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ORIGINAL ARTICLE

Effect of acemannan, an extracted polysaccharide from *Aloe vera*, on BMSCs proliferation, differentiation, extracellular matrix synthesis, mineralization, and bone formation in a tooth extraction model

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Abstract *Aloe vera* is a traditional wound healing medicine. We hypothesized acemannan, a polysaccharide extracted from *Aloe vera* gel, could affect bone formation. Primary rat bone marrow stromal cells (BMSCs) were treated with various concentrations of acemannan. New DNA synthesis, VEGF, BMP-2, alkaline phosphatase activity, bone sialoprotein, osteopontin expression, and mineralization were determined by [^{3}H] thymidine incorporation assay, ELISA, biochemical assay, western blotting, and Alizarin Red staining, respectively. In an animal study, mandibular right incisors of male Sprague–Dawley rats were extracted and an acemannan treated sponge was placed in the socket. After 1, 2, and 4 weeks, the mandibles were dissected. Bone formation was evaluated by dual-energy X-ray absorptiometry and histopathological examination. The in vitro results revealed acemannan significantly increased BMSC proliferation, VEGF, BMP-2, alkaline phosphatase activity, bone sialoprotein and osteopontin expression, and mineralization. In-vivo results showed acemannan-treated groups had higher bone mineral density and faster bone healing compared with untreated controls. A substantial ingrowth of bone trabeculae was observed in acemannan-treated groups. These data suggest acemannan could function as a bioactive molecule inducing bone formation by stimulating BMSCs proliferation, differentiation into osteoblasts, and extracellular matrix synthesis. Acemannan could be a candidate natural biomaterial for bone regeneration.

Keywords Acemannan · *Aloe vera* · BMSC · Differentiation · Mineralization · Tooth socket healing

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Introduction

Residual ridge resorption is a chronic, irreversible, and destructive event following tooth extraction, continuing even after socket healing. The precise etiology of this phenomenon remains unknown, and methods to induce socket healing and bone formation have been suggested to preserve residual alveolar bone [1].

Socket healing is characterized by three overlapping phases: blood clot formation, bone formation, and bone remodeling [2]. During bone formation, osteoprogenitors migrate to the wound site, proliferate, and differentiate into osteoblasts. Osteoblasts secrete local growth factors, extracellular matrix, and induce mineralization [3]. Bone

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marrow stromal cells (BMSCs) are considered a source of osteoprogenitor cells [4].

Aloe vera (*Aloe barbadensis* Miller) is a traditional medicine for wound healing [5]. Acemannan, β-(1–4)-acetylated polymannose, is the major polysaccharide extracted from *Aloe vera* gel. Previous in vivo studies demonstrated acemannan accelerated oral wound healing and reparative dentin formation [6, 7]. However, the effect of acemannan on bone formation has not been reported. Here, we investigated the effects of acemannan on BMSC proliferation, differentiation, extracellular matrix secretion, and in vitro nodule deposition. The effect of acemannan on tooth socket healing in a rat model was also determined.

Materials and methods

Preparation and characterization of acemannan

Aloe barbadensis Miller was obtained from a local Bangkok, Thailand supplier. *Aloe vera* was identified by Associate Professor Dr. Suchada Sookrong, Department of Pharmacognosy and Pharmaceutical, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The specimen (No. 051101) was deposited at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Acemannan was extracted from fresh *Aloe vera* pulp gel by homogenization, centrifugation, and alcohol precipitation as described [8]. To remove small proteins and monosaccharides, acemannan was placed into a 10,000-MWCO semi-permeable dialysis tubing (Thermo Scientific-Pierce Biotechnology) for 24 h. and then lyophilized. The molecular weight of acemannan was analyzed by HPLC (Shimadzu, Kyoto, Japan). Separation was performed using a column (Shodex Sugar KS-804) and compared with the P-82 standard. The monosaccharide composition and structure of the polysaccharide was analyzed by GC–MS and ^{13}C NMR as described [8]. The data obtained from these analyses were consistent with previous results indicating the polysaccharide extracted from fresh *Aloe vera* gel is acemannan [9] (See Online resource 1). The yield of acemannan extraction was roughly 0.2 %.

