Evaluation of the anti-inflammatory effects of surface-reaction-type pre-reacted glass-ionomer filler containing root canal sealer in lipopolysaccharide-stimulated RAW264.7 macrophages

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A prototype surface-reaction-type pre-reacted glass-ionomer (S-PRG) filler containing root canal sealer (S-PRG sealer) exhibits bioactive potential by releasing multiple ions. This study explored the suppressive effects and modes of action of S-PRG sealer extracts on proinflammatory cytokine expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Expression of proinflammatory cytokines was evaluated by RT-qPCR and ELISA. Expression of phosphorylated nuclear factor-kappa B (p-NF-kB) p65 was evaluated by western blotting. S-PRG sealer extracts significantly downregulated mRNA expression levels of interleukin (IL)-1 α , IL-6, and TNF- α in LPS-stimulated RAW264.7 cells; the extracts also reduced the levels of IL-6 protein and p-NF-kB. In order to verify that Zn²⁺ was responsible for downregulation of proinflammatory cytokine expression, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) was used as a heavy metal chelator with strong affinity for Zn²⁺. These effects were mitigated by TPEN. The application of ZnCl₂ reproduced the actions of S-PRG sealer extracts. These data suggest that S-PRG sealer has anti-inflammatory potential involving heavy metal ions such as Zn²⁺.

Keywords: S-PRG filler, Endodontic sealer, Inflammatory cytokine, NF-kB pathway, Zinc ion

INTRODUCTION

The ultimate objectives of root canal treatment are the complete eradication of bacterial infection from the root canal system and the prevention of bacterial invasion into the periradicular tissues¹. Thus, following thorough cleaning and shaping, the root canal space is hermetically sealed, most often with endodontic sealers and core materials. Thus far, several classes of endodontic sealers have been developed, including sealers based on zinc oxide eugenol, calcium hydroxide, glass ionomer, epoxy resin, methacrylate resin, and calcium silicate^{1,2}. These materials must meet several physical requirements including high sealing ability and low solubility to establish a long-lasting seal³⁻⁵. Moreover, endodontic sealers are usually applied in contact with diseased periradicular tissues; therefore, they should be biocompatible and offer beneficial biological actions, such as anti-inflammatory⁶ and pro-regenerative effects⁷. However, no available materials are considered ideal¹, and thus there is a need for the development of new materials with improved physical and/or biological properties.

Surface reaction-type pre-reacted glass ionomer (S-PRG) fillers comprise a class of bioactive filler materials that are produced by an acid-base reaction of porous silica glass-coated fluoroboroaluminosilicate glass fillers with a polycarboxylic acid solution⁸. S-PRG fillers release several ions (e.g., boron, aluminum, silicon, sodium, strontium, and fluoride) that confer these fillers with properties including anti-demineralizing⁹⁻¹¹, remineralizing¹², and anti-microbial abilities^{13,14}. Thus,

S-PRG fillers have been incorporated into various dental materials such as resin composites¹³, coating resins⁹, and dental sealants¹⁰.

A prototype S-PRG filler containing root canal sealer (S-PRG sealer) has been developed to incorporate the bioactive properties of S-PRG fillers into an endodontic sealer¹⁵; it also releases multiple ions (boron, aluminum, silicon, sodium, zinc, strontium, and fluoride)¹⁶. The S-PRG sealer is presumed to favor osteoblastic differentiation because extracts from the S-PRG sealer induce osteoblastic differentiation and mineralization of Kusa-A1, an osteoblastic cell line, through the calcium-sensing receptor-induced activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase¹⁶. Anti-inflammatory properties of the S-PRG sealer have also been described in a recent study: subcutaneous implantation of set S-PRG sealer led to reduced macrophage infiltration, compared with a silica filler-containing (non-SPRG filler-containing) sealer¹⁷. However, these results could be largely attributed to the difference in biocompatibility between the two materials, and thus anti-inflammatory effects of the S-PRG sealer have not yet been fully evaluated.

