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In Vitro Antibacterial Effect of the Combination of *Galla rhois* ethanol extracts and Sodium chlorate against Intramacrophage *Brucella abortus*

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ABSTRACT - This study investigated the antibacterial effects of GR ethanol extracts (GRE), sodium chlorate (SC) and a combination of GRE and SC (GS) on *Brucella abortus* (*B. abortus*). The antibacterial activities of GRE, SC and GS towards *B. abortus* were evaluated by incubating *B. abortus* with GRE, SC and GS. Following treatment with GRE, SC and GS, *B. abortus* survival and intracellular proliferation in macrophages were monitored. In the cellular cytotoxicity assay, GRE, SC and GS are not cytotoxic at concentrations less than 400 μg/ml, 15 mM and 0.6GS (1 of GS, GRE 1,000 μg/ml + SC 30 mM), respectively. The viability of *B. abortus* was markedly decreased in a dose-dependent manner in all treatment groups. In addition, *B. abortus* intracellular proliferation within macrophages was significantly reduced in cells treated with GRE (400 μg/mL), SC (15 mM) and 0.5GS (GRE 500 μg/mL + SC 15 mM) after 48 hr-incubation (GRE, $p < 0.01$; SC and 0.5GS, $p < 0.001$). Especially, in the treatment of GS, the synergistic effect of GRE and SC treatment on B. abortus in macrophage was observed. In conclusion, GS is useful as an antibacterial candidate against *B. abortus*, and can be applied in the field of meat and milk hygiene.

Key words : *Brucella abortus*, *Galla rhois*, sodium chlorate, the combination of GS and SC

Introduction

Brucellosis is one of the world's most common zoonotic diseases and is responsible for economic losses in livestock industries[1)]. In addition, the disease represents a considerable and increasing public health burden[2,3)]. Approximately, 500,000 cases of human brucellosis globally are reported to the World Health Organization annually (WHO)[4)]. This zoonotic disease is caused by various species of *Brucella* that infect many domestic animals, which can act as reservoirs for other animal species and human infection[5)]. The disease is a highly contagious zoonosis caused by ingestion of unsterilized milk and dairy products or meat from infected animals or close contact with their secretions[6,7)].

Brucellosis is caused by *Brucella* spp., which are *B. melitensis, B. abortus, B. suis, B. canis, B. neotomae* and *B. ovis*, and recently the recognized new species have been *Brucella ceti, Brucella pinnipedialis, Brucella microti and Brucella inopinata*, that contribute to abortion and infertility in various mammals, and well-known undulant fever in humans[4,8,9)]. These bacteria invade and replicate within professional phagocytes such as macrophages and non-professional phagocytes such as epithelial cells[8,9)].

Even today, treatment of brucellosis in human is largely based on the principles applied half a century ago, and few modifications have been made in the following years, despite the emergence of new antibiotic classes and different therapeutic approaches[10)]. As current recommended regimens for brucellosis treatment involve the use of several antibiotics to avoid relapses occurring and to prevent the prolonged use of these drugs, the combined antibiotics use may lead to problems of drug resistance arising and the vital effects with respect to health and safety[11,12)]. However, the alternatives for the brucellosis treatments have not been thoroughly surveyed yet. Thus, the research for alternative treatments of brucellosis is demanded.

Conventional herbal medicines have been used as remedies

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against infectious diseases in Asian countries long ago. *Galla rhois* (GR) has long been used in traditional Asian medicine to treat diarrhea, persistent coughing and spontaneous perspiration in man due to the antidiarrhetic, astringent and hemostatic properties of this product[8,13,14]. GR is a harmless natural material that contains a number of tannin-derived components, including methyl gallate and gallic acid[15]. Especially, the gallotannins are a class of hydrolysable tannin polymers that are formed from gallic acid, which seems to have anti-bacterial, anti-fungal, and anti-viral properties[16,17]. In the previous study[8], GR ethanol extracts (GRE) exhibited antibacterial and protective activities on *Brucella abortus* (*B. abortus*) *in vitro* and *in vivo*.

Sodium chlorate (SC) is used as an oxidising agent and for making chlorine dioxide used in water disinfection. Chlorate is found as a stable by-product in drinking water that has been disinfected with chlorine dioxide[18]. Previous studies had been carried out on the prevention and treatment of *Enterobacteriaceae* infections in animals using SC[19,20]. *B. abortus* such as *Escherichia coli* and *Salmonella* species has a respiratory nitrate reductase enzyme, which coincidentally catalyses the intracellular reduction of chlorate to chlorite, a cytotoxic product that kills the bacterium in tissue cells[21,22]. As most of the normal anaerobic gut bacteria lack respiratory nitrate reductase activity, chlorate selectively targets bacteria expressing respiratory nitrate reductase activity but not beneficial anaerobes lacking that enzyme[19,20].