For in vitro studies, acemannan was dissolved and autoclave sterilized. For the animal study, the acemannan solution was aseptically frozen and lyophilized, generating an acemannan sponge.

Bone marrow stromal cell (BMSC) isolation and culture

Bone marrow was obtained from the femurs of male Sprague–Dawley rats as described with minor modifications [10]. Briefly, after removing the ends of the femur, bone marrow was flushed out and resuspended in growth medium (DMEM supplemented with 10 % FBS, 100 IU/ml penicillin-G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin-B and 1 % L-glutamine (GIBCO®; Invitrogen™, NY, USA). Cells were pooled and cultured at 37 °C in a 5 % CO_2 atmosphere. On the 2nd day of culture, non-adherent cells were gently washed out. Growth medium was changed every 2 days. After 7–10 days in culture, BMSCs were subcultured. All experiments were performed using cells in the third–fifth passage. To induce differentiation and mineralization, BMSCs were cultured in a mineralizing medium (growth medium, 50 μg/ml ascorbic acid, 1 mM β-glycerophosphate, and 0.01 μM dexamethasone.

DNA synthesis assay

DNA synthesis was assayed by [^{3}H]-thymidine incorporation [6]. Briefly, BMSCs (6×10^4 cells/well) were treated with acemannan for 24 h. Control cultures received the same volume of growth medium only. During the last 4 h of incubation, cells were labeled with 0.25 μCi/well of [^{3}H]-thymidine (Amersham Biosciences, Little Chalfont, UK). The cells were washed with PBS, fixed with trichloroacetic acid, solubilized in NaOH, and neutralized with HCl. After mixing with scintillation fluid (OptiPhase HiSafe, Wallac, UK), the beta radiation was quantified by a liquid scintillation counter.

Alkaline phosphatase activity

Alkaline phosphatase activity was determined by biochemical assay [11]. After washing with PBS, cells were incubated with glycine buffer (100 nM glycine, 2 mM $MgCl_2$, and pH 10.5) containing 0.08 mg/ml *p*-nitrophenylphosphate at 30 °C for 15 min, and the reaction was terminated with NaOH. The amount of *p*-nitrophenol (*p*-NP) was measured at 405 nm. The ALPase activity was reported as *p*-NP production per minute normalized to total cellular protein.

Measurement of vascular endothelial growth factors (VEGF) and bone morphogenic protein-2 (BMP-2)

VEGF and BMP-2 levels were quantified by ELISA (R&D System, Minneapolis, USA) as per the manufacturer's instructions. Briefly, cells (5×10^4 cells/well) were grown to confluence in 24-well tissue culture plate. For VEGF measurement, cells were changed to serum-free medium for 3 h, two times. After that, the medium was replaced by various concentration of acemannan. A medium without acemannan was included as a control. After 48 and 72 h of

incubation, culture supernatant was collected and stored at −20 °C until used.

For determination of BMP-2, the cells were treated with acemannan in mineralizing medium. Test media were changed with fresh media every 3 days. Whole cell lysate was collected and stored at −20 °C until used. The sensitivity of the ELISA kits for VEGF and BMP-2 were 8.4 and 11 pg/ml, respectively.

Western blot analysis

Whole cell lysates (50 μg) of each sample were resolved by 10 % SDS-PAGE and transferred to a membrane (Immuno-Blot; Bio-Rad laboratories, USA). After blocking with 5 % skim milk, the membranes were immunoblotted with polyclonal anti-rat bone sialoprotein (BSP), osteopontin (OPN), or β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, USA). The signals were determined by SuperSignal® West Pico (Thermo Scientific-Pierce Biotechnology) as per the manufacturer's instructions.

