Inhibitory Effect of the Ethanol Extract of a Rice Bran Mixture Comprising *Angelica gigas*, *Cnidium officinale*, *Artemisia princeps*, and *Camellia sinensis* on *Brucella abortus* Uptake by Professional and Nonprofessional Phagocytes

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Received: May 2, 2017
Revised: August 9, 2017
Accepted: September 1, 2017

First published online
September 5, 2017

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pISSN 1017-7825, eISSN 1738-8872

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In this study, we evaluated the inhibitory effect of a rice bran mixture extract (RBE) on *Brucella abortus* pathogenesis in professional (RAW 264.7) and nonprofessional (HeLa) phagocytes. We fermented the rice bran mixture and then extracted it with 50% ethanol followed by gas chromatography-mass spectrometry to identify the components in RBE. Our results clearly showed that RBE caused a significant reduction in the adherence of *B. abortus* in both cell lines. Furthermore, analysis of phagocytic signaling proteins by western blot assay revealed that RBE pretreatment resulted in inhibition of phosphorylation of JNK, ERK, and p38, leading to decline of internalization compared with the controls. Additionally, the intensity of F-actin observed by fluorescence microscopy and FACS was remarkably reduced in RBE-pretreated cells compared with control cells. However, the intracellular replication of *B. abortus* within phagocytes was not affected by RBE. Taken together, these findings suggest that the phagocytic receptor blocking and suppressive effects of RBE on the MAPK-linked phagocytic signaling pathway could negatively affect the invasion of *B. abortus* into phagocytes.

Keywords: *B. abortus*, phagocyte, rice bran mixture extract, phagocytic signaling, pathogenesis

Introduction

Brucella spp. are facultative intracellular gram-negative bacteria that cause brucellosis in animals and humans. They can invade and replicate within a variety of phagocytes, such as macrophages, epithelial cells, and placental trophoblasts, displaying diverse interactions with host cells. However, the underlying mechanisms of host invasion are not fully understood [1, 2]. *Brucella* spp. have developed stealthy survival strategies, and thus there exists a need to develop new therapeutic interventions [3].

Vaccination seems to be the predominant strategy to eliminate this disease; however, there is no 100% efficacious vaccine for animals and humans. The current live-attenuated *Brucella* strains such as *B. abortus* S19 or *B. melitensis* Rev 1

strain used for cattle and goat still have some disadvantages, including abortion in animals when administered during pregnancy and being fully virulent for humans [4]. Additionally, elevated doses and extended use of antibiotics are still under debate [1]. Biological therapy should therefore be a major consideration due to the disadvantages and financial burden of conventional therapy in the treatment of infectious diseases [5].

Rice bran mixture extract (RBE) is mainly extracted from four traditional medicinal plants in Asia; namely, *Angelica gigas*, *Cnidium officinale*, *Artemisia princeps*, and *Camellia sinensis*. These have been reported as efficacious against bacterial infection, inflammation, and cancer. In particular, various pharmacological properties of *Angelica gigas* have been reported recently, such as antibacterial [6, 7], anticancer

[8, 9], antitumor [10], antioxidant, and neuroprotective activities [11]. In parallel, the ethanol extract from *Cnidium officinale* was shown to affect the release of proinflammatory mediators, TNF- α , IL-1 β , IL-6, and nitric oxide (NO), in murine peritoneal macrophages and splenocytes [12]. In addition, *Artemisia princeps* is widely used to maintain hemostasis and in the treatment of pain, inflammation, amenorrhea, uterine hemorrhage, hemorrhoids, and menopausal diseases. Its ethanol extract inhibits the proliferation, acid production, biofilm formation, and expression of virulence genes in antibiotic-resistant bacteria [13]. On the other hand, green tea, produced from the leaves of the plant *Camellia sinensis*, is one of the most popular beverages consumed worldwide and is a rich source of dietary flavonoids in humans. Recently, the antimicrobial activity of *Camellia sinensis* extract was demonstrated against multidrug-resistant bacteria [14].

Although various activities of the individual extracts on bacterial infections involved in host immune response have been evaluated, their effects on the infection with *B. abortus* have not been elucidated. Thus, in this study, we investigated the specific effects of RBE on *B. abortus* infection.

Materials and Methods

Bacterial Strain and Cell Culture

A smooth, virulent *B. abortus* 544 biovar 1 strain was cultured in Brucella broth (BD Biosciences, USA) at 37°C until stationary phase. RAW 264.7 and HeLa cells were grown in RPMI 1640 and DMEM, respectively, at 37°C in 5% CO₂ atmosphere containing 10% heat-inactivated FBS with or without 100 U/ml penicillin and 100 μ g/ml streptomycin.

