Salvia miltiorrhiza solution and its active compounds ameliorate human granulosa cell damage induced by H₂O₂

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Abstract. The dried roots or rhizomes of Salvia miltiorrhiza Bge are commonly used in Chinese medicine to promote blood circulation and regulate menstruation. Salvianic acid A and salvianolic acid B are the main active water-soluble compounds in Salvia miltiorrhiza solution. The present study investigated the protective effect of Salvia miltiorrhiza solution and its active compounds in H₂O₂-induced cell damage of the human ovarian granulosa tumor cell line (KGN) in vitro, as well as its underlying mechanism. Cell viability was detected using a Cell Counting Kit-8 assay. In addition, the levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and tumor necrosis factor-α (TNF-α) were measured. Western blotting was performed to detect the protein expression of cleaved caspase-3 and caspase-9. Furthermore, immunocytochemistry was used to detect the expression of TNF-α. It was demonstrated that Salvia miltiorrhiza solution, salvianic acid A and salvianolic acid B did not affect the viability of KGN cells. Additionally, salvianic acid A and salvianolic acid B significantly reduced the H₂O₂-induced increased MDA levels, and reversed the H₂O₂-induced suppression of SOD and GSH levels in KGN cells (P<0.05). Treatment with Salvia miltiorrhiza solution, salvianic acid A and salvianolic acid B significantly reduced the overexpression of cleaved caspase-3, cleaved caspase-9 and TNF-α compared with the H₂O₂-treated group (P<0.05). Therefore, the present results indicated that Salvia miltiorrhiza solution and its main water-soluble compounds, salvianic acid A and salvianolic acid B, ameliorated KGN cell damage induced by H₂O₂.

Introduction

Assisted reproductive technology has been widely used to treat patients with infertility complications (1,2). Clinical ovulation induction, ovulation collection and transplantation techniques for assisted reproduction have been optimized, which has improved pregnancy rates and successful deliveries (3). However, negative outcomes associated with assisted reproductive technology are associated with the effects of certain primary diseases, including severe endometriosis, polycystic ovary syndrome and old-age, which lead to a significant decline in the quality of eggs and embryos, and the clinical pregnancy rate (4). Oxidative stress damage in the follicular microenvironment contributes to the decline of oocyte quality in these primary diseases (5). Various gynecological diseases, such as endometriosis, polycystic ovary syndrome and reproductive aging, are common hormonal reproductive disorders leading to infertility, in which oxidative stress serves a crucial role (6).

Granulosa cells (GCs) are a type of ovarian cell that exist outside oocytes and in follicle walls. Their metabolic activity serves an important role in the quality of the oocyte) (7). GCs are involved in follicular development, oocyte maturation and atresia (7). The role of GCs in the process of follicular differentiation is vital, resulting in optimal conditions for oocyte development, ovulation, fertilization and embryo implantation (7).

Reactive oxygen species (ROS) are oxygen-derived molecules that include superoxide anions, H₂O₂ and hydroxyl radicals (8). ROS causes oxidative stress, damage to oocytes and damage to luteinized GCs (9). Increased ROS concentrations in GC reduces the number of retrieved oocytes (10) and in follicular fluid this correlates with poor oocyte and embryo quality (11). SOD is an antioxidant enzyme that acts as a scavenger in oxygen-free radical production, while MDA is...
produced by lipid peroxidation and can be used as a marker of oxidative stress and injury (12). In addition, GSH is a cellular antioxidant that protects cellular proteins from oxidative stress (12).

*Salvia miltiorrhiza* is a traditional Chinese herbal medicine (13). In Shen Nong’s Herbal Classic, the dried roots or rhizomes of *Salvia miltiorrhiza Bge* are described as a medicine to promote blood circulation and regulate menstruation (14). There are multiple potential biological effects of *Salvia miltiorrhiza* including antioxidation, anti-inflammation, inhibition of apoptosis and protective effects on organs such as the liver (15,16). *Salvia miltiorrhiza* solutions are the aqueous extracts of the dried roots or rhizomes of *Salvia miltiorrhiza Bge* (17). The major chemical components of the water-soluble fractions are salvinanoid acid A, salvinanolic acid B and lithospermic acids (14). The concentrations of salvinanoid acid A and salvinanolic acid B in *Salvia miltiorrhiza* solution are 2.15 and 1.01 mg/mL respectively, which have been previously detected by high-performance liquid chromatography-UV (18). *Salvia miltiorrhiza* solution, as a blood-activating drug, has achieved good clinical results in improving placental circulation in patients with recurrent abortion, as well as improving prognosis in the *in vitro* fertilization and embryo transfer cycle to treat infertility (19). In addition, previous studies have demonstrated that salvinanoid acid A and salvinanolic acid B attenuated damage induced by oxidative stress (20,21). Treatments with salvinanoid acid A and salvinanolic acid B reduce platelet-derived growth factor-induced ROS formation in rat hepatic stellate cells, possibly via the inhibition of nicotinamide adenine dinucleotide phosphate oxidase and are also effective against hepatic fibrosis in thioacetamide-intoxicated rats *in vivo* (22). Salvinianic acids prevent acute doxorubicin cardiotoxicity in mice via the suppression of oxidative stress (23). Furthermore, mixed aqueous extracts of *Salvia miltiorrhiza* inhibit hypertension via the inhibition of vascular remodeling and oxidative stress in spontaneously hypertensive rats (20). However, to the best of our knowledge, the effect of salvinanoid acid A and salvinanolic acid B on ovarian granulose cells has not been previously reported. Therefore, the aims of the present study were to investigate the protective effect of *Salvia miltiorrhiza* solution and its active compounds in H₂O₂-induced cell damage of the human ovarian granulosa tumor cells (KGN), and to identify the associated underlying mechanisms.