In the present study, GRE, SC and the combination of GRE and SC (GS) were investigated the antibacterial effect and inhibition of intracellular proliferation on *B. abortus*.

Materials and Methods

Cells and culture conditions

The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (ATCC, Rockville, USA) and grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 (hyclone, USA), containing 10% heat-inactivated fetal bovine serum (FBS). The cells were seeded (1×10^4 per well) in cell culture plates and incubated for 24 hr before infection for all the assays. Prior to the bacterial infection, the cells were washed three times with a sterile phosphate-buffered saline solution (PBS, pH 7.4) to eliminate antibiotics; this step was followed by the addition of fresh culture media (RPMI 1640 medium containing 10% fetal bovine serum (FBS)) without antibiotics.

Bacterial culture

B. abortus strains were derived from 544 (ATCC 23448), smooth, virulent *B. abortus* biovar strains, which were obtained from Animal and Plant Quarantine Agency (Anyang, Korea). *B. abortus* was maintained as frozen glycerol stocks and cultured in Brucella broth (Becton Dickinson, Sparks, MD.) without antibiotics for 3 days at 37°C. Bacteria were grown at 37°C with vigorous shaking until they reached the stationary phase, and bacterial growth rates were measured using the spectrophotometer (Beckman Coulter Korea, Seoul) at the wavelength of 600 nm.

GRE and SC solution preparation

GR powder was obtained from GS Bio (Jeonju, Korea), isolated from plant material as described previously[8]. Briefly, 1 kg of plant material was dried in an oven at 60°C for 3 days and extracted with ethanol twice at room temperature. The remaining residue was removed by filtration (Whatman no. 2, Sigma-Aldrich Korea, Yongin), and the filtrate was concentrated using a vacuum rotary evaporator (Iwai Co., Japan), followed by freezing of the dried powder. This crude, extracted powder was used in the present study.

SC was purchased from Sigma-Aldrich Korea (Yongin, Korea). To make 30 mM SC stock solution, 3.2 g of SC was dissolved in distilled water with a final volume of 1L. After the SC stock solution was suitably diluted with distilled water, the diluents were used in this study.

Cytotoxicity assay

To determine the cytotoxicity of GRE, SC and GS, RAW 264.7 cells (1×10^5 cells/mL) were cultured in the presence of different concentrations of GRE (0, 50, 100, 200, 300, 400 and 500 μg/mL), SC (0, 1.5, 3, 6, 9, 12 and 15 mM) and GS (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 (GRE 1,000 μg/mL + SC 30 mM)) in a 96-well cell culture plate for 48 hr. The cell viability was measured using a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cleavage assay that is a sensitive analysis for cytotoxicity that is based on the detection of mitochondrial enzymatic activity that reacts with a chromogenic substrate[23]. In brief, RAW 264.7 cells were inoculated in MTT solution (5 mg/mL in PBS) and incubated at 37°C in 5% CO_2 for 4 hr. After incubation, plates were centrifuged at 450 × g, and supernatants were removed. Acid/isopropanol (one portion of 4N HCl : 100 portions of isopropanol) was added to the wells and mixed completely to dissolve crystalline material. Product generation is measured at an optical density (OD) of 570 nm.

Bactericidal analysis

Bacteria grown to stationary phase were diluted with phosphate-buffered saline solution (PBS, pH 7.4) to a concentration of 2×10^4/mL and added to PBS containing different concentrations of GRE (0, 50, 100, 200 and 400 μg/mL), SC (0, 1.875, 3.75, 7.5 and 15 mM) and GS (0, 0.1, 0.2, 0.4, and 0.8 (1; GRE 1,000 μg/mL + SC 30 mM)), and

incubated at 37°C for 0, 1, 4, 8, 16, and 24 hr. After incubation and dilution, 100 μL of each diluent was plated onto Brucella agar and cultured for 3 days at 37°C to determine bacterial colony forming units (CFUs).

