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Protection of palmitic acid treatment in RAW264.7 cells and BALB/c mice during *Brucella abortus* 544 infection

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ABSTRACT

Background: We previously elucidated the protective mechanism of Korean red ginseng oil (RGO) against *Brucella abortus* infection, and our phytochemical analysis revealed that palmitic acid (PA) was an abundant component of RGO. Consequently, we investigated the contribution of PA against *B. abortus*.

Objectives: We aimed to investigate the efficacy of PA against *B. abortus* infection using a murine cell line and a murine model.

Methods: Cell viability, bactericidal, internalization, and intracellular replication, western blot, nitric oxide (NO), and superoxide (O₂⁻) analyses and flow cytometry were performed to determine the effects of PA on the progression of *B. abortus* infection in macrophages. Flow cytometry for cytokine analysis of serum samples and bacterial counts from the spleens were performed to determine the effect of PA in a mouse model.

Results: PA did not affect the growth of *B. abortus*. PA treatment in macrophages did not change *B. abortus* uptake but it did attenuate the intracellular survivability of *B. abortus*. Incubation of cells with PA resulted in a modest increase in sirtuin 1 (SIRT1) expression. Compared to control cells, reduced nitrite accumulation, augmented O₂⁻, and enhanced pro-inflammatory cytokine production were observed in PA-treated *B. abortus*-infected cells. Mice orally treated with PA displayed a decreased serum interleukin-10 level and enhanced bacterial resistance.

Conclusions: Our results suggest that PA participates in the control of *B. abortus* within murine macrophages, and the *in vivo* study results confirm its efficacy against the infection. However, further investigations are encouraged to completely characterize the mechanisms involved in the inhibition of *B. abortus* infection by fatty acids.

Keywords: *B. abortus*; macrophages; palmitic acid; spleen

INTRODUCTION

Brucellosis, caused by bacteria species of the genus *Brucella*, is a chronic infectious disease that remains endemic in many countries, leading to serious public health problems and economic losses [1]. Control and eradication programs are directed toward the reduction and elimination of the disease in small and large ruminants, and although vaccination is the

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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cornerstone of control programs in livestock that have been successfully used worldwide, shortcomings still occur. Furthermore, no serological test for the diagnosis of brucellosis has 100% sensitivity and specificity [2,3]. On the other hand, no human vaccine is currently available, and treatment consists of a combination of doxycycline and rifampin antibiotics for 42 days and is associated with a risk of relapse [4,5]. Essential to the etiologic agent's pathogenesis is its ability to colonize a wide range of mammalian cell types, although it primarily depends on phagocytic cells such as macrophages, dendritic cells, and placental trophoblasts – relying on an intracellular lifestyle for its infectious cycle and its ability to cause disease [6]. Host cells develop complex immune mechanisms to control invading pathogens and maintain a host resistance–*Brucella* virulence balance; however, *Brucella* has evolved multiple organized strategies to evade the host's immune response mechanisms, allowing it to persist and replicate inside host cells [7]. Furthermore, *Brucella* has different defensive antimicrobial resistance mechanisms against a wide array of phagocytic and inducible microbicidal functions, such as the production of reactive nitrogen intermediates and reactive oxygen intermediates through an oxidative burst process [6,7].

Alternatives to conventional antibiotics that are safe and effective against antibiotic-resistant pathogens are novel agents derived from medicinal plants. Previously, we described the *in vitro* and *in vivo* protective mechanisms of an essential oil from Korean red ginseng (RGO) against *B. abortus* infection and reported that one of the most abundant compounds present in RGO was palmitic acid (PA) [8]. PA is the most common saturated fatty acid (FA) in the human body and accounts for 20%–30% of the body's total FAs, which can be provided through the diet or synthesized endogenously from other FAs, carbohydrates, and amino acids. However, PA has been depicted negatively due to its potential unhealthy effects that can overshadow its multiple fundamental biological functions at cellular and tissue levels [9]. Another two compounds that were abundant in RGO were β -sitosterol and linoleic acid [8] and, previously, these two compounds were individually reported to positively participate in the control of *Brucella abortus* 544 infection in both *in vitro* and *in vivo* systems [10,11]. The present study aims to investigate the potential effects of PA against *B. abortus* infection.

MATERIALS AND METHODS

Palmitic acid preparation

Initially, the PA (molecular weight 256.43 g/mol; Sigma-Aldrich, USA) was dissolved in absolute ethanol (4 M). Further dilutions were obtained using an appropriate medium containing bovine serum albumin (BSA, GenDEPOT, USA) to a final working concentration of 0.1%.

Bacterial strain and growth condition

Brucella abortus 544 (ATCC 23448), a smooth virulent *B. abortus* biovar 1 strain, was kindly provided by the Laboratory of Bacteriology Division in Animal and Plant Quarantine Agency in the Republic of Korea and maintained on Brucella broth containing 1.5% agar (Becton Dickinson, USA) for 3 days at 37°C with aeration or grown in Brucella broth at 37°C with vigorous shaking until the stationary phase. Viable bacteria were measured by plating serial dilutions on Brucella agar.

