ORIGINAL RESEARCH

Kuntai Capsule Improves Ovarian Function in Rats with Premature Ovarian Failure After Transplantation of Cryopreserved Ovarian Tissue by Regulating Sex Hormones and Apoptosis

Kang Yu, MM; Chunli Wu, MM; Yan Lang, BM; Xin Zhou, MM; Guohui Ren, MM; Yanmin Li, MD

ABSTRACT

Objective • Our study aimed to investigate the therapeutic effects of the Kuntai capsule in improving ovarian function in rats with transplantation of cryopreserved ovary.

Methods • Two mice ovary cell lines were cultured with Kuntai capsule decoction, and cell apoptosis was detected by MTT assay. A total of 90 SPF Sprague Dawley rats were included in this study. Thirty rats were used as the control group (group A), and not treated with any surgical operation. The remaining 60 rats were subjected to surgery to collect ovarian tissues and to construct a premature ovarian failure model. Ovarian tissues were cryopreserved, thawed, and transplanted back to ovaries. Sixty rats with ovary transplantation were randomly divided into group B and group C. Rats in group B were treated with Kuntai capsule at a dose of 0.1 capsule per day while rats in group C were fed with normal food. Serum levels of estradiol (E2) and follicular stimulating hormone (FSH) were detected. Expressions of several cytokines, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), and apoptotic factors caspase-3 and p53, were also detected.

Results • Kuntai capsule decoction inhibited apoptosis of *in vitro* cultured mice ovary cells. Furthermore, the Kuntai capsule promoted the recovery of E2 and FSH to normal levels and regulated the abnormal expression of HGF, VEGF, and IGF-1 and apoptotic factors caspase-3 and p53 in rats with premature ovarian failure after homotopic transplantation of ovarian tissue.

Conclusion • The Kuntai capsule can improve ovarian functions by regulating sexual hormones and cell apoptosis in rats with premature ovarian failure after homotopic transplantation of cryopreserved ovary tissue. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Premature ovarian failure is defined as the loss of ovarian function before the age of 40 years, and it is believed that about 1-3% of females will develop this disease.[1] Ovarian transplantation between monozygotic twins is considered a promising treatment for patients with premature ovarian failure.[2] With the development of tissue transplantation technology, both fresh ovary and frozen ovary tissues are now used to improve ovarian function defects caused by various pathological conditions including premature ovarian failure.[3] However, the application of this technology is still challenged by the slow recovery of ovarian functions. For example, clinical studies have shown that it generally takes about half a year before menstrual cycles and levels of key sexual hormones return to normalcy.[3] Therefore, how to accelerate the recovery of ovarian function is a hot research field in the treatment of premature ovarian failure using ovary transplantation.

Chinese herbal medicine has been widely used in the treatment of a variety of ovarian diseases including premature ovarian failure, and satisfactory treatment outcomes have been observed.[4] Kuntai capsule is a Chinese materiamedica preparation containing six Chinese medicine ingredients including Rhizoma Coptidis, Radix Rehmanniae Preparata, Radix Scutellariae, Radix Paeoniae Alba, Donkey Hide Gelatin, and Poria. With the effects of eliminating worries, reducing pathogenic infection reactions, stabilizing the mind, and regulating Yin and Yang, the Kuntai capsule has been

widely used in improving peri-menopausal symptoms.[5] A recent study has shown that the Kuntai capsule has protective effects on ovarian function in mice with accelerated aging ovaries.[6] Therefore, it will be reasonable to hypothesize that the Kuntai capsule can also be used to improve ovary functions in rats with premature ovarian failure after homotopic transplantation of cryopreserved ovary tissue.

In this study, Kuntai capsule decoction was used to treat *in vitro* culture mice ovary cells to investigate its effects on cell apoptosis. Kuntai capsule was also used to feed rats with premature ovarian failure after homotopic transplantation of cryopreserved ovary tissue, and its effects on sexual hormones and cell apoptosis were explored.

