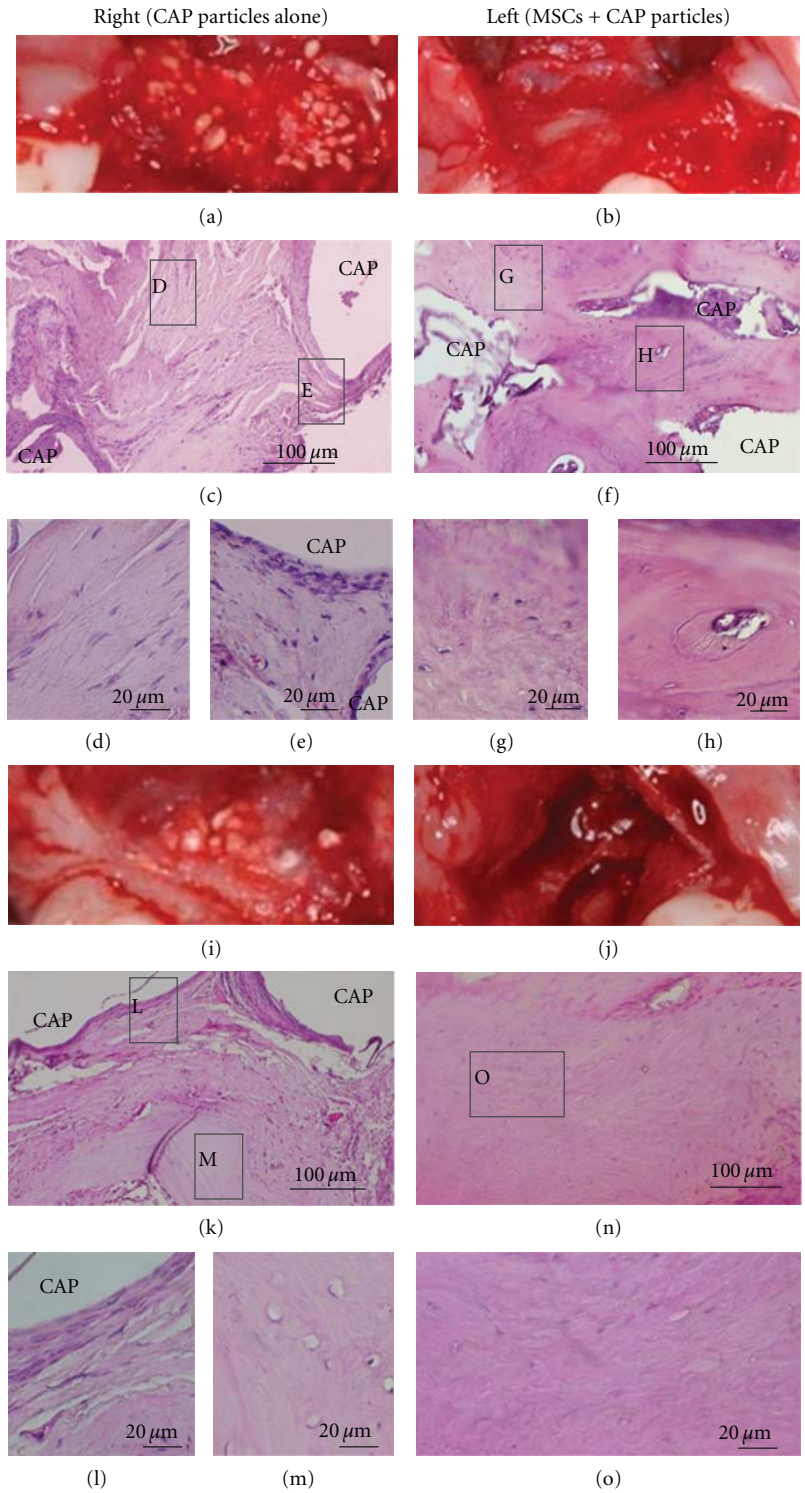

Figure 4: Histological observation before and after the transplantation of MSCs to artificial jaw cleft. The regenerated tissues were separated 3 and 6 months after the transplantation. Tissue sections were made and stained with hematoxylin and eosin (HE). Intraoral images 3 months after the transplantation on (a) the control side (CAP alone) and (b) the experimental side (MSCs and CAP particles). The tissue section showed fibroblastic cells and inflammatory cells around CAP particles on the control side (c, d, and e). New bone formation was shown widely on the experimental side (f, g, and h). Intraoral images 6 months after the transplantation on (i) the control side and (j) the experimental side. The tissue section showed that new bone formation had occurred locally on the control side (k, l, and m). On the other hand, new bone formation was observed in almost the whole area on the experimental side (n and o).

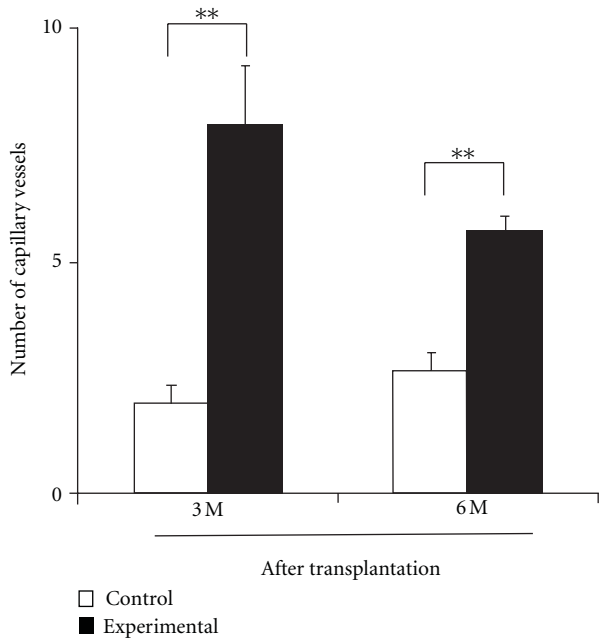

Figure 5: Number of capillary vessels in the regenerated area of artificial jaw cleft. Number of Capillary Vessels in Regenerated Area Was Counted on the Tissue Sections using a Phase Contrast Microscope. $N = 3$, $^{**}P < 0.01$.

β-TCP was suggested to cause disturbance of canine eruption and tooth movement after its transplantation [6]. Generally, alignment of tooth on the jaw cleft area by tooth movement or transplantation is performed after bone regeneration in the treatment of CLP patients [3, 16]. Therefore, the jaw cleft should be filled with bone having normal metabolism to allow tooth eruption or movement. For this purpose, transplant material with high solubility in a living body and that can be digested quickly is considered to be suitable.

In the present study, bone regeneration was accompanied by the absorption of CAP particles by the transplantation of CAP particles and MSCs on the experimental side. Meanwhile, absorption of CAP particles and new bone formation were much slower on the control side. In a living body, osteoclasts and osteoblasts at various differentiational phases act as one physiologic unit (basic multicellular unit; BMU), which controls bone remodeling repeatedly. The balance of bone absorption and formation is modulated by BMU activity, and new bone formation is activated after bone absorption. During the activation phase of bone remodeling, osteoclasts secrete acid and specific enzymes for continuous digestion of bone-salt and bone matrix proteins, respectively [17]. Therefore, it is speculated that digestion of transplant material plays a crucial role in new bone formation, and the difference in bone regeneration between the experimental and control sides may be due to the rate of CAP absorption in this study.

In the present study, the number of capillary *vessels* in regenerated tissue after transplantation was significantly greater on the experimental side than on the control side. It was suggested that MSCs express vascular endothelial growth factor (VEGF), which is advantageous for bone regeneration by inducing vascularization [18]. Since the enhancement of vascularization is favorable to induce osteoclasts in the bone regeneration area, it is assumed that transplantation of MSCs may contribute to angiogenesis, leading to enhancement of CAP digestion by osteoclast induction. The transplantation of MSCs may have relevance for not only their differentiation ability into osteoblasts but also for induction of capillary vessels. If this is true, the transplantation of undifferentiated MSCs has an advantage compared with the application of differentiated MSCs to osteoblasts. However, it remains unclear whether transplanted MSCs differentiated into osteoblasts or just *expressed* cytokines or growth factors after transplantation. Tracing of transplanted MSCs would be beneficial to resolve the above issue in future studies.

In addition, transplanted CAP particles on the control side were encapsulated with fibroblastic cells, and *this* may *be* another reason *for* the delay in digestion of CAP particles. However, it remains unclear why CAP particles on the experimental side were not encapsulated with fibroblastic cells. Therefore, the mechanism of CAP digestion should also be clarified in future studies.

A specific requirement in bone regeneration of jaw cleft is *the* guidance or movement of tooth into newly formed bone. In this study, sufficient volume of alveolar bone for tooth movement was formed in the artificial jaw cleft by the transplantation of MSCs with CAP particles. Orthodontic tooth movement is a result of bone remodeling, which has high relevance to angiogenesis [19]. Since the regenerated tissue on the control side has a small number of blood vessels as well as undigested CAP particles, difficulty of orthodontic tooth movement was speculated to be due to low bone remodeling.

In conclusion, it was shown that the transplantation of MSCs with CAP particles could regenerate bone in artificial jaw cleft. The transplantation of MSCs with CAP particles to jaw cleft may be a new treatment modality for CLP patients.

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Research Article

Evaluation of Osteoconductive and Osteogenic Potential of a Dentin-Based Bone Substitute Using a Calvarial Defect Model

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The aim of this study was to assess the osteoconductive and osteogenic properties of processed bovine dentin using a robust rabbit calvarial defect model. In total, 16 New Zealand White rabbits were operated to create three circular defects in the calvaria. One defect was left unfilled, one filled with collected autogenous bone, and the third defect was filled with the dentin-based bone substitute. Following surgery and after a healing period of either 1 or 6 weeks, a CT scan was obtained. Following sacrificing, the tissues were processed for histological examination. The CT data showed the density in the area grafted with the dentin-based material was higher than the surrounding bone and the areas grafted with autologous bone after 1 week and 6 weeks of healing. The area left unfilled remained an empty defect after 1 week and 6 weeks. Histological examination of the defects filled with the dentin product after 6 weeks showed soft tissue encapsulation around the dentin particles. It can be concluded that the rabbit calvarial model used in this study is a robust model for the assessment of bone materials. Bovine dentin is a biostable material; however, it may not be suitable for repairing large 4-wall defects.

1. Introduction

Allogenic dentin has been studied for its potential use as bone substitute [1–4]. Previously, a new method for processing bovine dentin was reported that resulted in a sterile bioactive material for repair and regeneration of bone [5]. This included processing the extracted bovine dentin mechanically and chemically with inorganic and organic solvents.

Initial biocompatibility tests involved *in vitro* testing on human gingival fibroblasts using the Alamar Blue assay and *in vivo* evaluation by implantation of the processed dentin into rat femur. The dentin product showed excellent biocompatibility in the rat femur model and stimulated the formation of new bone and was completely integrated with the surrounding bone. However, the rat femur model used in the previous study was a small 5-wall defect healing model. Healing in rat defects is known to be well underway by 9 weeks postoperatively. If the defect is 2 mm, then the defect will heal by bony union. If the defect is above 5 mm, then fibrous nonunion healing will occur [6]. The rat femur implantation test has high osteogenic potential due to its 5 bony walls that are mainly cancellous bone with surrounding marrow.

To obtain a rigorous assessment of the osteoconductive and osteogenic potential of the new dentin-based material, a robust rabbit calvarial defect model with 4-wall defects is preferred. The rabbit calvarial model is different from rat femur model as the defect in rabbit calvarium is similar to those created in the maxillofacial region in human, since morphologically and embryologically calvarium develops from a membrane precursor resembling the membranous bones of the face [7]. The defect has only 4 bony walls, and the surrounding bone is mainly cortical bone with a poorer

blood supply and less osteogenic cells being presented to the defect. Thus, it is regarded as a more robust model than the rat femur model.

The aim of this study was to assess the biocompatibility of the previously developed bovine dentin product using a robust rabbit calvarial defect model.

2. Materials and Methods

2.1. Ethical Approval. This study was approved by the University of Sao Paulo's Animal Research Ethics Committee. The animal experiments were carried out in the Department of Oral Maxillofacial Surgery, University of Sao Paulo in Brazil.

2.2. Bovine Dentin Processing. Extracted bovine dentin was processed mechanically and chemically with inorganic and organic solvents to produce a sterile powder with mixed particle size. Methods are described in detail in our previous report [5].

2.3. Animals and Anesthesia. In total, 16 New Zealand White rabbits weighing 3-4 kg were studied. All animals were kept in a single room and fed a dried diet and water ad libitum. The sample size chosen was based on our previous similar studies [8]. First 0.8 mL of 0.2% Acepran with the active ingredient of Acepromazine was injected intramuscularly. The animal was then induced into general anesthesia with a 3 mL solution containing a mixture of 2.2 mL Ketamine (União Química Farmacêutica Nacional S.A., Embuguaçú, São Paulo, Brazil) and 0.8 mL Xylazine. This was split into 2 equal doses of 1.5 mL intramuscular injections, administered with 3-minute intervals.

2.4. Surgical Procedure. The surgical field was disinfected with povidone-iodine 10%. A mid-sagittal incision was made after local infiltration of 2% lidocaine hydrochloride with 1 : 100,000 epinephrine 1.8 mL. Subperiosteal dissection was carried out, and the periosteum was reflected to expose the bony area. An 8 mm trephine was then used to create 3 circular defects as shown in Figure 1, and the bony discs were removed. It has been shown that 8 mm rabbit calvaria defects can be considered as critical size defects [9].

The defects were chosen randomly, and one defect was filled with the dentin substitute, one was filled with particulate autogenous bone, which was made from crushing the elevated bony discs using a bone mill, and finally the third defect had no filler placed and was left to heal with blood clot only. The defects were filled so as to be in consistent contour with the surrounding bone (Figure 1). No membrane was used in this study. The surgical site was then closed using a 5–0 Nylon suture.

Following the surgery, the animals were administered post-op analgesics (Tramal 0.02 mg/kg, Biolab Searle, São Paulo, Brazil) and anti-inflammatory medication (Profenids 3 mg/kg, Distribuidora Farmácia Brasil LTDA, Jandira, SP, Brazil) intramuscularly.

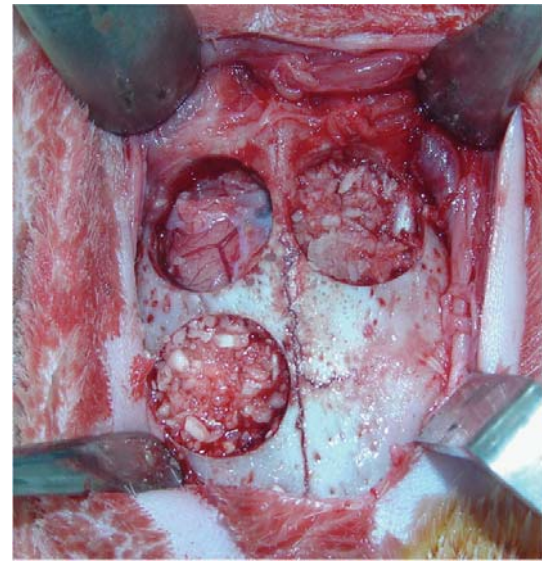

Figure 1: Surgical defects on rabbit calvarium filled with test materials. One defect was left unfilled (upper right), one filled with collected particulate autogenous bone (upper left), and the third defect was filled with the new bovine dentin bone substitute (lower right).

2.5. CT Scan. A multislice CT scan (Siemens Emotion) was then taken immediately postoperatively, and a multislice CT scan was taken immediately after sacrifice, using the same CT scanner.

2.6. Sacrificing Procedure. 7 rabbits were sacrificed 1 week postoperatively, and 9 rabbits were sacrificed 6 weeks postoperatively. The animals were killed using an overdose of xylazine and ketamine, and the tissues harvested from the graft sites were processed for histology.

2.7. Histology. The tissues were fixed by means of perfusion with paraformaldehyde 4% using a peristaltic pump (Masterflex Pump Controller, Cole-Parmer, USA). The biopsies were kept in 4.0% paraformaldehyde solution at 4°C for six hours in order to complete the fixation, then washed in phosphate buffer solution 0.1 M for 30 minutes, and moved on to EDTA 5.0% for decalcification. The pieces were rinsed in phosphate buffer for 24 hours and subsequently kept in solution containing sucrose 30% for two more days. After cryoprotection was concluded, each piece was fixed in a gel support (Tissue TeK—Laboratório FK Biotec, Bento Gonçalves, Brazil), frozen at −20°C, and sliced in a cryostate (Microm HM 505 N), 14 μm thick. The slices were made up in a semiserial fashion in a coronal orientation and assembled on polarized blades, where they were interspersed for the histological analysis. The histological slides were stained with Mallory's trichrome and examined under a light microscope. The histological appearance was assessed by more than one investigator blinded to the nature of the material the defect had been filled with. Where there were differences in the histological assessment of the tissues

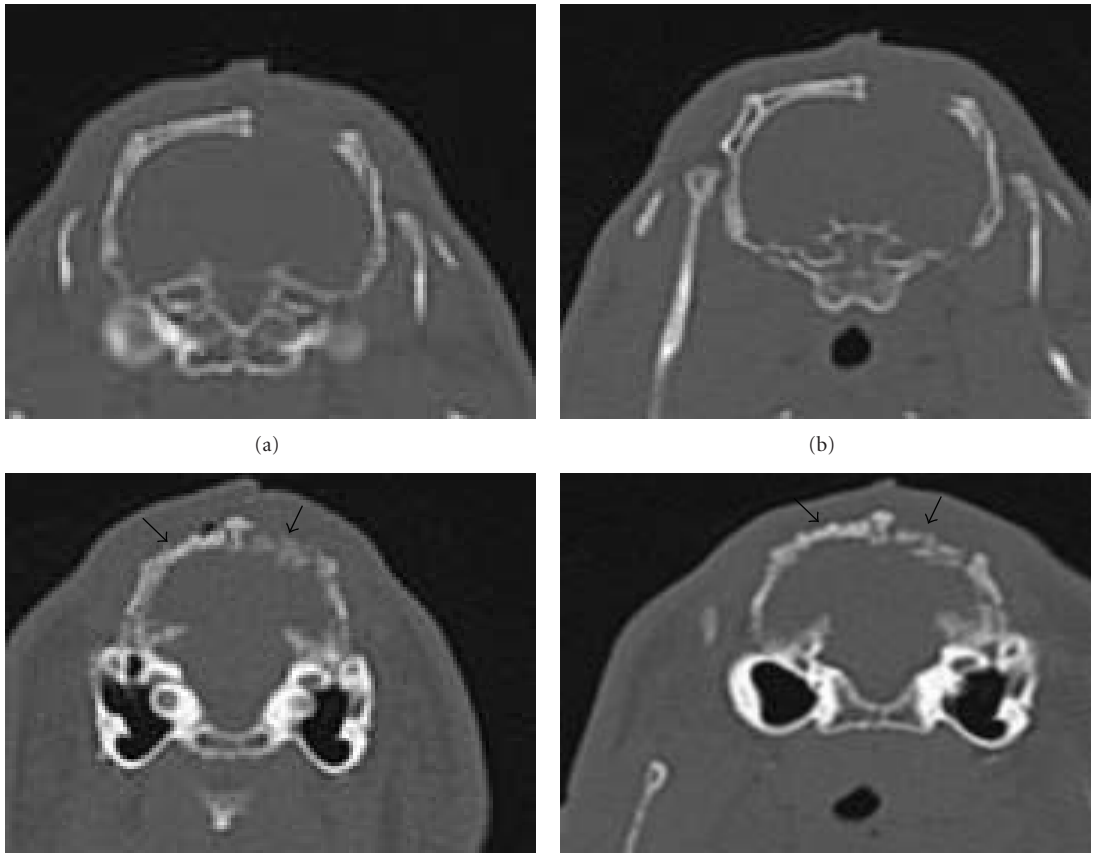

Figure 2: Post-op CT scan of (a) unfilled area immediately after surgery; (b) unfilled area 1 week post operatively; (c) dentin-filled defect (left arrow) and the autogenous bone-filled defect (right arrow) immediately after surgery; (d) dentin-filled defect (left arrow) and the autogenous bone-filled defect (right arrow) 1 week postoperatively.

between investigators, these were resolved by discussion and a consensus reached before blinding was removed.

3. Results

The skin wounds healed well with no sign of infection or adverse reaction to the implanted material.

3.1. CT Scan. The immediate postoperative and 1-week postoperative CT scans of defects are shown in Figure 2. After one week, there was no bony reunion or growth in the empty defect. In the filled defects, the dentin had a higher mineral content showing a higher density than the autologous bone when viewed on the CT scan both at the time of surgery and after 1 week.

The immediate postoperative and 6-week postoperative CT scans of defects are shown in Figure 3. After 6 weeks, it seemed the empty defect had some appositional bone growth but without any bony reunion. The filled defects both appeared to undergo organisation, with the dentin-filled defect being shown to be more opaque than the autologous graft after 6 weeks.

3.2. Histology. Histological views of the calvarial defects filled with the dentin product and autologous bone after 1 and 6 weeks are shown in Figure 4.

Histological examination of the defects filled with the dentin product revealed the presence of dentin particles after 1 week and 6 weeks. In some areas, the dentin had been partially resorbed showing features of fibrous tissue encapsulation.

Examination of the defects filled with autologous bone showed the presence of osteoblasts on the margin of the bone particles with the evidence of new bone formation. Some soft tissue invasion into between the bone particles was observed.

The untreated defects did not show any significant bony infill after 1 week or 6 weeks and were mainly filled with the soft tissue (figure not shown).

4. Discussion

Advances in tissue engineering and stem cell science have led to development of novel approaches for bone regeneration in maxillofacial region [10–12].

Dentin has been of an area of interest to study its potential use as a bone substitute since it has a higher mineral

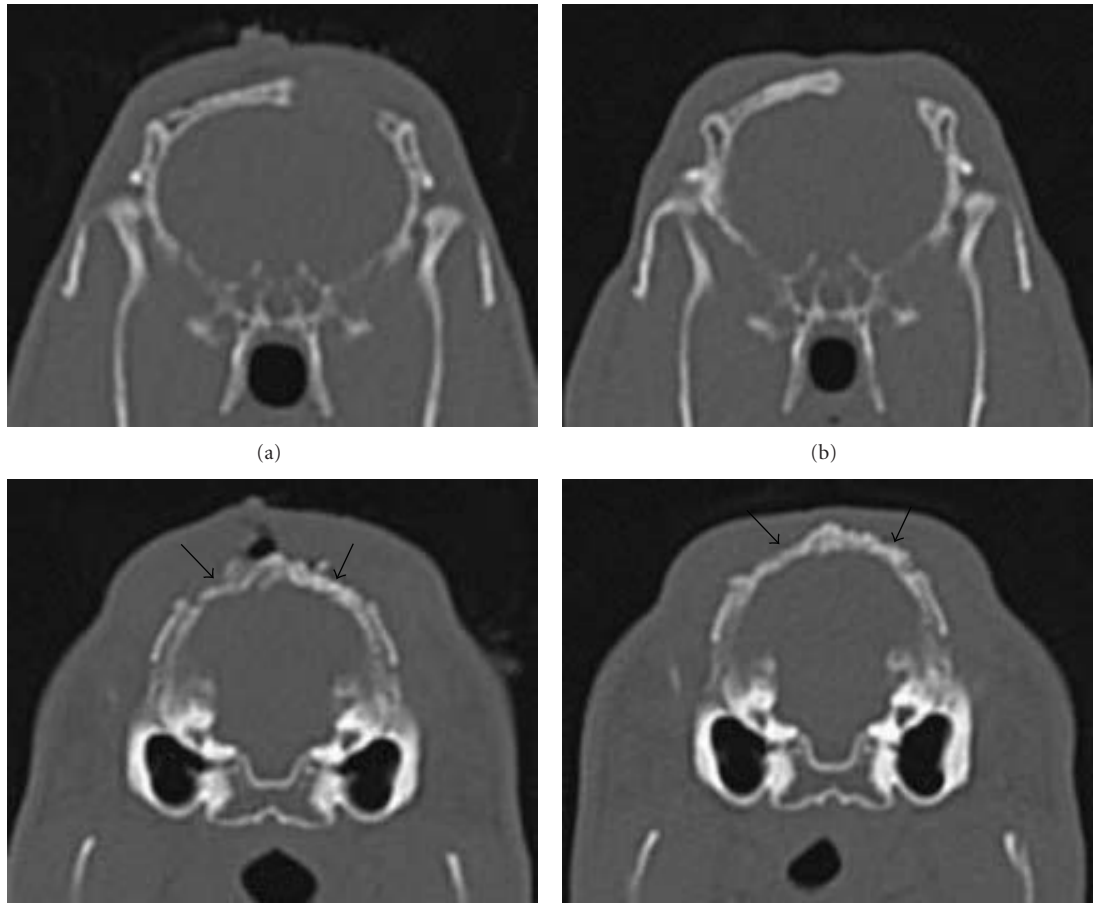

Figure 3: Post-op CT scan of (a) unfilled area immediately after surgery; (b) unfilled area 6 weeks postoperatively; (c) dentin-filled defect (right arrow) and the autogenous bone-filled defect (left arrow) immediately after surgery; (d) dentin-filled defect (right arrow) and the autogenous bone-filled defect (left arrow) 6 weeks postoperatively.

content than any bone-derived material, and it is also a readily available xenograft. Furthermore, there is a potential to utilize its organic component as well as its mineral component. For a long time, it has been known that proteins of similar weight to bone morphogenic proteins (BMPs) are abundant in tooth substance [13, 14] and that the BMPs are able to promote the differentiation of mesenchymal cells into odontoblasts and ameloblasts [15, 16]. Also dentin has the capability of promoting heterotopic bone formation [17]. These proteins may enhance the osteoinductive properties of the bone substitute if they are able to be retained during the processing of the material studied in this experiment. With the prospect of the possible use of bovine dentin as a graft material comes the opportunity to utilize a patient's own dentin from their extracted teeth, which is analogous to a previous study done using the extracted teeth from a rabbit to repair a calvarial defect made in the same animal once the dentin was processed [18]. The clinical scenarios for taking advantage of such an opportunity prior to implant placement are numerous.

