Acemannan sponges stimulate alveolar bone, cementum and periodontal ligament regeneration in a canine class II furcation defect model


Background and Objective: Periodontal disease is a common infectious disease, found worldwide, causing the destruction of the periodontium. The periodontium is a complex structure composed of both soft and hard tissues, thus an agent applied to regenerate the periodontium must be able to stimulate periodontal ligament, cementum and alveolar bone regeneration. Recent studies demonstrated that acemannan, a polysaccharide extracted from Aloe vera gel, stimulated both soft and hard tissue healing. This study investigated effect of acemannan as a bioactive molecule and scaffold for periodontal tissue regeneration.

Material and Methods: Primary human periodontal ligament cells were treated with acemannan in vitro. New DNA synthesis, expression of growth/differentiation factor 5 and runt-related transcription factor 2, expression of vascular endothelial growth factor, bone morphogenetic protein-2 and type I collagen, alkaline phosphatase activity, and mineralized nodule formation were determined using [3H]-thymidine incorporation, reverse transcription–polymerase chain reaction, enzyme-linked immunosorbent assay, biochemical assay and alizarin red staining, respectively. In our in vivo study, premolar class II furcation defects were made in four mongrel dogs. Acemannan sponges were applied into the defects. Untreated defects were used as a negative control group. The amount of new bone, cementum and periodontal ligament formation were evaluated 30 and 60 d after the operation.

Results: Acemannan significantly increased periodontal ligament cell proliferation, upregulation of growth/differentiation factor 5, runt-related transcription factor 2, vascular endothelial growth factor, bone morphogenetic protein 2, type I collagen and alkaline phosphatase activity, and mineral deposition as compared with the untreated control group in vitro. Moreover, acemannan significantly accelerated new alveolar bone, cementum and periodontal ligament formation in class II furcation defects.
Periodontal disease is a common chronic infectious disease causing the destruction of the periodontium: periodontal ligament (PDL), alveolar bone and cementum. Although conventional scaling and root planing therapy can halt the progression of this disease, and results in an increase in clinical periodontal attachment, this treatment is only effective in the early phase of the disease. Following scaling and root planing, periodontal tissue repair frequently results in a widened PDL space and incomplete regeneration of cementum and alveolar bone (1). Therefore, the ultimate goal of periodontal treatment is not only to cease and prevent further periodontal tissue destruction, but also to regenerate the periodontal apparatus (2,3).

Both anatomically and physiologically, the periodontium is a very complicated organ containing both soft tissue and hard tissue functioning together to support the teeth in the jaw. Therefore, the methods and agents used in periodontal tissue regeneration should stimulate all periodontal tissue types. Polysaccharides such as hyaluronic acid, chitosan, alginate and pectin have been proposed for use in tissue engineering and regenerative medicine (4-7). These natural materials have demonstrated biocompatibility, biodegradability, immunomodulation, antimicrobial, wound healing and osteogenic activities (4-8). Thus, they have the potential to be used as periodontal regenerative agents. Polysaccharides can be prepared in various forms such as gels, films, beads, sponges and scaffolds (4-7,9). Therefore, polysaccharides could function as either active molecules or scaffolds for periodontal regenerative therapy.

Acemannan is a biodegradable polysaccharide composed of β-(1,4)-acetylated polymannose extracted from Aloe vera gel. Acemannan has been shown to stimulate gingival fibroblast, dental pulp fibroblast, cementoblast and bone marrow stromal cell proliferation and differentiation in vitro (10–13). In vivo, acemannan enhanced oral ulcer and oral aphthous ulcer healing, reparative dentin formation and bone formation (10–12,14). Based on its bioactivity in inducing soft and hard tissue healing, acemannan is a candidate for use in periodontal tissue regeneration. However, the effect of acemannan on the regeneration of the periodontium has not been investigated. In this study, the effect of acemannan on the proliferation of periodontal ligament cells (PDLCS) and their differentiation to hard tissue forming cells was investigated. The effect of acemannan sponges on new PDL, alveolar bone and cementum formation in a canine furcation defect model was also evaluated.

### Conclusion

Our data suggest that acemannan could be a candidate biomolecule for periodontal tissue regeneration.

### Material and methods

#### Isolation and characterization of acemannan

Aloe vera (A. barbadensis Miller) was obtained from a local herbal supplier in Thailand. Aloe vera was identified by Assoc. Prof. Dr. Suchada Sukrong (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The specimen (no. 051101) was deposited in the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand).

