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The effect of rosehip extract on TNF-α, IL-1β, and IL-8 production in THP-1-derived macrophages infected with *Aggregatibacter actinomycetemcomitans*

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Inflammation is a protective mechanism against pathogens, but if maintained continuously, it destroys tissue structures. *Aggregatibacter actinomycetemcomitans* is a gram-negative, facultative anaerobic bacterium often found in severe periodontitis. *A. actinomycetemcomitans* invades epithelial cells and triggers inflammatory response in the immune cells. In this study, we investigated the effect of water-soluble rosehip extract on *A. actinomycetemcomitans*-induced inflammatory responses. A human monocytic cell line (THP-1) was differentiated to macrophages by phorbol 12-mystristate 13-acetate treatment. The cytotoxic effect of extract was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The effects of extract on bacterial growth were examined by measuring the optical densities using a spectrophotometer. THP-1-derived macrophages were infected *A. actinomycetemcomitans* after extract treatment, and culture supernatants were analyzed for cytokine production using enzyme-linked immunosorbent assay. Protein expression was measured by western blotting. Extract was not toxic to THP-1-derived macrophages. *A. actinomycetemcomitans* growth was inhibited by 1% extract. The extract suppressed *A. actinomycetemcomitans*-induced tumor necrosis factor-α, interleukin (IL)-1β, and IL-8 production. It also decreased mitogen-activated protein kinase (MAP kinase) and nuclear factor-κB (NF-κB) phosphorylation. Moreover, the extract inhibited the expression of inflammasome components, including nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 3, Absent in Melanoma 2, and apoptosis associated speck-like protein containing a CARD. And cysteine-aspartic proteases-1 and IL-1β expression were decreased by the extract. In summary, extract suppressed *A. actinomycetemcomitans* growth and decreased inflammatory cytokine production by inhibiting activation of MAP kinase, NF-κB, and inflammasome signaling. Rosehip extract could be effective in the treatment of periodontal inflammation induced by *A. actinomycetemcomitans* infection.

Keywords: *Aggregatibacter actinomycetemcomitans*, Inflammation, Cytokines, Periodontitis, Rosehip

Introduction

Periodontitis is an infection-driven chronic inflammatory disease involving gingival inflammation and the destruction of periodontal tissue [1]. Periodontal pathogens include a *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans* and are known to trigger the inflammatory responses [2]. *A. actinomycetem-*

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comitans is a gram-negative, facultative anaerobe, non-motile bacterium that is minor component of subgingival biofilms in periodontally healthy subjects but is found at higher levels in the diseased periodontal pocket [3,4]. *A. actinomycetemcomitans* produces an array of virulence factors that allow this bacterium to evade the host immune response, including two exotoxins, i.e., leukotoxin and cytolethal distending toxin [5]. Immune cells consisting of neutrophils and monocyte/macrophages were recruited into infection areas, triggering the production of a variety of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), and interleukin-8 (IL-8) [6,7]. In chronic inflammation, these cytokines are continuously accumulated and results in irreversible loss of attachment and disrupt alveolar bone [8,9].

For the treatment of periodontitis, antibiotics are mainly used as antimicrobial agents [10]. However, the continuous application of antibiotic agents sometimes causes antimicrobial resistance. The incidence of infections has been increasing due to the incorrect or prevalent overuse of antibiotics. Thus, natural constituents have been suggested to be worthy of replacing synthetic agents [11].

Rosehip is used in teas, jams, jellies and soups, and as a natural source of vitamin C [12,13]. Rosehip is being studied as a potential treatment to relieve osteoarthritis and protect joint cartilage [12,13]. However, the effect of rosehip on periodontitis has not been investigated. The purpose of this study is to examine the effect of rosehip on inflammatory response in THP-1 derived macrophages induced by *A. actinomycetemcomitans* infection for the possible suggestion of it for the treatment of periodontitis.

Materials and Methods

1. Reagents

The phorbol 12-mystristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). Rosehip (Ravah International, Mirada, CA, USA) was prepared by incubating 10 g of rosehip in 100 mL of 95℃ distilled water for 5 minutes, then filtrating the supernatant. Rosehip extract was concentrated 10 times by an amicon ultra centrifugal filter (MilliporeSigma, Burlington, MA, USA) at 2,000 g for 50 minutes.

