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Metabolomics-based analysis of the effect of *Cucumaria frondosa* intestinal and ovum hydrolysates on cytoxan-induced premature ovarian failure in mice

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ABSTRACT: Premature ovarian failure (POF) poses a significant health threat to women of reproductive age. This study investigated the protective effects of *Cucumaria frondosa* intestinal and ovum hydrolysates (CFH) in a mouse model of POF, utilizing metabolomic analysis to reveal potential underlying mechanisms. In the present study, female ICR mice treated with 100 mg/kg of cytoxan (CTX) were employed to assess the therapeutic effects of CFH on ovarian dysfunction. The ameliorative effects of CFH on POF were evaluated based on the estrous cycle, ovarian reserve, serum sex hormone levels, as well as the antioxidant and anti-apoptotic effects. The results indicated that oral administration of CFH effectively regulated the disordered estrous cycle, increased the number of follicles, significantly elevated serum estradiol (E2) and anti-Müllerian hormone (AMH) levels, while decreasing testosterone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in POF mice. Additionally, CFH supplementation resulted in increased superoxide dismutase (SOD) activity and decreased malondialdehyde (MDA) levels. Furthermore, CFH supplementation reduced ovarian granulosa cell apoptosis compared to the POF group. Metabolomic studies ultimately identified metabolites such as tryptophan, citric acid, and arachidonic acid as potential targets of CFH′s action on POF. Collectively, these findings suggest that CFH can improve POF and provide mechanistic insights from metabolomics, supporting the application of *Cucumaria frondosa* intestinal and ovum in the field of functional foods.

Keywords: *Cucumaria frondosa* intestinal and ovum hydrolysates; premature ovarian failure; cytoxan; sex hormones; metabolomics

1. Introduction

The search for methods to transform undervalued marine by-products and underutilized species into valuable components of functional foods and nutritional supplements has long been a topic of significant interest, warranting in-depth exploration and discussion [1]. In traditional Asian medicine, sea cucumbers are regarded as a medicinal marine treasure, playing a crucial role in the development of marine food due to their high protein content and potential therapeutic benefits [2,3]. *Cucumaria frondosa*, commonly referred to as the orange-footed sea cucumber, is a marine invertebrate belonging to the phylum Echinodermata and is distributed throughout the North Atlantic Ocean. Protein hydrolysates derived from *Cucumaria frondosa* exhibit antioxidant, angiotensin-converting enzyme (ACE) inhibitory, immunomodulatory, anti-aging, and

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anti-inflammatory properties [4,5]. Between 2008 and 2017, imports of *Cucumaria frondosa* rose from 4,516 to 9,922 metric tons, with a total value of $18.3 million [6]. The intestinal and ovum of *Cucumaria frondosa* are by-products generated during processing. Despite their high protein content, these by-products are frequently discarded as biowaste due to the current lack of advanced development techniques [7]. Protein hydrolysates derived from these by-products can convert large molecules into easily absorbable small molecules through enzymatic hydrolysis. This process not only releases and enhances the functionality of active components but also improves their physicochemical properties and safety. However, there remains a scarcity of *in vivo* studies on the functional development of protein hydrolysates derived from the intestinal and ovum of *Cucumaria frondosa*, underscoring the substantial commercial potential in exploring the functional activities of these hydrolysates.

Premature ovarian failure (POF), more precisely referred to as premature ovarian insufficiency (POI), is defined as the onset of ovarian failure in women before the age of 40, with a reported prevalence of approximately 1% in recent years [8]. POF is typically characterized by low levels of estradiol (E2) and elevated levels of follicle stimulating hormone (FSH), resulting in the premature depletion of ovarian function prior to menopause and leading to ovarian insufficiency [9]. In addition to the prominent symptoms of irritability, night sweats, amenorrhea, and infertility, POF increases the risk of osteoporosis and cardiovascular disease, posing significant threats to women's physical and mental health [10]. Currently, hormone replacement therapy remains the primary treatment; however, the associated risks of increased breast and endometrial cancer must not be overlooked [11]. Recent research has focused on the regulation of sex hormones by bioactive peptides in the context of premature ovarian failure. For instance, Wang et al. reported that deer blood hydrolysate reduced serum levels of FSH and luteinizing hormone (LH) while elevating E2 levels, increasing the number of primordial follicles and decreasing the number of atretic follicles in POF mice [12]. Furthermore, peptides derived from the sea cucumber (*Acaudina leucoprocta*) positively impacted POF mice by enhancing sex hormone synthesis through the upregulation of *StAR*, *Fshr* and *Cyp19a1* expressions in the ovaries [13].

In this study, we investigated the mitigating effects of *Cucumaria frondosa* intestinal and ovum hydrolysates (CFH) on mice with POF. We analyzed body weight and organ indices, estrous cycles, histopathology, hormone levels, antioxidant capacity, and ovarian apoptosis. Additionally, we employed metabolomics to elucidate the potential mechanisms involved. This research promotes the high-value utilization of marine by-products and offers new insights into the development of natural functional foods aimed at alleviating POF.

2. Materials and methods

2.1 Reagents and experimental materials

Cucumaria frondosa intestinal and ovum were obtained from the Yantai Haizhongbao Seafood Trading Center (Yantai, China). Flavourzyme (15,000 U/g), a food-grade enzyme, was sourced from Solarbio Biotechnology Co. Ltd. (Beijing, China). Cytoxan (CTX) and soy isoflavones (SIF, purity 40%) were

acquired from Shanghai Bichen Biochemical Technology Co., LTD and Shanghai Macklin Biochemical Technology Co., LTD (Shanghai, China). Superoxide dismutase (SOD) and malondialdehyde (MDA) kits were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for testosterone (T), E2, FSH, LH, and anti-Müllerian hormone (AMH) were provided by Jiangsu Aikang Science & Technology Co., Ltd (Jiangsu, China). All other reagents and chemicals utilized in this study were of analytical grade and were procured from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2 Preparation of CFH

Cucumaria frondosa intestinal and ovum were freeze-dried, powdered, and subjected to enzymolysis. The enzymatic conditions were as follows: a temperature of 35℃, a duration of 9 h, a solid-liquid ratio of 1:15, an enzyme dosage of 10,000 U/g (flavourzyme), and a pH of 5.5. Following hydrolysis, the mixture was heated in a boiling water bath for 15 min to terminate the hydrolysis process. After cooling the mixture in ice water, it was centrifuged at 10,000 g for 15 min at 4℃. The resulting supernatants were then lyophilized for 48 h at -65℃ to obtain the final product, CFH.

2.3 Determination of amino acid composition

Modifications were implemented to ascertain the amino acid composition utilized for CFH, following the method of Xu et al [14]. CFH was hydrolyzed in 6 M HCl at 110℃ for 22 h. The amino acid composition of CFH was analyzed using a high-speed amino acid analyzer (Hitachi L-8900, Japan).

2.4 Scanning electron microscope (SEM)

The samples were removed from glutaraldehyde and subjected to dehydration using a gradient of 50%, 70%, 90%, and 100% ethanol for 10 min at each concentration. Subsequently, the samples were dried in a supercritical dryer at the critical point for 1 h. Following this, the samples were affixed to the sample stage using conductive tape, sprayed with gold, and examined with a SEM (Hitachi SU8020, Japan) at an accelerating voltage of 5 kV.

2.5 Animals and experimental design

Forty-five female ICR mice, aged 8-10 weeks and weighing (30 ± 2) g, were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. The mice were housed in a sterile environment and allowed to acclimatize for one week prior to experimentation. The animal study protocol was approved by the Ethical Committee of Yantai University (protocol code 20240301s11, approved on 1 March 2024).

