Journal of Ethnopharmacology 198 (2017) 5–14

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

The host immune enhancing agent Korean red ginseng oil successfully attenuates *Brucella abortus* infection in a murine model

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ARTICLE INFO

Keywords:
Brucella abortus
Panax ginseng Meyer (Araliaceae)
Korean red ginseng oil
Macrophages
Cytokines

ABSTRACT

Ethnopharmacological relevance: *Panax ginseng* Meyer (Araliaceae), is one of the most valuable traditional Chinese medicines and is used for the treatment of various human diseases. In this study, we elucidated the protective mechanism of the essential oil from Korean red ginseng (RGO) against *Brucella* infection.

Materials and methods: The effects of RGO on *Brucella abortus* viability, NO production, uptake and intracellular growth in macrophages were investigated. Mice were intraperitoneally infected with *B. abortus* and orally treated with RGO for 14 days. The weights and bacterial numbers from each spleen were monitored, and the sera were evaluated for cytokine production.

Results: *B. abortus* viability was not affected, whereas NO production, internalization and intracellular replication were inhibited in RGO-treated macrophages. Bacterial adherence, F-actin polymerization and MAPK signaling protein phosphorylation (ERK1/2, JNK and p38α) were reduced and the co-localization of *B. abortus*-containing phagosomes with LAMP-1 was augmented in RGO-treated cells compared to untreated cells. RGO displayed protective effects against cell damage by inhibiting nitrite production during *B. abortus* infection in macrophages. Moreover, the spleen weight and bacterial burden were lower in the RGO-treated group than in the control group. The uninfected RGO-treated mice displayed increased TNF-α and IFN-γ production, whereas the *B. abortus*-infected RGO-treated mice showed reduced IL-10 production compared to the control.

Conclusion: RGO exhibits protective effects against *B. abortus* infection *in vitro* and *in vivo,* which emphasize the beneficial effects of RGO in the prevention and treatment of brucellosis.

1. Introduction

Brucellosis remains the most common bacterial zoonosis worldwide. It affects domestic animals, leading to reproductive disorders and a decrease in milk production in livestock. Although the disease causes a low mortality rate in humans, it can also develop into a disabling chronic illness with a common complication of osteoarticular manifestations (Lindahl et al., 2015). The disease is caused by the microorganism *Brucella,* which is transmitted to humans directly *via* contact with infected animals or indirectly through consumption of unpasteurized dairy products or inhalation of aerosols. The incidence is higher in people working on organized farms (Christopher et al., 2010; Jia and Joyner, 2015).

Brucella is a stealthy pathogen because it has no classical toxins and able to avoid activation of the innate immune response during infection. However, once taken up by phagocytes it establishes a replicative niche and inhibits programmed cell death (Rambow-Larsen et al., 2009). The pathogen may bind to distinct phagocytic or

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http://dx.doi.org/10.1016/j.jep.2016.12.026
Received 18 May 2016; Received in revised form 9 December 2016; Accepted 14 December 2016
Available online 22 December 2016
0378-8741/

unknown receptors in macrophages, resulting in zipper-like phagocytosis or through lipid raft microdomains (Lee et al., 2013a). *Brucella*-containing vacuoles (BCVs) interact with early endocytic compartments and eventually these vacuoles mature into endoplasmic reticulum (ER)-derived replicative organelles (Lee et al., 2014).

Ginseng (*Panax ginseng* Meyer, Family Araliaceae) was originally used as an herbal medicine in ancient China (Dharmananda, 2002). It is the best known traditional Chinese medicine and the records of its pharmacological activity can be found in the oldest Chinese Pharmacopea 'Shen Nong Ben Cao' which was written more than 2000 years ago (Jie et al., 1986). In oriental medicine, ginseng is considered sacred due to its shape and together with its various beneficial effects, is known as a noble, miraculous medicine prescribed to treat many symptoms (Baek et al., 2012). Mostly roots, as well as stems, leaves, and their extracts have been used for maintaining immune homeostasis and enhancing resistance to disease or microbial attacks through immune modulation (Kang and Min, 2012; Kim et al., 2015). Numerous studies confirmed the enhancing ability of ginseng extracts to the phagocytic activity of macrophages (Kang and Min, 2012). Recently, the biological roles of essential oils extracted from various herbs and plants have gained increasing attention due to their potential uses as alternative remedies for the treatment of many infectious diseases (Prabuseenivasan et al., 2006). Essential oil isolated from fresh ginseng has been shown to exert antibacterial and anti-inflammatory activities (Bak et al., 2012). Because brucellosis presents several challenges (particularly for the development of improved treatment regimens) and no current patient-friendly treatments or approved human vaccines are available, we investigated the protective efficiency of Korean red ginseng oil (RGO) against *B. abortus in vitro* and *in vivo* to find an alternative approach for brucellosis treatment.

