Intracellular Trafficking Modulation by Ginsenoside Rg3 Inhibits Brucella abortus Uptake and Intracellular Survival within RAW 264.7 Cells

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Introduction

Brucellosis is probably the most common zoonotic infection globally in humans and animals, caused by bacteria of the genus Brucella spp., which are facultative intracellular and gram-negative parasites. It is also known as “undulant fever” with symptoms and signs of fever, chills, sweats, headache, weight loss, testicular pain, epididymal-orchitis, and central nervous system abnormalities in humans [1]. In animals, it causes abortion at late gestation, orchitis, and infertility that is responsible for severe economic losses [2]. This pathogen is classified as a category (B) pathogen by the Centers for Disease Control and Prevention, USA and causes more than 500,000 human infection cases annually [3, 4]. In addition, it possesses the ability to invade and replicate within phagocytic and non-phagocytic cells. Recent studies revealed its functional mechanisms to adapt to the macrophage-inside environment, such as blocking receptors for innate immunity, avoiding fusion with lysosomes, inhibiting apoptosis, and downregulating antigen presentation [2, 5]. Currently, treatment regimens of two or more antibiotics are used to prevent relapses. However, antibiotic treatment may lead to increasing drug resistance [6]. In addition, vaccination is also an effective method to

Ginsenoside Rg3, a saponin extracted from ginseng, has various pharmacological and biological activities; however, its effects against Brucella infection are still unclear. Herein, the inhibitory effects of ginsenoside Rg3 against intracellular parasitic Brucella infection were evaluated through bacterial infection, adherence assays, and LAMP-1 colocalization, as well as immunoblotting and FACS for detecting MAPK signaling proteins and F-actin polymerization, respectively. The internalization, intracellular growth, and adherence of Brucella abortus in Rg3-treated RAW 264.7 cells were significantly decreased compared with the Rg3-untreated control. Furthermore, an apparent reduction of F-actin content and intensity of F-actin fluorescence in Rg3-treated cells was observed compared with B. abortus-infected cells without treatment by flow cytometry analysis and confocal microscopy, respectively. In addition, treating cells with Rg3 decreased the phosphorylation of MAPK signaling proteins such as ERK 1/2 and p38 compared with untreated cells. Moreover, the colocalization of B. abortus-containing phagosomes with LAMP-1 was markedly increased in Rg3-treated cells. These findings suggest that ginsenoside Rg3 inhibits B. abortus infection in mammalian cells and can be used as an alternative approach in the treatment of brucellosis.

Keywords: B. abortus, ginsenoside Rg3, intracellular growth, macrophage, inhibitory effect
control brucellosis, but there is no effective vaccine available for humans [7]. Because of safe, efficient, and economic properties, the use of traditional medicine such as natural plant extract has become a potent candidate for alternative therapy against brucellosis.

Panax ginseng is a famous medicinal herb and has been used widely in traditional medicine in Asia and around the world. It possesses many pharmaceutical benefits and thus the world ginseng market is estimated to be worth 2.08 billion US$ [8]. The major bioactive components of P. ginseng are the ginsenosides, which are triterpene saponins [9]. Various pharmacological effects have been demonstrated, including immunomodulatory, anticarcinogenic, anti-inflammatory, and antidiabetic effects as well as effects on the central nervous system [10]. A study by Arayan et al. [11] exhibited the inhibitory effect of ginseng saponin fraction-A (RGSF-A) to B. abortus infection.

We hypothesized that ginsenoside Rg3, which is one of the panaxadiol saponin components of RGSF-A, has the potential ability to control brucellosis. Hence, this study aimed to determine the effects of Rg3 on phagocytosis and intracellular trafficking of B. abortus in RAW 264.7 cells. The results in this study suggested that ginsenoside Rg3 possesses inhibitory ability against B. abortus infection in phagocytes and has potential as a therapeutic agent against brucellosis.

Materials and Methods

Ginsenoside Rg3 Preparation

Ginsenoside Rg3 was purchased from Ambo Institute (Korea). The powder was dissolved in phosphate-buffered saline solution (PBS, pH 7.4) and filtered through 0.45 μm membranes (Minisart; Sartorius Stedim Biotech, Germany).

