Am J Transl Res 2025;17(7):4879-4893
www.ajtr.org /ISSN:1943-8141/AJTR0161476

Original Article

Huatan Tongmai Yin regulates RFRP-3 to intervene in the "microbe-gut-HPO axis" to treat PCOS-IR rats

Hanmei Lin*, Hua Guo*, Kena Lu, Sheng Ban

*Department of Gynaecology, The First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning 530022, Guangxi, China. *Equal contributors.*

Received October 25, 2024; Accepted May 13, 2025; Epub July 15, 2025; Published July 30, 2025

Abstract: Objective: To explore the therapeutic effect of Huatan Tongmai Decoction on insulin-resistant polycystic ovary syndrome (PCOS-IR) and the potential mechanism. Methods: The PCOS-IR rat model was constructed and HE staining was used to observe the morphological changes of vaginal shedding cells and ovary. Serum sex hormone levels, fasting insulin (FINS), fasting blood glucose (FBG) and inflammatory factors were detected by enzyme-linked immunosorbent assay (ELISA). Intestinal flora was analyzed by 16SrDNA sequencing. The mRNA expression of RFRP-3 and GPR147 in hypothalamus tissues was detected by RT-qPCR. The expression of ZO-1 and Occludin in colon tissue and the expression of GPR54, MyD88, TLR4, GnRHR and KP in the hypothalamus were detected by Western blot. Results: Compared with the model group, the body weight of the Huatan Tongmai Yin group decreased, the estrus cycle returned to normal, the levels of serum T, LH, FINS, HOMA-IR and inflammatory factors decreased, the levels of FSH increased, the types of intestinal flora OTU were more abundant, ACE and Chao index increased, and the mRNA level of hypothalamus RFRP-3 was up-regulated. In addition, serum T, LH, FPG, FINS and HOMA-IR levels were decreased, FSH levels were increased, inflammatory factors were down-regulated, and hypothalamic GPR54, MyD88, TLR4 and GnRHR proteins were reduced in rats after overexpression of RFRP-3. Conclusion: Tantongmai Decoction can correct the imbalance of intestinal flora, improve intestinal mucosal barrier function, relieve chronic inflammation and insulin resistance, thus up-regulating RFRP-3 in the hypothalamus, inhibiting the release of GnRH and KP, reducing the secretion of LH and T, and effectively alleviating PCOS-IR by regulating "microbe-gut-HPO axis".

Keywords: Huatan Tongmai Yin, RFRP-3, microbiota-gut-HPO, PCOS-IR, mechanism

Introduction

Polycystic ovary syndrome (PCOS) is characterized as hyperandrogenism and insulin resistance (IR), obesity, infertility, and dyslipidemia, with an age-standardized point prevalence and annual incidence of 1677.8 and 59.8 cases per 100,000 people, respectively [1]. Currently, Western medicine treatments for PCOS-IR mainly include sex hormone-regulating drugs and insulin sensitizers [2]. However, these drugs often produce several adverse reactions, and the Hypothalamic-pituitary-ovary (HPO) axis function cannot be fundamentally restored. Therefore, it is of great clinical significance to explore new therapeutic strategies for PCOS-IR, especially based on the overall regulatory role of traditional Chinese medicine.

The HPO axis is crucial in PCOS-IR pathogenesis. Gonadotropin-releasing hormone (GnRH) promotes the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), regulates sex hormone and affects ovarian function [3]. In addition, RFRP-3 is a key physiological regulator in mammals with a homologous relationship with gonadotropin-inhibiting hormone (GnIH). RFRP-3 can regulate the activity of GnRH neurons and affect the dynamic balance of the HPO axis. Cheng et al. showed that RFRP-3 directly inhibited kisspeptin and regulated the HPO axis [4]. Enhancing the function of GnIH/RFRP-3 neurons may inhibit GnRH and GnRH receptors synthesis, thereby interfering with the release of pituitary LH and improving the sex hormone levels of PCOS-IR patients.

https://doi.org/10.62347/XGFS9323

Alterations in gut microbiota composition are implicated in the pathogenesis of PCOS-associated insulin resistance (PCOS-IR). Intestinal microbiota disruption contributes to ovarian hyperandrogenism and follicular developmental abnormalities through the induction of chronic low-grade inflammation and insulin resistance [5]. The intestinal flora involves in the brain-gut axis of PCOS-IR because brain-gut axis is an important bidirectional information conversion pathway which is mainly composed of the brain, intestines and intestinal microorganisms, including central, autonomic nervous and intestinal microbial pathways [6]. The intestinal flora and metabolites play a vital role in regulating inflammatory pathways, activating brain-gut peptide secretion, regulating intestinal mucosal permeability and promoting pancreatic islet cell proliferation [7]. Intestinal flora imbalance can lead to abnormal hormone levels related to the brain-gut axis, insulin resistance and compensatory hyperinsulinemia, thereby causing PCOS. Qi et al. [8] reported that, compared with other intestinal microbial levels, the level of Lactobacillus acidophilus in PCOS patients was significantly increased, while the levels of glycodeoxycholic acid and taurodeoxycholic acid were decreased. By increasing IL-22 levels, the patients' conditions were alleviated through improving intestinal microbiome composition and bile acid metabolism. Shen et al. [9] showed that berberine can improve the condition of PCOS-IR by regulating intestinal microorganisms and metabolites. However, at present, there are no studies investigating whether RFRP-3 mediates the "microbe-gut-HPO axis" and how it is involved in the occurrence and development of PCOS-IR, which provides a new direction for exploring the pathogenesis and therapeutic targets of PCOS.

Studies have indicated that traditional Chinese medicine may present unique advantages in the treatment of PCOS-IR [10]. Some studies have suggested that natural plant extracts may normalize hormones by reducing inflammation, regulating the HPO axis, regulating autophagy and other ways [11], so as to achieve the purpose of treating PCOS. Among them, Huatan Tongmai Drink is a compound preparation developed based on this theory. It is composed of pinellia [12], Tangerine peel, poria cocos, Salvia miltiorrhiza [13], red peony root, Xiangfu and other drugs. It has the effects of eliminating phlegm and dampness, promoting blood circulation, regulating menstrual flow and assisting pregnancy. However, the specific mechanism of its action, especially whether it plays a role by regulating the "microbe-intestine-HPO axis" mediated by RFRP-3, has not been studied. Therefore, this study systematically evaluated the therapeutic effect and mechanism of Huatan Tongmai Yin by constructing a PCOS-IR rat model, aiming to deepen the understanding of the pathogenesis of PCOS-IR, and provide a new experimental basis for the regulation of neuroendocrine and metabolic network by traditional Chinese medicine through the "microbe-gut-HPO axis".