Acemannan was isolated and characterized as previously described with some modifications (11,15). Briefly, fresh mature Aloe vera leaves were washed and the skin removed. The Aloe vera parenchyma were washed in running tap water for 30 min, and soaked in distilled water for 30 min. The parenchyma gels were blended using a homogenizer and centrifuged at 18,890 g for 60 min at 4°C. The supernatant was collected and mixed with absolute alcohol at a 1 : 3 ratio. The precipitated white opaque particles were collected by centrifugation at 12,090 g for 30 min at 4°C. After lyophilization, the pellets were ground and kept dry until use.

The molecular weight of the ground powder was analyzed using high-performance liquid chromatography connected to a reflective index detector (RID-10A; Shimadzu, Shimadzu Corporation, Tokyo, Japan). The separation was performed with a Shodex Sugar KS-804 column and compared with Shodex standard P-82 (Showa Denko K.K., Yokohama, Japan). The monosaccharide compositions were analyzed using gas chromatography-mass spectrometry and 13C-NMR spectroscopy as previously described (16,17). The data obtained were comparable to that of previous studies, indicating that the polysaccharide extracted from fresh Aloe vera gel was acemannan (15–17). The yield of acemannan extraction was approximately 0.2%.

#### Cell culture

All study protocols were approved by the Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University. PDLCS were isolated from third molars extracted from healthy young donors. The teeth were washed 3 x with phosphate-buffered saline (PBS). PDL tissue was removed using sterile surgical blades from the middle one-third of the root surface to avoid gingival and apical tissue contamination (18,19). The isolated tissue was cut into 1–2 mm³ pieces, placed into 60 mm culture dishes, and incubated with growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10,000 IU/mL penicillin G sodium, 100,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B and 1% l-glutamine) at 37°C, in an atmosphere containing 5% CO2. The growth medium was replaced every
other day. When the outgrown cells reached confluence, the cells were subcultured using 0.25% trypsin-EDTA solution. All experiments were performed using cells from the third to the fifth passage. All cell culture media were purchased from Gibco BRL™ (Invitrogen™, Grand Island, NY, USA).

DNA synthesis assay
New DNA synthesis was investigated using an [3H]-thymidine incorporation assay (20). Briefly, PDLCs (5 × 10^4 cells/well) were seeded into 24-well cell culture plates and cultured in growth medium for 16 h. The growth medium was then removed, and the cells were cultured in serum-free growth medium for 3 h, and treated with 0.25, 0.5, 1.0, 2.0 or 4.0 mg/mL acemannan for 24 h. After 20 h, the cells were labeled with 0.25 μCi/well of [3H]-thymidine (Amersham Biosciences, Little Chalfont, UK). Cells treated with the same volume of medium without acemannan served as a control group. After 24 h, the growth medium was then neutralized with 0.5 M HCl, the medium was then removed and the growth medium was replaced by osteogenic medium containing the same concentrations of acemannan as described above. Cells treated with medium without acemannan were included as a control group. After 24 h, the cells were washed 3 times with PBS, fixed with 70% ethanol, and stained with 100 mM cetylpyridinium chloride for 15 min. The absorbance of the released stain was measured at 570 nm (12,22).

Vascular endothelial growth factor, bone morphogenetic protein 2 and type I collagen measurement

Vascular endothelial growth factor (VEGF), bone morphogenetic protein 2 (BMP-2), and type I collagen levels were measured according to the manufacturers’ instructions (VEGF and BMP-2; R&D Systems, Minneapolis, MN, USA; type I collagen; Takara Bio Inc., Shiga, Japan). Briefly, PDLCs (5 × 10^4 cells) were seeded in 24-well plates and grown to 80% confluence. Then the medium was replaced by osteogenic medium containing the same concentrations of acemannan as described above. Cells treated with medium without acemannan were included as a control group. Culture supernatant was collected for VEGF, BMP-2 and type I collagen level determination. The sensitivities of the ELISA kits for VEGF, BMP-2 and type I collagen are 5 pg/mL, 11 pg/mL and 10 ng/mL, respectively. The assay was carried out in three independent experiments.