MATERIALS AND METHODS

Cell and cell culture

Two normal mice ovary cell lines CRL-9096 ™ and CRL-1737 ™ were purchased from the American Type Culture Collection (ATCC) USA and cultured under the conditions recommended by the ATCC. Cells were harvested at the logarithmic growth phase for subsequent study.

Preparation of Kuntai capsule decoction and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay Kuntai capsule was opened and the powder inside was mixed with 10 volumes of water and boiled for 30 mins. Dregs of a decoction were boiled with 5 volumes of water again for 30 mins. Decoctions were combined and concentrated. CRL-9096™ and CRL-1737 ™ were seeded into a 96-well plate (Thermo, USA) with 2×10^3 cells per well. Cells were cultured in culture media containing different dosages of Kuntai capsule decoction (0.1, 0.5, and 1 capsule/L). After culturing for 96 h, media were removed, and 200 μL of MTT (Sigma, China) was added to each well. Cells were cultured at 37 °C in darkness for 4 h. Then MTT solution was removed, and 150 μL of DMSO was added and incubated for another 10 mins. Finally, a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure OD values at 570 nm.

Animals

90SPF Sprague Dawley rats (200 and 250 g) were purchased from Taconic Biosciences (USA). All rats were cultured in the animal house of Weifang People's Hospital. All rats were raised under a light/dark rhythm of 12h/12h at 22°C ± 2°C with free access to water and food.

Collection, cryopreservation, and thawing of ovaries. Sixty rats were anesthetized by injection of 10% Chloral Hydrate (0.3 ml/100 g). Rats were fixed in spine position, and an incision was made on muscles above the ovary. Then reproductive tract was exteriorized, and partial ovary tissues were resected and collected. Ovarian tissues were processed and cryopreserved using the same method described by Jafarey, et al.[8] Ovarian tissues were stored in liquid nitrogen for 1 month. After that, ovarian tissues were thawed rapidly using a warm water bath (~100 ˚C/min to 35°C) for 2 mins. Ovarian tissues were washed by 10 ml HTF-HEPES + 1.0 M sucrose for one minute, then by 10 ml HTF-HEPES + 0.5 M sucrose for 3 minutes and 12% HTF-HEPES for 3 minutes to reduce osmotic damage.

Preparation of premature ovarian failure model and ovary transplantation. Three weeks after surgery, rats were recovered and subjected to premature ovarian failure model construction by subcutaneous injection of tripterygium glycoside using the methods described by Chen, et al.[9] Before transplantation, rats were anesthetized by injection of 10% Chloral Hydrate (0.3 ml/100 g). Ovary tissues were transplanted to ovaries at 6 -8 different positions under a microscope.

Grouping and treatment. Normal rats without surgery were treated as the control group (Group A). After transplantation, rats were randomly divided into two groups including group B and group C (n = 30). Rats in group B were fed with food containing Kuntai capsule decoction with a dose of 0.1 capsule per day, while rats in group C were fed with normal food.

Preparation of serum samples. Blood (1 ml) was extracted from each rat from the ear vein just before (0) and 2, 4, 6, 8, and 10 weeks after transplantation. Blood samples were kept at room temperature for 1 h, followed by centrifugation at 2500 rpm for 15 mins to separate serum samples. Measurement of estradiol (E2) and follicular stimulating hormone (FSH) homologous double antibody radioimmunoassay was performed using reagents provided by Beijing North Institute of Biological Technology (Beijing, P. R. China), and E2 was detected using an automatic chemiluminescence system (Bayer Co.).

qRT-PCR. Ovarian tissues were collected through surgery 10 weeks after transplantation, and total RNA was extracted from ovarian tissues using Trizol reagent (Invitrogen, USA). RNA samples were tested using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA), and only the ones with an A260-to-A280 ratio between 1.8 and 2.0 were used in reverse transcription to synthesize cDNA. Then, cDNA was used as a template to detect the expressions of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1) in ovarian tissue using primers provided by Sino Biological Inc (Beijing, China). PCR reaction conditions were: 95°C for 1 min, followed by 40 cycles at 95°C for 12 s and 55-60°C for 35 s. Ct values were processed using the 2-ΔΔCT method, and the relative expression level of each gene was normalized to endogenous control β-actin.