Cell culture and growth condition

RAW264.7 cells (ATCC TIB7-1, USA) maintained at 37°C in a 5% CO₂ atmosphere in RPMI 1640 containing 10% heat-inactivated fetal bovine serum along with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, USA) were seeded in 6- or 96-well tissue culture plates overnight prior to use at a concentration of 1×10^6 or 1×10^5 cells per well. Control cells were incubated with 0.1% ethanol and 0.1% BSA in fresh medium without antibiotics, and the medium was exchanged with fresh medium without antibiotics prior to all infection assays.

Cytotoxicity assay

RAW264.7 cells in a 96-well tissue culture plate were incubated with different concentrations of PA (0, 40, 80, 200, 400, 800, and 2,000 µM) in a total volume of 100 µL in each well at 37°C in a 5% CO₂ atmosphere for 48 h. Viability of cells was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) cytotoxicity assay. Briefly, at the end of incubation, cells were incubated in RPMI 1640 containing MTT (0.5 mg/mL) for 3 h under standard culture conditions. The MTT solution was then removed, and 150 µL of dimethyl sulfoxide (DMSO) was added in each well and incubated for 15 min. The absorbance of each well was measured at 540 nm. Viability of cells was calculated by dividing the mean absorbance of the treated cells by the mean absorbance of the control cells. The highest non-cytotoxic concentration of PA (800 µM) was used in succeeding experiments.

Bactericidal assay

Bacteria (10 µL) in PBS (2×10^4 colony forming units, CFU/mL) were added to a 96-well round-bottom plate with different concentrations of PA in PBS (0, 40, 200, 400, and 2000 µM) in a total mixture volume of 100 µL per well. The plate was then centrifuged at $200 \times g$ for 30 sec and then incubated at 37°C for 0, 2, and 24 h. At the end of incubation, each well was serially diluted in PBS and 50 µL was plated onto Brucella agar plates. CFUs were counted to calculate bacterial survival rates of the treated samples relative to that of the untreated samples where the control (untreated) survival rates were set to 100 percent.

Internalization and intracellular replication assay

RAW264.7 cells in a 96-well tissue culture plate were incubated with or without PA. For the internalization assay, cell pre-treatment with PA was done for at least 4 h, and the cells were then washed with PBS prior to infection with *B. abortus* at a multiplicity of infection (MOI) of 100 in a total volume of 100 µL medium. The plate was then centrifuged at $200 \times g$ for 5 min and incubated at 37°C in a 5% CO₂ atmosphere. At 0 or 30 min, cells were washed with PBS and then incubated in 100 µL medium containing gentamicin (100 µg/mL) per well for 30 min. At the end of incubation, cells were washed with PBS and then lysed in 100 µL distilled water per well. Serial dilutions using PBS were done, and 50 µL of the diluted solution was plated on Brucella agar plates. CFUs were assessed after 3 d of incubation to determine bacterial internalization efficiency. For the intracellular replication assay, cells were infected with *B. abortus* for 1 h prior to incubation with PA. The cells were washed with PBS and then incubated with 100 µL of medium containing PA and gentamicin (100 µg/mL) per well for 1 h at 37°C in a 5% CO₂ atmosphere. The gentamicin concentration was reduced to 30 µg/mL, and the cells underwent further incubation for 2, 24, or 48 h. Similar infection, washing, lysis, plating, and CFU assessment procedures as those of the internalization assay were performed. In addition, cells were incubated with PA for at least 4 h prior to infection using similar infection and incubation with PA procedures as those of the intracellular replication assay. At 48 h post-incubation, cell supernatants were collected for cytokine analysis.

Western blot analysis

RAW264.7 cells prepared in a 6-well tissue culture plate were incubated with or without PA for at least 4 h. An infection procedure similar to that of the internalization assay was used. At 30 min post-infection, cells were washed with cold PBS and lysed using 100 μ L RIPA buffer (iNtRON Biotechnology, Inc., USA) and a 1% protease inhibitor cocktail (Promega Corporation, USA) at 4°C for 10 min. Lysed cells were scraped and transferred into a 1.5 mL microcentrifuge tube, further incubated at 4°C overnight, and then centrifuged at 14,000 $\times g$ at 4°C for 30 min to collect the lysates. Preparation, separation, and transfer of proteins were performed as previously described [8]. Briefly, proteins were denatured by boiling for 5 min in 2 \times Laemmli sample buffer (Bio-Rad Laboratories, Inc., USA) mixed with 2-mercaptoethanol. Protein concentrations were measured using the Bradford protein assay (Bio-Rad Laboratories, Inc.) and then separated by performing SDS-PAGE in 8% SDS gel. The proteins were electrically transferred onto a nitrocellulose membrane (Merck Millipore Ltd., Germany) for 20 min using a semi-dry transfer system (ATTO, Japan). The membranes were then cut to the appropriate target protein size and incubated with anti-sirtuin 1 (SIRT1) (1:250, MyBioSource, Inc., USA) or β -actin (1:500, Cell Signaling Technology, Inc., USA) in 5% BSA (GenDEPOT, USA) at 4°C overnight. Incubation with a secondary antibody was done using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1,000, Thermo Scientific, USA) at room temperature for 1 h. After washing, membranes were exposed to a Molecular Imager[®] ChemiDoc[™] XRS+ system machine (Bio-Rad Laboratories, Inc.) using a luminol-coumaric acid-H₂O₂ detection solution (ATTO Corporation, Japan) and analyzed using Image Lab[™] software version 5.0. The blots were quantified relative to that of β -actin.