Following the acclimatization period, the molding phase commenced. The forty-five mice were divided into five groups: control, POF, SIF, CFH low dose (CFH-L), and CFH high dose (CFH-H). Thirty-six mice from the four groups, excluding the control group, were injected intraperitoneally with 100 mg/kg of CTX to induce POF. Three days later, the SIF, CFH-L, and CFH-H groups were gavaged with 400 mg/kg of SIF, 200 mg/kg of CFH, and 600 mg/kg of CFH, respectively, for a duration of four weeks. The estrous cycle was monitored during the 2^{nd} and 4^{th} weeks.

2.6 Observation of estrous cycle

At weeks 2 and 4, saline was applied to 2-3 cm of the mouse vagina using a cotton swab, and secretions were collected by gently rotating the swab. Following application, the swab was stained with 0.4% methylene blue, and the morphology of the epithelial cells and the leukocytes was observed under a light microscope (Nikon Eclipse E100, Japan) to assess the state of the mice during estrus.

2.7 Organ index and area of the ovary

Mice were anesthetized via intraperitoneal injection of a 1% pentobarbital sodium solution. Blood was collected from the hearts of the anesthetized mice, centrifuged at 3000 r/min for 10 min to isolate the serum, and subsequently stored at -80℃ until analysis. Euthanasia was performed through cervical dislocation, after which the bilateral ovaries and uteri were harvested. Excess adipose tissue was carefully excised using forceps, weighed, and the area of the ovaries was quantified using ImageJ software. Subsequently, the samples were stored at -80℃ or in 4% paraformaldehyde for further experiments.

2.8 Determination of biochemical parameters

Ovarian tissue and serum samples were collected and analyzed for T, E2, FSH, LH, AMH, SOD, and MDA according to the instructions provided with the assay kits.

2.9 Histopathology

Uterine and ovarian tissues were immersed in 4% paraformaldehyde, subsequently dehydrated, and embedded in paraffin. Sections of 5μm thickness were prepared, stained with hematoxylin and eosin (H&E), and observed under a light microscope (Nikon Eclipse E100, Japan).

2.10 TUNEL

Paraffin-embedded ovarian sections were deparaffinized and treated with proteinase K solution for 20 min, followed by rinsing with phosphate-buffered saline (PBS). The sections were then incubated with 0.3% methanol peroxidase for 15 min to inactivate endogenous peroxidase. After allowing the sections to air-dry slightly, they were incubated at room temperature for 10 min with a buffer drop. Excess buffer was removed, and TUNEL reaction solution was added dropwise. The sections were covered with coverslips and incubated for 1 h. Following this, the sections were rinsed with PBS and treated with proteinase K solution for an additional 20 min, after which they were washed three times with PBS and counterstained with DAPI for 15 min before sealing. Apoptotic cells in the ovarian tissue were visualized using a fluorescence microscope (Leica, Wetzlar, Germany).

2.11 LC-MS based untargeted metabolomics analysis

The serum samples from the control, POF, and CFH-H groups were mixed with an extraction solution containing a deuterium internal standard (MeOH: ACN, 1:1 (v/v)), then vortexed and sonicated for 10 min at 4℃ in a water bath. Following this, the extract was centrifuged at 4℃ for 15 min at 12,000 r/min. The supernatant was transferred to a new glass vial for analysis. Quality control (QC) samples were prepared by combining equal volumes of the sample supernatant. On-line assays were conducted using a Waters

ACQUITY UPLC BEH Amide column for liquid chromatographic separation on an ultra-high performance liquid chromatograph (UHPLC, Thermo Vanquish Flex, Massachusetts, USA). The liquid chromatography phase A consisted of an aqueous phase containing 25 mmol/L ammonium acetate and 25 mmol/L ammonia, while phase B was acetonitrile. Data were collected using an Orbitrap Exploris 120 mass spectrometer with the following parameters: sheath gas flow rate of 50 Arb, auxiliary gas flow rate of 15 Arb, capillary temperature set at 320℃, full MS resolution of 60,000, MS/MS resolution of 15,000, collision energy at SNCE 20/30/40, and spray voltage of 3.8 kV (positive) or -3.4 kV (negative). Data processing was performed via the website https://www.genescloud.cn/login.

2.12 Statistical analysis

All experimental results were expressed as mean ± standard deviation (SD). The experimental data were analyzed using GraphPad Prism 8.0. One-way ANOVA followed by Duncan's multiple range post hoc analysis was employed to assess differences between groups. A significance level of $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). All data included in this study have been derived from a minimum of three independent experiments.

3. Results

3.1 Structural characterization of CFH

3.1.1 Amino acid composition of CFH

Amino acids play crucial roles in energy metabolism, tissue repair, neurotransmitter and hormone synthesis, and immune system regulation in living organisms [15-17]. The results from this study indicated that CFH had an essential amino acid to non-essential amino acid (EAA/NEAA) ratio of 0.93. This ratio serves as an important indicator of protein quality across various dietary protein sources [18], with CFH exhibiting a desirable ratio exceeding 0.6. Furthermore, acidic amino acids (glutamic acid and aspartic acid) and basic amino acids (lysine, arginine, and histidine) accounted for 7.70% and 7.61% of the total amino acids, respectively, indicating that CFH possessed the capability to effectively scavenge free radicals.

Table 1 The amino acid composition of CFH.

Amino Acid	Abbreviation	Ratio (g/100 g)
Glutamic acid #	Glu	4.74
Lysine *	Lys	4.15
Aspartic acid #	Asp	2.96
Leucine *	Leu	2.34
Arginine #	Arg	2.29
Valine *	Val	2.15
Alanine #	Ala	2.02
Glycine #	Gly	2.01
Isoleucine *	Ile	1.76
Threonine *	Thr	1.69
Serine #	Ser	1.57
Phenylalanine *	Phe	1.18
Histidine *	His	1.17
Methionine *	Met	1.16
Tyrosine #	Tyr	1.13

Acidic amino acids	7.70
Basic amino acids	7.61
Essential amino acids *（EAA）	15.60
Non-essential amino acids #（NEAA）	16.72
Total amino acids	32.32

Note: Amino acids marked with * indicate essential amino acids, while those marked with # represent non-essential amino acids.

3.1.2 Microstructure of CFH

The microstructure of CFH was illustrated in Fig. 1. At magnifications of 5 k and 10 k, the surface of CFH appeared rough and not finely machined, suggesting that its uneven surface might provide additional binding sites. Furthermore, at a magnification of 50 k, an irregular groove structure could be observed.

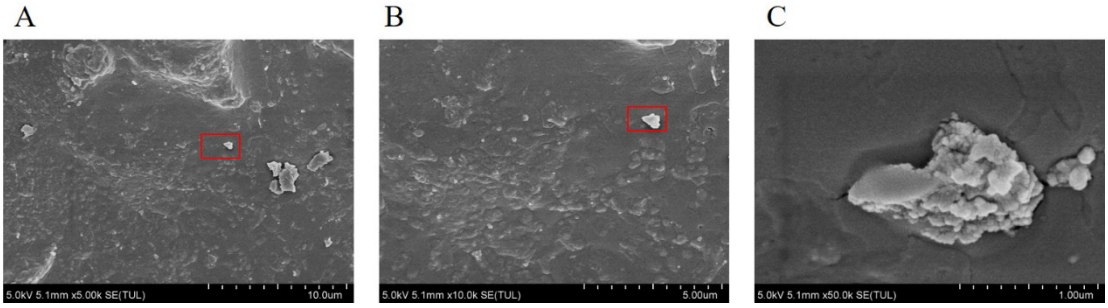

Fig. 1 SEM of CFH. (A) ×5 k; (B) ×10 k; (C) ×50 k.

