

Acemannan Stimulates Bone Sialoprotein, Osteocalcin, Osteopontin and Osteonectin Expression in Periodontal Ligament Cells *in Vitro*

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Abstract

The periodontium is composed of both soft and hard tissues, thus hard tissue regeneration is one of the most important processes in periodontal regeneration. Bone sialoprotein, osteocalcin, osteopontin and osteonectin are non-collagenous matrix proteins which play vital roles in the mineralization of hard tissue. Recent studies have demonstrated that acemannan, a polysaccharide extracted from *Aloe vera* gel, upregulated the expression of proteins involved in hard tissue regeneration. This study investigated effect of acemannan on bone sialoprotein, osteocalcin, osteopontin and osteonectin expression in human periodontal ligament cells. Primary periodontal ligament cells were isolated from impacted third molars and then treated with acemannan *in vitro*. The mRNA expression of bone sialoprotein and osteocalcin and the protein levels of osteopontin and osteonectin were determined using reverse transcription-polymerase chain reaction and western blot analysis, respectively. One-way analysis of variance and Dunnett multiple comparisons were performed to analyze the data. The results revealed that acemannan significantly increased the *in vitro* expression of bone sialoprotein, osteocalcin, osteopontin and osteonectin, which were 1.4, 1.2, 3.3 and 3.6 fold higher compared with the untreated control group, respectively. Our data indicate that acemannan is a bioactive molecule that can induce the expression of the non-collagenous matrix proteins; bone sialoprotein, osteocalcin, osteopontin and osteonectin in periodontal ligament cells.

Key words: Acemannan; Bone sialoprotein; Osteocalcin; Osteonectin; Osteopontin; Periodontal ligament cells

Introduction

Periodontal disease is a chronic infectious disease that results in the destruction of the periodontium, which consists of cementum, alveolar bone and periodontal ligament. Conventional periodontal treatment such as scaling and root planning can inhibit the progression of periodontal disease but the lost structures cannot be completely regained.¹ Thus, the reconstitution of the periodontium is a major goal of periodontal regeneration.

Bone sialoprotein (BSP), osteocalcin (OC), osteopontin (OPN) and osteonectin (ON) are markers of hard tissue formation. These proteins are the major non-collagenous constituents of the extracellular matrices of hard tissue.²⁻⁶ In addition to their roles of hard tissue formation, BSP, OC, OPN and ON are involved in the homeostasis and regeneration of the periodontium.^{4,7-12} The expression of BSP, OC, OPN and ON correlates with the differentiation of periodontal ligament cells (PDLCs) into osteoblast-like cells.¹³⁻¹⁵ Therefore, the use of biomolecules which induce extracellular matrix expression can be expected to facilitate periodontal regeneration.

Acemannan is a β -(1,4) acetylated polymannose polysaccharide extracted from *Aloe vera* gel.¹⁶ Acemannan has been shown to stimulate the expression of proteins involved in hard tissue formation such as OPN, alkaline phosphatase, BSP and bone morphogenetic protein-2.¹⁷⁻¹⁹ Moreover, acemannan has been reported to induce *in vitro* mineralization by cementoblasts, bone marrow stromal cells and dental pulp fibroblasts.¹⁷⁻¹⁹ However, the effect of acemannan on the upregulation of BSP, OC, OPN and ON expression in PDLCs has not yet been investigated. The aim of this study was to investigate the effect of acemannan on the expression of BSP, OC, OPN and ON in PDLCs.

Materials and Methods

Isolation and characterization of acemannan

Aloe vera (*A. barbadensis* Miller) was obtained from a local supplier and was validated by Assoc. Prof. Dr. Suchada Sukrong, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sci-

ences, Chulalongkorn University. The specimen (No. 051101) was deposited in the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand).