RBE Preparation

The fermented RBE was prepared as follows: the rice bran containing *Angelica gigas*, *Cnidium officinale*, *Artemisia princeps*, and *Camellia sinensis* (ratio of 80:8:6:6 (v/v)) was injected with enzymes of *Lactobacillus rhamnosus* (α -D-glucosidase, β -D-glucosidase, α -D-galactosidase, β -D-galactosidase, β -D-glucuronidase, and in major quantity α -L-rhamnosidase, since it is responsible for the hydrolysis of terminal non-reducing α -L-rhamnose sugar to reducible α -L-rhamnoside sugars, making it an important enzyme for the breakdown of sugars present in the rice bran for more bioavailability [15]; 6.2×10^5 colony forming unit (CFU)/ml) and *Pichia deserticola* (phospholipase A1, phospholipase A2, phospholipase C, phospholipase B, phospholipase D, and acyltransferase. Phospholipases were the major enzymes injected in rice bran mixture because they hydrolyze the breakdown of fatty acids and lecithin into bioavailable lipophilic substances forming lysolecithin that is responsible for recruitment of phagocytes; 3.7×10^4 CFU/ml) [16]. The hydrated enzymes of *Lactobacillus rhamnosus* and *Pichia deserticola* and rice

bran mixture were mixed well and then fermented at room temperature in a dark container [17]. The mixture was then extracted with 50% ethanol, filtered with Whatmann filter paper, and evaporated. Later it was freeze dried and the powdered form obtained was fermented RBE.

Gas Chromatography-Mass Spectrometry Analysis

Gas chromatography-mass spectrometry (GC-MS) was carried out as described previously [18]. Briefly, to identify its phytochemical constituents, small amounts of extracts (2.5 mg) were analyzed by GC-MS using an Agilent Technology 7890A-Gas Chromatograph system (Agilent Technologies, USA), coupled to XLMSD-5975C equipment operating in electrospray ionization mode. The relative percentage of each component was calculated by comparing its average peak area to the total area.

Bactericidal Assay

Bacteria were diluted with PBS to a concentration of 2×10^4 CFU/ml, added to PBS containing different concentrations of RBE (1, 10, and 100 μ g/ml), and incubated at 37°C for 0, 4, 8, and 24 h as previously described [3]. Each diluent was then plated on Brucella agar and incubated for 3 days at 37°C. The bacterial survival rates were expressed as the percentage of the survival rate of the treated sample relative to an untreated control, which was set at 100%.

Cytotoxicity Assay

Following overnight culture in 96-well plates at 2×10^4 cell/well, cells were treated with different concentrations of RBE (0.001, 0.1, 1, and 10 mg/ml) for 48 h at 37°C in 5% CO₂. The cytotoxic effect of RBE was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described [3].

Bacterial Uptake and Intracellular Replication Assay

These assays were performed as previously described [3]. To analyze bacterial internalization efficiency, cells were pretreated with different non-cytotoxic concentrations of RBE 4 h prior to infection. Following centrifugation at 150 $\times$ g for 10 min, bacteria were infected at multiplicities of infection of 100 and incubated at 37°C in 5% CO₂ for 0, 15, and 30 min. At the indicated time point, the cells were washed once with PBS and then incubated at 37°C in RPMI or DMEM containing 10% (v/v) FBS and gentamicin (30 μ g/ml) for 30 min to kill any remaining extracellular bacteria. The cells were washed three times with PBS, lysed with distilled water and plated on Brucella agar. Gentamicin protected percentage was calculated as the mean internalized bacteria divided by the originally infected bacteria. For the intracellular growth efficiency measurements, the infected cells were incubated at 37°C for 1 h, washed once with PBS, incubated on RPMI or DMEM containing 10% (v/v) FBS and gentamicin (30 μ g/ml) with different non-cytotoxic concentrations of RBE, and then incubated for 2, 24, or 48 h. Cell washing, lysis, and plating procedures were the same as those utilized in the internalization assay.

