PROTECTIVE EFFECTS OF CISTANCHES HERBA AQUEOUS EXTRACT ON
CISPLATIN-INDUCED PREMATURE OVARIAN FAILURE IN MICE

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Abstract

Background: Chemotherapeutic treatment of premenopausal women has been linked to premature ovarian failure (POF). Cistanches Herba (CH) is a commonly used male impotence and female infertility treatment in China; however, whether CH protects ovaries from chemotherapeutic drug-induced POF remains unclear. In this study, we investigated the protective effects of CH in a mouse model of chemotherapeutic drug-induced POF.

Materials and Methods: We administered low- and high-concentration CH to cisplatin-induced POF mice for 2 weeks and determined body and ovarian weights, as well as serum follicle-stimulating hormone (FSH) and estradiol concentrations, to evaluate ovarian function. In addition, we evaluated the protective mechanisms of CH by detecting the levels of apoptosis-related proteins and evaluating markers of mitochondrial function.

Results: In POF mice, we observed reduced body and ovarian weights; elevated serum FSH and attenuated estradiol concentrations; apoptosis of ovarian granulosa with concomitant changes in apoptosis-related proteins (including caspase-3, poly adenosine diphosphate-ribose polymerase, Bcl-2, and Bax); and mitochondrial dysfunction, such as a reduction in mitochondrial numbers, destruction of ultrastructural morphology, decrease in ATPase activity, and decreases in mitochondrial membrane potential and mitofusin-2 (a mitochondria dynamin-like GTPase). Significantly, CH reversed, to an extent, functional and morphologic injuries and ovarian tissue apoptosis by up-regulating the level of Mfn2 and the ratio of Bcl-2/Bax. Furthermore, CH reduced cisplatin-induced mitochondrial dysfunction in ovarian tissues.

Conclusion: The present findings showed that CH inhibited cisplatin-induced POF through interactions between Mfn2 and Bcl-2/Bax proteins and, possibly, by up-regulation of Mfn2 expression. Ultimately, CH protects ovarian tissues from cisplatin-induced apoptosis.

Keywords: Premature ovarian failure (POF), Cistanches Herba (CH), Cisplatin, Apoptosis, Mitofusin-2

Abbreviations: POF, premature ovarian failure; CH, Cistanches Herba (CH); HPLC, high-performance liquid chromatography; i.p., intraperitoneal injections; i.g., intragastric injections; FSH, follicle-stimulating hormone; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; TUNEL, TdT-mediated dUTP nick end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mfn2, mitofusin 2; cleaved-PARP, poly adenosine diphosphate-ribose polymerase; ΔΨm, mitochondrial membrane potential.

Introduction

Premature ovarian failure (POF) is a disease that results in premature menopause, or the cessation of ovarian function before the age of 40. This is characterized by amenorrhea, dysgenesis, hypoestrogenism, and increased levels of gonadotropins (Wang *et al.,* 2015). The etiology of POF involves immunologic, genetic, metabolic, environmental, and iatrogenic factors (Chen *et al.,* 2015). Chemotherapeutic agents can also lead to POF in women during their reproductive years, and can therefore affect their future fertility. The risk for developing POF appears to be dependent upon the exact nature of the chemotherapeutic regimen, and includes factors such as drug dosage and patient age (Morgan *et al.,* 2013). Recent studies have documented the induction of ovarian dysfunction as a result of cisplatin, including menstrual disorders, premature menopause, and infertility (Chen *et al.,* 2015；Li *et al.,* 2013). Moreover, experimental studies have shown that cisplatin disrupts estrous cyclicity in mice and rats, reduces ovarian weight, and causes ovarian cell apoptosis (Li *et al.,* 2013).

Cistanches Herba (CH) is a parasitic plant that primarily grows in North Africa and in Arabic and Asian countries. Within traditional Chinese medicine, CH is applied as a tonic and/or treatment for chronic renal diseases, impotence, female infertility, morbid leucorrhea, profuse metrorrhagia, and senile constipation (Li *et al.,* 2016; Leong *et al.,* 2015). Modern pharmacologic studies have demonstrated that CH exhibits anti-aging properties, improves learning and memorization abilities, treats symptoms of Alzheimer's disease, and enhances the immune system (Li *et al.,* 2016; Gu *et al.,* 2016). Furthermore, studies on CH and the regulation of reproductive functions have linked CH to increased sex hormone levels as a result of inducing testicular steroidogenic enzymes, up-regulating the gene expression of 3β-hydroxysteroid dehydrogenase (which is responsible for the synthesis of dihydrotestosterone), and reversing reproductive toxicity induced by Leigongteng (*Radix et Rhizoma Tripterygii*) by decreasing the reproduction-related genes *DDX3Y*, BCL6, and STAT3 (Wang and Chen *et al.,* 2016; Shimoda *et al.,* 2009; Li *et al.,* 2014). Moreover, recent studies have demonstrated the protective effects of CH extract and its ability to reduce apoptosis in muscle cells (Wat *et al.,* 2016). While CH was shown to improve ovarian weight and increase the expression of estrogen and progesterone receptors in a model of rat ovarian functional decline published over 20 years ago (Chen *et al.,* 1995), the exact regulating mechanisms in relation to the female reproductive system remain unknown.