Table 1. Nucleotide sequence of sense and antisense primers of GAPDH, GDF-5 and Runx2

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<th>Gene</th>
<th>Company name</th>
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Alkaline phosphatase activity assay

PDLCs were prepared and treated with acemannan as described above. Alkaline phosphatase (ALPase) activity was determined after 72 h. The cells were washed 3 × with PBS, and incubated with glycine buffer (100 mM glycine, 2 mM MgCl₂, pH 10.5) containing 0.35 mg/mL p-nitrophenyl-phosphate (Sigma-Aldrich, St. Louis, MO, USA) at 30°C for 30 min. The reaction was terminated with 1 M NaOH. ALPase activity was reported in terms of p-nitrophenol production which was measured at 405 nm and normalized to total cellular protein (nmol p-nitrophenol/min per μg) (21).

Mineralization staining

PDLCs were prepared and treated with acemannan as described above. Mineral deposition by cultured PDLCs was determined by alizarin red (AR) staining after 9 and 18 d. The cells were washed 3 × with PBS, fixed with 70% ethanol, and stained with 2% AR (pH 4; Wako Pure Chemical Industries, Osaka, Japan). After photographing the staining results, the stained mineral nodules were destained with 100 mM cetylpyridinium chloride for 15 min. The absorbance of the released stain was measured at 570 nm (12,22).

Preparation of acemannan sponges

Acemannan sponges were prepared by direct lyophilization as previously described (23). Briefly, 5% and 10% acemannan solutions (w/v) were frozen at −80°C for 16 h, lyophilized for 16 h and exposed to ultraviolet light for 1 h. The 5% and 10% acemannan were converted to cDNA. Then the target cDNA was amplified (Prime RT Premix and Prime Taq Premix; Genet Bio, Chungnam, Korea). The sense and antisense primer sequences used for GAPDH, growth/differentiation factor 5 (GDF-5) and runt-related transcription factor 2 (Runx2) are shown in Table 1.

The amplification cycles were composed of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel (570 bp for GDF-5, 229 bp for Runx2 and 307 bp for GADPH).
solutions generated 10 mg and 20 mg acemannan sponges, respectively. The sponges were kept in a desiccator at room temperature until used.

**Scanning electron microscopy and pore size analysis**

Acemannan sponges were sputter coated with gold-palladium and analyzed under scanning electron microscopy (SEM; JSM-5410LV; JEOL, Tokyo, Japan). Samples were analyzed in both longitudinal and transverse planes. Thirty pores were randomly selected. Pore diameter, circularity and pore size were measured using the IMAGE PRO-PLUS program, version 6.0 (MediaCybernetics, Rockville, MD, USA). Because the pore shapes were predominantly elliptical, the pore diameter was calculated by the average of the longest and shortest axis of each pore. Circularity was the ratio between the shortest and longest axis of each pore (24).

**Biocompatibility evaluation**

According to ISO, both extract test and direct contact assays were used as in vitro cytotoxicity tests (25,26). For the extract test, acemannan sponges were incubated in growth medium (1 sponge/2 mL) at 37°C with gentle agitation. The conditioned media were collected at 1 and 3 d of immersion.

PDLCs (5 × 10^4 cells) were seeded in 24-well plates and incubated until 80% confluent. The growth medium was removed and the cells were washed with PBS. The cells were then incubated with conditioned media for 72 h. Cells incubated with growth medium were used as a control group. Subsequently an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) viability test was performed as described (27). Briefly, the cells were washed twice with PBS and incubated with 0.5 mg/mL MTT solution for 10 min. The formazan crystals were dissolved in dimethyl sulfoxide and the optical density was determined by measuring the light absorbance at 570 nm. The background absorbance of dimethyl sulfoxide was subtracted from the sample absorbance (26).

For the direct contact assay, sponges were soaked in growth medium for 4 h. Then the sponges were placed in center of each well in 12-well culture plates. PDLCs (8 × 10^4) were seeded around the sponges. Cell morphology was observed under the phase contrast microscope at 0, 4, 24, 48 and 72 h after seeding (25).

**In vivo study**

Four young adult mongrel dogs (12 mo of age) were obtained from the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The protocol for the animal study was approved by the Animal Ethics Committee of the Faculty of Veterinary Science, Chulalongkorn University. The animals were adapted to a 12-h light/12-h dark cycle for 2 wk before the operation. During the experiment, the animals had access to food and water ad libitum. Two weeks before the operation, all subjects received scaling and root planing. Cefazolin (first generation cephalosporin) 25 mg/kg IV was used for pre-operative antibiotics prophylaxis. Sedation was achieved using propofol 1–4 mg/kg and maintained with isoflurane (2% in 100% oxygen). The operation area was locally anesthetized using 2% lidocaine with 1 : 100,000 norepinephrine.