3.2 The effect of CFH on basic condition in POF mice

3.2.1 Body weight changes and organ indices

In this study, there was no significant change in the body weight among the five groups of mice (Fig. 2B). The ovarian index was significantly decreased by CXT ($P < 0.01$); however, no significant differences were observed in the ovarian index among the commercially available SIF, CFH-L, and CFH-H groups when compared to the POF group (Fig. 2C). Additionally, there was no significant change in the uterine index across all five groups (Fig. 2D), a finding consistent with previous studies [19]. Furthermore, a significant reduction in ovarian area was observed in mice treated with CTX ($P < 0.0001$), which was restored by both SIF ($P < 0.001$) and CFH-L ($P < 0.01$). Although the area of CFH-H showed improvement, it was not statistically significant. These results provide preliminary evidence that CFH may ameliorate the ovarian changes induced by CHX.

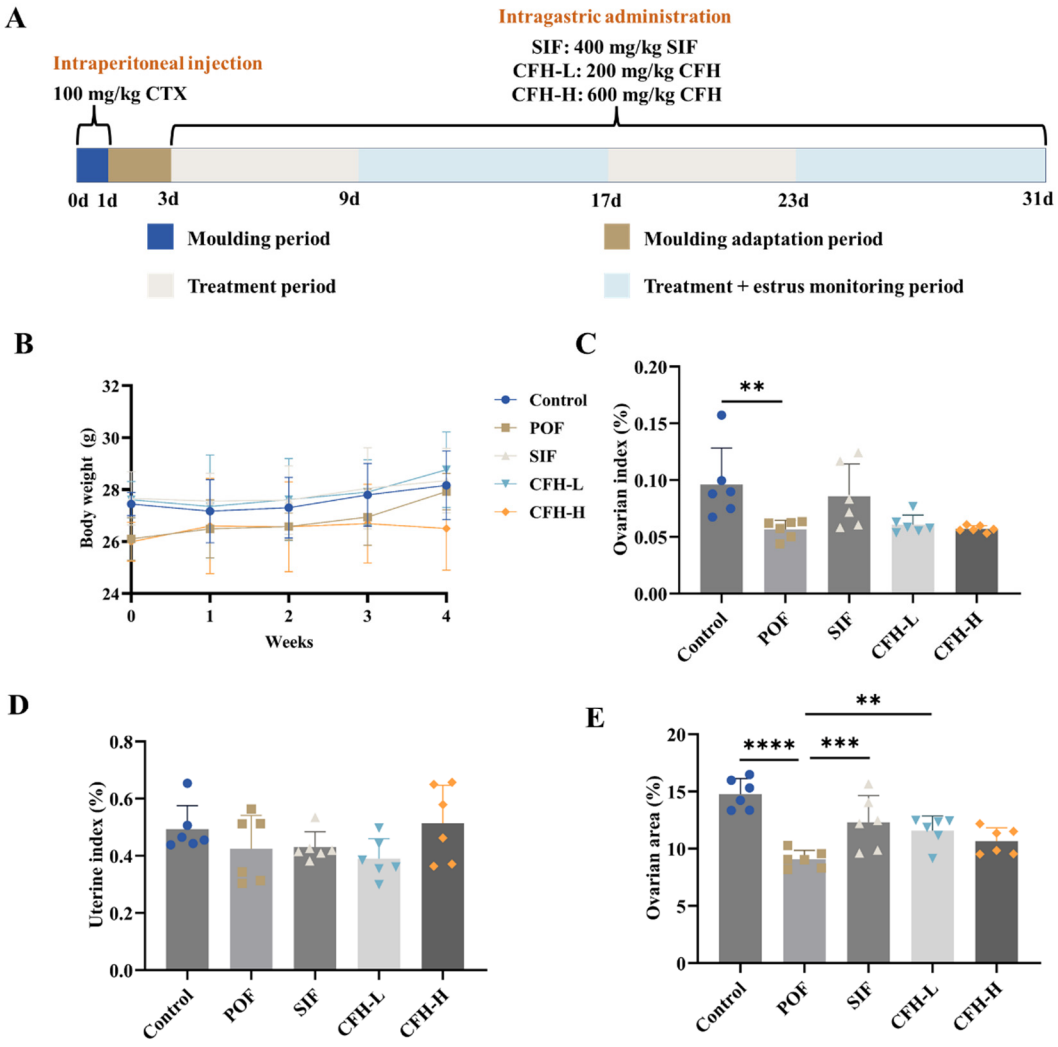

Fig. 2 Experimental design and the effects of CFH on the body weight and reproductive organs of POF mice. (A) Experimental design; (B) Body weight; (C) Ovarian index; (D) Uterine index; (E) Ovarian area. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

3.2.2 CFH maintained the normal estrous cycle

CTX has been identified as a model for inducing POF in mice, wherein the estrous cycle becomes disrupted, serving as a primary epistatic indicator of POF success [20]. Vaginal smears were utilized to observe epithelial cells and leukocytes in vaginal secretions, allowing for the classification of the estrous cycle into four distinct stages [21]: proestrus, characterized by oval-shaped epithelial cells containing nuclei; estrus, where epithelial cells exhibited irregular keratinization and nuclei were absent; metestrus, marked by the presence of leukocytes that phagocytosed keratinized epithelial cells; and diestrus, dominated by leukocytes (Fig. 3A). At week 2, mice treated with CTX exhibited a significant increase in the retention time of metestrus/diestrus stages; however, this effect was significantly reversed by the interventions of SIF ($P < 0.05$), CFH-L ($P < 0.01$), and CFH-H ($P < 0.05$) (Fig. 3B). Furthermore, the ability of the mice to complete a full estrous cycle was an important evaluative indicator, and the proportion of mice demonstrating a normal estrous cycle increased under the treatments with SIF, CFH-L, and CFH-H (Fig. 3C). In week 4, this effect not only persisted but also demonstrated an increased proportion of normal estrous mice receiving SIF and CFH-L

administration (Fig. 3D, E). Overall, both SIF and CFH exhibited positive restorative effects on the regulation of the estrous cycle in mice, while the effect of CFH-H was comparatively weaker than that of SIF and CFH-L.