Mineralization

After culturing in mineralizing media for 15 days, cells were washed with PBS, fixed with 70 % ethanol, and stained with 0.5 % AR solution, pH 4 (Wako Pure Chemical Industries, Osaka, Japan). To quantify the AR staining, AR was solubilized in 100 mM cetylpyridinium chloride. Absorbance of the released stain was measured at 570 nm and compared with an AR solution standard curve [12].

Animals

Fifty-two male Sprague–Dawley rats (8-weeks old; 200–250 g; National Laboratory Animal Centre, Nakhon Pathom, Thailand) were used in this study. They were given access to water and diet ad libitum and maintained at 25 ± 1 °C with a 12 h light/12 h dark cycle. Throughout the study, the animal remained healthy and thrived.

In vivo tooth socket healing assay

The protocol was approved by the Animal Ethic Committee, Faculty of Dentistry, Chulalongkorn University (No. 0832001). The procedures were performed as described with minor modifications [13]. Briefly, right lower incisors were consecutively cut 3 times at the middle of the crown length: on days 9, 6, and 3 before the experiment. The animals were anesthetized intraperitoneally with 80 mg/kg Zoletil® (chloral hydrate tiletamine and chloral hydrate zolazepam; Virbac Laboratories, Carros, France). The incisors were then carefully extracted and tooth sockets were randomly divided into four groups: Group I was the untreated control group; Groups II, III, and IV were implanted with 8, 16, and 32 mg/kg acemannan sponges, respectively, into the sockets.

Bone mineral density (BMD) analysis

On days 7 and 14 postoperatively, three animals from each group were killed. The remaining animals, 7 per group, were killed on day 30. The right lower jaws were dissected and fixed in 10 % neutral-formalin buffer. The bone mineral density (BMD) of the lower jaw was measured by dual-energy X-ray absorptiometry (DEXA, DCS-600R; Aloka Co., Ltd., Tokyo, Japan). The selected area of interest was the region from the apex of the incisal residual ridge to the mesial/anterior surface of first molar (Area A) and to the distal/posterior surface of third molar (Area B; Fig. 1). With the limitation of software, a large region of interest was applied. A similar amount of calcium in the three molars in all experiment animals was assumed [14].

Histopathological evaluation

After radiological analyses, the samples were demineralized, dehydrated, and embedded in paraffin. Samples at the third molar level of socket were sectioned at 6 μm thickness in a bucco-lingual direction and stained with hematoxylin and eosin (H&E). The sections were scanned and recorded by Dot slide digital virtual microscopy system with software version 2.3 (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using the SPSS program for Windows, version 11.5 (SPSS, Chicago, IL, USA). The in vitro experiments were carried out in three independent studies. The data were presented as mean ± standard error. The results were analyzed by one-way analysis of variance (ANOVA), followed by post hoc analysis with Dennett's multiple range tests. Values of $p < 0.05$ were considered as statistically significant.

Results

Acemannan induced BMSC proliferation and VEGF expression

After 24 h of incubation, acemannan at concentrations of 2, 4, and 8 mg/ml, significantly induced DNA synthesis of BMSCs in a dose-dependant manner compared with control ($p < 0.05$; Fig. 2a). The greatest effect of acemannan was observed at 8 mg/ml.