Class II furcation defects were created in the furcation areas of the maxillary and mandibular second and third premolars (P2 and P3) of each dog for a total of 32 defects (28,29). Briefly, a mucoperiosteal flap was raised. The alveolar crest in the furcation area was vertically reduced 5 mm from the cemento-enamel junction using atraumatic osteotomy. The mesial and distal roots served as the mesial and distal walls of the defect, respectively. The bucco-lingual depth of the defect was approximately two-thirds the diameter of the tooth crown. The average width and depth of the defects was 5 and 3 mm, respectively. All PDL tissue and cementum were removed from the root surface of the defect area using a curette. Reference notches were placed in the mesial and distal roots at the base of the defect using a 0.25 mm diameter round bur (Fig. 1A).

In each dog, the defects randomly received one of the following treatments: (i) blood clot in an untreated defect (negative control); (ii) 10 mg acemannan sponge (5% w/v); (iii) 20 mg acemannan sponge (10% w/v); and (iv) sham/no operation as a reference of the normal anatomy of the periodontium. The flap was repositioned and sutured with absorbable sutures (FSSB, Jestetten, Germany).

Two dogs were killed 30 and 60 d after the operation. Jaw blocks of the premolar regions, including bone, teeth and soft tissue, were removed, fixed in 10% neutral formalin buffer, and dehydrated with 4% nitric acid in 10% neutral formalin buffer. Tissue dehydration was carried out using ethanol–acetone dehydration and samples were routinely embedded in paraffin. Five micrometer sections were prepared from each tissue block in a lingual–buccal direction.

**Histomorphometric analysis**

Histomorphometric analysis was performed following the method of Kosen et al. (30) with some modifications. Five sections were selected from each specimen. The first section was from the mid-point of the furcation defect and the rest were obtained every 120 μm in a buccal direction from the initial section. The selected sections were stained with hematoxylin and eosin and photographed using the OLIVIA program (Olympus, Tokyo, Japan).

The following five histomorphometric measurements were determined for each stained section using the IMAGE PRO-PLUS program, version 6 (MediaCybernetics, Rockville, MD, USA) (Fig. 1C).

1. Defect area: the total area from the furcation to the apical border of the notches on the mesial and distal roots.
2. New bone: the percentage of newly formed alveolar bone area in relation to the defect area (a).
3. Defect length: the length of the root surface located between the notches on the mesial and the distal roots (b).
4. New cementum: the percentage of the length of the newly formed amorphous substance, cementoid-like tissue or cementum-like tissue on the root surface in relation to the defect length (c, d).
5. New PDL length: the percentage of the length of fibrous tissue between newly formed cementum and alveolar bone in relation to the defect length (e, f).

Statistical analysis
The data were collected and analyzed using the spss program for Windows, version 17.0 (SPSS, Chicago, IL, USA). The results were expressed as mean ± standard error. One-way analysis of variance and Dunnett multiple comparisons were performed in this study. Values of p < 0.05 were considered as statistically significant.

Results

Acemannan-induced periodontal ligament cell proliferation and mRNA expression of runt-related transcription factor 2 and growth/differentiation factor 5

The [³H]-thymidine incorporation assay showed that after 24 h, acemannan at concentrations of 2 and 4 mg/mL significantly increased new DNA synthesis in PDLCs compared with the negative control group (p < 0.05; Fig. 2A). Acemannan at a concentration of 4 mg/mL exhibited the maximum effect on DNA synthesis, which was approximately three-fold that of the untreated group.

Acemannan at concentrations of 1, 2 and 4 mg/mL significantly upregulated the mRNA level of Runx2 1.18, 1.17 and 1.25-fold, respectively, compared with the untreated group (Fig. 2B).

Acemannan also significantly increased the mRNA level of GDF-5. Acemannan at 1 mg/mL showed the maximum effect, an approximately 1.48-fold increase compared with the negative control group (Fig. 2C).