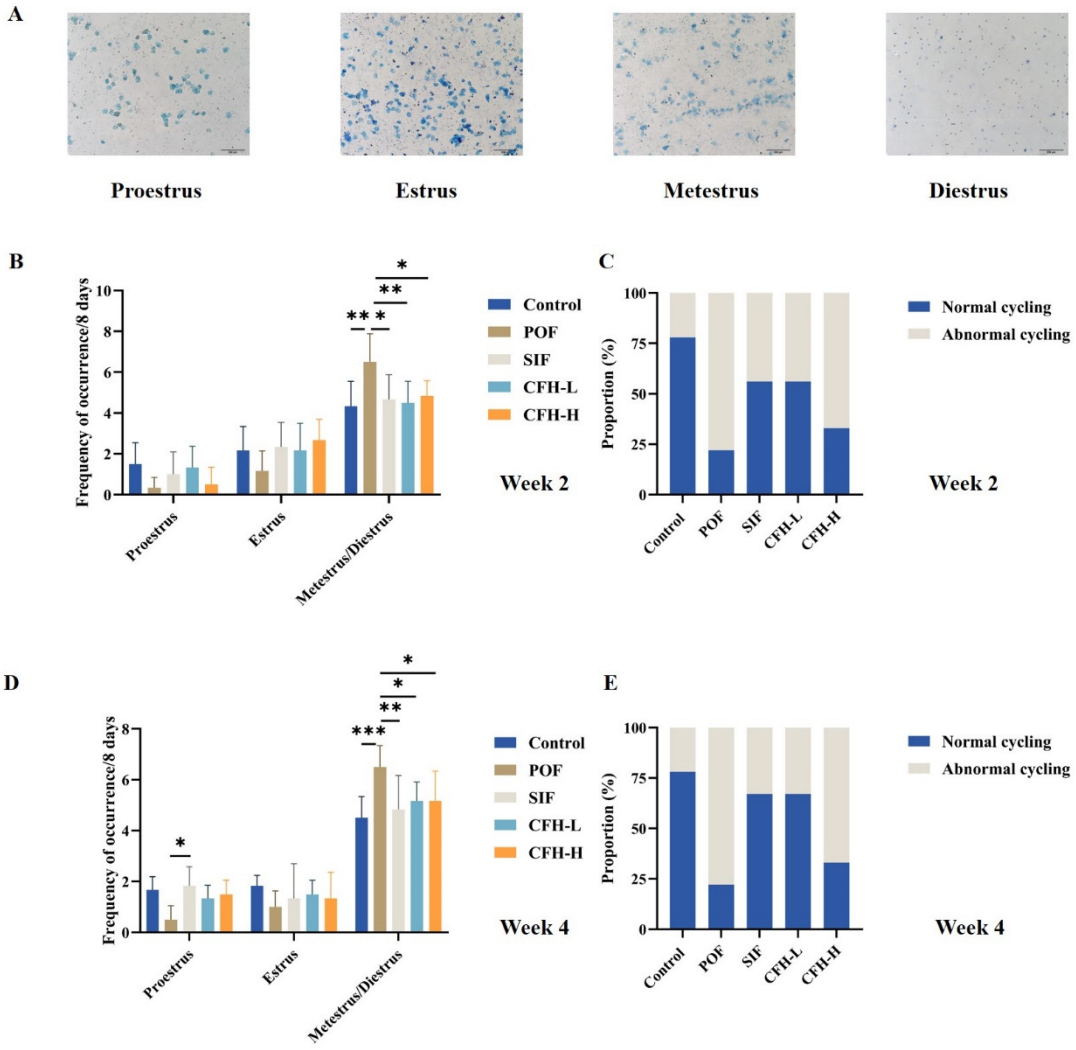

Fig. 3 The effect of CFH on the estrus cycle. (A) Stained smears representing the phases of the estrous cycle: proestrus, estrus, metestrus, and diestrus; (B) Estrus cycle in POF model mice at week 2; (C) Percentage of cycling mice exhibiting normal estrous cycles at week 2; (D) Estrus cycle in POF model mice at week 4; (E) Percentage of cycling mice exhibiting normal estrous cycles at week 4. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3.3 The effect of CFH on serum sex hormones

The symptoms associated with POF, including hot flashes, excessive sweating, nervousness, loss of libido, weakness, and dryness of the skin and mucous membranes, are primarily attributable to E2 deficiency. Previous studies have indicated that women with POF who exhibit estrogen deficiency may experience a substantial decrease in bone density [9]. In the current study, treatment with CTX resulted in a significant reduction in serum E2 levels in normal female rats ($P < 0.0001$, Fig. 4A). A similar pattern was observed for AMH ($P < 0.0001$, Fig. 4C). Notably, the decline in E2 levels was significantly ameliorated by the intervention of SIF ($P < 0.0001$) and CFH ($P < 0.0001$). With respect to AMH, SIF ($P < 0.001$) and CFH-L ($P < 0.001$) demonstrated superior efficacy compared to CFH-H ($P < 0.01$). These findings were consistent with previous research [19]. Low serum E2 levels, coupled with elevated pituitary gonadotropin FSH levels, are critical indicators for diagnosing POF in women who experience amenorrhea before the age of 40 [22]. The

present study corroborated these findings, revealing a significant increase in FSH levels in the POF group compared to the control group ($P < 0.001$), alongside a reduction in FSH levels caused by CTX in both the SIF group ($P < 0.01$) and the CFH-L group ($P < 0.01$). While CFH-H also exhibited some effect, it was not statistically significant (Fig. 4B). The immunomodulatory mechanism of the Kidney Replenishing and Blood Invigorating Formula also mitigates the elevation of FSH levels induced by CTX [23]. The number of follicles in women is established early in life, and premature follicular maturation can lead to diminished ovarian function [24]. During the gonadotropin-dependent phase of follicular growth, FSH plays a regulatory role, acting as a pro-growth factor in the sinus follicle, thereby promoting the differentiation of granulosa cells and the synthesis of E2 [25]. However, the ovotoxic effect of CTX disrupts the hormonal balance, resulting in decreased E2 levels and abnormally elevated FSH levels. AMH plays a crucial role in inhibiting FSH in differentiated cells, particularly through the inhibition of FSH-induced aromatase activity, with FSH significantly stimulating the expression of the aromatase gene (*CYP19*) [26]. In the present study, the inhibitory effect of AMH was clearly influenced by CTX. Reassuringly, the treatment with SIF and CFH effectively restored this hormonal balance. In the ovary, follicular membrane cells synthesize T under the stimulation of LH, which then enters the granulosa cells by diffusion [27,28]. Within the granulosa cells, T is converted to E2 by aromatase. However, following CTX-induced damage to the ovary, this feedback regulatory mechanism is disrupted, leading to a diminished capacity for the ovary to efficiently convert T to E2. Additionally, the impaired ovarian function, which results in elevated LH and FSH levels, fails to stimulate E2 secretion, further contributing to decreased E2 levels [29,30]. The results of the present study demonstrated a significant increase in both T and LH levels compared to the control group (Fig. 4D, E, $P < 0.0001$). Both SIF and CFH-L exhibited significant modulatory effects, successfully contributing to a significant decrease in LH and T levels ($P < 0.0001$). In addition, CFH-H also significantly reversed the effects of CTX on LH ($P < 0.0001$) and T ($P < 0.001$). In conclusion, the hormonal results indicated that SIF and CFH effectively maintained the regulation of ovarian hormone levels and protect ovarian health; however, the specific healing mechanism warrant further investigation.

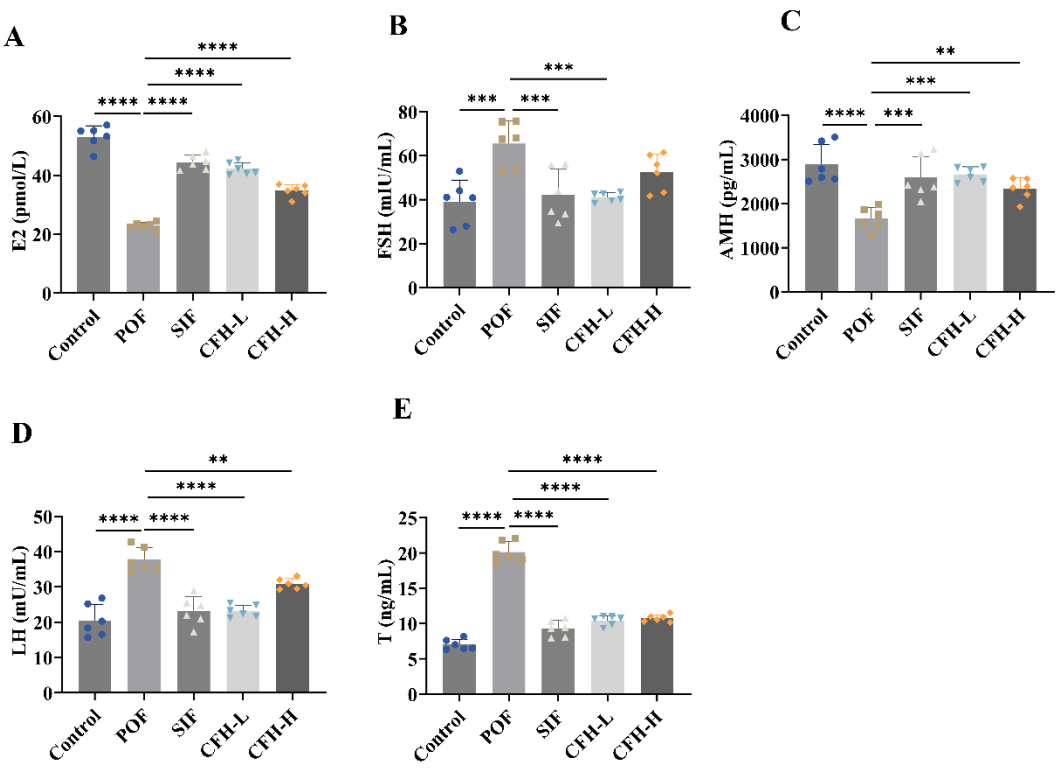

Fig. 4 The regulatory effect of CFH on serum sex hormone levels. (A) E2; (B) FSH; (C) AMH; (D) LH; (E) T. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