Western Blot. Ovarian tissues were collected through surgery 10 weeks after transplantation, and total protein was extracted from ovarian tissues using cell lysis buffer (Cell Signaling Technology). Protein samples were quantified using the bicinchoninic acid (BCA) method. Then, 30 μg of protein from each sample was subjected to 10 % SDS-PAGE gel electrophoresis, and proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk at room temperature for 1 h. After washing, membranes were incubated with primary

antibodies including rabbit anti-caspase-3 antibody (1: 2000, ab4015, Abcam), anti-p53 antibody (1: 2000, ab131442, Abcam), anti-bcl-2 antibody (1: 2000, ab59348, Abcam), and anti-GAPDH antibody (1: 1000, ab9485, Abcam) overnight at 4 °C. After washing, membranes were further incubated with anti-rabbit IgG-HRP secondary antibody (1:1000, MBS435036, MyBioSource) at room temperature for 1 h. Then membranes were washed again, and signals were detected using the enhanced chemiluminescence (ECL, Sigma-Aldrich, USA) method. The relative expression level of each protein was normalized to endogenous control GAPDH using Image J.

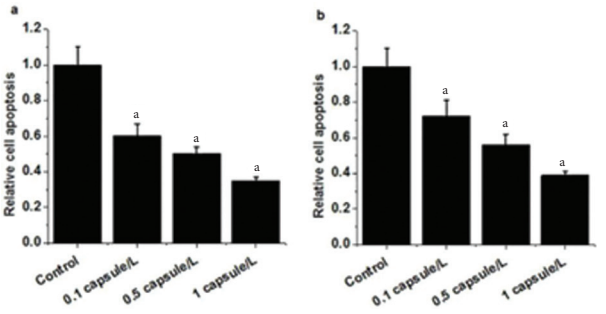
Statistical analysis

Statistical analyses were performed using SPSS19.0 (SPSS Inc., USA). Data with normal distribution data were expressed by ($x \pm SD$), and comparisons between two groups were performed by t test. $P < .05$ was statistically significant.

RESULTS

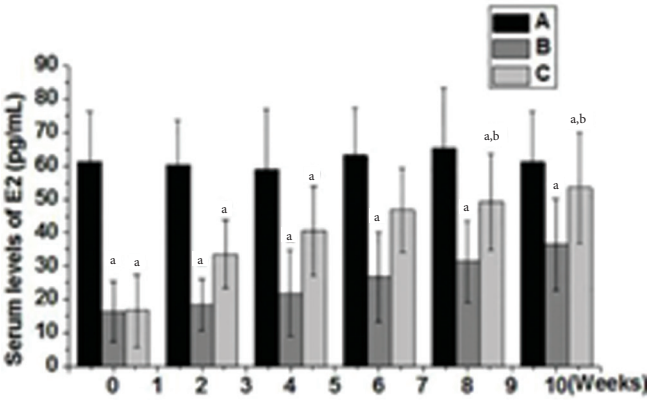
Kuntai capsule decoction inhibited apoptosis of mice ovary cell lines (CRL-9096 ™ and CRL-1737 ™). Cells were treated with different dosages of Kuntai capsule decoction, and cell apoptosis was detected by MTT assay. As shown in Figure 1, compared with control cells, cell apoptosis was significantly reduced in cells treated with Kuntai capsule decoction in a dose-dependent manner.

