Abstract. Brucellosis, caused by a facultative intracellular parasite \textit{Brucella species}, is the most common bacterial zoonotic infection worldwide. \textit{Brucella} can survive and proliferate in several phagocytic and non-phagocytic cell types. Human brucellosis has similar clinical symptoms with systemic diseases, which may lead to delay of diagnosis and increasing of complications. Therefore, investigating the proliferation of \textit{Brucella} in host cells is important to understand the pathogenesis of the disease. Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisin, has been recommended by World Health Organization as an anti-malarial drug. However, there have been few studies regarding its effectiveness against bacteria. In the present study, it was revealed that \textit{B. suis} vaccine strain 2 (S2) grew in BV2 cells without significant cytotoxicity, and less than 20 \( \mu \)M DHA had no inhibitory effects on BV2 cells. Furthermore, DHA reduced \textit{B. suis} S2 growth in BV2 cells, and increased the percentage of apoptosis and the expression of cleaved caspase-3 in \textit{B. suis} S2-infected cells. Collectively, the present data indicated that DHA induced the caspase-dependent apoptotic pathway to inhibit the intracellular \textit{B. suis} S2 growth.

Introduction

Brucellosis, caused by a facultative intracellular parasite \textit{Brucella species}, is the most common bacterial zoonotic infection worldwide (1,2). \textit{Brucella} can survive and proliferate in several phagocytic and non-phagocytic cell types, including macrophages, dendritic cells, placental trophoblasts \textit{in vivo}, and has the ability to replicate in a variety of mammalian cells, such as microglia, fibroblasts, epithelial and endothelial cells (3). In China, Brucellosis represents a major challenge from the last decade, and a high incidence of human brucellosis has been reported in northwestern China (4). Human brucellosis has similar clinical symptoms with systemic diseases and can lead to delay in diagnosis and may increase unexpected complications (5). Therefore, investigating the proliferation of \textit{Brucella} in host cells is important to understand the pathogenesis of the disease. In addition, earlier evidence indicates that \textit{Brucella} inhibits apoptosis of host cells to maintain an intracellular niche for its replication (6). Therefore, induction of apoptosis in the host cells may provide a useful therapeutic strategy for the treatment of Brucellosis.

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisin, has been recommended by World Health Organization as an anti-malarial drug (7). Previous studies have documented that DHA possesses the ability to prevent angiogenesis (8), cause cell cycle arrest (9), promote apoptosis, and inhibit cancer invasion (10). However, there have been few studies regarding its effectiveness against bacteria.

In the present study, it was investigated whether DHA inhibited the intracellular growth of \textit{Brucella suis} vaccine strain 2 (\textit{B. suis} S2) in murine microglia BV2 cells, and whether the inhibitory effect of DHA was associated with stimulation of apoptosis of BV2 cells.

Materials and methods

Cell line and bacteria. Murine microglia BV2 cells were obtained from China Center for Type Culture Collection and cultured with Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 2 mM glutamine, and 200 mM streptomycin/penicillin and maintained in 5% CO\textsubscript{2} at 37°C. \textit{B. suis} S2 was provided by Professor Xu from Ningxia...
Medical University (Yinchuan, China) and was grown in Soybean-Casein Digest Agar Medium (TSA) at 37°C in a 5% CO2 incubator. The single B. suis S2 colony was inoculated in sterilized tryptic soy broth (TSB) solution at 37°C in a 5% CO2 atmosphere until its use. Bacteria were harvested by centrifugation for 20 min at 2,000 x g at 4°C and washed twice with phosphate-buffered saline (PBS). Bacterial density in the cultures were estimated by the McFarland standards of bioMérieux, Inc. All bacterial experiments were carried out at the Biosafety Level 2 Laboratory (Key laboratory of pathogenic microorganisms, the general hospital of Ningxia Medical University).

In vitro infection and DHA treatment. A total of 2x10^5 BV2 cells were seeded in 6-well culture plates. After reaching 60% confluence, the cells were exposed to B. suis S2 at a multiplicity of infection (MOI) of 100 for 2 h in medium without antibiotics. Subsequently, BV2 cells were extensively washed to remove extracellular bacteria, and infection was maintained for 24 h in the presence of 100 µg/ml gentamicin to kill remaining extracellular bacteria (11). Two hours after infection, the cells were treated with different concentrations of DHA (0, 10, 20, 30, and 40 µM).

