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ORIGINAL ARTICLE

Astragaloside IV Inhibits *Salmonella*-Induced Meningitis Via Modulation of Bacterial Virulence and Host Response

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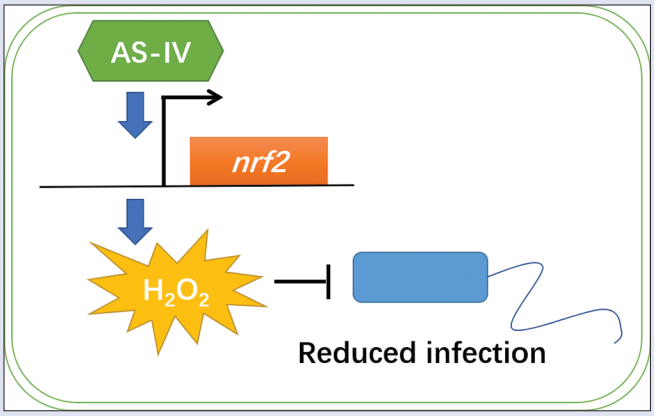
ABSTRACT

Objectives: To control *Salmonella*-induced meningitis, in this study, we aimed to investigate the potential anti-virulence activity and neuroprotective role of astragaloside IV (AS-IV) under *in vitro* and *ex vivo* conditions during coculturing with *Salmonella* cells. **Materials and Methods:** The expression of virulence factors encoding genes was tested under *in vitro* conditions using quantitative real-time polymerase chain reaction (PCR) and Western blot analysis. In addition, *Salmonella* cells and neuron cells were cocultured to examine the expression of virulence-associated genes and host immune response. **Results:** The results of this study indicate that AS-IV efficiently inhibited the production of virulence factors such as biofilm formation and expression of virulence-associated genes including *rpoS*, *phoP*, *sopA*, and *spvB* under both *in vitro* and *ex vivo* conditions. In addition, our results showed that AS-IV might boost the production of reactive oxygen species via Nrf2/ARE signaling pathway, which in turn inhibits the growth and virulence factors of *Salmonella* cells. **Conclusion:** In summary, the results of this study provide insights into the mechanisms of AS-IV against *Salmonella* infections, and we identified a promising therapeutic agent against *Salmonella*-induced meningitis.

Key words: Astragaloside IV, meningitis, Nrf2/ARE, salmonella cells, virulence

SUMMARY

- Anti-virulence activity and neuroprotective role of astragaloside IV (AS-IV) under in vitro and ex vivo conditions during coculturing of Salmonella and neuron cells. AS-IV efficiently inhibited the production of virulence factors such as biofilm formation and expression of virulence-associated genes including rpoS, phoP, sopA, and spvB under both in vitro and ex vivo conditions. These findings suggest that AS-IV might be a promising therapeutic agent against Salmonella-induced meningitis.



Abbreviations used: AS-IV: Astragaloside IV; PCR: Polymerase chain reaction; ROS: reactive oxygen species; CNS: Central nervous system; SPI: *Salmonella* pathogenicity island; TCM: Traditional Chinese medicine; Nrf2: Nuclear-factor-erythroid 2-related; CFUs: Colony-forming units; HPLC: High-performance liquid chromatography; OD: Optical density; FACS: Fluorescence-activated cell sorter; BCA: Bicinchoninic acid; CSF: Cerebrospinal fluid; SILAC: pulsed-stable isotope labeling by amino acids in cell culture.

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INTRODUCTION

Bacterial meningitis is a serious infectious disease of the Central Nervous system (CNS) which can occur in adults, children, and immunocompromised people.[1] It is transmitted mainly via food, inhalation, and blood transfusion, and can be fatal if not treated properly and timely.[2] A variety of microorganisms can cause this disease, of which *Cryptococcus neoformans* is the most common, followed by *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis,* and other Gram-positive bacilli, *Listeria monocytogenes*, and Group B *Streptococcus.*[3,4] Typical signs and symptoms include sudden onset of fever, headache, and stiff neck. Other symptoms may be observed such as nausea, vomiting, photophobia, and altered mental status.[5] Symptoms of bacterial meningitis could either progress quickly or over several days, and the later stage could be fatal.[2]

Although *Salmonella* is a rare cause of meningitis, *Salmonella* meningitis can still occur mostly in newborn babies and immunodeficient adults.[6] It has been reported that *Salmonella* meningitis can be transmitted to babies, pregnant women, and immunodeficient adults by pet reptiles.[7] In addition, *Salmonella typhimurium* can infect the CNS after oral inoculation in mice.[8] Following ingestion, *Salmonella* crosses the intestinal epithelium and disseminates into the systemic sites, such as the liver, spleen, bone marrow, gall bladder, and CNS.[9] Typical symptoms