Among different models, the rabbit calvarial model has been used for many years as a reliable method of evaluating bone substitutes. This model has been reported to be very suitable for the assessment of osteoconductive properties of biomaterials [19].

The rabbit model has several advantages, such as standardization of experimental conditions and experiment repeatability, size, inexpensiveness, and high bone turnover rate [20]. The calvaria and the facial bones are pure membranous bones, with the mandible and the greater wing of the sphenoid being exceptions. Subtle differences exist between the microscopic structures and functions of the calvaria in different species; however, embryonic development is very similar [7].

The skull is much more biologically inert due to its poor blood supply and relative deficiency of bone marrow, when compared to other bones [21]. There is no primary nutrient artery in the human calvaria, unlike many long bones that contain a primary nutrient artery. Since a large area of human skull is devoid of muscle insertions, the blood supply to the human calvaria is poorer than in other mammals [7]. The resultant effect means that even small defects in the adult human skull do not spontaneously repair. Due to this, the regenerative capacity of the calvaria of

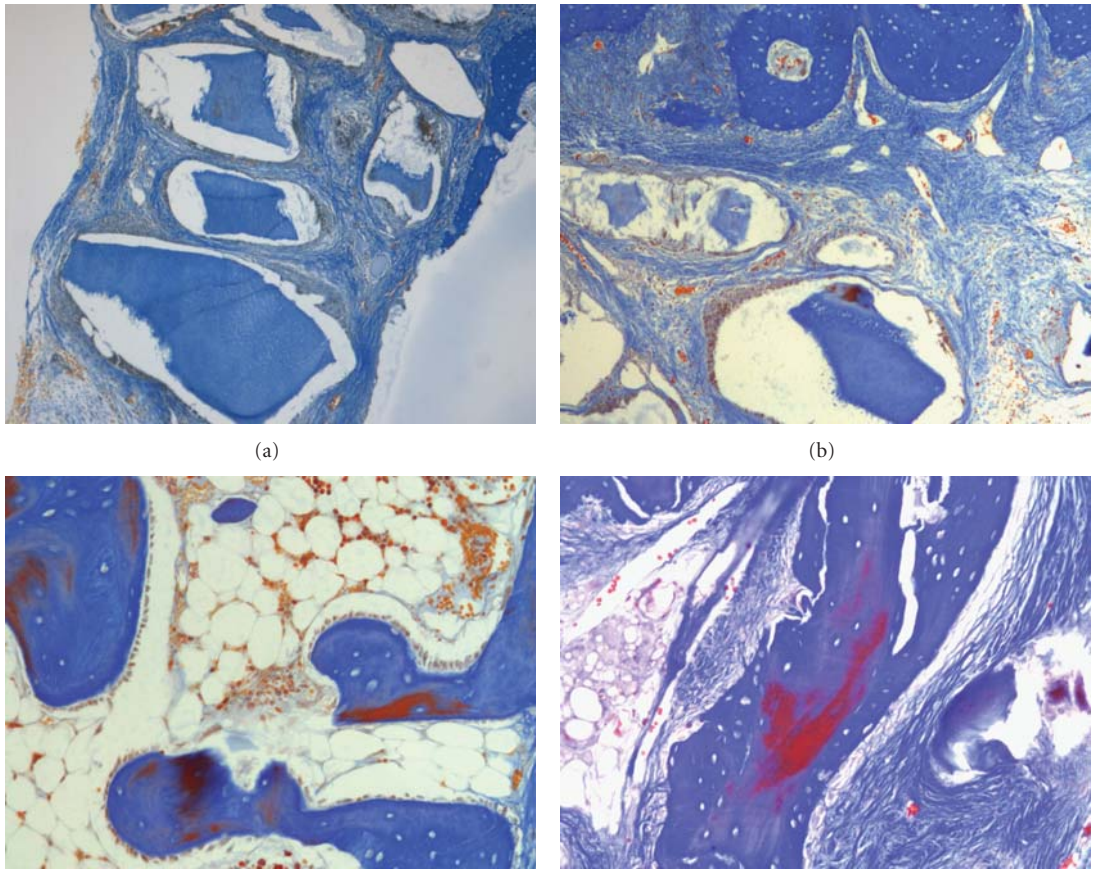

Figure 4: Mallory's trichrome-stained histological sections of defect areas filled with (a) processed dentin product, 1 week post-op, original magnification ×10; (b) processed dentin product, 6 weeks post-op, original magnification ×10; (c) autogenous bone, 1 week post-op original magnification ×20; (d) autogenous bone, 6 weeks post-op, original magnification ×20.

experimental animals can be considered better than that of humans. As previously mentioned, calvarial wound defect model has many similarities to the maxillofacial region. Physiologically, the cortical bone in the calvaria resembles an atrophic mandible [22]. Implantation into a skull defect is the most severe test for a bone implant [7], and thus the calvaria has been a frequent site for the testing of bone repair materials. This is unlike the rat femur, for example, which has mainly cancellous bone surrounding the 5-wall defect in the implantation test model.

There are no previously published studies that have used CT data to assess dentin when used as a bone substitute. Multislice computerised tomography data was used as a visual aid to determine the density and the form of the grafted area as it is a readily available noninvasive technique with high accuracy [23]. Furthermore, with newly available software, it is possible to reconstruct a 3D high-resolution image using the data from the scanned subject for easy visual, quantitive, and density analysis. Using the same CT scanner for all the scans, which was calibrated prior to scanning, it was possible to ensure the consistency in the recording of data in the subjects and obtain a realistic comparison of the dentin substitute to the neighbouring empty defect and the defects grafted with autologous bone. This was an ideal way of visualizing the differences in the graft density in this study.

Histological data has the benefit of allowing us to observe a number of features depending on the type of stain that is used. In this study, Mallory's trichrome staining was used. This was a suitable stain for identifying the newly formed bone following decalcification of the specimens. The histological features observed in this study were different from our previous findings using a rat femur implantation testing where complete integration of the bovine dentin to the bone was observed [5]. However, in this study, we unexpectedly found soft tissue encapsulation of the dentin particles. The dentin particles also appeared to be undergoing resorption within the fibrous tissue.

In the previous study using the rat femur model (5-wall defect), there was plenty of cancellous bone in the 5 bony walls, which had a higher blood supply and more osteoblastic cells to repair and deposit new bone around the particles. There was also less soft tissue contact with the graft material, and so, in a 5-wall defect, the likelihood of soft tissue invasion was very low. However, in a large rabbit calvarial defect (4-wall), most of the material was exposed to the soft tissue below and above and was only exposed to bone on the edge of

the wound. Also, there was less cancellous bone in a calvarial defect compared to rat femur.

A crestal incision was used to access the surgical site. This meant that the closure site was very close to the defects and material. In hindsight, a remote incision would have been more favorable to minimize the potential risk of skin cells invading the defects. Also, there may have been potential damage to the underlying dura which could account for the soft tissue invasion and encapsulation of the dentin particles.

Whilst this study may have given a negative result as for the suitability of bovine dentin for use in a 4-wall defect, there may still be the potential for its use in smaller 5-wall defects.

The results of histology also showed that there was evidence of some soft tissue invasion into defects filled with autologous bone. Previous studies [18] have shown that, when bone materials are used in a rabbit calvarial model with the presence of a membrane, the bone regeneration appeared more extensive and more matured histologically than bone materials without a membrane. Therefore, future studies in this area may be necessary to explore the means to reduce the soft tissue invasion and promote bone regeneration. This may include the modification of the existing dentin product and the use of guided tissue regeneration approaches.

5. Conclusions

The rabbit calvarial defect model used in this study is a suitable and robust model for the assessment of osteoconductive and osteogenic properties of bone materials and indicates that the processed bovine dentin product may not be an ideal bone material for large 4-wall defects. However, based on our previous rat femur implantation studies, the bovine dentin material may be suitable for the repair of smaller 5-wall defects. The findings of this study also confirm that the soft tissue invasion can also occur in large 4-wall rabbit calvarial defects filled with autologous bone.

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Review Article

The Current and Future Therapies of Bone Regeneration to Repair Bone Defects

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Bone defects often result from tumor resection, congenital malformation, trauma, fractures, surgery, or periodontitis in dentistry. Although dental implants serve as an effective treatment to recover mouth function from tooth defects, many patients do not have the adequate bone volume to build an implant. The gold standard for the reconstruction of large bone defects is the use of autogenous bone grafts. While autogenous bone graft is the most effective clinical method, surgical stress to the part of the bone being extracted and the quantity of extractable bone limit this method. Recently mesenchymal stem cell-based therapies have the potential to provide an effective treatment of osseous defects. In this paper, we discuss both the current therapy for bone regeneration and the perspectives in the field of stem cell-based regenerative medicine, addressing the sources of stem cells and growth factors used to induce bone regeneration effectively and reproducibly.

1. Introduction

Regenerative medicine is the medical field that creates functional tissues to repair and replace damaged or malfunctioning tissues and organs [1]. Tissues or organs generated from a patient's own cells would allow transplants without tissue rejection. Furthermore, regenerative medicine treatments have the potential to replace organ transplants or artificial organs. Because regenerative medicine generates tissues or organs using engineering technology, it is also called "tissue engineering." To make regenerative medicine successful, three elements are required: stem cells, scaffolds, and growth factors [1]. Translational research, which takes results from the laboratory and translates them to the clinics, and industry-academic collaborations also play important roles in making regenerative medicine suitable for practical use.

The human skeleton consists of approximately 200 bones, and it weighs approximately 2 kg. Bone is a tough supporting tissue and functions in both movement and the maintenance of postural stability by working cooperatively with muscles. Bones also play an important role in calcium metabolism. Despite its hard structure, bone actually exists in a constant state of dynamic turnover known as bone remodeling [2] (Figure 1). There are two types of bone structures, cortical bone and cancellous bone. The ratio of the cortical bone and the cancellous bone of an adult is 9 : 1. Approximately 3% of the cortical bone is remodeled per year, whereas more than 30% of the cancellous bone is remodeled per year. Thus, approximately 6% of all human bones will be remodeled in a year. The bone mass in an adult human reaches its maximal level (peak bone mass) during a person's twenties and then gradually declines thereafter, as the speed of bone resorption

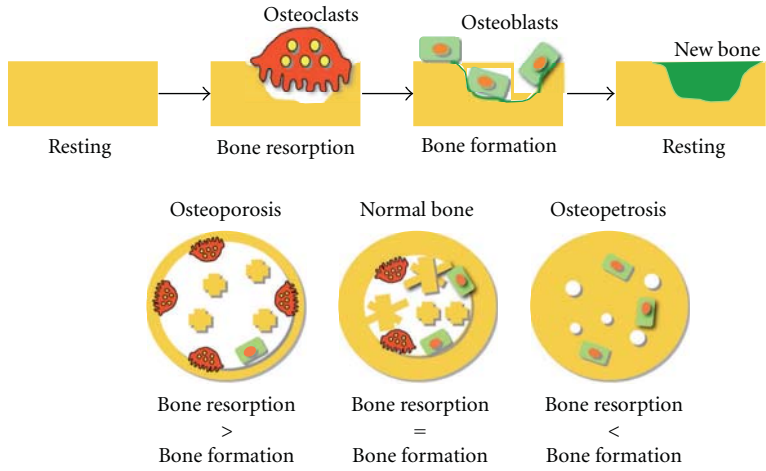

Figure 1: The schematic outlines of the bone remodeling cycle and the balance of bone resorption and bone formation. (a) In bone tissue, the osteoblasts are involved in new bone formation, while osteoclasts play a major role in bone resorption. The first step in the bone remodeling cycle is the resorption of existing bone by osteoclasts, followed by formation of the cement line in resorption lacunae and osteoblasts. Each cell type seems to be regulated by a variety of hormones and by local factors. (b) If the balance between bone formation and resorption is lost by the uncontrolled production of regulators, bone structure would be strikingly damaged, and the subject would be susceptible to osteoporosis and osteopetrosis.

exceeds bone formation with increasing age. Although bone mass in humans decreases by approximately 1% per year, the bone mass in women entering menopause will decrease by approximately 3% per year. At the remodeling sites, osteoblasts produce new bone, while osteoclasts resorb existing bone. Each cell type seems to be regulated by a variety of hormones and by local factors. If the balance between bone formation and resorption is lost by uncontrolled production of these regulators, the bone structure will be damaged, and the subject would be susceptible to osteoporosis and osteopetrosis [2] (Figure 1).

Bone defects often result from tumor resection, congenital malformation, trauma, fractures, surgery, or periodontitis in dentistry, as well as from diseases, such as osteoporosis or arthritis. The gold standard for reconstruction of large bone defects is the use of autogenous bone grafts [3]. This method has significant limitations, such as a lack of sufficient transplantable materials, donor site morbidity, inflammation, and resorption of the implanted bone. Although alternatives, such as the use of allografts or synthetic grafting materials, address these limitations, both alternatives are also limited by immunogenesis or lack of osteoinductivity [4].

The discovery of stem cells and recent advances in cellular and molecular biology have led to the development of novel therapeutic strategies that aim to regenerate tissues that were injured by disease. Recently, embryonic stem cells (ES cells) [5], induced pluripotent stem cells (iPS cells) [6], and somatic stem cells have been reported; however, there are many issues to overcome for the clinical use of ES and iPS cells, including ethical and safety problems, immunorejection, and tumorigenesis.

This review discusses the current therapies for bone regeneration and perspectives in the field of stem cell-based regenerative medicine, addressing sources of stem cells and growth factors to develop an efficient and high-quality bone derivation without any immunorejection, and tumorigenesis. We also discuss the potential use of regenerative medicine in dental tissue engineering.

2. Current Status of Bone Regeneration in Dentistry

Chronic dental disease and tooth loss often lead to the loss of hard tissue in the jaw. Patients with missing teeth by infections, or inflammation may experience bone resorption with loss of the affected part of the jaw. In addition to making a patient uncomfortable, this bone loss can cause unsightly disfigurements and may complicate the fitting of implants and other dental appliances. Although dental implants serve as an effective treatment to recover mouth function from tooth defects, many patients do not have adequate bone volume to build an implant. The outline of the current method to increase bone volume is described.

2.1. Bone Grafts and Artificial Bone Materials. Although the autogenous bone graft is the most effective clinical method for bone repair, it can be restricted by surgical stress to the site of bone extraction and the quantity of extractable bone. Demineralized and freeze-dried bone (DFDBa) extracted from the body is used for xenogamous bone grafts [3, 4].

Hydroxyapatite or various forms of β-tricalcium phosphate (β-TCP) are used as artificial bone materials [7]. Because these calcium phosphate materials do not have bone guidance capability, they are used together with autogenous bone grafts or other bone increasing methods, such as the guided bone regeneration (GBR) method and platelet-rich plasma (PRP).

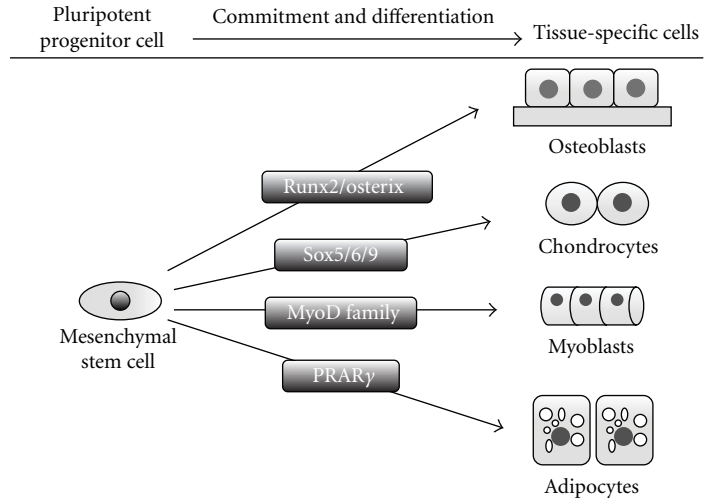

Figure 2: A schematic model for the differentiation of mesenchymal stem cells into tissue-specific cells by specific transcriptional factors. Mesenchymal stem cells can differentiate into osteoblasts, chondrocytes, myoblasts, and adipocytes. Each differentiation program is regulated by specific transcription factors: Runx2/Osterix, Sox5/6/9, MyoD family, and PPARγ, respectively.

2.2. Guided Bone Regeneration: GBR. Guided bone regeneration (GBR) encourages new bone growth to replace areas of damage in the jaw and can be used alongside guided tissue regeneration (GTR) to rebuild soft tissue in a patient's mouth [8]. The technologies and practices behind these techniques are subject to constant refinement, and clinical studies examine the possible application of these techniques to other regions of the body. GBR involves epithelial and connective tissue exclusion and space creation to allow the cells of the periodontal ligament to repopulate the root surface and to allow bone cells to grow into the area of the defect. GBR is usually performed together with a bone graft or PRP. Although this method induces self-regeneration of bone, it takes a long time to obtain adequate bone volume in many cases.

2.3. Distraction Osteogenesis. Distraction osteogenesis is a well-established technique used by orthopedic surgeons to repair long bone defects without the use of grafting materials and has gained acceptance over the past 15 years for the correction of various craniofacial deformities [9]. Several studies in various animal models demonstrated the application of osteodistraction at a number of different sites, including the mandible, the maxilla, the midface, and the cranial vault. There are several advantages of distraction osteogenesis over conventional osteotomy: operative times and blood loss are reduced, bone grafts are unnecessary, and bone is distracted in conjunction with the surrounding soft tissues and nerves. However, distraction osteogenesis has some disadvantages, such as technique-sensitive and equipment-sensitive surgery, and the possible need for a second surgery to remove distraction devices and patient compliance.

2.4. Platelet-Rich Plasma: PRP. In the field of dentistry, PRP has been used in different clinical procedures, such as sinus floor elevation, alveolar ridge augmentation, mandibular reconstruction, maxillary cleft repair, treatment of periodontal defects, and treatment of extraction sockets, where it has been applied alone or in addition to the autogenous bone, anorganic bone mineral, and organic bone substitutes [10]. The growth factors present in PRP are thought to contribute to the bone-healing process. The following growth factors are reported to be present in PRP: platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), insulin growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF). In addition, three blood proteins, fibrin, fibronectin, and vitronectin, are known to act as cell adhesion molecules for osteoconduction [11, 12]. Therefore, PRP may influence bone formation through a variety of pathways.

3. A Cell-Based Therapy for Bone Regeneration in Dentistry

In recent years, stem cell research has grown exponentially due to the recognition that stem cell-based therapies have the potential to improve the life of patients with several kinds of diseases, such as Alzheimer's disease and cardiac ischemia. These therapies have also a role in regenerative medicine, such as the repair of bone or tooth loss. Stem cells have the potential to differentiate into several cell types, including odontoblasts, neural progenitors, osteoblasts, chondrocytes, and adipocytes (Figure 2). Mesenchymal stem cells (MSC) are multipotent progenitor cells that were originally isolated from various tissues, including adult bone marrow, adipose tissue, skin, umbilical cord, and placenta. Bone marrow-derived MSCs have been used in clinical trials for the effective treatment of osseous defects. However, bone marrow aspiration is an invasive and painful procedure for the donor and is a difficult procedure for a general practitioner. Furthermore,

MSCs constitute heterogeneous cell types, and the potential for proliferation and differentiation of the MSCs depends on a patient's age, sex, or the presence of certain medical conditions, such as diabetes or hypertension [13].

Several cell populations with stem cell properties have been isolated from different parts of the tooth, including the pulp of both exfoliated and adult teeth, the periodontal ligament, and the dental follicle. Dental pulp stem cells (DPSCs) [14] and stem cells from human exfoliated deciduous teeth (SHED) [15] have generic mesenchymal stem cell-like properties, such as self-renewal and multilineage differentiation. DPSCs and SHED have the ability to generate not only dental tissue but also bone tissue. Because SHED exhibit higher proliferation rates and can be obtained with ease compared to bone marrow-derived MSCs, they might become an attractive source of autologous stem cells for bone regeneration. As described above, MSCs are heterogeneous cell populations; therefore, to induce bone regeneration effectively and reproducibly, it is important to understand the mechanisms by which growth factors or cytokines regulate osteoblast differentiation.

3.1. Regulation of Osteoblast Differentiation. Bone consists of hydroxyapatite crystals and various kinds of extracellular matrix proteins, including type I collagen, osteocalcin, osteopontin, bone sialoprotein and proteoglycans. Most of these bone matrix proteins are secreted and deposited by mature osteoblasts, which are aligned on the bone surface [2, 16]. The formation of hydroxyapatite crystals in osteoids is also regulated by osteoblasts. Therefore, the expression of a number of bone-related extracellular matrix proteins, the high enzyme activity of alkaline phosphatase (ALP) by responses to osteotropic hormones and cytokines are believed to be major characteristics of osteoblasts [2, 16].

It is well known that osteoblasts, chondrocytes, adipocytes, myoblasts, tendon cells, and fibroblasts are differentiated from common precursors in the bone marrow-derived MSCs. The lineages are determined by different transcription factors. The transcription factors Runx2, Osterix, or β-catenin regulate osteoblast differentiation, the Sox family of transcription factors (Sox9, Sox5, and Sox6) regulate chondrocyte differentiation, MyoD transcription factors (MyoD, Myf5 and Myogenin) regulate myogenic differentiation, and the C/EBP family (C/EBPβ, C/EBPδ, and C/EBPα) and PPARγ transcription factors regulate adipocyte differentiation (Figure 2). Runx2 directs multipotent mesenchymal cells to an osteoblastic lineage, and β-catenin, Osterix, and Runx2 direct them to mature osteoblasts after differentiation to preosteoblasts [2, 16, 17].

Several hormones and cytokines, such as bone morphogenetic proteins (BMP), TGF-β, Wnt, hedgehog, bFGF, and estrogen, are involved in the regulation of mesenchymal cell differentiation by stimulating intracellular signaling pathways. Among them, BMP is one of the most powerful cytokines to induce ectopic bone formation, and it strongly promotes the differentiation of mesenchymal cells into osteoblasts.

BMPs, members of the TGF-β superfamily, were originally identified by their ability to induce ectopic bone formation when implanted into muscle tissue, and they play a pivotal role in the signaling networks and processes associated with skeletal morphogenesis [16, 17]. BMP signals are transduced from the plasma membrane receptors to the nucleus through both the Smad pathway and non-Smad pathways and are regulated by many extracellular and intracellular molecules that interact with BMPs or components of the BMP signaling pathways. This bone-inducing ability of BMPs should be useful for the development of bone regeneration. However, BMPs cannot generate a sufficient clinical response to be used in bone regeneration. One possible reason might be that inflammatory cytokines inhibit both bone formation and osteoblast differentiation induced by BMPs. For example, the inflammatory cytokine tumor necrosis factor (TNF) α inhibits osteoblast differentiation in multiple models, including fetal calvaria, bone marrow stromal cells, and osteoblastic cells [18–20].

3.2. Inflammatory Cytokines Suppress Osteoblast Differentiation. Inflammatory cytokines, such as TNFα or interleukin-(IL-)1, contribute to local and systemic bone loss in inflammatory bone diseases, such as rheumatoid arthritis and periodontitis, and estrogen deficiency [21]. In patients with rheumatoid arthritis, TNFα and other cytokines are overproduced in inflamed joints by various cells that infiltrate the synovial membrane, and anti-TNF drugs, such as infliximab, etanercept, and adalimumab, have been shown to not only diminish signs and symptoms of disease but also to prevent joint damage [22]. Under these conditions, osteoblast-mediated bone formation cannot compensate for accelerated osteoclastic bone resorption, suggesting a direct inhibitory effect of inflammatory cytokines on osteoblasts.

Consistent with clinical and *in vivo* animal studies, the inhibitory effects of TNFα or IL-1β on bone formation *in vitro* were also observed with a neonatal rat calvarial organ culture system [16]. TNFα or IL-1β inhibited not only spontaneous osteoblast differentiation but also BMP-induced osteoblast differentiation, as measured by a change in the BMP2-induced expression of Runx and osteocalcin and a dose-dependent change in ALP activity. These responses were mediated *via* several signaling pathways, such as mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, and NF-κB.

3.3. Suppression of NF-κB Enhances BMP-2-Induced Osteoblast Differentiation. The transcription factor NF-κB has a key role in inflammation and immune responses. Previous studies have shown that inhibition of NF-κB suppresses inflammatory bone loss by inhibiting osteoclastogenesis in an arthritis model, suggesting that NF-κB is a major target of inflammatory bone diseases [23]. The importance of NF-κB in osteoblasts was revealed in a recent paper, where the authors expressed a dominant negative form of IKKβ to inhibit NF-κB in the mature osteoblasts of mice. Expression of this dominant negative IKKβ led to increased BMD and bone volume due to the increased activity of osteoblasts [24].

Inhibition of NF-κB by overexpression of the dominant negative form of IκBα (IκBαDN) leads to the induction of

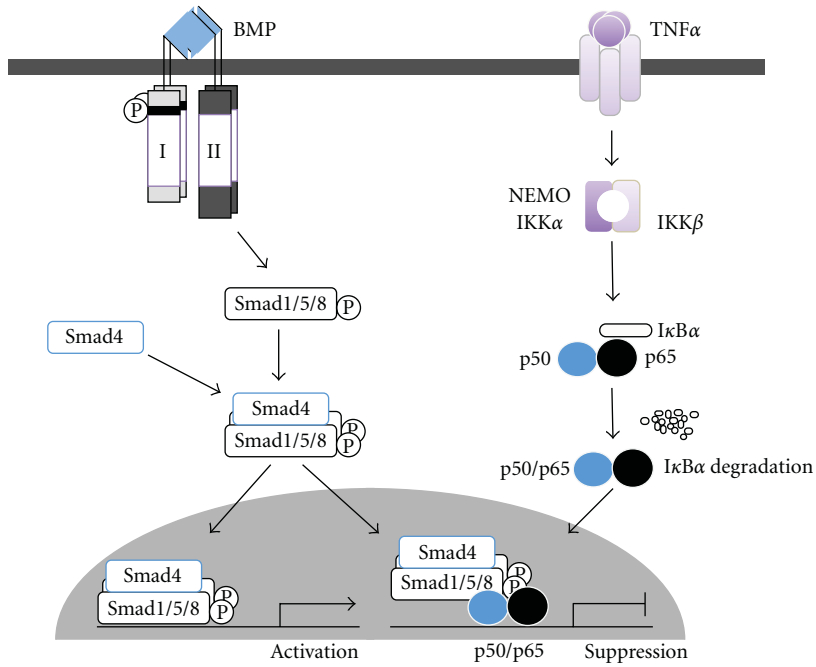

Figure 3: A model of NF-κB-mediated inhibition of BMP/Smad-mediated DNA binding activity. NF-κB, particularly the p65 subunit, binds the Smad1/Smad4 complex directly or indirectly, and that this binding interferes with the DNA binding of Smad proteins induced by BMP-2.