3.4 The effect of CFH on oxidative stress

CTX, along with its major metabolite acrolein, has been reported to cause excessive production of reactive oxygen species (ROS) in vivo, disrupting DNA structure and function in granulosa cells and inducing apoptosis in oocytes, ultimately leading to ovarian senescence and a diminished ovarian reserve [31,32]. This excessive ROS production is likely attributable to mitochondrial dysfunction. Research has indicated that mutations in mitochondrial transfer ribonucleic acid (mt-tRNA) genes or high variability in patients with POI result in impaired mitochondrial protein synthesis, significant reductions in ATP levels, and elevated ROS levels, as detected by PCR-Sanger sequencing. It is believed that these mt-tRNA mutations may contribute to mitochondrial dysfunction [33]. Wu et al. [34] demonstrated that dendrobium polysaccharides exert protective effects on mice suffering from natural aging-induced POF, effectively elevating SOD activity, reducing MDA levels, and attenuating oxidative damage while simultaneously enhancing antioxidant capacity in aging female mice. Furthermore, another study revealed that deer blood hydrolysate increased SOD levels and decreased MDA levels in POF-affected mice [12]. The present study yielded consistent results, demonstrating a significant decrease in SOD activity ($P < 0.01$) and an increase in MDA levels ($P < 0.01$) in serum due to CTX treatment. The oral administration of SIF, CFH-L, and CFH-H in POF mice exhibited notable antioxidant effects on both SOD activity and MDA levels (Fig. 5A, B).

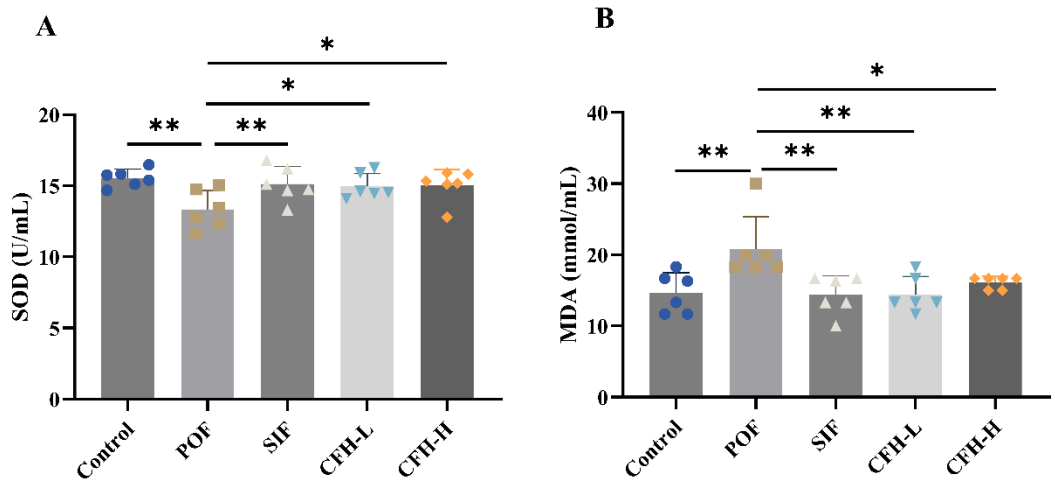

Fig. 5 The effect of CFH on oxidative stress. (A) SOD; (B) MDA. Statistical significance is indicated as follows: $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.5 The effect of CFH on ovarian performance in POF mice

3.5.1 The effect of CFH on ovarian morphology

As illustrated in Fig. 6, following treatment with CTX, the ovaries of the mice exhibited atrophy characterized by irregular follicular morphology. This was marked by a decrease in the number of growing follicles and an increase in atretic follicles. Notably, in the SIF, CFH-L, and CFH-H groups, there was an increase in follicles across all developmental stages, accompanied by a decrease in atretic follicles post-treatment. Furthermore, the CFH-H group displayed a slightly diminished effect compared to both the SIF and CFH-L groups.

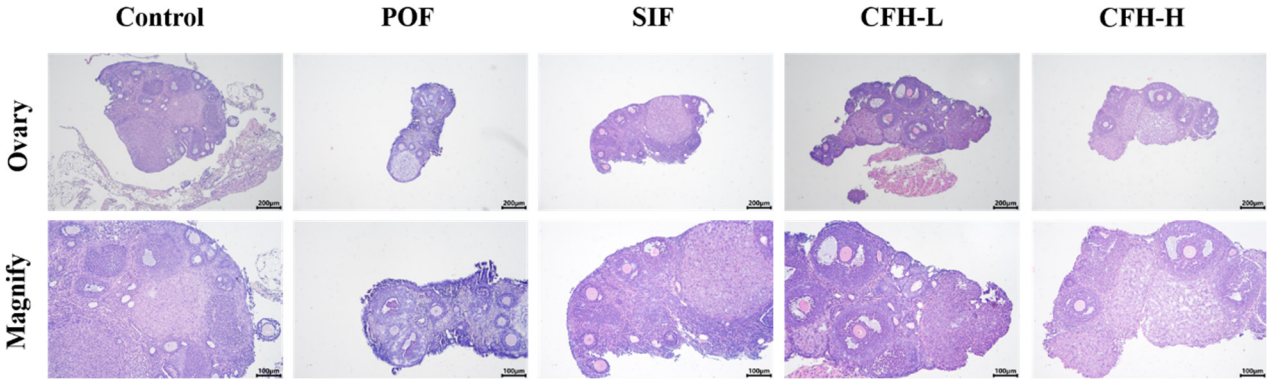

Fig. 6 Histological analysis of ovaries.

3.5.2 The effect of CFH on apoptosis of ovarian granulosa cells

Granulosa cells are the predominant somatic cell type within the ovary, playing a crucial role in the transition from primordial follicles to mature follicles. Apoptosis-induced ovarian atresia in these cells is a significant factor contributing to the decline of ovarian function [35]. Previous studies have demonstrated that treatment with oyster polypeptides reduced the apoptosis of the ovarian granulosa cells and down-regulated the mRNA expression levels of apoptosis-related genes [36]. Furthermore, Luo et al. [13] discovered that peptides derived from the sea cucumber (*Acaudina leucoprocta*) exert a regulatory effect on sex hormones in female mice with CTX-induced POF. TUNEL assay results indicated that sea cucumber peptides can inhibit

the apoptosis of ovarian granulosa cells. Consistent with these findings, the current study also observed a similar effect. Notably, the rate of granulosa cell apoptosis was significantly elevated in the POF group compared to the control group; however, following oral administration of CFH, there was a significant decrease in the apoptosis rate (Fig. 7A, B).