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develop within 10-14 days postingestion including fever, headache, muscle aches, stomach pain, constipation or diarrhea, and neuronal disorder.[10] The pathogenesis of *S. typhimurium* is linked with its specific virulence factors including a polysaccharide capsule named the Vi antigen, typhoid toxin, the *Salmonella* pathogenicity island (SPI)-1, and SPI-2 type III secretion systems.[9] Moreover, the development of the infection cycle is associated with the regulators of these virulence factors such as RpoS and two-component system PhoP/PhoQ, pH, and nutrients within hosts.[11]

Currently, vaccines are the most effective way to protect against certain types of bacterial meningitis.[12] Nowadays, at least three types of vaccines are available for the prevention of meningitis, including meningococcal vaccines against *N. meningitidis*, pneumococcal vaccines against *S. pneumoniae*, and Hib vaccines against *H. influenzae* type b.[13] In addition, bacterial meningitis can be treated with frontline antibiotics which may cause severe side effects such as permanent brain damage and hearing loss.[14] Therefore, it is urgent and necessary to search for alternative therapeutics for this disease.

For thousands of years, traditional Chinese medicine (TCM) has been considered important resources for the treatment against infectious diseases. The adoption of TCM to treat bacterial meningitis has been extensively studied in China.[15] Some unique functional ingredients such as *Radix astragali* (also Huangqi in Chinese), *Forsythiae fructus* (also Lianqiao in Chinese), and *Lonicerae japonicae* Flos (also Jinyinhua in Chinese) have been shown to protect bacterial infections via anti-inflammatory, antioxidative, and immunomodulatory effects.[15-17] For example, Chinese herbal monomer can exert neuroprotective effects via activation of nuclear-factor-erythroid 2-related (Nrf2) factor.[18,19]

Astragaloside IV (AS-IV), one of the main ingredients of Radix Astragali, possesses antioxidant, anti-inflammatory, and immunoregulatory effects.[20] For instance, AS-IV has been shown to increase the activity of antioxidant enzymes and decrease the production of reactive oxygen species (ROS) induced by high glucose in HK-2 cells via regulation of the Nrf2 pathway.[21] However, the antivirulence ability of this compound has been seldom studied. Therefore, in this study, we aimed to find out whether AS-IV acts against *Salmonella* meningitis via inhibition of virulence factor production and activation of the Nrf2 pathway at the cellular level.

MATERIALS AND METHODS

Bacteria growth conditions

Salmonella enterica serovar Typhimurium strain LT2 was a gift from Prof. Shaofu Qiu (Institute of Disease Control and Prevention, Academy of Military Medical Sciences, Beijing, China) and used in this study as previously described.[22] Bacteria were cultured in Luria–Bertani (LB) broth and on LB agar plates supplemented with 100 μg mL^{-1} streptomycin. Before animal infections, overnight bacterial cultures were pelleted in LB broth (BD, USA), washed in a buffer containing 0.1 M HEPES (pH 8.0) and 0.9% sodium chloride, and resuspended in the same buffer to ~10^7-10^9 colony-forming units (CFUs)/mL. The actual inoculum size was verified by plating on LB plates for CFU counts.

Reagents and chemicals

All chemical reagents including HEPES, sodium chloride, and antibiotics used in this study were purchased from Sigma-Aldrich (Shanghai, China). Solvents used in this study were of high-performance liquid chromatography grade.

Experimental animals

Experimental animals (C57BL/6, Jackson Laboratories) were female mice between 8 and 12 weeks of age (weight of 18–22 g). Mice were kept in pathogen-free conditions with free access to food and water, except where noted.

Animal infections and tissue samples

All animal experiments were conducted after obtaining approval from the Animal Ethics Board of Maoming People's Hospital and according to the guidelines set out by the Hospital Council on Animal Care (Approval number: 202004). Animal infections were performed as described by inoculating the mice with a gavage needle of approximately 10^6–10^8 wild-type *S. enterica* serotype Typhimurium strain LT2 in a 100 μL volume.[22] Food and water were restored 1 h postinoculation. The infected mice were checked twice daily for signs of clinical illness such as ruffled fur, hunched posture, or ataxia. The animals were anesthetized with isoflurane for 2 min, and the tissues including brain, spleen, and liver were harvested in preweighed tubes containing 500 mL of sterile buffered pharmaceutical-grade saline. The samples were homogenized with a Precellys 24 tissue homogenizer and adjusted for concentration (based on weight) before plating and enumeration of CFUs. LB agar plates containing 25 μg/mL streptomycin were used for colony purification.