Materials and methods

Establishment of the animal model

Ninety-eight SPF-grade SD female rats aged 6 weeks, were selected. Rats were purchased from Hunan Lake Jingda Experimental Animal Co., Ltd., with the experimental animal production license number: SCXK (Xiang) 2019-0004 and the quality certificate number: NO. 430727221102805413. Body weight was between 180-200 g. After 1 week of adaptation feeding, 23 rats were selected as the control group, and the remaining were constructed as the model group. Modeled rats were fed with a high-fat diet, including 61.5% ordinary feed, 12% lard, 5% sucrose, 5% peanuts, 5% milk powder, 10% eggs, 0.5% salt, and 1% sesame oil. One mg/(kg·d) letrozole was dissolved in 0.5% (mass fraction) sodium carboxymethyl cellulose solution to prepare a letrozole suspension for daily gavage. At the same time, control rats were fed with ordinary feed and received 0.5% (mass fraction) sodium carboxymethyl cellulose solution (CMC-Na) via gavage at a ratio of 1 ml/100 g body weight per day. Ten rats in each of the control group and the model group were randomly selected to verify whether the membrane was successfully formed. This experimental plan was approved by the Experimental Animal Welfare Ethics Committee of Guangxi University of Chinese Medicine, with the approval number: DW20220501-220.

Grouping treatment

Thirteen modeled rats were randomly given Huatan Tongmai Yin. The decoction consisted

of 15 g of Pinellia, 6 g of Tangerine Peel, 10 g of Angelica, 20 g of Millettia, 10 g of Peach Kernel, 15 g of Salvia Miltiorrhiza, 15 g of Atractylodes, 20 g of Poria, 15 g of Zedoaria, 10 g of Chuanxiong, 10 g of Corrugated Seed, 15 g of Cyperus, and 15 g of Atractylodes (the same batch of drugs was purchased from the First Affiliated Hospital of Guangxi University of Chinese Medicine). The medicinal materials were processed by extraction technology and the concentration was adjusted to 4.8 g/mL. Rats were gavaged with Huatan Tongmai Yin 24 g/(kg·d) for 21 consecutive days, stopped for 1 day, and then gavaged for another 21 days. Another 13 rats were randomly selected and gavaged with metformin 0.16 g/(kg·d) for 21 consecutive days, stopped for 1 day, and gavaged for 21 days. The remaining 26 modeled rats were randomly assigned into RFRP-3 overexpression group (n = 13) and virus empty group (n = 13). The RFRP-3 overexpression lentivirus group was injected with 5 μL of RFRP-3 lentivirus into the lateral ventricle, and the virus empty group was injected with 5 μL of empty RFRP-3 virus vector. Lentivirus overexpression vector and empty virus vector were constructed by Hanheng Biotechnology (Shanghai) Co., Ltd.

Enzyme-linked immunosorbent assay

Under anesthesia, 4-5 mL of blood was drawn from the abdominal aorta and centrifuged at 3,000 rpm for 15 minutes after standing for 1 hour. The upper serum was collected and stored at -80°C. The levels of FSH, LH, T, GnRH, FINS, FBG, CRP, IL-6, TNF-α, IL-1β, NF-κB, TLR4, and LPS in serum were measured by ELISA and HOMA-IR was calculated as HOMA-IR = FBG (mmol/L) × FINS (mIU/L)/22.5.

Detection of the diversity of intestinal flora in rats

The rectal area of rats was gently pressed to stimulate defecation, and feces were collected with sterile forceps, stored in sterile EP tubes, and then labeled. The samples were stored at -80°C. The intestinal 16S rDNA sequencing analysis was completed by Nanning Current Science Co., Ltd. The main process is as follows: CTAB extracted genomic DNA of the intestinal contents of rats in each group; agarose gel electrophoresis detected DNA concentration and purity, and the DNA template was diluted and amplified using the Barcode-specific primers with the V3-V4 region; the target band was purified using AxyPrep DNA Gel Extraction Kit nucleic acid purification kit; and the recovered product was detected and quantified using the Quantus™ Fluorometer (Promega, USA). After quality control and splicing of the original sequencing data, OTUs were clustered, and the number, distribution and species annotation of OUTs were further counted. The sample complexity (Alpha Diversity) and diversity (Beta Diversity), KEGG functional annotation and statistics were analyzed. R 4.2.1 was used to visualize the data.

HE staining

From the 7th day of membrane formation, vaginal smears were performed every morning to observe morphological changes of epithelial cells and monitor the estrous cycle of rats until the end of membrane formation.

The end point of experiment was euthanasia by intraperitoneal injection of pentobarbital sodium (150 mg/kg). Death was confirmed by pupil dilation and cardiac and respiratory arrest. All operations follow AVMA euthanasia guidelines. Ovarian tissue was removed and fixed with 4% paraformaldehyde. After routine dehydration and paraffin embedding, tissue sections were prepared and immersed in xylene, anhydrous ethanol and 75% alcohol in turn, and then washed. Then they were stained with hematoxylin and then decomposed with dilute hydrochloric acid and ammonia water to make the tissue blue. The sections were washed again and sealed with neutral gum and then observed under a microscope.

RT-qPCR

According to the instructions for use of the reagents, after extracting total RNA, the concentration of RNA samples (2 μL) was accurately measured with a spectrophotometer. GAPDH was used as the reference gene to detect RFRP-3 and GPR147 mRNA levels. The reference mixed sample was pre-denatured at 94°C for 5 minutes, followed by denaturation, extension, and amplification. The amplified product (10 μL) was further extracted and electrophoresed, and the results were finally analyzed using an electrophoresis gel imaging system. The primers are shown in **Table 1**.

Table 1. Primer information of target genes and the internal reference gene

Gene	Primer	Sequences (5' to 3')	Length (bp)	Anneal temperature
β-actin	F	CGTAAAGACCTCTATGCCAACA	100	57 °C
	R	TAGGAGCCAGGGCAGTAATC		
RFRP-3	F	GGAGGAACATAGAAGACAGAAGAAG	186	57 °C
	R	TGGCATAGAGCAATTCACTGGA		
GPR147	F	ACAATGCCAACACTCTCCTCTG	106	57 °C
	R	CAACTCACCAACTCCTCCTTCC		

Western blot

We took about 50 mg of tissue, ground it into powder with liquid nitrogen, added the appropriate amount of RIPA lysis buffer, and sonicated it for 1-2 min. Next, it was centrifuged at 4°C, 12000 rpm for 5 min to remove insoluble impurities, and the supernatant and total protein was quantified by a BCA kit (Biyuntian, Shanghai, China). Thirty ug of protein was taken as a sample for SDS-PAGE gel electrophoresis to separate proteins; after removing the gel, we transferred the membrane at 40 V for 2-3 h; removed the PVDF membrane, rinsed twice with TBST, added blocking solution, placed it on a shaker for 2 h, rinsed twice with TBST, added an appropriately diluted primary antibody and incubated it with the PVDF membrane overnight, rinsed three times with TBST, added an appropriately diluted secondary antibody and incubated with the PVDF membrane for 1 h, rinsed three times with TBST, covered the front of the membrane with an appropriate amount of ultrasensitive ECL (A:B = 1:1) chemiluminescent solution, and used an imaging system to expose and detect the PVDF membrane.

