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Original Article

Acemannan-containing bioactive resin modified glass ionomer demonstrates satisfactory physical and biological properties

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Abstract *Background/purpose:* Resin-modified glass ionomers (RMGIs) have been recommended as liner and cement to provide the teeth with mechanical support, a chemical barrier, and thermal insulation. Acemannan, the main polysaccharide extracted from Aloe vera, is a promising inductive material *in vitro* and *in vivo*. This study aimed to develop acemannan-containing bioactive resin-modified glass ionomers (RMGIs).
Materials and methods: Acemannan (3%, 5%, and 10%) was added to the three types of RMGIs (RU-HBM1/Fuji II LC/Vitrebond) to generate 3%, 5%, and 10% aceRMGIs (aceRU/aceFuji/aceVB). The materials were evaluated for depth of cure/flexural strength/cumulative fluoride ion release. Cell viability and vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2) secretion were determined using MTT/apoptosis/necrosis assays, and ELISA kits, respectively. RMGI without acemannan were used as controls.
Results: The aceRMGIs met the ISO requirements for depth of cure and flexural strength. Adding 10% acemannan increased the cumulative fluoride release in the RU and FJ groups, but slightly decreased it in the VB group ($P < 0.05$). The MTT assay revealed 10% aceRU and all aceFJ groups significantly increased cell viability compared with each control group ($P < 0.05$). Apoptosis/necrosis assay showed the biocompatibility of all aceRMGIs. Adding

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acemannan to RMGIs significantly induced VEGF expression in a dose dependent manner while 5% and 10% aceRU significantly induced BMP-2 expression compared with RU group ($P < 0.05$). *Conclusion:* We conclude that 5—10% acemannan in RMGI is the optimal concentration based on its physical properties and ability to induce pulp cell proliferation and growth factor secretion.

Introduction

Dental caries is a major oral health problem, especially in developing countries. Deep caries lesions are conventionally treated by completely removing the infected soft- and demineralized-dentin and restoring the lost tooth structure with a dental material. Resin-modified glass ionomer (RMGI) is used as a cavity liner to protect the underlying pulp from the chemical irritants, postoperative sensitivity, and thermal irritation of the overlying filling materials.[1] Fluoride release, good chemical tooth adhesion, biocompatibility, antimicrobial effect, and a controllable setting time make RMGI the lining material of choice. RMGI biocompatibility to pulpal tissue in long-term observation has been demonstrated.[2,3] To enhance biological properties without significantly affecting their physical properties of RMGI many modifications have been developed.[4,5]

Because the effect of fluoride ions on dentin regeneration remains unresolved, biomolecules, *i.e.*, growth factors, extracellular matrix, and hydroxyapatite have been proposed to promote odontoblast and pulp cell activity for dentin regeneration.[6—8] Although exogenous growth factors are promising agents for promoting dentin regeneration, these agents are obtained as recombinant proteins from transfected bacteria or immortal cells that have a risk of disease transmission.[9] Furthermore, they have a short half-life, are difficult to achieve a controlled release, leave a space between the tooth surface and dental material, and are high cost. Many investigators have been searching for alternative biomaterials to overcome these disadvantages.

Currently, various natural biomaterials *e.g.*, chitosan, silk fibroin, hyaluronic acid, and acemannan, have been proposed as pulp regenerative agents.[6,7,10,11] Acemannan is a polysaccharide extracted from Aloe vera. *In vitro*, acemannan stimulates dentin regeneration via increased pulp cell proliferation, differentiation, growth factor expression, extracellular matrix synthesis, and mineral deposition.[12] As an *in vivo* capping material, acemannan enhanced dentin regeneration in experimental rodents and canines, and clinical studies.[12—14] Therefore, we hypothesized that incorporating acemannan, a natural biomaterial, into RMGI would enhance the material's regenerative activity and retain its physical properties as a lining material/meet the related ISO requirements.

In the present study, acemannan was added to three types of resin-modified glass ionomer (RU-HBM1, Fuji II LC, and Vitrebond) to generate acemannan-containing RMGIs (aceRU, aceFJ, and aceVB). The depth of cure, flexural strength, fluoride ion release, cytotoxicity, apoptosis/necrosis, and growth factor secretion effect of the acemannan-containing RMGIs were investigated.

