Journal of Molecular Biomarkers & Diagnosis
ISSN: 2155-9929

Zhang et al., J Mol Biomark Diagn 2017, 9:1
DOI: 10.4172/2155-9929.1000373

Research Article Open Access

Human Placental Extracts Improve Ovarian Function by Reducing Follicular Atresia in Mice With CTX-Induced Premature Ovarian Failure

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Abstract

The details of the pathogenic mechanisms underlying premature ovarian failure (POF) remain unknown. Accumulating evidence suggests that primordial follicle inactivity, disorders affecting follicular survival and growth and follicular atresia may affect an individual's susceptibility to POF. The Rictor/mTORC2/Akt/Foxo3a pathway plays a central role in cytoskeletal construction and follicle survival. As a stronger alkylating agent that exerts immunosuppressive effects, cyclophosphamide

(CTX) is widely used in clinical practice, especially in cancer. However, it also has significant reproductive toxicity. CTX accelerates the development of ovarian follicles into mature follicles, resulting in a decreased follicular reserve and ultimately leading to ovarian failure or even POF. We have sought to research effective methods to reduce the damage caused by CTX.

Here, we investigated the protective role of human placental extracts on CTX-induced ovarian injury in mice. We describe the effects of HEP on histopathology, the number of atretic follicles, the weight of the ovary, serum hormone levels and apoptosis in granulosa cells. Our data show that ovarian injury can be effectively attenuated by HPE administration. Ovarian weight was higher, the number of atretic follicles was lower, the serum levels of the hormones E2 and P were higher, and the rate of apoptosis and the serum levels of the hormones LH and FSH were lower in granulosa cells in mice treated with HPE for 2 weeks than in the control group.

At the molecular level, our results demonstrated that HPE can be used to inhibit the expression of p-Rictor, Bad, Bax and PPAR and activate the expression of p-Akt and p-Foxo3a, thus preventing follicular granulosa cells from undergoing a higher rate of apoptosis and blocking atresia follicle formation. These effects alleviated CTX-induced ovarian injury by affecting the Rictor/mTORC2/Akt/Foxo3a signalling pathway.

Keywords: Premature Ovarian Failure (POF); Ovarian function; Follicular atresia; Cyclophosphamide (CTX)

Introduction

In premature ovarian failure (POF), the ovaries become dysfunctional or are lost, and persistent amenorrhea and sexual atrophy are observed in some affected women before the age of 40 [1]. POF can result from hereditary, metabolic, infectious, autoimmune, iatrogenic (radiotherapy and chemotherapy) and other causes [2]. Moreover, patients with hormonal disorders may exhibit vasomotor and psychiatric symptoms, such as a hectic fever with unsteady movement, perspiration, daze, heart palpitations, and decreased libido. The mechanisms involved in POF remain unclear, and clinical treatments, especially chemotherapy, play important roles as iatrogenic factors [3].

Because cyclophosphamide (CTX) is a cheap and effective therapy, it is widely used as a treatment for many conditions, including cancer, haematologic diseases, primary angiitis of the central nervous system, chronic inflammatory demyelinating polyneuropathy (CIDP), rheumatoid arthritis, nephrotic syndrome. CTX exerts a toxic effect by covalently binding to DNA, causing abnormal complementary base pairings, misstructured and dysfunctional cells and irreversible injury in the ovary [4].

CTX accelerates the maturation of ovarian primordial follicles into mature follicles, reducing ovarian reserves and thereby leading to ovarian failure in young female patients with POF [5]. Recently, the proliferation, development and maturation of ovary germ cells are regulated by the mechanistic target of rapamycin (mTOR) pathway, in which Rictor, mTORC2, Akt, and Foxo3a play key roles. Based on recent reports, a mouse model of POF was constructed using 4-vinylcyclohexene diepoxide (VCD) [6,7]. In these mice, Rictor and the molecular functions of its downstream effector mTORC2 were affected in that both of these are apoptosis-related proteins were overexpressed. These effects accelerated follicular atresia and apoptosis, leading to the loss of ovarian functions.