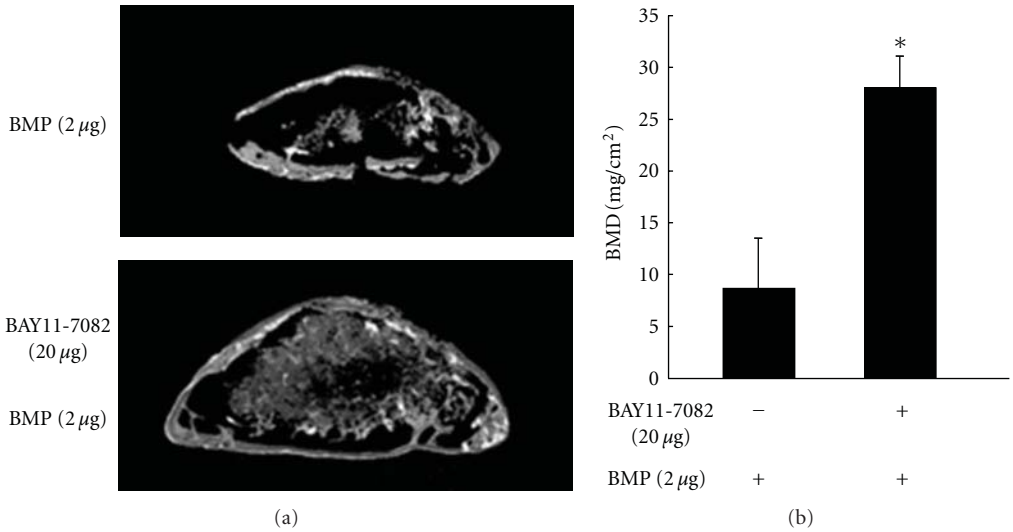

Figure 4: BMP2-induced ectopic bone formation *in vivo* is enhanced in the presence of a selective inhibitor of NF-κB, BAY11-7082. Two micrograms of BMP2 was implanted subcutaneously to induce ectopic bone formation in the presence or absence of BAY11-7082 in mice ($n = 8$). (a) μCT reconstruction images of ectopic bone in the presence or absence of BAY11-7082 in mice. Bar: 1 mm. (b) Bone mineral density (BMD) of the ectopic bone in the presence or absence of BAY11-7082 was measured by dual-energy X-ray absorptiometry (DXA). $^*P < 0.01$.

osteoblast differentiation [25]. The cell permeable NF-κB activation antagonist TAT-NBD blocks the activation of NF-κB by TNFα and could prevent TNFα from suppressing TGFβ-stimulated Smad luciferase activity, BMP2-induced Runx2 mRNA expression, and osteoblast differentiation in MC3T3-E1 cells, a mouse osteoblastic cell line [26]. Furthermore, the selective inhibition of NF-κB increased the bone formation and ameliorated osteopenia in ovariectomized mice [27]. We have previously shown that TNFα inhibited BMP-induced osteoblast differentiation through NF-κB activation by inhibiting Smad DNA binding [28] (Figure 3). Therefore, we examined whether the selective inhibitor of NF-κB, BAY11-7082, enhanced the ectopic bone formation induced by BMP2 in mice. BMP2-induced ectopic bones were enlarged and had enhanced radioplaques in the presence of BAY11-7082 compared with BMP2 treatment alone. The μCT image

of ectopic bones induced by BMP2 together with BAY11-7082 showed a thick outer bone filled with trabecular bone (Figure 4(a)). The bone mineral density (BMD) of these ectopic bones were also increased in the presence of BAY11-7082 (Figure 4(b)). These results strongly indicate that inhibition of NF-κB may promote BMP-induced bone regeneration in the treatment of bone diseases.

4. Conclusion

Although regenerative medicine has been tried in various fields, there is much demand for regenerative medicine in dentistry, particularly in bone regeneration. Depending on the state of periodontitis or jaw resection, it might take more than 6 to 12 months for occlusal reconstitution. Thus, the development of an efficient and high-quality bone derivation method is necessary. Cell-based therapy may pave the way to rejection-free regenerative treatment for bone defects. It is also likely that research concerning growth factor or cell-based therapies will continue to progress. However, there are also many problems, such as laws and costs of equipment, that must be solved. Although it is unclear when the technology of regenerative medicine will be put into practical use, it is important to follow the current status of regenerative medicine to keep abreast of the progression the technology.

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Research Article

Possible Involvement of Smad Signaling Pathways in Induction of Odontoblastic Properties in KN-3 Cells by Bone Morphogenetic Protein-2: A Growth Factor to Induce Dentin Regeneration

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We examined the effects of bone morphogenetic protein-2 (BMP-2) on growth, differentiation, and intracellular signaling pathways of odontoblast-like cells, KN-3 cells, to clarify molecular mechanisms of odontoblast differentiation during pulp regeneration process. After treatment with BMP-2, the cell morphology, growth, alkaline phosphatase (ALP) activity, and the activation and expression of BMP-induced intracellular signaling molecules, such as Smad1/5/8 and Smad6/7, as well as activities of dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP1), were examined. BMP-2 had no effects on the morphology, growth, or ALP activity of KN-3 cells, whereas it induced the phosphorylation of Smad1/5/8 and expression of Smad6/7. BMP-2 also induced the expressions of DSP and DMP-1. Our results suggest that KN-3 cells may express an odontoblastic phenotype with the addition of BMP-2 through the activation of Smad signaling pathways.

1. Introduction

When dental pulp is exposed to external stimuli such as bacterial infection and following restorative procedures, a wound healing process is induced, and surviving odontoblasts and odontoblast-like cells differentiated from progenitor or stem cells form reactionary and reparative dentin to block further external stimuli [1–3]. During the regeneration process of dental pulp, similar mechanisms to form new dentin are also induced. Previously, it was demonstrated that controlled release of fibroblast growth factor-2 (FGF-2) from gelatin hydrogel beads induced regeneration of dentin and pulp tissue on amputated dental pulp [4, 5]. Furthermore, applications of bone morphogenetic proteins-2 and -4 (BMP-2,-4) along with dentin powder [6] and BMP-2 along with dental pulp stem cells [7] induced regeneration of dentin on amputated dental pulp. Several studies have focused on clarification of a common mechanism existing in wound healing and regeneration of dental pulp in order to overcome the limitations of present methods to preserve vital dental pulp and develop effective pulp regeneration therapies; however, the molecular mechanisms of odontoblastic cells activated by BMP-2 are not fully understood.

BMPs are known to have diverse biological functions during embryonic development [8, 9] and osteogenesis [10, 11]. Notably, the regulation mechanisms of Runx2/Smad and other cascades controlled by BMP-2 during osteoblast and odontoblast differentiation from mesenchymal stem cells have been extensively studied [12–17]. In addition, the combination of BMP-2 with a collagen sponge was recently approved by the US Food and Drug Administration for clinical use such as oral maxillofacial surgery [18],

while further studies have focused on development of an effective method based on available clinical data as well as to understanding the effects of BMP-2.

We previously established a proliferating pulp progenitor cell line (KN-3 cells) from dental papilla cells of rat incisors [19]. KN-3 cells have high levels of alkaline phosphatase (ALP) activity and expression of Runx2 and dentin sialophosphoprotein (DSPP). The addition of the medium including ascorbic acid and β-glycerophosphate induced the formation of extracellular mineralized nodules, which was suppressed by bacterial lipopolysaccharides. These findings indicated that KN-3 cells exhibited typical odontoblastic properties [19]. To understand molecular mechanisms of the differentiation of KN-3 cells into functional odontoblast-like cells, we examined the effects of BMP-2 on the cell growth, differentiation, and the involvement of Smads signaling pathways in the responses of KN-3 cells on BMP-2.

2. Materials and Methods

2.1. Cell Culture. A rat clonal dental pulp cell line with odontoblastic properties (KN-3) was maintained in alpha-modification of Eagle's medium (α-MEM) (Invitrogen Life Technology, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FCS), 100 μg/mL of streptomycin, and 100 U/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37°C [19].

2.2. Morphological Analysis. KN-3 cells (3×10^4/well) were subcultured in 6-well plates for 24 hours in α-MEM containing 10% FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin, then treated with BMP-2 (100 ng/mL) in α-MEM containing 5% FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37°C. After 72 hours, the cells were observed by phase-contrast microscopy.

2.3. Cell Viability Assay. The cell proliferation reagent WST-1 was used for quantitative determination of cellular proliferation (Dojindo, Kumamoto, Japan). KN-3 cells (2×10^4/well) were subcultured in 96-well plates for 3 hours in α-MEM containing 10% FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin, then treated with BMP-2 (0–100 ng/mL) in α-MEM containing 5% FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37°C for 48 hours. WST-1 and 1-Methoxy PMS (10 μL/well) were added and incubation was performed for 2–4 hours, after which the viability of KN-3 cells was analyzed by measuring optical density with a microplate reader (Model 680; Bio-Rad laboratories, Inc., Tokyo, Japan) using a test wavelength of 450 nm.

2.4. Alkaline Phosphatase Activity. KN-3 cells (1×10^4/well) were subcultured in 96-well plates for 24 hours in α-MEM containing 10% FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin, and then treated with BMP-2 (100 ng/mL) in α-MEM containing 0.1% FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37°C. After 1, 3, 5, and 7 days, the cells were solubilized with 200 μL of Hank's salt solution containing 0.2% Nonidet P-40 (Pierce Biotechnology, Rockford, IL) for 10 minutes at 37°C. ALP activity of the lysate was measured using *p*-nitrophenylphosphate with the Lowry method. After 30 minutes of incubation at 37°C, the absorbance of *p*-nitrophenylphosphate at 405 nm was determined by using a microplate reader, and the specific activity of ALP (μg/μg of protein/30 minutes) was calculated. Protein contents were measured with a DC protein assay kit (Bio-Rad Lab, Hercules, CA).

2.5. Western Blot Analysis. KN-3 cells treated with BMP-2 were washed with phosphate-buffered saline (PBS; pH 7.2) and lysed in cell lysis buffer (50 mM Tris-HCl containing 2% SDS). Protein contents were measured using a DC protein assay kit (Bio-Rad, Hercules, CA). The samples were subjected to 10% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA). Nonspecific binding sites were blocked by immersing the membranes in 5% bovine serum albumin in PBS for 1 hour at room temperature, then they were incubated with the primary antibodies; rabbit antidentin sialoprotein (DSP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit antidentin matrix protein 1 (DMP-1) (Takara Bio, Inc., Shiga, Japan). Subsequently, the membranes were incubated with anti-rabbit and anti-mouse IgG secondary antibodies (GE Healthcare, Little Chalfont, and Buckinghamshire, UK). Immunodetection was performed using an ECL plus Western blot detection system (GE Healthcare), according to the manufacturer's instructions. Blots were stained with Coomassie Brilliant Blue and all lanes were confirmed to contain similar amounts of protein extract.

2.6. Statistical Analysis. Statistical differences were determined using one-way ANOVA computation combined with Scheffe test for multiple comparisons. All data are expressed as the mean ± SD.

3. Results

3.1. Cell Viability, Morphology, and ALP Activity of KN-3 Cells. Figure 1 shows the effects of BMP-2 (100 ng/mL) on cell viability, morphology, and ALP activity of KN-3 cells. The results of the WST-1 assay showed no significant differences in cell viability among several different concentrations of BMP-2 (Figure 1(a)). Also, there were no differences in the morphology of KN-3 cells before and after confluence between the presence and absence of BMP-2 (Figure 1(b)). The ALP activity of KN-3 cells after confluence increased in a time-dependent manner, regardless of exposure to BMP-2 (Figure 1(c)).

3.2. Intracellular Responses of KN-3 Cells. Figure 2 shows the effects of BMP-2 on the activation of Smad signaling pathways. The addition of BMP-2 (100 ng/mL) induced phosphorylation of Smad1/5/8 in KN-3 cells, which reached

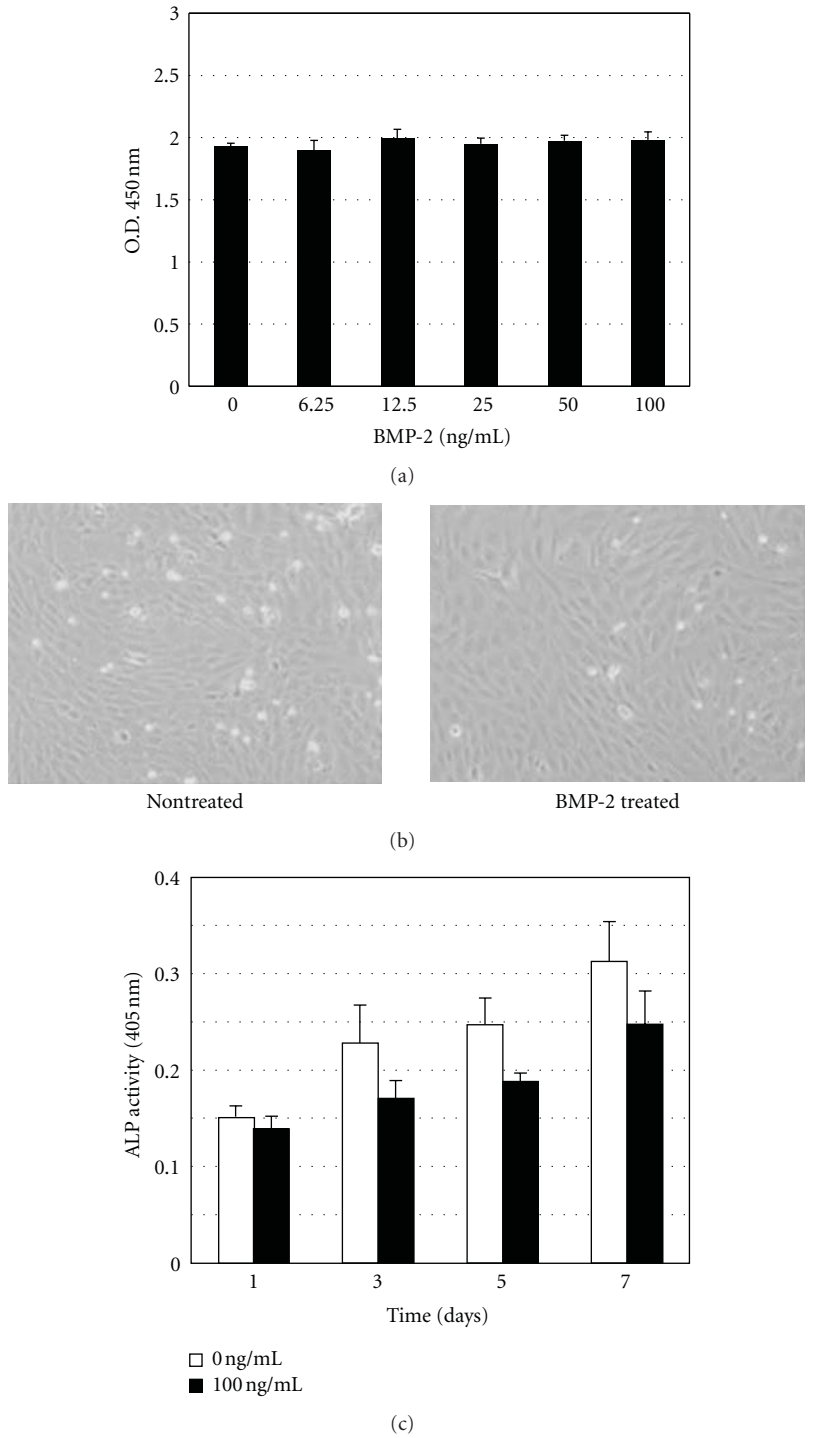

Figure 1: (a) Viability of KN-3 cells after exposure to BMP-2. Data are expressed as the mean ± standard deviation of triplicate cultures. Each experiment was performed 3 times, with similar results obtained in each. (b) Phase-contrast microphotographs of KN-3 cells treated with BMP-2 (100 ng/mL) for 72 hours. (c) Alkaline phosphatase activity of KN-3 cells treated with BMP-2 (100 ng/mL). BMP-2 was replaced with fresh medium every 3 days.

a maximum level within 30 minutes (Figure 2(a)). Also, the BMP-2-induced phosphorylation of Smad1/5/8 increased in a dose-dependent manner, and the maximum in the phosphorylation was at 100 ng/mL of BMP-2 (Figure 2(b)). The expressions of Smad6 and 7 appeared to increase at 24 hours and reached a maximum level within 72 hours after the addition of BMP-2 (100 ng/mL) and then continued throughout the culture period (Figure 2(c)).

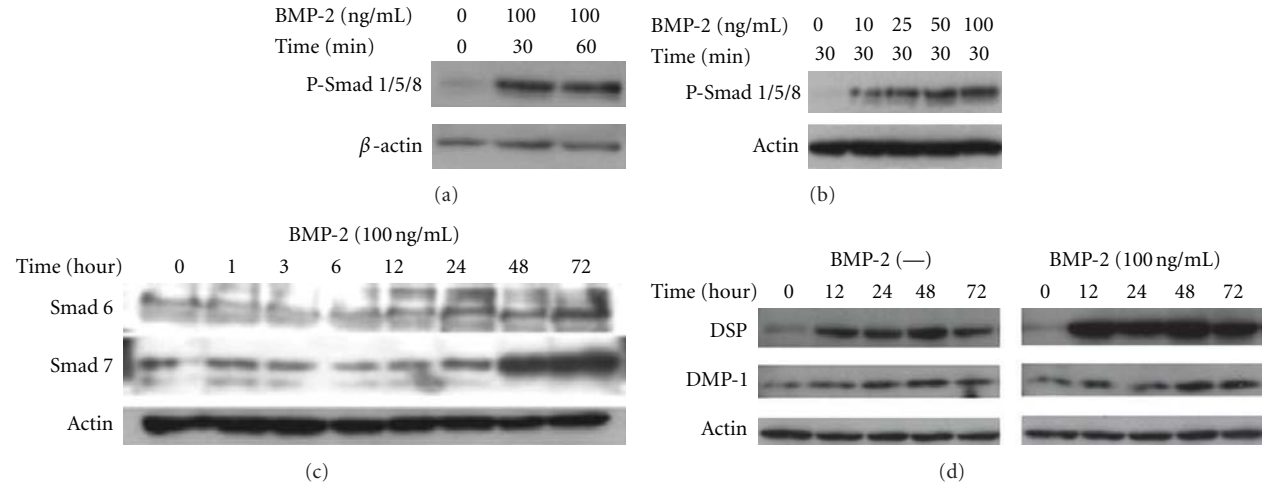

Figure 2: Western blot analysis of (a) and (b) phosphorylated Smad1/5/8, (c) inhibitory Smad6/7, and (d) DSP and DMP-1 extracted from KN-3 cells after treatment with BMP-2.

The expression of DSP and DMP-1, markers of odontoblast differentiation, were increased at 12 and 48 hours, respectively, following the addition of BMP-2 (100 ng/mL), the expression of DSP were increased throughout the culture period, but that of DMP-1 had been on the increase, but not significantly (Figure 2(d)).

4. Discussion

A number of studies have used primary dental pulp cells, existing pulp cell lines, and a few odontoblastic cells lines, and characterized their properties to elucidate tooth development, responses of dental pulp to external stimuli, as well as therapeutic approaches for the dentin-pulp complex. Previously, we established KN-3 cells from dental pulp and found that the cells had odontoblastic properties. In the present study, we examined the effects of BMP-2, the most bioactive molecules to induce osteoblast and odontoblast differentiation, on KN-3 cells *in vitro* in order to clarify further characteristics of KN-3 cells as odontoblastic-like cells. BMP-2 had no effects on cell growth, morphology, and ALP activity of KN-3 cells. Previously, we reported that KN-3 cells are able to form calcified nodules. It was also suggested that the outgrowth of odontoblastic process was induced by FGF-2 to a much greater degree than BMP-2, and that base membrane components such as laminin also had effects on the outgrowth of odontoblastic process [20, 21]. Furthermore, it was shown that MC3T3-E1 cells, osteoblastic cell line, differentiated into odontoblasts without upregulation of ALP activity [22], suggesting that an increase in ALP activity is not necessary for odontoblast differentiation. The present results support the notion that BMP-2 is not necessary for morphological change or activation of ALP in the process of differentiation of odontoblast-like cells.

In contrast, the present results revealed that BMP-2 induced activation of intracellular signaling pathways in KN-3 cells, as we found phosphorylation of Smad1/5/8, and upregulation of Smad6 and 7 in BMP-2-treated KN-3 cells. It is well known that the members of TGF-β including BMPs activate Smad proteins are intracellular signaling molecules, and that the Smad pathway is one of important signaling pathways in signal interpretation from the ECM during osteogenesis/dentinogenesis. There are 3 types of Smads: receptor regulated (R-Smads), common mediator (Co-Smads), and inhibitory (I-Smads) [23]. Following the phosphorylation R-Smads (Smad1, 5, and 8) by BMPs, heteromeric complexes are formed with Co-Smad (Smad4) and translocate to the nucleus where they regulate the transcription of target genes together with other nuclear cofactors. In the present study, we clearly detected that BMP-2-induced phosphorylation of Smad1/5/8 in KN-3 cells, as well as upregulation of Smad6 and 7. I-Smads (Smad6 and 7) provide feedback inhibition of BMP-receptor activation by blocking continued R-Smad phosphorylation by BMP receptors, suggesting that upregulation of I-Smads may be essential to limit BMP signaling for proper odontoblastic differentiation of KN-3 cells. Taken together, the present results are the first to demonstrate that KN-3 cells respond to BMP-2 signaling via activation of Smad signaling pathways.

We also examined whether BMP-2 has effects on the expressions of odontoblast specific molecules in KN-3 cells and found that those of DSP and DMP-1 were induced by BMP-2 in a time-dependent manner. DSP is a noncollagenous dentin matrix protein that is transcripted from the dentin sialophosphoprotein (DSPP) gene and known as an odontoblast-specific protein [24–27]. The previous study revealed that KN-3 cells cultured for 72 hours showed high expression level of DSPP [19], indicating that KN-3 cells are precursor cells, which have abilities to differentiate into odontoblast-like cells. DSP increase in BMP-2-untreated cells in the present study was resulted from the properties of KN-3 cells, and the enhanced expression of DSP on BMP-2-treated KN-3 cells indicates the upregulation of odontoblastic differentiation. DMP-1 is a noncollagenous protein expressed in mineralized tissues [24–26, 28]. Various lines of evidence indicate that DSP is induced in the

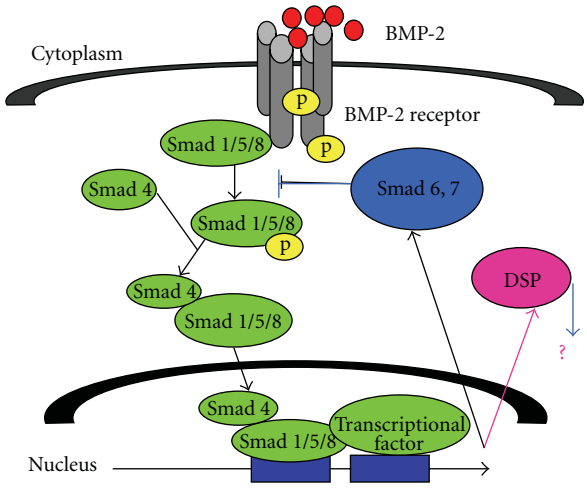

FIGURE 3: Proposed schema of Smad signaling pathways in KN-3 cells.

early stage of odontoblast differentiation, whereas DMP-1 is induced in the late stage and tightly bound to the mineral phase of dentin. In the present study, effects of BMP-2 on the early stage of KN-3 cell differentiation were analyzed, resulting in no significant difference in DMP-1 expression between BMP-2-treated and nontreated cells and the less expression of DMP-1 than that of DSP in BMP-2-treated cells.

Our results indicate that BMP-2 plays critical roles in induction of the odontoblast properties of KN-3 cells via activation of Smad signaling pathways. In previous studies [29], it was indicated that BMP-2 mediates DSPP expression and odontoblast differentiation and Smad signaling pathway plays a crucial part in the regulation of Dspp expression through the action of Smads, Dlx5, Runx2, and Msx2, suggesting that the expressions of DSP and DMP1 in KN-3 cells may be linked to the Smad pathway (Figure 3). We are continuing research to clarify the direct regulation of DSP and DMP-1, as well as mineralized nodule formation in the later stage of KN-3 cells differentiation, by BMP-2-Smad signaling pathway. Our results also show that KN-3 cells with odontoblastic properties are useful to clarify molecular mechanisms of odontoblasts against external stimuli such as growth factors, in order to develop appropriate regeneration therapy for the dentin-pulp complex.

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Review Article

Candidates Cell Sources to Regenerate Alveolar Bone from Oral Tissue

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Most of the cases of dental implant surgery, especially the bone defect extensively, are essential for alveolar ridge augmentation. As known as cell therapy exerts valuable effects on bone regeneration, numerous reports using various cells from body to regenerate bone have been published, including clinical reports. Mesenchymal cells that have osteogenic activity and have potential to be harvested from intra oral site might be a candidate cells to regenerate alveolar bone, even dentists have not been harvested the cells outside of mouth. This paper presents a summary of somatic cells in edentulous tissues which could subserve alveolar bone regeneration. The candidate tissues that might have differentiation potential as mesenchymal cells for bone regeneration are alveolar bone chip, bone marrow from alveolar bone, periosteal tissue, and gingival tissue. Understanding their phenotype consecutively will provide a rational approach for alveolar ridge augmentation.