Materials and methods

Acemannan extraction and characterization

Acemannan was isolated and characterized as previously described.[12] Briefly, fresh mature leaves of Aloe vera were washed with tap water and their skins were removed. The Aloe vera gels were washed in running tap water for 45 min and soaked in distilled water for 2 × 30 min to remove the yellow exudates. The parenchyma gels were homogenized and centrifuged at 10,000 rpm for 60 min at 4 °C. The supernatant was collected and precipitated with absolute alcohol. After lyophilization, the pellets were ground and kept in a desiccator. The polysaccharide was characterized by ^{1}H and ^{13}C NMR, and the analysis results were consistent with the prior studies,[12—14] confirming that the extracted polysaccharide was acemannan.

aceRU, aceFJ, and aceVB preparation

Light-cured resin-modified glass ionomer RU-HBM1(RU; Research Unit of Herbal Medicine, Biomaterial and Material for Dental Treatment, Chulalongkorn University, Bangkok, Thailand), GC Fuji II LC (FJ; GC Corporation, Tokyo, Japan), and Vitrebond (VB; 3M™ ESPE™, St. Paul, MN, USA) were used in this study. The materials' expiration dates were more than 6 months after the study was completed. Acemannan powder was added to the powder part of the RMGI at 3, 5, and 10 wt% and mixed using a rotator at 30 rpm overnight. The powder and liquid were used and mixed following the manufacturer's instructions (Table 1). A light activator (Halogen Curing Light, EliparTM 2500, 3 M ESPE, Minneapolis, MN, USA) was used to light-cure the materials at an intensity of 700 mW/cm^2. The light was calibrated before curing each new group.

Depth of cure

RU, aceRU, FJ, aceFJ, VB, and aceVB (n = 5) were loaded into a custom circular stainless-steel mold (6 × 4 mm^2; H × diameter) and placed on a glass slide. The materials were covered with a polyester film and a second glass slide was placed on top of the mold under gentle pressure to displace the excess material. The sample was light activated at the top for 20 or 30 s per the manufacturer's instructions. The specimen was removed from the mold and the uncured material was immediately removed with a plastic spatula. The height of the cured sample was determined with a digital caliper (Mitsutoyo Co., Kawasaki, Japan). According to ISO 9917—2:1998, the minimal depth of cure of an RMGI used as a liner or as a base is 1.0 mm.

Table 1 Powder and liquid components of experimental resin modified glass ionomer cements (RMGIs). HEMA, 2-Hydroxyethyl methacrylate; TEGDMA, Triethylene glycol dimethacrylate; ace, Acemannan.

Materials	Composition		Liquid	Recommended light cure duration (sec)	Powder/ Liquid ratio (g/g)	Manufacturer
	Powder					
	Glass	Acemannan (%wt)				
RU-HBM1 (RU)	Fluoroaluminosilicate glass	0	Polyacrylic acid (29%); HEMA (33%); TEGDMA (7%); Photoinitiator	20	1.6/1	Research unit of Herbal Medicine, Biomaterials and Material for Dental Treatment, Chulalongkorn University, Bangkok, Thailand
3% aceRU		3			1.65/1	
5% aceRU		5			1.68/1	
10% aceRU		10			1.76/1	
Fuji II LC (F; Lot# 000186)	Fluoroaluminosilicate glass	0	Polyacrylic acid; HEMA; TEGDMA; Photoinitiator	20	3.2/1	GC corporation, Tokyo, Japan
3% aceFJ		3			3.3/1	
5% aceFJ		5			3.36/1	
10% aceFJ		10			3,52/1	
Vitrebond (VB; Lot# N896231)	Fluoroaluminosilicate glass	0	Polyacrylic acid; HEMA; TEGDMA; Photoinitiator	30	1.4/1	3M™ ESPE™, St. Paul, MN, USA
3% aceVB		3			1.44/1	
5% aceVB		5			1.47/1	
10% aceVB		10			1.54/1	

Flexural strength

The flexural strength test was performed as previously described with some modifications.[15] Each material ($n = 5$) was placed into a customized stainless-steel mold ($24 \times 2 \times 2$ mm^3; L × W × H). A polyester film and a glass slide were placed on both sides of the mold. The material was cured using three overlapping activating-light exposures of 20 or 30 s each per the manufacturer's instructions. The specimen was light cured on the opposite side of the mold in the same fashion. The slides were clamped to the mold and the specimen was put in a water bath at 37 °C for 15 min. After removing the specimen from the mold, any flash was removed using sandpaper, and the specimen was immersed in distilled water at 37 °C for 24 h. The flexural strength was determined using a three-point bending testing device (Universal Testing Machine 8872, Instron, High Wycom, UK) with a crosshead speed of 50 N/min, a span of 20 mm, and a 1000 N load cell. The specimens were loaded until a fracture occurred. The flexural strength was calculated using the following formula: $\delta = 3FI/2bh$,[2] where δ = flexural strength (MPa), F = maximum load (N), I = span length between the supports (mm), b = specimen width (mm), and h = specimen height (mm). According to ISO 9917–2:2010, the minimal flexural strength of an RMGI used as a liner or as a base is 10 MPa.