Therefore, in the present study, our primary focus concerned the protective mechanisms of CH on the female reproductive system in a cisplatin-induced POF mouse model. We expect that our research results will contribute to identifying and detailing the pharmacologic effects of CH and the mechanisms underlying its actions. Moreover, our findings can be used in the clinical application of chemotherapeutic protective agents.

Materials and Methods
Preparation of CH Extract and Standardization

We obtained dried CH from the First Affiliated Hospital of Liaoning University of Traditional Chinese Medicine. We prepared water extracts by boiling 200 g of each sample in 2000 mL of distilled water for 2 hours. We then centrifuged the solution at 4000 r/min for 10 minutes. We removed residues using filtration, and evaporated the

combined filtrate by heating to obtain a desired concentration of 2.1 g of dried plant equivalent extract/mL (Liang *et al.,* 2011). We chemically analyzed the CH extract for acteoside and echinacoside using reverse-phase high-performance liquid chromatography (HPLC) based on previously published methods (Lu *et al.,* 2013; Fig. 1 shows the CH results). The standard compounds acteoside and echinacoside were purchased from GuangRun Bio Technology (China).
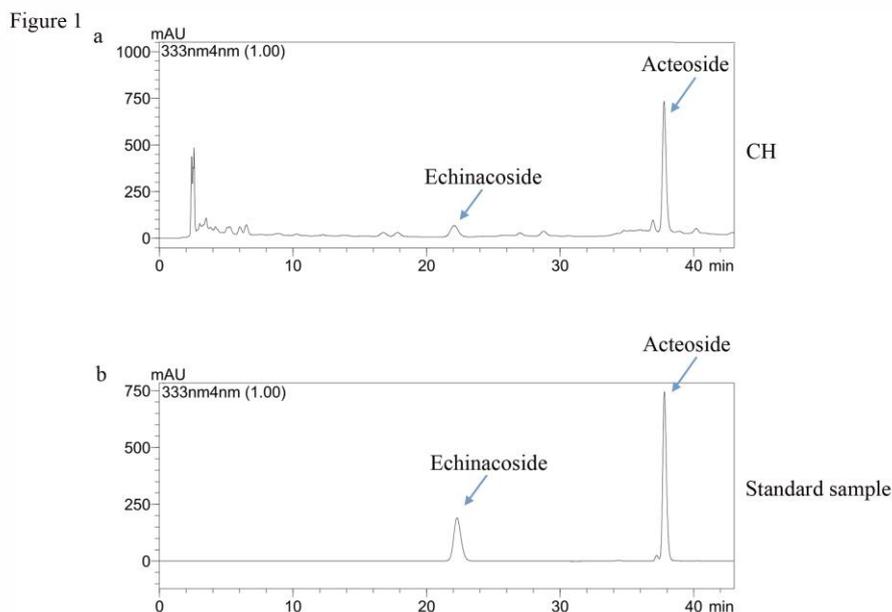

Figure 1: HPLC data for CH.

(a) and (b). CH (upper panel) and standard sample (lower panel). The peaks indicate the presence of echinacoside and acteoside, which validate the authenticity of CH.

Animals and CH Treatment

Ethics Approval and Consent to Participate

This study was approved by the Institutional Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine, and was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals."

We obtained 40 pathogen-free female Kunming mice at 8 weeks of age and weighing 28-30 g, from the Experimental Animal Center of Liaoning University of Traditional Chinese Medicine. The mice were acclimatized to standard laboratory conditions (temperature, 22-25°C; relative humidity, 50-60%; and a 12-hour photoperiod with lights on from 07:00-19:00).