16S rRNA identification

We identified the bacterial species based on 16S rRNA gene.[23] Briefly, polymerase chain reaction (PCR) was performed in a Bio-Rad (Hercules, CA, USA) PCR machine. Primer sets (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′) were adopted and the following cycling parameters were used: 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 90 s and 72°C for 30 s, followed by 72°C for 7 min. Amplified products were electrophoresed on 0.8% agarose gels to examine the presence of positive amplicons and sent for Sanger sequencing analysis with correct amplicon size. Then, 16S rRNA gene sequences were analyzed using DNAMAN (Lynnon Biosoft) and CLC Main Workbench (CLC Bio).

Biofilm assay

After growing in LB broth overnight, 1 μL of *Salmonella* culture was added into 99 μL of LB broth in a 96-well plate, and incubated at 30°C for 10 h. Then, the nonadherent bacterial cells were removed, and the plate was rinsed thrice with distilled water. Next, 100 μL of 0.1% crystal violet solution was added and rinsed thrice with distilled water and then 200 μL of 30% sodium acetate solution was added to dissolve all the crystal violet on the wall of the plate. Then, from each well, 125 μL of the solution was pipetted into a clean polystyrene microliter dish, and the optical density (OD) was read at 560 nm on a microplate reader (Synergy H4, Biotek). Each experiment was repeated thrice.

RNA extraction

Bacteria at exponential growth phase were collected and their total mRNA was isolated using Kit RNAfast200 (TaKaRa Biotechnology, China). The mRNA was quantified using an ND-2000 ultra-micro nucleic acid protein analyzer (NanoDrop, USA) and was subsequently stored at −80°C before its use.

Reverse transcription-polymerase chain reaction

For each reverse transcription-PCR (RT-PCR) reaction, 2 μg of total RNA was used to synthesize cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa RR047B, Da Lian, China). The quantification of the relative mRNA was detected by quantitative RT-PCR (qRT-PCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems) with the SYBR green Plus reagent kit (Roche A46109). The primers were

synthesized by Shanghai Jingan Biotechnology Co. Ltd. and are shown as follows:

rpoS-Fw (5′-3′): CAGTGTCAGCATTGTCTGTA,

rpos-rev (5′-3′): CAGCTCTACAAGCTTGCATT;

phoP-Fw (5′-3′): ATGCAAAGCCCGACCATGACG,

phoP-Rev (5′-3′): GTATCGACCACCACGATGGTT;

sopA-Fw (5′-3′): GGACTGTCATTGAATAATCAGC,

sopA-Rev (5′-3′): GGTTGAGGCTGGACTAC;

spvB-Fw (5′-3′): TCATACTCCAGCAGCAGACG,

spvB-Rev (5′-3′): AGCAGTTTTTATCGCCTGGA.

For all primer sets, the following cycling parameters were used: Initial denaturation at 95°C for 3 min followed by secondary 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 10 min. The relative fold change in transcription due to AS-IV treatment with respect to the control was calculated through the comparative cycle time (CT) approach[24] and all treatments were calibrated with the housekeeping gene, 16S rRNA.

siRNA transfection

In this study, Hiperfect (Qiagen, USA) was used for siRNA transfection according to the manufacturer's recommendations. NRF2-siRNA (SI03246950) and negative control-siRNA (1022076) were purchased from Qiagen (USA).

Cell viability assay

Cell viability assay was conducted based on the manufacturer's instructions (CCK-8 kit, APExBIO). Briefly, 20 μL of CCK-8 reagent was added to each well (containing 200 μL of medium) in a 96-well microplate, and the microplate was further incubated at 37°C for 4 h. Finally, $OD_{450\ nm}$ were recorded for different groups ($n = 3$). Cell viability in the control group was referred to as "100%," and the relative cell viability of other groups was calculated correspondingly.

Flow cytometry analysis

Fluorescence-activated cell sorter (FACS) cytometric analysis was conducted based on a previous study with the H_2O_2-activated green fluorescent dye dihydrodichlorofluorescein diacetate (H_2DCFDA, Molecular Probes) and with slight modifications.[25] This redox dye is readily taken up by the cells and cleaved by the cellular esterase's to form non-cell permeant H_2DCF, which can be activated by ROS to give green fluorescent DCF as the indicator of intracellular levels of oxidative stress. Cell suspensions were adjusted to an $OD_{600\ nm}$ of 0.5, pelleted by centrifugation, and resuspended in phosphate-buffered saline (PBS) containing 20 mM H_2DCFDA. The suspension was incubated for 4 h, diluted 1:100 times in PBS, and fluorescence levels of 50,000 cells were measured with a FACScalibur cytometer (BD Biosciences). Summit software (Dako Colorado) was applied for data analysis.