Fluoride release measurement

The fluoride release was evaluated as previously described with some modifications.[16] Each material type ($N = 5$) was loaded into a customized square stainless-steel mold ($4 \times 4 \times 2$ mm^3) and placed on a glass slide. The materials were covered with a polyester film and a second glass slide was placed on top of the mold under gentle pressure to displace excess material. The sample was light activated for 20 or 30 s per the manufacturer's instructions at the top and bottom. The samples were then hung in 5 ml deionized water at 37 °C. The conditioned solution was collected on days 1, 3, 7, 14, and 28 and evaluated for fluoride ion content using an Orion fluoride ion selective electrode (Thermo Fisher Scientific, Waltham, MA, USA) and new water was added.

Cell culture

The study protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (No. HREC-DCU 2019-073). Human dental pulp cells were isolated from teeth obtained from the Department of Oral Surgery, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. The teeth used for the study were freshly extracted caries-free and periodontitis-free permanent third molars of healthy patients. The teeth were rinsed with 1% chlorhexidine solution and opened with a chisel. The pulp tissue was aseptically removed and rinsed several times with sterile phosphate-buffered saline (PBS). The pulp tissue was minced into small $1 \times 1 \times 1$ mm^3 pieces and placed in 35-mm culture dishes (Nunc, Thermo Scientific Fisher, Roskilde, Denmark). The cells were cultured in growth media (DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, 25 μg/ml amphotericin B, and 2 mM L-glutamine; Invitrogen Thermo Fisher Scientific, Carlsbad, CA, USA). The cultures

were maintained at 37 °C in 95% humidified air and 5% CO_2. When the cells reached confluence, they were subcultured and transferred to 60-mm culture plates. These cells were considered the first passage cells. Cells from the third and fifth passages were used in the study.

Conditioned media preparation

The materials (RU, aceRU, FJ, aceFJ, VB, and aceVB) were prepared in an autoclaved stainless-steel mold (2×22 mm^3; n = 4). After UV exposure for 30 min on each side, each specimen was immersed in 1 ml 10% serum DMEM for the MTT assay, or 2% serum DMEM for the apoptosis/necrosis assay and ELISA at 37 °C with gentle agitation for 24 h. For the control group, the same volume of 10% or 2% serum culture media was incubated under the same conditions. The conditioned media were passed through 0.2 μm sterile filters (Acrodisc® Syringe Filters with Supor® Membrane, Pall Corporation, Ann Arbor, MI, USA) before use.

MTT cytotoxicity assay

The MTT test was performed as previously described with some modifications.[17] Briefly, 40,000 cells/well were seeded into a 48-well culture plate. After 24 h, the cells were washed twice with PBS and incubated with the conditioned medium of each sample for 24 h. Subsequently, the cells were washed twice with PBS and incubated with 1 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma Aldrich, St. Louis, MO, USA) solution for 30 min. The precipitated formazan crystals were dissolved in dimethyl sulfoxide and the optical density was determined by measuring the light absorbance at 570 nm. The assay was performed in three independent experiments.

Apoptosis/necrosis assay

The 3×10^4 cells/ml dental pulp cells were seeded in 8-well chamber slides (SPL Life Science, Gyeonggi-do, Korea) for 24 h. After being washed with PBS, the cells were incubated with the conditioned medium of each material for 24 h. After incubation, the cell viability was evaluated using an apoptosis/necrosis assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. The images were viewed and recorded using a fluorescence microscope (BX 40, Olympus, Tokyo, Japan).

ELISA assay

Pulp cells (30,000 cells/well) were seeded in a 48-well tissue culture plate at 5% CO2 and 37 °C for 24 h. The cells were washed with PBS and treated with the respective group's conditioned medium for an additional 24 h. The conditioned media obtained from the RU-, aceRU-, FJ-, aceFJ-, VB-, and aceVB-treated groups were collected at 24 and 72 h for VEGF and BMP2 protein level measurement (ELISA kits; R&D System, Minneapolis, MN, USA), respectively, per the manufacturer's instructions. The control groups were treated with the same volume of 2% FBS supplemented DMEM media. The sensitivity of the VEGF and BMP-2 kit was 8.4 and 11 pg/ml, respectively. The assay was performed in three independent experiments.

Statistical analysis

The data were collected and presented as mean ± SD for depth of cure, flexural strength, fluoride ion release, and as mean ± SE for the number of viable cells, VEGF, and BMP2 concentration. The data were analyzed using one-way ANOVA (SPSS program for Windows, version 22; SPSS, Inc., Chicago, IL, USA). Scheffe's multiple comparison test was used for post-hoc analysis. Significance was assumed at $P < 0.05$.