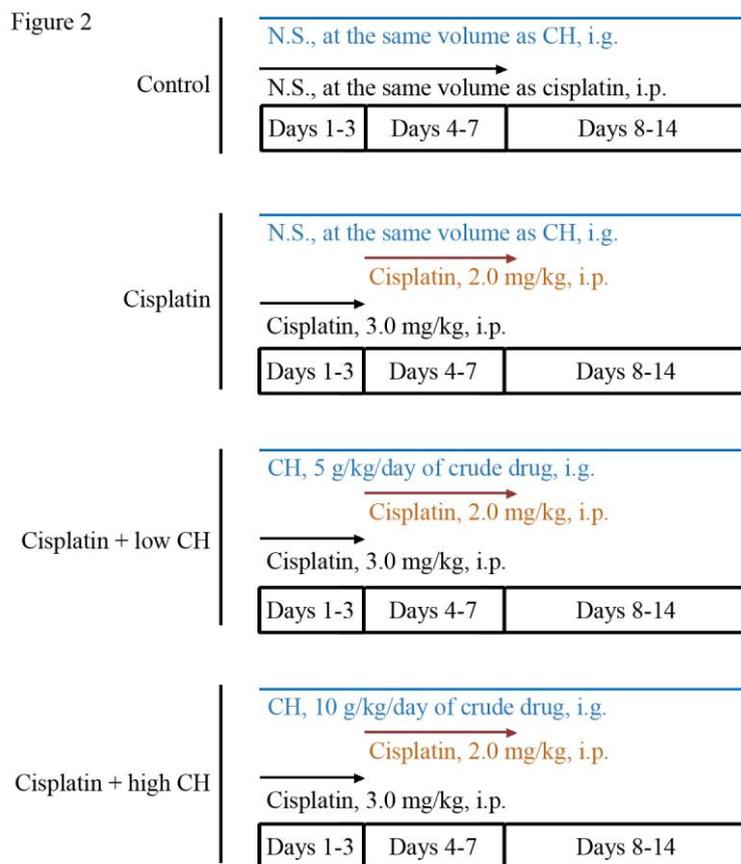We then randomly divided the mice into 4 groups (n=10/group) prior to treatment. Fig. 2 shows the different groups.

Figure 2

Control

N.S., at the same volume as CH, i.g.

N.S., at the same volume as cisplatin, i.p.

Days 1-3	Days 4-7	Days 8-14

Cisplatin

N.S., at the same volume as CH, i.g.

Cisplatin, 2.0 mg/kg, i.p.

Cisplatin, 3.0 mg/kg, i.p.

Days 1-3	Days 4-7	Days 8-14

Cisplatin + low CH

CH, 5 g/kg/day of crude drug, i.g.

Cisplatin, 2.0 mg/kg, i.p.

Cisplatin, 3.0 mg/kg, i.p.

Days 1-3	Days 4-7	Days 8-14

Cisplatin + high CH

CH, 10 g/kg/day of crude drug, i.g.

Cisplatin, 2.0 mg/kg, i.p.

Cisplatin, 3.0 mg/kg, i.p.

Days 1-3	Days 4-7	Days 8-14

Figure 2: Mouse protocol and treatments.

The mice were divided into 4 groups: (1) the control group, (2) the cisplatin group, (3) the cisplatin + low CH group, and (4) the cisplatin + high CH group. We administered CH or normal saline (N.S.) i.g. from Days 1 to 14, and administered different concentrations of cisplatin or N.S. i.p. from Days 1 to 7.

Group 1 was the control group of mice that received daily intraperitoneal (i.p.) injections of normal saline (N.S.) at the same volume as with cisplatin for the first week (Days 1 to 7). Additionally, the control group of mice were treated daily with N.S. by intragastric injections (i.g.) at the same volume as with CH for 2 weeks, starting at Day 1 and continuing until the termination of the experiment.

Group 2 was the cisplatin group of mice that received cisplatin (QILU Pharmaceutical, China) at a dose of 3.0 mg/kg for the first 3 days, and 2.0 mg /kg for the remaining 4 days of the first week (Luo *et al.,* 2013). We also treated the control group mice with N.S. i.g., for 2 weeks.

Group 3 was the cisplatin + low CH group of mice that received the same cisplatin treatment as those in Group 2 while also receiving a CH equivalent of 5 g/kg/day of crude drug (calculated as 10 times the human dose of 0.5 g/kg/day) daily for 2 weeks (Li *et al.,* 2014).

Group 4 was the cisplatin + high CH group of mice that received the same cisplatin treatment as those in Group 2 while also receiving a CH equivalent of 10 g/kg/day of crude drug (calculated as 20 times the human dose of 0.5 g/kg/day) daily for 2 weeks (Li *et al.,* 2014).

During the cisplatin treatments, we recorded the weights of the mice 3 times: (1) on Day 1 before any of the treatments began, (2) 6 hours after the termination of the cisplatin or N.S. i.p. treatment on Day 7, and (3) 6 hours after the termination of the experiments on Day 14. After the last weights were taken, blood was collected from each group via direct cardiac puncture, and the serum was isolated and stored at -80°C. We first weighed the ovarian tissues from

the mice in the 4 groups, and then either stored the ovarian tissues at -80°C for protein analysis, or fixed them in a 4% paraformaldehyde solution for morphologic analysis.