Western blot Analysis

Protein concentrations were measured using bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Equal amounts of proteins (40 μg) were loaded in each well and separated by 10% SDS-PAGE and blocked with 5% bovine serum albumin dissolved in TBST (Tris-buffered saline, 0.1% Tween 20) for 1 h, and then incubated with primary antibody for RNAP (1:2000; ab191598; Abcam, Cambridge, MA, USA) and custom made antibodies for RpoS, PhoP, SopA, and SpvB (1:1000; Abmart, Shanghai, China) at 4°C for 12 h. The membranes were washed four times for 6 min and incubated with the appropriate secondary antibody conjugates (Abcam, Cambridge, MA, USA) or horseradish peroxidase-conjugated protein antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Then, membranes were washed four times and stained with DAB Horseradish Peroxidase (Color Development Kit Beyotime, Shanghai, China). The proteins were detected with the Gel Visualize Alpha Innotech (Tanon-5200Multi, Tanon Science and Technology Co., Ltd., Shanghai, China). Protein levels were normalized to RNAP and quantified using densitometry via a Tanon Gel Imaging System (Tanon, Shanghai, China).

Preparation of drug extract

For the preparation of extract, 100 g of AS-IV powder (Tongrentang group Co, Ltd.) was extracted with 500 mL of ultrapure water at 50°C for 24 h. The aqueous extract was centrifuged twice at 25,000 rpm for 60 min. The supernatant liquor of the extract was concentrated in vacuo to 100 mL of the aqueous extract and then lyophilized (Zirbus, Germany). The lyophilized powder was stored at −50°C. The drug extract was then dissolved in PBS and filer-sterilized before using.

Determination of minimal inhibitory concentration and sub-minimal inhibitory concentration

The minimal inhibitory concentration (MIC) value of AS-IV for *Salmonella* was performed according to a previous study[26] and measured by two-fold dilutions in Mueller-Hinton broth (BD, Difco) with inoculum of 5×10^5 CFUs/mL. The final concentration of the active components in AS-IV was 512 mg/mL to 0.125 mg/mL. The MIC value was referred to as the lowest concentration of AS-IV allowing no visible growth, and the sub-MIC was defined as the highest concentration of AS-IV that did not inhibit bacterial growth by determining the cell density. For other experiments, *Salmonella* was cultured in a 20-mL conical flask with shaking at 37°C in AS-IV broth supplementing with appropriate concentrations of AS-IV. Bacterial cultures were sampled at intervals of 1 h. Cell density was calculated by measuring absorbance at 600 nm using a Synergy H1 microplate reader (BioTek, USA).

Statistical analysis

GraphPad Prism software version 7 was used for all statistical analyses (USA). The tests are indicated in Figure legends. Data are expressed as mean ± standard deviation and analyzed using the Student's *t*-test and variations are considered significant at $P < 0.05$.

RESULTS

Salmonella enterica serovar typhimurium caused the meningitis

To verify whether AS-IV might efficiently inhibit meningitis caused by *Salmonella*, we used *Salmonella*-infected meningitis mice model to examine the role of AS-IV in defense against bacterial infection. First, we confirmed that *Salmonella* might indeed be identified as a unique causal agent in inducing meningitis. Bacterial colonies were extracted from cerebrospinal fluid (CSF), spleen, and liver and isolated using LB agar containing streptomycin. Several colonies were formed after serial dilution and plating on overnight culture [Figure 1a]. We randomly chose two of these colonies from the CSF group and further purified them by secondary streaking on LB agar plates containing streptomycin. Single colonies appeared and were PCR-verified by 16S rRNA analysis and sent for confirmation by Sanger sequencing [Figure 1b]. It was shown that the unique bacterial isolate from CSF was *Salmonella* and *Salmonella enterica* serovar Typhimurium could cause meningitis in our study.