Acemannan enhanced vascular endothelial growth factor, type I collagen and bone morphogenetic protein 2 expression

After 24 h of incubation, acemannan at concentrations of 2 and 4 mg/mL significantly increased the expression of VEGF 1.93- and 1.72-fold, respectively, compared with the control group, while slightly increasing the expression of BMP-2 and type I collagen. However, after 48 and 72 h, acemannan significantly enhanced expression of type I collagen and BMP-2, respectively, compared with the control group. Acemannan exhibited a dose-dependent upregulation of type I collagen and BMP-2. Acemannan at concentrations of 2 and 4 mg/mL exhibited the maximum effect on BMP-2 and type I collagen expression, respectively (Fig. 3).

Acemannan stimulated alkaline phosphatase activity and mineral deposition

After 72 h of treatment, acemannan at concentrations of 2 and 4 mg/mL significantly enhanced PDLC ALPase activity 1.39- and 1.63-fold, respectively (Fig. 4A). AR staining indicated that, by 9 and 18 d, acemannan induced mineral deposition by PDLCs. More intense AR staining was observed in the acemannan-treated groups compared with the negative control group. The increase in mineralized nodules occurred in a dose-dependent manner. The greatest mineralization was observed at a concentration of 4 mg/mL (Fig. 4B and 4C).

Characterization and biocompatibility of acemannan sponges

SEM evaluation revealed that the concentration of acemannan used in...
the preparation of acemannan sponges determined their pore diameter and pore size. Increased concentration of acemannan resulted in larger pore diameters and pore areas (Fig. 5A). The pore geometry of both the 5% and 10% acemannan sponges was generally elliptical (Table 2). The MTT assay and direct contact assay results indicated that the acemannan sponges were biocompatible with PDLCs. The acemannan sponge extracts significantly enhanced cell proliferation compared with the control group (Fig. 5B). Moreover, the direct contact test showed that PDLs migrated towards, and proliferated around, the acemannan sponges (Fig. 5C).

**Acemannan-induced periodontal regeneration in a class II furcation defect model**

Following surgery, all dogs recovered uneventfully and gained weight over the experimental period (data not shown). The animals were examined for inflammation and foreign body interaction 30 and 60 d after the operation, and neither was detected in either control or acemannan-treated groups.

Histological analysis revealed that 30 d after treatment, the defects were partly filled with alveolar bone. The negative control and acemannan-treated groups all demonstrated new alveolar bone, cementum and PDL formation (Fig. 6A–C). These tissues extended from the pre-existing bone, cementum and PDL at the base of the defect. However, the amount of new bone and cementum was much greater in the acemannan-treated groups than in the control group. Serial histological sections demonstrated that bone formation progressed from the midpoint of the furcation defect in a buccal direction. Sixty days post-surgery, all groups demonstrated more alveolar bone, cementum and PDL formation than that seen after 30 d (Fig. 6D–F). Marked periodontal regeneration, including new bone, cementum, and PDL, was detected in the acemannan-treated groups.

Examining the newly formed bone at 30 d post-surgery more closely revealed woven bone with narrow trabeculae lined with osteoblasts. The trabeculae contained irregularly arranged osteocytes (Fig. 7A–C). Sixty days post-surgery, more new lamellar bone formation containing osteons and Haversian canal patterns was observed in the control and acemannan-treated groups (Fig. 7D–F). Thin cellular cementum and cementoid-like tissue partially covered the root surface after 30 d (Fig. 8A, a–c). The newly formed PDL was characterized by a cell-rich and vascularized dense connective tissue between the root surface and new bone. The PDL fibers were loose,

<ref>Fig. 2. Acemannan-induced PDLC proliferation and the Runx2 and GDF-5 mRNA expression in PDLCs after 24 h. Acemannan significantly enhanced PDLC proliferation at concentrations of 2 and 4 mg/mL (A). Acemannan at concentrations of 1, 2 and 4 mg/mL significantly upregulated mRNA expression of Runx2 (B). Acemannan at concentrations of 0.25, 0.5, 1, 2 and 4 mg/mL significantly upregulated mRNA level of GDF-5 (C). GAPDH served as internal control. *Compared with the untreated group; p < 0.05, n = 3. GDF-5, growth/differentiation factor 5; PDLC, periodontal ligament cell; Runx2, runt-related transcription factor 2.</ref>

Acemannan and periodontal regeneration
poorly organized and irregularly oriented (Fig. 8B, a–c). Sharpey’s fibers inserted into both the new cementum and alveolar bone were observed in some specimens by 30 d after the operation (Fig. 8B, b). At 60 d post-treatment, the width and length of the cementum in the acemannan-treated groups was greater than that of the untreated group (8A, d–f). The PDL fibers were denser and better organized compared with the specimens at 30 d (8B, d–f). However, the PDL space of all groups was wider than that of the pre-existing space.