Statistical methods

Data were analyzed by SPSS 26.0 and GraphPad Prism software. Normality testing of the data was conducted on continuous variables such as hormone levels, inflammatory factors, intestinal flora α diversity index, etc. All of them were in line with a normal distribution and were expressed as "mean ± SD". Bilateral independent sample t test was used for comparison between the two groups, single factor ANOVA was used for comparison between multiple groups, and LSD method was used for post-test. If there was no special requirement, $P<0.05$ was used as the test standard.

Results

Huatan Tongmai Yin can improve relevant indicators of PCOS-IR rats

After modeling, HE staining showed significant differences between the control group and model group. The vaginal exfoliated cells of rats in the control group consistently showed a stable estrous cycle, large and regular follicles, thick and compact granulosa cell layer, and well-developed corpus luteum, while the vaginal exfoliated cells of rats in the model group had fallen cells, with small bubbles, loose granule cell layer, and small number of corpus luteum showing that they were in interestrus for a long time, with irregular estrous cycle (**Figures 1** and **2**). Secondly, the serum T, LH, FPG, FINS, and HOMA-IR levels of rats in the model group were higher than in the control group, and FSH was decreased (**Table 2**; **Figure 3**). These results indicate that the PCOS-IR rat model using letrozole combined with a high-fat diet was successfully constructed.

We found that Huatan Tongmai Yin can effectively reduce body weight of PCOS-IR rats, achieving the same effect as metformin (**Table 3**). Modeled rats treated with drug intervention had restored and regular estrous cycles, and serum T, LH, FINS, and HOMA-IR significantly decreased, while FSH increased significantly (**Table 4**; **Figures 4** and **5**). HE staining of ovaries in the Huatan Tongmaiyin group showed several mature follicles and corpus luteum, and the granulosa cells showed an orderly multilayered structure. The number of mature ovaries, corpus luteum, and layers of granulosa cells were all better than those in the metformin group (**Figure 6**). The above results show that Huatan Tongmai Yin has similar effects to metformin and can effectively reduce the weight of PCOS-IR rats, enhance insulin sensitivity, alleviate hyperinsulinemia, and bring ste-

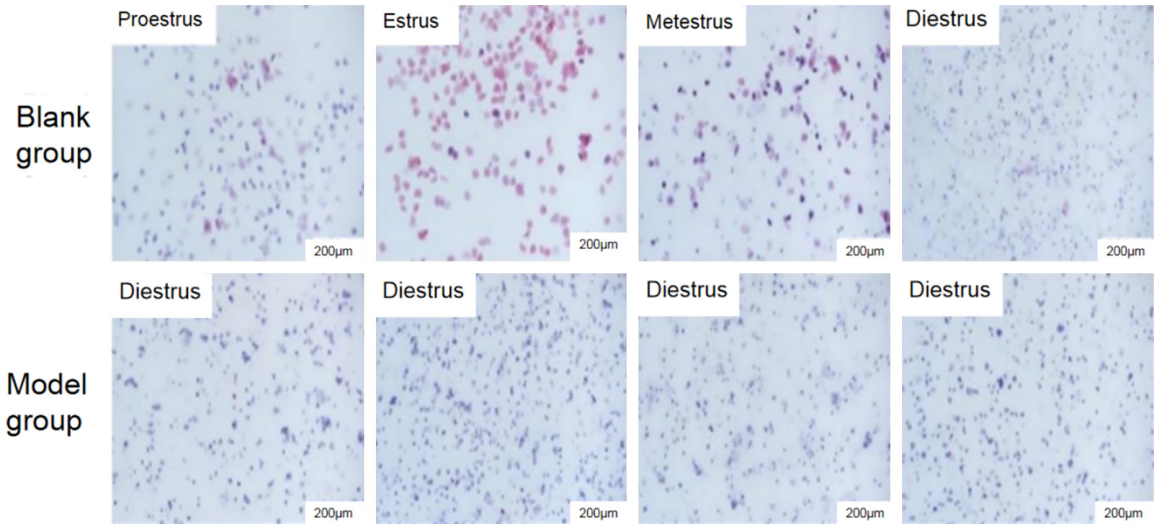

Figure 1. The changes of estrus cycle in rats were observed by HE staining of vaginal shed cells (HE staining, 40×). Note: Preestrus: A large number of nucleated epithelial cells can be seen, with clear cell boundaries; Estrus: keratinized epithelial cells without nuclei were seen, and the cells were squamous. Metestrus: mixed keratinized epithelial cells and white blood cells were seen. Diestrus: A large area of white blood cells is seen.

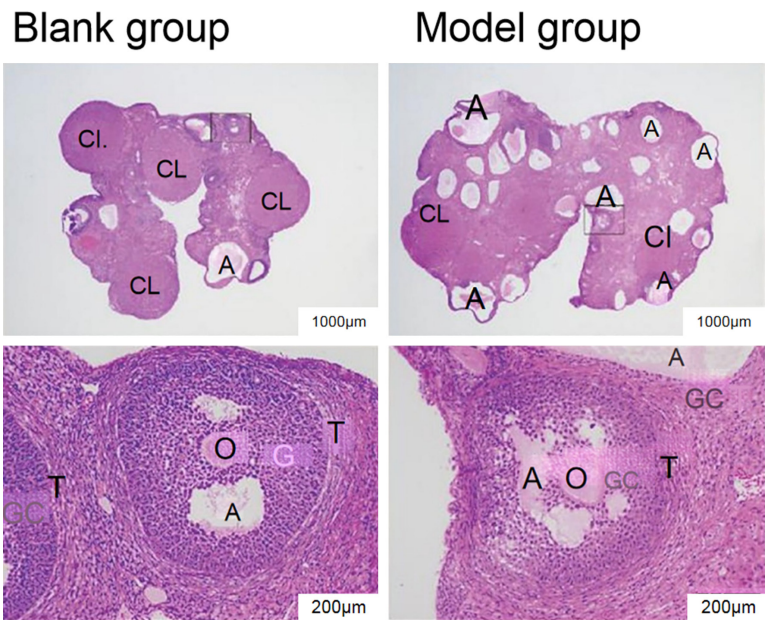

Figure 2. HE staining of rat ovarian tissue (HE staining, 40×, 100×). Note: Control group: follicles and corpus luteum at all levels can be seen, and granular cells are arranged neatly; Model group: Multiple cystic follicles were seen, the granular cell layer was thinner, and the number of corpus luteum was reduced. CL: Corpus luteum: corpus luteum; O: Oocyte; A: Antrum, antral follicle; T: Thecal cells, theca cells; GC: Granular cell.

roid hormone levels closer to normal range, which could correct endocrine and metabolic disorders. It is better than metformin in alleviating polycystic changes in rat ovaries and has certain advantages in the treatment of PCOS-IR.