Acemannan was extracted and characterized as previously described, with some modifications.^{20,21} Briefly, the skin was removed from fresh mature *Aloe vera* leaves. The *Aloe vera* parenchymas 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 10,000 rpm for 60 min at 4°C. The supernatant was collected and mixed with absolute alcohol at a ratio of 1:3. The precipitated white opaque particles were collected by centrifugation at 8,000 rpm 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 refractive index detector (RID-10A, 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 (GC-MS) and ¹³C-NMR spectroscopy as previously described.^{22,23} The data obtained were comparable to that of previous studies, indicating that the polysaccharide extracted from fresh *Aloe vera* gel was acemannan.²¹⁻²³ 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. Periodontal ligament cells (PDLCs) were isolated from impacted third molars removed from five healthy young donors. The extracted teeth were washed with phosphate buffered saline (PBS). Periodontal ligament (PDL) tissue from the middle one-third of the root surface was removed using sterile surgical blades to avoid gingival and apical tissue contamination.^{24,25} The

isolated tissue was cut into 1 - 2 mm.³ 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 a humidified atmosphere containing 5% CO₂. The growth medium was replaced every other day. The cells were subcultured using 0.25% trypsin - EDTA when the outgrown cells reached confluence. Cells from the third to the fifth passages were used in the experiments. All cell culture media were purchased from Gibco BRL™ (Invitrogen™, Grand Island, NY, USA).

Cytotoxicity assay

PDLCs (5 x 10⁴ cells) were seeded in 24-well plates and cultured until 80% confluent. The growth medium was removed and the cells were washed with PBS. The cells were then incubated in growth media containing acemannan at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0, or 8.0 mg/ml for 72 h. Cells incubated with growth medium alone were used as a control group. After 72 h, an MTT viability test was performed as previously described.²⁶ Briefly, the cells were washed twice with PBS and incubated with 0.5 mg/ml MTT solution for 10 min. The formazan crystals that developed were dissolved in dimethyl sulfoxide (DMSO) and the optical density was determined by measuring the light absorbance at 570 nm. The background absorbance of DMSO was subtracted from the sample absorbance.

RNA isolation and RT-PCR analysis

PDLCs were cultured in osteogenic medium (growth medium supplemented with 50 µg/ml L-ascorbic acid, 10 mM glycerophosphate and 100 nM dexamethasone) with acemannan at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0 or 8.0 mg/ml for 72 h. Cells treated with osteogenic medium without acemannan were included as a control group. After 72 h, total cellular RNA was collected using a Total RNA mini kit (Geneaid Biotech Ltd., Taipei, Taiwan). Total RNA (5 µg) was converted to cDNA and the target cDNA was amplified using Prime RT Premix and Prime Taq Premix, respectively (Genet Bio, Chungnam, Korea). The sense and antisense primer sequences used for *GAPDH*, *BSP* and *OC* are shown in Table 1.

The amplification cycles were composed of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel (326 bp for *BSP*, 317 bp for *OC* and 307 bp for *GADPH*).

Western blot analysis

PDLCs were cultured for 6 days in osteogenic medium with acemannan at the concentrations described above. Cells treated with osteogenic medium without acemannan served as a control group. Whole cell lysate was extracted with radio-immunoprecipitation assay lysis buffer (Perbio, Pierce, IL, US). Total protein concentration was measured using the Bradford microassay (BioRad, Hercules, CA, USA). Fifty µg of protein from each sample was resolved via 10% SDS-polyacrylamide gel electrophoresis and transferred to a Polyvinylidene fluoride membrane (Immuno-Blot™, Bio-Rad, CA, US). The membrane was then immunoblotted with anti-human OPN, ON or β-actin (Santa Cruz, Biotechnology, CA, US). Detection was performed using SuperSignal® WestPico (Thermo Scientific, Pierce, IL, US) according to the manufacturer's instructions. β-actin served as the internal control.

Statistical analysis

The data were collected and analyzed using the SPSS program for Windows, version 17.0 (SPSS, Chicago, IL, USA). The quantitative results were normalized to their respective controls, and are 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 statistically significant.

Result

Acemannan was biocompatible with PDLCs

After 72 h of incubation, the MTT assay results indicated that acemannan was biocompatible with PDLCs. Acemannan at concentrations of 0.5 - 8 mg/ml significantly enhanced cell proliferation compared with the control group. Acemannan at 4 mg/ml showed the maximum effect, a 1.47 fold increase compared with the control group ($p < 0.05$, Fig. 1).