1. Introduction

For increasing the success rate of implant surgery, various scaffolds and methods have been developed to augment atrophic alveolar ridge. Generally, autologous bone augmentation has been penetrating as a golden standard bone augmentation; however, most of the patients might not be feasible for extracting their own bone, just because it is a healthy part. To avoid aforementioned high-invasive treatments, cell therapy has recently been researched in this age of rapid advance.

Combination of mesenchymal cells and ceramic scaffold for bone regeneration has been documented [1]. Cultured mesenchymal cells introduced into ceramic scaffolds exhibits robust osteogenic potential, with bone forming into pore regions of scaffolds. After this report, numerous reports using various cells to regenerate bone and sophisticate reviews for bone regeneration of craniofacial site have been published [2–7]. Usage of tooth, including periodontal ligament or pulp, has also been reported that multipotential stromal cells which are composed above mentioned were exploited in bone or periodontal regeneration [8, 9]. Although bone augmentation is mostly fundamental to elderly, they unfortunately follow to edentulous patients in aging society. Thus, this paper focuses on adult mesenchymal cells that could be able to expand from edentulous jaw. Figure 1 shows the tissues we describe in this paper by sectional scheme of edentulous alveolar ridge.

2. Alveolar Bone Chips

Osteoblasts-like cells migrated from alveolar bone chips have generally high osteogenic activity. Essentially, mammalian bones are in the form of two different ways: long bones via endochondral ossification and flat bones via intramembranous ossification. Orofacial bone is mainly formed via intramembranous ossification, and a part of mandibular is formed via endochondral ossification. These bony types show considerable differences in protein composition [10]. Harvesting bony chips from various sites implicate that origins of the osteoblastic cells (from maxilla or mandibular, from cortical or trabecular bone) are distinct from each reports; furthermore osteogenic activity, expression

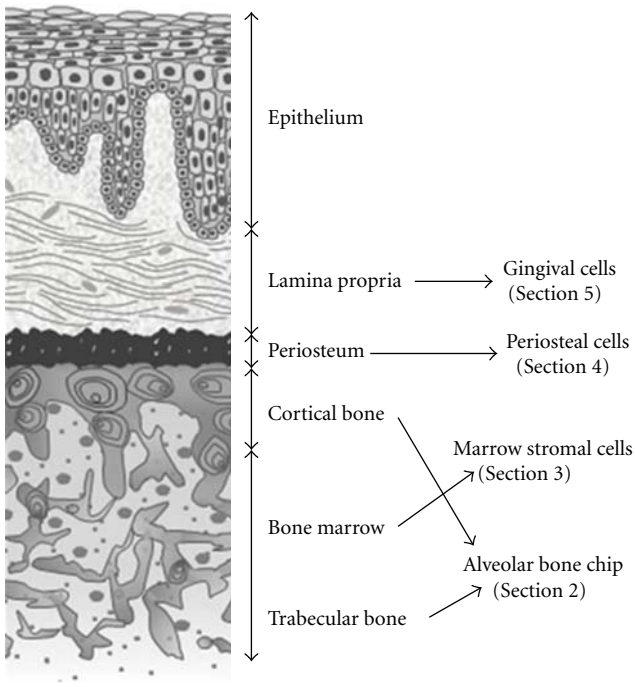

Figure 1: Sectional scheme of edentulous alveolar ridge. Figure shows the origin of candidate tissues and the cells we could harvest from alveolar bone chip, bone marrow, periosteum, and gingiva.

of surface antigens, or ability for ectopic bone formations might be different among each report, beside cell isolation protocols are different among each report. Majority of culture protocol of osteogenic cells from alveolar bone are wash bone specimens in PBS, scrape to remove attached soft tissue and periosteum, brake into small pieces, and wash with collagenase (1 to 2 mg/mL) dissolved in culture medium [11, 12]. In some reports, osteogenic cells were collected without collagenase [13–15]. However, despite harvesting bony chips from healthy site is essential when we use these in clinic, it is not feasible for all patients just because of the invasive operation. In addition, it is still not clear how amount of bony chips is enough to regenerate each part of alveolar ridge and which part of bone cells are suitable to keep augmented bone volume on long prognosis.

3. Bone Marrow from Alveolar Bone

The reason why iliac crest bone marrow is the most documented bone marrow transplantation is because they have been corrected for bone marrow transplantation in clinic as usual. Bone marrow stromal cells (BMSCs) have been reported their ability of multipotent differentiation to bone, cartilage, tendon, muscle, adipose tissue, and neuronal tissue [16–18]. Bone regenerative clinical studies using BMSCs, collected from iliac crest to reconstruct jaw defects, have been reported [19, 20]. Kawaguchi et al. reported that iliac crest BMSCs enhance periodontal tissue regeneration as well [21, 22]. Alveolar BMSCs, however, is essentially different from axial BMSCs from their differential potential or their gene expression pattern [23, 24]. Embryologically, alveolar tissues including alveolar bone marrow are originated from neural crest cells, but other bone marrows are from mesoderm [25, 26]. Cherubism [27], Treacher Collins syndrome [28], craniofacial fibrous dysplasia [29], and hyperparathyroid jaw tumor syndrome [30] affect only jaw bones, indicating that orofacial bone development differs from that of axial and appendicular bone formation. Whitaker's group have reported that membranous bone underwent less resorption than endochondral bone in monkey model [31], and they found the rapid vascularization on membranous onlay bone grafts in rabbit model [32]. In human alveolar cleft defects, chin bone was better incorporated, significantly less resorbed than iliac crest bone [33, 34]. In histomorphometry, autologous grafts obtained from calvarial sources for sinus lift procedure present a significantly higher degree of bone volume in contrast to bone harvested from the iliac crest [35]. In *in vitro* and *in vivo* study, Akintoye et al. have investigated skeletal site-specific phenotypic and functional differences between orofacial (maxilla and mandible) and axial (iliac crest) human BMSCs. Compared with iliac crest cells, orofacial BMSCs proliferated more rapidly with delayed senescence, expressed higher levels of alkaline phosphatase, and demonstrated more calcium accumulation *in vitro*. Orofacial BMSCs formed more bone *in vivo*, while iliac crest BMSCs formed more compacted bone that included hematopoietic tissue and were more responsive *in vitro* and *in vivo* to osteogenic and adipogenic inductions [36]. Comparing with the osteogenic properties of BMSCs attached on titanium for evaluating the avidity bone to implant exhibited that there was no difference in the affinity of maxilla and iliac crest BMSCs to titanium. Titanium-attached maxilla BMSCs, however, were apparently more osteogenically responsive than iliac crest cells based on calcium accumulation and gene expression of alkaline phosphatase and osteopontin [37]. Akintoye et al. have also studied the skeletal site-specific osteogenic response of BMSC to BMP-2 stimulation [38]. They reported orofacial BMSC displayed high expression of osteogenic markers in response to BMP-2 in contrast to the low response of adult iliac crest BMSC. They also reported that mandible BMSCs were more susceptible to bisphosphonates than iliac crest BMSC [39]; orofacial BMSC survived higher radiation doses and recovered quicker than iliac crest BMSC [40]. Osteoclastogenic potential of jaw and long-bone-derived osteoclasts have different dynamics, and this might primarily due to differences in the cellular composition of the bone site-specific marrow [41]. Aghaloo et al. established a protocol for rat mandible and long-bone marrow stromal cell isolation and culture. Upon implantation into nude mice, mandible BMSCs formed 70% larger bone nodules containing three-fold more mineralized bone compared with long-bone BMSCs [42]. Alveolar BMSCs are obtained from older individuals, and the donor age has little effect on their gene expression pattern [43]. According to aforementioned studies, usage of alveolar BMSCs might have high advantages for alveolar bone regeneration compared with iliac BMSCs; however, establishing the protocol of harvesting BMSCs in low invasive way is still unclear.

4. Periosteal Tissue from Oral Site

Usage of periosteal cells from periosteum was originally reported by Breitbart et al. on rabbit experiments [44]. Outgrowthed periosteal cells were cultured with dexamethasone contained medium, and cell/polyglycolic acid nonwoven fiver scaffolds complex showed significant bone formation on calvarial defect compared with scaffold only. Adult human periosteal cells from tibia include multipotent clonogenic cells [45], while, in the case of oral tissue, periosteal cells isolated from the mandibular angle of human with β-tricalcium phosphate granules have shown that combined treatment with bFGF and BMP-2 can make periosteal cells a highly useful source of bone regeneration [46]. In nude mouse subcutaneously model, acid-treated HA block cultured with human periosteal cells complex had significant osteogenic potential at the site of implantation *in vivo* [47], while in canine model for peri-implant bone regeneration, periosteum-derived cells in conjunction with e-PTFE membranes did not provide additional benefit [48]. Comparing with proliferated periosteal cells, Cicconetti et al. harvested marrow cells from maxillary tuberosity bone. They concluded that both periosteal cells and marrow stromal cells showed comparable phenotypic profiles and both cell populations formed bone upon ectopic *in vivo* transplantation [49]. Harvesting periosteal cells is relatively invasive treatment; for instance, it is still unclear the collection quantity for required bone regeneration and also periosteal tissue is hard to collect by general practitioners.

5. Gingival Tissue

During dental surgery, gingival tissue could be obtained frequently as a discarded biological sample. Wound healing within the gingiva is characterized by markedly reduced inflammation, scarless healing, rapid reepithelialization, contrary to the common scar formation present in skin [50, 51]. Recently, several reports have indicated the presence of progenitor cells in gingival connective tissue [52, 53]. Tang et al. reported gingival tissue contains tissue-specific mesenchymal stem cell population and is an ideal resource for immunoregulatory therapy, using human normal and hyperplastic gingival tissues [54]. The ratio of these progenitors in gingival fibroblasts, however, might be very low rather than bone marrow, periosteal tissue, or bone chips. Nevertheless, enrichment of progenitor cells that show characteristics with of differentiation as osteoblastic cells entail for usage in bone tissue engineering.

6. Conclusion

Bone marrow-derived cells, called mononuclear cells or marrow mesenchymal cells, are essentially different from osteoblastic cells derived from bone chip. In a case of small animal model, it is hard to separate mandibular bone marrow cells to bone lining osteoblastic cells [55]. In bulk, however, this bone/BMSCs possess unique stem cell properties that the size of alveolar bone has restrained the precise analysis of BMSCs phenotype. Furthermore, diversity of culture methods made us to confuse. As we use outgrowth cells from tissues on the other side, the population cultured cells is influenced by their migration ability. Treating tissue with enzyme to disperse cells from tissue, however, cell population is not dependent on migration ability. Thus, developing stable and universal methods to harvest and culture osteogenic cells for bone regeneration is prerequisite.

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Research Article

Application of Laser-Induced Bone Therapy by Carbon Dioxide Laser Irradiation in Implant Therapy

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This study evaluated the application of laser-induced bone therapy (LIBT) to reduce implant healing time in rat tibia. Twenty 10-week-old female Sprague-Dawlay rats were used. The rats received laser irradiation (laser group) or sham operation (control group) on either side of the tibia. Five days after invasion, titanium implants were inserted in proximal tibia. Five, 10, and 20 days after implant placement, tibiae were collected. After taking micro-CT and performing a torque test, the tibiae were decalcified and 8-μm-thick sections were prepared. Specimens were stained with hematoxylin and eosin. *Results*. Micro-CT images, removal torque values, and histomorphometric analysis data demonstrated a significantly accelerated bone formation in the laser group earlier in the healing process. *Conclusion*. The use of laser irradiation was effective in promoting bone formation and acquiring osseointegration of titanium implants inserted in rat tibia. LIBT may be suitable for use in implant therapy.

1. Introduction

Bone formation in peri-implant tissue is a key factor in acquiring osseointegration and maintaining implant stability. However, a longer period for healing and acquiring osseointegration is needed in order to add occlusal load. It has been suggested that a standard healing period of at least 3 months in the mandible and 6 months in the maxilla is needed before implant loading [1–3]. Thus, to speed up the rehabilitation process is still a challenging and important clinical aim.

The skeleton that adapts to mechanical usage and mechanical loading promotes bone formation and remodeling, which is commonly referred to as "Wolff's Law" [4, 5] and Frost's theory [6–8]. Mechanical strains such as pulsed electromagnetic fields [9, 10] and low-intensity pulsed ultrasound [11, 12] are widely accepted. Currently, lasers are commonly used in medical and dental treatment. Clinical applications of lasers are largely divided into low reactive level laser therapy (LLLT) and high reactive level laser therapy (HLLT). LLLT provides photobiological and photochemical effects. LLLT enhanced healing, especially in soft tissues [13, 14] such as healing of ulcers [15] and other wounds [16]. Recent research demonstrated that the enhancement of functional attachment of bone-to-titanium implants and promotion of bone mineralization could be achieved by LLLT [17–21]. This would allow the implant to be loaded after a shorter period, reducing the treatment time. A positive effect of LLLT on osseointegration of implants and maturation of peri-implant bone was mainly obtained with Gallium Aluminum Arsenide (GaAlAs) laser [17, 18, 20]. HLLT is useful for cutting biologic materials and producing coagulation necrosis in target tissues with a subsequent reaction in the surrounding tissue. When HLLT is applied to hard tissues such as tooth or bone, carbon dioxide laser (CO_2 laser) induces extreme cracking and charring of surrounding enamel, dentin, and bone. After HLLT CO_2 laser irradiation on rat tibia, disappearance and shrinkage of osteocytes within the lacunae have been observed [22–27]. New bone formation was also observed at the tibial wall adjacent to the marrow cavity under the laser-irradiated cortex [27, 28]. However, HLLT thermal damage results in extensive cell mediated resorption of bone or sequestration of dead bone [29], thus severely limiting the use of HLLT on bone. Currently, CO_2 laser is common laser used in dental clinics and it also enables

rapid and precise tissue destruction, reduces bleeding and postoperative pain, and results in low morbidity, minimal scarring, and wound contracture [30]. However, the application of CO_2 laser in dental treatment is restricted to the treatment of soft tissue.

This time, we hypothesized that bone formation occurring within the marrow mediated by HLLT CO_2 laser irradiation would accelerate the osseointegration process and reduce healing time. To evaluate our hypothesis, an animal study using functional and morphological analysis was performed.

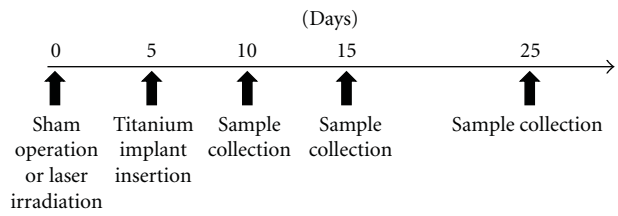

Figure 1: Experimental protocol. Time schedule of bur/laser injury, implant placement, and subsequent healing periods.

2. Material and Methods

2.1. Experimental Design. The care and use of animals followed "The Guidelines for the Care and Use of Animals" approved by Ohu University in accordance with the principles of the NIH guidelines (approval date: 5/14/2009; Approval number: 2). Twenty 10-week-old female Sprague-Dawley rats were purchased from Crea Japan (Tokyo, Japan) and used as the experimental model in this study. The right tibia of all rats was treated with laser irradiation, and left tibia was sham operated. Five days after laser irradiation and sham operation, titanium implants were placed. Briefly, under general anesthesia, the surface of the proximal metaphases of the tibiae was exposed by an incision approximately 10 mm in length. Under constant saline irrigation, a bicortical implant bed was drilled with a dental bur at a rotary speed not exceeding 1500 rpm, and the implant was placed until the screw thread completely penetrated the bone cortex. After installation, the soft tissue was replaced and sutured. After these procedures, the animals were housed with free access to water and provided a diet. Care was taken to avoid unnecessary stress and discomfort to the animal throughout the experimental period. Five animals were sacrificed 5, 10, or 20 days after implantation, and tibiae containing implants were collected (Figure 1).

2.2. CO_2 Laser. In this experiment, CO_2 laser (NANOLASER GL-III, GC Co., Tokyo, Japan, and OPELASER Lite, Yoshida Co., Tokyo, Japan) was used at a wavelength of 10.6 μm, a diameter of 1.70 mm, output of 1.0 W, and a continuous wave form. The laser beam was focused by maintaining 10 mm delivery tip-to-target surface distance. Total irradiated energy was 220.4 J/cm^2.

2.3. Titanium Implant. Screw-shaped implants made from commercially pure titanium were used in this study (Nishimura Co., Ltd., Fukui, Japan). The total length of each implant was 2 mm, thread diameter 1.4 mm, and pitch 0.6 mm. Implants were cleaned in absolute ethanol in an ultrasonic bath and sterilized by autoclaving.

2.4. Microtomographic Histomorphometry (Micro-CT). After sacrificing the rats, tibiae with titanium implants were collected, and microtomographic histomorphometry was performed with a high-resolution micro-CT system (TOSCANER-30000, Toshiba IT and Control Systems Co., Tokyo, Japan). The Computed tomography parameters were as follows: (1) the image pixel size was set to 1024 × 1024; (2) the slice thickness was set to 0.05 mm; (3) the image magnification was set to 10x; (4) the X-ray tube voltage was set to 100 kV; (5) the anode electrical current was set to 80 μA. Three dimensional images were reconstructed using the microreconstruct software (Simplant Pro, Materialise Dental Japan Inc., Tokyo, Japan.).

2.5. Torque Test. After stabilization of the implanted tibia, the force needed to unscrew the implants was measured using a Tohnichi Torque driver FTD2-S (Tohnichi Mfg. Co., Ltd., Tokyo, Japan). It has a round dial gauge with a pointer to read the peak value. Peak value when the rupture occurred between implant and bone was recorded, and the mean torque measurements were calculated for each implant inserted into the tibia specimen.

2.6. Histomorphometric Procedure. After removing the titanium implants, collected tibiae were fixed in 10% phosphate-buffered neutral formalin (pH 7.4) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), decalcified in 0.5 mol/L EDTA (pH 7.5) (Wako Pure Chemical Industries) for 2 weeks at 4°C, dehydrated in an ethanol (Wako Pure Chemical Industries) series, washed in xylene (Wako Pure Chemical Industries), and then embedded in paraffin. Decalcified 8-μm thick sections were made and stained with hematoxylin and eosin (H&E) for general morphological analysis.

2.7. Statistical Analysis. For biomechanical testing analysis, a two-way analysis of variance was used to examine the influence on osseointegration of (1) laser or sham operated and (2) the length of the healing period. Differences with a P value less than 0.05 were considered significant.

3. Results

3.1. Osteoid Formation (Figure 2). To examine the correlation between total irradiated energy and osteoid formation capacity, we evaluated four kinds of energy densities, 88.2, 220.4, 441.0, and 661.5 J/cm^2. Five days after laser irradiation, a char layer, empty osteocytic lacunas were observed. The osteocytic lacunae in most of the cortical bone appeared to be devoid of osteocytes because the typical blue-purple staining of osteocytic nuclei was absent on H&E staining.

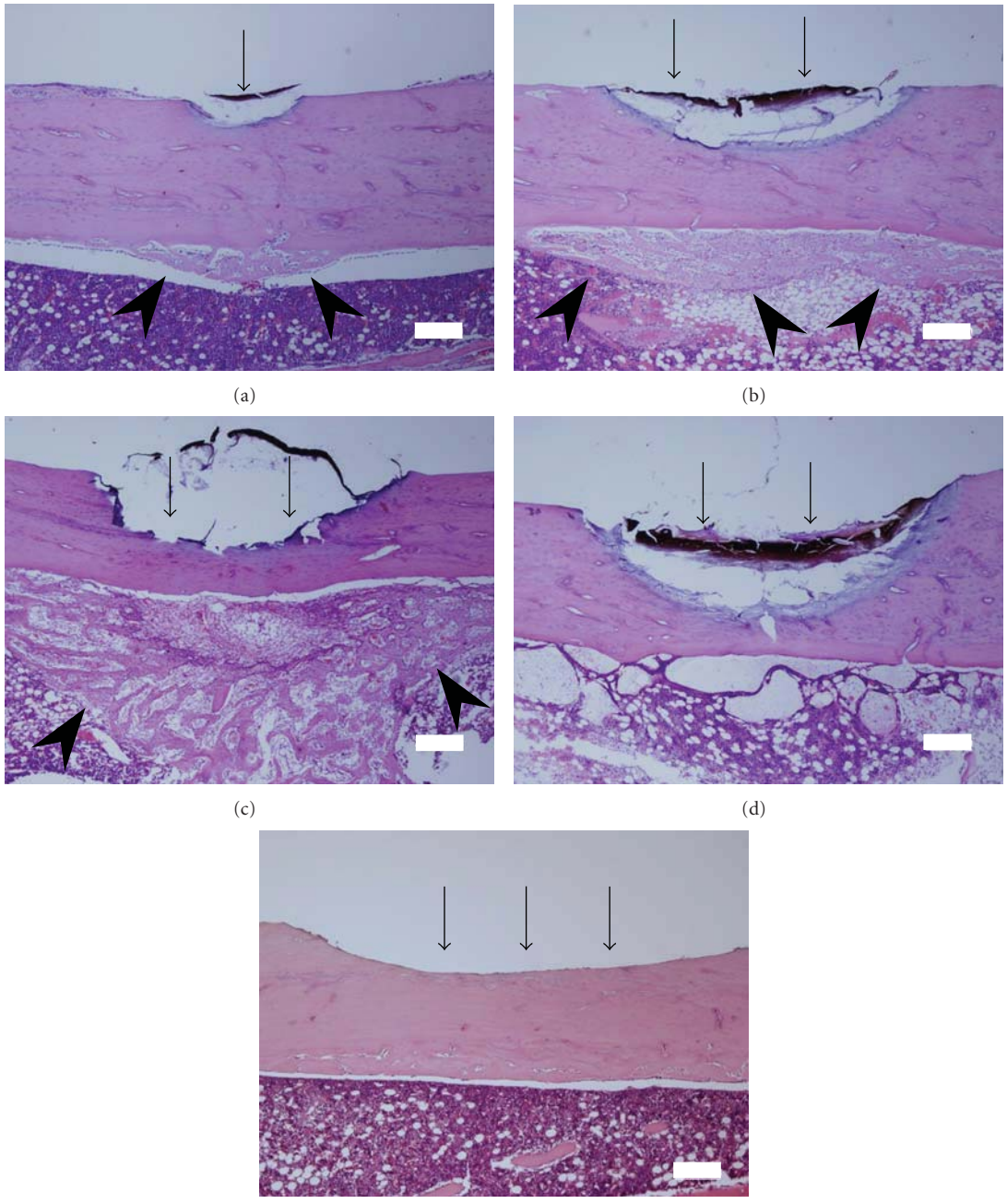

Figure 2: Comparison of osteoid formation in the bone marrow space in the laser-irradiated group and bur-injured group 5 days after treatment. (a) laser-irradiated tibia. Energy density = 88.2 J/cm^2, (output: 1.0 W, irradiation time: 2 sec). (b) laser-irradiated tibia. Energy densities = 220.5 J/cm^2, (output: 1.0 W, irradiation time: 5 sec). (c) laser-irradiated tibia. Energy densities = 441.0 J/cm^2, (output: 1.0 W, irradiation time: 10 sec). (d) laser-irradiated tibia. Energy densities = 661.5 J/cm^2, (output: 1.0 W, irradiation time: 15 sec). (e) bur-injured tibia. (a–d) laser-irradiated tibia showed an ablation defect, carbon deposits, and numerous empty osteocytic lacunae. Moreover, newly formed trabecular bone was observed on the marrow side of the laser-treated site (a–c). (e) bur-injured tibia showed a slight amount of reactive bone formation on the endosteal surface. H&E stain with 40x magnification. Bar = 200 μm. Arrow indicates laser-irradiated or bur-injured site. Arrow heads indicate osteoid formation site.

Moreover, as energy densities increased, the depth of ablation and width of surface damage increased (Figures 2(a)–2(d)). Osteoid formation just under the irradiated cortical bone was observed in the 88.2, 220.5, and 441.0 J/cm^2 groups tibia (Figures 2(a)–2(c)). In the 441.0 J/cm^2 group tibia, no osteoid formation was observed (Figure 2(d)). In the bur injured tibia, a cortical bone defect and a small amount of reactive bone formation in the bone marrow space were observed (Figure 2(e)). From this observation, we used 220.5 J/cm^2 to this experiment.

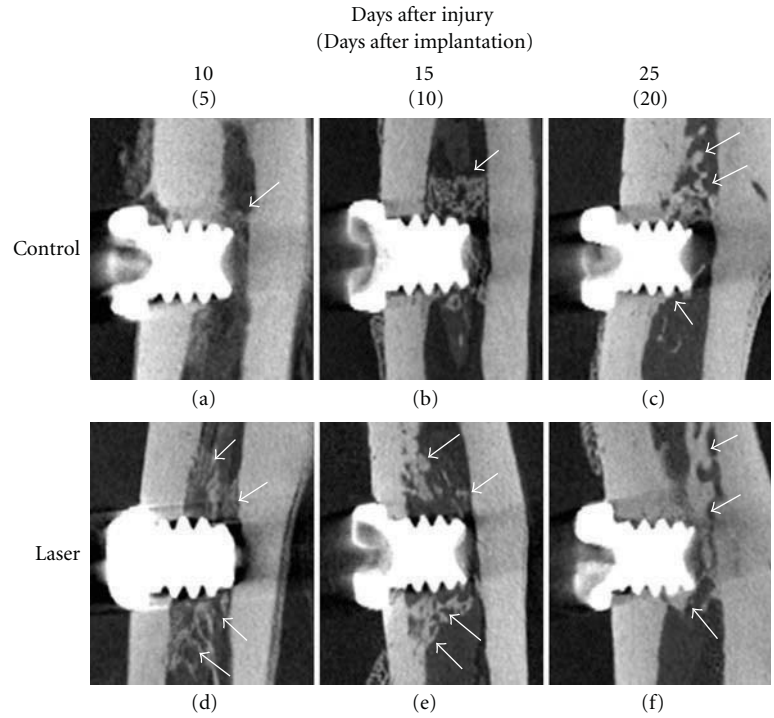

Figure 3: Micro-CT images. (a, d): 5 days after implantation (10 days after sham operation or laser irradiation). (b, e): 10 days after implantation (15 days after sham operation or laser irradiation). (c, f): 20 days after implantation (25 days after sham operation or laser irradiation). (a–c): control group tibia. (d–f): laser group tibia. Arrow indicates newly formed bone.