Human placental extracts (HPE) perform many functions, such as enhancing immunity, regulating endocrine processes, extending youth, repairing damaged skin, eliminating ageing skin, beautifying skin, restraining inflammation, and performing anti-allergic functions [8].

Thus, in this study, we had the following aims:

(i) To explore the influence of HPE on ovarian function and histopathological analysis, to determine the number of atretic follicles, the weight of the ovary and body, and serum hormone levels, and to

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Received December 12, 2017; **Accepted** December 28, 2017; **Published** December 30, 2017

Citation: Zhang B, Yu L, Liu YM, Zhao XK, Zhu LL, et al. (2018) Human Placental Extracts Improve Ovarian Function by Reducing Follicular Atresia in Mice With CTX-Induced Premature Ovarian Failure. J Mol Biomark Diagn 9: 373. doi: 10.4172/2155-9929.1000373

quantify apoptosis in granulosa cells in a mouse model of CTX-induced ovarian injury and

(ii) To use Western blot analysis to detect the expression levels of p-Rictor, mTORC2, p-Akt, p-Foxo3a, Bad, Bax, and PPAR to investigate the molecular mechanisms by which HPE alleviates the ovarian injuries induced by CTX.

Materials and Methods

Animal experiments

Female C57BL mice 36, 8 weeks old, 16~20 g, SPF grade, Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the IACUC (institutional animal care and use committee) of University of Alabama of Birmingham (Permit Number:2015. 011-0003). The mice were individually housed in plastic cages at room temperature (22°C) and maintained on an artificial cycle of 12-h light and 12-h dark. Surgical preparations involved anesthetization with a xylazine/ketamine mixture. The mice were then sacrificed by cervical dislocation. Ovarian tissue was precisely dissected, immersed in liquid nitrogen, and stored at -80°C until further analysis. All procedures were approved by institutional ethics committee and performed in accordance with the guidelines on animal ethics. The cisplatin (Cyclophosphamide Baxter 0054-4130-25) was administered intraperitoneally once daily at doses of 2.0 g/g for 14 days. Control animals received injections of phosphate buffered saline(PBS) at doses of 2.0 g/g, HPE animals received injections of HPE (National medicine permission number H20046260) at doses of 1.2 ml/kg. After induction of POF, different doses of HPE (Group CH+++: high dose 2.4 ml/kg, Group CH++: medium dose 1.2 ml/kg and Group CH+: low dose 0.6 ml/k) were administered intraperitoneally once daily to mice in the treatment groups for 14 days. Control, model and HPE groups were given the same volume of saline (1.2 ml/kg) daily throughout the treatment period.

Sample collection and preparation for pathological evaluation

After the final administration of HPE or saline, mice were fasted for 12 h and then anesthetized with 10% chloral hydrate. Then 5 mL blood samples were collected from an artery in the rat abdomen. Blood samples were centrifuged at 3000 r/min and 4°C for 15 min to obtain serum samples. After blood collection, ovary samples were removed and weighed. Then samples were fixed in 4% paraformaldehyde (Sigma-Aldrich) for subsequent paraffin embedding. Ovary specimens were sectioned at 5 μm and then stained with hematoxylin and eosin (H&E).

ELISA assay

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum hormone levels of estrogen (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and progesterone according to the manufacturer's instructions. Briefly, 100mL of mouse E2 or FSH at concentrations of 8,000, 4,000, 2,000, 1,000, 500, 250, and 125 pg/mL or 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 ng/mL or diluted mouse plasma were added to each antibody percolated microtest wells and incubated for 60min. After 3 times of washing, the HRP-conjugated detection antibodies were added followed by substrate solution. The absorbance was determined at a wavelength of 450 nm.