Inhibitory effects for intracellular proliferation of *B. abortus*

For the inhibitory efficiency measurements, RAW 264.7 cells were infected with *B. abortus* according to the method by Lee *et al*. (2004)[8]. After infected cells were incubated at 37°C for 1 hr, cells were washed once with media and then incubated with RPMI 1640 media containing 10% FBS with gentamicin (30 μg/mL) for 30 min to kill any remaining extracellular bacteria. Referred on the results of cytotoxicity assay, infected cells were treated with GRE (400 μg/mL), SC (15 mM) and 0.5GS (GRE 500 μg/mL + 15 mM) and incubated for 2, 24 or 48 hr. To evaluate the number of viable bacteria at different periods of time, the infected cells were extensively washed three times with PBS and then lysed with distilled water. The number of viable bacteria was determined by counting the CFU from serial dilutions of cell lysates that were spread on Brucella agar plates in triplicate.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD) for the triplicate experiments. The significance between the control group and experimental groups was determined by Student's *t*-test. A difference at the level of $p < 0.05$ was considered to be statistically significant.

Results

Cytotoxic effects on murine macrophage

Table 1 showed the cytotoxicity of GRE, SC and GS against RAW 264.7 cells. The cells were cultured in the presence of different concentrations of GRE (0, 50, 100, 200, 300, 400 and 500 μg/mL), SC (0, 1.5, 3, 6, 9, 12 and 15 mM) and GS (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1 (GRE 1,000 μg/mL + SC 30 mM) for 48 hr.

Cell viability was assessed by the MTT assay and correlated with OD values and the results of trypan blue exclusion assays. The OD values of the cultures treated with 500 μg/mL of GRE and 0.8 and 1 of GS (GRE 1,000 μg/mL + SC 1.6 mg/ml) were significantly lower than those treated with 0 μg/mL of GRE and 0 of GS, respectively ($p < 0.05$), but there was no significant difference between those treated

Table 1. The cytotoxicity of GRE, SC and GS against RAW 264.7 cells by MTT assay

GRE		SC		GS	
Con. (μg/ml)[1)]	OD[2)]	Con. (mM)	OD	Con.	OD
0	1.46 ± 0.14	0	1.41 ± 0.15	0	1.45 ± 0.14
50	1.54 ± 0.18	1.5	1.56 ± 0.17	0.1	1.53 ± 0.25
100	1.42 ± 0.13	3	1.35 ± 0.14	0.2	1.44 ± 0.15
200	1.45 ± 0.15	6	1.40 ± 0.15	0.4	1.39 ± 0.13
300	1.38 ± 0.14	9	1.33 ± 0.13	0.6	1.35 ± 0.15
400	1.25 ± 0.17	12	1.27 ± 0.14	0.8	1.23 ± 0.11*
500	1.17 ± 0.12*	15	1.20 ± 0.15	1[3)]	1.15 ± 0.12*

[1)]Con. : concentration.
[2)]Optical density (OD) values are presented as the mean ± standard deviation of the mean.
[3)]The concentration of GS (GRE 1,000 μg/ml + SC 30 mM) was represented as 1.
*$p < 0.05$, significant difference as compared with the respective value of untreated cells.

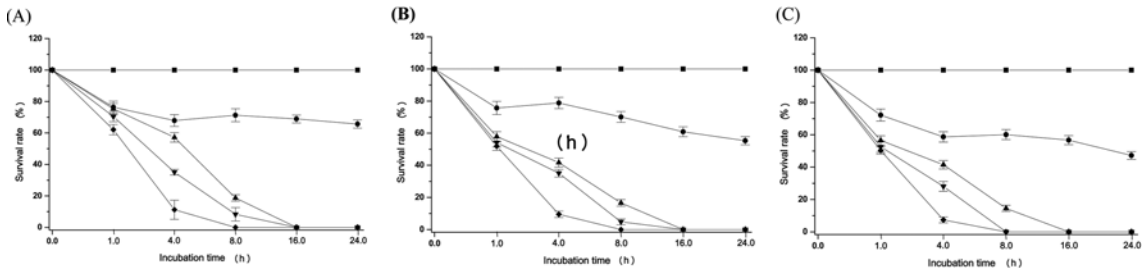

Fig. 1. Bactericidal effects of GRE, SC and GS on *Brucella abortus*. Different concentrations of (A) GRE (0 (■), 50 (●), 100 (▲), 200 (▼) and 400 (◆) μg/mL), (B) SC (0 (■), 1.875 (●), 3.75 (▲), 7.5 (▼) and 15 (◆) mM) and (C) GS (0 (■), 0.1 (●), 0.2 (▲), 0.4 (▼), and 0.8 (◆) (1; GRE 1,000 μg/mL + SC 30 mM)) were diluted in PBS and incubated with *B. abortus* for 1, 4, 8, 16 and 24 hr. Bacterial viability was monitored by measuring CFUs on culture plates, and the rate of bacterial viability was compared to the zero time point in the untreated PBS. The data represent the mean ± S.D. of triplicate experiments.

with 15 mM of SC and 0 mM of SC. These data indicated that GRE, SC and GS are not cytotoxic at concentrations less than 400 μg/ml, 15 mM and 0.6GS (1 of GS, GRE 1,000 μg/ml + SC 30 mM), respectively.