Huatan Tongmai Yin improves PCOS-IR rats and changes the RFRP-3 expression by interfering with the "microbiome-intestinal-HPO" axis

Intestinal flora analysis found that the Huatan Tongmai Yin group had the richest OTU types (**Figure 7**), and the ACE, Chao, Richness and PD_whole_tree indices were all increased (**Figure 8**). We further evaluated inflammatory factors and found that CRP, IL-6, TNF-α, IL-1β, LPS, TLR4, and NF-κB in rats in the model group were significantly increased, while the Huatan Tongmai Decoction group and metformin group had significantly reduced CRP and IL-6, TNF-α, IL-1β, LPS, and NF-κB levels, with TNF-α showing a downward trend (**Tables 5** and **6**). In terms of improving intestinal barrier function, ZO-1 and Occludin expression in the colon tissue of rats in the model group was reduced. However, Occludin expression in the colon tissue of Huatan Tongmai Yin group and metformin group increased significantly; ZO-1 in the Huatan Tongmai Yin group showed an upward

Table 2. Comparison of serum sex hormone levels in rats in each group (n = 10, $\bar{x}$±s)

Group/Indicator	LH (ng/L)	FSH (IU/L)	T (nmol/L)
Control group	40.22±2.89	31.21±1.41	7.68±0.47
Model group	53.00±1.93[a]	15.68±1.50[a]	10.95±0.74[a]

Note: LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone. In the same row, superscripts with different letters indicate significant differences ([a]*P<0.05*).

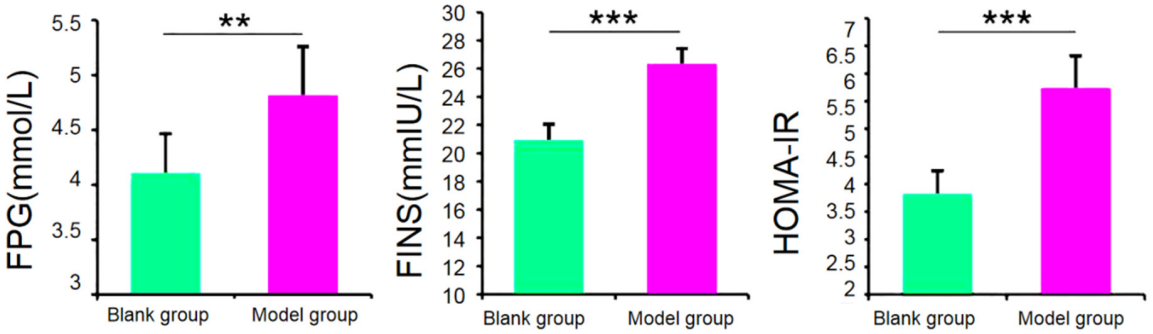

Figure 3. Comparison of FPG, FINS, and HOMA-IR levels in rats. Note: FPG: fasting blood glucose; FINS: fasting insulin; HOMA-IR: insulin resistance index. Two-tailed t-test was used to perform significance statistics between groups. ** represents *P<0.01*, *** represents *P<0.001*.

Table 3. Comparison of body weight of rats in each group (n = 13, $\bar{x}$±s)

Group/Indicator	Control group	Model group	Huatan Tongmai Yin group	Metformin group
Initial weight (g)	200.86±2.79[a]	201.23±2.79[a]	199.79±2.89[a]	202.75±4.15[a]
Body weight after modeling (g)	289.32±15.62[b]	376.98±17.13[a]	367.31±21.26[a]	362.05±24.87[a]
Weight after experiment (g)	289.95±9.55[c]	416.80±19.09[a]	373.76±31.83[b]	366.60±27.25[b]

Note: In the same row, superscripts with different letters indicate significant differences (*P<0.05*).

Table 4. Comparison of serum sex hormone levels in rats in each group before and after intervention treatment (n = 13, $\bar{x}$±s)

Group/Indicator	LH (ng/L)	FSH (IU/L)	T (nmol/L)
Control group	41.25±3.36[c]	31.26±1.43[a]	8.07±1.02[c]
Model group	51.23±3.87[a]	15.39±1.52[d]	10.47±1.23[a]
Huatan Tongmai group	45.07±2.35[b]	21.9±1.46[c]	9.29±0.41[b]
Metformin group	46.36±2.69[b]	26.25±1.99[b]	9.51±0.59[b]

Note: LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone. In the same row, superscripts with different letters indicate significant differences (*P<0.05*).

trend (**Figure 9**). The expression of RFRP-3 and GPR147 mRNA was significantly down-regulated in the model group, while their expression after treatment with Huatan Tongmai Yin and metformin was reduced. The expressions of RFRP-3 and GPR147 mRNA in the thalamus were significantly upregulated (**Figure 10**). These results show that Huatan Tongmai Decoction has the function of improving the intestinal barrier function, inflammatory state, intestinal flora OTU types and Alpha and Beta diversity in PCOS-IR rats. Its mechanism of action may be through "microorganism-intestinal-HPO" axis, which is considered to be closely related to RFRP-3.

Effect of RFRP-3 on the "microbiome-gut-HPO axis" in PCOS-IR rats

In the above experiments, we noticed the close changes between RFRP-3 and PCOS-IR. In order to further explore the underlying mecha-

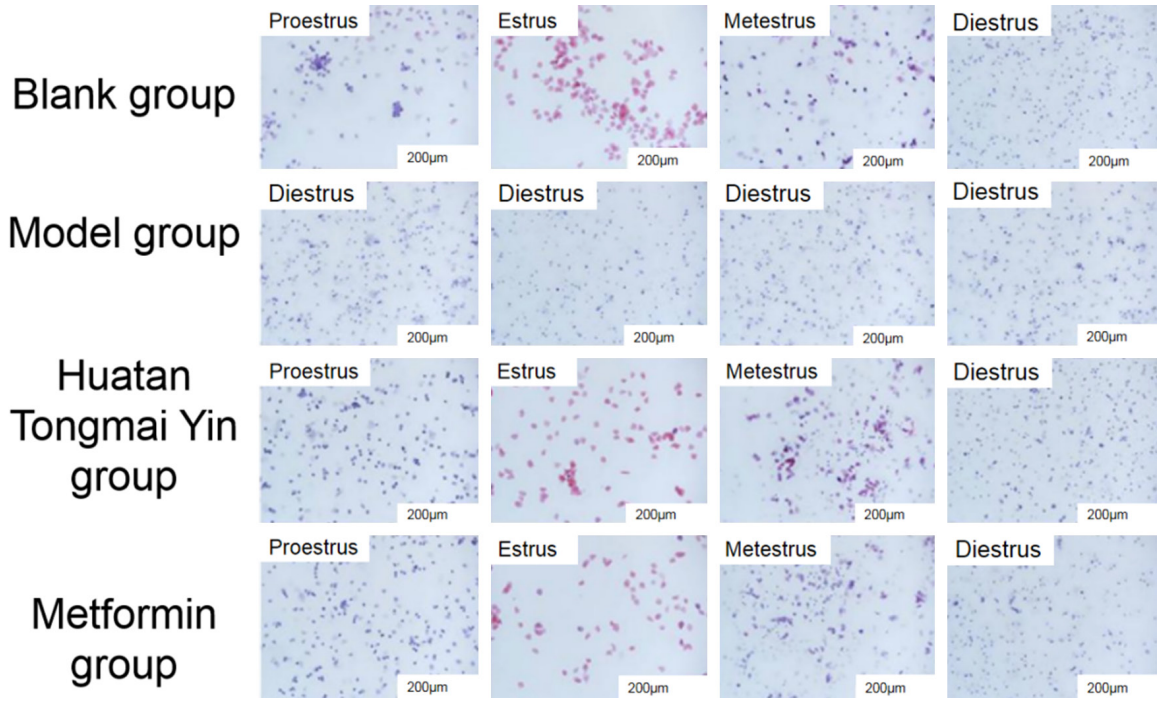

Figure 4. Comparison of changes in estrous cycles of rats in each group (HE staining, 40×). Note: Proestrus is proestrus; Estrus is estrus; Metestrus is metaestrus; Diestrus is interestrus.