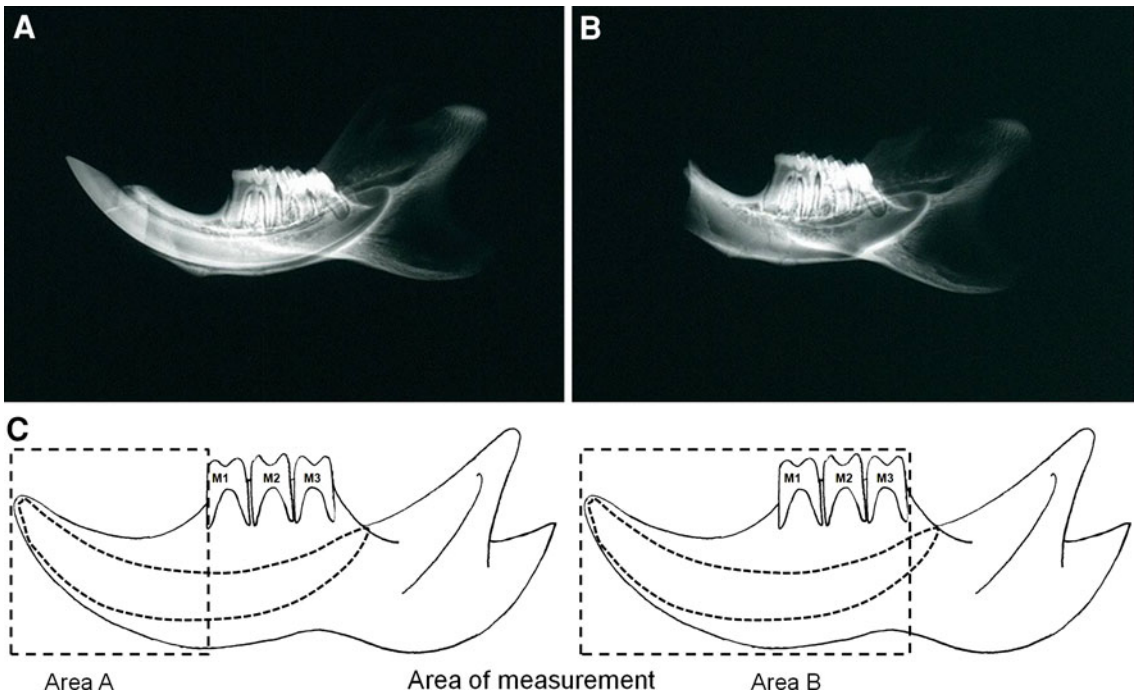

Fig. 1 The radiographic image of rat mandible before (**a**) and after (b) extraction of the lower incisor. The outline of lower jaw, incisor socket, and bone mineral density (BMD) measurement area (**c**). The BMD was determined from the residual ridge of incisor to the mesial/anterior surface of first molar (*rectangular area* of *dash line* of Area A) and from the residual ridge of incisor to the distal/posterior surface of third molar (*rectangular area* of *dash line* of Area B)