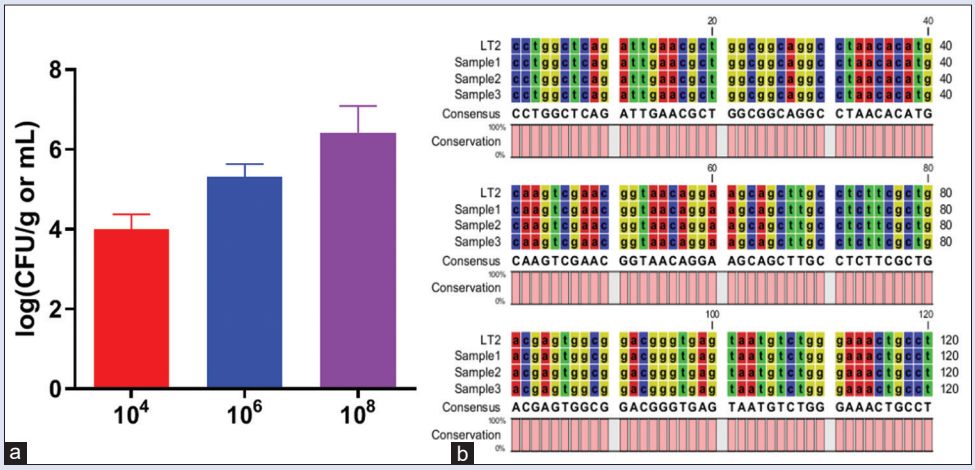

Figure 1: *Salmonella enterica* serovar typhimurium caused the meningitis in mice. (a) Organ colonization of *Salmonella* from cerebrospinal fluid, spleen and liver. Samples were collected and bacterial colonies were counted. (b) 16S rDNA sanger sequence alignment using CLC workbench. Three samples were randomly selected. No mutation was identified

Astragaloside IV inhibits the *in vitro* production of virulence factor

To understand the mechanisms underlying the potential inhibitory properties of AS-IV against *Salmonella*, we performed the growth curve analysis in the presence or absence of different concentrations of AS-IV (0, 1%, 2%, 3%, and 4% is the MIC). According to the results, *Salmonella* growth kinetics were unaffected at all tested concentrations (data not shown). It was known that mechanisms underlying *Salmonella* infection are largely dependent on the virulence factors such as pathogenicity islands, endotoxins, and adhesins. In addition, regulatory elements such as two-component systems are also involved in this process.[9] To investigate whether AS-IV inhibits the production of these virulence factors, we examined the effect of AS-IV on the formation of biofilm of *Salmonella*, a trait mainly dependent on virulence factor genes on the SPI-2 island.[27] We found that AS-IV could greatly attenuate the production of biofilm formation in a time-dependent manner [Figure 2a], indicating that AS-IV inhibited the ability of adhesion during cellular contact and virulence gene expression.

To further confirm that AS-IV could mitigate the gene expression related to pathogenesis, we extracted the bacterial RNA and performed qRT-PCR analysis to examine the influence of AS-IV on their transcription level. Interestingly, all four genes (*rpoS*, *phoP*, *sopA*, and *spvB*) showed dramatic decrease in the expression level, which indicates that AS-IV inhibits the expression of virulence genes of *Salmonella* under *in vitro* conditions [Figure 2b].

Furthermore, we extracted the total proteins of AS-IV-treated cells and compared with that of nontreated control cells using Western blot analysis. According to our results, the selected proteins (RpoS, PhoP, SopA, and SpvB) were greatly downregulated in the AS-IV-treated group as compared to that of nontreated control cells [dimethyl sulfoxide (DMSO), Figure 2c]. Figure 2d shows the results.

Altogether, we have confirmed that AS-IV could efficiently inhibit the gene expression of virulence factors under *in vitro* conditions.

Astragaloside IV protects PC12 cells against *Salmonella*-induced injury via inhibition of production of virulence factor

AS-IV might inhibit the production of virulence factors produced by *Salmonella in vitro*; therefore, we hypothesized if it could function *in vivo* in the presence of neurons. First, we infected neuron cell line PC12 with *Salmonella* for 2 h and then we added AS-IV into the cell culture medium for inhibition analysis. We analyzed the cell viability in the presence and absence of AS-IV, we found cell viability in the AS-IV group was much better compared to nontreatment control [Figure 3a], which indicates that it inhibits the *Salmonella* virulence and protects PC12 cells from *Salmonella*-induced injury. Our results showed substantial reduction in cell death after 24 h of incubation with AS-IV. To further understand the underlying inhibitory mechanism, we extracted bacterial mRNA at 5 different time points and performed qRT-PCR to analyze the gene expression level of virulence-related genes (*rpoS*, *phoP*, *sopA*, and *spvB*). Figure 3b shows that AS-IV efficiently inhibited the gene expression in a time-dependent manner, and all four tested genes were significantly downregulated by AS-IV treatment.

Furthermore, we performed Western blot analysis of AS-IV treated cells and compared it with that of untreated control cells. We found that four selected proteins (RpoS, PhoP, SopA, and SpvB) were greatly downregulated in the AS-IV-treated group as compared to that of the untreated control group [DMSO, Figure 3c and d].