Results

Depth of cure and flexural strength

The depth of cure and flexural strength of the RU, aceRU, FJ, aceFJ, VB, and aceVB are presented in Table 2. FJ had the highest curing depth and flexural strength, while VB had the lowest. Incorporating acemannan in the RMGIs reduced the depth of cure and flexural strength in a concentration-dependent manner. Adding acemannan up to 10% by weight significantly decreased the depth of cure in the RU, FJ, and VB groups compared with their control by 17.45%, 9.67%, and 11.64%, respectively ($P < 0.05$), which had the lowest depth of cure. According to ISO 9917—2:1998, the minimum depth of cure of a light-activated dental RMGI is 1 mm. Therefore, the aceRU, aceFJ, and aceVB groups all met the required depth of cure.

The flexural strength data indicated that there was no significant difference between RU and aceRUs ($P > 0.05$). Incorporating acemannan at 5% and 10% in the FJ group and 10% in the VB group significantly reduced their flexural strength compared with that of the control group ($P < 0.05$). According to ISO 9917—2:2017, an RMGI used as a liner or base should have a flexural strength equal or

Table 2 Depth of cure (mm.) and flexural strength (MPa) of RU, 3%-, 5%-, and 10%- aceRU, FJ, 3%-, 5%-, and 10%-aceFJ, and VB, 3%-, 5%-, and 10%-aceVB groups. The data are expressed as mean ± SD (n = 5). * Indicates significant difference compared with its control ($P < 0.05$). RU, RU-HBM1; FJ, Fuji II LC; VB, Vitrebond; ace, Acemannan.

Material	Depth of cure (mm.)	Flexural strength (MPa)
RU	3.61 ± 0.05	23.93 ± 3.96
3% aceRU	3.62 ± 0.05	24.91 ± 3.29
5% aceRU	3.63 ± 0.03	24.57 ± 3.07
10% aceRU	2.98 ± 0.22*	23.27 ± 2.58
FJ	5.48 ± 0.22	41.52 ± 1.97
3% aceFJ	5.66 ± 0.17	39.01 ± 0.84
5% aceFJ	5.38 ± 0.22	35.3 ± 1.7*
10% aceFJ	4.95 ± 0.08*	32.06 ± 1.22*
VB	1.46 ± 0.03	22.07 ± 2.95
3% aceVB	1.43 ± 0.07	19.91 ± 3.01
5% aceVB	1.39 ± 0.06	18.78 ± 1.47
10% aceVB	1.29 ± 0.03*	16.24 ± 1.36*

Table 3 Accumulative fluoride ion release of aceRU groups, aceFJ groups, and aceVB groups. The materials were hung in deionized water and replaced with fresh water on days 1, 3, 7, 14, and 28. The condition solutions were collected and analyzed (n = 5). The data were represented as mean ± SD.* Significant difference from its control group ($P < 0.05$). RU, RU-HBM1; FJ, Fuji II LC; VB, Vitrebond; ace, Acemannan.

	Accumulative Fluoride ion release (ppm)				
	Day 1	Day 3	Day 7	Day 14	Day 28
RU	16.8 ± 3.1	29.2 ± 1.25	63.8 ± 7.82	81.89 ± 5.17	94.43 ± 4.39
3% aceRU	11.6 ± 1.44	32.8 ± 4.27	55.83 ± 6.49	73.10 ± 7.84	83.24 ± 6.75
5% aceRU	16.5 ± 7.33	32.96 ± 9.74	65.63 ± 11.56	81.96 ± 8.03	89.94 ± 8.65
10% aceRU	18.03 ± 4.45	38.06 ± 9.32	48.6 ± 12.4	56.15 ± 12.12*	62.13 ± 12.27*
FJ	2.68 ± 0.03	6.87 ± 2.32	12.18 ± 0.08	19.43 ± 0.13	29.25 ± 0.08
3% aceFJ	2.69 ± 0.05	6.94 ± 0.15	12.76 ± 2.03	18.48 ± 0.08*	27.46 ± 0.04*
5% aceFJ	3.87 ± 0.05*	9.87 ± 0.07*	17.28 ± 0.02*	27.11 ± 0.1*	39.06 ± 0.05*
10% aceFJ	4.97 ± 0.04*	12.51 ± 0.05*	21.94 ± 0.15*	32.94 ± 0.22*	46.78 ± 0.17*
VB	5.88 ± 0.01	15.57 ± 0.01	27.12 ± 0.04	39.82 ± 0.16	54.02 ± 0.16
3% aceVB	4.26 ± 0.04*	10.97 ± 0.02*	18.73 ± 0.05*	27.14 ± 0.03*	36.41 ± 0.16*
5% aceVB	5.31 ± 0.01*	13.16 ± 0.03*	21.69 ± 0.03*	30.65 ± 0.06*	40.37 ± 0.04*
10% aceVB	5.79 ± 0.09	14.47 ± 0.08*	24.87 ± 0.08*	36.17 ± 0.08*	49.02 ± 0.13*

greater than 10 MPa. From our data, the aceRU, aceFJ, and aceVB groups exceeded the flexural strength criterion.