This study was performed to obtain insights regarding the anti-inflammatory functions of the S-PRG sealer. We used macrophages as the target cell type because they are the predominant immunocompetent cells throughout the development of periapical lesions^{18,19}. Moreover, they produce many proinflammatory and bone-resorbing cytokines that are involved in the process of active lesion expansion^{20,21}. Thus, the specific aim of this study was to explore the suppressive effects and modes

of action of S-PRG sealer extracts on proinflammatory cytokine expression in lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages²²⁾. The null hypothesis was that S-PRG sealer extracts would have no effect on proinflammatory cytokine expression in LPS-stimulated RAW264.7 cells.

MATERIALS AND METHODS

Cell culture and chemicals

RAW264.7 mouse macrophages²²⁾ around passage 20 (Riken Bioresource Research Center, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) containing heat-inactivated (56°C for 30 min) 10% fetal bovine serum (FBS; HyClone/GE Healthcare, Chicago, IL, USA) and 1% antibiotics (penicillin-streptomycin-amphotericin B suspension; FUJIFILM Wako Pure Chemical) at 37°C in 5% CO₂ with 100% humidity. Cells were then subcultured carefully before they reached confluence. LPS (*Escherichia coli* O111: B4; Sigma-Aldrich, St. Louis, MO, USA; 100 ng/mL) was used to induce the expression of inflammatory cytokines and NF-κB signaling in RAW264.7 cells. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN; 50 μM, Dojindo, Kumamoto, Japan) is a chelator²³⁾ with strong affinities for Zn²⁺, Fe²⁺, and Mn²⁺ and low affinity for Ca²⁺. TPEN was used to eliminate heavy metal ions. Other chemicals used in this study were as follows; B(OH)₃ (300 μg/mL), SrCl₂ (15 μg/mL), NaF (45 μg/mL), and ZnCl₂ (20 μg/mL) (FUJIFILM Wako Pure Chemical). The concentrations of these solutions were determined based on the concentrations of corresponding ions released from S-PRG sealer extracts¹⁶⁾.

Preparation of sealer extracts

S-PRG sealer (Shofu, Kyoto, Japan) and Canals N (CN; a zinc oxide-non-eugenol sealer, Showa Yakuhin Kano, Tokyo, Japan) were used in this study. The compositions of the root canal sealers are described in Table 1. The sealers were mixed in accordance with the manufacturer's instructions under aseptic conditions and used to fill sterile polypropylene discs (8 mm diameter, 3 mm height). S-PRG sealer and CN were allowed to set for respective durations of 72 h and 24 h, both at 37°C and 100% humidity. Set samples were placed in DMEM (10 mL), then shaken at room temperature for 24 h and sterilized through a membrane filter (0.45 μm pore size, Sartorius, Göttingen, Germany).

Cell viability assay

RAW264.7 cells (3×10³ cells/well, 100 μL, 96-well plate) were cultured for 24, 48, and 72 h in the presence or absence (control) of S-PRG sealer extracts. Cell viability was measured using a WST-8 assay (CKK-8, Dojindo). Briefly, 10 μL of CKK-8 solution was added to each well of a 96-well plate, then incubated for 1 h; the optical density of each well at 450 nm (OD450) was measured using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland).

Reverse transcription-quantitative polymerase chain reaction

RAW264.7 cells (2×10⁵ cells/well, 12-well plate) were cultured in DMEM without FBS for 24 h, then cultured with samples for 3 h. Total RNAs were extracted at 3 h after application of the samples with a QuickGene-Mini80 kit (Kurabo Biomedical, Osaka, Japan). The isolated RNA (150 ng) was reverse transcribed to cDNA by reverse transcriptase (PrimeScript™ RT Reagent

Table 1 Root canal sealers used in this study

Product	Composition	Lot No.
S-PRG sealer	Powder: Zinc oxide-based inorganic compound filler, S-PRG filler, additive	140901P
	Liquid: Polycarboxylic acid sodium salt, water, other	121301L
Canals N	Powder: Zinc oxide, bismuth subcarbonate	9006UA
	Liquid: Fatty acids, propylene glycol	9007UA

