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Anti-inflammatory effect of black rice in *Aggregatibacter actinomycetemcomitans* stimulated macrophages via inhibition of NF-κB pathway

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Periodontitis, which results from infection and inflammation, is one of the most common inflammatory diseases. Some studies have shown that plant extracts can inhibit inflammation in periodontitis. However, the anti-inflammatory effects of black rice extract (BRE) have not yet been investigated. Here, we investigated the anti-inflammatory activity of BRE *in vitro* on *Aggregatibacter actinomycetemcomitans* (*A. a*) stimulated RAW 264.7 macrophages. The results showed that BRE did not affect cell viability at concentrations ranging from 0.015 to 0.5 mg/mL, but toxicity was observed >1 mg/mL. *A. a*-induced nitric oxide (NO) production and iNOS protein expression were inhibited by treatment with BRE. In addition, BRE inhibited the gene expression of pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α, and blocked NF-κB p65 translocation as well as IκBα and IKK α/β phosphorylation. Moreover, the DPPH and ABTS radical scavenging activity results showed that BRE exhibits strong antioxidant activity in a dose-dependent manner. This study found that BRE exerts anti-inflammatory activity via inhibition of NF-κB signaling and antioxidant activity. The above finding suggests that BRE can be used as a potential therapeutic agent for treating inflammation in periodontitis.

Keywords: Antioxidant activity, Antiradical activity, *Oryza sativa*, Periodontitis, Purple rice

Periodontitis is a prevalent disease characterized by inflammation of the periodontium. It occurs due to bacterial infection of the tissue surrounding the teeth. When the damage spreads to the soft tissue and bone that supports the tooth, it is referred to as advanced periodontal disease[1]. As periodontal inflammation continues to progress, the teeth surrounding the bone are destroyed and eventually need to be extracted. Periodontitis almost always begins as a result of inflammation due to gingivitis[2].

There are several well-known risk factors for periodontitis, including a number of genetic indicators, diabetes, pregnancy and environmental factors such as smoking[3]. Furthermore, periodontitis is caused by infection with periodontopathic bacteria such as *Prophyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans* (*A. a*)[4]. Among the periodontal pathogenic bacteria, *A. a* is an exogenous bacterium highly associated with periodontitis[5]. It is a Gram-negative bacillus, and its infection induces the immune-inflammatory response, which consequently causes oral inflammatory disease[6]. Periodontitis has also been associated with increased inflammation in the body, as indicated by raised levels of pro-inflammatory cytokines such as IL-6 and IL-1β[7].

Therefore, to prevent and treat periodontitis, plants and their extracts are used to reduce inflammation and irritation. Research has reported that extracts from aloe vera and calendula contain several anti-inflammatory and antimicrobial agents[8]. These plant extracts contain chemicals such as bioflavonoids, anthocyanin, and other substances that exhibit anti-inflammatory activity[9].

Black rice is one type of rice belonging to the species *Oryza sativa* L and is consumed as food in Asian countries. Regarding its health benefits, due to the high content of bioactive agents such as anthocyanin and other phenolic compounds, black rice has been suggested to be a beneficial food[10]. One study has reported evidence of the anti-inflammatory properties of black rice extract (BRE) as a functional material[11]. Plant extracts have been widely used for their antioxidant and anti-inflammatory effects as well as their safe preclinical and clinical profiles.

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Regarding their anti-inflammatory effects, plant extracts have been shown to interfere with NF-κB-related regulation[12,13]; NF-κB typically translocates into the nucleus and regulates the gene expression of pro-inflammatory proteins, such as the interleukin family and inducible nitric oxide synthase (iNOS)[14].

However, the anti-inflammatory activity of BRE on periodontal pathogenic *A. a* associated inflammation and its molecular mechanism have not been reported. Therefore, in this study, we investigated the anti-inflammatory effects of BRE on periodontal pathogenic *A. a* stimulated RAW 264.7 macrophages and explored the related molecular mechanism.