Altogether, we identified that AS-IV efficiently protected neuronal cell line against *Salmonella* infection via inhibition of production of virulence factors.

Astragaloside IV functions partially via the Nrf2/ARE pathway

According to previous literature, Nrf2/ARE signaling pathway is involved in the neuroprotective mechanism by TCM such as Tao hong si wu decotion and Gualou Guizhi granule.[18,28-31] Therefore, to understand the mechanism of host response by AS-IV, we studied Nrf2/ARE signaling pathway to examine whether it is involved in AS-IV protection against *Salmonella*-induced meningitis. First, we determined the transcription of *Nrf2* mRNA and found that the expression of *nrf2* was significantly upregulated as compared to that of untreated control group [Figure 4a]. Western blot analysis also confirmed our result at the translational level [Figure 4b]. Both data indicated that AS-IV functions via Nrf2/ARE pathway.

To search for more evidence of Nrf2/ARE engagement in AS-IV protection, we transiently knocked down the expression of *nrf2* at the cellular level and then investigated the transcription of bacterial virulence

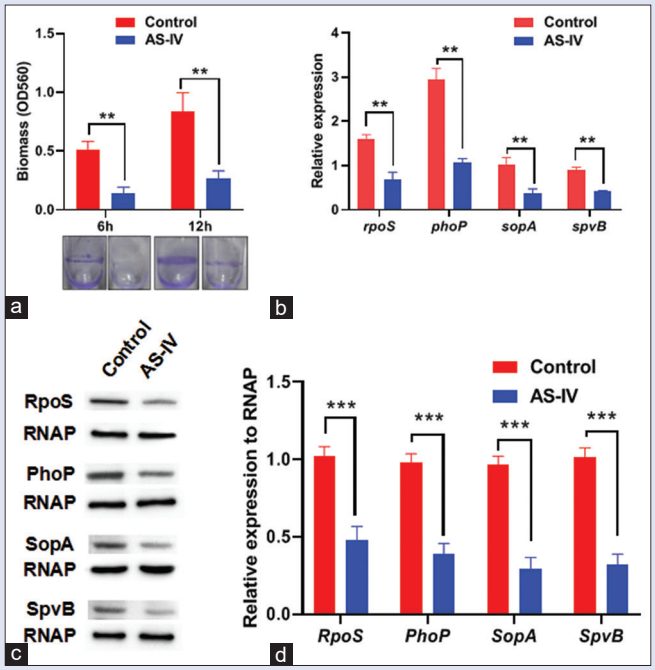

Figure 2: Astragaloside IV inhibits the virulence factor production *in vitro*. (a) Biofilm formation analysis of astragaloside IV treatment of *Salmonella* cells. Two different time points were documented and analyzed using statistical methods (Student's *t*-test, **, $P < 0.01$). CV staining was used and three independent experiments were performed to calculate the final biomass formation and representative pictures were shown in bottom panel. (b) The relative expression of *rpoS*, *phoP*, *spoA* and *spvB* using quantitative reverse transcription polymerase chain reaction. Results were expressed as mean ± standard deviation ($n = 3$), and $P < 0.05$ was considered statistically significant. **, $P < 0.01$. (c) Astragaloside IV treated cells was collected and evaluated by western blotting analysis for RpoS, PhoP, SpoA and SpvB, respectively. RNAP was used as a housekeeping control. (d) The relative quantification of immunoblot results from C. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$

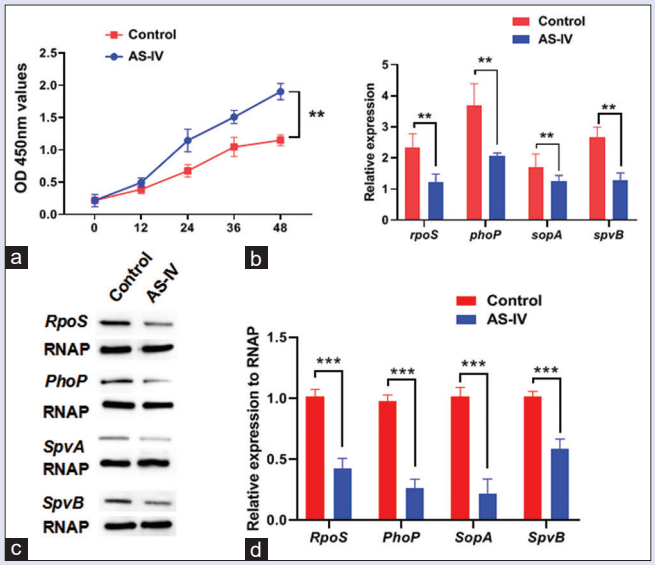

Figure 3: Astragaloside IV protects PC12 cells against *Salmonella*-induced injury via inhibiting the virulence factor production. (a) Cell viability was evaluated using CCK-8 method. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$. (b) The relative expression of *rpoS*, *phoP*, *spoA* and *spvB* using quantitative reverse transcription polymerase chain reaction. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$. (c) Astragaloside IV treated cells was collected and evaluated by western blotting analysis for RpoS, PhoP, SpoA and SpvB, respectively. RNAP was used as a housekeeping control. (d) The relative quantification of immunoblot results from C. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$