3.2. Micro-CT Observation of Tibia (Figure 3). Ten days after injury (5 days after implantation), little radiopacity was observed around the implant body in the bur-implant group tibia. In the laser group tibia, evident radiopacity was observed around the titanium implant.

Fifteen days after injury (10 days after implantation), beginning of evident radiopacity was observed around titanium implant body in the control group. In the laser group tibia, increased radiopacity was observed around the titanium implant.

Twenty-five days after injury (20 days after implantation), obvious radiopacity, though thinner than that in the laser group, was observed around the implant body. In the laser group tibia, thick radiopacity was observed around the implant body.

3.3. Removal Torque Test (Figure 4). Functional attachment of the integration between implants and bone was evaluated using a torque test with torque drivers.

Ten days after injury (5 days after implant placement), the average removal torque was 0.63 ± 0.18 N cm for the control group and 1.36 ± 0.15 N cm for the laser group. There was a significant difference between the laser and control groups.

Fifteen days after injury (10 days after implant placement), the average removal torque was 0.68 ± 0.15 N cm for the control group and 1.65 ± 0.21 N cm for the laser group. There was a statistically significant difference between the laser and control groups.

Twenty-five days after injury (20 days after implant placement), the average removal torque was 0.89 ± 0.16 N cm for the control group and 1.87 ± 0.28 N cm for the laser group. There was a significant difference between the laser and control groups. Moreover, there were significant differences between 25-day value of control group and 10- or 15-day value of laser group.

3.4. Histological Findings (Figure 5)

3.4.1. Day 10 (Figures 5(a) and 5(d)). Ten days after injury (5 days after titanium implant insertion), hematoma, soft tissue, and little bone fragment formation were observed around the inserted titanium implant in the control group tibia (Figure 5(a)). In the laser group tibia, a large amount of bone matrix had formed along the bone-implant interface (Figure 5(d)).

3.4.2. Day 15 (Figures 5(b) and 5(e)). Fifteen days after injury (10 days after titanium implant insertion), formation of woven bone was observed around the inserted titanium implant in the control group tibia (Figure 5(b)). In the laser group, most of the implant surface was in direct contact with the new woven bone (Figure 5(e)).

3.4.3. Day 25 (Figures 5(c) and 5(f)). Twenty-five days after injury (20 days after titanium implant insertion), the implant surface was also covered with newly formed bone in the control group tibia. But the trabecular bone was thinner than

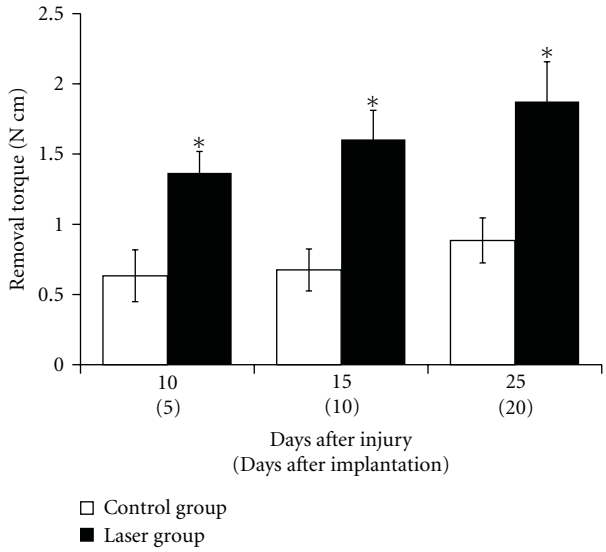

Figure 4: Removal torque examination of titanium implant. Removal torque values were measured at different time points. Mean ± SE (N cm) of torque forces in laser group and control group. Five days after implantation, significant difference was observed between the control and laser groups. Until twenty days after implantation, there was still a significant difference between the two groups. $^*P < 0.05$.

that in the laser group (Figure 5(c)). In the laser group, most of the implant surface was covered with thick newly formed bone lamella, which was connected with preexisting bone by newly formed trabeculae. Moreover, osteocytes were apparent in the newly formed bone matrix surrounding the implant (Figure 5(f)).

4. Discussion

CO_2 laser emits a beam of energy that lases tissues in a noncontact mode. HLLT CO_2 laser irradiation is known to produce a photobiodestructive reaction inducing cellular vaporization, whereas LLLT CO_2 laser therapy generates a photobioactive reaction (PAR) stimulating cellular proliferation and differentiation [31]. CO_2 laser has a wavelength of 10.6 μm, which falls within the specific absorption spectrum for calcium hydroxyapatite, 9.0 to 11.0 μm [32]. Moreover, previous report indicated that when laser irradiation was performed on the cortical bone, only CO_2 laser could induce newly bone formation in the marrow cavity [26]. Based on this observation, the mineral components of bone are expected to exhibit maximal absorption of the laser energy. It seems reasonable to assume that CO_2 laser irradiation of the bone tissue would be suitable for bone regeneration therapy. However, it is commonly accepted that CO_2 laser is unable to use hard tissue treatment.

The bone tissue that received laser irradiation demonstrated acceleration of bone formation. Bone formation induced by CO_2 laser has also been reported previously [24–26, 33, 34]. As cellular mechanisms of this reactive bone formation have been obscure, we speculated that osteocytes have been capable of playing a part. In fact, Tasumi et al. reported that bone formation in tibiae of transgenic mouse model in which specific ablation of osteocytes have been accelerated drastically [35]. They pointed out that mature osteocytes express *Sclerostin*, the negative regulator of osteoblastic bone formation by antagonizing BMP and Wnt signal. Moreover, the expression of *Sclerostin* was decreased followed by osteocytes ablation in the transgenic mouse model, which may cause stimulation of bone formation. In this present study, judging from osteocytic appearances with pyknotic, shrunken, and displaced cells within their lacunae, laser irradiation locally damaged osteocytes. Damaged osteocytes mediated by laser irradiation also decreased *sclerostin* expression; the bone formation could be stimulated. However, it is necessary to analyze what is the negative and/or positive regulator of osteocyte-derived factors induced by laser irradiation. Further investigation will be required to clarify this point.

However, laser irradiation also induced not only damage of cortex but also induced the inflammatory reaction and degeneration of bone marrow. In this study, we inserted titanium implant to the damaged cortex and bone marrow. To achieve the early healing and lording, osseointegration of screw neck to cortical bone is important. Additional studies are needed to clarify the effective irradiation energy to minimize the heat damaged cortical bone and bone marrow inflammatory reaction in order to apply this method to dental implant therapy.

To confirm whether laser-induced bone therapy (LIBT) was effective in implant therapy, we inserted a titanium implant into woven bone and evaluated the bone formation. Although an implant has the ability to induce bone formation around itself, we hypothesized that bone formation occurring within the marrow prior to implant insertion would accelerate the osseointegration process and reduce healing time. To evaluate our hypothesis, we used histomorphological and clinical parameters to evaluate the degree of bone formation and osseointegration. Micro-CT image analysis [36], removal torque measurements [37–43], and histomorphometric evaluation [21, 43–47] are currently considered standard analyses in implant research. In the early healing stage, advancement of histomorphological changes and significantly increased functional analysis data were observed in the laser-implant group. These results may be attributed to the laser-mediated bone formation in the marrow area. These results suggest that preoperative HLLT treatment may promote formation of bone tissue with a tighter mesh of trabeculae, which promotes early osseointegration.

Many signaling molecules, such as growth factors and hormones, are involved in bone metabolism [48, 49], especially around the titanium implant [50]. Further investigations are needed to focus on the underlying biological mechanisms, which induce osseointegration in implant therapy when using LIBT.

In this experimental model, the application of LIBT before implant insertion may promote bone formation and facilitate osseointegration of titanium implants (Figure 6). The introduction of LIBT in implant treatment seems

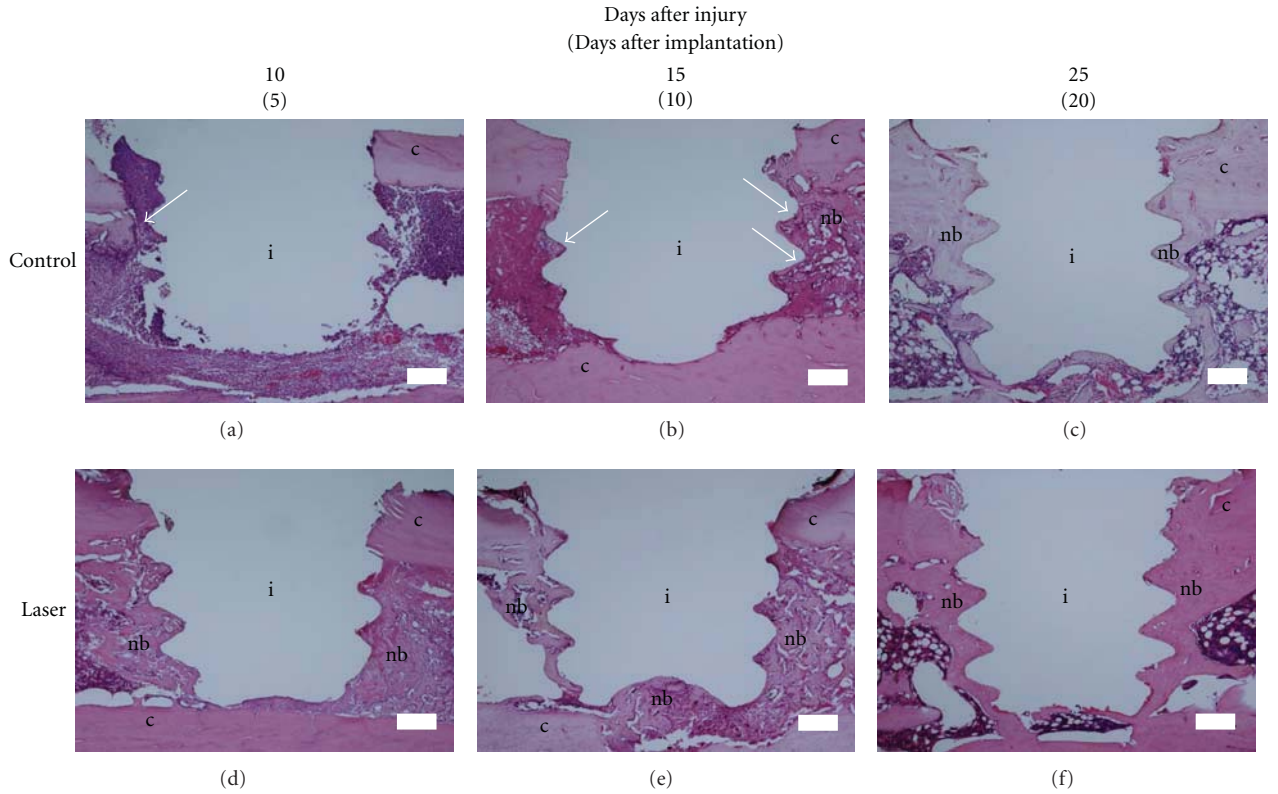

Figure 5: H&E staining observation. (a, d): 5 days after implantation (10 days after sham operation or laser irradiation). (b, e): 10 days after implantation (15 days after sham operation or laser irradiation). (c, f): 20 days after implantation (25 days after sham operation or laser irradiation). (a–c): control group tibia. (d–f): laser group tibia. In the early healing period (10 days after injury), in the control group, there was limited bone formation around the implant body, while in the laser group, there was obvious bone formation along the inserted implant. Fifteen days after injury, in the control group, tibia showed little osteoid formation around the implant body. In the laser group, thick and abundant bone formation was evident along the inserted implant body. Twenty-five days after injury, in the control group, tibia showed newly formed cortical bone, but the trabecular bone was thinner than that in the laser group tibia. In the laser group, thick cortical bone was connected to the previously existing cortex. H&E stain with 40x magnification. c: cortical bone, i: implant cavity, nb: newly formed bone, bar = 200 μm.

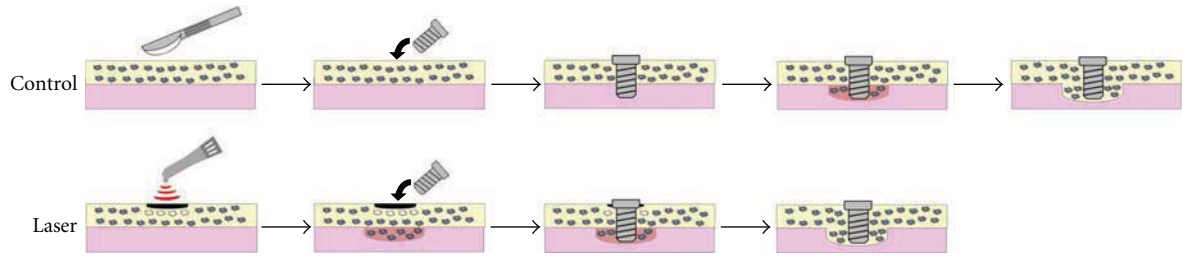

Figure 6: Summary of the healing processes in the control group and laser group. Laser irradiation-induced bone formation in the bone marrow and laser group showed earlier osseointegration. The control group also acquired osseointegration, but the healing period was longer than that of the laser group.

feasible and may be of therapeutic benefit in accelerating healing.

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Research Article

Periradicular Tissue Responses to Biologically Active Molecules or MTA When Applied in Furcal Perforation of Dogs' Teeth

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The aim of this study was the comparative evaluation of inflammatory reactions and tissue responses to four growth factors, or mineral trioxide aggregate (MTA), or a zinc-oxide-eugenol-based cement (IRM) as controls, when used for the repair of furcal perforations in dogs' teeth. Results showed significantly higher inflammatory cell response in the transforming growth factorβ1 (TGFβ1) and zinc-oxide-eugenol-based cement (IRM) groups and higher rates of epithelial proliferation in the TGFβ1, basic fibroblast growth factor (bFGF), and insulin growth factor-I (IGF-I) groups compared to the MTA. Significantly higher rates of bone formation were found in the control groups compared to the osteogenic protein-1 (OP-1). Significantly higher rates of cementum formation were observed in the IGF-I and bFGF groups compared to the IRM. None of the biologically active molecules can be suggested for repairing furcal perforations, despite the fact that growth factors exerted a clear stimulatory effect on cementum formation and inhibited collagen capsule formation. MTA exhibited better results than the growth factors.

1. Introduction

Perforations represent pathologic or iatrogenic communications between the root canal space and the periradicular tissue. Common causes of perforations are resorptive defects, caries, or iatrogenic events that occur during or following an endodontic treatment [1]. Several factors may interfere with the repair of the defect and at the same time affect prognosis [2]. Convincing evidence exists [2], demonstrating that the location of the perforation in relation to the gingival sulcus has a definitive role in the healing outcome. More specifically, a furcation perforation has poor prognosis leading to periradicular breakdown and consequent loss of the periodontal attachment, while frequently results in tooth loss [3, 4]. At the same time, it has been demonstrated that immediate restoration of the perforation prevents bacterial infection of the wound site [2, 5]. Wound healing with reparative mineralized tissue formation represents the optimal end result of a treatment; tissue regeneration depends on several host and treatment factors. One of the reasons why perforations tend to have such poor prognosis may be the fact that none of the materials used can accomplish tissue regeneration at the treated site.

A plethora of materials has been utilized for the restoration of perforations, including amalgam, gutta-percha, composite resins, glass ionomers, zinc oxide eugenol cements such as IRM and a reinforced zinc oxide cement based on a mixture of eugenol and ethoxy benzoic acid (Super-EBA), and more recently mineral trioxide aggregate (MTA) [6–9]. Although amalgam has been invariably used in restorative dentistry and apical retrofilling techniques for over a century, its safety and integrity have both been questioned in recent years due to the following phenomena associated with its use: release of ions, mercury toxicity, corrosion and electrolysis, marginal leakage, delayed expansion, and tattoo formation [6, 10]. If on the other hand, restoration of perforations

could be achieved by using biological factors with proven properties in tissue repair, the above side effects can be eliminated.

Growth factors are proteins that play a role in regulating a variety of cellular processes such as cell proliferation, differentiation and angiogenesis and are crucial molecules for wound repair and tissue regeneration. Upon binding to their transmembrane receptors, a cascade of intracellular biochemical signals are transmitted. These signals involve tyrosine kinase or serine/threonine kinase phosphorylation pathways and result in the activation or repression of various subsets of genes. Proteins of the transforming growth factor-beta (TGF-β) superfamily, which includes bone morphogenetic proteins (BMPs), together with the formerly known as acidic and basic fibroblast growth factors FGF1 and FGF2, have been found to be localized in the bone, cementum, and healing tissues, providing clues of their potential role on these sites. Similar results have been found for the insulin-like growth factors (IGF-I and -II) and cementum-derived growth factor (CGF) [11–13]. Moreover, osteogenic protein-1 (OP-1), which is also known as BMP-7 definitely seems to increase alkaline phosphatase activity in a dose-dependent fashion, while failing to promote periodontal ligament fibroblast mitogenesis [14].

Application of dentin matrix components and growth factors in deep cavities has stimulated upregulation of biosynthetic activity of primary odontoblasts (reactionary dentin formation). Pulp-capping studies with a broad spectrum of biological agents, including growth factors and extracellular matrix molecules, showed formation of osteodentin and/or tertiary dentinogenesis (reparative dentin formation) [15–20].

Apart from the growth factors mentioned above, mineral trioxide aggregate has been recommended as a root-end filling material, but it has also been used in pulp capping, pulpotomy, apical barrier formation in teeth with open apexes, repair of root perforations, and root canal filling because of its good sealing ability and biocompatibility [21–23].

The aim of the present study was the comparative evaluation of the inflammatory reactions and tissue responses to four different growth factors, MTA and IRM as control, when used for the repair of furcation perforations in dogs' teeth.

2. Materials and Methods

Six healthy male Beagle dogs aged 18–30 months with intact dentitions were used. The experimental protocol was conducted in accordance with the ethical guidelines laid down by the Research Committee of Aristotle University of Thessaloniki (European Communities Directive 24 November 1986-86/609/EEC), for the animal care in experimental procedures and approved by the Ethical Committee of the School of Dentistry, Aristotle University of Thessaloniki, Greece. All measures were taken to minimize pain and animal discomfort.

Each animal was pretreated with 1 mg/kg xylazine (Rompun; Bayer, Germany) intramuscularly, and general anaesthesia was induced with an intramuscular injection of 6 mg/kg thiopentone, (Thiopental; Biochemie, Austria). The animals were intubated with a cuffed endotracheal tube, and anaesthesia was maintained under state using halothane (1.5–2.5%) in oxygen, delivered through a semiclosed breathing circuit.

2.1. Experimental Procedure. Permanent premolars and molars of both jaws were selected. All teeth were scaled and polished with a rubber cup, cleaned with 5% iodine solution on the day of the operative procedure, to achieve clear visualization of the treatment outcome. Teeth were isolated with cotton rolls, while saliva flow was controlled with high-speed evacuation.

The cavities were prepared using a tungsten carbide pear-shaped bur (ISO, no. 330 LSS. White, Lakewood, NJ, USA) at ultrahigh speed with copious water spray. Access to the pulp chamber was gained with a sterile water-cooled carbide bur. Pulp tissue was extirpated with Hedstrom files. The root canals were instrumented, irrigated with sodium hypochlorite (1%), dried with sterile paper points, and obturated with thermoplasticized gutta-percha (Obtura, Spartan) and AH26 sealer. The coronal pulp chamber was thoroughly cleaned from all debris with water spray and sterile cotton pellets soaked with saline. A 1.4-mm-diameter perforation was created in the center of the pulp chamber floor of the experimental and positive control teeth using a sterile round bur (ISO size 4) at low speed. The depth of the intentionally created and standardized perforation was 2 mm into the interradicular bone of each selected tooth. This was guided by use of a rubber stopper as a marker on the shank of the bur. Bleeding was controlled with sterile cotton pellets soaked in 2% lidocaine with 1 : 50,000 epinephrine. A new bur was used for each tooth.

The perforations were washed with sterile saline and dried with cotton pellets, light pressure was applied to control hemorrhage. The perforations were treated immediately with the following lyophilized human recombinant bioactive molecules which were reconstituted in a solubilized basement membrane preparation (BD Biosciences Greece), or MTA (ProRootMTA, Dentsply Tulsa), or zinc and eugenol hard cement IRM (Dentsply, Germany) according to manufacturers' instructions.

(i) 14 perforations were treated with basement membrane preparations soaked in 25 μL of solution containing 10 mg of OP-1(recombinant human, R&D Systems) per mL PBS (0.1% albumin control solution).

(ii) 10 perforations were treated with basement membrane preparations soaked in 25 μL of solution containing 1 mg of TGFβ1 (recombinant human, Sigma) per mL PBS (0.1% albumin control solution).

(iii) 10 perforations were treated with basement membrane preparations soaked in 25 μL of solution containing 10 mg of bFGF(recombinant human, R&D Systems) per mL PBS (0.1% albumin control solution).

(iv) 6 perforations were treated with basement membrane preparations soaked in 25 μL of solution containing 10 mg of IGF-I (recombinant human, R&D Systems) per mL PBS (0.1% albumin control solution).

Preparation of solutions containing biologically active materials, establishment of their doses, and quantitative assessment of adsorbed molecules have been performed as described previously by Tziafas et al. [19].

Restorations were performed with amalgam fillings. The tissue responses were assessed at 3 and 8 weeks aftter operation. At the termination of the experimental periods, the animals were sacrificed by using an excess dose of pentobarbital sodium and jaws were removed. Specimens were subsequently created containing the operated teeth and the surrounding tissues. Briefly, teeth were fixed in 10% neutral-buffered formalin solution for two weeks and demineralized using Morse's solution (50% formic acid +20% sodium citrate) for two months. Finally, teeth were embedded in paraffin and serially sectioned at 7 μm thickness. All sections coming through the furcal perforation site were stained either with Mayer's hematoxylin-eosin stain or using modified Brown-Brenn's technique and following a standardized protocol. Brown-Brenn's technique was used in order to examine if there was bacterial presence at the wound site. A number of specimens were stained with Mallori's trichrome to detect newly formed bone matrix.

2.2. Histological Assessment. The stereotypic connective tissue reactions and the periodontal-specific reparative tissue response to the combined effect of perforation preparation and restoration were evaluated under microscopy according to the following criteria.

Inflammatory Cell Response. Inflammatory cell infiltration of the amputated tissue area was classified as (a) absent, if no inflammatory cells were detected; (b) slight, if a few scattered inflammatory cells were detected; (c) moderate or severe if a large amount of inflammatory cells were present or abscess formation.

Epithelium Proliferation. The junctional epithelial responses were classified as (a) absent if no epithelium was detected; (b) partially organized if traces of epithelium were present; (c) organized if presence of continuous epithelium barrier was visible.

Hard Tissue Resorption. The changes were classified as "yes" or "no" based on whether there was tissue resorption in both bone and cementum adjacent to the amputated area.

Hard tissue repair. The changes were classified as "yes" or "no" based on whether there was tissue repair in both bone and cementum adjacent to the amputated area.

Comparisons among different treatment groups were achieved using the Kruskal Wallis and Mann-Whitney U nonparametric statistics. A comparison was considered significant at the $P = 0.05$ cutoff level.

3. Results

The rate of inflammatory cell filtration, the proliferation of epithelial tissue at the amputated location, resorption rate of bone and cementum and the formation of bone, cementum and connective tissue were compared among the control groups (Figures 1 and 2), the IGF-I group (Figure 3), the bFGF group (Figure 4), the OP-1 group (Figure 5), and the TGFβ1 group (Figure 6). Results are summarized on Table 1.

Statistical analyses showed the following.

3.1. Inflammatory Cell Infiltration

(1) There was no statistical difference as far as inflammatory cell infiltration is concerned among the growth factor treated and the IRM (control) groups and the MTA groups at the 3 weeks after operation time point, (Kruskal-Wallis, $X^2(5) = 10.054$, $P = 0.074$).

(2) A significantly higher inflammatory cell response (Kruskal-Wallis, $P = 0.007$) was observed in the TGFβ1 group (Mann-Whitney, $P = 0.003$) and the IRM (control) group (Mann-Whitney, $P = 0.001$) compared to the MTA groups at the 8 weeks after operation time point.

(3) A significantly higher inflammatory cell response was observed in the TGFβ1 group (Mann-Whitney, $P = 0.004$) between the 3 weeks and the 8 weeks after operation time point.

3.2. Proliferation of Epithelial Tissue

(1) Significantly higher rates of epithelial proliferation (Kruskal-Wallis, $P = 0.007$) were observed in the IGF group compared to the MTA group (Mann-Whitney, $P = 0.005$) at the 3 weeks after operation time point.

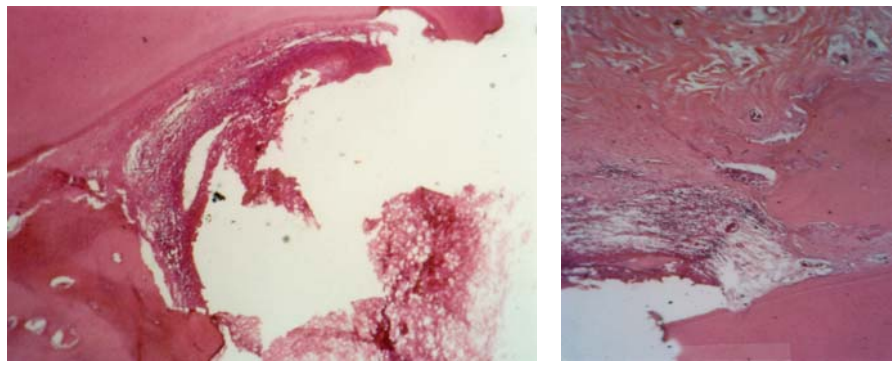
(2) Significantly higher rates of epithelial proliferation (Kruskal-Wallis, $P < 0.001$) were observed in the TGFβ1 group (Mann-Whitney, $P = 0.002$), in the bFGF group (Mann-Whitney, $P = 0.002$), and in the IGF group (Mann-Whitney, $P = 0.005$) compared to the MTA group at the 8 weeks after operation time point.