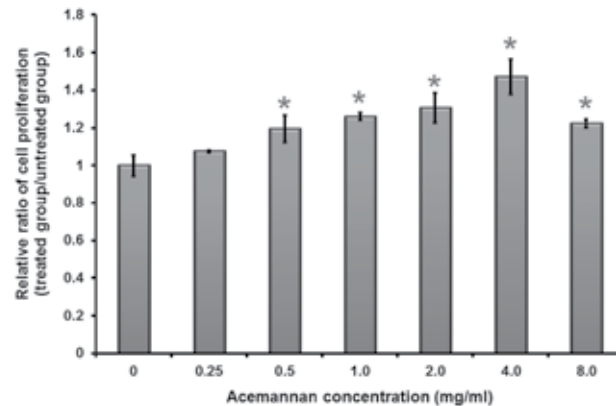


Figure 1 Acemannan was biocompatible with PDLCs. Acemannan significantly increased PDLC proliferation after 72 h of incubation (MTT assay). *Compared with the untreated group; ($p < 0.05$), $n = 5$.

Acemannan induced bone sialoprotein and osteocalcin mRNA expression

After 72 h of incubation, RT-PCR analysis revealed that acemannan at concentrations ranging from 1–8 mg/

ml significantly enhanced the expression of *BSP*. Acemannan at a concentration of 4 mg/ml exhibited the maximum effect, which was 1.45 fold higher compared with the negative control group ($p < 0.05$, Fig. 2A).

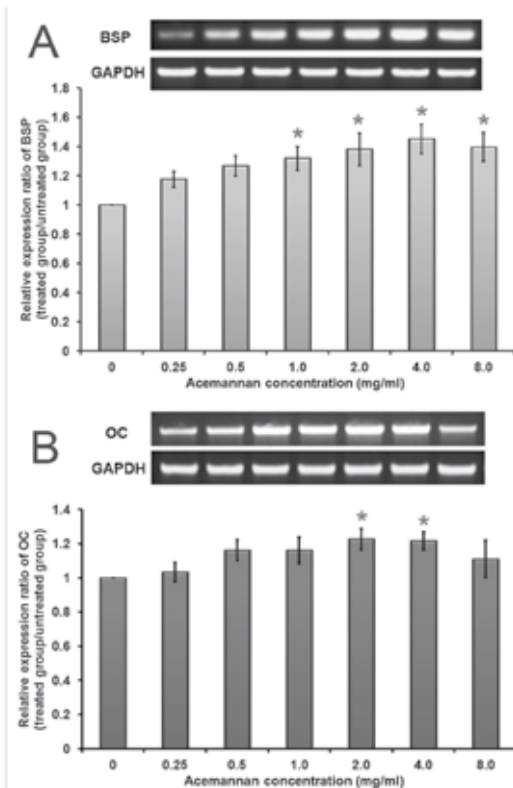


Figure 2 Acemannan significantly induced *BSP* and *OC* mRNA expression. Acemannan significantly upregulated (A) *BSP* and (B) *OC* mRNA expression in PDLCs after 72 h of incubation. GAPDH served as internal control. *Compared with the untreated group; ($p < 0.05$), $n = 5$.

Acemannan at concentrations ranging from 0.25 - 4 mg/ml dose-dependently increased the expression of OC, with concentrations of 2 and 4 mg/ml significantly upregulating the mRNA levels of OC, 1.23 and 1.22 fold, respectively, compared with the untreated group ($p < 0.05$, Fig. 2B).

Acemannan stimulated osteopontin and osteonectin expression

After 6 days of treatment, acemannan at concentrations of 0.25, 0.5, 1, 2, 4 and 8 mg/ml significantly upregulated

OPN expression in a dose-dependent manner. Acemannan at a concentration of 8 mg/ml exhibited the maximum effect on OPN expression, which was 3.32 fold higher than that of the untreated group ($p < 0.05$, Fig. 3A). Acemannan at concentrations of 0.25, 0.5, 1, 2, 4 and 8 mg/ml also significantly enhanced ON levels. The maximum effect of acemannan on ON expression was seen at 4.0 mg/ml, which was 3.59 fold higher compared with the negative control group ($p < 0.05$, Fig. 3B).