Flow cytometry analysis

Flow cytometry analysis was performed to measure the apoptotic status of granulosa cell. Briefly, 100 μL cells at 5×10^5/mL were transferred into 5 mL flow tubes. Annexin V/fluorescein isothiocyanate (FITC) (5 μL) was added, and apoptotic rates were detected based on the fluorescence of Annexin V/FITC with a flow cytometer (Coulter Epics XL; Beckman Coulter, Fullerton, CA, USA).

Western blotting

The protein concentration of the whole ovary lysate was determined by BCA kit (Pierce, Rockford, IL). Lysates were electrophoresed on a disulfide-reduced 12% SDS PAGE, transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA), probed and stripped followed by re-probing with indicated antibodies, and developed with the enhanced chemiluminescent (ECL) system (Pharmacia Biotech, Piscataway, NJ). The expression of GAPDH protein was used as a loading control. For densitometric analysis of band intensity, a specific band on the ECL-developed film was subjected to densitometric analysis (Adobe Photoshop). The densitometric readings were pooled and averaged from three independent experiments. The background of densitometric reading on the ECL-developed film was subtracted.

Data analysis

Data from at least three independent duplicates were expressed as mean ± SD. Differences between two groups were analyzed with Student's t-test. For animal studies, each experimental and control group contained 5 to 8 animals and repeated twice. A p value of < 0.05 was considered statistically significant. All statistical analyses were done with the SPSS 20.0 software program (SPSS Inc, Chicago, IL, USA).

Results

HPE protects follicles in POF mice

In histopathologically stained sections of ovaries from control mice and HPE mice, large numbers of follicles were observed at various stages of development. In contrast, fewer or no follicles were observed in ovarian sections from POF mice and CH+ mice, which showed larger atretic follicle. In ovarian sections from CH++ and CH+++ mice, fewer atretic follicle and mature follicles resembling those in control mice were observed (Figure 1A). The number of follicles (atretic or normal follicles) in each group was counted from 5 sections of each ovary. The results showed a significantly higher number of atretic follicles in the ovaries of the POF group than that in the CH++ and CH+++ groups. There were fewer normal follicles in the POF group than that in the CH++ and CH+++ groups (Figure 1B).

Compared with the control group, the number of normal follicles in POF, CH +, CH ++ and CH +++ groups was significantly decreased (**P<0.01, *P<0.05 vs. the control group); Compared with the POF group, the number of normal follicles in HPE, CH ++ and CH +++ group was significantly increased (## P<0.01, #P<0.05 vs. the POF group); Compared with the Control group, the number of atretic follicles in POF and CH + groups was increased significantly (P<0.05 vs. the control group); Compared with the POF group, the number of atretic follicles in the HPE, CH ++ and CH +++ groups was significantly reduced (P<0.05 vs. the POF group).

HPE induced an increase in serum E2 and progesterone levels and decreased the serum levels of FSH and LH in POF mice

In order to investigate the levels of serum sex-related hormones in mice, we analysed the serum levels of the oestradiol E2, FSH, LH and progesterone. The results showed that FSH and LH levels were significantly higher, and E2 and progesterone levels were significantly

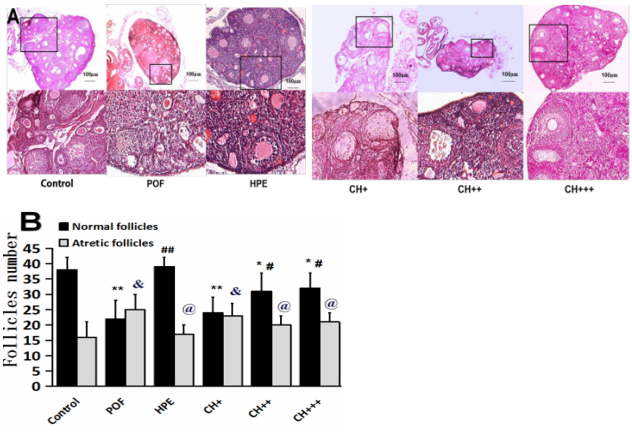

Figure 1: A. Histological comparison of ovarian morphology; B. Comparison of the total number of classified follicles.