Bacteria and Cell Culture

The wild-type B. abortus strains were derived from 544 (ATCC 23448), a smooth, virulent B. abortus biovar 1 strain. B. abortus was cultured in Brucella broth (Becton Dickinson, USA) or Brucella broth containing 1.5% agar. Murine macrophage RAW 264.7 cells (ATCC; TIB-71) were grown at 37°C with 5% CO₂ in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (all provided by Gibco, Invitrogen, USA). Cells were seeded in cell culture plates and incubated overnight before infection for all assays.

Bactericidal Analysis

Bacteria grown to stationary phase were diluted with PBS to a concentration of 2 × 10⁵ colony forming units (CFU)/ml and added to PBS containing different concentrations of Rg3 (0.01, 0.1, 1 mg/ml), and incubated at 37°C for 0, 1, 4, 8, and 24 h. After incubation, each diluent was plated onto Brucella agar and cultured for 3 days at 37°C to determine CFUs. The bacterial survival rates were expressed as previously reported [11].

Cytotoxicity Assay

The cytotoxic effect of Rg3 on RAW 264.7 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells (1 × 10⁵ cells/ml) were incubated with different concentrations of Rg3 (0, 0.001, 0.01, 0.1, 1 and 10 mg/ml) in a 96-well culture plate for 1 h. After 1 h, the cells were incubated in medium with MTT solution (5 mg/ml) at 37°C in 5% CO₂ for 4 h. The product was measured at an optical density (OD) of 540 nm.

Bacterial Uptake and Intracellular Replication Assay

RAW 264.7 cells were pretreated with the highest non-cytotoxic concentration of Rg3 (0.1 mg/ml) for 3 h before infection. Bacteria were deposited onto cells at multiplicities of infection (MOIs) of 10, centrifuged at 1,500 rpm for 10 min at room temperature, and incubated at 37°C in 5% CO₂ for 0 and 30 min. The infected cells were washed with PBS and then incubated at 37°C in RPMI 1640 containing 10% FBS with gentamicin (30 μg/ml) for 30 min to kill any remaining extracellular and adherent bacteria. The infected cells were washed with PBS and lysed with distilled water. Bacterial CFUs were measured by serial dilutions on Brucella agar plates in triplicates.

For intracellular growth assay, the infected cells were incubated at 37°C for 1 h, washed with PBS, incubated on RPMI 1640 containing 10% FBS containing gentamicin (30 μg/ml) with Rg3 (0.1 mg/ml), and then incubated for 2, 24, or 48 h. Cell washing, lysis, and plating procedures were the same as the analysis of bacteria in the internalization assay. All assays were conducted in triplicates.

Bacterial Adherence Assay

RAW 264.7 cells were cultured in 12-well plates with 18-mm-diameter glass coverslips (Fisher Scientific, USA) (1 × 10⁵ cells per well). The cells were pretreated with Rg3 (0.1 mg/ml) for 3 h. During the last 40 min of pretreatment, cytochalasin D (0.5 mg/ml) was added to the cells to inhibit bacterial internalization. After that, the cells were infected with B. abortus at an MOI of 10 and centrifuged at 150 ×g for 10 min and then incubated at 37°C in 5% CO₂ for 30 min. The cells were washed with PBS, fixed with 4% paraformaldehyde, and incubated at 37°C for 30 min. The samples were permeabilized at −20°C in methanol for 10 sec. Preparations were stained with anti-B. abortus polyclonal rabbit serum (1:500) for 1 h at 37°C and then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:1,000; Sigma-Aldrich, USA) for 1 h at 37°C. Finally, the samples were washed and mounted with fluorescent mounting medium (Dako North America, Inc., USA). All images were collected using a microscope (Olympus IX70, Japan). One hundred macrophages were randomly selected, and the adherent bacteria on the cells were counted.
F-actin Staining

The cells were pretreated with Rg3 (0.1 mg/ml) for 4 h. B. abortus cells were harvested and suspended in PBS, adjusted to 1 x 10^6 CFU/ml. Then, B. abortus cells were pelleted and suspended in 1 ml of FITC (0.5 mg/ml) (Sigma-Aldrich) in 50 mM sodium carbonate-100 mM sodium chloride at pH 9.0 for 20 min at room temperature and then washed with PBS [12]. FITC-conjugated B. abortus cells were deposited onto the cells and then incubated at 37°C for 20 min. The infected cells were fixed with 4% paraformaldehyde for 1 h at 37°C, permeabilized with 0.1% Triton X-100 for 10 min at 22°C, and incubated with blocking buffer (2% goat serum in PBS) for 30 min at 37°C. The cells were incubated with 0.1 μM rhodamine-phalloidin (Cytoskeleton, USA) for 30 min at 22°C.