**Materials and methods**

**Cell culture and treatment.** KGN cells (cat. no. bncc37610; Beijing Beina Chuanglian Biotechnology Research Institute) were cultured in RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 IU/ml penicillin G; 100 mg/ml streptomycin; Beijing Solarbio Science & Technology Co., Ltd.) in a humidified incubator at 37°C with 5% CO₂. The H₂O₂ group consisted of KGN cells grown to 80% confluence, which were then treated with 200 µM H₂O₂ for 24 h at room temperature. The treatment group consisted of KGN cells grown to 80% confluence that were pretreated with *Salvia miltiorrhiza* solution (0.2, 1 and 5%), salvinanoid acid A (3, 10 and 30 µM; Beijing SLF Chemical Research Institute) and salvinanolic acid B (3, 10 and 30 µM; Nanjing Spring and Autumn Biological Engineering Co., Ltd.) for 4 h at 37°C, followed by treatment with 200 µM H₂O₂ for 24 h at 37°C (24). *Salvia miltiorrhiza* solution (1.5 g/ml) was purchased from Shineway Pharmaceutical Co., Ltd. (cat. no. Z13020777). Stock solutions of *Salvia miltiorrhiza* solution (0.2, 1 and 5%), salvinanoid A and salvinanolic acid B (3, 10 and 30 µM) were dissolved in 0.1% DMSO and stored at -40°C. All solutions were freshly prepared from stock solutions prior to each experiment and the final concentration of DMSO was <0.1%.

**Cell Counting Kit-8 (CCK-8) assay.** A CCK-8 assay kit (cat. no. EP328-500t; Beijing Zoman Biotechnology Co., Ltd.) was used to investigate the viability of KGN cells. The CCK-8 assay was performed according to the manufacturer’s instructions. KGN cells were cultured in 96-well plates at a density of 5,000 cells/well. When grown to 80% confluence, cells were treated with drugs for 24 h at 37°C. Cells treated with 0.1% DMSO served as the control group. Then, 10 µl CCK-8 solution was added to each well for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader. Cell viability was expressed as the percentage of the drug group to the control group (100%). Data represents the mean of three independent experiments.

**Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and tumor necrosis factor-α (TNF-α) analysis.** Following *Salvia miltiorrhiza* solution, salvinanoid acid A and salvinanolic acid B treatment, KGN cells were collected and expressions of MDA and GSH were detected, along with SOD activity. MDA concentration was measured using a lipid peroxidation MDA assay kit (cat. no. A003-1; Nanjing Jiancheng Bio-Engineering Institute Co., Ltd.) and SOD activity was measured with a SOD assay kit (cat. no. A001-3; Nanjing Jiancheng Bio-Engineering Institute Co., Ltd.). GSH concentration was measured with a GSH assay kit (cat. no. A006-2; Nanjing Jiancheng Bio-Engineering Institute Co., Ltd.). The cell culture supernatant was obtained by centrifugation at 326.5 x g. TNF-α concentration in the cell culture supernatant was measured with a TNF-α ELISA kit (cat. no. ARG80120; Arigo Biolaboratories), according to the manufacturer’s protocol.

**Western blot analysis.** Following cell treatment as described above, KGN cells were harvested and cells were lysed using cell lysis buffer RIPA (cat. no. 89900; Thermo Fisher Scientific, Inc.). Total protein concentration was quantified using a Pierce bichinonic acid protein assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). A total of 90 µg protein was used for analysis. Protein electrophoresis was performed using 12% SDS-PAGE and a PVDF membrane that was blocked by 5% non-fat milk at room temperature for 1 h. Western blotting was used to detect the protein expression of cleaved caspase-3 and cleaved caspase-9 in KGN cells. Primary antibodies included: Anti-cleaved-caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), anti-cleaved-caspase-9 polyclonal (1:1,000; cat. no. A2636; ABEclonal Biotech Co., Ltd.) and anti-β-actin monoclonal (1:10,000; cat. no. AC026; ABEclonal Biotech Co., Ltd.). The secondary antibodies used were goat anti-rabbit immunoglobulin G-horseradish peroxidase (1:5,000; cat. no. 5220-0336; Kirkegaard & Perry Laboratories;
Seracare). Protein bands were developed using an EZ-ECL kit (cat. no. 20-500-120; Biological Industries) and analyzed with a Tanon 2500 chemiluminescence imaging system (Tanon Science and Technology Co., Ltd.).