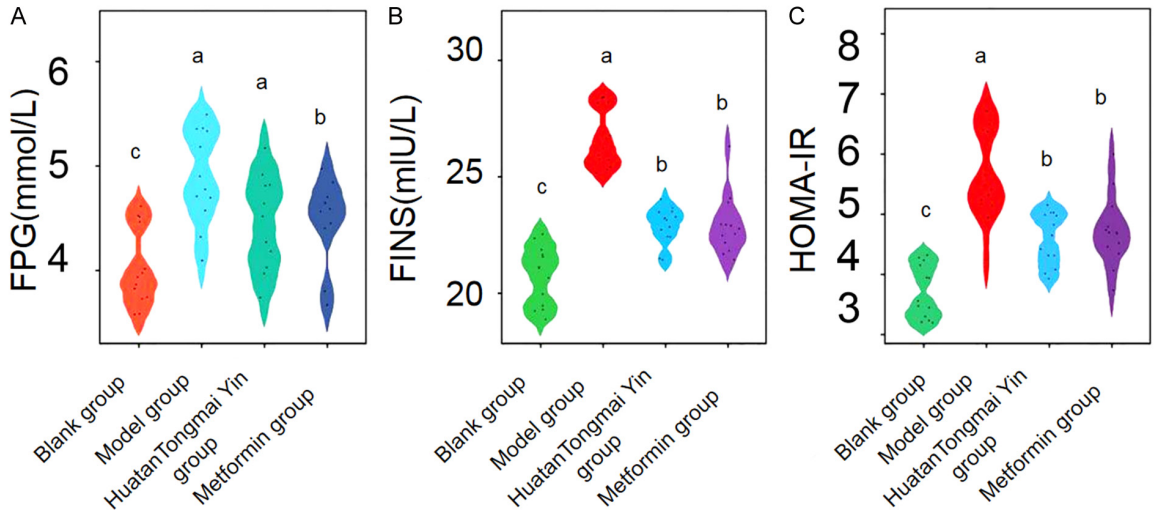

Figure 5. Comparison of FPG, FINS, and HOMA-IR levels in rats in each group. Note: Comparison of FPG (A), FINS (B) and HOMA-IR (C) of rats in each group. FPG: fasting plasma glucose; FINS: fasting insulin; HOMA-IR: insulin resistance index. Superscripts with different letters indicate significant differences (*P<0.05*).

nism, RFRP-3 was used to intervene in the model group. The detection of RFRP-3 expression in each group showed that RFRP-3 expression was significantly decreased in model group and increased in the RFRP-3 overexpressing virus group, indicating that the experimental construction of the RFRP-3 overexpressing virus group was successful (**Figure 11**). The results showed that RFRP-3 could significantly reduce the risk of PCOS-IR rats. Serum T, LH, FPG, FINS, and HOMA-IR levels increased FSH (**Tables 7** and **8**). At the same time, it can significantly down-regulate CRP, IL-6, TNF-α, IL-1β, LPS, TLR4, and NF-κB in

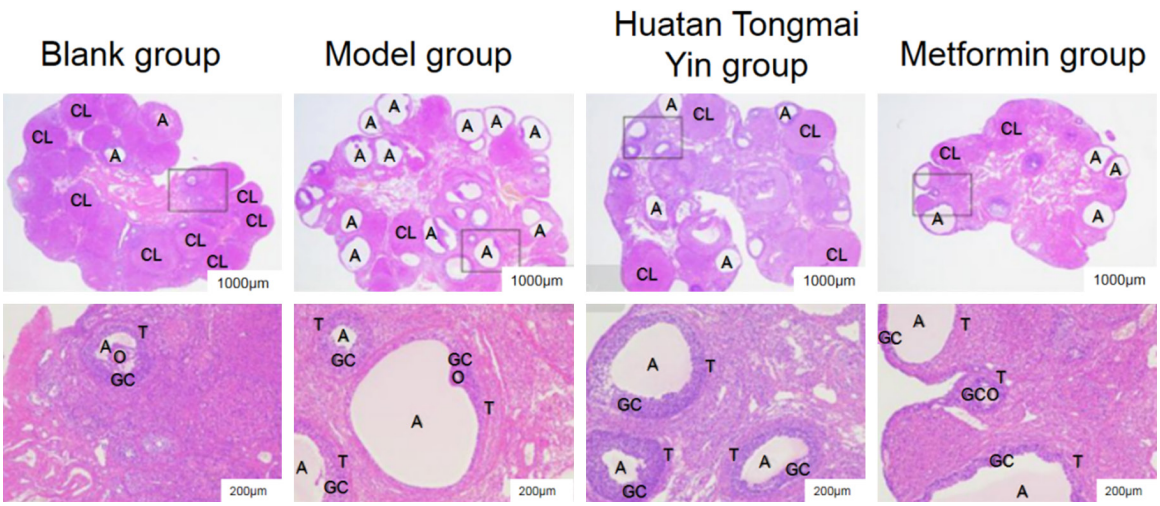

Figure 6. Comparison of morphological changes in ovarian tissue of rats in each group (HE staining, 40×, 100×). Note: CL: Corpus luteum: corpus luteum; O: Oocyte: oocyte; A: Antrum, antral follicle; T: Thecal cells: theca cells; GC: Granular cell.