Fluoride ion release of RMGIs and aceRMGIs

The cumulative fluoride ion release and overall measurement times of the RMGIs are presented in Table 3. Each material group demonstrated a continuous release of free fluoride ions for up to 28 d. The free fluoride ion release of each RMGI and aceRMGI had a unique pattern (Table 3). RU had the highest cumulative fluoride release, while FJ had the lowest cumulative fluoride release at all observation time points. In the RU group, 10% aceRU had the highest fluoride ion release on the first day of incubation ($P > 0.05$), and then significantly decreased at day 14 and day 28 ($P < 0.05$). The RU, 3%aceRU, and 5%aceRU free fluoride ion release were not significantly different ($P > 0.05$). In the FJ group, adding acemannan resulted in increased free fluoride ion release in a concentration-dependent manner. The 10% aceFJ had the highest cumulative free fluoride ion release ~1.3–1.6-fold compared with the control group at all observed time points ($P < 0.05$). The cumulative fluoride ion release in the FJ groups in descending order was 10% aceFJ, 5% aceFJ, 3%aceFJ, and FJ. In the VB group, adding acemannan resulted in a slightly decreased free fluoride ion release ($P > 0.05$).

Cytotoxicity and growth factors secretion on pulp fibroblasts

No cytotoxicity was observed in any aceRMGI group compared with its control. The aceRU and aceFJ groups demonstrated a significantly increased cell number in a concentration-dependent manner (Fig. 1). The percentage mean number of viable cells for 10% aceRU was 110.35% and significantly increased compared with the RU group ($P < 0.05$; Fig. 1a). The 3%-, 5%-, and 10%-FJ groups had a 1.18-, 1.25-, and 1.32-fold, respectively, greater cell viability than the FJ group ($P < 0.05$; Fig. 1b). However,

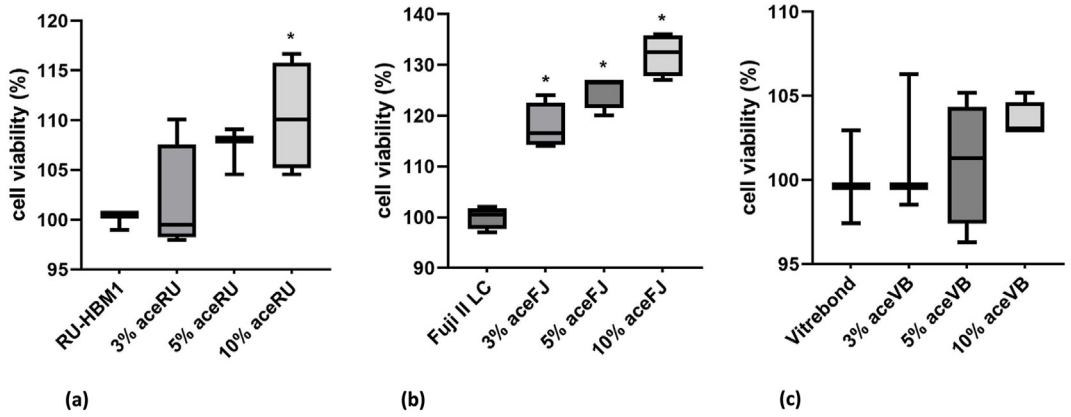

Figure 1 Dental pulp cells are incubated with RMGIs and aceRMGIs conditioned media for 24 h. MTT assay was performed to determine the cell viability. Percentages cell viability of (a) aceRU-, (b) aceFJ-, and (c) aceVB-treated groups compared with control, respectively, were shown. *Significantly different from the control of each group ($P < 0.05$).