(3) Significantly higher rates of epithelial proliferation (Kruskal-Wallis, $P < 0.001$) were observed in the TGFβ1 group (Mann-Whitney, $P < 0.001$), in the bFGF group (Mann-Whitney, $P = 0.002$), and in the IGF group (Mann-Whitney, $P < 0.001$) compared to the MTA group between the 3 weeks and the 8 weeks after operation time point. In addition, significantly higher rates of epithelial proliferation were observed in the IGF group between the 3 weeks and the 8 weeks after operation time point.

3.3. Resorption of Bone. Significantly higher rates of bone resorption (Kruskal-Wallis, $P < 0.001$) were observed in the TGFβ1 group (Mann-Whitney, $P < 0.001$), in the bFGf group (Mann-Whitney, $P = 0.002$), and in the IGF group (Mann-Whitney, $P < 0.001$) between the 3 weeks and the 8 weeks after operation time point. The observation was apparent for both in both time points examined.

Table 1: Periodontal tissue reactions, including inflammatory cell response, epithelium proliferation, bone and root resorption, and matrix formation, classified according to criteria of histological assessment (see in the text).

Type of treatment	n	Inflammatory cell infiltration			Epithelium response			Resorption		Matrix formation		
		No	slight-severe	moderate	No	local	complete	Bone	cementum	Bone	cementum	soft tissue
IRM	7/7	0/0	5/6	2/1	7/4	0/2	0/1	0/0	0/0	0/0	0/0	4/5
MTA	6/6	4/5	1/1	1/0	6/6	0/0	0/0	0/0	0/0	6/6	0/2	3/7
OP-1	7/7	0/2	5/4	2/1	2/6	4/0	1/1	1/1	5/1	5/4	0/4	0/0
TGFβ1	5/5	1/0	4/0	0/5	1/0	2/3	2/2	2/0	2/0	0/5	0/4	0/0
bFGF	4/6	2/1	2/1	0/4	4/0	0/3	0/3	0/0	0/0	4/6	0/6	0/0
IGF	3/3	0/0	2/2	1/1	0/0	1/0	2/3	0/0	0/0	2/1	2/3	0/2
Carrier	4/4	0/0	1/1	3/3	2/2	2/2	0/0	0/4	0/4	0/4	0/4	3/4



(a) (b)

Figure 1: Periodontal tissue response in the dog following a 3- (a) or 8- (b) week application of IRM (control treatment). Note the moderate inflammatory cell infiltration and the superficial necrotic zone. New bone formation and a site of root resorption can be also seen after 8 weeks (magnification ×100).

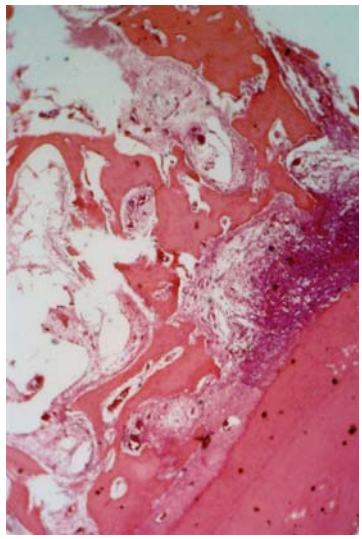


Figure 2: Severe inflammatory infiltration around perforation treated with carrier alone after 3 weeks (magnification ×100).

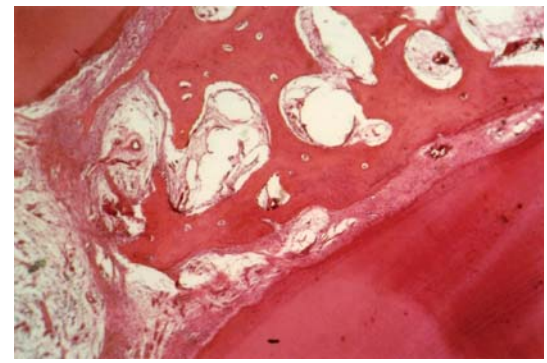
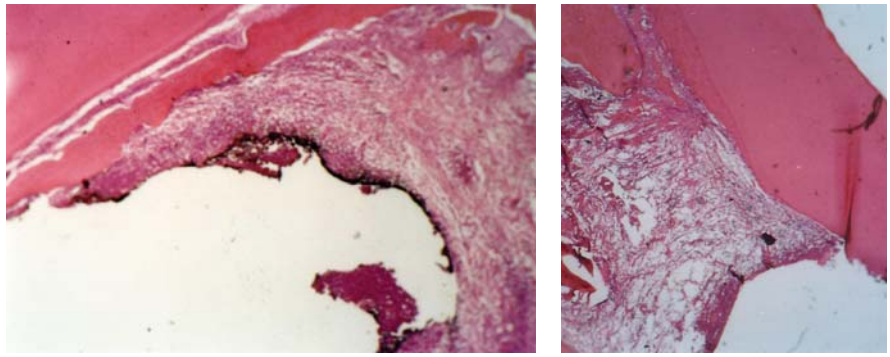


Figure 3: Periodontal tissue response in the dog following an application of IGF-I for 3 weeks. The complete organization of epithelium is evident (magnification ×100).

3.4. Resorption of Cementum

(1) A significantly high rate of cementum resorption (Fisher's Exact Test, $P = 0.004$) was observed in the OP-1 group compared to the MTA group (Fisher's Exact Test, $P = 0.021$) and the IRM (control) group (Fisher's Exact Test, $P = 0.021$) at the 3 weeks after operation time point.

(2) No difference among the growth factor treated groups of teeth and the IRM treated control group and the MTA group was observed at the 8-weeks post-operation time point.



(a) (b)

Figure 4: Periodontal tissue response in the dog following an application of bFGF for 8 weeks. Absence of inflammatory infiltration and formation of connective tissue can be seen (magnification ×100).

(3) A significantly high rate of cementum resorption (Fisher's Exact Test, $P = 0.004$) was observed in the OP-1 group between the 3 weeks and the 8 weeks after operation time point.

3.5. Formation of Bone

(1) Significantly higher rates (Fisher's Exact Test, $P < 0.001$) of bone formation were observed in the MTA group (Fisher's Exact Test, $P = 0.001$) and the bFGF group (Fisher's Exact Test, $P = 0.003$) compared to the IRM (control) group at the 3 weeks after operation time point. In addition, significantly higher rates of bone formation were observed in the MTA group (Fisher's Exact Test, $P = 0.002$) compared to the TGFβ1 group at the 3 weeks after operation time point.

(2) Significantly higher rates (Fisher's Exact Test, $P < 0.001$) of bone formation were observed in the MTA group (Fisher's Exact Test, $P = 0.001$), the bFGF group (Fisher's Exact Test, $P = 0.001$), and the TGFβ1 group (Fisher's Exact Test, $P = 0.001$) compared to the IRM (control) group at the 8 weeks after operation time point.

(3) Significantly higher rates (Fisher's Exact Test, $P < 0.001$) of bone formation were observed in the MTA group (Fisher's Exact Test, $P < 0.001$), the bFGF group (Fisher's Exact Test, $P < 0.001$), and the OP-1 group (Fisher's Exact Test, $P = 0.001$) compared to the IRM (control) group between the 3 weeks and the 8 weeks after operation time point.

3.6. Formation of Cementum

(1) No difference among the growth-factor-treated groups of teeth and the IRM treated control group and the MTA group was observed concerning the cementum formation at the 3 weeks after operation time point (Fisher's Exact Test, $P > 0.05$).

(2) Significantly higher rates (Fisher's Exact Test, $P = 0.001$) of cementum formation were observed in the bFGF group (Fisher's Exact Test, $P = 0.001$) compared to the IRM (control) group at the 8-weeks post-operation time point.

(3) Significantly higher rates (Fisher's Exact Test, $P = 0.001$) of cementum formation were observed in the IGF group (Fisher's Exact Test, $P < 0.001$) and the bFGF group (Fisher's Exact Test, $P = 0.002$) compared to the IRM (control) group between the 3 weeks and the 8 weeks after operation time point.

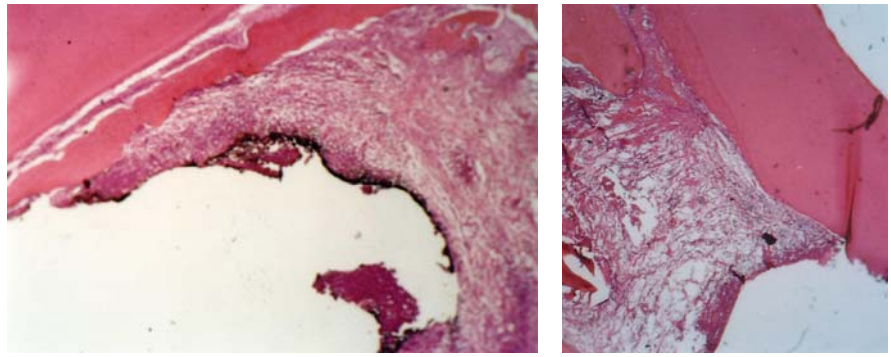
4. Discussion

Until today, and to the best of our knowledge, there are no research studies available that evaluated the role of growth factors in healing furcal perforations and included a control group except from one [24]. Kim et al. investigated the effects of repair of apical perforations after applying calcium hydroxide containing growth factors to the lesions.

As it was shown in our results, successful treatment of furcation perforations can be very difficult. They often result in secondary periodontal involvement with eventual tooth loss [25] and the poor prognosis associated with the treatment, often represents a challenge when evaluating repair materials.

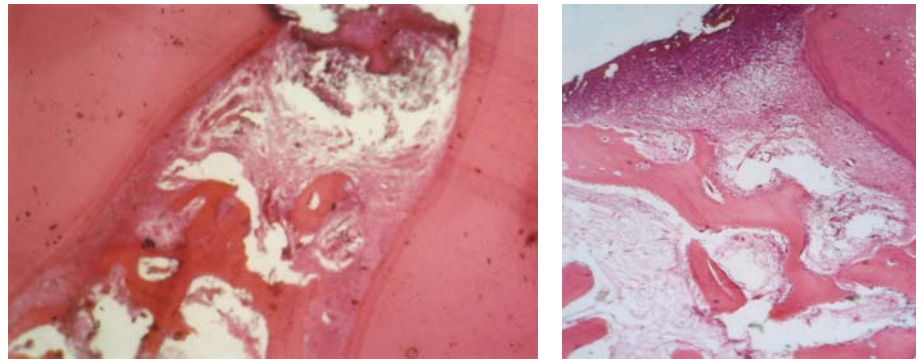
In this study all perforation defects and the entire access cavities were filled with amalgam because of its durability, ease of manipulation, and good clinical performance. Weldon et al. [9] showed that filling the furcation perforation and access cavity with the same material produced less leakage and therefore prognosis might be improved. Premolars and molars have to bear great stresses from chewing and grinding, and an amalgam-filled molar will resist cracking or breaking. Besides, amalgam is well-tolerated by connective tissue and bone.

Compared to other research studies that have not examined an agent with reported effects on wound healing, when evaluating several agents, this study examined a number of growth factors for their potential to repair perforations comparing data also for MTA. The lack of such a control group has resulted in inconclusive and contradictory studies



(a) (b)

Figure 5: Periodontal tissue response in the dog following a 3- (a) or 8- (b) week application of OP-1. Note absence of inflammatory cell infiltration, traces of new bone formation, and superficial incomplete zone of mineralized matrix (magnification ×100).



(a) (b)

Figure 6: Periodontal tissue response in the dog following a 3- (a) or 8- (b) weeks application of TGF-β1. Note the severity of inflammatory cell infiltration and the newly deposited cementum (magnification ×100).

in the past which failed to show a beneficial effect of treatment of the tooth with growth factors.

At the same time, exogenous application of growth factors, which has been widely used in other fields, is a rapidly developing area of dental research. Its basic aim is to regenerate all oral structures, including teeth and periodontal ligament. OP-1, TGF-β1, IGF1, and FGF2 were used in our study due to their involvement in oral facial tissue development and regeneration [12–14, 18–20, 22, 26–29]. Especially, OP-1 has well-documented osteoinducting properties by recruiting mononuclear phagocytes to different situations of craniofacial complex [30]. Furthermore, it has demonstrated potent effects on the stimulation of periodontal wound healing including new bone and cementum [12].

BMPs and TGF-β1 have been demonstrated to participate in the processes of both reactionary and reparative dentinogenesis [19, 20, 31]. In vitro experiments and genetic studies in animals have also demonstrated that TGFβ1 is a very important factor involved in dental repair events [31]. FGF exhibits potent angiogenic activity [32] and has demonstrated its ability to induce the growth of immature periodontal ligament cells [29].

IGF-1 regulates DNA and protein synthesis in periodontal ligament fibroblasts in vitro and enhances soft-tissue wound healing in vivo in human tissues [33, 34]. Furthermore, it has anabolic effects when administered to hypophysectomized rats and may be a locally acting mediator of pituitary hormone actions [35].

In comparison to the growth factors, MTA showed better results, which can be attributed to its ability to create an ideal environment for healing [21]. Andelin et al. [36] investigated the presence of dentin sialoprotein (DSP) after pulp capping with either MTA or OP-1. Their results revealed more staining for DSP in the calcified bridge of teeth that were capped with MTA than with OP-1. On the basis of the DSP-positive staining of MTA-capped pulps, the authors concluded that hard tissue produced by MTA has a closer similarity to dentin. Kuratate et al. [37] showed upregulation of osteopontin, nestin, and 5-bromo-2-dexyuridine assay (BrdU) immunopositive cells after pulp capping with white MTA (tooth colored) in rats, but the mechanism of action responsible for the bioactivity of MTA requires further investigation. However, it should be declared that MTA appears not only to demonstrate acceptable biocompatible behavior,

but also exhibits acceptable in vivo biologic performance when used as repair material. It should also be noted that the supported data have been overwhelming from either in vitro or animal studies [22].

Using the canine as an experimental animal for our study has certain advantages: premolars and molars of the dog are large and therefore they offer good accessibility and visibility. However, the relationship between furcation area and bone margin of the dog's tooth is not directly comparable to that of the human tooth [8, 38]. Furcation is often as close as 1-2 mm from the cementoenamel junction in the dog. The furcation lies more deeply within the alveolus in humans while the epithelization and the formation of connective tissue is less common. Thus, any technique shown to produce favorable results in dogs may have a more favorable response in humans, where the distance from the cementoenamel junction to the furcation is greater. Thus, it was not surprising to see epithelial proliferation and connective tissue in cases of tissue inflammation in furcal perforation of dogs' teeth [7, 39].

The present experimental data suggest that the application of growth factors in the furcal perforations resulted in stimulation of bone or cementum in the amputated tissue area, while they exhibit an effect on the formation of connective tissue capsule. Capsule formation was detected in the control teeth treated with IRM. Present observation is in accordance with previous reports on the periodontal tissue reactions to IRM, suggesting that the choice of IRM as an internal quality control was successful. In general, slight-to-moderate cell infiltration was found in control groups.we should note that no new bone or cementum was found in this group of teeth. IRM is traditional zinc-oxide-eugenol cement reinforced with polymethyl-methacrylate to improve its mechanical properties that has been broadly investigated in dental research [40, 41]. We used IRM as a control material due to its known behavior to the lesions therefore, it would set a baseline for any effects observed with the growth factor treatment groups.

A number of critical parameters need to be considered when the proliferative and wound healing effects of a growth factor are being examined: the optimum concentration needs to be determined for the specific tissue where the peptide is to be applied, and the carrier needs to be compatible with the site injected, protein instability needs to be considered.

During this study, we used growth-factor-reduced (GFR) Matrigel Matrix as a carrier. It is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which contains laminin, collagen type IV, heparan sulfate proteoglycan, entactin, growth factors, and other components. By employing a modified method, a more defined preparation of Matrigel is obtained where the levels of endogenous growth factors, excluding TGF-β are greatly reduced. This modified form was used due to its reduced levels of EGF, PDGF, and IGF-1, which have been shown to influence cell behavior. It was chosen because of its ease of use, as it gels rapidly at 22–35°C, when it comes in contact with the animal body in the liquefied phase.

5. Conclusions

In conclusion, perforations located in the furcation still remain a challenging as much as demanding field to investigate, since none of the biologically active materials used in the present study can be suggested for repairing perforations. The fact that growth factors exerted a stimulatory effect on bone and cementum formation in the dog and inhibited collagenous capsule formation suggest that they could potentially be used as a treatment strategy for the most problematic area on the endodontic tissues, the furcal perforations. MTA exhibited better results than the growth factors and besides the fact that it can be easily manipulated, it is highly recommended in repairing perforations. Next interesting step is to examine if the effects of application of growth factor mixture with MTA on tissue healing and regeneration is better than MTA alone.

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Review Article

Current Status and Future Development of Cell Transplantation Therapy for Periodontal Tissue Regeneration

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It has been shown that stem cell transplantation can regenerate periodontal tissue, and several clinical trials involving transplantation of stem cells into human patients have already begun or are in preparation. However, stem cell transplantation therapy is a new technology, and the events following transplantation are poorly understood. Several studies have reported side effects and potential risks associated with stem cell transplantation therapy. To protect patients from such risks, governments have placed regulations on stem cell transplantation therapies. It is important for the clinicians to understand the relevant risks and governmental regulations. This paper describes the ongoing clinical studies, basic research, risks, and governmental controls related to stem cell transplantation therapy. Then, one clinical study is introduced as an example of a government-approved periodontal cell transplantation therapy.

1. Introduction

Since the 1980s, periodontal ligament cells have been considered a reliable source for periodontal regeneration. Nyman et al. reported that periodontal ligament (PDL) tissue possesses periodontal regenerative properties [1]. Based on this regenerative concept, several procedures have been introduced for the selective proliferation of PDL stem cells, such as guided tissue regeneration and enamel matrix derivative [2]. However, the desired regenerative outcomes have not been attained, especially for patients with severe periodontal defects. Experimental approaches to overcome the limitations of existing therapies have included the ex vivo expansion of stem cells derived from PDL, bone marrow, adipose tissue, and alveolar periosteum for transplantation as stem cell replacement therapy in animal studies. These studies have indicated that the transplantation of stem cells can be an effective treatment for periodontal defects [2]. As a consequence of these successful animal studies, the clinical application of stem cells for the regeneration of periodontal tissue has begun. However, the efficacy and safety of such cell-based therapies have not been fully evaluated, and the risks of stem cell therapies have been underscored by several clinicians and researchers. In this paper, we first review the current research targeting cell-based therapies for periodontal regeneration and then discuss the risks and governmental controls of stem cell transplantation therapy. Last, we will introduce our ongoing clinical study that was approved by the regulatory authority of the Japanese government.

2. Current Progress in Periodontal Cell Transplantation Therapy

2.1. Periodontal Ligament-Derived Mesenchymal Stem Cells (PDL-MSCs). Previous studies that reported the regenerative properties of PDL using animal models indicated the existence of stem cells in PDL tissue [3, 4]. Liu et al. reported that autologous PDL-MSCs enhanced regeneration of periodontal tissue, including alveolar bone, cementum and PDL in a minipig [5]. Feng et al. transplanted autologous PDL progenitors to three patients who suffered from periodontal disease. The results showed periodontal regeneration with no adverse effect [6]. Tissue engineering techniques have been

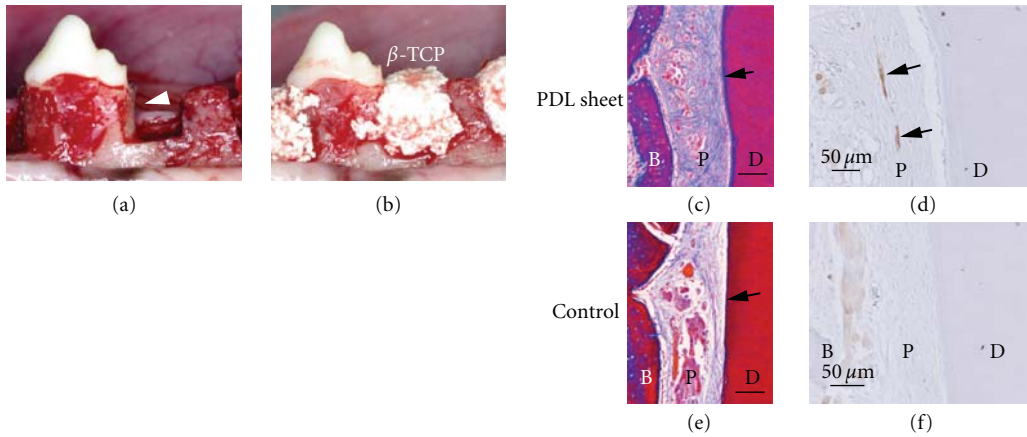


Figure 1: Periodontal tissue regeneration using PDL cell sheets. (a) Sheets of polyglycolic acid (arrowhead) with or without the cell sheets were applied onto the root surfaces of canine mandibular premolars. (b) Bone defects were filled with β-TCP. (c, e) Thick, newly formed cementum (arrow) and dense collagen fibres were observed in the PDL of the PDL sheet-transplanted group. (d, f) The immunohistochemical detection of neurofilament protein revealed that newly formed nerve fibres were only observed in the group given the PDL sheets (arrow). Scale bars: 100 μm in (c) and (e); 50 μm in (d) and (f). B: newly formed bone, P: periodontal ligament, and D: root dentin. Modified from [13] with permission.

applied to improve these cell-based therapies. Okano et al. developed a temperature-responsive cell culture technique to permit the harvest of adherent cultured cells by simply lowering the temperature [7, 8]. Our group produced PDL-derived cell sheets using this temperature-responsive culture dish and found that the cell sheets had a potential to promote regeneration of periodontal tissue, which was composed of bone, cementum, and PDL, in vivo (Figure 1) [9–12].

Allogeneic transplantation of PDL-MSCs into bone defects in minipigs has been shown to result in periodontal regeneration without significant immunological rejection [5]. It has also been shown that porcine PDL-MSCs possess a low immunogenicity and immunosuppressive function [14]. These data could shed light on the potential of allogeneic transplantations using PDL-MSCs at the clinical level and, thus, broaden the range of opportunities for cell transplantation therapy.

2.2. Periosteal Cells. Periosteal cells have been reported to be a potential source of cells for the regeneration of periodontal tissue [15, 16]. Recently, Mizuno et al. reported that cultured autologous periosteal cell membranes induced regeneration of periodontal tissues including bone, cementum, and periodontal ligament in a canine model of a class III furcation defect [17]. Following the results of these studies, clinical trials for cell transplantation therapy using periosteal cells were conducted. Human periosteal cell sheets with platelet-rich plasma (PRP) and hydroxyapatite (HA) were transplanted into 30 patients who suffered from chronic periodontitis, and this treatment was found to enhance periodontal regeneration [18].

2.3. Bone-Marrow-Derived Mesenchymal Stem Cells (BM-MSCs). Bone-marrow-derived mesenchymal stem cells (BM-MSCs) have the potential to differentiate into various types of tissue, including bone, cartilage, adipose, muscle, and periodontal tissue [19–22]. Recent in vivo studies have shown that BM-MSCs could induce periodontal regeneration [21, 23]. Clinical trials using BM-MSCs with PRP have also been conducted, and the results indicated that periodontal regeneration could be induced by this approach as well [24].

2.4. Adipose-Derived Stem Cells (ADSCs). Adipose-derived stem cells (ADSCs) are a useful source for cell transplantation therapy because this tissue is abundant and easy to obtain compared with other sources. ADSCs have been shown to be capable of differentiating into various tissue types [26–29]. It is reported that ADSCs mixed with PRP had the potential to regenerate periodontal defects in vivo [30–32], which supports the use of ADSCs in cell transplantation therapy for periodontal regeneration.

2.5. Gingival Fibroblast. To recover gingival recession, a cell transplantation therapy using gingival fibroblasts has been developed. For this technique, gingival fibroblast sheets were obtained by culturing gingival fibroblasts on collagen sponges [33]. The autologous gingival fibroblast sheets were then transplanted to 14 sites in 4 patients suffering from periodontal disease in order to achieve root coverage. The results demonstrated regeneration of gingival tissue and suggested that gingival fibroblasts could be readily harvested and prepared for transplantation [34].

2.6. ES/iPS Cells. Because embryonic stem (ES) cells and induced pluripotent (iPS) cells can differentiate into all types of somatic cells, so-called pluripotency, they are thought to be the ultimate cellular source for regenerative therapies. Recently, clinical trials using ES cells have begun in the United States. However, tumorigenesis is a common concern

with this application of ES/iPS cells. Thus, it is highly desired to achieve commitment of the ES/iPS cells to a differentiated state prior to transfer. Tada et al. demonstrated the differentiation of ES/iPS cells into a mesodermal lineage [35] and Duann et al. demonstrated alveolar bone regeneration by iPS cells in an animal model [36]. Furthermore, recent reports have indicated the sparse existence of pluripotent cells among MSCs [37, 38], which expands the possibilities for the use of pluripotent cells in cell transplantation therapy. However, little is known about the mechanisms and pathways that control the differentiation. Further studies are therefore needed in regard to periodontal regenerative therapy.

3. Characteristics and Future of MSC

Because the PDL, bone marrow, adipose, and periosteal tissues are thought to contain MSCs, cells from these tissues can be used to regenerate mesenchymal tissue, including alveolar bone and periodontal ligament. MSCs were first identified from bone marrow as plastic-adherent fibroblastic cells and shown to possess a multipotency to differentiate into multiple mesenchymal lineages, such as osteocytes, chondrocytes, and adipocytes [39, 40]. As early as 1995, MSCs from multiple sources were transplanted into patients because of their proliferative property, immunomodulatory, and trophic effects [41].

Cells extracted enzymatically from tissue tend to possess proliferative capacities and multipotency. In the dental field, the multipotency of dental pulp cells was first described by Gronthos et al. [42]. This group introduced a method for digesting tissue with enzymes that dramatically expanded stem cell research in the dental field. Compared to conventional outgrowth methods, enzyme treatment allows for the harvest of a variety of cell types, such as fibroblastic cells, vascular cells, and nerve cells, among others [42]. Furthermore, the characteristics of these cells have been shown to become homogenised during cultivation.