Bacterial Adherence Assay

The bacterial adherence assay was performed as previously described [2]. Briefly, phagocytes were cultured in 12-well plates with 18-mm diameter glass coverslips (Fisher Scientific, USA), at a concentration of 1×10^5 cells per well overnight before infection. Cells were pre-incubated with RBE (0.1 and 1 mg/ml) for 4 h, and during the last 40 min of pre-incubation, 2.5 μ l of cytochalasin D (500 μ g/ml) was added. Cells were then infected with *B. abortus* and centrifuged at $150 \times g$ for 10 min at RT and incubated at 37°C in 5% CO₂ for 30 min. The cells were washed three times with PBS, fixed with 4% paraformaldehyde, and incubated at 37°C for 30 min. The adherent bacteria on the cell surface within 30 min of infection were monitored and the cells were washed three times with PBS and then permeabilized at -20°C in methanol for 10 sec. The adherent bacteria were then stained with anti-*B. abortus* polyclonal rabbit serum (1:500) in PBS for 1 h at 37°C and then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:500; Sigma-Aldrich, USA) in PBS for 1 h at 37°C. The preparations were washed and then mounted in DakoCytomation fluorescent mounting medium (Dako North America, Inc., USA). Fluorescence images were collected using a microscope (IX70; Olympus; Japan). One hundred macrophages were randomly selected and the bacteria that adhered to the cells were counted.

Immunoblot Analysis

The lysates of cells were identified by western blot analysis as previously described [19]. After electrophoresis, the separated proteins were transferred onto Immobilon-P membranes (Milipore, USA) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol, pH 8.3) for 60 min using a semi-dry electroblot assembly (Bio-Rad, USA). The membranes were blocked with 5% skim milk (Difco, USA) for 30 min at room temperature and subsequently washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), and then incubated with primary antibodies (1:1,000 dilution) in blocking buffer at 4°C overnight. The membranes were washed with 0.05% PBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:5,000 dilution; Sigma) in blocking buffer for 1 h at room temperature and finally washed with 0.05% PBS-T. The proteins were detected with ECL solution (Thermo Scientific, USA).

F-Actin Staining

RAW 264.7 or HeLa cells were stained for F-actin and examined by immunofluorescence microscopy as previously described [2]. Briefly, 2-day cultured bacteria were harvested and stained for 20 min at room temperature with 1 ml of FITC (0.5 mg/ml) in 50 mM sodium carbonate-100 mM sodium chloride at pH 9.0. The cells were infected with FITC-conjugated bacteria for 15 min and then fixed with 4% paraformaldehyde for 1 h at 37°C. The cells were then 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. The cells were incubated with 0.1 μ M rhodamine-

phalloidin for 30 min and washed three times with PBS. F-actin organization was observed by fluorescence microscopy.

FACS Assay

The relative content of F-actin in *B. abortus*-infected and uninfected cells with or without pretreatment of RBE (1 mg/ml) was evaluated as previously described [2]. Briefly, RAW 264.7 or HeLa cells were cultured in 6-well plates and pretreated with RBE for 4 h prior to infection. The cells were infected for 15 min and fixed with paraformaldehyde at 22°C for 30 min, permeabilized, and stained with 20 μ g/ml lysophosphatidylcholine containing 1 μ M tetramethyl rhodamine isothiocyanate-phalloidin for 1 h at 22°C. The cells were centrifuged at $300 \times g$ for 5 min at 4°C and washed three times with PBS. The F-actin content was quantified by FACS analysis using a FACS Calibur flow cytometer (BD Biosciences) and is represented on log-scale histograms depicting 10,000 cells. The average F-actin content of a population was expressed as the mean fluorescence intensity.

Statistical Analysis

The data are expressed as the mean $\pm$ standard deviation (SD). Student's *t*-test was used to statistically compare the groups. The results with $p < 0.05$ were considered statistically significant.

Results

Chemical Composition Analysis of RBE

Following the extraction of fermented rice bran mixture with 50% ethanol, GC mass spectrometry was performed to identify the chemical composition of RBE. As shown in Table 1, fatty acids and their derivatives are the primary components of RBE.

Table 1. Chemical composition of the rice bran mixture extract analyzed by gas chromatography-mass spectrometry.

Components	%
1,4-Dihydrophenanthrene	0.25
3-Methylenecyclohexyl methyl ether	0.19
Caffeine	0.65
Benzoic acid, 3,5-dihydroxy	0.36
9-Hydroxybutyl-3,9-diazabicyclo	0.53
L-Proline	0.69
Hexadecanoic acid	13.21
9,12-Octadecadienoic acid	37.07
6-Octadecenoic acid	39.87
Linoleic acid, ethyl ester	1.62
10-Octadecenoic acid, methyl ester	0.36
Pyrrolo[1,2-a]pyrazine-1,4-dione	0.27
(2,4-Dichloro-6-nitrophenoxy) acetic acid	0.14
But-2-enoic acid	0.47

Fig. 1. Cytotoxic effect of the rice bran mixture extract on phagocytes, assessed by MTT assay. Data are presented as the mean $\pm$ SD of triplicates.