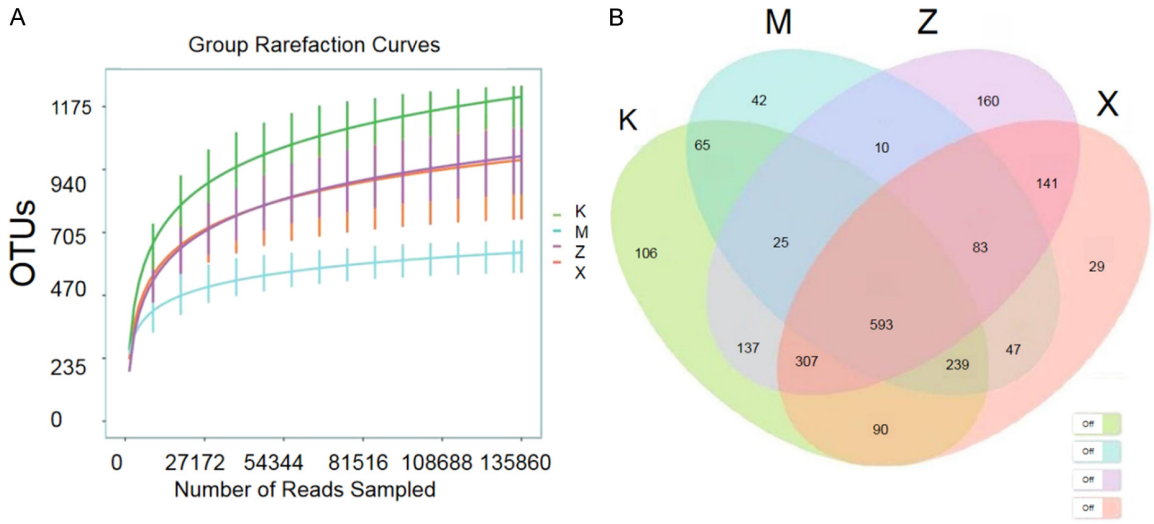

Figure 7. OTUs statistical analysis results chart. Note: K is the control group; M is the model group; Z is the Huatan Tongmai Decoction group; and X is the metformin group. (A) is a dilution curve analysis chart. (B) is a Venn diagram of the intestinal flora of rats in each group. The overlapping part of (B) represents the number of common species, and the non-overlapping part represents the number of species unique to the corresponding group.

PCOS-IR rats (**Tables 9** and **10**), and significantly reduce the symptoms of PCOS-IR rats. The protein expression of GPR54, MyD88, TLR4, and GnRHR in the mouse hypothalamus also had a downward trend in regulating the protein expression of KP (**Figure 12**). This experiment is the first to explore the therapeutic effect of RFRP-3 on PCOS-IR rats, proving that RFRP-3 can interfere with "microbiome-gut-HPO axis" by inhibiting TLR4/MyD88/NF-κB pathway and reducing inflammatory factors, to achieve the effect of treating PCOS-IR.

Discussion

Polycystic ovary syndrome is a metabolic disorder with hyperandrogenism, ovulation disorder, and insulin resistance as its main pathological characteristics [14]. Since its exact pathogenesis is not clear yet, Western medicine treatment usually chooses metformin to enhance insulin sensitivity, combined with clomiphene or letrozole to promote ovulation, and progesterone to adjust the menstrual cycle [15], which often does not achieve a complete cure,

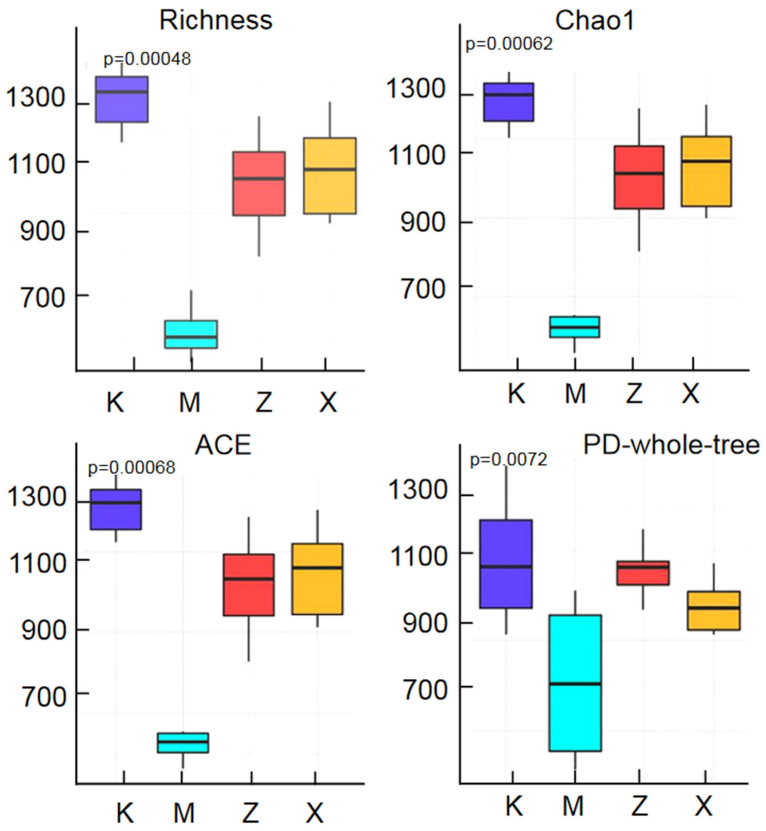

Figure 8. AlPha diversity index analysis. Note: K is the control group; M is the model group; Z is the Huatan Tongmai Decoction group; and X is the metformin group. Richness index is the species richness index, which is the sum of the number of species with an abundance greater than 0 in the community; Chao1 is the bacterial species richness index, which is used to estimate the number of OTUs contained in the sample; ACE, used to estimate the number of OTUs contained in the community Index of the number of OTUs; PD_Whole_Tree is a diversity index calculated based on phylogenetic trees.

and is also accompanied by gastrointestinal side effects. "Gut-brain" axis dysfunction is an important pathogenic factor of PCOS-IR, and regulating the imbalance of the brain-gut axis is a new research direction in the treatment of PCOS-IR. RFRP-3 is a biological short peptide known to inhibit the HPO axis, inhibits GnRH activity through kisspeptin neurons, and affects the secretion of LH and FSH through multiple pathways [16]. In order to explore whether Huatan Tongmai Yin can mediate the "microbiome-gut-HPO" axis through the expression of RFRP-3, thereby improving the condition of PCOS-IR, we first constructed a PCOS-IR rat model and detected vaginal shedding cells, ovarian HE staining, serum levels of sex hormones and insulin-related indicators. The results show that Huatan Tongmai Yin can effectively regulate these indicators, suggesting its potential therapeutic effect on PCOS-IR. The mechanism may be as follows: Pinellia in Huatan Tongmai Decoction is rich in β-sitosterol, which can reduce local ovarian inflammation by inhibiting the expression of IL-1β/COX-2 [12]. At the same time, Angelica in the formula is rich in active substances such as ferulic acid, which can inhibit the excitability of GnRH neurons in the hypothalamus and improve insulin sensitivity [17]. Meanwhile, in this study, it was found that Huatan Tongmai Yin and metformin produced similar improvement effects when intervening PCOS-IR rats, but the Huatan Tongmai Yin group still showed certain advantages, which may be attributed to the fact that the combination of angelica and Astragalus inhibits CYP19A1 activity. Furthermore, it can reduce local ovarian androgen synthesis more effectively than metformin [18]. At the same time, the main component of the compound, Whitractonide A, can specifically promote the increase of short-chain fatty acid producing bacteria, while metformin had no significant effect on such bacteria [19], so Huatan Tongmai Yin has more advantages in restoring ovarian cycle function and maintaining long-term metabolic stability. However, the specific mechanism needs to be further explored. Intestinal flora not only maintains ovarian function, but also regulates endocrine levels. It can convert conjugated estrogens and metabolites produced by the liver into free states, thereby regulating estrogen levels [20]. Its dysregulation may promote androgen production in the ovaries and interfere with normal follicular development by inducing chronic inflammation. Changes in metabolic and clinical parameters in PCOS-IR patients are associated with changes in gut microbiota composition. Studies have shown that compared with healthy people, the intestinal flora of PCOS-IR