Table 2 Primer sequences

Genes	Forward Primers (5'-3')	Reverse Primers (5'-3')	Accession No.	Size (bp)
β-actin	GTAAAGACCTCTATGCCAACACAGT	AATGATCTTGATCTTCATGGTGCTA	NM_007393	122
IL-1α	ATTTAACCAAGTGGTGCTGAGATAG	CACCTTACACCTACCAGAGTGATTT	NM_010554	137
IL-6	TCTGGCTTTGTCTTTCTTGTTATCT	TGGATGCTACCAAACTGGATATAAT	NM_031168	130
TNFα	ATCATCTTCTCAAAATTCGAGTGAC	CTAGTTGGTTGTCTTTGAGATCCAT	NM_013693	235
PPARα	GCCTGTCTGTCTGGGATGT	GGCTTCGTGGATTCTCTTG	NM_001113418	216
IL-10	GATCATCATGTATGCTTCTATGCAG	GATTTCTCCCCTGTGAAAATAAGAG	NM_010548	140

Kit, Takara Bio, Kusatsu, Japan). Real-time polymerase chain reaction (PCR) analysis was performed with cDNA, primers, and Taq polymerase (GoTaq qPCR Master Mix, Promega, Madison, WI, USA) using a real-time PCR detection system (CFX96; Bio-Rad, Hercules, CA, USA). Specific primers were used to detect mRNAs for interleukin (IL)-1 α , IL-6, tumor necrosis factor (TNF) α , peroxisome proliferator-activated receptor (PPAR) α , and IL-10 (Table 2). Beta-actin was used as an internal control. Relative gene expression values were calculated with $\Delta\Delta CT$ -based fold-change calculations.

Western blotting

RAW264.7 cells (1×10^5 cells/well, 24-well plate) were cultured in DMEM without FBS for 24 h, then cultured with samples for 10 min. Cells were then lysed in 100 μ L of a radioimmunoprecipitation assay buffer containing proteinase and phosphatase inhibitors (cOmplete and PhosSTOP; Sigma-Aldrich). Lysates were loaded into ready-to-use sodium dodecyl sulfate-polyacrylamide gels (e-Pagel; ATTO, Tokyo, Japan) and subjected to electrophoresis; separated proteins in the gels were transferred to polyvinylidene difluoride membranes (GE Healthcare) using a semi-dry transfer system (0.15 mA, 1 h; WSE-4040, ATTO). Membranes were then incubated at 4°C overnight with the following primary antibodies: rabbit anti-nuclear factor-kappa B (NF- κ B) p65 (#8242, 1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-NF- κ B p65 (#3033, 1:1000, Cell Signaling Technology), and a horseradish peroxidase-conjugated rabbit anti-alpha tubulin (PM054-7, 1:4000; MBL, Nagoya, Japan). After membranes had been washed with Tris-buffered saline containing Tween 20 (1% v/v), they were incubated at room temperature for 1 h with a horseradish peroxidase-labeled antirabbit IgG secondary antibody (#1706515, 1:5000, Bio-Rad). Horseradish peroxidase substrate (Immobilon, Merck Millipore, Burlington, MA, USA) was applied as a chemiluminescent reagent, and the developed membranes were imaged using the LAS-3000 Mini Imaging System (Fuji Film/GE Healthcare, Tokyo, Japan). The corresponding bands were measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and their ratios were calculated.

Enzyme-linked immunosorbent assay (ELISA) analysis

RAW264.7 cells (1×10^5 cells/well, 12-well plate) were cultured in DMEM without FBS for 24 h, then cultured with samples for 24 h. Synthesis of IL-6 in the medium was measured with an ELISA kit (IL-6 Duo Set, R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's protocol. 3,3',5,5'-tetramethylbenzidine (SureBlue TMB Microwell Peroxidase Substrate, KPL, Milford, MA, USA) was used as a chromogenic substrate, and 0.6N sulfuric acid was used as a stop solution. Color change was measured with a microplate reader (wavelength: 450 nm, Sunrise, Tecan) and the protein concentrations were calculated using a standard curve.