2. Bacterial and cell culture

A. actinomycetemcomitans (ATCC 33384) was cultured in tryptic soy broth (TSB) (BD, Franklin Lakes, NJ, USA) with 1% yeast (LPS Solution, Seoul, Korea) at 37℃ in a 5% CO_2 incubator. *A. actinomycetemcomitans* was collected by centrifugation at 5,000 rpm for 5 minutes and resuspended in RPMI media (Gibco, Carlsbad, CA, USA) to infect the cells at a multiplicity of infection (MOI) of 1:50 and 100. THP-1-derived macrophages were cultured in RPMI supplemented with 10% fetal bovine serum and differentiated into macrophage cells by a 50 nM PMA treatment overnight. The differentiated cells were pretreated with rosehip extract for 30 minutes, and then infected with *A. actinomycetemcomitans* in cytokine assay and Western blot analysis.

3. Cell cytotoxicity assay

To determine the cytotoxicity of rosehip extract on THP-1-derived macrophages, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. THP-1-derived macrophages were seeded at 5 $\times$ 10^4 cells per well in a 96-well plate and treated with rosehip extract for 24 hours. To determine cell viability, cells were incubated with an MTT solution (1 mg/mL) at 37℃ for 4 hours. The purple formazan crystal was dissolved in dimethylsulfoxide and the absorbance was measured using a spectrophotometer at 570 nm.

4. Growth curve

A. actinomycetemcomitans was grown in tryptic soy broth (TSB) broth at 37℃ in an atmosphere containing 5% CO_2. *A. actinomycetemcomitans* was mixed with TBS broth containing various concentrations of rosehip extract in 96 wells. The optical density of each well was measured against the standard medium on a microplate reader at a wavelength of 650 nm.

5. Cytokine assay

The production of cytokines released on the culture supernatants were assessed with an enzyme-linked immunosorbent assay (ELISA) kit (BioLegend, San Diego, CA, USA) following the manufacturer's instructions. THP-1-derived macrophages were seeded at 2 $\times$ 10^5 cells per well in a 24-well plate and infected with *A. actinomycetemcomitans* for 24 hours after treatment with rosehip extract for 30 minutes. The cytokine level was measured at 450/570 nm using an ELISA reader (Tecan, Männedorf, Switzerland).

6. Western blot analysis

THP-1-derived macrophages were seeded at 1 × 10^6 cells per well in a 6-well plate and infected with *A. actinomycetemcomitans* for 30 minutes after treatment with rosehip extract for 30 minutes. Cell were harvested and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). The proteins samples were separated using 12% and 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto membranes (MilliporeSigma). The membranes were incubated with a primary antibody and secondary antibody. The immunolabeled proteins were visualized with an ECL chemiluminescence kit (GE Healthcare, Piscataway, NJ, USA) and a LAS-4000 lumino-image analyzer (Fuji Film, Tokyo, Japan). The band intensities of the immunoblot were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and are presented as the ratio relative to the intensity of β-actin. Phospho-nuclear factor kappa B (NF-κB), NF-κB, phospho-mitogen-activated protein kinase (MAP kinase) family, MAP kinase family, apoptosis-associated speck-like protein containing a CARD (ASC), Caspase-1, AIM2 and IL-1β antibodies were purchased from Cell Signaling Technology. Anti-NLRP3 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

7. Statistical analysis

All results are expressed as the mean ± standard deviation and an unpaired, one-tailed Student's t-test was performed for the comparison within the groups using the SPSS 13.0 statistical software program (SPSS Inc., Chicago, IL, USA). Differences were considered significant when *p*-values were < 0.05.

Results

1. Effect of rosehip extract on the viability of THP-1-derived macrophages and the growth of *A. actinomycetemcomitans*

To examine the effect of rosehip extract on viability of THP-1 derived macrophages and the growth of *A. actinomycetemcomitans*, rosehip extract was added to THP-1-derived macrophages and *A. actinomycetemcomitans*, respectively. THP-1-derived macrophages treated with all concentration of rosehip extract did not show any cytotoxicity by MTT assay (Fig. 1A). Although not statistically significant at 10%, cell proliferation tends to decrease, so the next highest concentration of rosehip extract 5% was used in subsequent experiments.

A. actinomycetemcomitans showed similar growth pattern between rosehip extract-treated group and non-treated control group at 8 hours. At late logarithmic phase, 1% and more than 1% rosehip extract significantly decreased bacterial growth (Fig.