2. Materials and methods

2.1. Korean red ginseng oil preparation

The supercritical CO_2 extract preparation was performed according to previous studies (Bak et al., 2012a, 2012b). The red ginseng oil (RGO) was dissolved in dimethylsulfoxide (DMSO) and diluted using sterile phosphate-buffered saline solution (PBS, pH 7.4) with Tween 20 (0.5% v/v for easy diffusion) as described in earlier studies (Hammer et al., 1999; Prabuseenivasan et al., 2006). RGO was kindly provided by Research Institute of Technology, Korea Ginseng Corporation (Taejon, Korea).

2.2. Analysis by gas chromatography (GC) with a flame ionization detector (FID)

For fatty acid (FA) analysis, the methyl esters of FAs were prepared according to Korea Food Standard Codex 2016 (Korea Food Industry Association, KFIA, 2016) using BF_3/MeOH (14% boron trifluoride) and analyzed by a gas chromatograph (6890N, Agilent Technologies, USA) with FID and a SP-2560 column (100 m×0.25 mm I.D. and 0.2 μm film thickness, Sigma-Aldrich, MO, USA). The temperatures for injector, column and detector were 225, 100 for 4 min and raised to 240 at a rate of 3 °C/min, and 285 °C, respectively. Split ratio was 200:1 and the carrier gas was helium at a flow rate of 0.75 ml/min. For sterol analysis, determination of sterol fractions was done according to Functional Food Codex 2012 (Korea Food Drug and Administration, KFDA, 2012) analyzed by a gas chromatograph (6890N, Agilent Technologies, USA) with FID and a DB-1 column (30 m×0.32 mm I.D. and 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA). The internal standard used was 5-α-cholestane (Sigma-Aldrich, MO, USA). The temperatures for injector, column and detector were 270, 200 for 1 min and raised to 300 for 14 min at a rate of 5 °C/min, and 320 °C, respectively. Split ratio was 10:1 and the carrier gas was nitrogen at a flow rate of 1.2 ml/min.

Table 1
Groups and oral administration in mice.

Group	Treatment	Volume (μl)	Animals
Uninfected			
Negative control	1% DMSO and 0.5% Tween 20 mixed in PBS	100	5
RGO-treated	RGO (10 μg/ml)	100	5
Brucella-infected			
Positive control	1% DMSO and 0.5% Tween 20 mixed in PBS	100	5
RGO-treated	RGO (10 μg/ml)	100	5

2.3. Bacterial strain

B. abortus 544 (ATCC 23448) was cultivated in Brucella broth (Becton Dickinson, Sparks, MD, USA) at 37 °C with vigorous shaking until stationary phase was reached. Viable bacterial counts were assessed by plating serial dilutions onto agar plates.

2.4. Cell culture

RAW 264.7 cells (ATCC TIB7-1, Rockville, MD, USA) were routinely grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HI-FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (all provided by Invitrogen, Grand Island, NY, USA) and incubated at 37 °C with 5% CO_2 atmosphere. The cells were seeded into tissue culture plates at a concentration of 1×10^5 cells per well and incubated for 24 h. Prior to bacterial infection, the culture medium was changed to fresh medium without antibiotics.

2.5. Cytotoxicity assay

RAW 264.7 cells were pre-treated with different RGO concentrations (0, 5, 10, 20, 40, 50, 100, 200, 400, 500 and 1000 μg/ml) in a 96-well cell culture plate for 48 h. A colorimetric 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) staining method was used to analyze cytotoxicity as previously described (Lee et al., 2013b). Fresh culture medium with 0.1% DMSO and 0.5% Tween 20 was used as a control in all *in vitro* experiments performed in this study.

2.6. Bactericidal assay

Bacteria (2×10^4 colony forming units (CFU)/ml) were added to different RGO concentrations (0, 5, 10 and 50 μg/ml) and incubated at 37 °C for 0, 2, 4, 8 and 24 h as previously described (Reyes et al., 2016). After incubation, each sample was serially diluted on Brucella agar and incubated at 37 °C for 3 days to determine the CFUs.

2.7. Nitrite assay

RAW 264.7 cells were cultured overnight and then pre-treated with RGO (10 μg/ml) in a 96-well cell culture plate for 4 h. The cells were either treated with lipopolysaccharide (LPS) (Sigma-Aldrich, Co., St. Louis, MO, USA) (10 μg/ml) or infected with *B. abortus* at a multiplicity of infection (MOI) of 100 for 1 h and then incubated in medium with or without RGO (10 μg/ml) for 24 h. Nitrite accumulation in the culture medium was measured as an indicator of nitric oxide (NO) production based on the Griess reaction (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions and as previously described (Baek et al., 2012).

2.8. Bacterial infection assay

To analyze the bacterial internalization efficiency, previously de-