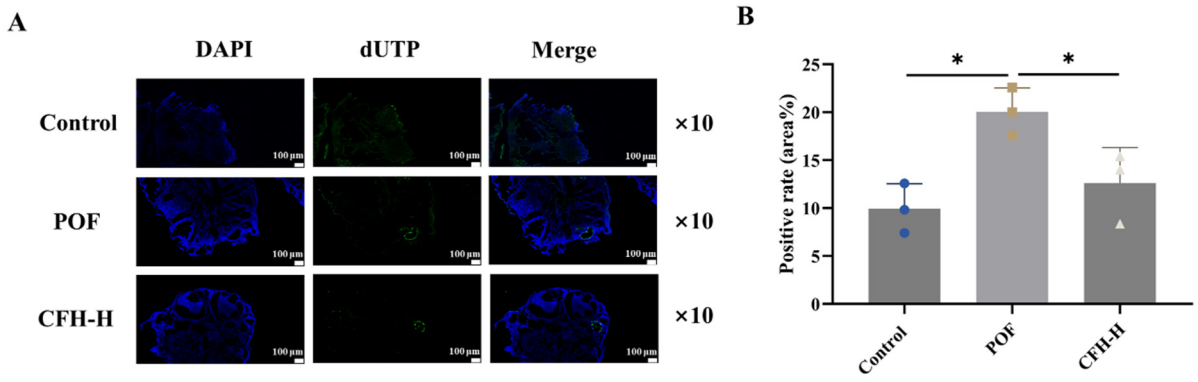

Fig. 7 Effect of CFH on apoptosis of ovarian granulosa cells. (A) TUNEL staining to detect ovarian apoptosis (scale bar: 100 μm); (B) Apoptotic optical density of ovarian granulosa cells (n = 3). Statistical significance is indicated as follows: $^{*}P < 0.05$.

3.6 The effect of CFH on serum metabolic profiles in POF mice

The underlying mechanism of CFH was investigated using untargeted metabolomics. Results from the partial least squares discriminant analysis (PLS-DA) model indicated distinct clustering among the control, POF, and CFH groups in the positive ion mode, indicating significant differences in the serum metabolomes of the three groups. Furthermore, the permutation test results revealed that all red dots were positioned below the right-most red dots, confirming the accuracy and reliability of the model′s results (Fig. 8A). Similar findings were observed under the negative ion model (Fig. 8B). Subsequently, univariate statistical analysis was employed to identify differential metabolites between the control and the POF groups, as well as between the POF and CFH groups (Fig. 8C). The VIP value data obtained from the partial least squares discriminant analysis model were utilized, with screening criteria set for significantly different metabolites: $P < 0.05$, and VIP > 1. The data in Fig. 8D indicated that the serum of normal female mice exhibited a significant up-regulation of 16 metabolites influenced by CTX, while 17 metabolites were significantly down-regulated. Additionally, in the serum of POF mice following CFH intervention, 32 metabolites were significantly up-regulated and 10 metabolites were significantly down-regulated.

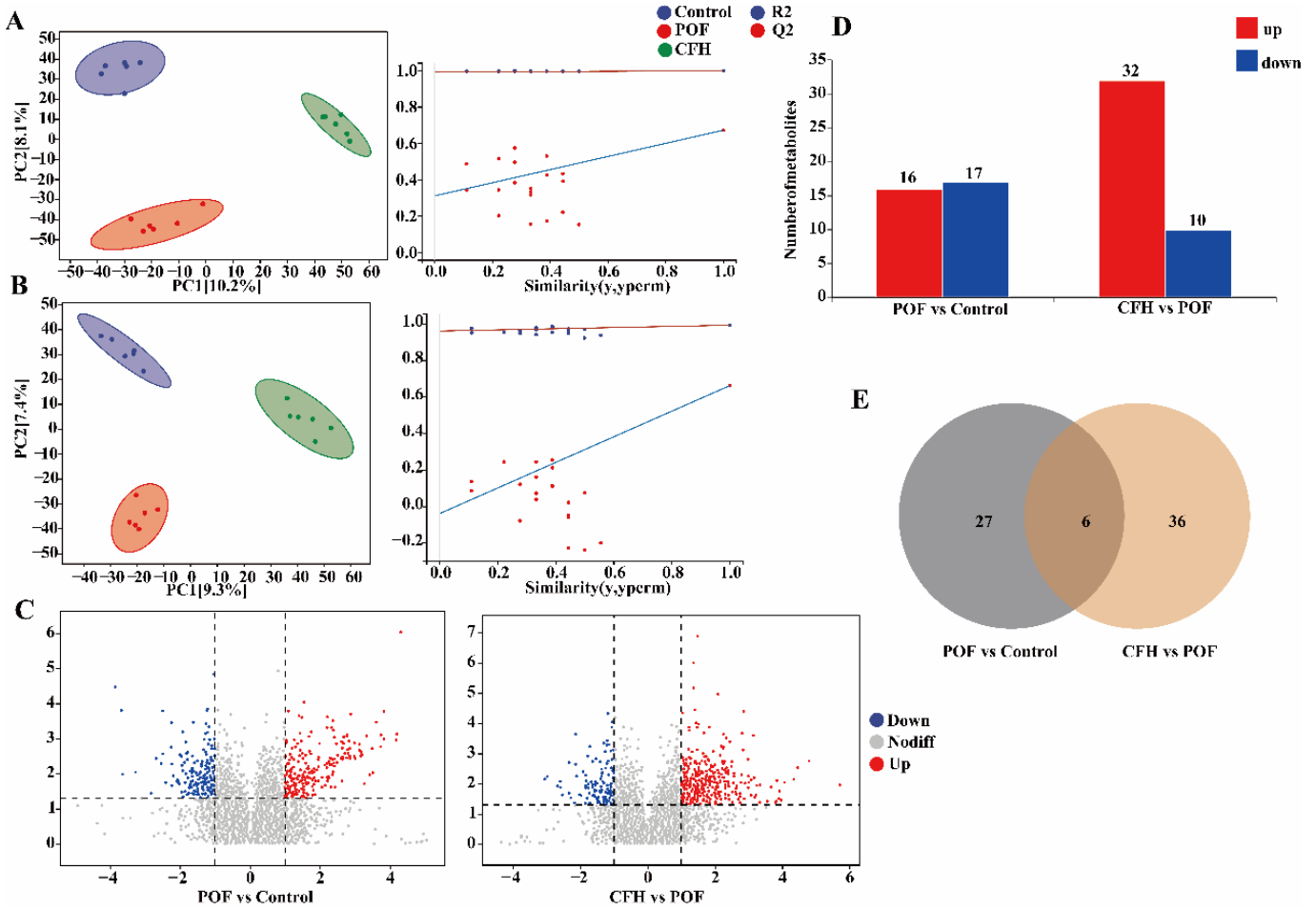

Fig. 8 Effect of CFH on serum metabolic profiles in POF mice. (A) Positive Ion Modelling PLS-DA and Displacement Test; (B) Negative Ion Modelling PLS-DA and Displacement Test; (C) Univariate statistical analysis (POF vs Control and CFH vs POF); (D) Bar chart depicting differential metabolite screening; (E) Venn diagram (POF vs Control and CFH vs POF).

Based on the results of the differential substance screening, we quantified the number of shared and unique differential substances among the comparison groups. The findings were illustrated using a Venn diagram (Fig. 8E), revealing a total of six identified metabolites: citric acid, phenylacetylglycine, tryptophan, N2-Malonyl-D-tryptophan, arachidic acid, and protoporphyrinogen IX. Notably, in comparison to the control group, the POF group exhibited significant decreases in citric acid, phenylacetylglycine, tryptophan, N2-Malonyl-D-tryptophan, and arachidic acid, while protoporphyrinogen IX was significantly elevated. Furthermore, CFH supplementation significantly reversed the alterations in these metabolites, with the exception of phenylacetylglycine, which increased following CFH administration (Fig. 9A-F). This study further employed metabolic pathway enrichment analyses to determine the metabolite changes in POF mice after CFH supplementation. The results indicated that treatment of CTX led to significant alterations in taste transduction, serotonergic synapse, bile secretion, synaptic vesicle cycle, lysine degradation, and the cAMP signaling pathway in female mice. In the CFH group, significant changes were observed in taste transduction, D-Amino acid metabolism, central carbon metabolism in cancer, biosynthesis of amino acids, ABC transporters, as well as alanine, aspartate, and glutathione metabolism (Fig. 9G, H).