The histomorphometric analysis indicated that the application of acemannan to the furcation defects induced greater periodontal tissue regeneration than in the control group (Fig. 9). There were significant differences in the mean percentage of new bone formation between the acemannan-treated groups and untreated control group at 30 and 60 d post-implantation (Fig. 9A). Acemannan also significantly induced cementum and PDL formation after 60 d of treatment (Fig. 9B and 9C). At 60 d post-surgery, the values of new bone, cementum, and PDL length formation found in the 10 mg acemannan sponge-treated group were slightly higher than those of the 20 mg group.

Discussion

Tissue engineering and regenerative medicine in combination with periodontal therapy can be used to overcome the limitations of conventional treatment and regenerate new periodontal tissue. The PDL is a fibrous connective tissue connecting the alveolar bone and tooth root cementum. In addition to anchoring the teeth in the jaw, the PDL is a key contributor of the cells involved in periodontal regeneration (31). Many studies have demonstrated that the PDL contains stem cells that participate in periodontal tissue homeostasis and regeneration. These cell populations contain progenitors of the fibroblast and osteoblast/cementoblast cell lineages, which are involved in periodontium regeneration. Under suitable inductive conditions, PDLCs proliferate and differentiate to osteoblast-like and cementoblast-like cells, express bone-associated protein markers and generate mineralized nodules and ectopic hard tissue (32,33). These data suggest that PDLCs have the potential to regenerate all types of periodontal tissues.

In the present study, acemannan functioned as a bioactive molecule, stimulating PDLC proliferation, expression of Runx2, GDF-5, BMP-2, VEGF, type I collagen, ALPase activity, and mineral deposition. Runx2 is a transcription factor considered to be a regulator of osteoblast/cementoblast differentiation and function (34,35). BMP-2, GDF-5 and VEGF are important growth factors for periodontal tissue healing and regeneration. BMP-2, GDF-5 and VEGF are one of the most potent growth factors inducing osteogenic/cementogenic differentiation (36–38). GDF-5 has been demonstrated...
to stimulate PDL development, new cementum formation and bone regeneration (39,40). GDF-5 and its receptor have been detected in PDLCs (41). Intrabony defects treated with BMP-2 or GDF-5 exhibited enhanced periodontal healing/regeneration with new alveolar bone, cementum and PDL formation (37,39,42). VEGF has been shown to induce angiogenesis by increasing endothelial cell proliferation and migration (43). BMP-2 and VEGF have been observed to stimulate dental follicle cells to differentiate toward an osteoblast/cementoblast phenotype (44,45). Therefore, acemannan may accelerate periodontal tissue healing/regeneration by stimulating PDLC expression of BMP-2, GDF-5 and VEGF.

In addition to enhanced growth factor synthesis, acemannan induced the expression of type I collagen, ALPase activity and mineral deposition by PDLCs. Type I collagen is the predominant extracellular matrix protein in the PDL, cementum and alveolar bone (46,47), providing physical support and acting as a template for mineral deposition in hard tissue.
ALPase is an osteogenic/cementogenic differentiation early phase marker (48). Increased levels of ALPase activity in periodontal tissues correlated with periodontal tissue regeneration (49, 50). Mineral deposition is a unique characteristic of hard tissue forming cells. Our data suggest that acemannan can induce extracellular matrix synthesis and the differentiation of PDLCs into hard tissue forming cells, osteoblasts and cementoblasts, which generate bone and cementum, respectively.