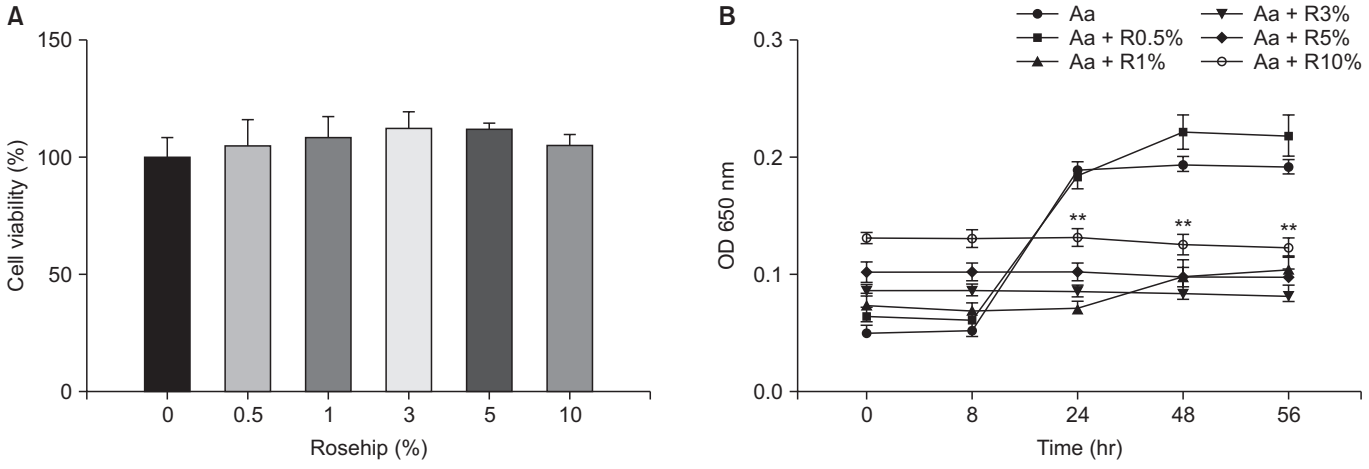

Fig. 1. The viability of THP-1-derived macrophages and *Aggregatibacter actinomycetemcomitans* growth to rosehip extract. (A) THP-1-derived macrophages were treated with various concentrations of rosehip extract for 24 hours, and the cytotoxicity was measured by MTT assay. Data are presented as mean ± SD (n = 6). (B) *A. actinomycetemcomitans* was grown in TSB containing 1% yeast extract with or without rosehip extract for the indicated times. The OD of each tube was measured at a wavelength of 650 nm by spectrophotometer. Data represent mean values ± SD (n = 6).
Aa, *A. actinomycetemcomitans*; R, rosehip; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; TSB, tryptic soy broth; OD, optical density.
**$p < 0.01$ versus *A. actinomycetemcomitans* infection.

1B).

Based on these results, 5% of rosehip extract was used for the next experiments.

2. Effect of rosehip extract on inflammatory cytokines production induced by *A. actinomycetemcomitans* in THP-1-derived macrophages

We investigated the effects of rosehip extract on inflammatory cytokines production induced by *A. actinomycetemcomitans* infection. *A. actinomycetemcomitans* infection (MOI 50 and 100) significantly increased the secretion of both TNF-α, IL-1β, and IL-8. The secretion of all three cytokines was significantly inhibited by rosehip extract. The results indicate that rosehip extract can be effective in inhibiting the production of inflammatory cytokines induced by *A. actinomycetemcomitans* infection in THP-1-derived macrophages.

3. Effect of rosehip extract on MAP kinase and NF-κB activation induced by *A. actinomycetemcomitans* in THP-1-derived macrophages

To determine whether rosehip extract has anti-inflammatory properties by regulating the MAP kinases and NF-κB expression, the phosphorylation of c-Jun-N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), and NF-κB was measured in *A. actinomycetemcomitans*-infected THP-1-derived macrophages. *A. actinomycetemcomitans* infection (MOI 50) significantly increased the phosphorylation of both MAP kinase and NF-κB expression. Rosehip extract inhibited the phosphorylation of JNK, p38, and ERK. Rosehip extract also suppressed the phosphorylation of NF-κB in *A. actinomycetemcomitans*-infected THP-1-derived macrophages. These results indicate that the anti-inflammatory properties of rosehip extract are dependent on MAP kinase and NF-κB pathway.