Statistical analysis

Results are shown as means $\pm$ standard deviations. One-way analysis of variance (ANOVA) followed by the Tukey–Kramer or Dunnett's test was used for multiple comparisons; the F-test followed by Student's *t*-test was used for comparisons of two groups. Statistical analyses were performed using Prism software version 6 (GraphPad, San Diego, CA, USA). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

First, we examined the effects of S-PRG sealer extract on RAW264.7 cell growth. In the presence of twofold diluted S-PRG sealer extract, cell growth was significantly depressed at 24, 48, and 72 h, compared with control (no

Fig. 1 Effects of S-PRG sealer extract on viability of RAW264.7 cells.

The viability of RAW264.7 cells was evaluated in the presence of various dilutions of S-PRG sealer extract at 24 h, 48 h, and 72 h. SPRG 1/2: twofold diluted S-PRG sealer extract, SPRG 1/4: fourfold diluted S-PRG sealer extract, $n=4$, * $p < 0.05$.

Fig. 2 Effects of S-PRG sealer extract on IL-1 α , IL-6, and TNF α mRNA expression levels in LPS-stimulated RAW264.7 cells.

mRNA expression levels in LPS-stimulated RAW264.7 cells were determined at 3 h after the addition of S-PRG sealer extract. SPRG: fourfold diluted S-PRG sealer extract, $n=3$, ** $p < 0.01$.

sealer extract) and fourfold diluted S-PRG sealer extract ($p < 0.05$, Fig. 1). However, cell growth in the presence of fourfold diluted S-PRG sealer extract was generally similar to growth in the control condition (Fig. 1).

Next, we investigated the effect of S-PRG sealer

extract on proinflammatory cytokine expression in LPS-stimulated RAW264.7. As shown in Fig. 2, LPS treatment upregulated the mRNA expression levels of IL-1 α , IL-6, and TNF α in RAW264.7 cells; these expression levels were significantly downregulated by the application of fourfold diluted S-PRG sealer extract ($p < 0.01$, Fig. 2). As shown in Fig. 3, extracts from CN failed to show a reducing effect on LPS-induced

Fig. 3 Effects of Canals N (CN) extracts on IL-1 α , IL-6, and TNF α mRNA expression levels in LPS-stimulated RAW264.7 cells.

mRNA expression levels were determined at 3 h after the addition of CN or S-PRG sealer extracts. CN: Canals N extract, SPRG: fourfold diluted S-PRG sealer extract, $n=3$, $*p < 0.05$.

Fig. 4 Effects of B(OH)₃, SrCl₂, ZnCl₂, NaF, and S-PRG sealer extract on IL-1 α , IL-6, and TNF α mRNA expression levels in LPS-stimulated RAW264.7 cells.

mRNA expression levels were determined at 3 h after the addition of B(OH)₃, SrCl₂, ZnCl₂, NaF, and S-PRG sealer extract. SPRG: fourfold diluted S-PRG sealer extract, $n=3$, $*p < 0.05$ versus LPS.

upregulation of IL-1 α , IL-6, and TNF α mRNA expression levels in RAW264.7 cells.

S-PRG sealer extract contains various ions released from the sealer¹⁶⁾. Therefore, we examined whether the cytokine mRNA downregulation by sealer extract in LPS-stimulated RAW264.7 cells could be reproduced by B(OH)₃, SrCl₂, ZnCl₂, and NaF solutions. The

concentrations of the corresponding ions in these solutions were adjusted to the levels present in the fourfold diluted S-PRG sealer extract. ZnCl₂ significantly downregulated the IL-1 α , IL-6, and TNF α mRNA expression in RAW264.7 cells, compared with the levels in LPS-stimulated RAW264.7 cells ($p < 0.05$, Fig. 4). In contrast, B(OH)₃, SrCl₂, and NaF solutions

Fig. 5 Effects of TPEN on IL-1 α , IL-6, and TNF α mRNA expression levels in LPS-stimulated RAW264.7 cells in the presence of S-PRG sealer extract and ZnCl₂. mRNA expression levels were determined at 3 h after the addition of S-PRG sealer extract (A) and ZnCl₂ (B), together with TPEN. SPRG: fourfold diluted S-PRG sealer extract, $n=3$, * $p < 0.05$.