Table 5. Serum inflammatory factor levels of rats in each group (n = 13, $\bar{x}\pm s$)

Group/Indicator	CRP (μg/L)	TNF-α (ng/L)	IL-1β (ng/L)	IL-6 (pg/mL)
Control group	2430.52±113.9^{d}	405.99±17.62^{d}	42.94±1.82^{d}	125.08±6.42^{d}
Model group	3161.39±114.81^{a}	556.83±20.63^{a}	57.76±2.41^{a}	162.26±6.19^{a}
Huatan Tongmai group	2833.66±125.9^{b}	495.6±18.92^{b}	52.1±2.23^{b}	148.07±5.45^{b}
Metformin group	2728.52±112.54^{c}	464.73±21.56^{c}	48.46±2.66^{c}	138.37±5.87^{c}

Note: CRP, C-reactive protein; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6. Superscripts with different letters indicate significant differences (*P<0.05*).

Table 6. Serum LPS, TLR4 and NF-κB levels of rats in each group (n = 13, $\bar{x}\pm s$)

Group/Indicator	LPS (ng/L)	NF-κB (ng/L)	TLR4 (ng/mL)
Control group	110.94±5.13^{d}	785.36±32.48^{b}	31.06±1.87^{c}
Model group	144.18±6.56^{a}	850.25±33.55^{a}	35.46±1.39^{a}
Huatan Tongmai group	134.36±7.66^{b}	820.89±35.58^{a}	32.8±1.38^{b}
Metformin group	123.03±4.03^{c}	824.07±46.38^{a}	32.19±1.79^{b,c}

Note: LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; NF-κB, nuclear factor κB. Superscripts with different letters indicate significant differences (*P<0.05*).

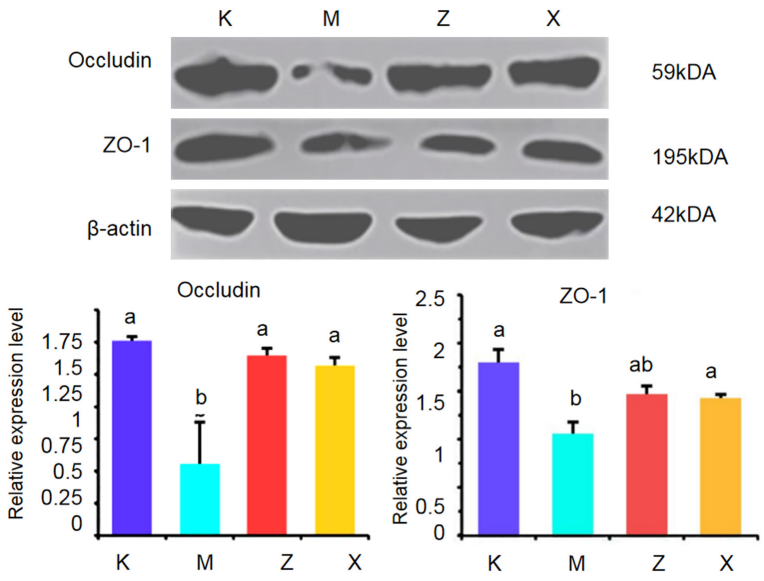

Figure 9. Expression of Occludin and ZO-1 proteins in the colon of rats in each group. Note: K is the control group; M is the model group; Z is the Huatan Tongmai Decoction group; and X is the metformin group. Superscripts with different letters indicate significant differences (*P<0.05*).

patients is significantly reduced [21]. Zhao et al. [22] found that the modified Banxia Xiexin Decoction can increase the abundance of beneficial flora in PCOS-IR rats, reduce pathogenic bacteria, and regulate the diversity of the flora. Similarly, research by Zhu et al. [23] showed that Guizhi Fuling Pills can relieve inflammation and improve the symptoms of PCOS-IR while regulating intestinal flora.

This study observed the potential of Huatan Tongmai Decoction in treating PCOS-IR through the "microbiome-intestinal-HPO" axis. After establishing PCOS-IR rat model, the rats were given Huatan Tongmai Yin and we found that the follicular morphology of PCOS-IR rats was improved, the corpus luteum was increased, the pathological structure was improved, the levels of LH, FSH and FINS were regulated, and HOMA-IR was reduced. In addition, changes in the intestinal flora index were also observed, indicating that the therapeutic effect of Huatan Tongmai Yin is related to "microorganism-intestinal-HPO". One possible reason is that Huatan Tongmai Yin contains pinellia polysaccharide. Its water-soluble polysaccharide can inhibit macrophage infiltration, downregulate the expression of IL-1β and COX-2 to reduce inflammatory response, and increase mucin 2 (MUC2) secretion and tight junction proteins expression to maintain stability of the intestinal mucus barrier [24]. In addition, β-sitosterol in Pinellia ternata can upregulate tight junction proteins, promote the secretion of regenerative islet-derived protein IIIγ and secretory IgA, enhance the physical and biochemical barrier functions of intestinal epithelium, and reshape intestinal flora [25]. In addition, Atractylodes macrocephala, another component of Huatan Tongmai Yi, contains atractylodesin A, which can inhibit intestinal

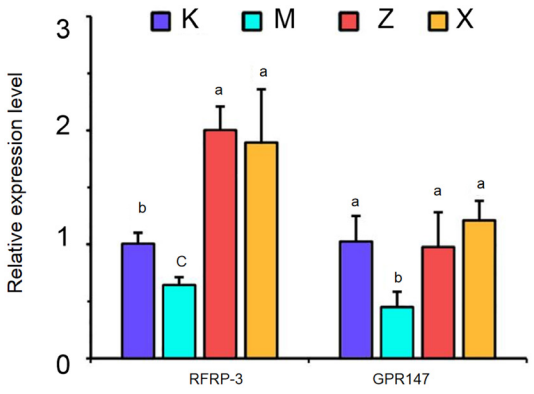

Figure 10. mRNA expression levels of RFRP-3 and GPR147 in hypothalamic tissue. Note: K is the control group; M is the model group; Z is the Huatan Tongmai Decoction group; and X is the metformin group. Superscripts with different letters indicate significant differences (*P<0.05*).

inflammatory response, reverse damaged mucin synthesis through the TLR4/MyD88/NF-κB signaling, repair intestinal barrier, and regulate intestinal tract flora [26]. It can be seen that Huatan Tongmai Yin can reduce the inflammatory response of PCOS-IR, improve the intestinal barrier and regulate intestinal microorganisms through multiple ways. The brain-gut axis plays a role in regulating gut microbiota. Changes in intestinal flora can affect the HPO axis through the brain-gut axis, forming a "microbiome-gut-HPO" axis, thereby affecting the condition of PCOS-IR. In the HPO axis, the pituitary gland receives GnRH signals from hypothalamus and secretes LH and FSH, thereby regulating follicle maturation and sex hormone levels [27]. HPO axis imbalance is considered an important pathological feature of PCOS-IR.