4. Effect of rosehip extract on inflammasome activation in THP-1-derived macrophages induced by *A. actinomycetemcomitans*

To examine the effects of rosehip extract on IL-1β secretion, in addition to pro-IL-1β expression, the expression of inflammasome components were examined. Rosehip extract inhibited the pro-IL-1β protein expression which was induced by *A. actinomycetemcomitans* infection. Furthermore, rosehip extract suppressed the expression of both the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) and Absent in Melanoma 2 (AIM2) that was induced by *A. actinomycetemcomitans* infection. Therefore, rosehip extract can inhibit IL-1β secretion via the down-regulation of NLRP3 and AIM2 inflammasome activation.

Discussion

Inflammation is one of the main characteristics of periodontitis [8]. Periodontitis is caused by a disrupted response of the immune system against several periodontal pathogens [8]. The main etiologic bacteria of periodontitis were *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, and *T. forsythia* [14]. Excessive inflammatory expression of inflammatory cells including monocytes and macrophages adversely affects the gingiva surrounding bone cells and periodontal ligament cells, destroying the alveolar bone [2]. Thus, we examined the effect of rosehip on inflammatory response in THP-1 derived macrophages induced by *A. actinomycetemcomitans* infection to suggest effective agent for the control of periodontal inflammation.

Rosehip has been used for medicine that contained many bioactive constituents [15]. Their anti-oxidant activity is due to the content of polyphenols, vitamins C, E, B and carotenoids and these compounds may have synergistic effects [16]. The proper intake of vitamin C may improve periodontal health in adults [17]. Vitamin deficiency is a local cause of periodontitis and vitamin intake is recommended as an oral supplement [18]. The anti-oxidant activity of rosehip has been reported to be effective in various disease including rheumatoid arthritis, osteoporosis, diabetes, and hyper-lipidaemia [19]. Therefore, we selected rosehip as a candidate material for periodontal inflammation treatment and its effect on inflammatory response induced by *A. actinomycetemcomitans* was examined.

Drug solubility is one of the important parameters to achieve a systemic circulation for desired pharmacological response [20]. However, most chemical substrates developed in pharmaceutical industry are insoluble in water. In the experiment, we made the rosehip extract into a water-boiled solution which can absorb well into the oral cavity. In addition, it has advantages in terms of time and cost in manufacturing when considering future oral applications.

At first, we examined the viability of THP-1-derived macrophages and *A. actinomycetemcomitans* growth in the presence of rosehip extract (Fig. 1). Rosehip extract did not show any cytotoxic effect on THP-1-derived macrophages at all

concentrations up to 10%. As for bacterial growth, from 1%, rosehip extract inhibited *A. actinomycetemcomitans* growth. These results indicated that water-soluble rosehip extract has antibacterial effect on *A. actinomycetemcomitans*.

Next, the effects of rosehip extract on inflammatory cytokine production induced *A. actinomycetemcomitans* in THP-1-derived macrophages were examined. Rosehip extract significantly suppressed the production of TNF-α, IL-1β, and IL-8 induced by *A. actinomycetemcomitans* infection as shown in Fig. 2. IL-8 produciton is an early response of macrophages after phagocytosis. IL-8 is a pro-inflammatory chemokine that attracts and activates neutrophils in inflammatory regions [21]. The response of neutrophils to IL-8 is characterized by migration of the cells, the release of granule enzymes including nicotinamide adenine dinucleotide phosphate oxidase and elastase [16]. Connective tissue was degraded by neutrophil enzymes, released upon activation. Excessive degradation of the connective tissue surrounding the teeth has adverse effects such as the teeth loss [16]. And secretion of pro-inflammatory cytokines such as IL-1β and TNF-α is one of the most important initial responses of macrophage to resist periodontal pathogens [8]. These cytokines activate osteoclasts and cause bone resorption [22]. In this regard, over-expression of IL-1β and TNF-α should be regulated to prevent periodontitis progressions [9].

Next, The MAP kinase and NF-κB signaling pathway are one of the best understood immune-related pathways in macrophages. The inhibition of JNK, p38, ERK, and NF-κB activation significantly reduced the expression of TNF-α and IL-1β in bone marrow derived macrophage cell [23]. And, lipopolysaccharide stimulated THP-1 cells increased the production IL-8 via NF-κB signaling [24]. Upon bacterial invasion, MAP kinase and NF-κB has been identified as common signaling pathways. Therefore, we investigated if rosehip extract modulated these pathways. As shown in Fig. 3, rosehip extract suppressed the phosphorylation of JNK, p-38, ERK, and NF-κB which were increased by *A. actinomycetemcomitans* infection in THP-1-derived macrophages. Moreover, rosehip extract reduced the pro-IL-1β expression (Fig. 4) which could be resulted from the inhibition of MAP kinase and NF-κB phosphorylation.