Bactericidal and Cytotoxicity Assays

The bacterial survival rate of RBE-treated *B. abortus* was not significantly different when compared with that of the controls (data not shown), suggesting that RBE has no bactericidal effect. Additionally, as shown in Fig. 1, treatment and incubation of RAW and HeLa cells with different concentrations of RBE (0.001, 0.1, 1 or 10 mg/ml) revealed that cell survival at a concentration of 1 mg/ml was not decreased compared with untreated cells. These results indicated that RBE did not cause any cytotoxicity to RAW and HeLa cells at or below 1 mg/ml. Thus, concentrations of 0.1 and 1 mg/ml were used for subsequent experiments.

Inhibitory Effect of RBE on Internalization but Not on Intracellular Growth of *B. abortus*

To verify the effects of RBE on the invasion of *B. abortus* in RAW 264.7 and HeLa cells, phagocytes were pretreated with different concentrations of RBE (0, 0.001, 0.1, and 1 mg/ml) and then infected with *B. abortus* for the indicated times (0, 15, or 30 min). The results indicated that pretreatment of RBE at concentrations of 0.1 and 1 mg/ml caused a significant reduction in *B. abortus* invasion of phagocytes in a concentration-dependent manner compared with that of untreated cells (Fig. 2). However, RBE did not cause any effect on the intracellular growth of *B. abortus* in either of the cell lines (data not shown).

Effect of RBE on *B. abortus* Phagocytosis Via Modulation of Adherence and F-Actin Polymerization

Adherence is a critical step of *Brucella* invasion; thus, we tested whether RBE has effect on the adherence of *B. abortus* to phagocyte surface membranes. As shown in Fig. 3, pretreatment with 0.1 and 1 mg/ml of RBE caused significant

Fig. 2. Effect of the rice bran mixture extract (RBE) on *B. abortus* invasion.

RAW 264.7 (A) and HeLa (B) cells were pretreated with or without RBE at different concentrations and then infected with *B. abortus*. Log CFUs were determined at the indicated times. Data are presented as the mean $\pm$ SD of triplicates. Asterisks indicate a significant difference ($p < 0.05$).

Fig. 3. Effect of the rice bran mixture extract (RBE) on bacterial adherence.

Phagocytes were pre-incubated with or without RBE and infected with *B. abortus* for 15 min. The infected cells were stained with different antibodies and 100 macrophages were randomly selected and the bacteria that adhered to the cells were counted. Data are presented as the mean $\pm$ SD of triplicates. Asterisks indicate a significant difference ($p < 0.05$).

reductions of bacterial adherence of approximately 35.2% and 48.6%, respectively, in RAW 264.7 cells, whereas reductions of 47% and 63.5%, respectively, were observed in HeLa cells. In addition, observation by fluorescence microscopy revealed that F-actin polymerization in *B. abortus* invasion was attenuated in the RBE-treated cells compared with untreated control cells (Fig. 4A). In order to quantitatively evaluate the effect of RBE on the F-actin content during *B. abortus* invasion, FACS analysis was performed for phagocytes with or without pretreatment of RBE. Interestingly,

RBE pretreatment led to a remarkable decrease in F-actin fluorescence intensity compared with the *B. abortus*-infected control cells, whereas no difference in F-actin fluorescence was found between the uninfected control cells and the RBE-treated uninfected cells for both cell lines (Fig. 4B).

Downregulation of Phagocytic Signaling by RBE

The phosphorylation levels of ERK1/2, p38a, and JNK in the RBE-treated phagocytes at 15 min post-infection were reduced by approximately 35%, 52%, and 24%, respectively, in RAW 264.7 cells (Fig. 5A) and reduced by 62%, 8%, and 37%, respectively, in HeLa cells (Fig. 5B) compared with those in the infected control cells. These findings indicate that RBE could negatively regulate the activation of mitogen-activated protein kinase (MAPK), resulting in the inhibition of *B. abortus* invasion into professional and nonprofessional phagocytes.