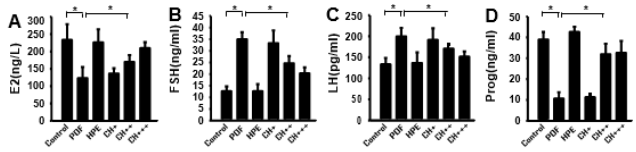

Figure 2: Determination of serum hormone levels.

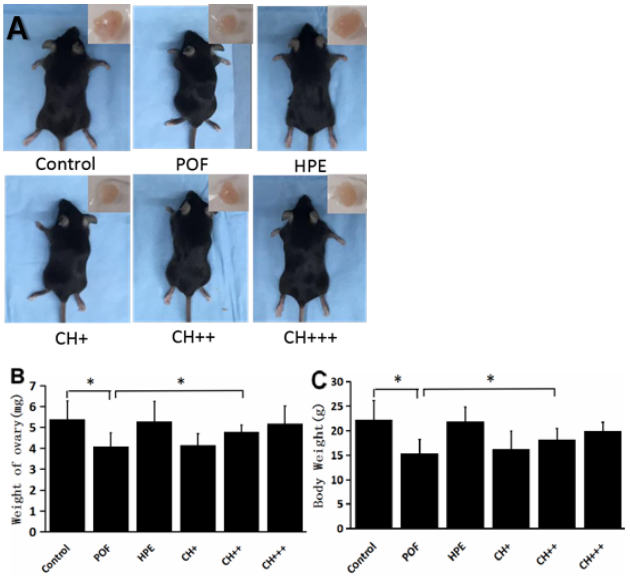

Figure 3: Weight of ovary and body.

lower (P<0.05), in the POF model group than in the control group. After the mice were treated with a medium or high dose of HPE, serum FSH and LH levels were significantly lower and serum E2 and progesterone levels were significantly higher than in the POF group. However, there was no significant difference between the control and HPE groups (P>0.05) (Figure 2).

A. Compared with the control group, serum levels of E2 was significantly decreased in POF, CH +, CH + + groups (*P<0.01). Compared with the POF group, serum levels of E2 was significantly increased in HPE, CH++ and CH+++ groups (*P<0.01).

B. Compared with the control group, serum levels of FSH was significantly increased in POF, CH +, CH + + and CH+++groups (*P<0.01). Compared with the POF group, serum levels of FSH was significantly decreased in HPE, CH++ and CH+++ groups (*P<0.01).

C. Compared with the control group, serum levels of LH were significantly increased in POF, CH + and CH + + groups (*P<0.01). Compared with the POF group, serum levels of FSH was significantly decreased in HPE, CH++ and CH+++ groups (*P<0.01).

D. Compared with the control group, serum levels of Progesterone was significantly decreased in POF, CH + and CH + + groups (*P<0.01). Compared with the POF group, serum levels of Progesterone were significantly increased in HPE, CH++ and CH+++ groups (*P<0.01).

Increased ovarian and body weight in POF mice treated with medium or high dose of HPE

Body weight of POF mice was significantly lower than in the control group. Following treatment with a medium or high dose of HPE, body weight of mice was significantly higher than in the POF group. Ovarian weight of the POF group was significantly lower than in the control group. Post-treatment ovarian weight was also significantly higher than in the POF group. However, in the mice treated with a low dose of HPE, there was no difference between the POF and the low dose group(P>0.05) (Figure 3).

A. Gross morphology of mice and ovaries of each group.

B. Compared with the control group, the ovarian weight of POF and CH + groups was significantly decreased, (*P<0.01). Compared with the POF group, the ovarian weight of HPE, CH++ and CH+++ groups was significantly increased (*P<0.01) C. Compared with the control group, the body weight of POF, CH + and CH++ groups was significantly decreased (*P<0.01). Compared with the POF group, the bodyweight of HPE, CH++ and CH+++ groups was significantly increased.