Fluorescence-Activated Cell Sorting Assay for Quantifying F-actin Polymerization

RAW 264.7 cells were pretreated with Rg3 (0.1 mg/ml) for 4 h. The relative content of F-actin polymerization in B. abortus-infected or -uninfected cells for the indicated time was determined by FACS assay. After treatment, the cells were infected for 30 min, fixed with 4% paraformaldehyde at 22°C for 30 min, permeabilized, and then stained with 20 μg/ml lysophosphatidylcholine containing 1 mM tetramethyl rhodamine isothiocyanate-phalloidin (Sigma-Aldrich) for 1 h at 22°C. The cells were centrifuged at 300 x g for 5 min at 4°C and washed. The F-actin content was quantified as log-scaled fluorescence histograms from 10,000 cells using a FACSAlibur flow cytometer (BD Biosciences, USA). The average F-actin content of a population was expressed as the mean of the fluorescence intensity. This experiment was conducted in triplicates.

Immunoblot Analysis

The immunoblot analysis method was performed as described by MacPhee [13]. RAW 264.7 cells were treated with Rg3 (0.1 mg/ml) and infected with B. abortus for the indicated time. The cells were then washed with ice-cold PBS and lysed using ice-cold radioimmunoprecipitation assay buffer with 1% protease inhibitor cocktail for 30 min at 4°C. Samples were separated by SDS-PAGE and electrically transferred onto Immobilon-P membranes (Millipore, USA) using 1× transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) with a constant current of 2 mA per cm^2 for 1 h in a semi-dry electroblot assembly (Bio-Rad, USA). The membrane was blocked with 5% skim milk in 1× Tris-buffered saline-Tween 20 (20 mM Tris-HCl, 150 mM NaCl, TWEEN 0.1%, pH 7.6) for 30 min at room temperature and incubated with phospho-specific antibodies for ERK 1/2 (Thr183/Tyr185), JNK (Thr183/Tyr185), and p38 (Thr180/Thr182) overnight at 4°C. Pan antibodies and β-actin antibody were applied to verify equivalent amounts of protein loading per lane. The membrane was washed and incubated with horseradish peroxidase-conjugated protein G (Thermo Scientific, USA; 1:1,000 dilution) in 5% blocking buffer for 1 h. Finally, the signal was detected with a luminal-coumaric acid-H_2O_2 detection solution (Atto Corporation, Japan) and Molecular Imager ChemiDoc XRS + system machine (Bio-Rad Laboratories, USA). The immunoblot signals were quantified using NIH ImageJ software.

LAMP-1 Staining

RAW 264.7 cells were infected and incubated for 1 h at 37°C, washed, and then incubated in RPMI 1640 containing FBS and gentamicin (30 μg/ml) and treated with Rg3 (0.1 mg/ml) for 2 h. Fixation, permeabilization, and blocking procedures were performed similarly to that of F-actin staining. Afterwards, all the staining procedures were performed for 1 h at 37°C: anti-LAMP-1 rat monoclonal antibody (1:100), Texas red-goat anti-rat IgG (1:1,000), anti-B. abortus rabbit serum (1:500), and FITC-conjugated goat serum (1:500). Finally, the cells were washed and mounted. Fluorescence images were captured using a laser scanning confocal microscope (Olympus FV1000) and processed using FV10-ASW Viewer 3.1 software. The extent of LAMP-1 acquisition of bacteria was analyzed as previously described [11].

Fig. 1. Effect of Rg3 on survival rate of B. abortus and RAW 264.7 cells.

The antibacterial activities of Rg3 on B. abortus (A), and cytotoxicity activity of Rg3 on RAW 264.7 cells (B). Data represent the mean ± SD of duplicate samples from three identical experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (***, p < 0.001).

Statistical Analysis

The results obtained were expressed as the mean ± standard deviation (SD). Student’s t-test was used to make a statistical comparison between the groups. Results with $p < 0.05$ were considered statistically significant.