Bactericidal effects of GRE and GS on *B. abortus*

Fig. 1 presented the bactericidal effect of GRE, SC and GS on *B. abortus*. The bacteria were incubated with different concentrations of GRE (0, 50, 100, 200 and 400 μg/mL), SC (0, 1.875, 3.75, 7.5 and 15 mM) and GS (0, 0.1, 0.2, 0.4, and 0.8 (1; GRE 1,000 μg/mL + SC 30 mM)) in PBS at 37°C for 0, 1, 4, 8, 16, or 24 hr.

Bacterial survival rates were calculated by counting CFUs and expressed as a percentage of the survival rate of the treated sample relative to an untreated control (0 μg/mL), which was set to 100%. Bacterial survival rates were markedly decreased in a dose-dependent manner in all treatment groups (Fig. 1A, GRE; Fig. 1B, SC; Fig. 1C, GS) (Fig. 1). In all concentrations except for the lowest dose in all groups, the survival of bacteria treated with drugs showed rapid decreases with increasing incubation time.

Inhibitory effects of GRE, SC and GS on bacterial intracellular proliferation

To determine whether GRE, SC and GS inhibit the replication of *B. abortus* in macrophages, RAW 264.7 cells were treated with GRE (400 μg/mL), SC (15 mM) and 0.5GS (GRE 500 μg/mL + SC 15 mM) using the same conditions as those used for the cytotoxicity assay, and incubated for 2, 24 and 48 hr after *B. abortus* infection (Fig. 2).

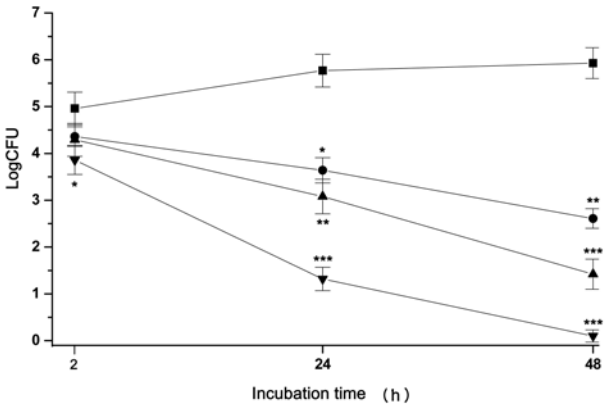

Fig. 2. Effects of GRE, SC and GS on the intracellular replication of *Brucella abortus*. RAW 264.7 macrophages were infected with *B. abortus* at MOIs of 10, and the infected cells were incubated with saline (■), GRE 400 μg/mL (●), SC 15 mM (▲) and 0.5GS (1; GRE 1,000 μg/mL + SC 30 mM) (▼) for 2, 24 and 48 hr. Intracellular growth efficiency was evaluated by Log_{10}CFU. Data are the averages of triplicate samples from three identical experiments, and the error bars represent the standard deviations. Statistically significant differences relative to the untreated control sample are indicated by asterisks ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

At 24 hr post-treatment, the intracellular replication of *B. abortus* in GRE, SC and GS was significantly inhibited as compared to that of the untreated control (GRE, $p < 0.01$; SC and GS, $p < 0.001$). At 48 hr post-treatment, *B. abortus* intracellular replication in GRE, SC and GS was significantly prohibited as compared to that of the untreated control (GRE, $p < 0.01$; SC and GS, $p < 0.001$). These results indicate that GRE, SC and GS have an inhibitory activity for the proliferation of *B. abortus* in murine macrophages.

Discussion

Brucellosis is one of the recognized zoonoses and is considered by the Food and Agricultural Organization (FAO), WHO and the Office International des Epizooties (OIE) as the most widespread zoonosis globally[24]. Vaccination has been used for the control of brucellosis. However, there is currently no safe or efficient vaccine that can be used to control human brucellosis[25], and treatment for brucellosis with antibiotics also remains controversial and requires prolonged therapy with at least two agents[26]. In addition, *Brucella* has developed various strategies to evade innate and adaptive host immune responses, aimed at establishment of an intracellular niche for long-term survival and replication[27]. These facts may indicate that the conventional antibiotic regimen is not suitable for brucellosis treatment, and together with the rapid emergence of antibiotic resistance and the delayed use of antibiotics, could lead to serious problems. Furthermore, there have been few reports examining the use of traditional plants and non-residual-chemical compounds for the treatment of brucellosis. Therefore, conventional treatments for brucellosis should be increasingly regulated, and alternative, sophisticated treatments that abrogate the invasion and intracellular trafficking of *Brucella* need to be identified.