Materials and Methods

Black rice extraction

Black rice was purchased form the local market in the Jeonbuk province of Korea. Voucher specimen (No.: 3-21-19) was kept in the Herbarium of the Department of Oral Biochemistry, School of Dentistry in Wonkwang University. Black rice (900 g) was extracted with 3 L of 95% ethyl alcohol for one week at room temperature, filtered, and evaporated. The BRE yield was 21.82 g (2.4%). Black rice was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich Co., St Louis, MO, USA). It was diluted prior to the experiments, and the final concentration of DMSO was adjusted to 0.5% (v/v). The negative control group was adjusted to 0.5% DMSO alone.

Measurement of black rice extract composition

Total polyphenol

The total polyphenol content analysis was conducted with reference to the Folin-Ciocalteu method with some modification[15]. Briefly, 50 µL of standard solution was mixed with 25 µL folin-ciocalteu reagent and 125 µL 20% sodium carbonate solution, and the mixture reacted in the dark at room temperature for 40 min. Then, the absorbance was measured at 725 nm using a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). The total polyphenol content was calculated by quantifying gallic acid (Sigma-Aldrich Co. Louis, MO, USA) from the prepared standard curve.

Total flavonoid

The total flavonoid content analysis was measured as follows with reference to the Davis method as in Shruti *et al*.[16]. BRE solution was mixed with methanol, 10% aluminum (III) solution and potassium acetate (1M). Then, the mixture was incubated at room temperature for 10 min. The absorbance of the mixture was measured at 430 nm using a microplate reader (SpectraMax 190). The total flavonoid content was calculated from the standard curve prepared by quantifying quercetin (Sigma-Aldrich Co., Louis, MO, USA).

Total anthocyanin

Total anthocyanin content was analyzed by the pH differential method[17]. The diluted sample was mixed with 0.025 M KCl buffer (pH 1.0) and 0.4 M NaOAc buffer (pH 4.5), respectively. Then, the absorbance was measured at 510 nm and 700 nm, respectively. The total anthocyanin content was calculated with the following formula:

$$\text{Anthocyanin content (mg/L)} = \frac{A \times B \times C \times 1{,}000}{\varepsilon 1}$$

where A (absorbance) = $(A_{510}\text{-}A_{700})_{pH1.0} - (A_{510}\text{-}A_{700})_{pH4.5}$, B = (Molecular weight of cyanidine-3 glucoside), and C=Dilution factor of sample, ε = 26,900 M^{-1} cm^{-1}.

Cell culture

The RAW 264.7 murine macrophage cell line (kindly supplied by G. Nussbaum) was grown as described previously in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, as well as penicillin and streptomycin (both at 100 g/mL).

Bacteria culture

Aggregatibacter actinomycetemcomitans (*A. a*) was purchased from ATCC (American Type Culture Collection) and grown on tryptic soy-serum-bacitracin-vancomycin (TSBV) at 37°C under anaerobic conditions (5% CO_2, 10% H_2 and 85% N_2). The bacteria was grown until the log phase and harvested. Then, it was washed and re-suspended with phosphate-buffered saline (PBS). The bacteria were inactivated with 0.5% formalin for 30 min. To adjust the bacterial number, the optical density (OD) of the formalin-inactivated bacterial suspension was measured, and PBS was added to reach a dilution of OD = 1.

Cell viability

RAW 264.7 macrophages were seeded in a 96-well cell culture plate at 1×10^5 cells per well. After overnight culture, the cells were pre-treated with various concentrations of BRE for 2 h, followed by treatment with inactivated *A. a* (1×10^8 CFU). After 24 h of cultivation, cytotoxicity was measured using a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-

(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit according to the manufacturer's protocol.