HPE treatment inhibits apoptosis in mouse ovarian granulosa cells

Atretic follicles formation is associated with the excessive apoptosis of ovarian granulosa cells. We investigated the rate of apoptosis in ovarian granulosa cells by flow cytometry. The results showed that the rate of apoptosis was significantly higher in ovarian granulosa cells in the POF group than in the control group. After mice were treated with a medium or high dose of HPE, the rate of apoptosis was significantly lower in mouse ovarian granulosa cells than in the POF group (P<0.05, Figure 4). These results revealed that inhibiting the excessive apoptosis of ovarian granulosa cells may be the mechanism by which HPE treats POF.

A. Flow cytometry analysis was performed to measure the apoptotic status of granulosa cell.

B. Compared with control group, the apoptotic rate of ovarian granulosa cells in POF, CH +, CH ++ and CH +++ groups was significantly higher (*P<0.01). Compared with POF group, the apoptotic rate of ovarian granulosa cells in HPE, CH ++ and CH +++ groups was significantly lower (*P<0.01).

HPE inhibited the protein expression of p-Rictor, Bad, Bax, and PPAR and promoted the protein expression of p-AKT and p-Foxo3a in a mouse model of POF

The Rictor/mTORC2/Akt/Foxo3a signalling pathway is vital to

follicular atresia and apoptosis. We next examined the expression of related signalling molecules. The results showed that in the POF group, the protein levels of p-Rictor, Bad, Bax and PPAR were higher in ovarian follicles, while the protein levels of p-AKT and p-Foxo3a were lower. However, treatment with HPE significantly reduced the levels of the p-Rictor, Bad, Bax and PPAR proteins and increased the protein levels of p-AKT and p-Foxo3a (Figure 5).

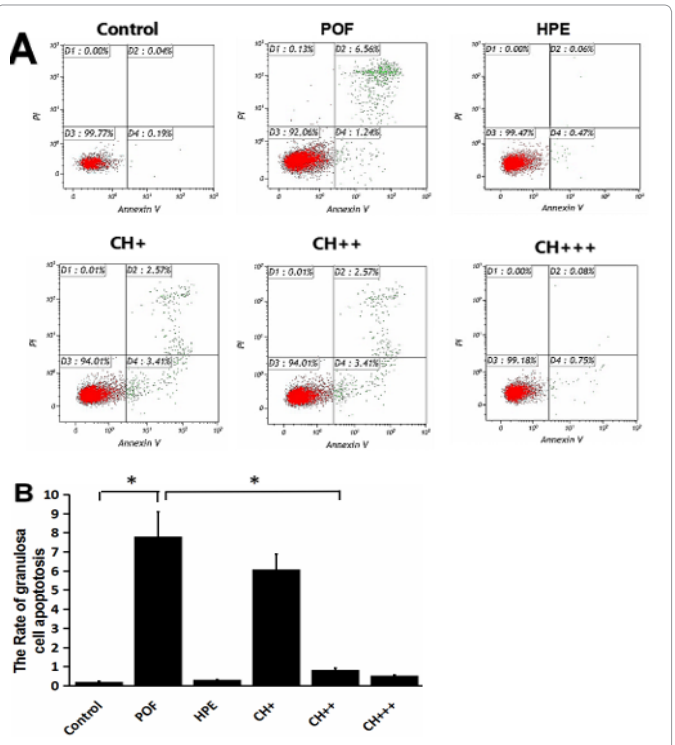

Figure 4: Apoptosis of granulosa cell.