Results

Effect of Rg3 on Bacterial and Macrophage Viability

The bacterial survival rates were not affected when *B. abortus* was treated with different concentrations of Rg3 (0.01, 0.1, and 1 mg/ml) at various time (0, 1, 4, 8, and 24 h). Therefore, the results suggest that Rg3 has no bactericidal effect against *B. abortus* (Fig. 1A). RAW 264.7 cells were incubated with various concentrations of Rg3 (0, 0.001, 0.01, 0.1, 1, and 10 mg/ml) for 48 h. The OD value (cell survival) at higher concentrations (1 and 10 mg/ml) decreased, which indicated a cytotoxic effect. Conversely, the OD values at the concentration of 0.1 mg/ml did not decrease significantly compared with untreated control cells. These results indicate that Rg3 did not have any cytotoxic effect at a concentration at or below 0.1 mg/ml (Fig. 1B). Thus, the highest non-cytotoxic concentration of Rg3 (0.1 mg/ml) was used in all subsequent experiments.

Effect of Rg3 on Bacterial Internalization and Intracellular Growth

The results showed that the uptake of *B. abortus* in Rg3-treated cells was significantly decreased compared with the untreated control cells after both 0 and 30 min of infection ($p < 0.05$) (Fig. 2A). Similarly, the intracellular replication of *B. abortus* in Rg3-treated cells was significantly reduced at 2, 24, and 48 h post-infection, as compared with that of the untreated control cells ($p < 0.05$) (Fig. 2B).

![Fig. 2. Effect of Rg3 on the invasion and intracellular growth of *B. abortus*.](image)

Bacterial internalization efficiency (A), and bacterial intracellular growth efficiency (B). Data represent the mean ± SD of duplicate samples from three identical experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

![Fig. 3. Effect of Rg3 on the adherence of *B. abortus* to macrophages.](image)

Fluorescence images were collected using a microscope equipped with a camera. One hundred macrophages were randomly selected, and the bacteria that adhered to the cells were counted. Data represent the mean ± SD of triplicate samples in at least three independent experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (*, $p < 0.05$).
Effect of Rg3 on Phagocytosis of \textit{B. abortus} in Macrophages

The number of adherent bacteria to Rg3-treated cells (30 ± 1) was significantly decreased compared with untreated control cells (73.33 ± 1.53), showing a reduction rate of 59.09 ± 0.37% (p < 0.05) (Fig. 3).

The results showed that the inhibitory effect of Rg3 on F-actin polymerization was apparent in Rg3-treated cells compared with the untreated control cells, and displayed a reduction in filopodia and lamellipodia scattering at the periphery of the cells (Fig. 4A). Moreover, F-actin fluorescence intensity in Rg3-treated cells was significantly reduced (p < 0.01) compared with the \textit{B. abortus}-infected untreated cells. There was no significant difference in F-actin fluorescence between the uninfected Rg3-treated cells and the uninfected control cells (Fig. 4B).

Effects of Rg3 on Phagocytic Signals and the Intracellular Trafficking of \textit{B. abortus}

Western blot analysis demonstrated that Rg3 treatment led to a reduction in the phosphorylation levels of ERK 1/2 and p38 in \textit{B. abortus}-infected treated cells compared with control cells at 30 min post-infection by 32.91% and 38.53%, respectively (Fig. 5). No difference in the phosphorylation levels of JNK was found between the \textit{B. abortus}-infected treated and untreated control cells.

Colocalization of LAMP-1 with \textit{B. abortus}-containing...
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phagosomes (BCPs) in Rg3-treated cells increased by 2.65-fold after 2 h of incubation compared with untreated control cells ($p < 0.001$) (Fig. 6).

**Discussion**

Brucellosis is still considered a serious zoonosis that causes enormous disease burden in livestock and humans, and heavy economic losses. *Brucella* spp. have the ability to replicate inside various mammalian cell types with intracellular lifestyle; restrict exposure to the host innate and adaptive immune response drive the unique feature of pathology in infected hosts and insulate the hosts from the effects of antibiotics [14]. Moreover, nonspecific influenza-like symptoms in humans as well as the appropriate therapy deficiency in acute infection duration causes the translocation of *Brucella* to various tissues and organs that leads to chronic disease, which is difficult to treat [4, 15]. Treatment of brucellosis by using traditional plants and natural products has been massively reported. *P. ginseng* is very popular because of its pharmacological benefits. Ginsenosides and acidic polysaccharide are active constituents in most ginseng species [16]. The recent studies proved that both ginsenosides (RGSF-A) and acidic polysaccharides had inhibitory effects on *Brucella* infection [11, 17].