Similar to the present results, another study showed that rosehip extract suppressed IL-1β-induced NF-κB activation by inhibition of IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation in chondrocytes treated with IL-1β [25]. Rosa mosqueta oil supplementation also prevented the obese phenotype observed in high fat diet-fed mice by downregulating inflammatory cytokine secretion [26]. Thus, the inhibition of MAP kinase and NF-κB activation could suggest the anti-inflammatory propeties of rosehip.

Among various cytokines, IL-1β is closely related with periodontal inflammation. IL-1β release is strictly regulated by two signal pathways [27]. The first signal pathway for IL-1β activation was initiated by NF-κB activation, and the subsequent pathway is the caspase-1-dependent secretion of IL-1β via inflammasome activation [28]. The IL-1β release requires in-

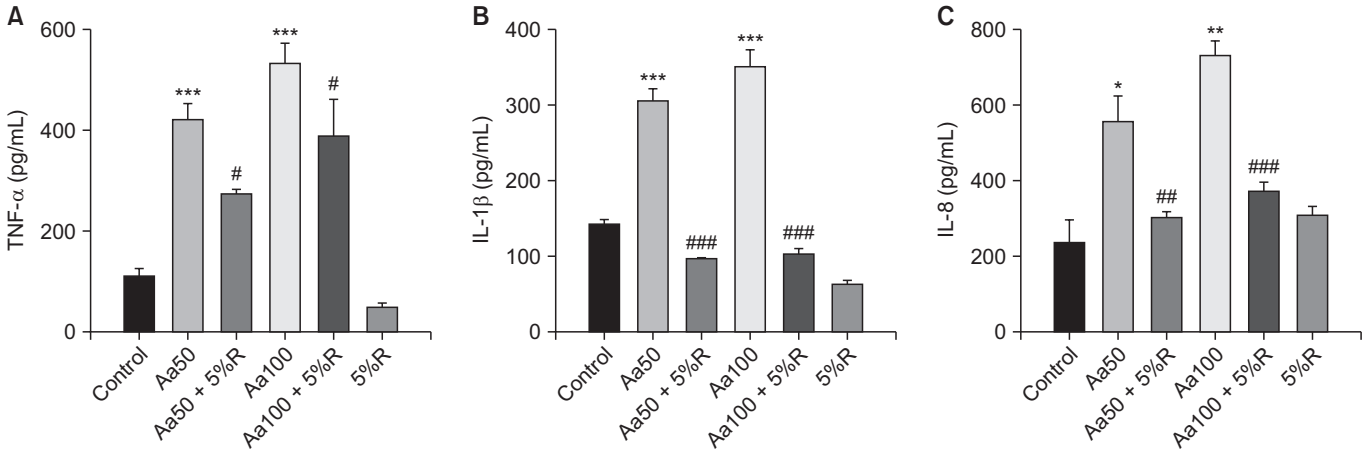

Fig. 2. Inhibitory effect of rosehip extract on inflammatory cytokine secretion induced by *Aggregatibacter actinomycetemcomitans*. (A–C) THP-1-derived macrophages were pretreated with 5% rosehip extract for 30 minutes and infected with *A. actinomycetemcomitans* (MOI 50 and 100) for 24 hours. Culture supernatant was assayed for TNF-α, IL-1β, and IL-8 by ELISA. Data represent mean values ± SD (n ≥ 3).
Aa, *A. actinomycetemcomitans*; R, rosehip; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1-beta; IL-8, interleukin-8; MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus *A. actinomycetemcomitans* infection.