Nitric oxide (NO) quantification

RAW 264.7 macrophages were seeded in a 24-well cell culture plate at 5×10^5 cells per well. After overnight culture, the cells were pre-treated with various concentrations of BRE for 2 h, followed by treatment with inactivated *A. a* (1×10^8 CFU). After 24 h of cultivation, the cell-cultured supernatant (200 μL) was collected and mixed with 200 μL of Griess reagent solution (Promega, Madison, WI, USA). Then, absorbance was measured at a 540 nm wavelength with microplate reader. For quantitative calculation, $NaNO_2$ was used for the calibration curve.

Real-time PCR

The cells were pre-treated with various concentrations of BRE for 2 h and then treated with inactivated *A. a* (1×10^8 CFU). To obtain the total RNA, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacture's protocol. After RNA quantification, cDNA was synthesized using 1 μg RNA with a cDNA synthesis kit (PrimeScript RT Reagent Kit; TaKaRa, Shiga, Japan). RT-PCR was performed using the AccuPower PCR PreMix Kit (Bioneer, Daejeon, Korea) on GeneAmp PCR system 2400 (Applied Biosystems, Thermo Fisher Scientific). Specific primers are shown in Table 1[18]. The relative mRNA levels were calculated using the ΔΔCq method. β-actin was used as an internal control.

Western blot

The cells were pre-treated with various concentrations of BRE for 2 h and then treated with inactivated *A. a* (1×10^8 CFU). To extract total protein, cells were lysed with PhosphoSafe Protein Extraction Reagent (Novagen, Madison, WI, USA). Then, 30 μg of protein was resolved by sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). After membrane blocking with 5% skim milk, primary antibodies (1:1000) were added. Secondary antibodies conjugated with horseradish peroxidase (HRP) were used at a dilution of 1:5000 in 3% skim milk in TBST solution. The bands were exposed using lumino reagent (Milipore Corporation, Billerica, MA, USA).

Table 1 — Primer sequence of pro-inflammatory cytokine genes

Genes	5′-3′	Sequences
IL-1β	F	GAA AGA CGG CAC ACC CAC CCT
	R	GCT CTG CTT GTG AGG TGC TGA TGT A
IL-6	F	GAT GGA TGC TAC CAA ACT GGA
	R	TCT GAA GGA CTC TGG CTT TG
TNF-α	F	CCA CCA CGC TCT TCT GTC TAC
	R	AGG GTC TGG GCC ATA GAA CT
β-actin	F	CAT CAC TAT TGG CAA CGA GC
	R	GAC AGC ACT GTG TTG GCA TA

[F: Forward; R: Reverse]

Measurement of antioxidant activity

DPPH free-radical scavenging method

Free-radical scavenging activity was measured using the modified method previously described[19] for the DPPH scavenging assay. Briefly, 0.1 mL of BRE solution was added to an equal volume of 0.4 uM DPPH/methanol solution. The mixture was incubated in dark conditions for 30 min, and the absorbance was measured at 517 nm. The percentage of scavenging activity was expressed as the percentage of DPPH free-radical inhibition by the sample and was calculated by the following formula:

$$\text{Scavenging activity (\%)} = \frac{((\text{OD control} - \text{OD Sample})}{\text{OD control)}} \times 100$$

ABTS free-radical scavenging method

The antioxidant activity of BRE against 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) was determined by a modified method[20]. Briefly, 2 mM ABTS and 2.45 mM potassium persulfate were prepared and kept in the dark for 16 h at room temperature. The mixture was diluted with methanol until its absorbance value reached OD = 1-1.5 (at 734 nm). The percentage of scavenging activity was expressed as the percentage of ABTS free-radical inhibition by the sample and was calculated by the following formula:

$$\text{Scavenging activity (\%)} = \frac{((\text{absorbance of control} - \text{absorbance of sample}))}{\text{absorbance of control}} \times 100$$

Statistical analysis

Statistical analyses were carried out using Student's t-test and one-way analysis of variance (ANOVA). The data are presented as the mean ± standard deviation (SD) of the mean. $P < 0.05$ was considered statistically significant.