Figure 1. Detection of Apoptosis of Mice Ovary Cell Lines by MTT assay



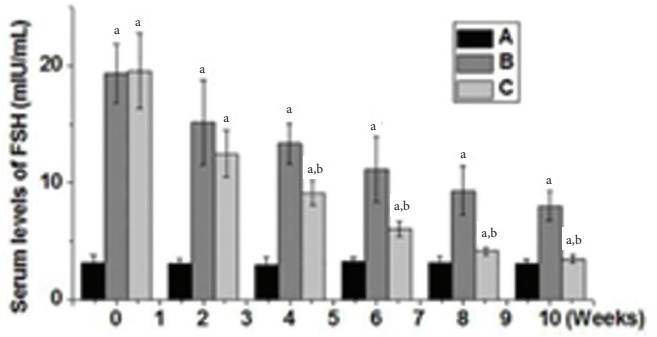
[a]Compared with Group A, $P < .05$

Figure 2. Serum Levels of E2 (pg/mL) in 3 Groups of Rats



[a]Compared with Group A, $P < .05$
[b]Compared with Group B, $P < .05$

Figure 3. Serum Levels of FSH (mIU/mL) in 3 Groups of Rats



[a]Compared with Group A, $P < .05$
[b]Compared with Group B, $P < .05$

Effects of Kuntai capsule on sexual hormones in rats with cryopreserved ovarian transplantation

E2 and FSH are two important sexual hormones that are directly related to ovarian function. As shown in Figures 2 and 3, compared with control rats, serum E2 level was significantly reduced, while serum FSH level was significantly increased in rats with premature ovarian failure. After ovary transplantation, serum E2 level gradually increased (Figure 2), while serum FSH level gradually decreased (Figure 3) in both groups B and C. No significant differences in serum levels of E2 and FSH were found between groups B and C before transplantation. However, the serum level of E2 was higher in group C than in group B at each time point after transplantation, and significant differences were found between the two groups 8 and 10 weeks after transplantation. No significant differences in serum level of E2 were found between groups A and C 10 weeks after transplantation. In contrast, the serum level of FSH was lower in group C than in group B at each time point after transplantation, and significant differences were found between two groups 4, 6, 8, and 10 weeks after transplantation. No significant differences in serum levels of FSH were found between groups A and C 10 weeks after transplantation.

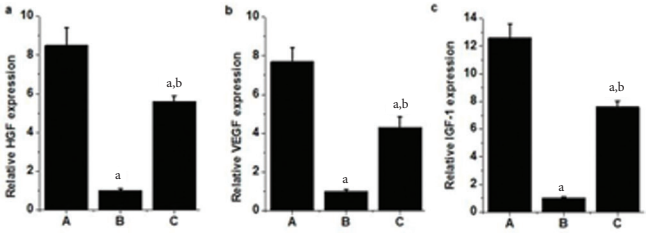
Effects of Kuntai capsule on the expression of cytokine in ovarian tissue

Several important cytokines including HGF, VEGF, and IGF-1 are also important for ovarian function. In this study, ovarian tissues were collected 10 days after transplantation, and expression levels of HGF, VEGF, and IGF-1 in ovarian tissue were measured by qRT-PCR. As shown in Figure 4, compared to group A, the expression levels of HGF, VEGF, and IGF-1 were significantly decreased in groups B and C ($P < .05$). In addition, expression levels of HGF, VEGF, and IGF-1 were significantly higher in group C than in group B ($P < .05$).