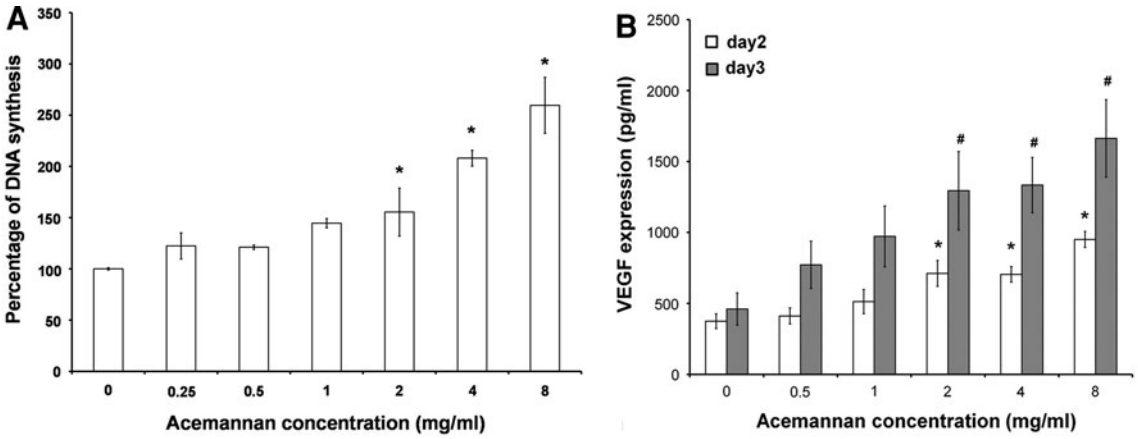

Fig. 2 Acemannan promoted BMSCs proliferation and VEGF expression. **a** Cell proliferation was evaluated by incorporation of [^{3}H]-thymidine after 24 h of incubation with various concentrations of acemannan. Acemannan significantly stimulated DNA synthesis in BMSCs at concentration of 2, 4, and 8 mg/ml. *Significant difference compared with the untreated group as control; $p < 0.05$, $n = 3$. **b** Acemannan at concentration of 2, 4, and 8 mg/ml significantly enhanced VEGF expression on days 2 and 3 of incubation. *Significant difference compared with the untreated group on day 2; #significant difference compared with the untreated group on day 3; each at $p < 0.05$, $n = 3$. BMSCs, bone marrow stromal cells VEGF, vascular endothelial growth factor

On day 2 of treatment, acemannan at concentrations of 2, 4, and 8 mg/ml significantly induced VEGF expression in a dose-dependent manner by 1.6-, 1.6-, and 2.2-folds, respectively, compared with the untreated group. On day 3, the productive effect of acemannan was maintained. Acemannan at concentrations of 2, 4, and 8 mg/ml continued to significantly increase VEGF secretion 2.8-, 2.9-, and 3.6-folds, respectively, compared with the control group (Fig. 2b).

Acemannan increased ALPase activity, expression of BMP-2, OPN, and BSP, and mineralization

Compared with the untreated group, acemannan enhanced BMSC ALPase activity after 3 days of incubation in a dose-dependant manner, peaking at concentrations of 0.5 and 1 mg/ml. The difference was significant for 0.5 and 1.0 mg/ml acemannan. At higher concentrations (2–8 mg/ml), the ALPase activity declined from its peak, reaching control level at 8 mg/ml (Fig. 3a).

After 6 days of incubation, acemannan at 1 and 2 mg/ml significantly stimulated BMP-2 expression approximately 4.7- and 2-folds compared with the untreated group (Fig. 3b). The level of OPN and BSP expression also increased in a dose-dependent manner. Acemannan at a concentration of 8 mg/ml significantly upregulated OPN and BSP expression 5- and 2.9-folds, respectively, compared with control (Fig. 3c).

At 15 days of treatment, acemannan induced mineral deposition by BMSCs. Large areas of nodule formation were observed in the acemannan-treated groups. Quantitative analysis revealed the AR concentration significantly increased in all experimental groups by 1.7-, 2-, 2.2-, 2.7-, and 3.1-folds, respectively, compared with the control group (Fig. 3d).

Acemannan stimulated bone mineral density (BMD) and tooth socket healing after extraction

By DEXA analysis, the bone of the rat sockets implanted with 16 and 32 mg/kg acemannan sponges showed a