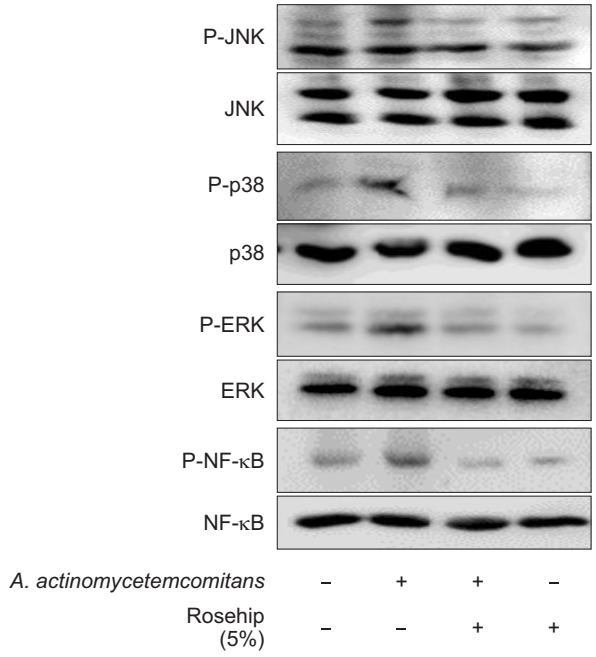

Fig. 3. Inhibitory effect of rosehip extract on MAP kinase and NF-κB activation induced by *Aggregatibacter actinomycetemcomitans*. THP-1-derived macrophages were pretreated with 5% rosehip extract for 30 minutes and infected with *A. actinomycetemcomitans* (MOI 50) for 24 hours. The expression of NF-κB and MAP kinase was analyzed by immunoblot analysis.
MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; MOI, multiplicity of infection; JNK, c-Jun-N-terminal kinase; ERK, extracellular signal-regulated kinase.

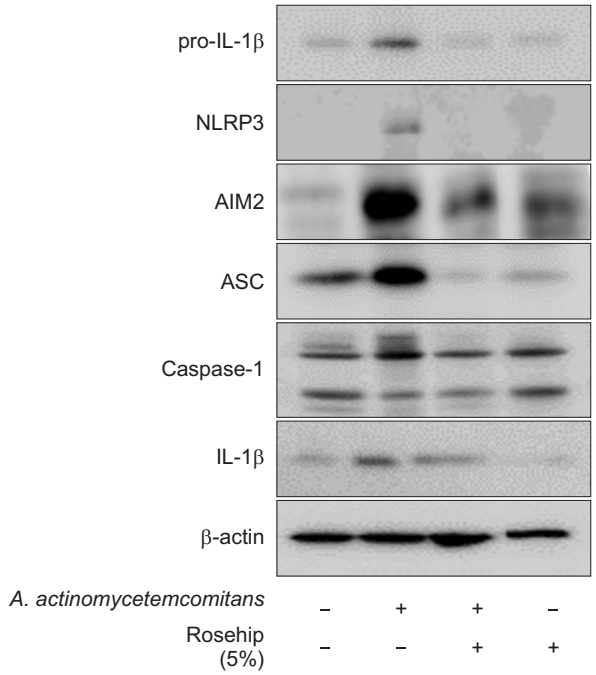

Fig. 4. Inhibitory effect of rosehip extract on the activation of inflammasome pathway induced by *Aggregatibacter actinomycetemcomitans*. THP-1-derived macrophages were pretreated with 5% rosehip extract for 30 minutes and infected with *A. actinomycetemcomitans* (MOI 50) for 24 hours. Expression of pro-IL-1β, NLRP3, AIM2, ASC, Caspase-1, and IL-1β was analyzed by immunoblot analysis.
IL-1β, interleukin-1-beta; NLRP3, NOD-like receptor pyrin domain-containing protein 3; AIM2, Absent in Melanoma 2; ASC, apoptosis-associated speck-like protein containing a CARD; MOI, multiplicity of infection.

flammasome activation, which converts pro-IL-1β to IL-1β. Inflammasome is composed of NLR or AIM2 family receptors and procaspase-1 [19]. Previously, we have reported an increased IL-1β secretion through inflammasome activation that was activated by *A. actinomycetemcomitans* infection [29].

In this study, rosehip extract inhibited NLRP3 and AIM2 inflammasome activation leading to the suppression of caspase-1 activation and IL-1β secretion (Fig. 4). Thus, the suppressive effects of rosehip extract on *A. actinomycetemcomitans*-induced IL-1β secretion were mediated by inflammasome pathway. This study is the first report to explain the action mechanism of rosehip to exert anti-inflammatory effect via inhibition of inflammasome activation.

In summary, our data showed that rosehip extract inhibited the inflammatory response against *A. actinomycetemcomitans* infection. Therefore, rosehip possibly can be applied for the treatment of periodontal inflammation.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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