gene expression. As can be seen from Figure 4c, the expression of *nrf2* was indeed downregulated in the wild-type background, indicating that it was really knocked down. Furthermore, we investigated the production of the ROS in the *nrf2* null mutant and found that the ROS level was mitigated by AS-IV when compared to the untreated control group [Figure 4d]. Moreover, we extracted the RNA from coculture and used *Salmonella*-specific primers to determine the expression of virulence-related genes including *spoA*. As can be seen from Figure 4e, we found that the expression of *spoA* was lower than the control group, indicating that *nrf2* was involved in the regulation of virulence traits in *Salmonella*. Western blot analysis further confirmed the qRT-PCR results [Figure 4f].

Altogether, our results confirm that the Nrf2/ARE pathway was involved in the increased production of ROS and decreased production of virulence factors and the inhibition might function through ROS.

DISCUSSION

Salmonella-associated bacterial meningitis has been frequently reported in developing countries, although it was a rare type of bacterial meningitis in industrialized ones.[32] In this study, we focused on this type of bacterial meningitis due to its lack of efficient countermeasures and pharmaceutical treatment, especially in terms of TCM. Although it has been proven that vaccines and antibiotics have been useful in the prevention and treatment of *Salmonella* meningitis,[2,13,32] the lack of efficient epitopes and the possibility of developing resistance are posing challenges to the control of this disease.[33,34] To this end, we used PC12 cells as a cell model of *Salmonella* meningitis to search for efficient compounds to combat this infection and provided insights into further experimentation of promising candidates.

To control *Salmonella*-associated infections, several TCM have been tested and proved to be promising.[15-17] However, underlying inhibitory mechanisms are still lack of evidence. In this study, we investigated one of the main ingredients of TCM Radix Astragali and found its main ingredient AS-IV has anti-virulence effect in addition to its previously reported antioxidant, anti-inflammatory, and immunoregulatory effects.[20] We have confirmed that AS-IV could efficiently inhibit the expression of virulence-associated genes such as these involved in pathogenesis island SPI-2 and its regulatory elements.[9] We observed these effects in both *in vitro* and *ex vivo* experiments. In addition, we also noticed that AS-IV could efficiently diminish the ability of bacterial cells to attach surfaces, suggesting that it could probably inhibit the colonization of *Salmonella* cells during the invasion.

Interestingly, we have noticed that AS-IV could target one of the extensively studied two-component systems PhoP/PhoQ. This system has been shown to be necessary for the virulence of *Salmonella* spp.[35,36] PhoQ is a membrane-bound sensor kinase that upon sensing specific environmental signals such as Mg^{2+} triggers a phosphorylation cascade to activate the response regulator PhoP. Activation of PhoP can affect a large number of genes transcriptionally and this occurs during *in vivo* invasion of mammalian cells.[35,37] Our results indicate that AS-IV