Fig. 6 Effects of S-PRG sealer extract and TPEN on NF-kB signaling and IL-6 expression in LPS-stimulated RAW264.7 cells.

(A) Expression levels of p-NF-kB, NF-kB, and alpha tubulin (control) in LPS-stimulated RAW264.7 cells in the presence of S-PRG sealer extract and TPEN were evaluated by western blotting, and the intensities of bands were measured by ImageJ software. SPRG: fourfold diluted S-PRG sealer extract. All experiments were performed three times and representative results are shown. (B) Expression levels of IL-6 in LPS-stimulated RAW264.7 cells in the presence of S-PRG sealer extract and TPEN were evaluated by ELISA. SPRG: fourfold diluted S-PRG sealer extract, Ratio: the ratio of pixel intensity of p-NF-kB *versus* NF-kB. $n=3$, *** $p < 0.001$.

Fig. 7 Effects of S-PRG sealer extract on PPAR α and IL-10 mRNA expression levels in LPS-stimulated RAW264.7 cells. mRNA expression levels were determined at 3 h after the addition of S-PRG sealer extract. SPRG: fourfold diluted S-PRG sealer extract, $n=3$, $*p<0.05$.

failed to inhibit the increased expression levels of proinflammatory cytokines (Fig. 4).

We then sought to verify whether Zn²⁺ was responsible for the cytokine downregulation caused by S-PRG sealer extract. TPEN, a heavy metal chelator²³ with a strong affinity for Zn²⁺, was added to the sealer extract to remove the Zn²⁺ activity. As shown in Fig. 5, TPEN mitigated the reducing effects of S-PRG sealer extract and ZnCl₂ solution ($p<0.05$).

We next examined the effects of S-PRG sealer extracts on NF- κ B signaling and on IL-6 protein synthesis in LPS-stimulated RAW264.7 cells. NF- κ B signaling is a major signaling cascade involved in LPS-induced proinflammatory cytokine synthesis²⁴. As shown in Fig. 6A, phosphorylation of NF- κ B promoted by LPS stimulation was downregulated by treatment with fourfold diluted S-PRG sealer extract. LPS-stimulated IL-6 protein synthesis from RAW264.7 cells was significantly downregulated by treatment with fourfold diluted S-PRG sealer extract ($p<0.001$; Fig. 6B). Moreover, TPEN partially abolished the reducing effects of the sealer extract on both NF- κ B phosphorylation (Fig. 6A) and IL-6 protein synthesis ($p<0.001$, Fig. 6B) in LPS-stimulated RAW264.7 cells.

We also examined the effects of S-PRG sealer extract on the mRNA expression of PPAR α and IL-10 in LPS-stimulated RAW264.7 cells. PPAR α is a nuclear transcription factor known to downregulate proinflammatory cytokine expression^{25,26}, and IL-10 is a typical anti-inflammatory cytokine²⁷. The mRNA expression levels of PPAR α and IL-10 did not differ between control and LPS-stimulated RAW264.7 cells, whereas LPS stimulation in the presence of S-PRG sealer extracts induced significant increases in the mRNA expression levels of PPAR α and IL-10 ($p<0.05$, Fig. 7).

DISCUSSION

In this study, we focused on whether and how the S-PRG sealer extract influences proinflammatory cytokine expression in LPS-stimulated RAW264.7 cells. The main findings were that S-PRG sealer extract induced the downregulation of IL-1 α , IL-6, and TNF α mRNA

expression levels; the extract also reduced IL-6 protein levels. These downregulation effects were partially abolished by the addition of TPEN and mimicked by the addition of ZnCl₂ (instead of the sealer extract). Thus, the findings led to rejection of the null hypothesis that S-PRG sealer extracts would have no effect on proinflammatory cytokine expression in LPS-stimulated RAW264.7 cells. Our findings indicate that the cytokine reducing action of S-PRG sealer extract is greatly dependent on Zn²⁺ released from the sealer; however, other mechanisms (e.g., pathways dependent on PPAR α and IL-10) may have some roles as alternative anti-inflammatory mechanisms.