Detection of Serum Estradiol and follicle-stimulating hormone (FSH)

We measured serum estradiol and FSH plasma levels using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA), at an absorbance of 450 nm. We performed each experiment 3 times.

Hematoxylin and Eosin (H&E) Histology

After fixation, we embedded the ovarian tissues in paraffin. We then sectioned and stained the ovarian tissues with H&E for general histomorphometric analysis.

Immunohistochemistry

We dewaxed and rehydrated the paraffin slides containing ovarian tissues using xylol and ethanol, and used the mitofusin 2/Mfn2 antibody (Abcam, Massachusetts) and UltraSensitive ™ SP IHC kit (Maixin Biotech, China) for immunohistochemistry. Two independent and unbiased researchers reviewed and analyzed the results using the MetaMorph/DP10/BX41 image analysis system (UIC/Immunohistochemistry Olympus, US/Japan).

TdT-mediated dUTP nick end labeling (TUNEL) Assay of Cellular Apoptosis

We evaluated cellular apoptosis with conventional TUNEL assay using the In Situ Cell Death Detection Kit, POD (Roche, USA), counting the number of TUNEL-positive cells using 10 different high-magnification views of each ovarian tissue slide. Additionally, we counted the number of positive cells for every 1,000 cells. The apoptosis rate was calculated as a percentage (%) = number of positive cells/1,000 × 100% (Wang and Zhao *et al.,* 2016).

Western Blotting Analysis

The ovarian tissue samples were homogenized in a lysis buffer (DINGGUO, China), and protein levels were detected using a Pierce BCA Protein Assay Kit (Thermo, USA). We fractioned equal amounts of separated protein using SDS-PAGE, and transferred the separated proteins to a PVDF membrane (Bio-Rad, USA). The blots were incubated overnight at 4°C with antibodies against cleaved-caspase 3 (SAB, USA), cleaved poly adenosine diphosphate-ribose polymerase (cleaved-PARP, SAB, USA), Bcl-2 (Cell Signaling, USA), Bax (Cell Signaling, USA), or Mfn2 (Abcam, UK). We then used horseradish peroxidase-conjugated secondary antibodies (ZSGB BIO, China) in conjunction with an ECL chemiluminescence detection system (Amersham, UK). Quantification of staining was by scanning densitometry, and we normalized the densitometric values for measured proteins to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control.

Transmission Electron Microscopy Study

The ovarian tissues were fixed, post-fixed, and embedded as previously described (Lu *et al.,* 2014). We then observed the ultrathin sections under a transmission electron microscope (model RILI H-7500; Hitachi, Japan) at 80 kV.

ATPase Activities Assay

Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities were measured electrophotometrically by measuring the amount of inorganic phosphate release. We then weighed, homogenized, and centrifuged the ovarian tissues, using the supernatant for the ATP assay kit (Nanjing Jiancheng Bioengineering Institute, China). The results obtained were measured at 636 nm. We ultimately calculated the ATP contents in the samples based on the formula provided in the manufacturer's instructions.

Measurement of Mitochondrial Membrane Potential (ΔΨm)

We assessed mitochondrial membrane potential using fluorescence microscopy after first using the JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime Biotechnology, China). The ovarian tissues were weighed, cut, ground, and filtered to acquire a single cell suspension according to the method described by Chen et al (2015). The cell precipitates were then incubated in JC-1 fluorescent dye in a 5% CO_2 incubator for 30 minutes at 37°C. After washing the cell precipitates 3 times, we analyzed the mitochondrial membrane potential using a fluorescence microscope (Leica, Germany); and measured the green signal of the JC-1 at 530 nm, whereas the measurement of the red signal was at 590 nm.

Statistical Analysis

Each individual experiment was conducted 3 times, and SPSS 15.0 was used for statistical analyses. Data are expressed as means ± SD. We analyzed the different groups using 1-way ANOVA and Tukey's method for post-hoc comparisons. A *P* value < 0.05 was considered to be statistically significant.

Results
Effects of CH on Ovarian Weight and Morphology

The results of our study showed that cisplatin decreased the body weight of the mice, and significantly reduced ovarian weight compared to the same indices in the control group. Significantly, treatment with different doses of CH effectively increased both body and ovarian weights (Figs. 3a and 3b).