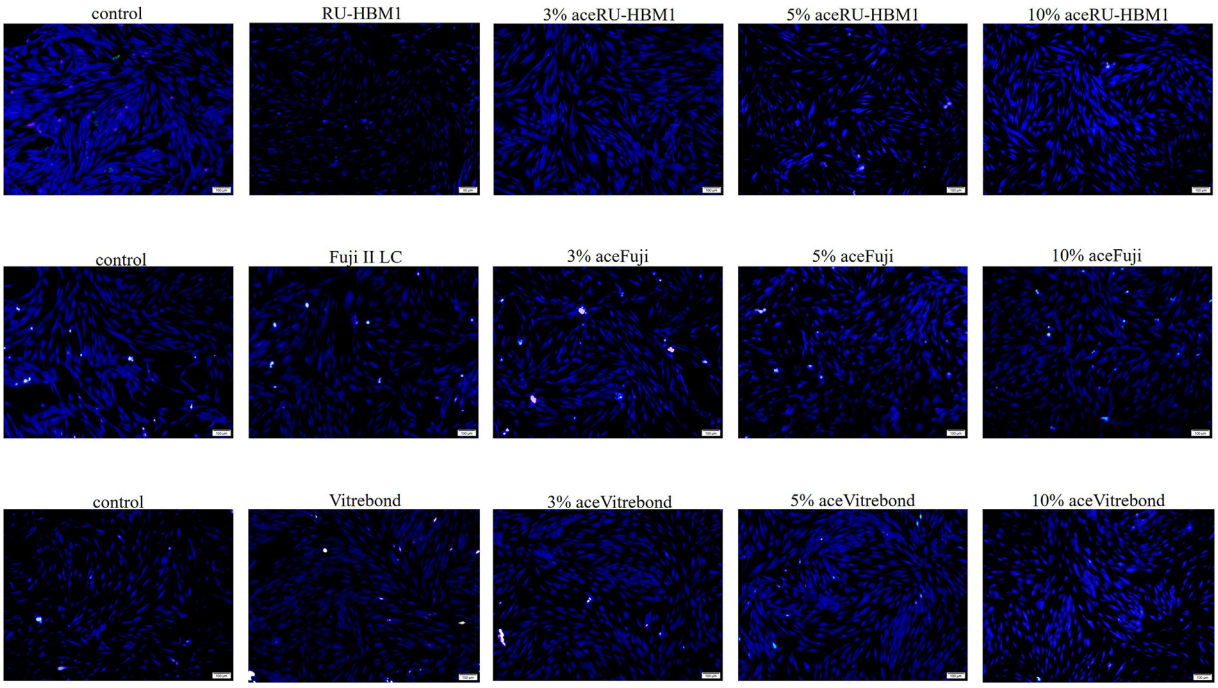

Figure 2 The representative images of live, apoptotic, and necrotic pulpal cells treated with RMGIs and ace RMGIs conditioned media for 24 h. Fluorescent staining demonstrated cells that are live (blue; CytoCalcein Violet 450 staining), apoptotic (green; Apopxin Green staining), and necrotic (red; 7-Aminoactinomaycin D staining), respectively.

there was no significant difference in cell viability between the VB and aceVB groups (Fig. 1c).

The apoptosis/necrosis assay results demonstrated that most of the cells appeared healthy post-treatment with the conditioned media obtained from the RMGIs and aceRMGIs. A few apoptotic and necrotic cells were observed. No significant difference between the RGMI and aceRMGI-treated groups was detected (Fig. 2).

The ELISA data indicated that the 3%-, 5%-, and 10% aceRMGIs groups induced significantly increased VEGF secretion compared with its respective RMGI and untreated control groups in a concentration-dependent manner ($P < 0.05$; Fig. 3a, b, and 3c). The maximum VEGF induction was observed in the 10% aceRU, 10% aceFJ, and 10% aceVB groups was 10.26-, 14.35-, and 4.55-fold, respectively, higher than their RMGI groups. Only 5% and 10% aceRU significantly induced BMP-2 synthesis by 1.77- and 2.02-fold, respectively, compared with the RU group ($P < 0.05$; Fig. 4a). There was no significant difference between the FJ and aceFJ, or between the VB and aceVB groups (Fig. 4b and c).

Discussion

The ultimate goal of operative dentistry is to regenerate the trauma- and caries-caused tooth structure destruction via inducing endogenous pulp tissue regeneration rather than restoring the tooth with an inert synthetic material. Several studies have reported the benefit of adding bioactive substances into dental restorative materials to stimulate dentin-pulp repair/regeneration.[18–20] Light-activated

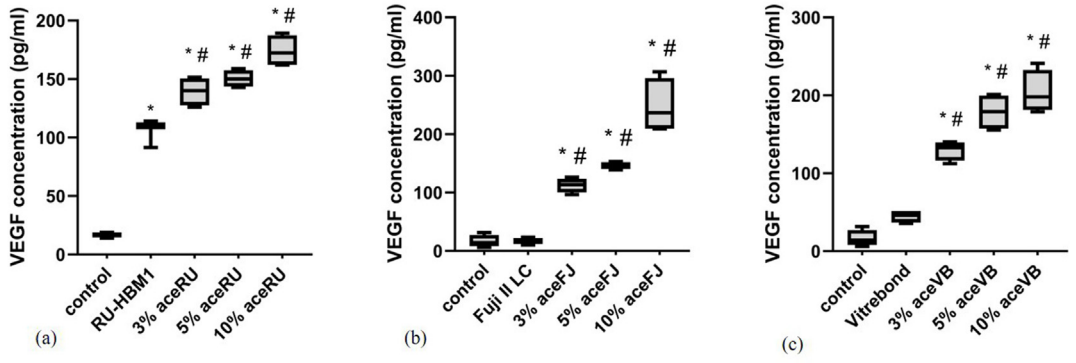

Figure 3 Dental pulp cells are treated with aceRMGIs conditioned media for 24 h. VEGF expression of (a) aceRU (b) aceFJ (c) aceVB. *, # Significantly different from the untreated control and its RMGI of each group, respectively ($P < 0.05$).