Although fourfold diluted S-PRG sealer extract showed minimal cytotoxicity in RAW264.7 cells, twofold diluted extract showed significant cytotoxicity (Fig. 1). This is consistent with the finding that undiluted S-PRG filler eluate caused cell death in human gingival fibroblasts, presumably through releasing BO₃³⁻, Sr²⁺, and F⁻ in high concentrations²⁸. Moreover, S-PRG sealer contains polycarboxylic acid in the liquid component, which could have contributed to the slight cytotoxicity of this sealer²⁹.

LPS is involved in the pathogenesis of periapical lesions through the induction of proinflammatory cytokines from activated macrophages³⁰. Proinflammatory cytokines (e.g., IL-1 α , IL-6, and TNF α) induce osteoclastogenesis and osteoclastic bone resorption, both of which are involved in periapical alveolar bone destruction²¹. This study revealed that S-PRG sealer extracts downregulated the proinflammatory cytokine expression in LPS-stimulated RAW264.7 cells (Fig. 2). This result implies that S-PRG sealer can partially alleviate periradicular inflammation and bone destruction by negatively controlling proinflammatory activity in macrophages. This is consistent with the finding that a root canal dressing containing S-PRG fillers promoted healing in experimentally induced rat periapical lesions³¹, potentially because S-PRG fillers cause downregulation of proinflammatory cytokines.

To reveal the mechanisms underlying the anti-inflammatory action of S-PRG sealer extract, we focused on the involvement of various ions that are released

from S-PRG sealer¹⁶⁾. It has been reported that a boron-containing organic compound reduces the levels of proinflammatory factors (e.g., IL-1 β and IL-6) in LPS-stimulated RAW264.7 macrophages³²⁾. NaF reduces the mRNA expression levels of IL-1 β , IL-8, and TNF α in IL-1 β -stimulated human gingival fibroblasts³³⁾. SrCl₂ also significantly inhibits the expression of proinflammatory cytokines (e.g., TNF α , IL-1 β , and IL-6) by downregulation of the NF- κ B pathway³⁴⁾. ZnCl₂ downregulates the mRNA expression levels of IL-1 β , IL-6, and IL-8 in immortalized human oral keratinocytes³⁵⁾. In the present study, B(OH)₃, SrCl₂ and NaF (with ion concentrations adjusted to the concentrations in diluted S-PRG) did not reduce the mRNA expression levels of proinflammatory cytokines in LPS-stimulated macrophages. In contrast, ZnCl₂ significantly downregulated the mRNA expression levels of IL-1 α , IL-6, and TNF α (Fig. 4). These findings suggested that Zn²⁺ has an important role in the anti-inflammatory effects of S-PRG sealer. Accordingly, we applied TPEN, a heavy metal chelating agent with high affinity for Zn²⁺ as an intracellular chelator^{23,36)}, which has been widely used³⁷⁾ to investigate the role of Zn²⁺. As shown in Fig. 5, the inhibitory actions of S-PRG sealer extract and ZnCl₂ on the mRNA expression levels of IL-1 α , IL-6, and TNF α in LPS-stimulated RAW264.7 cells were abrogated by the addition of TPEN. Therefore, our findings implied that heavy metal ions, particularly Zn²⁺, have important roles in the S-PRG sealer extract-induced suppression of inflammatory cytokines. In addition, application of TPEN to LPS-stimulated RAW264.7 cells induced downregulation of proinflammatory mediator expression (data not shown). This phenomena may be caused by depletion of intra-cellular heavy metal ions, which are essential for normal cell function³⁸⁾. CN, which is a zinc oxide-containing root canal sealer, achieves satisfactory biocompatibility by replacing eugenol with fatty acids from a Grossman sealer³⁹⁾ and releases¹⁶⁾ a small amount of Zn²⁺. However, extracts of CN failed to downregulate the mRNA expression levels of IL-1 α , IL-6, and TNF α (Fig. 3). This is presumably because the concentration of Zn²⁺ released from set CN may be insufficient to induce cytokine downregulation.