For clinical considerations, each type of MSC has advantages and disadvantages. PDL-MSCs have been shown to exhibit a greater regenerative capacity for PDL tissue compared to BM-MSCs and periosteal cells in canine one wall defect, model for severe periodontal defect [13]. Because clinicians routinely extract teeth, autologous PDL-MSCs can easily be obtained from patients. However, PDL-MSCs cannot be applied to patients who lack teeth to be extracted. Periosteal cells are more easily and abundantly harvested than PDL-MSCs and BM-MSCs, although periosteal cells require a longer culture period to obtain a sufficient number of cells for transplantation as compared to PDL-MSCs [17, 43]. BM-MSCs have been transplanted into many patients in various clinical studies and are thought to be suitable for cell transplantation therapy due to their multipotency and high cellular proliferation rate. However, the collection of bone marrow requires patients hospitalised. Although there are many data from animals and cells study, there is not enough clinical data to compare the efficacy of each type of MSC and the efficacy on severe periodontal defect including horizontal defect. Thus, it is currently difficult to draw conclusions about which MSC is most suitable for human periodontal regenerative therapy.

Recent studies have revealed that MSCs can be extracted from other tissues. One group of researchers demonstrated that MSCs are localised in the perivascular compartment [44]. Park et al. reported that PDL-MSCs could also be obtained from inflamed PDL tissue [45]. Because inflamed PDL tissue can be collected during periodontal treatment, it is possible that this technology will broaden the range of indications for cell transplantation therapy using PDL-MSCs.

4. Guarantees of Safety and Efficacy for Stem Cell Transplantation Therapy

Stem cell transplantation therapy is a promising technology that can regenerate periodontal tissue. However, the efficacy and safety of cell transplantation therapies are not well understood. There have been several clinical reports and reviews published that describe the risks and side effects of stem cell therapies. To protect patients from such risks, regulatory bodies in several countries have begun to establish guidelines and regulations for the control of stem cell therapies. Therefore, it is imperative that clinicians who aim to undertake stem cell therapies understand the associated risks and regulations. In the following section, we provide an overview of the risks related to stem cell therapies and then describe our work on a clinical study that was approved by the regulatory authorities of the Japanese government.

5. Risks of Stem Cell Transplantation Therapy

Many scientists suppose and indicate the risks of stem cell transplantation [46–48]. Indeed, severe side effects from stem cell therapies have been reported. One major concern about stem cell transplantation therapy is the tumorigenic property of the stem cells themselves. Notably, the formation of a transplanted cell-derived brain tumour in a patient who received neural stem cell transplantation was reported [49], although the precise mechanisms responsible for the formation of this tumour remain unclear. For MSC-based transplantation, no tumour formation has been reported during the course of clinical studies involving periodontal cell therapies [46, 50]. However, because the tumorigenic potentials of cells are thought to vary by cell type and application site, the risk of tumour formation must be investigated in the context of each type of cell transplantation therapy.

Other risks associated with stem cell transplantation therapy have been suggested, including those associated with cell harvesting, cell culture, the site of administration, and the interaction between the transplanted cells and the recipient's immune system [48, 51]. Because stem cell transplantation therapy is a new type of treatment, there are few published data on the fate of stem cells after transplantation. Therefore, it is important to confirm the safety of these approaches before applying them to patients, and all data related to the clinical course of each cell transplantation therapy should be recorded. As a result, these strategies should help to reduce patient risk.

6. Governmental Control over Cell Transplantation Therapy

In recent years, many new cell therapies have been developed, and their numbers continue to grow. However, there are many cell therapies that are inadequately designed, lacking evidences of efficacy, safety, and patient protection. For the protection of patients from such cell therapies, the governing bodies of Japan, the European Union, and the United States have been establishing regulations over the control of the quality of cell transplantation therapies based on Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP).

GCP is an international ethical and scientific quality standard that applies to clinical studies and governs their design and patient protection [52]. According to GCP, data and plans, including the evidence, potential risks and benefits of the cell transplantation therapy, the protection of patients' rights, the scientific propriety of the protocol, and data management, must be approved by the institutional review board and independent ethics committee. GMP governs the cell culture processes for cell transplantation therapy [53]. GMP requires that the materials, protocols, tools, and environments used for cell transplantation therapy are guaranteed as safe and effective. GMP also requires a record of all cell culture procedures to be kept in the laboratory for traceability.

Conforming to these regulations requires significant amounts of work and carries a high cost [54]. Cell transplantation therapy under such regulations are often too expensive and too late for patients who are in critical need. Therefore, existing mainstream regulations are sometimes criticised as overly strict and repressive. Indeed, patients who wish to receive cell transplantation therapy have been known to travel to countries where little or no regulation of these procedures is required to receive the therapy; this is referred to as "stem cell tourism" [54–56]. However, the safety and efficacy of many of these therapies have either been questioned or entirely invalidated. Several such cell therapies have been shown to exhibit no effect, and several patients have died from diseases or complications resulting from the cell transplantations [55, 57–61]. It is therefore important for clinicians to implement cell transplantation therapies only after their efficacy and safety have been validated.

7. Preparation for Clinical Trial

Our clinical study of periodontal regenerative therapy using cell sheet technology has been prepared according to the guidelines of the Japanese Ministry of Health, Labour, and Welfare. As we previously demonstrated that PDL cell sheets were effective for periodontal tissue regeneration in animal models [9, 11, 62], we have prepared for clinical trial with this transfer approach. The methods of autologous cell transplantation and cell culture with autologous serum were selected to avoid potential infections from animals or other people. To reduce the risk of tumour formation and to enhance hard tissue formation, the cells were cultured in medium that promoted osteoblastic differentiation after they had been seeded onto temperature-responsive culture dishes.

We optimised a protocol for harvesting human PDL cells that would retain their proliferative, osteogenic, and multipotency [43]. Regarding the validation of the quality of the cell sheets, we found that periostin and alkaline phosphatase (ALP) could be used as quality-control markers of PDL and osteoblast differentiation, respectively [43]. Next, using a canine model for a preclinical study, we investigated the periodontal regenerative capacity of cell sheets that were prepared according to this protocol. The results indicated that the combined use of autologous PDL cell sheets and β-tricalcium phosphate was able to regenerate alveolar bone and periodontal ligament tissue at defective sites [12].

We also established a culture protocol for the preparation of cell sheets that can be applied to patients. PDL cell culture was performed in a clean suite known as the Cell Processing Center (CPC). The CPC is composed of several rooms with controlled air flow in addition to separated personnel and material line of flow to maintain the cleanliness of the room and to prevent any possible contamination between personnel and cells [63]. All of the work in the CPC was carried out according to the Standard Operating Procedure (SOP), a cell culture working protocol that ensures the quality of the cell sheets. The work in the CPC was always performed by 2 individuals; one performed the culture procedures and the other directed the procedures to avoid human error and to keep a record of all of the procedures (Figure 2) [63].

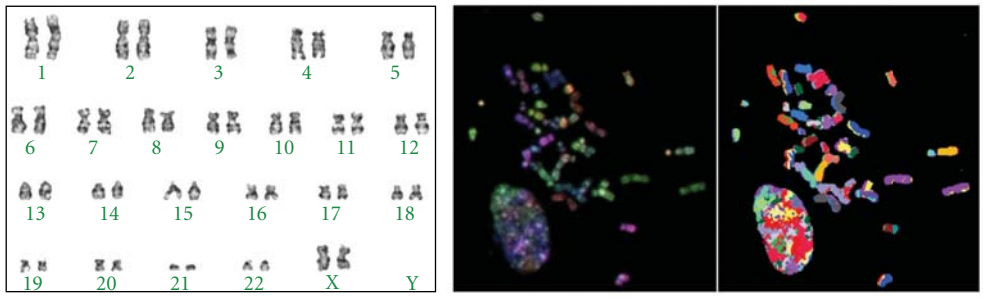
After establishing this SOP, we investigated the safety of the cell sheets that were prepared in this manner. We demonstrated that the cell sheets were free of contamination by bacteria or mycoplasma [25]. Because several studies had indicated that transformation of cells occurred during culture [64, 65], both in vitro and in vivo tumorigenicity tests were carried out [25]. Karyotype testing revealed that no chromosomal abnormalities related to tumorigenicity or other diseases had occurred in the cultured human PDL cells (Figure 3). By the soft agar test, cells from the PDL cell sheets exhibited no evidence of tumorigenic potential in vitro. Furthermore, the injection of cells isolated from PDL cell sheets into immunodeficient mice caused no tumour formation, whereas the injection of cancer cells reliably caused tumours to form (Figure 4) [25].

Next, we confirmed the in vivo and in vitro regenerative properties of the cell sheets that had been prepared according the SOP. For in vitro analysis, the cell sheets were found to be composed of adequate numbers of living cells that expressed periostin and ALP [25]. We then transplanted cell sheets with dentin blocks into immunodeficient mice and confirmed the formation of PDL- and cementum-like tissue around the dentin block (Figure 4) [25]. We also prepared a document that contained a full record of these procedures and the resulting data for submission to the Japanese Ministry of Health, Labour, and Welfare. This clinical study of PDL cell sheet transplantation was approved in January 2011.

As previously mentioned, the generation of cell transplantation materials according to the specified regulations is an enormously expensive and time-consuming process. For example, the CPC cost 2 million US dollars to build and 15,000 US dollars per year to maintain [63]. The performance of the safety and efficacy tests and the preparation of

(a) (b)

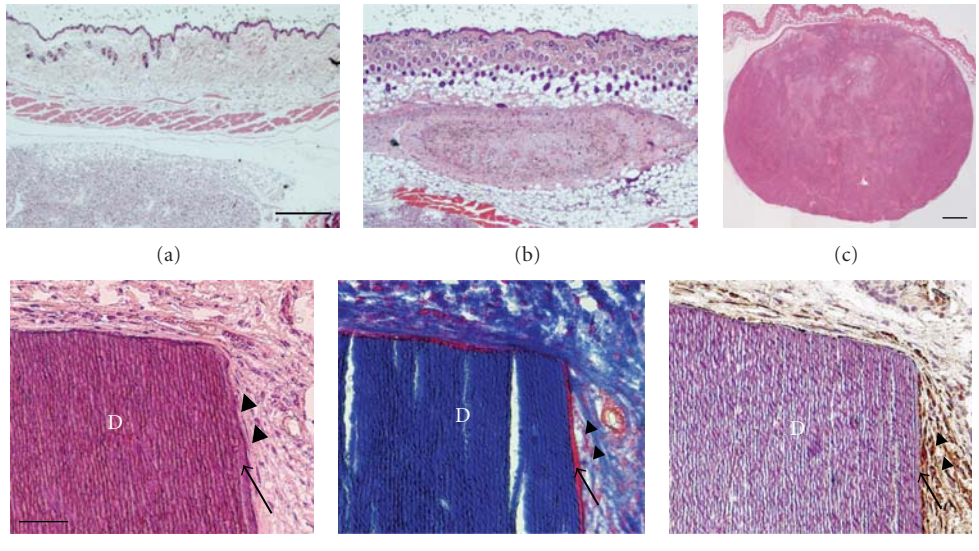
Figure 2: CPC of Tokyo Women's Medical University. (a) Individuals who work in the CPC wear sterile clothing, gloves, caps, and masks to avoid releasing possible contaminants. (b) All tubes and dishes were labelled to avoid misidentification.



G-band M-FISH

(a) (b)

Figure 3: *Karyotype of cultured human PDL cells*. (a) G-banding staining revealed that the number, banding, and shape of the chromosomes were normal. (b) Multiplex fluorescence in situ hybridization (M-FISH) that visualises each chromosome in a different colour showed that no chromosomal aberrations, including translocation, had occurred.



(a) (b) (c)

(d) (e) (f)

Figure 4: Tumorigenic and regenerative properties of human PDL cell sheets. (a)–(c) Tumorigenesis in vivo. Immunodeficient mice were injected with medium only (negative control), human PDL cells, or HeLa S3 cells (positive control). Mice injected with human PDL cells or the medium control did not form tumour-like tissue (a, b), whereas mice injected with HeLa S3 cells exhibited tumour formation (c). (d)–(f) Histology and immunohistochemistry of implanted human PDL cell sheets with dentin blocks. Newly formed cementum- (arrow) and PDL-like tissue (arrowheads) were observed around the dentin blocks (d, e). Immunostaining with an antibody against human vimentin revealed the presence of human PDL cells around the dentin block (f). Scale bars: 500 μm in (a) and (b); 1 mm in (c); 50 μm in (d)–(f). Modified from [25] with permission.

the necessary paperwork also represent significant burdens for the clinicians and associated university staff. Nevertheless, we believe that governmentally regulated cell transplantation therapy remains the best option for patients. One clinical solution may be the formation of collaborations between academic medical centres with existing CPCs. In this way, the outside clinician would collect tissue from a patient and send it to the university, where the cells could be harvested and cultured in a CPC and then sent back to the clinician for the cell transplantation therapy. For this purpose, Nozaki et al. developed a special container capable of maintaining an internal temperature of 37°C for more than 30 hours without a power supply. Using this container, cell sheets could be harvested without losing viability after 8 hours of transportation [66]. Another solution may be the use of closed or automated cell culture systems. There are several GMP-compliant automated cell culture systems currently available. Although the necessary equipment is expensive, the total cost would probably be far less than that of building and maintaining a CPC.

8. Conclusion

In this paper, we have provided an overview of the state of periodontal regenerative therapy using stem cells. Many patients suffer from periodontitis, and clinicians have struggled to regenerate the lost alveolar bone. Stem cell therapy is a promising nascent therapy that may allow the regeneration of lost periodontal tissue. Although there are many issues that need to be resolved before stem cell therapies become common, clinicians should continue to keep a watchful eye on the progression.

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Review Article

Local Regeneration of Dentin-Pulp Complex Using Controlled Release of FGF-2 and Naturally Derived Sponge-Like Scaffolds

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Restorative and endodontic procedures have been recently developed in an attempt to preserve the vitality of dental pulp after exposure to external stimuli, such as caries infection or traumatic injury. When damage to dental pulp is reversible, pulp wound healing can proceed, whereas irreversible damage induces pathological changes in dental pulp, eventually requiring its removal. Nonvital teeth lose their defensive abilities and become severely damaged, resulting in extraction. Development of regeneration therapy for the dentin-pulp complex is important to overcome limitations with presently available therapies. Three strategies to regenerate the dentin-pulp complex have been proposed; regeneration of the entire tooth, local regeneration of the dentin-pulp complex from amputated dental pulp, and regeneration of dental pulp from apical dental pulp or periapical tissues. In this paper, we focus on the local regeneration of the dentin-pulp complex by application of exogenous growth factors and scaffolds to amputated dental pulp.

1. Limitations of Conventional Therapy for Preservation of Dental Pulp

Dental pulp is sometimes affected by external stimuli such as caries infection or traumatic injury. Preservation of dental pulp and maintenance of its viability are essential to avoid tooth loss, and dentists carry out restorative procedures with pulp capping to regulate inflammatory responses of dental pulp, or cement lining on a cavity floor to block external stimuli. Reversible damage induces pulp wound healing, and direct pulp capping and pulpotomy with calcium hydroxide are known to be effective to induce pulp wound healing mechanisms.

After external stimuli such as cavity preparation, apoptosis of pulp cells including odontoblasts is induced [1–5], followed by pulp wound healing including reactionary and reparative dentinogenesis. Reactionary dentin is formed by surviving odontoblasts, whereas reparative dentin is formed by odontoblast-like cells that are differentiated from pulp cells of residual dental pulp, resulting in a reduction in dental pulp size and vitality [6–8].

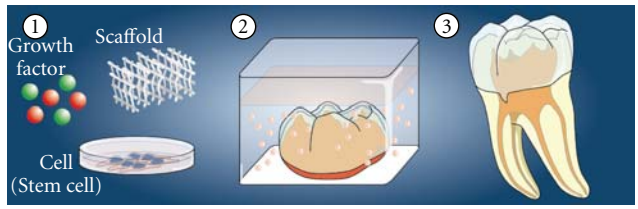
When the external damage to dental pulp induces irreversible changes of the pulp, dentists carry out pulpectomy. Generally, a root canal after pulpectomy is tightly filled with biomaterials such as gutta-percha to prevent reinfection by bacteria. However, a tooth without vital dental pulp has lost its defensive ability, which is often followed by the severe damage such as the progression of deep radicular caries or tooth facture, resulting in extraction of

the tooth. Furthermore, a treated tooth is often reinfected by bacteria because of its complicated anatomical structure or inadequate treatment by a dentist, resulting in formation of a lesion around the root apex with bone resorption. The success rate of the endodontic retreatment is lower than that of pulpectomy [9–12]. To overcome these limitations of the present endodontic treatment, the preservation of dentin-pulp complex is the clear strategy. However, when a dentin defect and the resultant exposure of dental pulp tissue reach a critical size, no treatments available are able to preserve and maintain the vitality of dental pulp. It is considered important to develop regeneration therapy for dental pulp or the dentin-pulp complex.

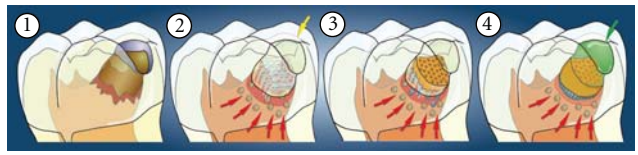
2. Regeneration of the Dentin-Pulp Complex

It is well known that growth factors, such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs), stem cells, and scaffolds, are essential for tissue engineering to regenerate tissues [13]. During regeneration processes, stem cells differentiate into specific cells for tissue defects, growth factors such as BMPs and FGFs induce proliferation and differentiation of stem cells, and scaffolds with properties of extracellular matrix temporally support structures for cell growth, differentiation, and tissue formation. In studies to develop the regeneration therapy of the dentin-pulp complex, three strategies that utilize these essential three factors have been proposed; regeneration of the entire tooth, local regeneration of the dentin-pulp complex in dentin defect area from residual dental pulp, and regeneration of dental pulp from apical dental pulp or periapical tissues including the periodontal ligament and bone (Figure 1).

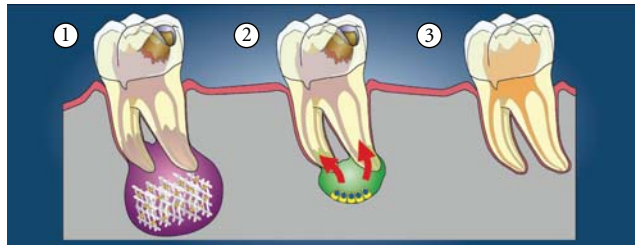
2.1. Regeneration of Entire Tooth. Regeneration of the entire tooth is accepted as a model of organ replacement and regeneration therapy. Recently, it was reported that tooth germs can be bioengineered using a three-dimensional organ-germ culture method, in which dental epithelial and mesenchymal cells isolated from tooth germs were cultured in three-dimensional scaffolds for the replacement therapy. Scaffolds consisted of synthetic polymers such as poly (lactide-co-glycolide) (PLGA) and bioceramics such as hydroxyapatite, tricalcium phosphate and calcium carbonate hydroxyapatite were examined in the three-dimensional organ-germ culture [14–21]. It was also reported that bioengineered teeth were generated from three-dimensionally arranged dental epithelial and mesenchymal cells in collagen gels by *in vitro* cell aggregate and manipulation method, and that the bioengineered tooth germ generated a structurally correct tooth showing penetration of blood vessels and nerve fibers *in vivo* transplantation into mouse maxilla, resulting in a successful fully functioning tooth replacement [22–25]. These bioengineered teeth, however, were reconstructed with dental epithelial and mesenchymal cells from genuine tooth germs. Further research will be needed to regenerate the entire tooth from other cell sources such as induced pluripotent stem (iPS) cells.



(a) Regeneration of the entire tooth



(b) Local regeneration of dentin-pulp complex from residual dental pulp



(c) Local regeneration of dentin pulp from apical pulp or periapical tissues

Figure 1: Strategies for regeneration of the dentin-pulp complex with three factors for tissue regeneration; growth factors, scaffolds, and cells (stem cells or progenitor cells). (a) Regeneration of the entire tooth. (b) Local regeneration of the dentin-pulp complex in the dentin defect area from residual dental pulp. (c) Local regeneration of dental pulp from apical dental pulp or periapical tissues.

2.2. Local Regeneration from Residual Dental Pulp. Local regeneration of the dentin-pulp complex from residual dental pulp has been mainly delivered by researchers who are engaged in clinical practice. Several studies have reported the use of local applications of bioactive molecules such as BMPs and recombinant fusion ameloblastin to exposed pulp [26–28]. However, local application of bioactive molecules without scaffolds only induces reparative dentin formation toward residual dental pulp, which is the same result provided by conventional therapy such as pulp capping.

Induction of appropriate pulp wound healing and formation of new dentin in dentin defects are essential for the local regeneration of the dentin-pulp complex and vital pulp therapies to form new dentin in defects. Several papers demonstrated the local regeneration of dentin-pulp complex in different methods. It was reported that BMP-4 with dentin powder induced dentinogenesis in dentin cavity with pulp exposure [29]. In this research, stem or progenitor cells were induced from residual pulp through the exposure site at the bottom of the cavity. It was also reported that ultrasound-mediated gene delivery of growth factors such as growth/differentiation factor 11 (GDF-11)/BMP-11 into dental pulp stem cells by *in vivo* sonoporation induced reparative dentinogenesis [30–32], and that the *ex vivo* gene therapy by the transplantation of pulp stem/progenitor cells

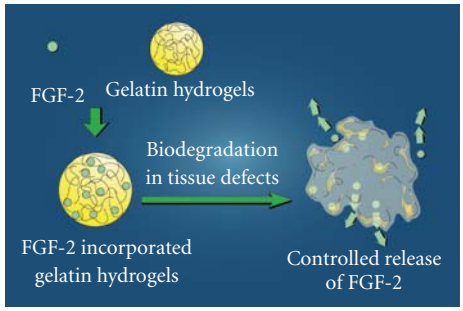


Figure 2: Controlled release of FGF-2. Gelatin hydrogels has an ability to incorporate growth factors such as FGF-2. After implantation of gelatin hydrogels incorporating FGF-2 with scaffolds, such as collagen sponge, FGF-2 is gradually released from gelatin hydrogels biodegraded by proteinase at tissue defect area. The controlled released FGF-2 can induce tissue regeneration.

transfected with some growth factors such as GDF-11/BMP-11 stimulated reparative dentinogenesis [33–36].

FGF-2 is known to play a role in both physiological and pathological conditions [37–39]. It was previously demonstrated that a gradual and continual release of biologically active FGF-2 was achieved by *in vivo* biodegradation of gelatin hydrogels that incorporated FGF-2 [40–43] (Figure 2). Recently, we used FGF-2, gelatin hydrogels, and collagen sponge as a scaffold to induce local regeneration of rat dentin-pulp complex. We implanted free FGF-2 or gelatin hydrogels incorporating FGF-2 with collagen sponge into dentin defects above the amputated pulp of rat molars, and we found that a noncontrolled release of free FGF-2 only accelerated reparative dentin formation in the residual dental pulp, whereas a controlled release of FGF-2 from gelatin hydrogels induced formation of DMP-1-positive and nestin-negative osteodentin in the pulp proliferating in the dentin defects. Furthermore, the controlled release of an appropriate dosage of FGF-2 from gelatin hydrogels induced the formation of the dentinal bridge-like osteodentin on the surface of the regenerated pulp (Figure 3). These results suggest that our method inducing the regeneration of dentin and pulp into defect area from the amputated pulp is different from the conventional therapy that induces reparative dentinogenesis toward the amputated pulp [44, 45].

2.3. Local Regeneration from Periapical Tissues. Studies on regeneration of dental pulp from the apical area began from the identification of stem cells in the apical areas of developing teeth in which root formation is incomplete. It is suggested the existence of a new population of mesenchymal stem cells residing in the apical papilla (SCAPs) of incompletely developed teeth, and these cells have the ability to differentiate into odontoblast-like cells [46–48]. SCAPs play important roles in continued root formation, and they have been suggested to participate in pulp wound healing and regeneration. It is also known that bone-marrow-derived mesenchymal stem cells (BMMSCs) have multipotent abilities to differentiate into several cell types and undergo osteogenic differentiation. Periapical tissues include periodontal ligament, and bone marrow, which is the source of BMMSCs [49–54]. Localization of SCAPs and BMMSCs in the apical area indicate the possibility of the induction of these stem cells for the regeneration of the dentin-pulp complex.

3. Scaffolds for Regeneration of Dentin-Pulp Complex

It is important to select appropriate scaffolds for successful tissue regeneration. It is well known that essential properties of scaffolds are mechanical properties such as porous three-dimension structure, and mechanical strength, as well as biological properties such as biocompatibility and biodegradation [55]. In recent research and clinical approach, the following biomaterials are utilized for tissue regeneration therapy; polyethylene terephthalate, poly(L-lactide-*co*-D, L lactide), polylactic acid, polyglycolic acid, PLGA, polyvinyl alcohol, collagen, hyaluronic acid, hydroxyapatite, tricalcium phosphate, silk fibroin, bioactive glasses, and ceramic materials [56]. Of the variety of biomaterials tested, collagen sponge has been found to be well suited for the regeneration of bone defects, as collagen is a major component of the extracellular matrix. Also in the research field of tooth regeneration therapy, several lines of studies analyzed and discussed which three-dimensional scaffolds were suitable for the regeneration of dentin-pulp complex [57–60].

Recently, we have been focusing on the application of hyaluronic acid for local regeneration of the dentin-pulp complex. Hyaluronic acid is one of the glycosaminoglycans present in the extracellular matrix and plays important roles in maintaining morphologic organization by preserving extracellular spaces, and it has been reported to have excellent potential for tissue engineering [61–65]. The roles of hyaluronic acid in some biological processes, including inhibition of inflammation and pain, and differentiation of osteoblastic and osteoclastic cells, were recently studied [66–68]. In addition, some researchers have reported that intra-articular hyaluronic acid treatment for patients with osteoarthritic knees reduced painful symptoms and improved joint mobility [69, 70].