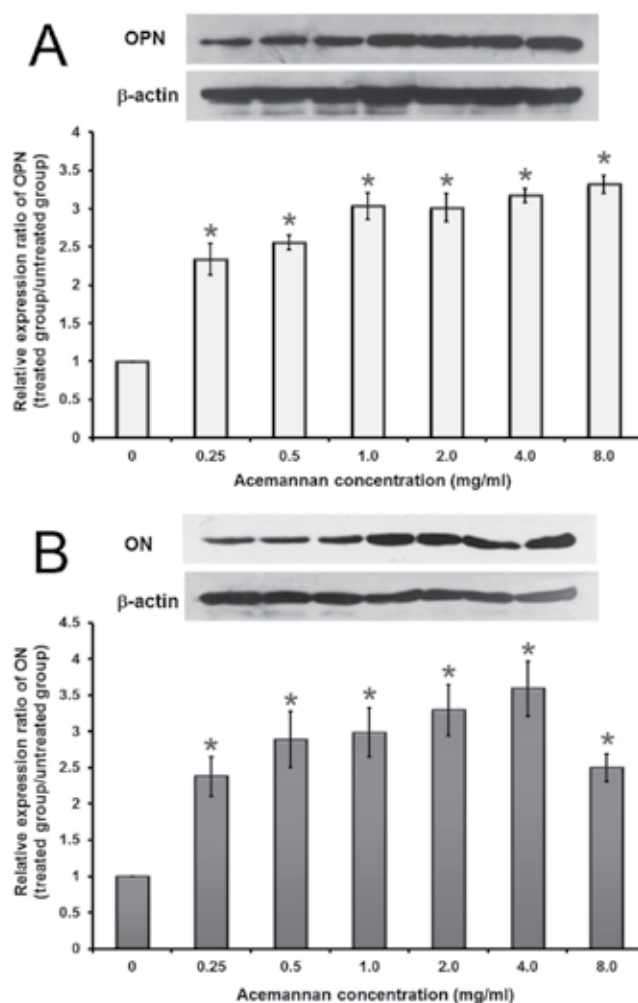


Figure 3 Acemannan promoted the expression of OPN and ON. Acemannan significantly induced (A) OPN and (B) ON expression after 6 days of treatment. β -actin served as the internal control. *Compared with the untreated group; ($p < 0.05$), $n = 5$.

Table 1 Nucleotide sequence of sense and antisense primers of *GAPDH*, *BSP* and *OC*

Gene	Company name	Base pair	Primer	
<i>GAPDH</i>	Bio Basic Inc	307	forward	GTCATCCATGACAACTTTGG
			reverse	GGAAGGCCATGCCAGTGACG
<i>BSP</i>	Bio Basic Inc	326	forward	GGGGTCTTTAAGTACAGGCCACG
			reverse	TTGTTATATCCCCAGCCTTCTTG
<i>OC</i>	Bio Basic Inc	317	forward	ACCATGAGAGCCCTCACACT
			reverse	AGAGCGACACCCTAGAC

Discussion

The PDL, which is a fibrous connective tissue connecting the alveolar bone and tooth root cementum, is a key contributor of cells for periodontal regeneration.²⁷ Many studies have demonstrated that the PDL contains stem cells that participate in periodontal tissue homeostasis and regeneration.²⁸⁻³¹ These cell populations contain some progenitors of the osteoblast and cementoblast cell lineages that can regenerate alveolar bone and cementum, respectively. Under suitable inductive conditions, PDLs proliferate and differentiate into osteoblast-like and cementoblast-like cells, express bone-associated protein markers and generate mineralized nodules and ectopic hard tissue.^{29,31} These data suggest that PDLs have the potential to regenerate PDL, cementum and alveolar bone.