Nitric oxide analysis

Nitric oxide (NO) analysis was done as previously reported [8]. RAW264.7 cells prepared in a 96-well tissue culture plate were incubated with or without PA for at least 4 h. Infection, washing, and further incubation with PA was done using similar procedures as those of the intracellular replication assay. Culture supernatants were collected at 2, 24, and 48 h post-incubation to measure nitrite accumulation as an indicator of NO production by using Griess reagent (Promega) according to manufacturer's instruction. The absorbance was read at 540 nm using an ELISA plate reader, and the amount of NO production was measured in comparison with a reference curve by using 0–100 μ M sodium nitrite.

Superoxide analysis

Cell preparation, incubation, and infection procedures were similar to those of the NO analysis. Cells were analyzed at 2, 24, and 48 h post-incubation for superoxide anion (O₂⁻) production by performing the nitro blue tetrazolium (NBT) reduction assay as previously reported [12]. Briefly, 50 μ L NBT (1 mg/mL) was added per well and incubated at 37°C for 45 min. To stop the reaction, 100 μ L 1 N HCl was added, followed by washing with PBS, and 150 μ L DMSO was added. Finally, 10 μ L 1 N NaOH was added, and the color developed was measured at 650 nm using an ELISA plate reader. Data represent the NBT reduction and were obtained by subtracting the average OD of the infected cells from the average OD of the non-infected cells.

Animal experiment

Eight-week-old specific pathogen-free female BALB/c mice (Samtako Bio Co. Ltd., Korea) were acclimatized for at least one week before being randomly assigned to four groups of five mice. Two groups were not infected, and another two groups were infected intraperitoneally with *B. abortus* 544 (2 \times 10⁴ CFU in 100 μ L PBS). Prior to infection, mice were treated orally

using a gavage needle with 100 μ L of PA (800 μ M) or vehicle (PBS containing 0.1% ethanol and 0.1% BSA) for 3 d. Oral treatment was continued for 14 d post-infection. All animal groups were observed for clinical symptoms during the entire experimental period. Mice were sacrificed at 14 d post-infection, and blood samples were collected from the heart. Spleens were collected, weighed, and a 0.05 g part was homogenized in PBS to determine the number of CFUs by serially plating 50 μ L onto Brucella agar plates. The total body weight of each animal was measured for the non-infected groups. The protection unit was computed by subtracting the average \log_{10} CFU/g organ of the control group from the average \log_{10} CFU/g organ of the treatment group. All animal procedures were approved and conducted under the guidelines of the Animal Ethical Committee of Chonbuk National University (Authorization Number CBNU-2018-101). The four groups of five mice each were housed in metabolic cages (Daejong Instrument Industry Co., LTD, Korea) under standard conditions at $23 \pm 1^\circ\text{C}$ with a 12 h light/dark cycle and provided with free access to feed and water.

ELISA

To check for hepatocellular injury, serum samples from the non-infected mice were analyzed to measure the alanine aminotransferase 1 (GPT) concentration using GPT (Mouse) ELISA kit (BioVision Inc., USA) according to the manufacturer's instruction. Briefly, a 100 μ L serum sample was added to each well of a pre-washed micro ELISA plate and incubated at 37°C for 1.5 h. The content was discarded, and 100 μ L biotin-detection antibody (1:100) was added. The plate was incubated at 37°C for 2 h and then washed. After washing, 100 μ L HRP-streptavidin conjugate (1:100) was added per well and the plate incubated at 37°C for 30 min. The solution was discarded, and the plate washed. After washing, 90 μ L TMB substrate was added and the plate incubated at 37°C for 30 min in the dark. Finally, 50 μ L of the stop solution was added, and the absorbance was read at 450 nm within 20 min. Mouse GPT concentration was determined by interpolation from a standard curve by using 0–100 mIU/mL of a GPT standard solution.