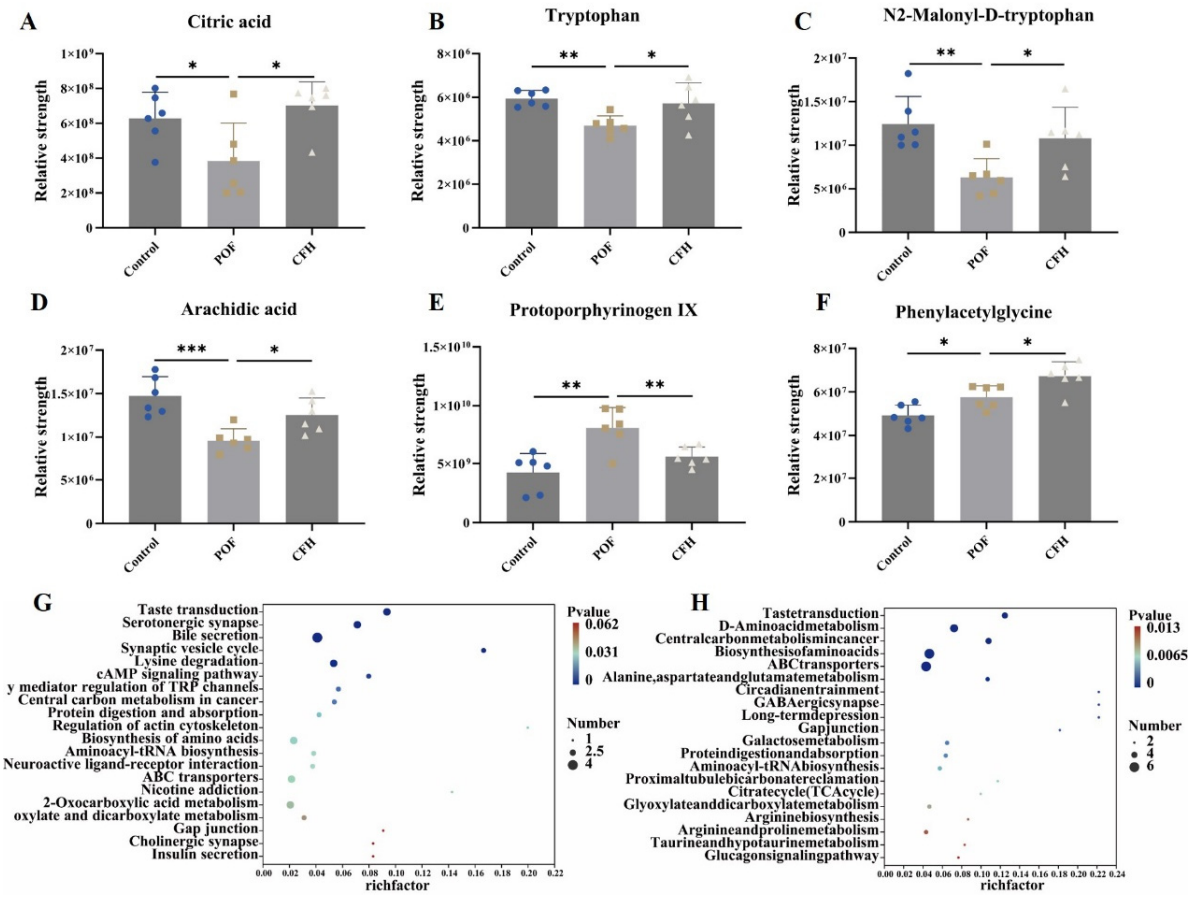

Fig. 9 Effect of CFH on serum metabolites and metabolic pathways in POF mice. (A-F) Common differential metabolites; (G-H) Enriched metabolic pathways (POF vs Control and CFH vs POF) (sort by *p*-value). Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

The metabolite N2-Malonyl-D-tryptophan, identified in mouse serum as a derivative of tryptophan, may account for the observed trends in results associated with tryptophan. However, the enrichment analysis did not reveal a specific metabolic pathway for its presence. To further investigate this hypothesis, we conducted a conjoint analysis, which demonstrated a positive correlation between the metabolite N2-Malonyl-D-tryptophan and tryptophan. Additionally, D-fructose revealed a positive correlation with phenylacetylglycine, arachidonic acid, and citric acid, while exhibiting a negative correlation with protoporphyrinogen IX (Fig. 10). Detailed information regarding the metabolites is provided in Table 2.

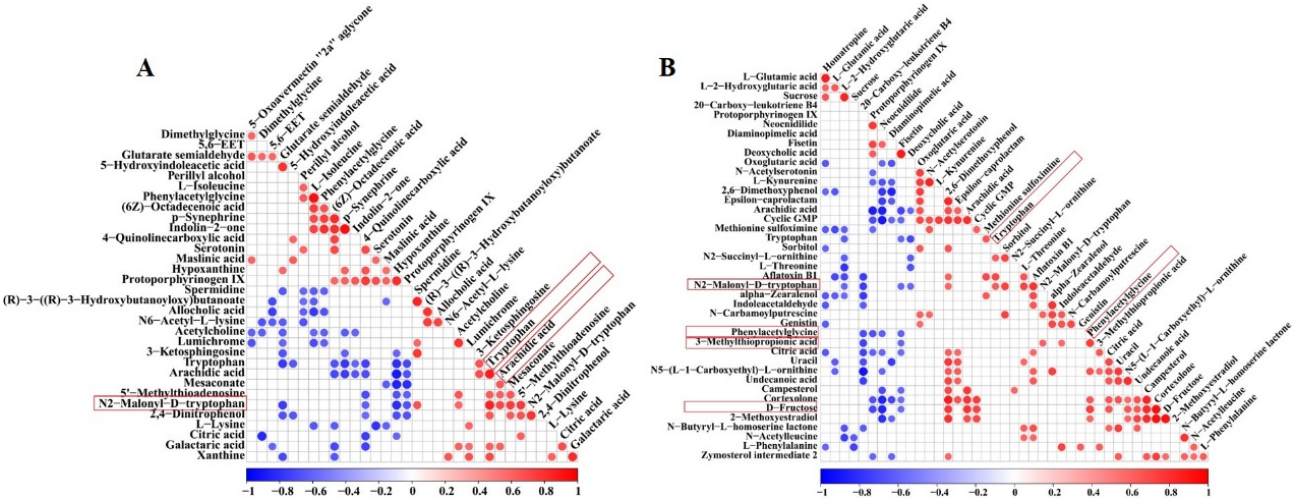

Fig. 10 Pearson correlation analysis of differential substance. (A) POF vs Control; (B) CFH vs POF.

Table 2 Detailed information regarding the identified metabolites.

Names	Experimental m/z	Formula	KEGG ID	HMDB ID	*p*-value (CFH vs POF)
Citric acid	191.0191	C6H8O7	C00158	0000094	0.0125
Phenylacetylglycine	192.0666	C10H11NO3	C05598	0000821	0.0202
Tryptophan	203.0825	C11H12N2O2	C00806	0013209	0.0386
N2-Malonyl-D-tryptophan	271.0707	C14H14N2O5	C03414	-	0.0247
Arachidic acid	311.2951	C20H40O2	C06425	0002212	0.0135
Protoporphyrinogen IX	568.3334	C34H40N4O4	C01079	0001097	0.0097
D-Fructose	180.0966	C6H12O6	C00095	0000660	0.0127

4. Discussion

This research provides a novel perspective on realizing the by-products value of *Cucumaria frondosa* intestinal and ovum, grounded in the therapeutic efficacy of CFH in mice with POF. The CTX-induced POF mouse model is widely employed to investigate ovarian health [37]. In this study, we utilized a 100 mg/kg CTX-induced POF mouse model to assess the effects of enzymatically prepared CFH at doses of 200 mg/kg and 600 mg/kg on the estrous cycle, hormone levels, antioxidant capacity, and the inhibition of granulosa cell apoptosis in POF mice. All these efforts yielded the anticipated positive results. Additionally, we sought to elucidate the underlying mechanism of CFH through serum untargeted metabolomics.