In the present study, PDLs at the 3rd-5th passages were used because early passage PDLs exhibit greater cell activity and osteogenic capacity. A study has shown that the expression of Runt-related transcription factor 2 (*Runx-2*), growth differentiation factor 5 (*GDF-5*), ALPase and type I collagen, which play important roles in periodontal regeneration, decreased as passage number increased.³² Moreover, the highest level of *STRO-1*, a recognized mesenchymal stem cell marker, was found at the 1st passage and *STRO-1* levels decreased rapidly in subsequent passages.³² The proliferative rate of PDLs

was highest at the 4th passage and decreased with further passaging.³³ These data indicated that early passage PDLs, which have the highest potential to proliferate and differentiate, should be used in our study. However, the number of cells in the 1st and 2nd passages was insufficient for the experiments. As a result, cells from the 3rd - 5th passages were employed.

The MTT assay is an acceptable and reliable method to evaluate the cytotoxicity of biomaterials. This test not only assays cell/biomaterial interaction but also relates to the number of viable cells.^{26,34} The result of an MTT assay correlates with a proliferative assay such as a ³H-thymidine incorporation assay.³⁴ In the present study, we evaluated the cytotoxicity of acemannan. The results of the MTT test revealed that acemannan was biocompatible with PDLs. Indeed, the results showed that acemannan significantly upregulated PDL proliferation compared with the negative control.

In this study, RT-PCR technique was used to detect the effect of acemannan on *OC* expression. With its low molecular weight, *OC* is not readily detected by western blot analysis. Small protein molecules commonly transfer through the nitrocellulose membrane and are not retained. As a consequence, no band or a weak signal is observed.³⁵⁻³⁷ In this study, *OC* could not be detected by western blot (data not shown). Thus, western blot may not be a technique that is amenable to the accurate detection of *OC* protein. Enzyme-linked

immunosorbent assay (ELISA), a more costly technique, could be an alternative method for the detection of small proteins.

To reduce the effect of cell proliferation on mRNA or protein expression, total RNA or total protein of each sample was measured before RT-PCR or western blot analysis, respectively. Then, equal amounts, rather than equal volumes, of total mRNA or total protein from every group were used in the assays. Moreover, β -actin and GAPDH were used as the internal controls in the RT-PCR and western blot analysis, respectively.

In previous studies, acemannan increased the expression of many osteogenic markers after 3 days of incubation.¹⁷⁻¹⁹ Although OC function occurs in the late stage of hard tissue formation,^{38,39} its mRNA expression can be detected earlier than its protein expression. Therefore, in the present study, three days of treatment with acemannan was chosen for the RT-PCR experiment. We found a significant difference in OC expression between the negative control and acemannan-treated groups.

Protein expression involves the translation process that converts mRNA to protein and requires more time than mRNA expression. A previous study has shown that after incubating dental pulp cells with acemannan for 3 days, western blot analysis of OPN expression demonstrated no significant difference between the untreated control and treated groups.¹⁸ However, acemannan was reported to significantly increase OPN protein expression in cementoblasts and bone marrow stromal cells after 6 days of incubation.^{17,19} Consequently, 6 days of treatment with acemannan was chosen to be used for the western blot analysis in our study. The results revealed a significant difference between the untreated and treated groups.

The effect of acemannan on OPN and BSP expression observed in the present study corresponds to previous studies. Acemannan has been reported to increase OPN expression in cementoblast and bone marrow stromal cells.^{17,19} Moreover, acemannan significantly upregulated BSP expression in bone marrow stromal cells.¹⁷ While acemannan has been shown to induce the expression of several non-collagenous extracellular matrix

genes, the effect of acemannan on OC and ON levels had not yet been investigated. Our study is the first to show that acemannan could stimulate the expression of OC and ON.

Currently, the molecular mechanisms whereby acemannan affects 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. A possible pathway is that acemannan binds to a mannose receptor. Mannose receptor is expressed by various types of cells such as macrophages, monocytes, dendritic cells, endothelial cells, retinal pigment epithelium, kidney mesangial cells and perivascular microglia.⁴⁰⁻⁴⁶ Moreover, recent studies reported that mannose receptor was also expressed by fibroblasts.^{45,47} However, the expression of mannose receptor in PDLs has not been reported yet.