We observed follicles at different stages of maturation in the ovaries of the control mice, and the number of follicles significantly decreased (especially for small follicles and Graafian follicles) in the atrophied ovaries of the cisplatin group. After administration of CH, ovarian morphology significantly improved compared to that of the cisplatin group. Specifically, we observed increased numbers of follicles and degrees of maturation (Fig. 3c).
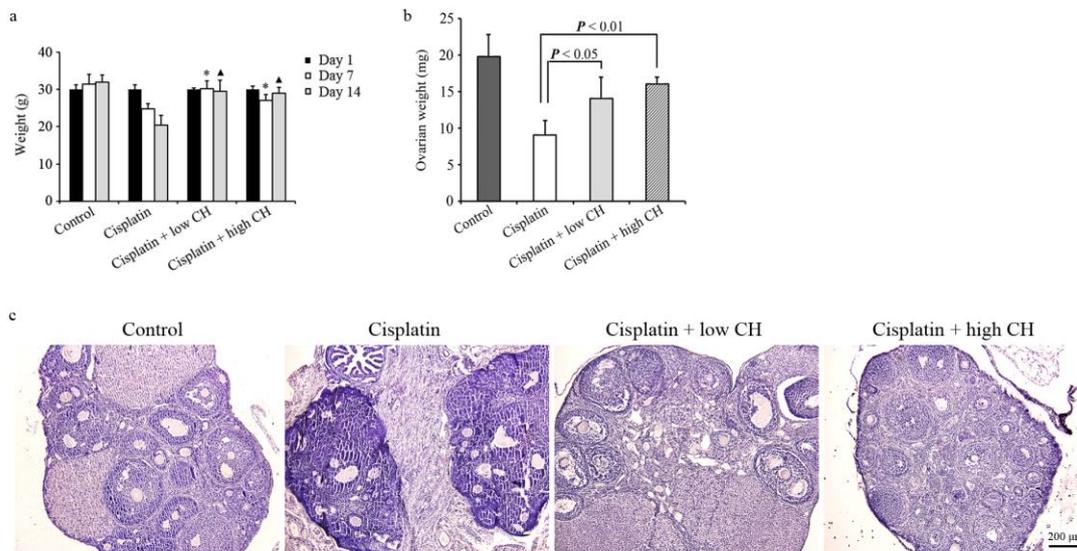
Figure 3

Figure 3: Effects of CH on ovarian weight and morphology after cisplatin treatment.
(a) Weights of the mice in the 4 groups at Days 1, 7, and 14; and analysis of differences using Tukey's method. $*p <$ 0.05 vs. the cisplatin group; $^{\blacktriangle}p < 0.01$ vs. the cisplatin group. (b) Ovarian weights of the 4 groups at Day 14.
(c) Morphology of ovarian tissues using H&E staining.

Effects of CH on the Production of FSH and Estradiol

We investigated peripheral FSH and estradiol concentrations, 2 important indices of ovarian function, in order to observe the effects of CH on POF induced by cisplatin. We observed a significant elevation in FSH in the cisplatin group compared to the control group; however, the FSH levels in the cisplatin + low and cisplatin + high CH groups decreased ($P < 0.05$) (Fig. 4a). Moreover, estradiol was significantly reduced in the cisplatin group compared to the control, and treatment with CH increased estradiol levels (Fig. 4b).
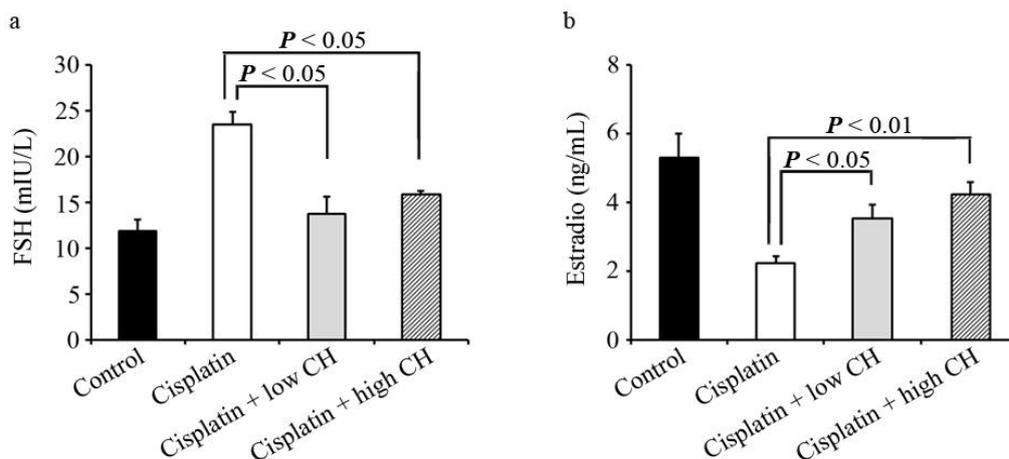
Figure 4

Figure 4: Effects of CH on serum FSH and estradiol concentrations after cisplatin treatment.
(a) FSH levels. (b) Estradiol levels. Note: ANOVA was followed by Bonferroni's test. Each bar represents the mean ± SD ($n = 10$/group).