Effects of Kuntai capsule decoction on expression of pro-apoptotic factors caspase-3 and p53, and anti-apoptotic factor bcl-2 in ovarian tissue

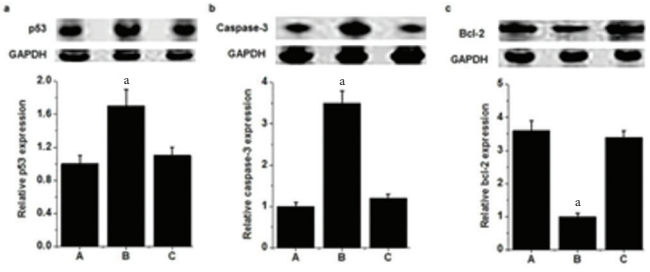
Expressions of pro-apoptotic factors caspase-3 and p53, and anti-apoptotic factor bcl-2 in ovarian tissue 10 weeks after transplantation were detected by Western blot. As

Figure 4. Effects of Kuntai Capsule Decoction on the Expression of Several Cytokines in Ovarian Tissue. (a) Effects of Kuntai Capsule Decoction on Expression of HGF in Ovarian Tissue; (b) Effects of Kuntai Capsule Decoction on Expression of VEGF in Ovarian Tissue; (c) Effects of Kuntai Capsule Decoction on the Expression of IGF-1 in Ovarian Tissue.



[a]Compared with Group A, $P < .05$
[b]Compared with Group B, $P < .05$

Figure 5. Effects of Kuntai Capsule Decoction on the Expressions of Pro-Apoptotic Factors Caspase-3 and p53, and Anti-Apoptotic Factor bcl-2 in Ovarian Tissue. (a) Effects of Kuntai Capsule Decoction on the Expression of Pro-Apoptotic Factor p53 in Ovarian Tissue; (b) Effects of Kuntai Capsule Decoction on the Expression of Pro-Apoptotic Factor Caspase-3 in Ovarian Tissue; (c) Effects of Kuntai Capsule Decoction on the Expression of Pro-Apoptotic Factor bcl-2 in Ovarian Tissue



[a]Compared with Group A, $P < .05$

shown in Figure 5, the expression levels of pro-apoptotic factors caspase-3 and p53 were significantly higher, while the expression level of bcl-2 was significantly lower in group B than in group A ($P < .05$), indicating the increased cell apoptotic rate in ovarian tissues after ovary transplantation.

DISCUSSION

Premature ovarian failure, also known as primary ovarian insufficiency, is a type of intractable female infertility induced by endocrine disorder and characterized by the loss of normal ovarian function before the age of 40 years.[9] As two key sexual hormones, E2 and FSH usually play opposite roles in many critical biological and pathological processes.[10] Reduction in E2 levels and increase in FSH levels have also been proven to be involved in the development of premature ovarian failure.[11] Although various factors, including pelvic radiotherapy, chemotherapy, diethylstilbestrol exposure in utero, and ovarian surgery, have been proven to be closely correlated with the onset and development of premature ovarian failure, etiology is unknown in nearly 90% of the cases.[12]

At present, hormone therapy is the main treatment of premature ovarian failure, which can improve hormone levels and relieve symptoms.[13-14] However, long-term application of hormones can produce significant side effects, such as an increased risk of endometrial cancer.

In recent years, more and more studies have found that hormones combined with traditional Chinese medicine can enhance therapeutic effects, improve ovarian function, and reduce side effects.[15-18] Many studies have shown that traditional Chinese medicine has been gradually applied to the treatment of premature ovarian failure and has obvious advantages. Pharmacological studies have shown that the salient effect of the Kuntai capsule on the ovary may be related to the estrogenic activity of Radix Rehmanniae Praeparata and Radix Paeonia Alba.[19] Studies have shown that Kuntai capsules can increase serum estrogen levels and lead to vaginal cell maturation;[20] increase the ovarian volume and restore ovarian function of menopausal animals; and increase the wet weight and tonify the uterus.[21] In addition, the Kuntai capsule can reduce menopausal symptoms and signs in menopausal and postmenopausal patients. These indicate that the role of the Kuntai capsule may be related to the enhancement of ovarian function. Furthermore, studies also show that although it has an effect like estrogen, the Kuntai capsule does not have many side effects.[22] In this study, significantly increased serum levels of FSH and significantly decreased serum levels of E2 were detected in rats with premature ovarian failure. Previous studies have shown that ovarian tissue transplantation can induce the recovery of sexual hormones to normal levels.[3] Consistent with previous studies, the serum level of E2 gradually increased, while the serum level of FSH gradually decreased after ovarian tissue transplantation. However, the serum level of E2 was still significantly lower, while the serum level of FSH was significantly higher, in rats with premature ovarian failure than in normal rats 10 weeks after transplantation, which is consistent with the findings of a previous clinical study which states that the recovery of sexual hormones to normal levels took almost half a year.[3] In contrast, the Kuntai capsule significantly accelerated the recovery of FSH and E2 levels to normal levels. These findings suggest that the Kuntai capsule can improve ovarian functions by regulating sexual hormones.