Fig. 4. Effect of the rice bran mixture extract (RBE) on *Brucella* phagocytosis by the modulation of F-actin polymerization. F-actin polymerization and bacterial co-localization (A) and FACS analysis for F-actin content (B) were determined. Data are presented as the mean $\pm$ SD of triplicates. Asterisks indicate a significant difference ($p < 0.05$). Scale bar = 5 mm.

Fig. 5. Effect of the rice bran mixture extract (RBE) on activation of the signaling of phagocytes during *B. abortus* infection. Immunoblot analysis of total cell lysates was evaluated using phospho-specific antibodies against ERK1/2, JNK, and p38a in infected or uninfected RAW 264.7 (A) and HeLa (B) cells pretreated with or without RBE.

Discussion

Brucella spp. are causative agents of human and animal brucellosis [1]. The prolonged use of conventional antibiotics is required for the treatment of this disease; however, this is still a controversial issue. Thus, the application of traditional medicine and natural products for the control of brucellosis seems to be a valid alternative for brucellosis treatment. The elucidation of the precise mechanisms with respect to how a natural product modulates *Brucella* infection is required [2].

Although the individual extracts from RBE, namely, *Angelica gigas*, *Cnidium officinale*, *Artemisia princeps*, and *Camellia sinensis*, have been reported to have a variety of pharmacological effects, such as antibacterial, anticancer, antitumor, antioxidant effects [8–13], the potential therapeutic effects of RBE on *B. abortus* infections have yet to be clinically examined. This study elucidated the specific effect of RBE on *B. abortus* infection in professional phagocytes (RAW 264.7) and nonprofessional phagocytes (HeLa).

First, we hypothesized that RBE can directly kill *B. abortus*. Hence, we treated free-living *Brucella* with RBE at different concentrations. However, our results revealed that RBE has no bactericidal activity on *B. abortus*. We then assessed its potential on internalization and intracellular growth of *Brucella*. Intriguingly, the bacterial uptake in RBE-treated cells was significantly diminished compared with the controls (Fig. 2); however, RBE treatment did not alter intracellular growth of *B. abortus* within phagocytes. Thus, we focused on elucidating the mechanism by which RBE could inhibit *Brucella* uptake by phagocytes.

The invasion of *Brucella* into host cells is likely the most crucial event in bacterial infections and depends on specific cell-to-bacteria interactions, which are directly mediated by a specific cell surface adhesion mechanism [2]. Therefore, we performed adherence assays to examine the effect of RBE on bacterial adhesion to their target cells. Our results showed that RBE pretreatment caused significant reduction of bacterial adherence to RAW 264.7 and HeLa cells, indicating that RBE interferes with *Brucella*-host surface interaction, which is an important step for subsequent phagocytosis. On the other hand, we also found that RBE hindered F-actin polymerization, which has been reported to be an essential event for phagocytic uptake of microbial pathogens in both epithelial cells and macrophages [2, 20, 21].

Several studies have demonstrated that MAPK plays an important role in the phagocytosis of bacteria and remodeling of the actin cytoskeleton [22, 23]. In this study, we proposed that the inhibitory effects of RBE on the

B. abortus invasion and F-actin polymerization resulted from the attenuation of MAPK (ERK1/2, p38a, and JNK) activation. To test this hypothesis, we evaluated the phosphorylation of MAPKs in phagocytes in the presence or absence of RBE during *B. abortus* invasion. As expected, the results revealed that RBE attenuated the activation of MAPKs (ERK1/2, p38, and JNK in RAW 264.7, whereas only ERK1/2 and JNK in HeLa) at 15 min post-infection. Taken together, these findings suggest that the suppressive effects of RBE on the MAPK-linked phagocytic signaling pathway could negatively affect the invasion of *B. abortus* into both professional and nonprofessional phagocytes.

Furthermore, the chemical composition of RBE analyzed by GC-MS revealed that RBE is mainly composed of short-chain fatty acids (SCFAs), suggesting the important role of SCFA-stimulated signaling during *Brucella* invasion. These findings paralleled previous observations that demonstrated that SCFAs inhibit *Salmonella* invasion through nutritional, regulatory, and immune-modulatory effects on the host physiology [24, 25].

In summary, our findings reveal that treatment of RBE could remarkably inhibit the invasion of *B. abortus* into professional and nonprofessional phagocytes through suppressing MAPK activation as well as F-actin polymerization. Thus, the medical effects of RBE could be extended to control brucellosis by applying it as a new therapeutic against *Brucella* infection.

Acknowledgments

This work was supported by the Strategic Initiative for Microbiomes in Agriculture and Food, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

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