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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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 periapical tissues. 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. These materials must meet several physical requirements including high sealing ability and low solubility to establish a long-lasting seal. Moreover, endodontic sealers are usually applied in contact with diseased periapical tissues; therefore, they should be biocompatible and offer beneficial biological actions, such as anti-inflammatory and pro-regenerative effects. However, no available materials are considered ideal, 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 fluoroborosilicofluoride glass fillers with a polycarboxylic acid solution. S-PRG fillers release several ions (e.g., boron, aluminum, silicon, sodium, strontium, and fluoride) that confer these fillers with properties including anti-demineralizing, remineralizing, and anti-microbial abilities. Thus, S-PRG fillers have been incorporated into various dental materials such as resin composites, coating resins, and dental sealants.

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)-1α, 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'-tetakis(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-κB pathway, Zinc ion

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of action of S-PRG sealer extracts on proinflammatory cytokine expression in lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages\textsuperscript{22). 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 macrophages\textsuperscript{22} 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% CO\textsubscript{2} 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 chelator\textsuperscript{23} with strong affinities for Zn\textsuperscript{2+}, Fe\textsuperscript{2+}, and Mn\textsuperscript{2+} and low affinity for Ca\textsuperscript{2+}. TPEN was used to eliminate heavy metal ions. Other chemicals used in this study were as follows; B(OH)\textsubscript{3} (300 μg/mL), SrCl\textsubscript{2} (15 μg/mL), NaF (45 μg/mL), and ZnCl\textsubscript{2} (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 extracts\textsuperscript{16}).

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 (3×10\textsuperscript{3} 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 (CCK-8, Dojindo). Briefly, 10 μL of CCK-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 (2×10\textsuperscript{5} 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 | GTAAAGACCTCTATGCCCAACACAG | AATGATCTTTGATCTTCATGTTGCTA | NM_007393 | 122       |
| IL-1α   | ATTTAACCAAGTTGGTGCTGAGTAG | CACCATACACCTACCAAGGATGATT | NM_010554 | 137       |
| IL-6    | TCTGGGCTTTGTCTTCTGGTATCT | TGGATGCTACAAACTGGGATAAT | NM_031168 | 130       |
| TNFα    | ATCATCTTCATCATAATTCGAGTGC | CTAGTTGGTTGTCTTTGAGATCCAT | NM_013693 | 235       |
| PPARα   | GCCTGCTCTGCGGGGATGT     | GGCTTCGGGATTTCTTCTTG    | NM_001113418 | 216       |
| IL-10   | GATCATCATGTATGCTCTATGCAG | GATTCTCCCTCGTGAAAAATAAGAG | 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)-1α, 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 ΔΔCT-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 µL 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-kB) p65 (#8242, 1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-NF-kB 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 anti-rabbit IgG secondary antibody (#1706515, 1:5000, Bio-Rad). Horseradish peroxidase substrate (Immobilon, 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±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...
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-1α, IL-6, and TNFα 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](image1.png)

**Fig. 3** Effects of Canals N (CN) extracts on IL-1α, IL-6, and TNFα 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](image2.png)

**Fig. 4** Effects of B(OH)$_3$, SrCl$_2$, ZnCl$_2$, NaF, and S-PRG sealer extract on IL-1α, IL-6, and TNFα mRNA expression levels in LPS-stimulated RAW264.7 cells. mRNA expression levels were determined at 3 h after the addition of B(OH)$_3$, SrCl$_2$, ZnCl$_2$, NaF, and S-PRG sealer extract. SPRG: fourfold diluted S-PRG sealer extract, $n=3$, $^*p<0.05$ versus LPS.
upregulation of IL-1α, IL-6, and TNFα mRNA expression levels in RAW264.7 cells.

S-PRG sealer extract contains various ions released from the sealer. 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-1α, IL-6, and TNFα 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

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Fig. 5 Effects of TPEN on IL-1α, IL-6, and TNFα 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 PPARα 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 Zn\textsuperscript{2+} was responsible for the cytokine downregulation caused by S-PRG sealer extract. TPEN, a heavy metal chelator\textsuperscript{23} with a strong affinity for Zn\textsuperscript{2+}, was added to the sealer extract to remove the Zn\textsuperscript{2+} activity. As shown in Fig. 5, TPEN mitigated the reducing effects of S-PRG sealer extract and ZnCl\textsubscript{2} solution (p<0.05).

We next examined the effects of S-PRG sealer extracts on NF-κB signaling and on IL-6 protein synthesis in LPS-stimulated RAW264.7 cells. NF-κB signaling is a major signaling cascade involved in LPS-induced proinflammatory cytokine synthesis\textsuperscript{24}. As shown in Fig. 6A, phosphorylation of NF-κB 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-κB 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 PPARα and IL-10 in LPS-stimulated RAW264.7 cells. PPARα is a nuclear transcription factor known to downregulate proinflammatory cytokine expression\textsuperscript{25,26}, and IL-10 is a typical anti-inflammatory cytokine\textsuperscript{27}. The mRNA expression levels of PPARα 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 PPARα 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-1α, IL-6, and TNFα 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 ZnCl\textsubscript{2} (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 Zn\textsuperscript{2+} released from the sealer; however, other mechanisms (e.g., pathways dependent on PPARα 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 BO\textsubscript{3}\textsuperscript{−}, Sr\textsuperscript{2+}, and F\textsuperscript{−} in high concentrations\textsuperscript{28}. Moreover, S-PRG sealer contains polycarboxylic acid in the liquid component, which could have contributed to the slight cytotoxicity of this sealer\textsuperscript{29}.

LPS is involved in the pathogenesis of periapical lesions through the induction of proinflammatory cytokines from activated macrophages\textsuperscript{30}. Proinflammatory cytokines (e.g., IL-1α, IL-6, and TNFα) induce osteoclastogenesis and osteoclastic bone resorption, both of which are involved in periapical alveolar bone destruction\textsuperscript{31}. 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 periapical 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 lesions\textsuperscript{31}, 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 sealer. It has been reported that a boron-containing organic compound reduces the levels of proinflammatory factors (e.g., IL-1β and IL-6) in LPS-stimulated RAW264.7 macrophages. NaF reduces the mRNA expression levels of IL-1β, IL-8, and TNFα in IL-1β-stimulated human gingival fibroblasts. SrCl2 also significantly inhibits the expression of proinflammatory cytokines (e.g., TNFα, IL-1β, and IL-6) by downregulation of the NF-κB pathway. ZnCl2 downregulates the mRNA expression levels of IL-1β, IL-6, and IL-8 in immortalized human oral keratinocytes. 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-1α, IL-6, and TNFα (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, which has been widely used 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-1α, IL-6, and TNFα 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 function. CN, which is a zinc oxide-containing root canal sealer, achieves satisfactory biocompatibility by replacing eugenol with fatty acids from a Grossman sealer and releases a small amount of Zn2+. However, extracts of CN failed to downregulate the mRNA expression levels of IL-1α, IL-6, and TNFα (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-κB 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-κB 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 expression. This notion is supported by a previous finding that the blockade of NF-κB phosphorylation results in substantial decreases in the expression levels of proinflammatory cytokines in LPS-stimulated RAW264.7 cells and dental pulp cells. NF-κB is also involved in osteoclast differentiation and the downregulation of NF-κB signaling contributes to osteoclastogenesis inhibition. Furthermore, S-PRG filler containing root canal dressing reportedly decreases the number of osteoclasts and the degree of inflammatory response in periapical lesions.

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