Results

Black rice extract on RAW 264.7 macrophage viability

When the cells were treated with BRE concentrations (0.015-0.5 mg/mL), there were not any negative effects on cell viability (Fig. 1). However, when the concentration of BRE reached 1 mg/mL, significant cytotoxicity toward the RAW 264.7 cells was observed. No effect on cell viability was

observed at concentrations lower than 0.5 mg/mL. However, the cells treated with the higher concentration (1 mg/mL) of BRE showed a reduced viability of 42%. Based on these results, non-toxic concentrations of BRE (0.015 and 0.500 mg/mL) were used in the subsequent experiments.

Black rice composition

The total phytochemical concentrations are shown in Table 2. The total phenolic content was found to be 29.5 ± 1.2 mg GAE/g, the total flavonoid content was 8.9 ± 0.3 mg GAE/g, and the anthocyanin content was 15.4 ± 2.5 mg/mL in BRE.

Inhibitory effects of BRE on NO production and iNOS expression

NO production and iNOS expression were evaluated to determine whether BRE exhibits anti-inflammatory activity against *A. a* induced inflammation in RAW 264.7 macrophages. When treated with *A. a*, NO production was higher than that in the control group. With BRE pre-treatment (0.125-0.5 mg/mL), NO production significantly decreased in a dose-dependent manner (Fig. 2A). The protein expression of iNOS was additionally evaluated, and the Western blot results (Fig. 2 B & C) showed that treatment with BRE significantly decreased iNOS expression in a dose-dependent manner.

Inhibitory effects of BRE on pro-inflammatory cytokines

To evaluate the inhibitory effects of BRE on pro-inflammatory activity, the expression levels of cytokines (TNF-α, IL-6 and IL-1β) were examined by RT-PCR. As shown in Fig. 3A, BRE treatment significantly reduced IL-6 gene expression at concentrations ranging from 0.125 to 0.5 mg/mL in RAW 264.7 cells. In addition, the mRNA expression of IL-1β and TNF-α were also markedly reduced by treatment with BRE (0.5 mg/mL) (Fig. 3 B-C).

Inhibitory effects of BRE on NF-kB translocation

To explore whether the NF-κB pathway is involved in the anti-inflammatory mechanism of BRE, the

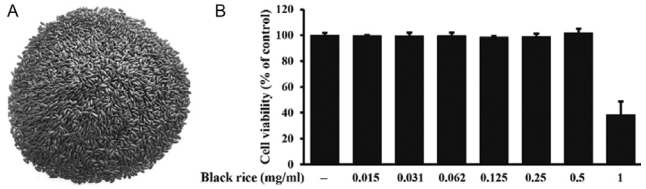

Fig. 1 — Effect of black rice extract on the viability of RAW 264.7 cells. (A) Black rice; and (B) Cells (1×10^5 cells) were incubated with or without *A. actinomycetemcomitans* (1×10^8 CFU) for 24 h in the presence or absence of black rice extract at the indicated concentrations. [Cell viability was determined by an MTS assay as described in Materials and Methods. Each column represents the mean ± S. D. from three independent experiments]

Table 2 — Phytochemical concentrations in black rice extract

Phytochemical	Black rice extract
Total polyphenol (mg GAE/g)	29.5 ± 1.2
Total flavonoid (mg GAE/g)	8.9 ± 0.3
Total anthocyanin (mg/L)	15.4 ± 2.5