RGSF-A contains a high percentage of panaxadiol saponins, and Rg3 is one of the characteristic panaxadiol saponin constituents of RGSF-A [18]. Therefore, to gain more insight into the inhibitory effects of RGSF-A on *Brucella* infection, we hypothesized that the Rg3 component of RGSF-A possesses the ability to control *Brucella* infection. Consistent with our expectation, our results showed that Rg3 reduced phagocytosis and intracellular survival of *B. abortus* in macrophages.

Adhesion to the host cell is one of the key steps in the infection process of the bacterial pathogen. Thus, disrupting bacterial adhesion is necessary to impair

![Fig. 5. Effect of Rg3 on the activation of intracellular signaling pathways required for the phagocytosis of *B. abortus*. Immunoblot analysis of total cell lysates was assessed by phosphospecific and pan antibodies against ERK1/2 and p38α in RAW 264.7 cells at the indicated time.](image)

![Fig. 6. Effect of Rg3 on intracellular trafficking of *B. abortus*. The percentage of LAMP-1 colocalized with *B. abortus*-containing phagosomes (A) and the LAMP-1 positive (arrows) or negative bacteria (arrow heads) are shown (scale bars = 5 μm) (B). The results are representative of three separate experiments. Data represent the mean ± SD of triplicate experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (****, $p < 0.001$).](image)
bacterial infection. Previous studies have demonstrated the antiadhesive effects of acidic polysaccharide from *P. ginseng* against *Helicobacter pylori* on gastric epithelial cells as well as *Actinobacillus actinomycetemcomitans*, *Propionibacterium acnes*, and *Staphylococcus aureus* [19]. In this study, bacterial adhesion was significantly decreased in the Rg3-treated cells compared with controls, which led to interfering with the *Brucella*-host cell interaction during infection. According to the adherence-hindering effect, the internalization of *B. abortus* in Rg3-treated cells was significantly reduced compared with the control. A recent study found that Rg3 inhibited proliferation of human melanoma cell line A375 by downregulating the EGFR/MAPK signaling pathway [20]. In agreement with the previous study, this study showed that Rg3 attenuated the activation of MAPKs (ERK 1/2 and p38) in *B. abortus*-infected cells. Moreover, it has been proven that the MAPK signaling pathway is involved in promoting actin polymerization [21] and polymorphonuclear neutrophil phagocytosis [22]. Studies of Kusumawati *et al.* [23] and Guzman-Verrti *et al.* [24] elucidated that F-actin polymerization is also involved in the phagocytosis of *Brucella* in macrophages and epithelial cells. In the present study, we proposed that Rg3 could interrupt phosphorylation of MAPKs that could negatively affect F-actin polymerization. Consistent with our expectation and previous studies, Rg3 also reduced F-actin polymerization, as shown in the fluorescent microscopy and FACS experiments. These findings indicated that the inhibitory effects of Rg3 on the invasion of *Brucella* into macrophages could be due to its negative effect on MAPK-linked phagocytic signaling pathways that could disrupt polymerization of actin.

A key aspect of the virulence of *Brucella* is its ability to survive within host cells by maturation of the BCPs, inhibiting its fusion with lysosome, which forms an organelle with degradative and antimicrobial components essential for the killing of internalized microorganisms, and driving the pathogen towards the intracellular multiplication niche at the endoplasmic reticulum [25, 26]. In the present study, Rg3 treatment enhanced the colocalization of the lysosomal marker LAMP-1 with the BCPs, which suggested that intracellular growth of *B. abortus* was reduced up to 48 h post-infection in Rg3-treated macrophages.

In conclusion, ginsenoside Rg3 exhibited suppressive effects on *B. abortus* infection through the inhibition of bacterial phagocytosis and intracellular replication in macrophages. Therefore, we propose that Rg3 treatment can be considered as a safe and natural alternative therapy for the control and treatment of brucellosis.

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### References