GR possesses positive health benefits, including anti-tumorigenic, anti-oxidative, anti-inflammatory and anti-microbial properties and GRE in the previous study significantly inhibits the growth of intracellular pathogenic bacteria such as *Clostridium perfringens*[16,28,29]. Additionally, many previous studies reported that SC had a bactericidal activity against *Enterobacteriaceae* because of the chlorite from which the chlorate is reduced by a respiratory nitrate reductase[19-22].

In the previous study[8], the cytotoxic effect of GRE was not detected at the concentrations between 0 and 400 μg/mL by trypan blue exclusion assays. In this study, GRE at the doses of below 400 μg/mL was occurred non-toxic to cells same with the above study. In addition, Lee *et al*. (2012)[30] reported that the survival rate of *B. abortus* in pure culture was reduced to 0% at 8 hr post-incubation by *Phellinus*

baumii ethanol extracts (PBE) at the concentration of 400 μg/mL. Furthermore, Lee *et al*. (2011)[8] reported that the survival rate of *B. abortus* was markedly decreased into 0% at 8 hr post-incubation with the dose of GRE 400 μg/mL. In the present study, GRE, SC and GS reduced the survival rates of *B. abortus* into 0% at 8 hr post-incubation with the dose of 500 μg/mL, 15 mM and 0.8GS, respectively. Compared to the above results, antibacterial effect of SC and GS on *B. abortus* was similar with that of PBE and GRE. Meanwhile, infected-macrophage cells treated with PBE 100 μg/mL and GSE 200 μg/mL in the previous studies displayed the reduction of intracellular proliferation rate of *B. abortus* into more than 99% compared to the untreated-control. On the other hand, 0.5GS-treated cells in this study reduced the intracellular proliferation rate of *B. abortus* into about 0%. With the consideration of the treated-dosage, the antibacterial effect of GS on *B. abortus* might be higher than that of PBE and GRE.

The previous study reported that the tannin components of GRE directly inhibit the growth of intracellular replication in macrophages[8]. In addition, chlorate from SC is reduced into chlorite, a cytotoxic product by the respiratory nitrate reductase that *B. abortus* such as obligatory intracellular bacteria has a reductase enzyme for the nitrogen fixation within cells[21,22]. Therefore, a strong synergistic antibacterial effect in GS was observed against *B. abortus* with the associations between the tannin components of GRE and chlorate derived from SC. Then GS was useful as an antibacterial candidate against *B. abortus*.

In conclusion, this study emphasizes the idea that GS has an antibacterial effect against *B. abortus*, and possibly other intracellular pathogenic bacteria. In the future, it will be necessary to determine the antibacterial usefulness of GS in the application of foods such as milk, dairy products and meat from animals infected with *B. abortus*.

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요 약

본 연구는 오배자 에탄올 추출물 (GRE), 염소산나트륨 (SC) 그리고 오배자 에탄올 추출물과 염소산나트륨 합제 (GS)의 *B. abortus*에 대한 항균효과를 확인하기 위해 수행되었다. GRE, SC 그리고 GS를 *B. abortus*에 처리하여 배양한 후, *B. abortus*의 생존수를 확인하였으며, 마우스 탐식세포 내 감염된 *B. abortus*의 증식 억제효과를 경시별 (2, 24, 48시간)로 조사하였다. GRE, SC 그리고 GS는 각각 400 μg/mL 이하, 15 mM 그리고 0.6GS (GS 1, GRE 1,000 μg/mL + SC 30 mM) 이하의 농도에서 세포독성을 나타나지 않았다. 모든 처리구에서 *B. abortus*의 생존율은 용량-의존적으로 현저하게 감소하는 결과를 나타내었다. 또한, GRE (400 μg/mL), SC (15 mM) 그리고 0.5GS (GRE 500 μg/mL + SC 15 mM)를 처리한 세포에서 배양 48시간 후에, *B. abortus*의 증식이 통계적으로 유의성 있게 감소하였으며 (GRE, $p < 0.01$; SC and 0.5GS, $p < 0.001$), 특히, GS를 처리한 경우, *B. abortus*의 세포내 증식이 GRE와 SC의 상승작용에 의한 강력한 항균효과를 나타내었다. 결론적으로, GS는 *B. abortus*에 대한 항균물질로서 유용할 뿐만 아니라, 식육과 우유 위생 분야에 적용할 수 있을 것으로 생각된다.

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