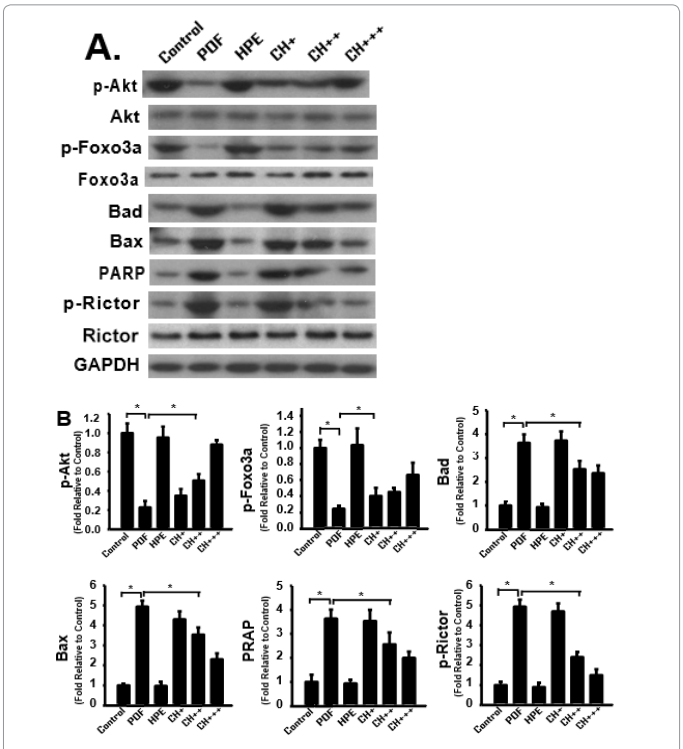

Figure 5: HPE inhibited protein expression of p-Rictor, Bad, Bax, PPAR and promoted protein expression of p-AKT and p-Foxo3a in POF model mice.

A. Ovaries were harvested at day 1 after the final administration of HPE or saline, equivalent amount of whole ovary detergent lysates was western blotted with indicated antibodies. Per lane represents individual animals (5- animals per group).

B. Densitometry analysis of p-Rictor, Bad, Bax, PPAR, p-AKT and p-Foxo3a. Data were pooled and represented as mean ± SD, n=5-8 animals per group. *p<0.01.

Discussion

The mechanisms involved in POF remain unclear. A variety of causes, such as a decrease in the number of original follicles, increased and accelerated follicular atresia, and disordered follicular maturation, can lead to POF [9,10]. Because CTX exerts strong toxic effects in the gonads, its medical uses often lead to POF. The use of CTX is a common contributor to iatrogenic effects and can cause conditions including menstrual disorders, amenorrhea, infertility, and decreased libido. POF decreases each follicular stage and is associated with interstitial fibrosis and necrosis in addition to endocrine changes that can increase the serum hormone levels of E2 and P and decrease the serum hormone levels of LH and FSH [11,12].

It has been reported that the mTOR signalling pathway regulates the growth and development of follicles. mTOR is a highly conserved Ser/Thr protein kinase that contains two unique functional domains for mTORC1 and mTORC2 [13-17]. MTORC1 consists of mLST8 and Raptor and regulates cell growth, proliferation, and metabolism. Activated MTORC1 can initiate the downstream phosphorylation of S6K1 and 4E-BP1, thereby promoting the production of egg cells and protein synthesis. MTORC2 is composed of mSIN1 and mLST8 and its core functional component, Rictor. Rictor regulates the functions of its downstream targets Akt, Rho, Rac and Cdc42, which themselves regulate egg cell survival and the construction of the cytoskeleton [18-21].

At present, the upstream and downstream cytokine functions of mTORC1 have been extensively studied. However, the biological functions of mTORC2 are not yet completely understood. Researchers set up a mouse model of POF using 4-vinylcyclohexene diepoxide (VCD) and explored its effects on a variety of factors (e.g., phosphorylation in response to Rictor overexpression, the phosphorylation of Akt and the inhibition of Foxo3a expression) and on Rictor and its downstream mTORC2 signalling molecules (e.g., the Rictor/mTORC2/Akt/Foxo3a signalling axis) [22-25]. However, some proteins associated with apoptosis (e.g., Bad, Bax and PARP) were overexpressed, resulting in the acceleration of follicular atresia and an increase in apoptosis and leading to the loss of ovarian physiological functions. In the mice, POF was induced by CTX, and the results were similar, including a decrease in the expression in the anti-apoptotic protein Bcl-2 and an increase in the expression of the pro-apoptotic protein Bax. These effects induced apoptosis in follicular granulocyte cells and increased follicular atresia [26,27].