Dental pulp is a type of connective tissue derived from the dental papilla, and contains large amounts of glycosaminoglycans [71, 72]. Previously, the contribution of hyaluronic acid to the initial development of dentin matrix and dental pulp [73], *in vivo* application of hyaluronic acid gels on the wound healing processes of dental pulp, and the application of gelatin-chondroitin-hyaluronan tricopolymer scaffold to dental bud cells were reported [74, 75].

To clarify whether hyaluronic acid sponge (molecular weight 800 kDa) is useful as a scaffold for wound healing and regeneration of dental pulp, we compared *in vitro* and *in vivo* effects of hyaluronic acid sponge and collagen sponge on KN-3 odontoblast-like cell line and amputated dental pulp of rat molars. KN-3 cells, which were established from dental pulp of rat incisors, have odontoblastic properties such as high alkaline phosphatase activity and calcification ability [76]. We found that KN-3 cells adhered to both hyaluronic

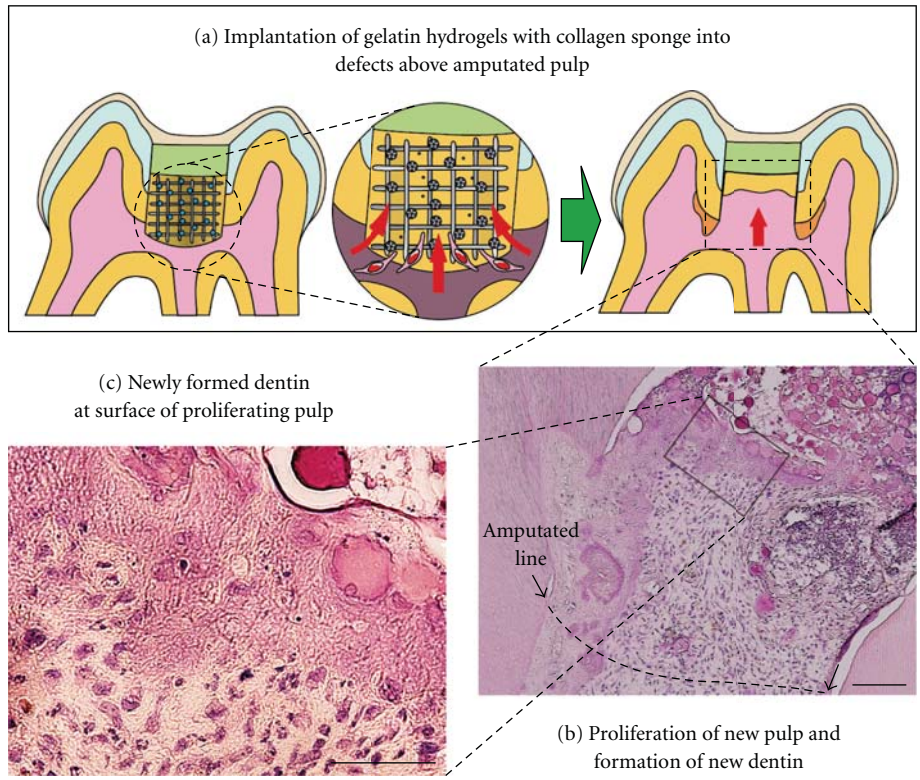


Figure 3: Local regeneration of the dentin-pulp complex in dentin defect area by implantation of gelatin hydrogels incorporating FGF-2. (a) Gelatin hydrogels incorporating FGF-2 with collagen sponge are implanted into dentin defect area. Controlled release of FGF-2 from biodegraded gelatin hydrogels can induce pulp stem cells or progenitor cells, as well as vessels, into collagen sponge at defect, resulting in the regeneration of pulp in the defect area and the formation of regenerative dentin on surface of the new pulp. (b) Histological photograph of proliferating pulp and newly regenerated dentin at surface of proliferating pulp. (c) High magnification of the regenerated dentin.

acid and collagen sponges during the culture period. *In vivo* results following implantation of both sponges in dentin defect areas above the amputated pulp showed that dental pulp proliferation and invasion of vessels into the hyaluronic acid and collagen sponges were well induced from the amputated dental pulp. These results suggest that hyaluronic acid sponge has an ability to induce and sustain dental pulp tissue regenerated from residual amputated dental pulp. In addition, we found that the inflammatory responses of KN-3 cells and the amputated dental pulp to hyaluronic acid sponge were lower than those against collagen sponge, suggesting that hyaluronic acid sponge has biocompatibility and biodegradation characteristics as well as an appropriate structure to make it suitable as a scaffold for dental pulp regeneration [77] (Figure 4).

It is also important to clarify neuronal differentiation and neurite outgrowth during regeneration of the dentin-pulp complex. We examined the effects of hyaluronic acid gel on neuronal differentiation of PC12 pheochromocytoma cells, which respond to nerve growth factor (NGF) by extending neurites and exhibit a variety of properties of adrenal medullary chromaffin cells. We applied diluted solutions of 800 kDa hyaluronic acid to NGF-exposed PC12 cells, and noted inhibition of NGF-induced neuronal differentiation of PC12 cells via inhibition of ERK and p38 MAPK activation, caused by the interaction of hyaluronic acid to its receptor, RHAMM [78].

Our results demonstrated that hyaluronic acid sponge is useful for local regeneration of the dentin-pulp complex, whereas hyaluronic acid gel inhibits the differentiation or neurite outgrowth of neurons. *In vivo,* our results showed that hyaluronic acid sponge is gradually biodegraded during the regeneration processes, leaving soluble hyaluronic acid in the regenerated dental pulp. Next, we intend to clarify the biological and physiological behaviors of hyaluronic acid throughout the regeneration the of dentin-pulp complex.

4. Future Challenges to Achieve Local Regeneration of the Dentin-Pulp Complex

In our strategy, growth factors and scaffolds are exogenously supplied as bioactive materials, while the source of stem cells that are able to differentiate into odontoblast-like cells and pulp cells is dependent on the residual dental pulp. The vitality of the residual dental pulp is a critical point to achieve local regeneration of the dentin-pulp complex. It is generally accepted that the pulp wound healing proceeds well under conditions of low inflammatory responses by the dental pulp. In addition, regulation of dental pulp infection is another critical point regarding the success of such regeneration

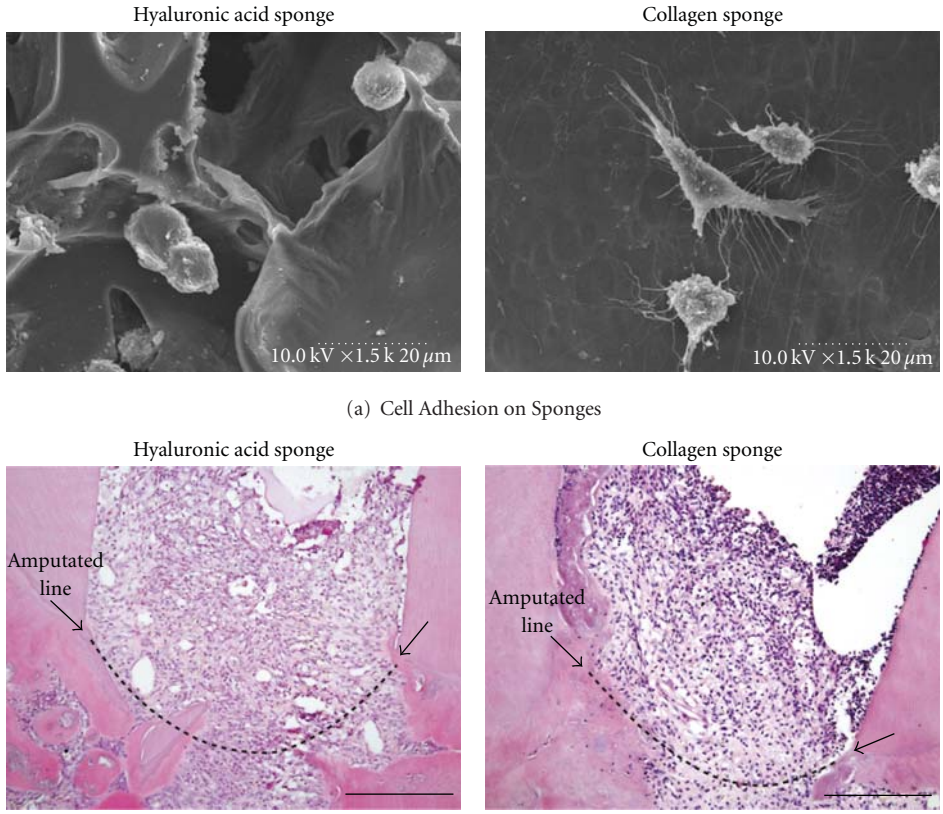


FIGURE 4: Application of hyaluronic acid sponge for local regeneration of the dentin-pulp complex. (a) KN-3 cells, odontoblastic progenitor cells, adhered to hyaluronic acid sponge, as well as collagen sponge. (b) Histological changes of amputated dental pulp after implantation of hyaluronic acid sponge *in vivo*. Amputated dental pulp well proliferated into hyaluronic acid and collagen sponges. Compared with collagen sponge, hyaluronic acid sponge significantly suppressed inflammatory reaction of dental pulp.

therapy. The resin bonding system is commonly used as one of materials showing favorable adhesion to enamel and dentin, and composite resin system with antimicrobial ability was reported [79–81]. These restorative materials may inhibit further bacterial invasion after tissue regeneration of dentin-pulp complex. Furthermore, when we try to induce revascularization and SCAPs and BMMSCs from the apical area into scaffolds at the root canal to regenerate dentin-pulp complex, disinfection of infected root canal systems, as well as proper apical enlargement to permit the induction from periapical tissues, should be successfully established [82]. Local regeneration of the dentin-pulp complex will be accomplished when the regulation mechanisms of pulp inflammation and infection, as well as pulp wound healing and regeneration mechanisms, are clarified.

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Review Article

Periosteum: A Highly Underrated Tool in Dentistry

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The ultimate goal of any dental treatment is the regeneration of lost tissues and alveolar bone. Under the appropriate culture conditions, periosteal cells secrete extracellular matrix and form a membranous structure. The periosteum can be easily harvested from the patient's own oral cavity, where the resulting donor site wound is invisible. Owing to the above reasons, the periosteum offers a rich cell source for bone tissue engineering; hence, the regenerative potential of periosteum is immense. Although the use of periosteum as a regenerative tool has been extensive in general medical field, the regenerative potential of periosteum is highly underestimated in dentistry; therefore, the present paper reviews the current literature related to the regenerative potential of periosteum and gives an insight to the future use of periosteum in dentistry.

1. Introduction

Reconstruction of lost tissues is a long cherished goal in medical field. A lot of research has been done in the past, and still research is going on to explore tools and techniques for regeneration of lost tissues as a result of the disease process. The use of various grafts and recent tissue engineering techniques including stem cell research are testimony to the ever increasing need for most suitable treatment option to replace/repair lost tissues due to various pathologic processes. The use of autogenous periosteum in general medical treatment has been extensive and has shown promising results [1–3]; on the contrary in dentistry, the use of periosteum as a regenerative tool has been limited and highly underrated; therefore, the purpose of this paper is to highlight the current status of use of periosteum in dentistry as well as suggesting its future use in various treatment options related specifically to dental field.

2. Periosteum: What Justifies Its Use?

The periosteum is a highly vascular connective tissue sheath covering the external surface of all the bones except for sites of articulation and muscle attachment (Figure 1) [4]. The periosteum comprises of at least two layers, an inner cellular or cambium layer, and an outer fibrous layer [1]. The inner layer contains numerous osteoblasts and osteoprogenitor cells, and the outer layer is composed of dense collagen fiber, fibroblasts, and their progenitor cells [5]; osteogenic progenitor cells from the periosteal cambium layer may work with osteoblasts in initiating and driving the cell differentiation process of bone repair characterized by the development of the initial fracture callus and subsequent remodeling. Periosteum can be described as an osteoprogenitor cell containing bone envelope, capable of being activated to proliferate by trauma, tumors, and lymphocyte mitogens [6]. Research on the structure of periosteum has shown that it is made up of three discrete zones [7]. Zone 1 has an average thickness of 10–20 um consisting predominantly of osteoblasts representing 90% of cell population, while collagen fibrils comprise 15% of the volume. The majority of cells in zone 2 are fibroblasts, with endothelial cells being most of the remainder. Zone 3 has the highest volume of collagen fibrils and fibroblasts among all the three zones. Fibroblasts take up more than 90% of the cells in zone 3. The morphology of fibroblasts is variable across the three zones (Figure 2).

The structure of periosteum varies with age. In infants and children it is thicker, more vascular, active, and loosely attached as compared to adults where it is thinner, less active, and firmly adherent [8]. In all age groups, the cells of the periosteum retain the ability to differentiate into fibroblasts,

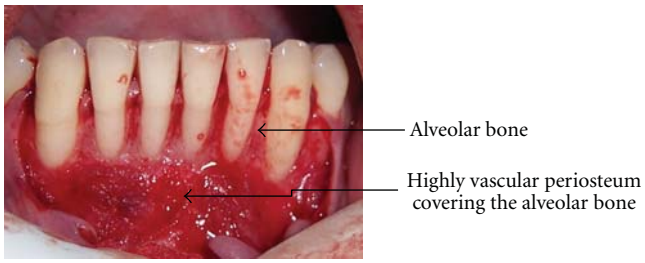

Figure 1: Highly vascular periosteum covering the alveolar bone.

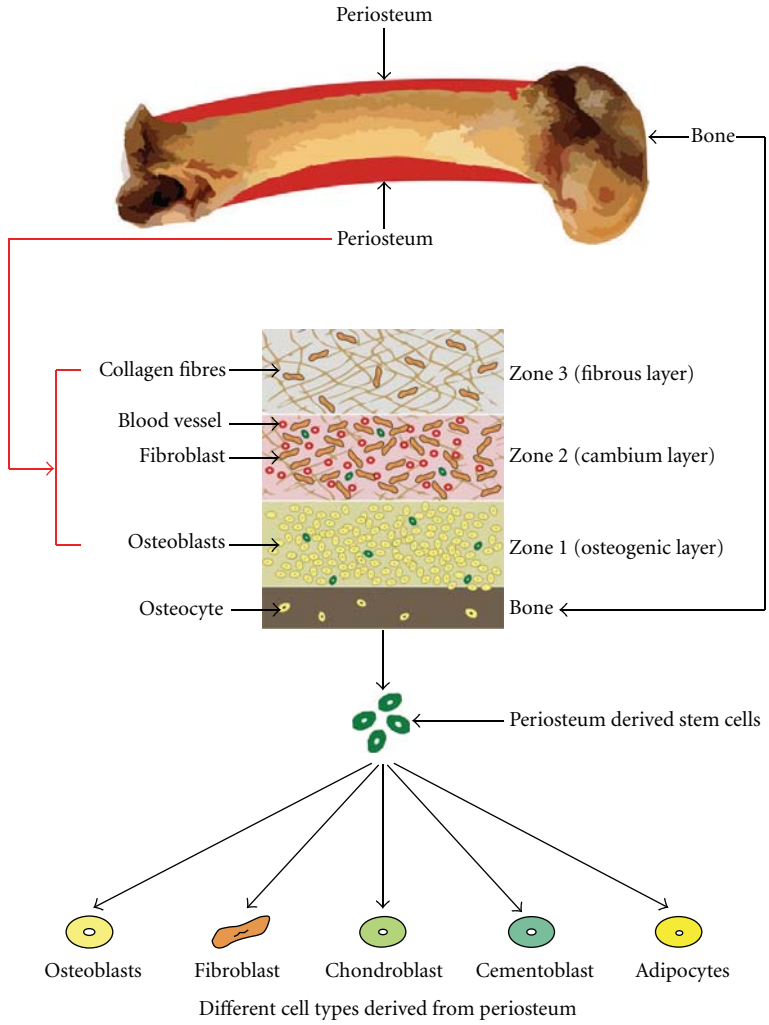

Figure 2: The three different Zones of periosteum; Zone 1 has an average thickness of 10–20 um consisting predominantly of osteoblasts; the majority of cells in Zone 2 are fibroblasts, with endothelial cells being most of the remainder. Zone 3 has the highest volume of collagen fibrils among all the three zones. The bottom of the figure shows regenerative capacity of the periosteum to form different cell types.

osteoblasts, chondrocytes, adipocytes, and skeletal myocytes. The tissues produced by these cells include cementum with periodontal ligament fibers and bone. The periosteum has a rich vascular plexus and is regarded as the "umbilical cord of bone" [9]. The vasculature system of the periosteum was first studied in detail by Zucman and later by Eyre-Brook [10]. Bourke's studies showed that the capillaries supplying blood to bone reside within the cortex linking the medullary and periosteal vessels; a recent study has even shown that periosteal cells release vascular endothelial growth factor [11] which promote revascularization during wound healing. Recently, studies have reported the existence of osteogenic progenitors, similar to mesenchymal stem cells (MSCs), in the periosteum [12, 13]. Under the appropriate culture conditions, periosteal cells secrete extracellular matrix and form a membranous structure [14]. The periosteum can be

easily harvested from the patient's own oral cavity, where the resulting donor site wound is invisible. Owing to the above reasons, the periosteum offers a rich cell source for bone tissue engineering; hence, the regenerative potential of periosteum is immense.

3. Periosteum as a Tool in Medicine and Dentistry

Developing bone substitutes for bone defect repair has inspired orthopedic surgeons, bone biologists, bioengineering researchers to work together in order to design and develop the promising products for clinical applications. Duhame in the year 1742 can be considered the first investigator to study the osteogenic potential of periosteum and published his findings in the article "Sur le Development et la Crueded Os des Animaux" [15]. A century later, another French surgeon, Ollier, discovered that the transplanted periosteum could induce de novo bone formation. One of the earliest experimental studies to demonstrate osteogenic potential of periosteum was that of Urist and McLean who reported that periosteum produced bone when transplanted to the anterior chamber of the eye of the rat [16]. Skoog subsequently introduced the use of periosteal flaps for closure of maxillary cleft defects in humans; he reported the presence of new bone in cleft defects within 3–6 months following surgery [17]. Since then, surgeons have reported the successful use of maxillary periosteal flaps [18, 19] as well as periosteal grafts from the tibia or rib. Melcher observed that new bone is laid down in parietal bone defects of rats and was deposited by periosteum that had not been previously elevated or disturbed in any other way [20], while other investigators have suggested that the contact between the periosteal flap or graft and the underlying bone is crucial to stimulation of osteogenesis [21, 22]. More recently, the osteogenic/chondrogenic capacity of periosteum and related mechanisms have been confirmed, and the underlying biology is better understood through a number of studies [23–40].

The use of periosteum in dentistry is not new. Various research papers have been published explaining the osteogenic potential of human periosteal grafts [41, 42]. The use of periosteum as a GTR has been suggested by many studies [43–46], although long-term results are still awaited to establish the regular and the most effective use of periosteal grafts as barrier membranes. The need for a graft, which has its own blood supply, which can be harvested adjacent to the recession defect in sufficient amounts without requiring any second surgical site and has a potential of promoting the regeneration of lost periodontal tissue is a long-felt need. The adult human periosteum is highly vascular and is known to contain fibroblasts and their progenitor cells, osteoblasts and their progenitor cells, and stem cells. In all the age groups, the cells of the periosteum retain the ability to differentiate into fibroblasts, osteoblasts, chondrocytes, adipocytes, and skeletal myocytes. The tissues produced by these cells include cementum with periodontal ligament fibers and bone; in addition the presence of periosteum adjacent to the gingival recession defects in sufficient amounts make it a suitable graft. Recent papers published have shown promising results with the use of periosteum in the treatment of gingival recession defects (Figure 3) [47, 48]; moreover, with the advancement in tissue engineering techniques the periosteal derived stem cells have been grown effectively to reconstruct lost tissues. Periosteum-derived progenitor cells may serve as an optimal cell source for tissue engineering based on their accessibility, ability to proliferate rapidly, and capability to differentiate into multiple mesenchymal lineages. The periosteum is a specialized connective tissue that forms a fibrovascular membrane covering all bone surfaces except for that of articular cartilage, muscle, and tendon insertions and sesamoid bones. Cells residing within the periosteum may be excised from any number of surgically accessible bone surfaces; in addition, when properly stimulated, the periosteum has the potential to serve as a bioreactor supporting a dramatic increase in the progenitor cell population over the course of a few days. Further, once the cells are removed from the periosteum, they have the potential to proliferate at much higher rates than bone marrow, cortical bone, or trabecular bone-derived progenitor cells [49].

In addition to their robust proliferation aptitude, it is well established that periosteum-derived progenitor cells have the potential to differentiate into both bone and cartilage. Further, their potential for regenerating both bone and cartilage constructs is superior to that of adipose-derived progenitor cells and comparable with that of bone marrow-derived mesenchymal stem cells. A recent study by De Bari et al. indicates that periosteal progenitor cells are able to differentiate not only into bone and cartilage cells but also into adipocyte and skeletal myocyte cells [50]. There is a growing requirement for dentists to regenerate alveolar bone as a regenerative therapy for periodontitis and in implant dentistry. Concerning the donor site, it is easier for general dentists to harvest periosteum than marrow stromal cells, because they can access the mandibular periosteum during routine oral surgery [51]; also the regenerative potential of periosteum has been effectively used in "osteodistraction" which has the benefit of simultaneously increasing the bone length and the volume of surrounding tissues. Although distraction technology has been used mainly in the field of orthopedics, early results in humans indicated that the process can be applied to correct deformities of the jaw. These techniques are now utilized extensively by maxillofacial surgeons for the correction of micrognathia, midface, and fronto-orbital hypoplasia in patients with craniofacial deformities [52].

4. Conclusion

The use of periosteum can revolutionize the success of various dental treatments which require either bone or soft tissue regeneration; particularly the future use of periosteum must be explored in periodontal and implant surgical procedures. Although the regenerative potential of periosteum has been proved by numerous studies, till date the use of periosteum-derived grafts has still not become a standard tool in the

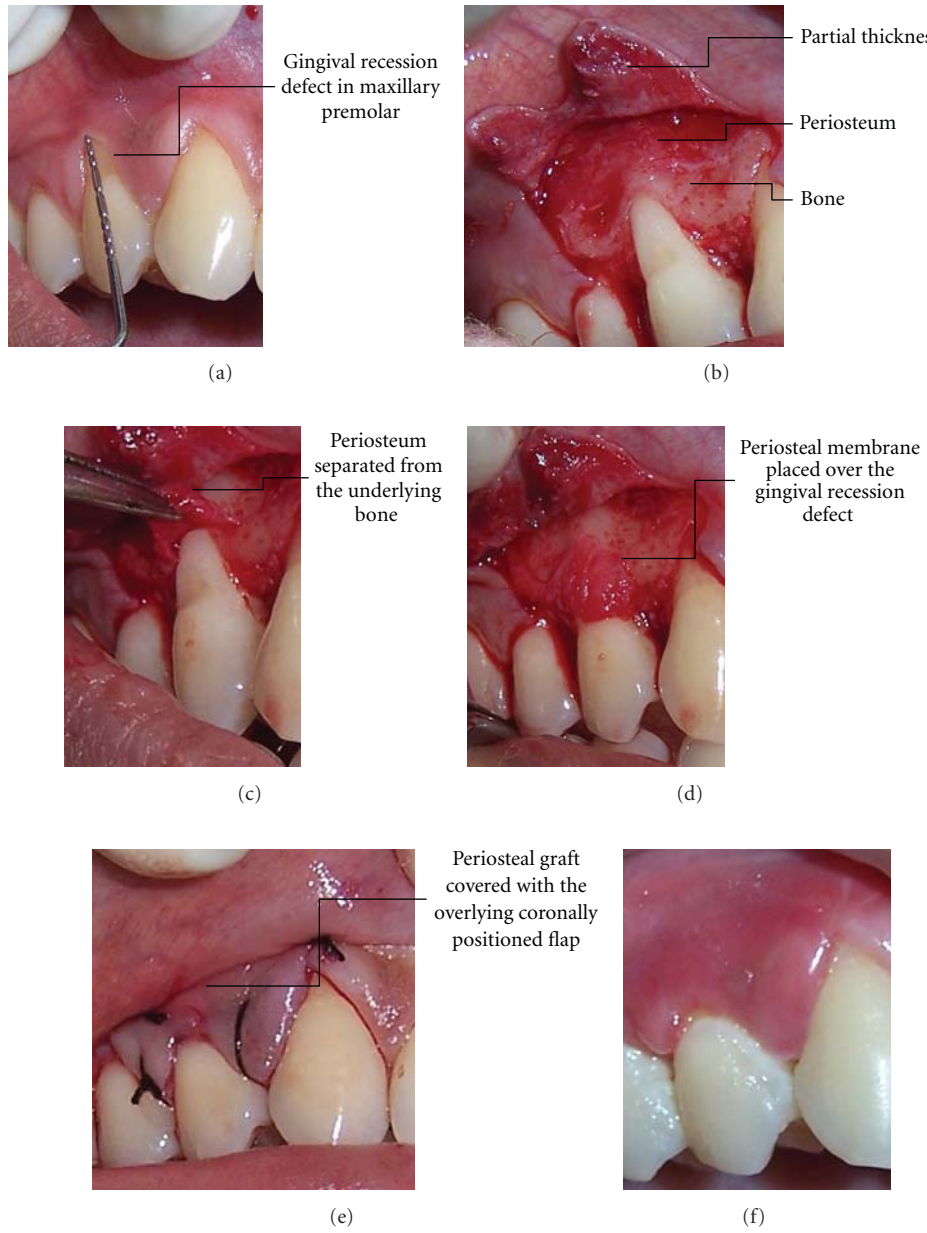

Figure 3: The use of periosteum for the treatment of gingival recession defect. (a) Clinical photograph showing gingival recession defect in relation to the maxillary first right premolar. (b) A partial thickness flap lifted to expose the underlying periosteum covering the alveolar bone. (c) The periosteum which is separated from the underlying bone. (d) The periosteum is used as a pedicle graft for covering the recession defect. (e) The periosteal graft is covered with the overlying coronally advanced flap which is sutured using 4–0 silk suture. (f) Satisfactory treatment outcome.

armamentarium of dental surgeons, and it may still need some time, and further research before the full regenerative potential of periosteum is utilized.

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