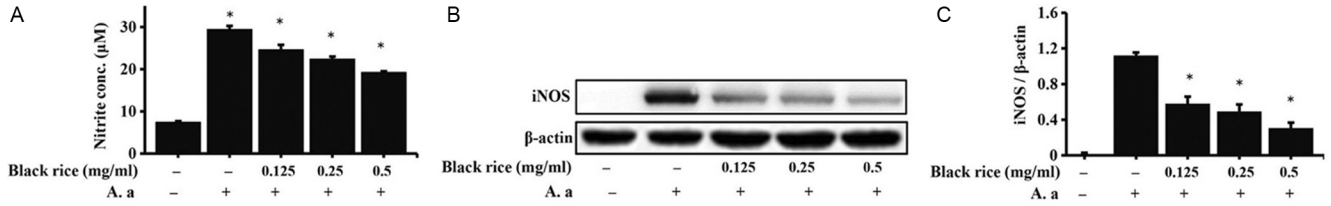

Fig. 2 — Effects of black rice extract on nitric oxide (NO) production and iNOS levels in *A. actinomycetemcomitans* activated RAW 264.7 cells. Cells were pre-incubated with black rice extract for 2 h and then incubated for 24 h with *A. actinomycetemcomitans*. (A) NO was determined by a Griess reaction; (B) The levels of iNOS were determined by Western blot; and (C) Image analysis of Western blot. [Data represent the mean ± SD from three independent experiments. **P* <0.05; compared with the *A. actinomycetemcomitans* treated group]

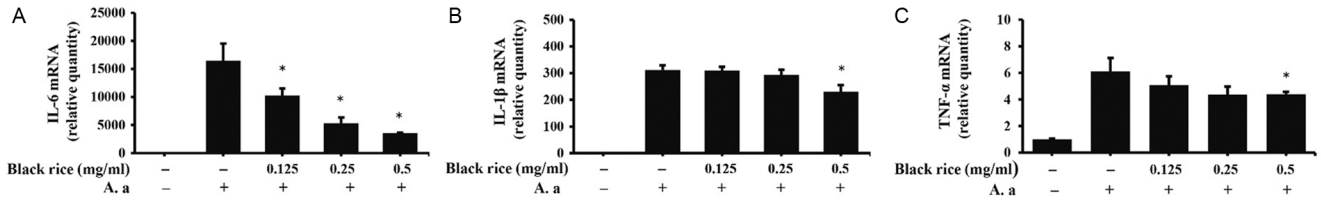

Fig. 3 — Effects of black rice extract on (A) IL-6; (B) IL-1β; and (C) TNF-α mRNA expression in *A. actinomycetemcomitans* activated RAW 264.7 cells. Cells were pre-incubated with black rice extract for 2 h and then incubated for 24 h with *A. actinomycetemcomitans*. IL-6, IL-1β and TNF-α mRNA levels were normalized to β-actin mRNA levels and expressed as fold-increase. [Data represent the mean ± SD from three independent experiments. **P* <0.05; compared with the *A. a*-treated group]

translocated NF-κB molecules were investigated by Western blotting. Fig. 4 shows that translocated NF-κB levels increased with *A. a* stimulation, but this was inhibited by BRE (0.125 -0.5 mg/mL) in a dose-dependent manner.

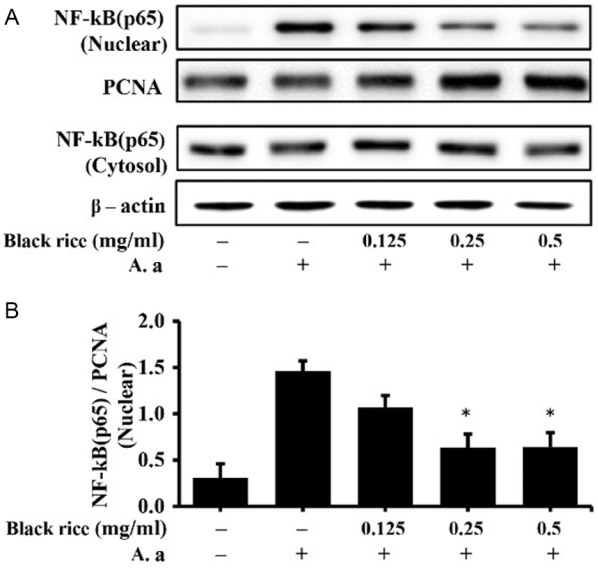

Fig. 4 — Effects of black rice extract on the nuclear translocation of NF-kB (p65) in *A. actinomycetemcomitans* activated RAW 264.7 cells. The cells were pre-incubated with black rice extract for 2 h and then incubated for 1 h with *A. actinomycetemcomitans*. (A) Cytosolic extract and nuclear extract were isolated, and the levels of NF-kB were determined by Western blot; and (B) Image analysis of Western blot. NF-kB levels were normalized to PCNA. [Data represent the mean ± SD from three independent experiments (*P <0.05; compared with the *A. actinomycetemcomitans* treated group]