Colonial-forming unit assay (CFU assay). The TSA was prepared according to the manufacturer’s instructions and autoclaved at 120°C for 30 min. On a clean bench, ~25 ml of TSA was poured into a 90-mm sterile culture plate, inverted after coagulation, and used for bacterial culture. BV2 cells were infected with B. suis S2 at MOI 100 for 24 h, washed with PBS twice and lysed in 0.3% Triton-X100 for 10 min. The sample was inoculated on TSA media for 72 h, and the cell suspension was diluted to count the number of colonies. The visible colonies with a diameter greater than 1 mm were manually counted with an optical microscope.

Cell viability assay. Cell viability was determined by Cell-counting Kit-8 (CCK-8; BestBio) assay according to the manufacturer’s protocol (12). In brief, cells (1x10^5) were seeded in 96-well culture plates and cultured overnight. The cells were treated with different concentrations of DHA (0, 10, 20, 40, and 80 µM) for 24 h, and the CCK-8 solution was added.
to each well and incubated for an additional 4 h. The absorbance at 450 nm was measured by Microplate reader (BioTek Instruments, Inc.) (13).

Lactate dehydrogenase (LDH) assay. The toxicity of BV2 cells after B. suis S2 infection was determined by the LDH assay using a commercially available kit (Sigma-Aldrich; Merck KGaA) (14). Briefly, the BV2 cells were infected with B. suis S2 at MOI 100 or exposure to different concentrations of DHA (0, 10, 20, 30 and 40 µM) for 24 h. Finally, the supernatant was collected for the LDH assay.

Flow cytometric assay. The BV2 cells were infected with B. suis S2 and then stained with Annexin V/PI according to manufacturer's protocol (BestBio). The apoptosis in cells was analyzed by flow cytometric analysis (FCM).

Western blot analysis. Cells were harvested, washed twice with PBS, and lysed in RIPA buffer (Nanjing KeyGen Biotech Co., Ltd.). Protein concentration in the lysates was determined by the bicinchoninic acid (BCA) method according to the manufacturer's recommendations (Nanjing KeyGen Biotech Co., Ltd.). Protein (30 µg) was separated on 12% Mini-Protean TGX gels and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane according to standard protocols. Caspase-3 (ID product code ab184787; dilution in 1:1,000), cleaved caspase-3 (ID product code ab49822; dilution in 1:500) or GAPDH (ID product code ab181602; dilution in 1:5,000; All from Abcam) antibodies were used for protein detection. Goat anti-mouse polyclonal antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies; ZB-2305; dilution 1:5,000) was used as a secondary antibody. The blot was developed using the Western Lightning Ultra chemiluminescent substrate from Bio-Rad Laboratories, Inc., and detected using EpiChem3 darkroom (UVP BioImaging Systems).

Antimicrobial susceptibility testing. The single clone was added to 5 ml TSB, incubated in a 37°C incubator for 24 h, and the concentration of the bacteria suspensions was adjusted to ~10⁸ CFU/ml with TSB. TSB (200 µl) and bacteria suspensions were used as a negative control and positive control, respectively. After loading the drug and bacteriostatic solution, the plate was incubated at 37°C in an incubator for 24 h, and the absorbance at 580 nm was measured (15).

Results

Replication of B. suis S2 in murine microglia BV2 cells. In order to investigate the growth of B. suis S2 in BV2 cells, BV2 cells were infected in vitro with MOI 100. It was revealed that there was a significant intracellular increase in CFUs 24 h post-infection (Fig. 1A), indicating the active growth of B. suis S2 in the murine microglial cells. In addition, there was no apoptosis of BV2 cells under light microscopy (data not shown) after bacterial infection, and the levels of LDH were not significantly altered 24 h post-infection (Fig. 1B).

Inhibition of caspase-dependent apoptosis after B. suis S2 infection. Western blot analysis revealed that the expression of cleaved caspase-3 was significantly decreased after B. suis S2 infection, but gradually increased as the infection period
Effects of DHA on BV2 cell viability. To observe the biological effects of DHA on BV2 cells, the cells were treated with different concentrations of DHA for 24 h. The results revealed that concentrations <40 µM DHA exhibited no inhibitory effects on cell viability (Fig. 3A). Moreover, cells were treated with 20 µM DHA for various intervals, and no significant changes in cell viability were observed (Fig. 3B). The LDH assay demonstrated that a dose of DHA <20 µM had no inhibitory effects on BV2 cells (Fig. 3B).

DHA reduces B. suis S2 growth in BV2 cells. The present data revealed that intracellular growth of B. suis S2 could inhibit cell apoptosis of BV2 cells (unpublished data). Furthermore, CFU assay results of the treated BV2-infected cells with 10-40 µM DHA revealed a significant and marked reduction in the B. suis S2 growth in the BV2 cells (Fig. 4).