Cytokine analysis

Culture supernatants and serum samples were processed to measure the levels of cytokines involved in the course of brucellosis by using a Cytometric Bead Array mouse inflammation kit (BD Biosciences, USA) according to manufacturer's instruction. Data acquisition was accomplished using a FACSCalibur flow cytometer (BD Biosciences). Briefly, a 50 μ L serum sample was prepared in a 15 mL conical polystyrene round-bottom tube (Falcon; Corning Science, Mexico), and 50 μ L of mixed capture beads were added. A 50 μ L mouse inflammation PE detection reagent was added and incubated at room temperature for 2 h in the dark. After incubation, 1 mL of wash buffer was added, and the mixture centrifuged at $200 \times g$ for 5 min. The supernatant was discarded, and 300 μ L of wash buffer were added to resuspend the pellet. Mouse inflammation standard dilutions ranging from 0–5,000 pg/mL were prepared to calculate the concentration of each cytokine per sample. The collected data were analyzed by BD Life Sciences (Korea).

Statistical analysis

The data are expressed as mean \pm SD values of 2–6 replicate samples from at least three independent experiments. Experimental groups consisted of five mice per group. Statistical analyses and graph constructions were performed using GraphPad InStat software version 3 (GraphPad Software, Inc., USA). Student's *t*-test was used to compare results between groups, and differences with a $p < 0.05$ were considered significant.

RESULTS

Influence of PA on bacterial survivability, internalization, and intracellular replication

Macrophages/monocytes are the primary target cells of *Brucella* for replication, persistent infection, and subsequent dissemination within the host [13]. The results indicate that 800 μM is the highest non-cytotoxic concentration among the different concentrations of PA (0, 40, 80, 200, 400, 800, and 2,000 μM) tested in the present study (Fig. 1A); hence this concentration was used in subsequent experiments. On the other hand, none of the different concentrations of PA (0, 40, 200, 400, and 2000 μM) directly inhibited the growth of *B. abortus* at 0, 2, and 24 h post-incubation (Fig. 1B), suggesting that PA had no bactericidal effect against *Brucella*. PA treatment of RAW264.7 cells for at least 4 h prior to infection showed no statistical difference from the control cells in the number of CFUs of internalized bacteria at 0 and 30 min post-infection (Fig. 1C), but there was lower intracellular bacterial CFUs at 24 ($p < 0.01$) and 48 ($p < 0.05$) h post-incubation (Fig. 1D), indicating that PA had no inhibitory effects on *Brucella*'s internalization but did on intracellular replication in RAW264.7 cells. On the other hand, SIRT1 expression was checked during treatment with or without infection, and the results showed a modest induction of SIRT1, although the differences between the control and the treatment groups were not significant (Fig. 1E).

Influence of PA on nitrite accumulation, superoxide anion production, and cytokine release

The results revealed that in the non-infected cells, PA treatment significantly reduced nitrite production at 2 ($p < 0.01$), 24 ($p < 0.05$), and 48 ($p < 0.05$) h post-incubation (Fig. 2A) while in