Inhibitory effect of black rice extract on the phosphorylation of IκBα and IKKα/β

Figure 5 shows that phosphorylated IκBα and IKKα/β were enhanced in *A. a*-stimulated RAW 264.7 cells. However, the phosphorylation of IκBα and IKKα/β was significantly reduced by treatment with BRE in a dose-dependent manner.

Effect of black rice on antiradical activity

BRE was shown to serve as a potent antioxidant agent that can scavenge free radicals in a dose-dependent manner (Fig. 6A). ABTS radical

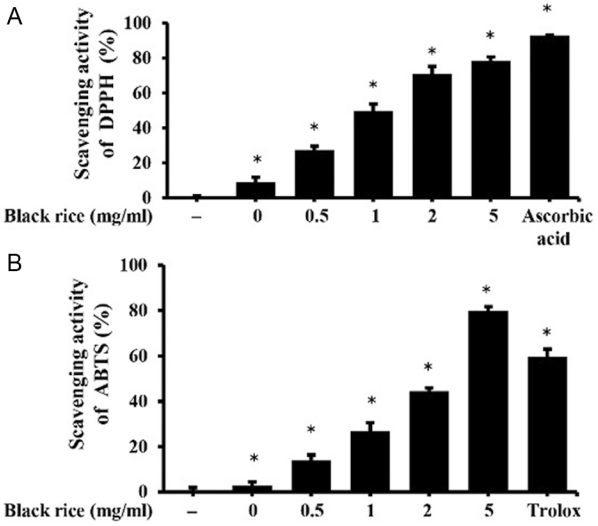

Fig. 6 —Scavenging activity of black rice on (A) DPPH; and (B) ABTS radicals. [Data represent the mean ± SD from three independent experiments. *P <0.05; compared with the control group]