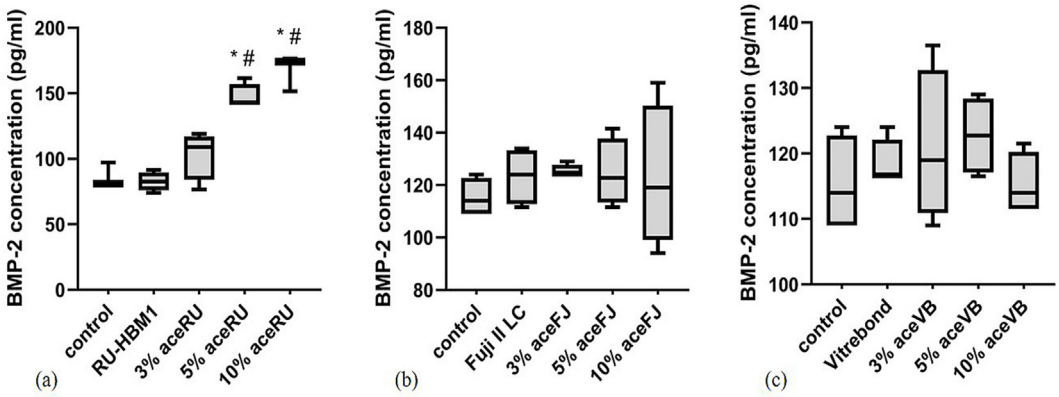

Figure 4 Dental pulp cells are treated with aceRMGIs conditioned media for 72 h. BMP2 expression of (a) aceRU (b) aceFJ (c) aceVB. *, # Significantly different from the untreated control and its RMGI of each group, respectively ($P < 0.05$).

dental resin-modified glass ionomers are frequently recommended as cavity liners due to their safety, adjustable setting time, simplicity of handling, and free fluoride release and reservoir.[2,21] The current study developed new bioactive RMGI prototypes by adding 3%, 5%, and 10% acemannan (%w/w in the powder part) to three types of RMGIs ((RU-HBM1; RU), (Fuji II LC; FJ), and (Vitrebond; VB)). Our results indicated that the aceRMGIs and RMGIs met the ISO physical requirements for an RMGI liner for depth of cure and flexural strength. These findings corresponded to previous studies that demonstrated that RU, FJ, and VB met the requirements for depth of cure and flexural strength per ISO 9917–2:2017 and ISO 9917–2:1998.[22–24]

The depth of cure is the thickness of a material that completely polymerizes from the top to the bottom after adequate light activation. Adding a low concentration of acemannan into the three RMGIs did not alter the depth of cure while adding up to 10% acemannan significantly reduced the curing depth. Increased acemannan concentration may interfere with the light direction and absorb the photon energy, thereby reducing the light source's depth of penetration and decreasing polymerization.[25–27] However, the mean depth of cure of the aceRMGIs surpassed the minimal depth of cure of light-activated RMGI ISO standard.

For brittle materials, which are considerably weaker in tension than in compression, flexural strength is a crucial physical characteristic. Dental materials are subjected to a variety of multidirectional masticatory forces in clinical situations. Our results illustrated that adding acemannan up to 10% did not alter the RU flexural strength, which suggests the amount of acemannan does not interfere with the setting reaction of this material. The possible explanations are that the polymerization of the aceRUs can be achieved at a 2 mm depth of the specimen, and acemannan molecules can locate in the three-dimensional structure of the material without interfering with the polymerization reactions.[28] Incorporated 10% natural polymer chitosan unaffected its adhesive bond strength of RMGI.[29] Our results illustrated that adding 10% acemannan to Fuji II LC and Vitrebond significantly reduced its flexural strength compared with their control groups. Decreased mechanical compressive strength of RMGIs after adding bioactive glass had been reported.[30] However, based on our data, the aceRMGIs met the ISO flexural strength requirements of a light-activated RMGI.