Although our in vitro results indicated acemannan induced PDLC

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<th>Parameters</th>
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<th>10% Acemannan sponge</th>
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<tr>
<td>Pore diameter (μm)</td>
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Fig. 5. Scanning electron microscopy analysis of the 5% (a, c) and 10% (b, d) acemannan sponges. Note the increased pore size in the 10% sponges (A). The acemannan sponge extracts significantly increased periodontal ligament cell proliferation (MTT assay) *Compared with the untreated group; p < 0.05, n = 3. (B). Acemannan sponge biocompatibility with periodontal ligament cell. Direct contact test of the 5% (a–c) and 10% (d–f) acemannan sponges was analyzed via the phase contrast microscope at 0 (a, d), 24 (b, e), and 72 h (c, f) after seeding. #Sponge (C).
activity, which could lead to periodontal tissue regeneration, acemannan in solution may not be appropriate for applying to periodontal pockets to induce periodontal regeneration because in solution acemannan would be diluted by crevicular fluid. Because of its physical properties as a polysaccharide, acemannan can be prepared as a sponge. SEM analysis revealed acemannan sponges contained interconnected pores with diameters ranging from 100 to 260 μm. This diameter range is suitable for PDLC attachment and growth (51). After being inserted into a periodontal defect, an acemannan sponge would absorb and maintain serum or interstitial fluid from the surrounding tissue, which is enriched with growth factors and nutrients that promote tissue healing. We found that acemannan sponges were biocompatible and exhibited biological activity as shown by their ability to stimulate PDLC migration and proliferation. This suggests that an acemannan sponge could function in vivo by inducing PDLC proliferation and activity and act as a scaffold permitting PDLC infiltration and growth.

Currently, the precise molecular mechanisms governing the effects of acemannan on cellular activity remain unknown. Based on its structure, sugar composition and molecular weight, acemannan could bind to a specific cell surface receptor and then initiate downstream intracellular signaling pathways to stimulate proliferation and differentiation. Acemannan induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in dental pulp cells. Preincubation with the specific p38 MAPK inhibitor SB203580 resulted in a 50% decrease in the phosphorylation level of p38 MAPK as compared with the acemannan-treated group (52). Periodontal ligament cell proliferation, gene expression, osteogenic differentiation, and mineralization have all been shown to be regulated by p38 MAPK (53). Therefore, acemannan may activate periodontal ligament cell proliferation and differentiation via the MAPK pathway. Another possible pathway is via acemannan binding to the mannose receptor. The mannose receptor family is composed of Endo180 (CD280), the M-type phospholipase A2 receptor, and the DEC-205/gp200-MR6 subfamily. These receptors contain C-type lectin-like domains that recognize mannose, fructose or N-acetylglucosamine at the end of a polysaccharide chain (54,55). After binding, the ligand–receptor complex is internalized and subsequently releases the ligands inside the cell. To better understand the molecular mechanisms of acemannan activity, future study is required.

In periodontal disease, the involvement of a furcation defect is considered a complex and severe condition, causing extensive and rapid attachment loss and tooth loss. The success of furcation defect treatment is often limited (56,57). To demonstrate the effect of acemannan on periodontal regeneration, an in vivo class II furcation defect model was chosen. An advantage of a class II furcation...
defect model is that it limits the severe gingival recession problems often found in class III furcation defect models. Severe gingival recession can result in the loss of test material from the defect and exposure of the defect area leading to microbial contamination (58). However, class II furcation defect models still have limitations in their use in periodontal regeneration studies. The lingual wall of a class II defect is in contact with intact alveolar bone, cementum and PDL, which is not the case in a class III defect. Consequently, the lingual defect area has a greater healing rate than the buccal area. Sectioning along the mesiodistal plane of class II furcation defects may lead to some difficulties in interpreting the histological results. To account for this, we employed criteria used in a previous study to select the sections to be examined (30). In a buccal direction beginning at the midsection, a section every 120 μm of each defect was selected and measured. The mean of the histomorphometric results obtained from each distance was used in the statistical analysis.

A study by Jittapiromsuk et al., reported that a dose of 300–600 μg acemannan was effective as a direct pulp capping material for a pinpoint pulpal exposure area of 1 mm² (12,59). The volume of the class II furcation defects in the present study was 37 mm³. For use in a three-dimensional defect, we calculated that the appropriate amount of acemannan sponge per defect should be 11–22 mg. Therefore, 10 and 20 mg acemannan sponges were prepared and placed in the defects. To minimize the effect of bone density variations between the upper and lower jaw, and between each animal, every group was represented in each jaw of each animal. In this preliminary study, a convenient sample of dogs and defects was used to evaluate the effect of acemannan on periodontal regeneration in canines. Histomorphometric analysis revealed that in all groups, more bone than cementum was regenerated. This may be because cementum has no direct blood supply, lymphatic drainage or innervation. Thus, cementum lacks sources of important factors for remodeling, repair and regeneration as compared with bone, which contains a blood supply system. Consequently, cementum repair and regeneration is lower and more unpredictable than bone regeneration (20,60,61). In our histological evaluation, the new cementum, which covered the denuded root dentin surface, exhibited various patterns; amorphous eosinophilic substance, cementoid-like tissue and cellular cementum. This finding corresponds to a previous report (62).