DHA (20 µM) inhibits bacterial multiplication by inducing apoptosis. The effects of DHA on B. suis S2 multiplication in BV2 cells were analyzed by the flow cytometric assay. The results revealed that 20 µM DHA treatment increased the level of apoptosis in B. suis S2-infected BV2 cells (Fig. 5). Furthermore, western blot analysis revealed that 20 µM of DHA treatment increased the expression of cleaved caspase-3 in B. suis S2-infected BV2 cells (Fig. 6). The present data indicated that DHA inhibited bacterial multiplication through induction of apoptosis in BV2 cells.

DHA does not directly inhibit or kill B. suis S2. To confirm whether DHA affects B. suis S2 growth directly or indirectly through apoptosis, an antibacterial susceptibility test was performed. The results revealed that different concentrations of DHA had no effect on bacterial growth (Fig. 7), indicating that DHA does not directly inhibit or kill B. suis S2. DHA did not exhibit any direct effects on bacterial growth, which supports our theory that the mechanism involved in the reduction of intracellular replication of BV2 cells by DHA may be achieved by promoting apoptosis of BV2 cells.

Discussion

Brucella is an intracellular bacterium that can cause chronic infections. It has been reported that infection of Brucella inhibits apoptosis of host cells (16,17). Therefore, it is necessary to investigate the mechanism underlying infection and develop therapeutic strategies for Brucellosis.

In the present study, it was demonstrated that B. suis S2 grew in BV2 cells without significant cytotoxicity, which is consistent with the characteristics of intracellular parasites. Previous studies revealed that Brucella outer membrane protein OMP25 inhibited apoptosis of host cells (6). Similarly, this study confirmed that the caspase-dependent apoptotic
pathway of BV2 cells was significantly inhibited after *B. suis* S2 infection, which provides a favorable condition for the intracellular replication of *B. suis* S2. This result is consistent with our findings on the mechanism of sustained infection for intracellular parasites, such as *Mycobacterium tuberculosis* (18).

Apoptosis, programmed cell death, is indispensable for the development and homeostasis of multicellular organisms (19). Caspase-3 is one of the activated caspases that catalyzes the cleavage of many key cellular proteins during apoptosis (20). In the present study, western blot analysis revealed that the expression of cleaved caspase-3 was significantly decreased in BV2 cells after *B. suis* S2 infection, but gradually increased as the infection period increased. These observations indicated that *Brucella* may inhibit apoptosis of host cells for the infection. The present results are in agreement with the findings of *Brucella* in other types of cells (21). Collectively, these data indicated that inhibition of host cell apoptosis may be a common strategy for *Brucella*-sustained infection. The underlying mechanisms of the DHA effect on bacteria remain unknown. Some studies suggest that the strategy of *Brucella* for infection establishment includes the following: i) to avoid intracellular destruction by limiting the fusion of the type IV secretion system-dependent vacuole-containing vacuoles to lysosomes; ii) inhibiting apoptosis of infected monocytes; and iii) inhibition of dendritic cell maturation, inhibition of antigen presentation and activation of naive T cells (22). It is our aim to investigate the mechanisms in our future studies.

Conversely, DHA displays potent anti-viral and anti-parasitic activities (23,24), however, the molecular mechanisms are not fully understood yet. Artemisinin was also effective on bacteria. For example, oral administration of artesunate was revealed to reduce death from sepsis caused by *E. coli* (25,26). In combination with antibiotic oxacillin, oral administration of artesunate revealed a high effectiveness against mouse sepsis induced by methicillin-resistant aureus (MRSA). On the basis of previous findings, the inhibitory effects of DHA were evaluated on *B. suis* S2-infected cells. It was revealed that DHA inhibited *B. suis* S2 growth in BV2 cells. Furthermore, DHA increased the percentage of apoptosis and the expression/proteolytic degradation of caspase-3 in *B. suis* S2-infected cells. It is therefore concluded that DHA induces caspase-dependent apoptotic pathway activation to inhibit intracellular *B. suis* S2 growth.

*Brucella* is an intracellular parasitic bacterium. In the present study, BV2 cells were infected with *B. suis* S2, and CFU experiments revealed that *B. suis* S2 grows in BV2 cells. After treatment with DHA, apoptosis of BV2 cell was increased. Additional experiments demonstrated that DHA had no direct effects on *B. suis* S2, which is similar to the effect of antibiotics on bacteria. Therefore, it is concluded that the mechanism involved in the reduction of intracellular replication of BV2 cells by DHA may be achieved by promoting apoptosis of BV2 cells.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JY, HL, ZhaW and ZheW conceived and designed the experiments. JY, HL, ZhaW and ZheW conducted all the experiments. LY, QL, XN and TX contributed reagents, materials and analysis tools. XN and TX performed the experiments and were involved in the preliminary work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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