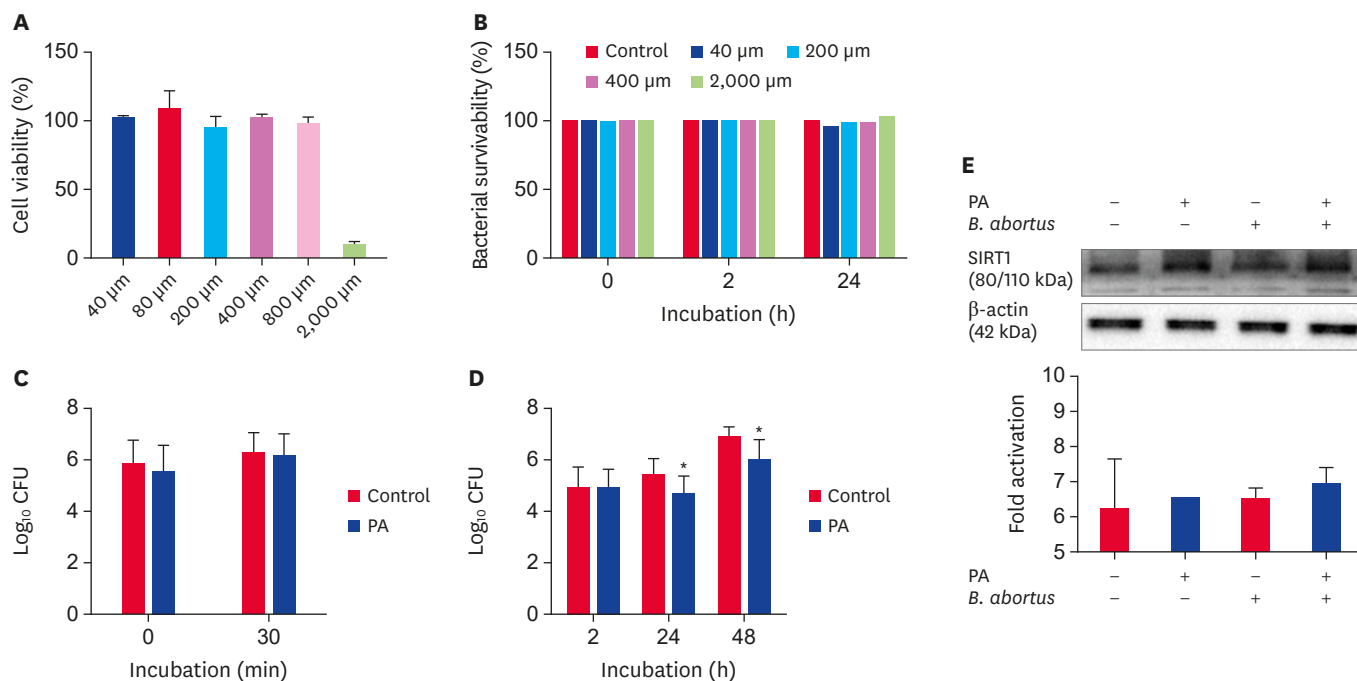


Fig. 1. Effect of PA on the uptake and intracellular survival of *Brucella abortus*. (A) RAW264.7 cells and (B) *B. abortus* were incubated with different concentrations of PA to determine cytotoxic (0, 40, 80, 200, 400, 800, and 2,000 μM) and bactericidal (0, 40, 200, 400, and 2,000 μM) effects, respectively. The highest non-cytotoxic concentration of PA was used to evaluate its (C) internalization and (D) intracellular survival efficacy against *B. abortus*. (E) SIRT1 expression was evaluated during PA treatment with or without *B. abortus* infection. Data represent mean \pm SD values of at least three independent experiments. Statistically significant differences in comparison to the untreated control group are indicated by asterisks (* $p < 0.05$). PA, palmitic acid.

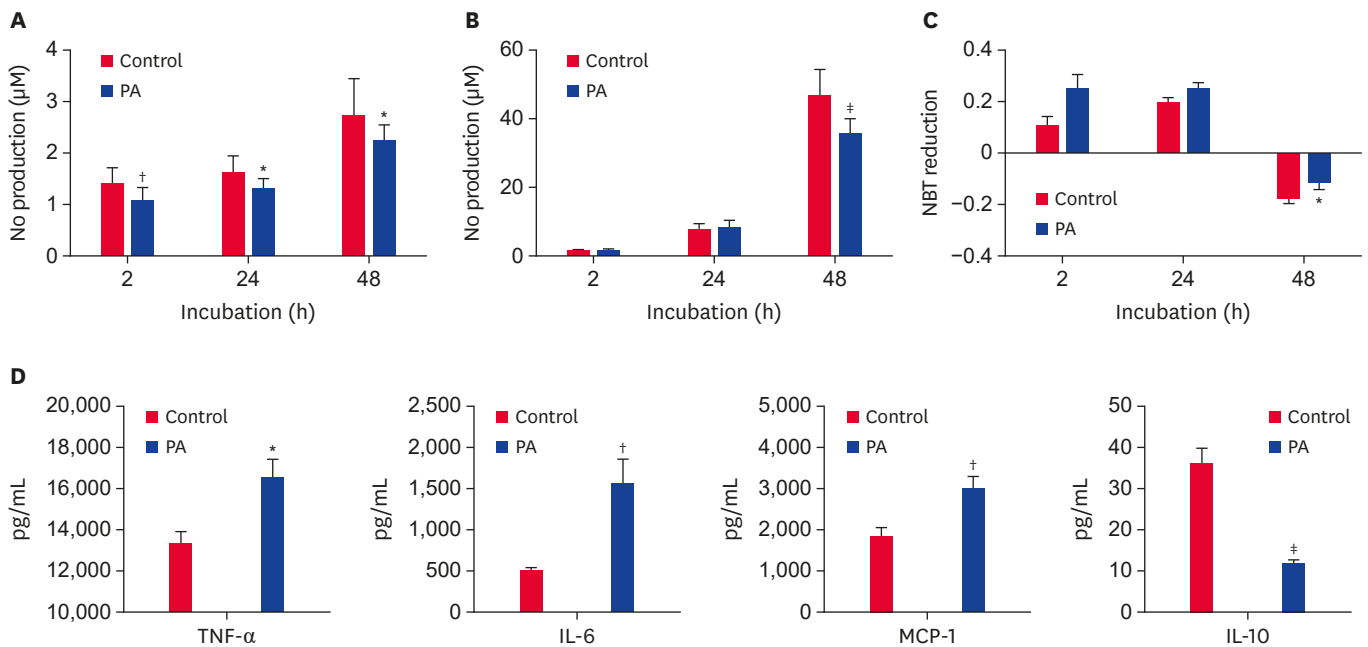


Fig. 2. Effect of PA on nitrite, superoxide, and cytokine production in RAW264.7 cells. RAW 264.7 cells were pre-incubated with PA (800 µM) for at least 4 h and then infected at a multiplicity of infection of 100 for 1 h. The cells were then further incubated with PA at indicated times to evaluate nitrite accumulation in (A) non-infected and (B) *B. abortus*-infected cells, (C) superoxide production and (D) TNF-α, IL-6, MCP-1, and IL-10 serum levels. Data represent mean ± SD values of at least three independent experiments. Statistically significant differences in comparison to the untreated control group are indicated by asterisks (* $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$).