We also found that p-NF- κ B expression was downregulated by S-PRG sealer extract in LPS-stimulated RAW264.7 cells (Fig. 6A). This result implies that the reducing action of S-PRG sealer extract involves inhibition of NF- κ B signaling, which is a major signal transduction pathway in LPS/Toll-like receptor inflammatory signaling, as well as a key signaling cascade for proinflammatory cytokine expression²⁴⁾. This notion is supported by a previous finding that the blockade of NF- κ B phosphorylation results in substantial decreases in the expression levels of proinflammatory cytokines in LPS-stimulated RAW264.7 cells⁴⁰⁾ and dental pulp cells⁴¹⁾. NF- κ B is also involved in osteoclast differentiation⁴²⁾ and the downregulation of NF- κ B signaling contributes to osteoclastogenesis inhibition⁴³⁾. Furthermore, S-PRG filler containing root canal dressing reportedly decreases the number of osteoclasts and the degree of inflammatory response in periapical lesions³¹⁾.

Zn²⁺ reportedly downregulates the I kappa B kinase- α (IKK- α)/IKK- β /NF- κ B signaling pathway through the induction of A20, a zinc finger protein known to suppress TNF α and IL-1 β ⁴⁴⁾. These mechanisms may be involved in the negative regulation of proinflammatory cytokine expression. In contrast, TPEN-induced selective Zn²⁺ deficiency upregulates NF- κ B activation and increases the expression levels of NF- κ B controlled vascular endothelial growth factor, IL-6, and IL-8 in prostate cancer cells⁴⁵⁾; moreover, Zn²⁺ deficiency enhances the production of proinflammatory cytokines in promyeloid cells⁴⁶⁾. Furthermore, Zn²⁺ enhances the expression of PPAR α ⁴⁷⁾, which belongs to the steroid receptor superfamily of nuclear transcription factors⁴⁸⁾ and acts as a negative regulator of NF- κ B signaling and proinflammatory cytokines (e.g., IL-1 β and TNF α)⁴⁹⁾. Mechanisms involving PPAR α could be associated with the negative regulation of NF- κ B by S-PRG sealer extract, because we found that S-PRG sealer extract induced PPAR α mRNA expression in LPS-stimulated RAW264.7 cells (Fig. 7). Taken together, our findings suggest that the reducing effect of Zn²⁺ on NF- κ B signaling is associated with the S-PRG sealer extract-induced downregulation of proinflammatory cytokines in LPS-stimulated RAW264.7 cells (Fig. 2).

TPEN induced increased IL-6 synthesis in LPS-stimulated RAW264.7 cells that were treated with S-PRG sealer extract; however, IL-6 synthesis was much higher in LPS-stimulated RAW264.7 cells that had not been treated with S-PRG sealer extract (Fig. 6B). This finding indicates that additional non-Zn²⁺-related mechanisms may be present. To address this, we examined IL-10, which is an important endogenous suppressor of infection-stimulated periapical lesions that acts through the inhibition of proinflammatory factors (e.g., IL-1 α)⁵⁰⁾. We revealed that the S-PRG sealer extract promoted IL-10 mRNA expression in LPS-stimulated RAW264.7 cells (Fig. 7). Further studies are necessary to explore the mechanisms governing the anti-inflammatory functions of S-PRG sealer.

Our study revealed that S-PRG sealer extract downregulated the expression of proinflammatory cytokines in macrophages. Such an anti-inflammatory property would be beneficial in alleviating chronic inflammation remaining in the periapical tissue. The anti-inflammatory property would also contribute to reducing excessive postoperative inflammation that can be induced through e.g., foreign body responses. Before clinical application, however, further studies are warranted on several other properties of S-PRG sealer, such as sealing ability, dimensional stability, and other physical properties.

CONCLUSION

S-PRG sealer extract downregulated the expression levels of proinflammatory cytokines including IL-1 α , IL-6, and TNF α , as well as p-NF- κ B expression; these effects may be mediated by Zn²⁺-dependent mechanisms.

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CONFLICTS OF INTEREST

The authors declare no financial conflicts of interest related to this study.

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