Cryopreserved ovary tissue transplantation has been proven to be a safe and effective treatment to improve ovarian function in patients with premature ovarian failure. However, the recovery of ovarian function after transplantation is usually slow.[3] In this study, auto-transplantation of ovarian tissues was performed on rats with premature ovarian failure. With anti-atretogenic and mitogenic effects, HGF promotes follicle maturation and inhibits granulosa cell apoptosis.[23] As an angiogenic cytokine, VEGF fulfills its biological roles by inducing the formation of new vessels and promoting endothelial cell proliferation.[24] IGF-1 is a growth hormone mediator that can induce the proliferation of and inhibit the apoptosis of granulosa cells, and promote the formation of follicular antrum.[25,26] Besides,

p53 and caspase-3 are two thoroughly studied pro-apoptotic factors, while bcl-2 is an anti-apoptotic factor.[27,28] All these factors may affect ovarian functions after transplantation.

In this study, expressions of HGF, VEGF, and IGF-1 mRNA, as well as bcl-2, were significantly reduced in rats with premature ovarian failure 10 weeks after ovary transplantation, indicating that ovary transplantation itself may be insufficient to achieve the rapid recovery of normal expression patterns of those anti-apoptotic factors. Kuntai capsule treatment accelerated the recovery of normal expression of those factors, and the expression level of bcl-2 returned to normal 10 weeks after treatment. Similarly, expressions of pro-apoptotic factors p53 and caspase-3 were significantly increased in rats with premature ovarian failure even 10 weeks after ovary transplantation. 10-week Kuntai capsule treatment reduced the expression levels of those pro-apoptotic factors to normal levels. These results suggest that the Kuntai capsule can improve ovarian function in premature ovarian failure by regulating abnormal expressions of apoptotic factors.

As for limitations, all data in this study were obtained from within our hospital and the study population was limited in scope and could not represent the results of different hospitals in different regions. Therefore, the relationship between empowerment education and disease prognosis needs to be explored in depth after further improvement of the experimental protocol.

CONCLUSION

In conclusion, Kuntai capsule decoction can inhibit cell apoptosis of *in vitro* cultured mice ovary cells and recover E2 and FSH to normal levels. In addition, Kuntai capsule decoction can also regulate abnormal expressions of key cytokines and apoptotic factors in rats with premature ovarian failure after homotopic transplantation of ovarian tissue. Therefore, we conclude that the Kuntai capsule can improve ovarian functions by regulating sexual hormones and cell apoptosis in rats with premature ovarian failure after homotopic transplantation of cryopreserved ovary tissue. Considering the safety of Chinese herbal medicine, the combination of Kuntai capsule and hormone therapy may be a new direction for the treatment of premature ovarian failure.

ETHICAL CONSIDERATIONS

The protocol was approved by the Committee on the Ethics of Laboratory Animals of Weifang People's Hospital. This study was carried out in strict accordance with the recommendations for the Care and Use of Laboratory Animals of Weifang People's Hospital.

AVAILABILITY OF DATA AND MATERIALS

The datasets used during the present study are available from the corresponding author upon reasonable request.

AUTHOR DISCLOSURE STATEMENT

All authors declare that they have no conflicts of interest.

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Not applicable

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