**Immunocytochemistry.** KGN cells were seeded in 96-well plates at a density of 5x10^3 cells/well on glass coverslips in a 24-multiwell plate. Drug treatment was administered 15 h after cells were seeded. The cellular detection and localization of TNF-α (TNF-α rabbit polyclonal antibody; cat. no. bs-2081R; BIOSS; 1:100) was determined using an immunocytochemistry assay. Cells were fixed in 4% paraformaldehyde overnight at 4˚C. Fixed cells were then washed three times with PBS and subsequently incubated with 0.3% triton at room temperature for 10 min. Samples were then blocked with 10% goat serum (cat. no. 04-009-1A; Biological Industries) for 1 h at room temperature and incubated with primary antibody against TNF-α overnight at 4˚C. Cells were then washed three times with PBS and incubated with secondary antibody (goat anti rabbit/mouse horseradish peroxidase kit; cat. no. SP-9000; ZSGB BIO) for 1 h at 37˚C. After washing three times with PBS, slides were incubated with 3,3’-diaminobenzidine tetrahydrochloride (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) for 1 min at room temperature and immediately washed with water after color development. Slides were then counter-stained with 0.2% hematoxylin for 5 min at room temperature. Slides were mounted with 1% hydrochloric acid Alcohol and then observed under a light microscope, at 400x magnification.

**Statistical analysis.** Data were analyzed using Origin 9.1 software (OriginLab Corp.) and presented as the mean ± SEM. Each n value represented data from one culture well. Unless otherwise indicated, n=8-12 culture wells were used for each group in each experiment, with repetitions conducted using ≥3 independent dissections for each experiment. Statistical analyses were performed using one-way ANOVA followed by Bonferroni’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Effects of *Salvia miltiorrhiza* solution and its active compounds on the viability of KGN cells. In Shen Nong’s Herbal Classic, the dried roots or rhizomes of *Salvia miltiorrhiza* (Fig. 1A) and salvianolic acid B (Fig. 1B) are described as a medicine to promote blood circulation and regulate menstruation (14). Salvianic acid A (Fig. 1A) and salvianolic acid B (Fig. 1B) are the main water-soluble compounds in *Salvia miltiorrhiza* solution (18).

The present study investigated the effects of *Salvia miltiorrhiza* solution and its active compounds, salvianic acid A and salvianolic acid B, on the viability of KGN cells by performing a CCK-8 assay (Fig. 1C). KGN cells were treated with *Salvia miltiorrhiza* solution or its main active compounds for 24 h. As presented in Fig. 1C, neither *Salvia miltiorrhiza* solution (0.2, 1 and 5%) nor salvianic acid A or salvianolic acid B (3, 10 and 30 µM) affected the viability of KGN cells when compared with the control group (100%; Fig. 1C). Therefore, the present results indicated that *Salvia miltiorrhiza* solution, salvianic acid A and salvianolic acid B exerted no toxic effects on granulosa cells.
group; Fig. 2B), while Salvia miltiorrhiza solution (0.2, 1 and 5%), salvianic acid A or salvianolic acid B (3, 10 and 30 µM) treatment significantly inhibited the H$_2$O$_2$-induced increase of MDA levels in a dose-dependent manner (P<0.05; Fig. 2B). It was also revealed that 200 µM H$_2$O$_2$ treatment significantly decreased SOD activity by 57.4% (P<0.05 vs. control group; Fig. 2A), while Salvia miltiorrhiza solution (0.2, 1 and 5%), salvianic acid A and salvianolic acid B (3, 10 and 30 µM) significantly attenuated the suppression of SOD activity compared with the H$_2$O$_2$ group (P<0.05; Fig. 2A). In addition, GSH activities were significantly decreased in the H$_2$O$_2$-treated group (P<0.05 vs. control group; Fig. 2C). Western blotting data demonstrated that treatment with 200 µM H$_2$O$_2$ significantly increased the protein expression of cleaved caspase-3 by 0.97-fold and cleaved caspase-9 by 0.85-fold compared with the control group (P<0.05; Fig. 3). Treatment with Salvia miltiorrhiza solution significantly attenuated the increased expression of cleaved caspase-3 and cleaved caspase-9 induced by H$_2$O$_2$.
Salvia miltiorrhiza solution, salvianic acid A and salvianolic acid B suppresses the H$_2$O$_2$-induced increased expression of TNF-α in KGN cells. To investigate whether Salvia miltiorrhiza inhibits the increased protein expression of H$_2$O$_2$-induced TNF-α, KGN cells were pretreated with Salvia miltiorrhiza solution or its main active compounds for 4 h, followed by the addition of 200 µM H$_2$O$_2$ for 24 h. As presented in Fig. 4B, treatment with 200 µM of H$_2$O$_2$ significantly enhanced the protein expression of TNF-α in KGN cells compared with the control group (Fig. 4A). It was also revealed that Salvia miltiorrhiza solution, in a concentration-dependent manner, significantly decreased the H$_2$O$_2$-induced upregulation of TNF-α (Fig. 4C). Furthermore, salvianic acid A and salvianolic acid B significantly attenuated TNF-α protein upregulation compared with the H$_2$O$_2$ group (Fig. 4D and E). The present study also investigated the release of TNF-α, and demonstrated that Salvia miltiorrhiza solution, salvianic acid A and salvianolic acid B significantly attenuated TNF-α release induced by H$_2$O$_2$ (Fig. 4F).