Currently, hormone replacement therapy is widely used to treat POF even though it causes a variety of adverse reactions. However, these treatments have many side effects, and their long-term safety and efficacy should be improved. The development of new treatments aimed at restoring ovarian function and improving patient quality of life remains a problem for domestic and international researchers [28].

HPE is a low molecular peptide with biology activity that is extracted from the human placenta, which contains more than 8000 kinds of nutrients. The molecular weight of HPE is only 3000 daltons. HPE has few side effects and can be absorbed by the body via intramuscular or intravenous infusion. *In vitro* experiments showed that HPE accelerates osteoblast mineralization to promote fracture healing [29]. HPE has also been shown to interfere with colon cancer cell apoptosis, block cell cycle progression, and inhibit cell proliferation and migration, all of which may inhibit tumour cell proliferation [8].

In this study, a mouse model of POF was induced by CTX, and we show that this model simulated the ovarian dysfunction caused by CTX chemotherapy drugs in clinical trials. We analysed the pathology of mice ovaries, counted atresia follicles, evaluated ovarian weight, determined serum sex hormone levels, measured ovarian granulosa cell apoptosis, and explored the pathogenesis caused by CTX-induced POF. Moreover, we treated mice with CTX-induced POF with different doses (e.g., high, medium and low doses) of HPE to evaluate its curative effects and explore the molecular mechanisms underlying its activities to further improve POF.

The results showed that CTX led to a decrease in body weight and ovarian atrophy. In the treated mice, pathological examinations showed that the number of normal follicles was significantly lower, the number of atretic follicles was significantly higher, the rate of apoptosis in ovarian granulosa cells was higher, serum E2 and progesterone levels were lower, and FSH and LH levels were higher, resulting in a phenotype similar to that of typical POF. The proteins for P-Rictor, Bad, Bax, and PPAR were overexpressed, whereas the proteins for p-Akt and p-Foxo3a were lower. These proteins are associated with the Rictor/mTORC2/Akt/Foxo3a signalling axis, and these effects could result in excessive follicular apoptosis and may therefore represent one of the mechanisms underlying POF.

A comparison between the control group and the groups treated with high and medium doses of HPE for 14 days showed that treatment with HPE increased weight gain in both the ovary and the body, reduced the number of atretic follicles, increased the serum levels of the hormones E2 and P, reduced the serum levels of the hormones LH and FSH, and decreased apoptosis in granulosa cells. We found that HPE inhibited the protein expression of p-Rictor, Bad, Bax, and PPAR and activated the expression of p-Akt and p-Foxo3a, thereby protecting follicular granulosa cells from undergoing over-apoptosis, resulting in the prevention of follicular atresia and the alleviation of CTX-induced ovarian injury.

Conclusion

Our results provide convincing evidence that the mouse POF model can be alleviated by dose dependently HPE, and thus will be useful to relieve symptoms in POF. But this study has some limitations. First, although POF model was successfully alleviated, the number of studies mice was small. Second, HPE is the compound providing large range of effective role. More studies will be needed to confirm which ingredient effectively protect health. Third, only a few biological studies were performed. Furthermore, although relative markers of POF were evaluated in this study, necessary biological studies can be added according to the purpose and direction of future experiments.

Author Contributions

Conceived and designed the experiments: BZ, YL, MM. Performed the experiments: BZ, YL, MC. Analyzed the data: BZ, YL, MM, MC. Contributed reagents/materials/analysis tools: BZ, YL, MM, MC. Wrote the paper: BZ, YL.

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