PA, palmitic acid; NBT, nitro blue tetrazolium; TNF, tumor necrosis factor; IL, interleukin.

the *Brucella*-infected cells, nitrite production was observed to significantly decrease at 48 ($p < 0.001$) h post-incubation (**Fig. 2B**) suggesting a protective role of PA against cell damage. On the other hand, O_2^- production in *Brucella*-infected PA-treated cells was significantly increased at 48 h post-incubation compared to that of the control cells (**Fig. 2C**), suggesting that PA promotes O_2^- production during *Brucella* infection in RAW264.7 cells. Furthermore, PA-treated cells showed increased production of tumor necrosis factor (TNF)-α ($p < 0.05$), interleukin (IL)-6 ($p < 0.01$), and MCP-1 ($p < 0.01$) but reduced IL-10 ($p < 0.001$) levels (**Fig. 2D**), indicating that PA treatment could favor production of pro-inflammatory cytokines. The interferon (IFN)-γ and IL-12 cytokines were not detected. Overall, these findings suggest that PA inhibited intracellular growth of *Brucella* associated with reduced nitrite accumulation, augmented O_2^- production, and induced pro-inflammatory cytokine production in RAW264.7 cells.

Influence of PA on *B. abortus* infection in mice

Mice were monitored for clinical symptoms during the entire treatment period, and at the end of the experiment, the total average body weight and GPT level were assessed in the control groups. The results showed that the total average body weight, as well as the GPT serum level of the treatment group (20 ± 0.97 g and 82.59 ± 5.52 mLU/mL, respectively), were not different from those of the control group (20 ± 1.32 g and 82.67 ± 3.98 mLU/mL, respectively) indicating the absence of a possible negative effect of PA treatment in mice. Furthermore, total average spleen weights were not different between the treatment and non-infected control groups (**Fig. 3A**). Further, the total average spleen weight of the *Brucella*-infected PA-treated group was not different from that of the control group (**Fig. 3A**); however, the average bacterial \log_{10} CFU/g organ in the treatment group was significantly reduced, showing a protection unit of 1.34 ($p < 0.01$) (**Fig. 3B**). None of the cytokines tested for the