Fluoride release is considered one of the advantages of an RMGI. Free fluoride ions can either coprecipitate with Ca^{2+} ions in saliva to form calcium fluoride and deposit on the tooth surface or migrate into the tooth substructure to form fluorapatite.[31] The fluoride ion release pattern from RMGI can be divided into two phases: an initial rapid releasing phase and a long-term diffusion phase.[32–34] The maximum fluoride release of the materials was detected on the first day and then gradually decreased time-dependently.[35] In the present study, we found that each RMGI and aceRMGI had a unique pattern of fluoride release in which adding 10% acemannan in RU and FJ increased fluoride release, while the release of free fluoride ions in the VB group was slightly reduced due to adding acemannan. The possible explanations for these findings are that the highest fluoride release from the 10% aceRU and 10% aceFJ at the first 24 h could be the initial rapid releasing effect due to the water solubility of acemannan. Higher concentrations of acemannan increase the amount of the polysaccharide molecules located on the material's surface. Acemannan would enhance the surface hydrophobicity of a material for water contaction and fluoride ions release compared with the control RMGI.[36]

The pulp is the soft tissue exposed to the unpolymerized residual monomer from a lining substance through the dentinal tubules. In the present study, the biocompatibility of the aceRMGIs on human pulp fibroblasts was investigated using MTT and apoptosis/necrosis assays. Our data indicated that the RMGIs and aceRMGIs were not cytotoxic to the pulp cells after a 24 h exposure. Few necrotic and apoptotic cells were detected after exposure to the conditioned media obtained from the RMGIs and aceRMGIs. The 10% aceRU and the aceFJ groups significantly induced pulp cell proliferation compared with their respective control group. The 5% and 10% aceVB also slightly increased cell numbers compared with the VB group. These findings suggest that aceRMGIs are biocompatible with pulp cells and have a proliferative effect *in vitro*. This data supports that acemannan induces pulp cell proliferation.[12,37] It also corresponds with findings that adding bioactive compounds to glass ionomer cement boosts cell viability.[38]

From our results, the cell viability treated with RU-HBM1 data were inconsistent compared with that of the live cell fluorescent staining. Due to the limitations of the study, an exact explanation of RU-HBM1's differential effect on cell viability and live cell fluorescent staining cannot be made. However, this disparity might be due to a difference in the underlying mechanism of these techniques. The MTT assay is used to evaluate the *in vitro* cytotoxicity and cell proliferation based on mitochondrial dehydrogenase activity changing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to produce formazan crystals.[39] After dissolving with DMSO, the optical density is determined and compared with a standard curve for estimating the number of living cell. However, this technique has some limitations. Numerous chemical compounds can interfere with the MTT assay, e.g., ascorbic acid, vitamin A, cholesterol, 2-deoxyglucose, 3-bromopyruvate, lonidamine, TiO_2, polyphenol.[40–47] The apoptosis/necrosis assay is a powerful technique that can identify apoptotic, necrotic, and healthy cells. Based on the phosphatidylserine sensor, Apopxin Green, 7-Aminoactinomaycin D Red, and CytoCalcein Violet are used to detect cell apoptosis, necrosis, and living cell, respectively.[48,49] With this assay's sensitivity and specificity, the biological effect of the investigated substances on cell viability, apoptosis, and necrosis can be simultaneously analyzed from the same cell population.

Growth factors play a role in controlling revascularization and proliferation, as well as odontoblast differentiation, in pulp tissue regeneration. For pulp-dentin regeneration, VEGF and BMP-2 are important and act synergistically.[50] VEGF promotes revascularization and endothelial cell proliferation in the initial stages of healing, whereas BMP-2 regulates odontogenic differentiation and drives the mineralization of the newly created matrix.[51,52] In the present study, we investigated the dentin regenerative effect of aceRMGIs via stimulating VEGF and BMP-2 secretion. Our result illustrated that adding acemannan to RMGIs significantly increased VEGF expression in a dose-dependent manner. Our data revealed that 5% and 10% aceRU significantly induced BMP-2 expression compared with the RU group. These data also coincided with findings where acemannan induced dentin regeneration *in vitro* and *in vivo*.[12–14] The proprietary restrictions about the composition and type of these material formulations do not allow us to precisely explain this phenomenon.

Our data revealed that acemannan improved biocompatibility, cell proliferation, and growth factor secretion while maintaining the physical properties of RMGIs as a lining material that met ISO requirements. RMGI seems to be a polymer-based vehicle with practical physical quality to carry biomolecule acemannan. The results of the present study suggest that 5–10% acemannan in RMGI is the optimal concentration based on its physical properties, cytotoxicity, and pulp regenerative activity. However, i*n vitro* circumstances may not perfectly replicate the *in vivo* environment because the complex orchestration of the effects of the host immune system, circulating blood system, and inflammation is absent in the *in vitro* environment.[53] In conclusion, adding acemannan to RMGIs compromises some mechanical properties and enhances some biological activities of the materials. This suggests the potential of RMGI containing acemannan as a lining material for dentin-pulp regeneration.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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