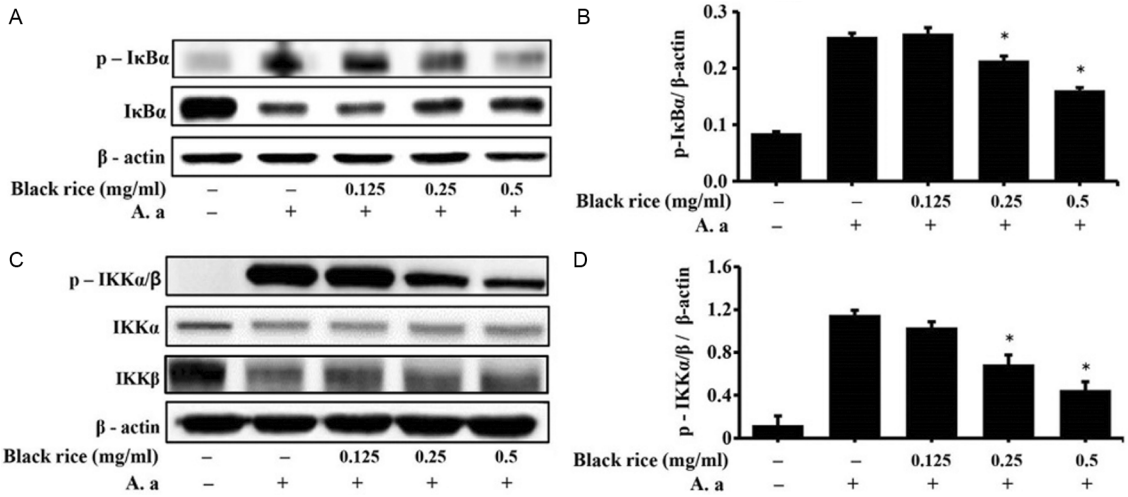

Fig. 5 — Effects of black rice extract on the phosphorylation of IkBα in *A. actinomycetemcomitans* activated RAW 264.7 cells. Cells were pre-incubated with black rice extract for 2 h and then incubated for 1 h with *A. actinomycetemcomitans*. (A & B) Cytosolic extract was isolated, and the levels of IkBα and p-IkBα were determined by Western blot and its image analysis; (C & D) Cytosolic extract was isolated, and the levels of p-IKKα/β, IKKα and IKKβ were determined by Western blot and and its image analysis. [The expression levels were normalized to β-actin. Data represent the mean ± S.D. from three independent experiments. *P <0.05; compared to the *A. a*-treated group]

scavenging activity is also an indicator of hydrogen-donating ability, and the results of this assay also showed that BRE exhibits strong scavenging activity in a dose-dependent manner (Fig. 6B).

Discussion

Phenolic compounds act as free-radical terminators due to their hydroxyl group, which is a powerful chain-breaking antioxidant[21]. Flavonoids also exert antioxidant activity as a result of several functions, including free-radical scavenging and chelation of metal ions such as iron and copper, which are essential to free-radical-generating enzymes[22]. Anthocyanin is a coloured pigment contained in berries, blackcurrants, and other red- to blue-coloured fruits. It exhibits strong antioxidant and/or anti-inflammatory activity[23]. The present study evaluated the total concentrations of polyphenols, flavonoids, and anthocyanin to elucidate the phytochemical contents of BRE. As shown in Table 2, BRE has potential anti-inflammatory and antioxidant activity because BRE contains a large amount of polyphenols, flavonoids, and anthocyanin. Then, BRE may be useful in inhibiting periodontal disease. However, its anti-inflammatory activity against *A. a* induced inflammation in macrophages have not yet been studied. Therefore, this study attempted for the first time to elucidate the mechanism by which BRE inhibits inflammatory cytokine and mediator expressions after infection with periodontopatic bacteria.

Inflammation is generally caused by several factors, such as bacterial infection and trauma, and is also the initial response of the immune system to bacterial infection. It is caused by cytokines such as IL-6 and IL-1β. These cytokines are highly expressed in periodontal inflammation sites[24] and are closely associated with the clinical severity of periodontitis. Furthermore, TNF-α is also one of the important inflammatory factors, and can stimulate the phagocytosis of macrophages in the injured site[25]. TNF-α is crucial for inducing synergy in NO production in LPS-stimulated macrophages, and it induces inflammatory responses[26].

In this study, we estimated whether BRE has inhibitory effects on the inflammation induced by *A. a* in RAW 264.7 macrophage cells. The *A. a* induced inflammatory response was evaluated by increased NO product and increased IL1β, and IL6 expression. In Fig. 3, our data has shown that BRE decreased the expressions of these proinflammatory cytokines.

NO is an important mediator and regulator of inflammatory reactions[27]. During inflammation processes, an excessive amount of NO is produced in macrophages[28]. In addition, NO is produced as a free radical by iNOS through the catalysis of oxidation[29]. Interestingly, several studies have reported that NO production was increased by bacteria-infected macrophages[30] because lipopolysaccharides (LPS) in the bacterial cell walls activate the inflammatory response, just as NO does[31]. Lots of studies reported that *A. a* LPS plays an important role in alveolar bone loss through the induction of NO production in macrophage cells[32]. In our research, the results showed that NO and iNOS were reduced by BRE treatment and proposed that BRE may inhibit *A. a* induced inflammation through inhibition of NO and iNOS.