Oxidative stress, identified as a pivotal factor for POF, leads to elevated levels of ROS in the body, which subsequently inflicts damage on DNA, proteins, and lipids within cells. This results in the deterioration of the ovarian microenvironment, ultimately promoting abnormal apoptosis of granulosa cells and a decline in oocyte quality. Arginine, a conditionally essential amino acid, serves as a vital substrate for protein synthesis and plays a significant role in immune function and antioxidant defense within the body. Recent reports indicate that Arginine can enhance ovarian antioxidant capacity during the luteal phase in ewes via the Nrf2/Keap1 signaling pathway [38]. Furthermore, glutathione (GSH), an important endogenous antioxidant, is synthesized from cysteine, glycine, and glutamic acid [39]. In an in vitro fertilization environment, supplementation with these amino acids promotes GSH synthesis, enhances antioxidant defense during oocyte maturation and early embryo development, and mitigates ROS accumulation [40]. CFH, which is rich in various amino acids, can serve as an effective supplement to alleviate oxidative stress damage in mouse ovaries.

Metabolites, as downstream products of molecular regulation, can directly illuminate the functions of upstream genes and proteins; their dysregulation may contribute to POF [41]. Citric acid is synthesized in the body through the combination of acetyl coenzyme A and oxaloacetate, representing the initial step in the citric acid cycle, also known as the Krebs cycle or tricarboxylic acid cycle [42]. Cellular energy metabolism is intrinsically linked to the citric acid cycle, as organisms require energy for growth and development. A deficiency in energy is likely to result in the presence of small and medium-sized ovaries [43]. Furthermore, a study investigating the role of citric acid, a metabolite associated with ovarian cancer, highlighted its ability to promote pyroptosis in ovarian cancer via the CASP4/TXNIP-NLRP3-GSDMD pathway, noting that citric

acid exhibited the most significant decline among metabolites in ovarian cancer [44]. The enrichment results indicate not only a substantial increase in citric acid levels with CFH but also a notable enrichment of the tricarboxylic acid cycle.

Reduced serum levels of tryptophan metabolites, similar to those observed in patients with ovarian cancer, are attributed to enhanced tryptophan degradation [45]. Some studies have indicated that tryptophan metabolite levels in follicular fluid are significantly decreased in female patients exhibiting reduced ovarian reserve. These studies postulate that tryptophan and its metabolites exert a protective effect on the ovary, suggesting that reductions in tryptophan and its degraded metabolites may impair follicular mass, oocyte maturation, and granulosa cell function in women with diminished ovarian reserve [46]. Notably, the decline of tryptophan in POF mice in this study was effectively reversed following the administration of CFH. Furthermore, this investigation into decreased ovarian reserve in women necessitates animal studies to further validate whether the tryptophan metabolites IPA and IAA can improve ovarian reserve function, with our animal results providing supportive data for this inquiry. Additionally, given that antioxidant properties are among the most significant biological attributes of tryptophan and its indole metabolites [47], it is highly plausible that the enhanced antioxidant capacity observed in POF mice after CFH intake resulted from the restoration of tryptophan levels.

Arachidic acid is catalyzed by lipoxygenase (LOX) to produce hydroxyeicosatetraenoic acids (HETEs), which are involved in inhibiting apoptosis, stimulating angiogenesis, enhancing cell proliferation, and promoting tumor metastasis [48]. Research has indicated that arachidonic acid can stimulate cholesterol biosynthesis and increase E2 levels by activating *CYP17α1* and *HSD3β1* [49,50]. Protoporphyrinogen IX, which is negatively correlated with arachidic acid, serves as a precursor to hemoglobin, an essential component of red blood cells. Elevated hemoglobin levels can increase the risk of blood clots, as increased blood viscosity may lead to clot formation, resulting in severe complications such as stroke or heart attack [51]. In the present study, CTX was found to significantly upregulate serotonin levels in normal mice. The metabolic pathways that were notably regulated in the POF group, as indicated by the enriched results, include taste transduction, serotonergic synapse, bile secretion, synaptic vesicle cycle, and the cAMP signaling pathway. These changes are primarily attributed to either the upregulation of serotonin, the downregulation of acetylcholine, or a combination of both. High levels of serotonin are a significant factor in metabolic syndrome and are associated with increased cardiovascular mortality. Regarding the increase in phenylacetylglycine in CFH, this can be inferred from the joint analysis results, which demonstrate a strong positive correlation between phenylacetylglycine and D-fructose. Notably, it has been shown that phenylacetylglycine increases in response to fructose-induced alterations in urinary metabolic profiles in mice [52]. The findings of this study suggest that the increase in D-fructose can be attributable to CFH administration. Additionally, D-fructose exhibited correlations with citric acid, arachidic acid, and protoporphyrinogen IX. Therefore, based on the existing literature, the relationships of the metabolic pathways both upstream and downstream, along with the correlation results of the three metabolites, support a

preliminary hypothesis regarding the metabolic mechanism underlying the efficacy of CFH in treating POF (Fig. 11).

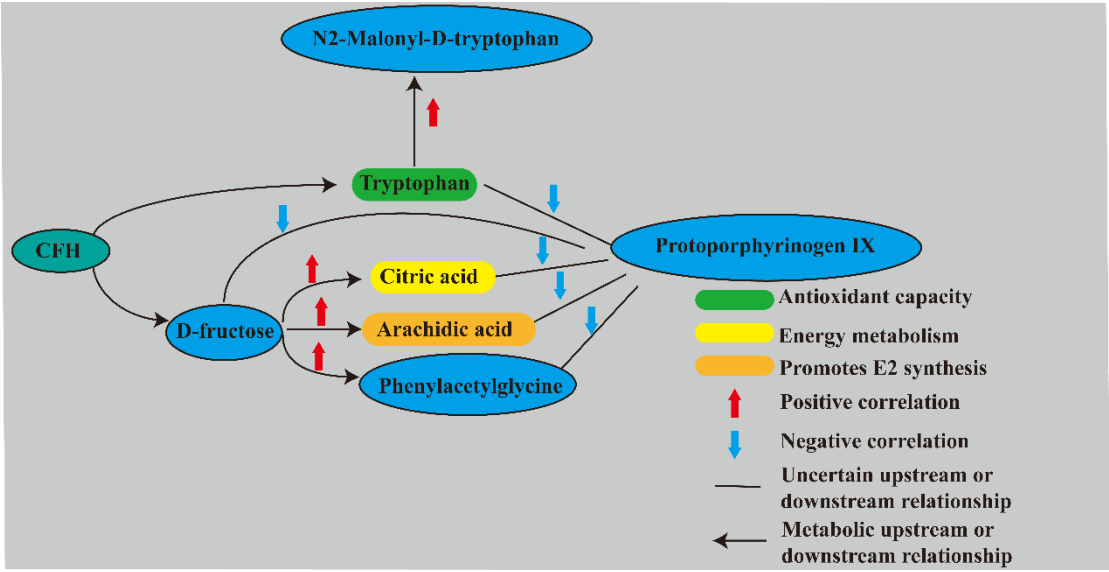

Fig. 11 The mechanism of CFH for POF based on metabolomics results.

5. Conclusion

In conclusion, CFH supplementation effectively modulated the hormonal dysregulation induced by CTX, resulting in an increased number of follicles and enhanced ovarian antioxidant and anti-apoptotic capacities. Based on the results of serum untargeted metabolomics, the effects of CFH on POF mice can be attributed to the enriched regulation of amino acid metabolism, energy metabolism, and lipid metabolism, including metabolites such as tryptophan, citric acid, and arachidonic acid. The therapeutic effects of CFH on POF contribute to the development of functional foods derived from the intestinal and ovum of *Cucumaria frondosa*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could potentially bias the work reported in this paper.

Data availability

The data presented in this study are available on request from the corresponding author.

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