CH Reversed Ovarian Tissue Apoptosis Induced by Cisplatin

Because apoptosis of ovarian granulosa cells is an initiating factor in the occurrence of POF and plays a key role in the overall condition (Chen *et al.*, 2015), we further analyzed cellular apoptosis in the 4 groups using TUNEL assay to detect any protective effects of CH in ovarian tissues. We observed that TUNEL-positive granulosa cells (dark brown in color) appeared in the cisplatin group, and that the number of positive cells in the cisplatin + low CH and cisplatin + high CH groups was lower than in the cisplatin group (Fig. 5a).

We also analyzed the activation of caspase 3, which is characteristic of the induction of apoptosis, as well as the inactivation of PARP (a DNA repair factor), using western immunoblotting. We observed caspase 3 activation and PARP inactivation/cleavage in the cisplatin group (Fig. 5b), and a diminution in the levels of the anti-apoptotic protein Bcl-2 in the cisplatin group compared to the control group. Additionally, the levels of the apoptosis-promoting protein Bax were higher in the cisplatin group compared to the control group (Fig. 5c). Notably, treatment with different doses of CH effectively reversed the activation of caspase 3, the inactivation of PARP, and the ratio of Bcl-2/Bax (Figs. 5b and 5c); and this reversal was similar to that observed for the TUNEL assay results.
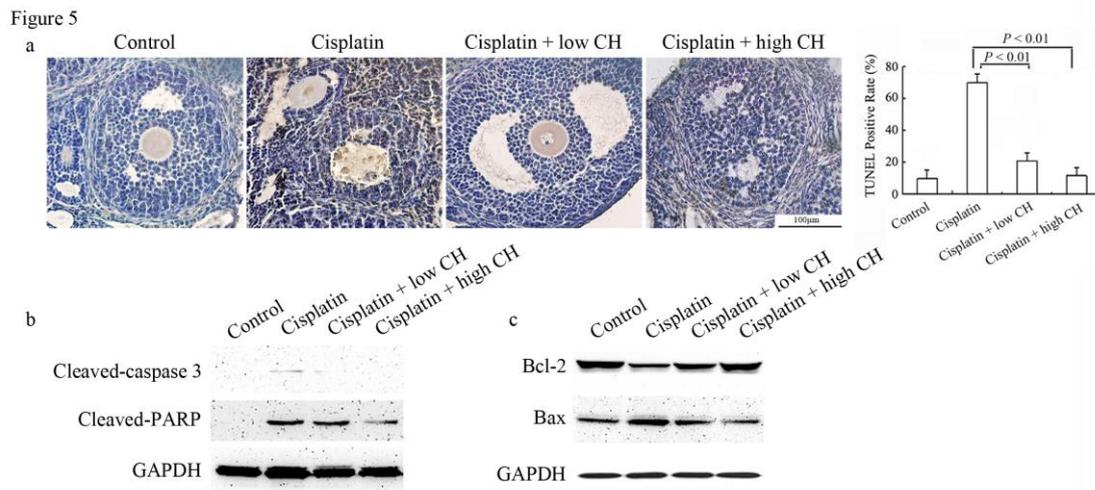

Figure 5: Effects of CH on ovarian cell apoptosis in POF resulting from cisplatin treatment.

(a) Apoptosis was determined by using TUNEL assay in ovarian tissues. We observed light staining of the granulosa cells in the cytotrophoblasts of the control group and in the 2 CH treatment groups, with deeper-staining granulosa cells extant in the cisplatin group. A comparison of the apoptotic cell rates is shown in the right-most panel. (b) Representative images of Western blots for caspase 3 and cleaved PARP. (c) Representative images of Western blots for Bcl-2 and Bax.

Protective Effects of CH in POF: Association with Reversing Mitochondrial Dysfunction

Chen et al. (2015) established that cisplatin induces mitochondrial dysfunction, resulting in POF. To further examine the protective effects of CH, we used transmission electron microscopy to detect mitochondrial morphology in ovarian tissue. In the control group, mitochondria were clear and numerous. Moreover, the mitochondria were round, oval, or rod-like in shape with clear and regularly arranged cristae. In contrast, the cells in the cisplatin group showed obvious structural abnormalities, including shrunken size and nuclear deformities. The number of mitochondria was also reduced in the cisplatin group; and the mitochondria exhibited swelling, vacuolization, and disorganization of the cristae. It should be noted that the physical appearance of the cells in the cisplatin + low and high CH groups were

noticeably restored, and had an increased number of mitochondria and a relatively regular arrangement of mitochondrial cristae (Fig. 6a).