Effect of palmitic acid on *B. abortus* infection

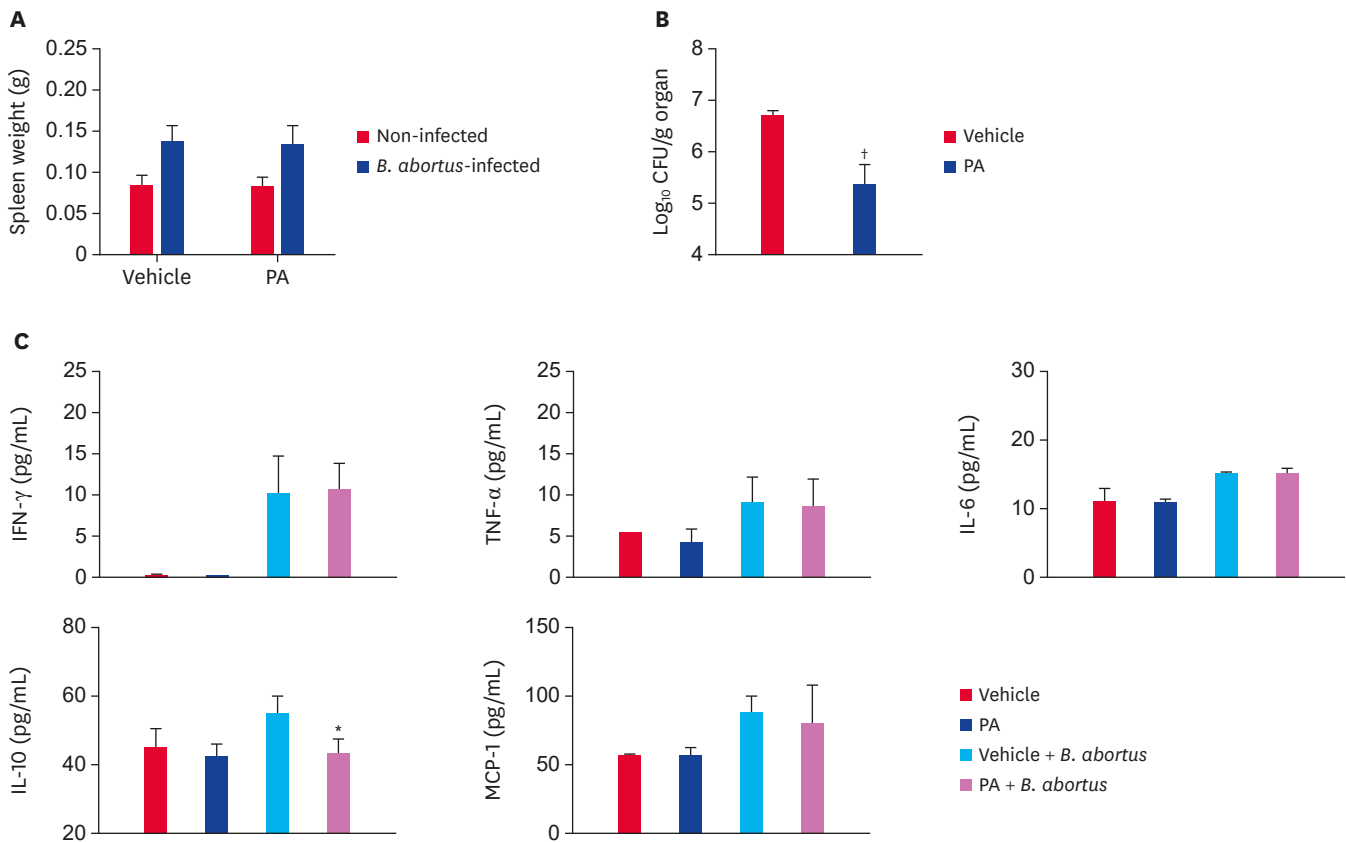


Fig. 3. Effect of PA in BALB/c mice during *Brucella abortus* infection. Mice were orally treated with PA (800 μ M; 100 μ L volume) using a gavage needle once daily for 3 d prior to infection until 14 d post-infection. At the end of the experiment, (A) spleen weights, (B) bacterial splenic proliferation, and (C) IFN- γ , TNF- α , IL-6, IL-10 and MCP-1 serum levels were evaluated. Data represent mean \pm SD values of five animals per group. Statistically significant differences in comparison to untreated mice are indicated by asterisks (^{*} $p < 0.05$; [†] $p < 0.01$). PA, palmitic acid; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin.

immune response analysis were differentially changed between treatment and non-infected control groups except for IL-12 for which its level was not detected. Only IL-10 ($p < 0.05$) was significantly reduced in *Brucella*-infected PA-treated mice compared to that of the control group (**Fig. 3C**). This result suggests that PA treatment in mice enhances resistance to *Brucella* infection and is accompanied by attenuated IL-10 induction during *Brucella* infection.

DISCUSSION

Macrophages and monocytes are an early barrier used by the host for defense and are the primary target of *Brucella* for replication, persistence, and dissemination within the host via the targeting of intrinsic and extrinsic pathways to prevent apoptosis, providing a hospitable intracellular niche for multiplication [13].

In a study done on chicken macrophages, HD11 cells, PA was one of the most predominant FA in these cells when HD11 cells were incubated in medium with or without 25 μ M of stearic acid (SA), α -linolenic acid (ALA), arachidonic acid, or docosahexanoic acid (DHA) for 24 h [12]. The uptake of *Salmonella* Typhimurium in these cells was unaffected, but bacterial clearance was significantly higher with ALA and DHA and was not associated with increased

NO or O_2^- production. Similarly, PA did not affect *Brucella* uptake in RAW264.7 cells, but it did contribute to decreased intracellular survival of the bacteria. It was reported that there was a moderate reduction in the uptake of *Staphylococcus aureus* in PA-enriched mouse peritoneal macrophages, but FA modification had no influence on the ability of these macrophages to kill intracellular bacteria nor generate superoxide anions [14]. These results suggest that the activity of PA may differ depending on host cell and pathogen types. In the present study, the reduced intracellular survival of *Brucella* can be partly attributed to the increased O_2^- production. Stimulation of macrophages by *Brucella* is a significant action in the disease progression in which the cells serve as an effector system of cytokines, chemokines, and free radicals, including NO which reacts to O_2^- to produce reactive nitrogen species (RNS). RNS are highly damaging and induce programmed cell death and apoptosis [15]. The reactive oxygen species (ROS) that are produced during this stage are used by leukocytes against bacterial infections [15]. NO and ROS have been directly and indirectly indicated as important effectors against several intracellular bacteria, including *Brucella*, in which mice deficient of NO synthase 2 or cytochrome B-245 beta chain that are responsible for NO and ROS production, respectively, display enhanced susceptibility to *Brucella abortus* S2308 infection, suggesting a crucial role in *Brucella* immunity during the early stage of infection [16].