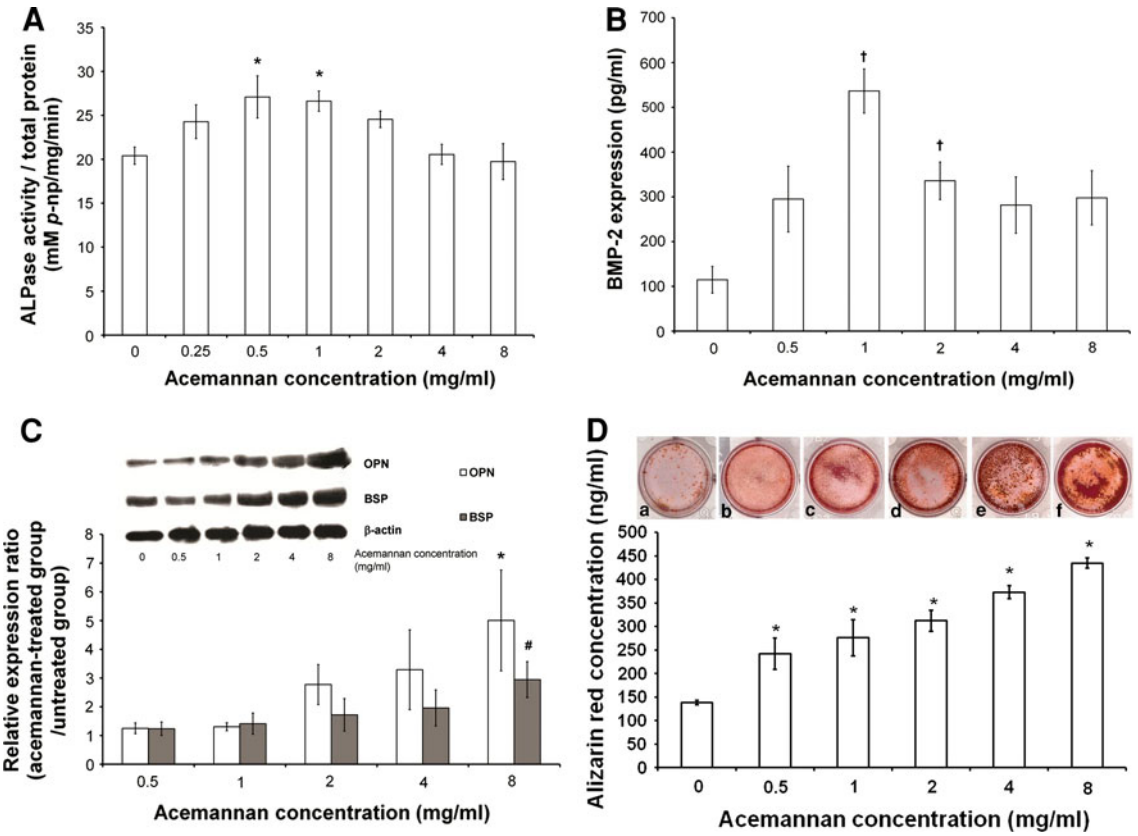

Fig. 3 Acemannan stimulated ALPase activity, BMP-2, OPN and BSP expression and mineralization of BMSCs. **a** Acemannan significantly enhanced ALPase activity of BMSCs at concentration of 0.5 and 1 mg/ml at 3 days of incubation. *Significant difference compared with the untreated group; $p < 0.05$, $n = 5$. **b** Acemannan at concentration of 1 and 2 mg/ml significantly stimulated the production BMP-2. †Significant difference compared with the untreated group on day 6; $p < 0.05$, $n = 3$. **c** Acemannan at concentration of 8 mg/ml increased OPN and BSP expression on day 6 of treatment. Levels of OPN and BSP expression were analyzed by western blot when β-actin served as internal control. *,#Significant difference compared with the untreated group; $p < 0.05$, $n = 3$. **d** Acemannan promoted BMSCs mineralization at 15 days of treatment. The acemannan treated groups, 0.5, 1, 2, 4, and 8 mg/ml (*b–f*), clearly exhibited stronger and larger areas of staining than that of untreated group (*a*). By quantitative AR staining, the acemannan treated groups, 0.5, 1, 2, 4, and 8 mg/ml significantly promoted mineralization. *Significant difference compared with the untreated group; $p < 0.05$, $n = 3$. *ALPase* alkaline phosphatase, *BMP-2* bone morphogenic protein-2, *OPN* osteopontin, *BSP* bone sialoprotein, *AR* Alizarin Red

significant increase in BMD compared with the untreated group ($n = 7$; Fig. 4a). Eight mg/kg acemannan sponges slightly increased BMD, but not significantly, compared with untreated group.

To confirm the above finding, tooth socket healing was evaluated by histopathological examination from the first–fourth week after extraction. By 1 week, the socket space of both control and acemannan-treated groups contained a blood clot, which exhibited neutrophils and macrophages. After 2 weeks, the control group showed remnants of hemorrhage with blood cyst. A few spindle-shaped cells and new blood vessels were detected at the socket periphery. In the acemannan-treated groups, extensive ingrowth of bone trabeculae was observed. Osteoblasts and osteocytes were found at the surface or embedded in the bone trabeculae, respectively (see Online resource 2).