The mannose receptor family is composed of Endo 180 (CD280), the M-type phospholipase A2 receptor and the DEC-205/gp200-MR6 subfamily. These receptors contain C-type lectin-like domains (CTLDs) which recognize mannose, fructose or N-acetylglucosamine at the end of a polysaccharide chain.^{48,49} 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 the current study, a dose-dependent increase was not found in the expression of BSP, OC or ON. Their expression in the 8 mg/ml-treated group was lower than that of the 4 g/ml-treated group. This pattern was also found in previous studies, where mRNA or protein expression slightly decreased at high concentrations of acemannan, such as 8 or 16 mg/ml, compared with the lower concentrations.¹⁸⁻²⁰ This may be the result of the self-aggregation of acemannan that can occur in aqueous solution.⁵⁰ Based on the molecular structure of acemannan, hydrogen bonds and entanglement between polysaccharide chains may develop at higher concentrations. These processes could alter the 3D conformation and decrease

the solubility of acemannan, which may lessen the binding capacity of acemannan to its cell surface receptors. Consequently, the effect of acemannan on cellular activity decreased.^{23,51,52} Another possibility is an increase of osmotic pressure. At the higher concentrations, the increase in osmotic pressure of the acemannan solution could affect cell permeability.^{53,54} This may have resulted in acemannan at a concentration of 8 mg/ml having a reduced effect on BSP, OC and ON expression than that of 4 mg/ml. Our results indicate that the optimum dose of acemannan for stimulating PDLs proliferation and non-collagenous extracellular matrix secretion is 4 mg/ml.

In the present study, acemannan functioned as a bioactive molecule, stimulating the PDL expression of BSP, OC, OPN and ON. These non-collagenous proteins are implicated in the mineralization of bone and cementum.^{2,4, 10-12} OC modulates the morphology and growth of hydroxyapatite crystals.⁵⁵⁻⁵⁷ BSP, which has ability to bind hydroxyapatite and cell-surface integrins, is considered as a potential nucleator of hydroxyapatite crystal.^{5,6} OPN functions as a bridge, enabling bone cells to adhere to the mineralized matrix,^{2,58} while ON serves as a link between type I collagen and apatite crystals and free calcium ions. The osteonectin-collagen complex nucleates mineral deposition.³ Moreover, ON expressed in the PDL plays an important role in PDL homeostasis. ON controls tissue collagen content and regulates the size of the collagen fibers of the PDL.⁴ BSP and OPN play essential roles in the differentiation of cementoblast progenitor cells to cementoblasts.¹² BSP, OC and OPN are strongly expressed in regenerated periodontal tissue, especially in newly formed cementum.⁸⁻¹¹ Periodontal regeneration studies have shown that OPN has a role in the adhesion of newly regenerated cementum to pre-existing denuded dentin.^{7,9} Therefore, the expression of BSP, OC, OPN and ON indicates that PDLs have the potential to maintain the homeostasis and regenerate the periodontium.¹³⁻¹⁵

Acemannan has been reported to stimulate the proliferation and differentiation of gingival fibroblasts, dental pulp fibroblasts, cementoblasts and bone marrow stromal cells *in vitro*.¹⁷⁻²⁰ In addition to its soft tissue healing effect, acemannan stimulated reparative dentin and

alveolar bone formation *in vivo*.^{17-20,59} Based on its bioactivity in inducing soft and hard tissue healing, and its ability to stimulate the expression of the non-collagenous extracellular matrix proteins; BSP, OPN, OC and ON, acemannan has the potential to induce PDL regenerative activity.

Conclusion

In conclusion, acemannan increased the mRNA levels of *BSP* and *OC* and the protein expression of *OPN* and *ON* *in vitro*. Our data suggest that acemannan is an herbal biomolecule that can induce the expression of non-collagenous matrix proteins associated with periodontal regeneration.

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