NF-κB is an important transcription factor, and it regulates cell proliferation, differentiation, and immune responses. NF-κB also regulates the expression of iNOS and pro-inflammatory cytokines[33]. Normally, NF-κB is bound by the inhibitor of κB (IκB), and NF-κB activation is initiated by the signal-induced degradation of IκB proteins and occurs primarily via the activation of IκB kinase (IKK), which is composed of a heterodimer of IKKα and IKKβ subunits[34]. The present study suggested that BRE inhibits the expression of pro-inflammatory cytokines by inhibiting the NF-κB pathway (Fig. 4) and also indicated that BRE interferes with the NF-κB pathway upstream of IκBα and IKKα/β phosphorylation (Fig. 5).

The antioxidant capacity of a plant extract is related to its levels of phenolic compounds, flavonoids, and anthocyanin[35]. In the present investigation, DPPH and ABTS assays were used for the evaluation of BRE antioxidant activity. The DPPH assay is normally used to determine the free-radical scavenging potential of compounds. In Fig. 6, BRE displayed the strong scavenging activity.

Phenolics are powerful antioxidants that can scavenge reactive oxygen species (ROS) and chelate transition metals[36]. Therefore, the phenolic content of a plant extract can be considered an indicator of its antioxidant properties[37]. Flavonoids also exert

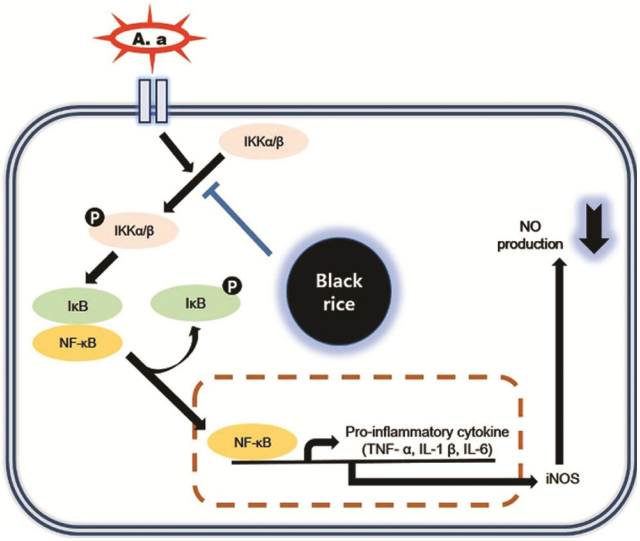

Fig. 7 — A schematic diagram of inhibition mechanism by black rice extract in the *A. actinomycetemcomitans* activated inflammatory response.

antioxidant activity, which is related to several of their functions via free-radical scavenging[38,39]. In addition, anthocyanin also behaves as an antioxidant through the direct trapping of ROS and the inhibition of superoxide production[40,41]. According to the analysis of the phytochemical concentrations in BRE (Table 2), the polyphenol, flavonoid, and anthocyanin levels were found to be higher in BRE than control. The antioxidant activity of BRE showed a relatively positive correlation with the levels of these phytochemicals. Our data suggest that BRE exhibits strong antioxidant activity due to its large concentrations of polyphenols, flavonoids, and anthocyanin. Figure 7 provides a schematic presentation of anti-inflammatory effects of BRE on periodontal pathogenic *A. a* stimulated RAW 264.7 macrophages and the related molecular mechanism.

Conclusion

This study demonstrated the anti-inflammatory and antioxidant effects of BRE. BRE inhibited pro-inflammatory signaling in *Aggregatibacter actinomycetemcomitans* (*A. a*) induced RAW 264.7 macrophages via inhibition of NF-κB signaling. Upstream of NF-κB signaling, BRE inhibited the phosphorylation of IKKs, IκBα and IKKα/β, decreased the translocation of NF-κB p65 to the nucleus, and decreased the expression of iNOS, IL6, IL-1β and TNF-α. Furthermore, BRE exhibits antioxidant activity. Although further *in vivo* study is required, these results indicate the anti-inflammatory and antioxidant activities of BRE and their molecular mechanisms. Overall, these findings suggest that BRE could be a potential therapeutic agent in the prevention of inflammatory periodontitis disease.

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Conflict of Interest

Authors declare no competing interests.

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