We measured the ATP content of all ovarian tissue samples to evaluate the degree of mitochondrial dysfunction. We found that Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities were decreased in the cisplatin-treated group; whereas, treatment with CH significantly restored the activities of these enzymes (Na^+-K^+-ATPase activity: cisplatin vs. cisplatin + low CH, $P < 0.05$; cisplatin vs. cisplatin + high CH, $P < 0.01$. Ca^{2+}-Mg^{2+}-ATPase activity: cisplatin vs. cisplatin + low CH, $P < 0.05$; cisplatin vs. cisplatin + high CH, $P < 0.01$) (Fig. 6b). When we linked mitochondrial membrane potential ($\Delta\Psi$m) to mitochondrial function using JC-1 fluorescent dye, we found a significant reduction (represented as green coloring) in the $\Delta\Psi$m of the cisplatin group compared to the control group (red). Treatment with different doses of CH effectively reversed decreases in $\Delta\Psi$m, similar to those observed with the ATPase activity assay (Fig. 6c).

Previous studies have suggested that mitochondrial dysfunction is correlated with a reduction in levels of Mfn2 (a conserved dynamin-like GTPase located in mitochondria) in ovarian tissues (Chen *et al.,* 2015). As such, we examined the expression of Mfn2, and determined that Mfn2 was exclusively present in the cellular cytoplasm of all groups; however, we also observed weaker immunostaining signals in ovarian tissues from the cisplatin group relative to the control group and the 2 CH-treated groups (particularly in the granulosa cells) (Fig. 6d). To assess the differential expression of Mfn2 in the 4 groups, we first determined Mfn2 levels in the ovarian tissues using Western immunoblotting, and our results were consistent with those from the immunohistochemical assay (Fig. 6e).
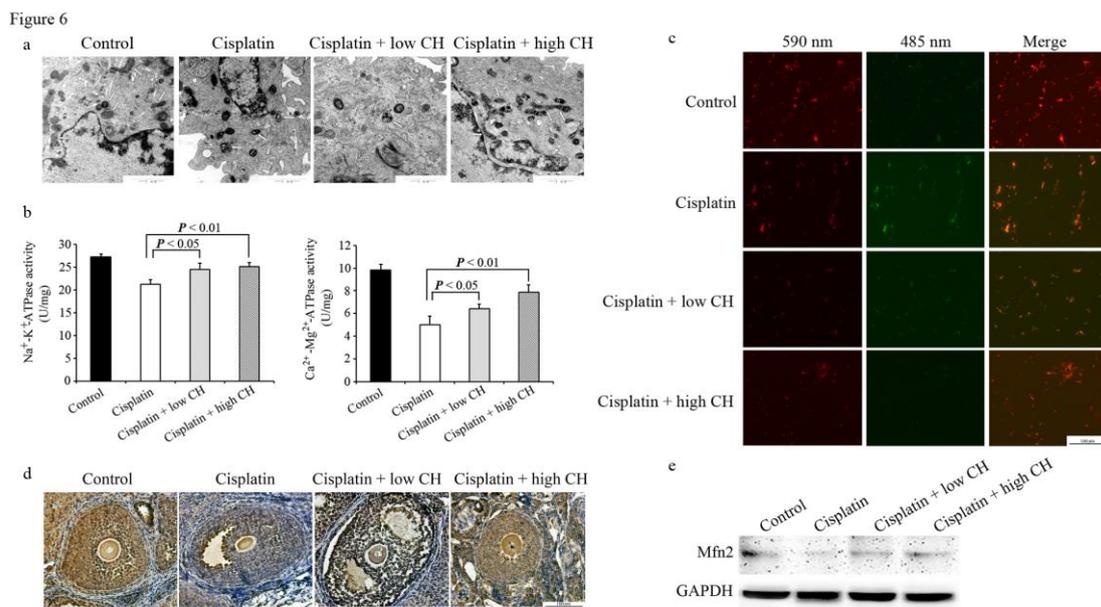

Figure 6: Protective effects of CH in POF are associated with reversing mitochondrial dysfunction.

(a) Ultrastructure of ovarian tissue as detected by transmission electron microscopy. In the control group, the mitochondria were greater in number and clearer than in the CH-treated groups. The mitochondria in the control group were round, oval, or rod-like, with clear and regularly arranged cristae. The mitochondria in the cisplatin group were reduced in number, swollen, vacuolated, and showed disorganized cristae. The mitochondria in the cisplatin + high CH group were increased in number, and the appearance and overall morphology of mitochondria and cristae were markedly restored. "⟹" indicate mitochondria.

(b) Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities were assessed electrophotometrically by measuring the amount of inorganic phosphate release. (c) Mitochondrial membrane potential ($\Delta\Psi$m) as detected by using JC-1.

(d) Mfn2 distribution was exhibited by immunohistochemistry. (e) Representative images of Western immunoblotting for Mfn2.