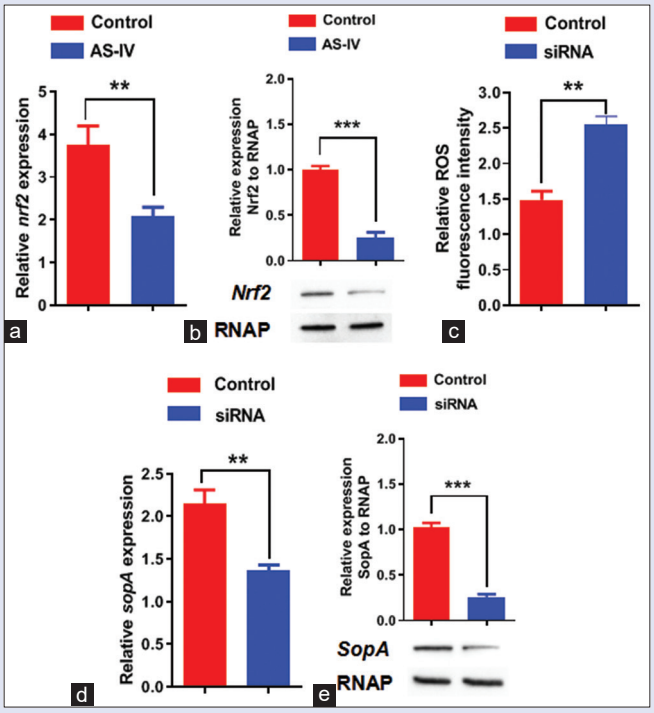

Figure 4: Astragaloside IV functions partially through Nrf2/ARE pathway. (a) The relative expression of *nrf2* using quantitative reverse transcription polymerase chain reaction. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$. (b) Astragaloside IV treated cells was collected and evaluated by western blotting analysis for Nrf2. RNAP was used as a housekeeping control. The relative quantification of immunoblot results was shown. (c) Relative ROS fluorescence intensity of 50,000 log phase grown cells treated with 20 mM H_2DCFDA is given. The assays were performed three times with independent cultures, the deletion of *nrf2* reduced the RFI. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$. (d) The relative expression of *spoA* using quantitative reverse transcription polymerase chain reaction. Results were expressed as mean ± standard deviation ($n = 3$). $^{**}P < 0.01$. (f) Cells was collected and evaluated by western blotting analysis for SpoA. RNAP was used as a housekeeping control

efficiently mitigated the expression of PhoP both under *in vivo* and *ex vitro* conditions. The detailed mode of action may be envisioned that AS-IV could block the possible positive feedback loop via inhibition of phosphorylation cascade. This needs further investigation.

Another interesting finding is that AS-IV induced the expression of Nrf2/ARE signaling cascade in the neuronal cell line, which enabled an efficient control of bacterial virulence *in vivo*. However, we tested the expression of other virulence-related genes such as *phoP* and *spvB* in *nrf2* null mutant and found no significant changes in the expression during our RT-PCR analysis (data not shown), suggesting that the potential interaction of *spoA* and *nrf2* in an unknown way. In addition, our results indicate that AS-IV targeted the host Nrf2/ARE signaling pathway to enhance its activity to increase the production of ROS and then damage or diminish the existence of bacterial cells when in contact with AS-IV under *in vivo* conditions. It has been previously reported that Nrf2/ARE pathway is involved in the protection of cells from death induced by oxidative stress.[38] Activation of Nrf2/ARE pathway would thus confer resistance to a variety of oxidative stress-related neurogenerative insults such as these infections caused by bacterial invasions. It has been reported that AS-IV acted as a potential antioxidant against diabetic ketoacidosis in juvenile mice through the activation of JNK/Nrf2 signaling pathway,[39] indicating the role played by AS-IV with respect to Nrf2 and its antioxidant properties. This also suggests that the protective role of AS-IV against *Salmonella*-induced meningitis is dependent on both its antivirulence and antioxidant properties.

One of the unanswered questions is the exact transcriptional and translational changes after incubation with AS-IV in *Salmonella*-infected cells. To understand this, we propose to implement omics tools to further investigate these effects and find out more physiological targets for therapeutic use. For example, dual-RNA seq might be used to identify the whole transcript me of *Salmonella* and neuronal cells which gives more insights into the altered signaling pathways in contact with AS-IV.[40,41] In addition, pulsed-stable isotope labeling by amino acids in cell culture (SILAC) quantitative proteomics might be adopted to study the new protein's abundance in AS-IV treated bacterial and mammalian cells.[42] All these information will further facilitate the treatment of *Salmonella*-associated meningitis. Finally, the introduction and administration of AS-IV in mammalian models would additionally provide insights into its clinical use in future.

CONCLUSION

Salmonella-induced meningitis has posed a healthcare threat to both infants and adults. In this study, we found that AS-IV might efficiently inhibit the production of virulence factors such as biofilm formation of *Salmonella*. The expression of virulence-associated genes including *rpoS*, *phoP*, *sopA*, and *spvB* was significantly downregulated in *in vitro* and *ex vivo* conditions by exposure to AS-IV treatment. In addition, our results showed that AS-IV could boost the production of ROS via Nrf2/ARE signaling pathway, which in turn inhibits the growth and production of virulence factors of *Salmonella* cells during coculturing with human neuronal cells. Our results clearly demonstrate that AS-IV could inhibit *Salmonella*-induced meningitis through the modulation of bacterial virulence and host response. More detailed mechanistic analysis should be performed in the future to untangle the underlying mode of action of AS-IV against bacterial infections. Altogether, our results show promising results for AS-IV as a therapeutic agent against *Salmonella*-induced meningitis.

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Conflicts of interest

There are no conflicts of interest.

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