By the fourth week, while some new bone ingrowth had occurred, a large unfilled area remained at the center of the control sockets (Fig. 4b*a1*). Contrarily, the acemannan-treated groups were mostly healed with new bone formation with numerous bone trabeculae bridges projecting across the socket (Fig. 4b*b1*). Moreover, in the acemannan-treated groups, the bone matrix was thicker and denser than that of the control group (Fig. 4b*a2*, *b2*).

Discussion

Acceleration of socket healing, especially during the bone formation phase, could minimize alveolar residual ridge resorption and enhance the quality/quantity of alveolar bone [15]. BMSCs are considered to be mesenchymal stem cells or osteoprogenitor cells [4]. Under appropriate conditions, BMSCs migrate and differentiate into osteoblasts and produce new bone. Therefore, in our study, BMSCs were utilized to investigate the potential effects of acemannan on bone formation, including proliferation, differentiation, and mineralization.

In the present study, acemannan significantly stimulated BMSC proliferation, ALPase activity, expression of VEGF, BMP-2, OPN, BSP, and mineralization. VEGF induces both new capillary formation and osteoblast differentiation [16]. ALPase, BMP-2, OPN, and BSP are considered BMSC osteoblastic differentiation markers [17]. ALPase is considered an initial marker of osteoblast differentiation and induces mineralization [18]. OPN and BSP are major non-collagenous proteins that play important roles in bone. OPN functions by linking hydroxyapatite crystal to bone matrix, while BSP functions as a hydroxyapatite nucleator [19, 20]. Therefore, acemannan may induce BMSC differentiation