**Discussion**

The present results indicated that the survival of granulosa cells was not affected by different concentrations of Salvia miltiorrhiza solution and its main water-soluble compounds. In addition, it was revealed that these compounds exerted no toxic effects on granulosa cells and significantly attenuated the H$_2$O$_2$-induced increase of MDA levels and H$_2$O$_2$-induced decrease of SOD and GSH activity. These compounds also reduced the H$_2$O$_2$-induced increased expression of cleaved caspase-3, cleaved caspase-9 and TNF-α. Therefore, the present results indicated that Salvia miltiorrhiza solution and its main water-soluble compounds ameliorated KGN cell damage induced by H$_2$O$_2$, suggesting that these may protect against oxidative stress in the granulosa cells around oocytes. Thus, Salvia miltiorrhiza may facilitate the treatment of infertility.

Oxidative stress has been implicated in many reproductive disorders including endometriosis, polycystic ovarian syndrome, infertility and aging (25). The degradation of oocyte quality caused by oxidative stress injury is the key factor leading to infertility and adverse fertility outcomes (26). ROS serves an important role in the induction of meiosis in the oocyte and high levels of ROS have been revealed to impair oocyte maturation (27). A previous study indicated that oocyte quality may be affected by an increase in ROS, which was associated with advancing maternal age (25). The clearance
of oxidative stress products from follicular fluid is decreased in older women (25). In addition, glutathione, transferase and catalase in ovarian follicular fluid are significantly decreased in older women undergoing in vitro fertilization (25). Therefore, oxidative stress induces granulosa cell discordant function and influences oocyte quality (28).

The antioxidant effect of *Salvia miltiorrhiza* has been extensively studied. Salvianic acid A reduces liver fibrosis by regulating the caspase-3/cleaved caspase-3 signaling pathway (29). Salvianolic acid B promotes anticonvulsant and anti-apoptotic effects in a pentyleneetetrazole-induced seizure model by activating the AKT/cAMP response element-binding protein/brain-derived neurotrophic factor signaling pathways, including the inhibition of cleaved caspase-3 overexpression (30). Furthermore, TNF serves a crucial role in the amplification of luteolytic signals, mediating vascular regression, and promoting apoptosis and necroptosis of luteal cells (31). Furthermore, it has been demonstrated that salvianolic acid B inhibited TNF-α to extenuate cholestatic liver injury *in vivo* and *in vitro*, indicating that the anticholestatic effects of salvianolic acid B may be associated with the inhibition of inflammation and the maintenance of bile acid homeostasis (32). These previous studies indicate that salvianic acid A and B serve an important role in the progression of oxidative stress and apoptosis via caspase and TNF-α (33). However, the molecular mechanism underlying the anti-oxidative effects of *Salvia miltiorrhiza* in KGN cells is not fully understood. In the present study, *Salvia miltiorrhiza* solution, salvianic acid A and salvianolic acid B significantly attenuated the H₂O₂-induced increase of MDA levels, and the H₂O₂-induced suppression of SOD and GSH activities in KGN cells. These compounds also attenuated the increased expression of cleaved caspase-3, cleaved caspase-9 and TNF-α. However, further research is required to investigate the effects of *Salvia miltiorrhiza* on ovary function using *in vivo* studies, such as the effects on theca cells, angiogenesis and ovarian...
functions. Furthermore, the effects of *Salvia miltiorrhiza* on the follicular microenvironment, the endometrium in infertility and the regulation of the pelvic microenvironment require further investigation.

*Salvia miltiorrhiza* solution and its main water-soluble compounds, salvianic acid A and salvianolic acid B, may ameliorate oxidative stress damage in 

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