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Discussion

Ovarian cellular function is characterized by rapid turnover, and there is a potential similarity to cancer cells because both cell types are primary targets for chemotherapeutic agents (Yucebilgin *et al.,* 2004). Among the numerous cell types in the ovary, the apoptosis of granulosa cells is thought to be an initiating factor in the occurrence of POF (Chuai *et al.,* 2012; Del Mastro *et al.,* 2011; Zhang *et al.,* 2015). In the present study, we observed typical granulosa cell apoptosis in the follicles of cisplatin-treated mice, following other pathogenic changes such as ovarian weight gain, a reduction in follicular number, and hormonal changes (i.e., an increase in FSH and decrease in estradiol concentrations in blood), consistent with previous reports (Chen *et al.,* 2015; Morgan *et al.,* 2013; Li *et al.,* 2013).

While the adverse effects of chemotherapeutic agents on ovaries are obvious, chemotherapy is still an appropriate and effective treatment for various cancers. Thus, it is essential to prevent adverse chemotherapeutic effects as much as possible in female cancer patients. The results from this study showed that CH protected ovaries from cisplatin-induced ovarian damage; e.g., mice that received oral CH treatments while simultaneously receiving cisplatin, or that received CH treatments for an additional week after the end of the cisplatin administration, did not experience the ovarian weight loss induced by cisplatin. In addition, treatment with CH significantly reversed cisplatin-induced apoptosis of ovarian follicles, inhibited the cisplatin-induced augmentation in FSH, and attenuated the cisplatin-induced diminution in estradiol. These findings are in accordance with the results achieved by a classical herbal prescription used in traditional Chinese medicine, the Tongmai Dasheng Tablet. The Tongmai Dashen Tablet uses a tripterygium-glycoside-induced POF model, in which CH is a crucial component (Fu *et al.,* 2012). However, the mechanism(s) underlying the protective effects of CH remains unknown.

Cisplatin plays a vital role in the apoptosis of granulosa cells in ovarian follicles, which in turn can lead to overall follicular atresia (Chen *et al.,* 2015; Li *et al.,* 2013). DNA damage induced by cisplatin generates oxygen free radicals, and overproduction of oxygen free radicals induces oxidative stress, which is in turn responsible for cisplatin-related tissue toxicity (Cepeda *et al.,* 2007). Granulosa cells, which typically exhibit rapid turnover, are extremely sensitive to cytotoxic chemotherapeutic agents; and this can inhibit the growth of sufficient numbers of mature oocytes required for normal fertility. In addition, oxidative stress can lead to disordered mitochondrial metabolism, which is characterized by a decline in mitochondrial membrane potential. Previous studies have demonstrated that CH protects against myocardial ischemia/reperfusion injuries by increasing mitochondrial respiration and inducing mitochondrial uncoupling, thereby eliciting a cellular antioxidant response (Leong *et al.,* 2015; Wong *et al.,* 2013). In our study, CH effectively increased the number of mitochondria, and restored mitochondrial morphology, ATPase activities (Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity), and membrane potential ($\Delta\Psi m$).

Recent studies have shown that the functional ovarian damage observed in POF induced by cisplatin is related to inadequate expression of Mfn2 (Chen *et al.,* 2015). Mfn2, located on the outer mitochondrial membrane, is involved in the process of mitochondrial fusion and is necessary for the maximization of ATP production (Chen *et al.,* 2015; Koshiba *et al.,* 2004). Low in-vitro expression of Mfn2 attenuated cleavage rates in mouse zygotes and subsequent blastocyst formation, and caused mitochondrial dysfunction as confirmed by alterations in ATP and mtDNA levels, as well as mitochondrial membrane potential (Zhao, *et al.,* 2015). In addition, Mfn2 plays a vital role in cellular activities and apoptosis; e.g., a deficiency in Mfn2 induced cellular apoptosis in mouse embryos, hippocampal neurons, and rat spinal cord tissue (Zhao, *et al.,* 2015; Peng *et al.,* 2015; Zhang *et al.,* 2015). Furthermore, the depletion of Mfn2 can lead to apoptosis, usually via Bcl-2-signaling pathways. The latter may in turn promote mitochondrial fission during apoptosis by inhibiting fusion or accelerating fission events (Peng *et al.,* 2015). In our study, cisplatin-induced apoptosis within the ovary, especially in granulosa cells, was followed by Mfn2 down-regulation; however, CH then

up-regulated the expression of Mfn2 in the ovarian tissues of cisplatin-treated mice, which was in concordance with the changes in anti-apoptotic molecules such as Bcl-2 and in pro-apoptotic molecules such as Bax.

Conclusion

In this study, we demonstrated that CH inhibited cisplatin-induced POF, and determined that CH up-regulated Mfn2 expression and altered mitochondrial membrane structure via interaction with Bcl-2/Bax proteins; thereby protecting ovarian tissues from cisplatin-induced apoptosis. CH may therefore be a useful alternative treatment for women in reducing chemotherapy-induced POF.

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