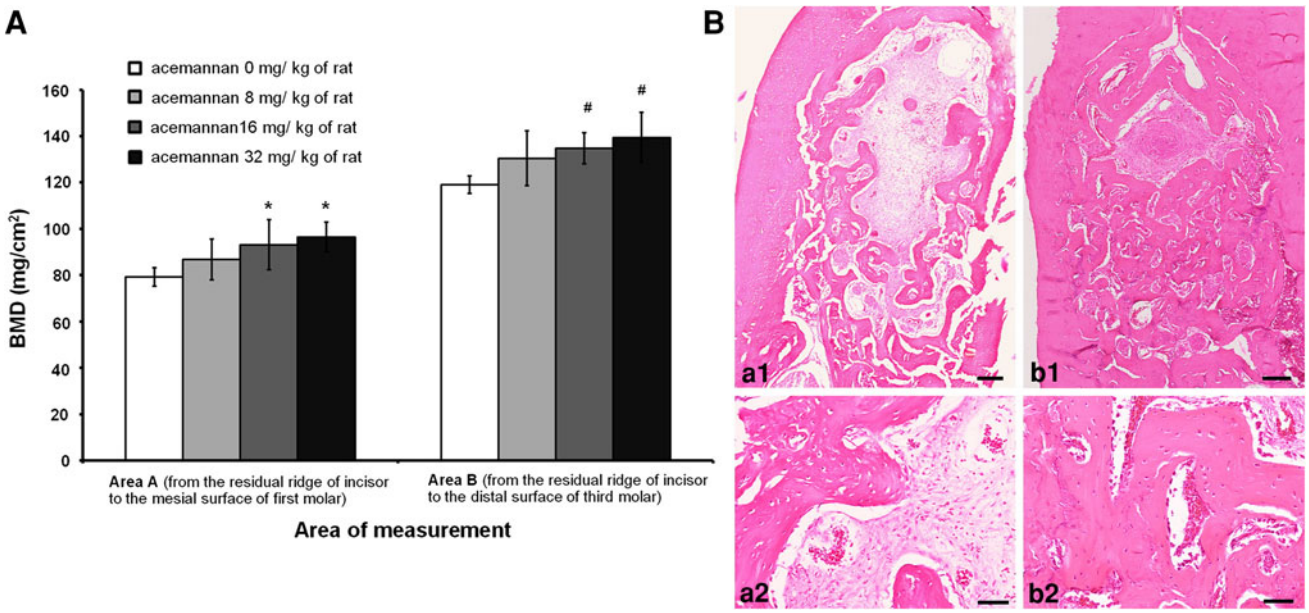

Fig. 4 Acemannan stimulated BMD and healing in tooth socket at 4 weeks after extraction. **a** The BMD of the new bone formation (rectangular area from the residual ridge of incisor to the mesial/anterior surface of first molar and rectangular area from the residual ridge of incisor to the distal/posterior surface of third molar) was analyzed by DEXA. $^{*,\#}$Significant difference compared with the untreated socket; $p < 0.05$, $n = 7$. **b** Histopathology of the tooth socket. In control group, a large unfilled area is seen in the socket (*a1*). Thin and loose bone matrix are also observed (*a2*). In acemannan-treated group (16 mg acemannan/socket/kg of rat), numerous bone trabeculae bridges are projecting and spanning the socket (*b1*). Thick and dense bone matrix of bone trabeculae are observed (*b2*). H&E staining; *bar* 200 μm (*a1*, *b1*) and 50 μm (*a2*, *b2*)

and bone formation via upregulating VEGF, BMP-2, extracellular matrix synthesis, and mineral deposition.

To explore the bioactivity of acemannan on bone formation, a rat tooth socket model was used. This model was previously utilized for investigating effect of candidate substances on bone formation [1]. To maintain the chewing ability of the rats, only the lower right incisor was extracted. The untreated socket was taken as a negative control because it corresponds to the clinical situation existing after conventional atraumatic extraction. To evaluate effect of acemannan on bone formation, DEXA and histopathological examination were employed. DEXA was used for quantitatively determining the BMD in the healing socket. The BMDs from apex of the incisal residual ridge to the mesial/anterior surface of first molar and to the distal/posterior surface of third molar were measured to confirm the effect of acemannan on bone formation in the socket. Sections at the third molar level were examined because these contain the base of incisor socket, which is the initial site of healing [21]. From our data, higher BMD values of the acemannan-treated groups (16 and 32 mg/kg sponges) demonstrated a higher rate of new bone formation than that of the untreated group. Osteoblasts and osteocytes were more numerous than that of control group. Taken together, these findings suggest the potential osteoinductive activity of acemannan on bone formation. Even though DEXA has been shown to provide valid and reliable information [22], this is a two-dimensional measurement technique. Therefore, to confirm this finding, a three-dimensional micro-CT measurement should be performed to describe the bone formation in extraction socket.

In our study, the data revealed different maximal effective concentrations of acemannan on BMSCs' protein expression. ALPase and collagen are early stage markers of osteoblastic differentiation while OPN, BSP, and osteocalcin are late stage markers [17]. In late stage of osteoblastic differentiation, ALPase expression level is downregulated. Therefore, the different maximal effective concentrations of BMSC's protein expression observed in the present study may reflect difference in differentiation stage of BMSCs induced by various concentration of acemannan. The other possible explanation is these proteins are regulated by distinct signaling pathways. Pre-osteoblastic cells responded to differently to TGF-β at different concentrations [23]. A low dose of TGF-β upregulated collagen type I and ALPase, while a high dose enhanced bone sialoprotein and osteocalcin.

Many cell types in addition to BMSCs have been proposed to play a major role in tooth socket healing including perivascular progenitor stem cells, undifferentiated ecto-mesenchymal cells in the PDL, periosteum, and periodontal ligament cells [24]. Within the limitations of our data, we cannot rule out the possible role of those cells in tooth socket healing.

It should be noted that in our study the tooth socket model was performed in a healthy socket with intentional extraction. In residual ridge resorption patients, the tooth is normally extracted due to pathologic conditions such as caries or periodontal disease. Therefore, to verify its osteoinduction potential, a further study of acemannan on bone formation in tooth sockets under pathological conditions, ectopic bone formation, and calvaria defect should be performed.

In summary, our in vivo and in vitro data indicated that acemannan accelerates bone formation, inducing BMSC proliferation, differentiation, expressions of VEGF, BMP-2, OPN, BSP, and mineralization.

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Conflict of interest None.

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