The later adaptation phase of the acute inflammatory response following TLR4 stimulation requires FA oxidation for energy and is dependent on SIRT1 [17]. SIRT1 is known to have a critical role in cellular metabolism and the response to oxidative stress in mammals, important for the maintenance of human health, and is known to be important in preventing viral diseases [18]. SIRT1 activators have also been indicated to have potential application in the design of effective therapies against *Mycobacterium tuberculosis* infection [18]. Herein, we investigated the influence of PA in the expression of SIRT1 during *Brucella* infection. The PA concentration used in the present study did not significantly change SIRT1 expression, although a modest increase was observed during treatment.

PA has been shown to function as a ligand for Toll-like receptor 4 (TLR4) on human monocyte-derived dendritic cells in response to direct molecular interactions between PA and adaptor protein MD-2 for the induction of pro-inflammatory immune responses [19]. Furthermore, the blood concentration of PA is elevated in obese patients, resulting in inflammatory responses where TLR2 and TLR4 play an important role, and metabolic products of PA increase ROS generation [20]. In addition, PA has been reported to activate TLR4-mediated pro-inflammatory signaling pathways via MyD88-dependent NF- κ B activation [21], which has led to increased expression of cytokines including TNF- α and MCP-1 in macrophages and monocytes as well as IL-6 in mouse C2C12 myoblasts [22-24]. MyD88 is important for host defense and reported to be required for efficient clearance of *B. abortus* infection in mice [25]. TLR4 has a dominant effect with regards to *Brucella* macrophage activation and host control infection in comparison to TLR2, reinforcing the role of TLR4 in activating immune response and suggesting that the difference in protection induced by S vaccine strains may be dependent on innate immune system activation and pro-inflammatory cytokine production via this receptor [26]. It has been illustrated that a TLR2 and TLR4 agonist, recombinant *Brucella* cell-surface protein 31(rBCSP31), induces cytokine production, macrophage function as well as the T-helper 1 (Th1) immune response [27]. The control of infection in mice by another species of *Brucella*, *B. microti*, was reported to be TLR2- and TLR4-dependent [28]. Possibly, the PA treatment in the present study induced TLR2 and TLR4, activating a downstream signaling pathway and subsequent cytokine production for the control of *B. abortus* since high levels of pro-inflammatory cytokines such as TNF- α and IL-6

and MCP-1 and reduced anti-inflammatory cytokine IL-10 were observed in cells treated with PA after 48 h of incubation during *B. abortus* infection.

Cytokines are important in inflammation, and many researchers have focused on cytokine-mediated inflammatory reactions in brucellosis [27]. *Brucella* is known to downregulate production of pro-inflammatory cytokines, particularly TNF- α , to promote its intracellular survival and evade immune recognition [29]. TNF- α is a crucial cytokine that can activate macrophages and protect humans and animals against *Brucella* persistence [29]. A low level of MCP-1 was observed in MyD88 knockout macrophages compared with that of C57BL/6, a potent leukocyte chemoattractant critical for Th1 cell activation; hence, the low level could enhance host susceptibility to murine brucellosis [30]. Previously, we reported that IL-6 contributes to host resistance against *B. abortus* by activating bactericidal activity in macrophages [31]. On the other hand, *in vitro* neutralization of IL-10 reduced, by up to 10-fold, the bacteria in the spleens of BALB/c mice infected with *B. abortus* 2308, suggesting that this cytokine downregulates immune response to the bacteria by inhibiting the anti-*Brucella* effector functions of macrophages and production of IFN- γ by spleen cells [32]. Furthermore, we previously reported that IL-10 attenuates lysosome-mediated killing of intracellular *B. abortus* in RAW264.7 cells [33]. Interestingly, only a reduction in serum IL-10 production was observed in mice orally treated with PA, which may also contribute to the reduced number of bacterial CFU in the spleens of BALB/c mice. Splenic weight was unaffected by the treatment, and a direct bactericidal effect of PA against the pathogen can be ruled out since no inhibitory effect was observed during the *in vitro* experiment. Overall, it can be implicated that the efficacy of RGO against *B. abortus* is further enhanced by PA.

In conclusion, the results of the present study suggest that PA potentially contributes to the control of *B. abortus* proliferation in murine macrophages and BALB/c mice. The inhibitory effect on the progression of the infection was attributed to reduced bacterial intracellular survival accompanied by reduced nitrite accumulation, increased O₂⁻ production, induced pro-inflammatory cytokine secretion, and reduced IL-10 in macrophages. The *in vivo* experiments showed attenuated IL-10 serum level with a high protection unit calculated from bacterial CFU in the spleens of PA-treated mice, suggesting that PA treatment could benefit the host during brucellosis. However, the complete underlying mechanisms revealing how PA treatment results in the control of *Brucella* infection are still undetermined; hence, further studies are recommended.

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