Ankylosis is the pathological fusion between tooth root and alveolar bone. Our study did not reveal any instances of ankylosis in either the control or experimental groups. Ankylosis has been proposed to be caused by an imbalance between new alveolar bone and periodontal tissue formation.
which impairs or hinders periodontal regeneration. In vivo, ankylosis is associated with the application of recombinant human BMP-2. The possible explanation is that BMP-2 is strongly osteoinductive with low periodontal regeneration activity, while acemannan can stimulate both soft and hard tissue regeneration (10–14,63,64).

In some of our samples, we found a small space, known as slit formation, located between newly formed cementum and dentin. Although the mechanism of slit formation is still unclear, this phenomenon is commonly seen, indicating a weak cohesion between cementum and dentin (58, 65–67). The formation of a smear layer on the surgically denuded dentin surface before new cementum synthesis has been proposed as its cause (66,67). Electron microscopy revealed the smear layer as an electron-dense, granular and non-collagenous layer present between these tissues (65,68). This smear layer can inhibit the reattachment between newly formed cementum and dentin. Another possible cause of slit formation is tissue shrinkage during the paraffin sectioning process that can break the weak attachment between these two tissues (66). Recent studies have shown that bone sialoprotein and osteopontin play a role in adhesion between these two tissues (69,70).

Currently, a number of bioactive substances and techniques have been introduced as clinical periodontal regeneration therapies such as GEM21, EMDGAIN and guided tissue regeneration (65,66,71–74). GEM21 is a mixture of recombinant human platelet derived growth factor BB and beta-tricalcium phosphate, while EMDGAIN is a purified extract of porcine enamel matrix proteins, largely consisting of amelogenin. Guided tissue regeneration is a technique that uses membranes as a barrier to support the ingrowth of periodontal tissue and inhibit the invasion of gingival epithelium into the periodontal defect. There have been many modifications made to the membrane, including gene, protein or cell therapy approaches. All of these materials have been reported as successful in animal and clinical periodontal regeneration studies (71,72).

Based on the source and composition of acemannan, the sponges may be an alternative biomaterial for patients who wish to avoid the use of recombinant protein or have restrictions on the source of a material. Moreover, in sponge form, acemannan is easy to insert into periodontal defects. An acemannan sponge can be conveniently combined with various types of periodontal surgery techniques. Unlike a solution or gel, an

**Fig. 8.** Histology of cementum and PDL regeneration in class II furcation defects at 30 d (a–c) and 60 d (d–f) post-surgery of control groups (a, d), 10-mg acemannan sponge groups (b, e), and 20-mg acemannan sponge groups (c, f). The width and length of the cementum in the acemannan-treated groups was greater than that of the untreated group (A). At 60 d post-surgery, the PDL fibers were denser and more organized at 30 d post-surgery (B). NB, new bone; PB, pre-existing bone; NC, new cementum; PC, pre-existing cementum; PDL, PDL space; D, dentin; black arrow, Sharpey’s fibers; white arrow, the apical limit of the defect. Scale bar = 50 μm.
Acemannan sponge remains in the defect for several weeks and gradually releases its bioactive molecules to promote regeneration of the surrounding tissue. We found in our in vivo study that the sponge itself is able to stop excessive bleeding. The blood and tissue fluid held in the sponge can be a source of growth factors and nutrients for tissue regeneration. The ability to physically remain in the surgical defect might help to decrease epithelial downgrowth and provide space for periodontal tissue regeneration.

With some modification, acemannan can be prepared as a scaffold and be used as a cell carrier in tissue engineering techniques. However, more animal and clinical studies regarding the efficiency of acemannan on periodontal regeneration and a comparison with these other materials and techniques are required.

We note that the class II furcation defects in our study were iatrogenically created in healthy periodontal tissue. Therefore, there was neither microorganism invasion nor the chronic inflammation and tissue destruction as occurs in periodontal defects. To confirm our data and verify its clinical applicability, further in vivo studies of acemannan in bacteria-induced periodontal defects should be performed.

**Conclusion**

In conclusion, acemannan increased PDLC proliferation, growth factor and extracellular matrix synthesis, differentiation and mineralization in vitro, and enhanced periodontal regeneration in class II furcation defects. Taken together, our data suggest that acemannan could be a candidate herbal biomolecule for periodontal tissue regeneration.

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