"Metabolic endotoxemia" caused by intestinal flora imbalance may be the trigger for PCOS. Intestinal flora serves as a storage reservoir for LPS. When the flora is imbalanced, such as an increase in the number of Desulfovibrio [28, 29], it can induce an increase in LPS levels, increase intestinal permeability, and promote the entry of endotoxins into the systemic circulation [30]. In addition, studies have proven that TLR4 [31] can recognize LPS and activate the NF-κB pathway, releasing pro-inflammatory factors such as CRP, TNF-α, IL-1β, and IL-6, resulting in the presence of a large number of inflammatory factors in the blood circulation and ovarian tissue of PCOS patients [32-34]. These factors can affect the expression of gonadotropin FSH and LH receptors in the pituitary gland, further regulate the levels of estrogen and progesterone in the ovary, and affect the neuroendocrine activity of HPO axis [35]. When the level of TNF-α in the body increases, the secretion of adiponectin (which has the effect of increasing insulin sensitivity and can stimulate cell proliferation and steroid production) decreases, resulting in decreased insulin sensitivity and increased steroid production, ultimately leading to hyperandrogenism.

In this study, PCOS-IR rats were administered Huatan Tongmai Yin and metformin. Subsequently, the inflammatory factors (CRP, IL-6, TNF-α, IL-1β, LPS, TLR4, NF-κB), intestinal tissue proteins (ZO-1, Occludin), and intestinal flora were measured. The results showed that the Huatan Tongmai Decoction group had the richest types of OTUs, all indices increased, and it could significantly downregulate CRP, IL-6, TNF-α, IL-1β, LPS, and NF-κB, and also had an effect on TNF-α. Occludin expression in colon tissue increased; ZO-1 showed an upward trend. These results demonstrate that Huatan Tongmai Decoction improves intestinal barrier function, reduces inflammatory markers, and modulates intestinal flora (including OTU types and Alpha/Beta diversity) in PCOS-IR rats. Its mechanism of action may be through "microorganism-intestinal-HPO" axis which is considered closely related to RFRP-3. Neuropeptide-RF amide-related peptide-3 (RFRP-3) is the only neuropeptide found so far in the mammalian brain that exerts a negative regulatory effect on hypothalamic-pituitary-gonadal axis [36]. Studies have found that RFRP-3 acts on hypothalamic neurons and the pituitary gland, inhibiting GnRH and LH synthesis [37].

Consistent with our findings, a study also showed that polycystic ovary syndrome rats had lower levels of RFRP-3 compared with normal rats [38]. RFRP-3 sends inhibitory signals to the reproductive axis through mammalian GPR147, a well-known HPO axis inhibitor that regulates LH secretion by acting on GnRH neurons [39]. Peragine et al. [40] found that RFRP-3 can affect the sexual maturation of mice through GnRH. In addition, Xiong et al. [41] demonstrated that large doses of exogenous RFRP-3 can affect bovine oocytes by regulating apoptosis rate and steroidogenesis.

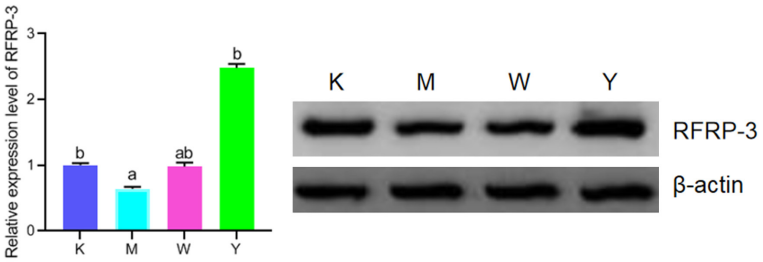

Figure 11. Expression of RFRP-3 in hypothalamus of rats in each group. Note: K, control group; M, model group; Y, RFRP-3 overexpression group; W, empty virus group. Superscripts with different letters in the same row indicate significant differences (*P<0.05*).

Table 7. Comparison of sex hormone levels in rats in each group (n = 13, $\bar{x}\pm s$)

Group/Indicator	LH (ng/L)	FSH (IU/L)	T (nmol/L)
Control group	41.25±3.36^{c}	31.26±1.43^{a}	8.07±1.02^{c}
Model group	51.23±3.87^{a}	15.39±1.52^{d}	10.47±1.23^{a}
RFRP-3 overexpression group	44.95±2.28^{b}	28.88±1.4^{b}	9.11±0.67^{b}
Virus empty group	50.89±2.39^{a}	18.12±1.45^{c}	10.64±0.52^{a}

Note: LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone. In the same row, superscripts with different letters indicate significant differences (*P<0.05*).

Table 8. Comparison of FPG, FINS, and HOMA-IR levels in rats in each group (n = 13, $\bar{x}\pm s$)

Group/Indicator	FPG (mmol/L)	FINS (mIU/L)	HOMA-IR
Control group	4.02±0.36^{b}	20.73±1.21^{d}	3.71±0.42^{c}
Model group	4.78±0.47^{a}	26.65±1.15^{a}	5.66±0.64^{a}
RFRP-3 overexpression group	4.3±0.52^{b}	22.33±0.98^{c}	4.26±0.55^{b}
Virus empty group	4.68±0.44^{a}	25.53±0.86^{b}	5.31±0.51^{a}

Note: FPG, fasting blood glucose; FINS, fasting insulin; HOMA-IR, insulin resistance index. Superscripts with different letters indicate significant differences (*P<0.05*).

We found significantly down-regulated RFRP-3 and GPR147 mRNA in the model group, while they were up-regulated after treatment with Huatan Tongmai Decoction. The active ingredients in Huatan Tongmai Yin, such as the alkaloids in Pinellia ternata and Angelica sinensis, can act on the hypothalamus, activate RFRP-3, inhibit the kisspeptin/GPR54 pathway, and inhibit GnRH-induced LH secretion in pituitary cells [42].

To further investigate the potential mechanism by which RFRP-3 alleviates PCOS-IR via the "microbiome-gut-HPO axis", we performed RFRP-3 overexpression in rats in the model group. The results showed that RFRP-3 could improve sex hormone and insulin-related indicators in PCOS-IR rats, reduce the inflammatory state, reduce the expression of GPR54, MyD88, TLR4, and GnRHR in the hypothalamus, and it also had a down-regulation trend in the protein expression of KP. This experiment is the first to explore the therapeutic effect of RFRP-3 on PCOS-IR rats, proving that RFRP-3 can interfere with the "microbiome-gut-HPO axis" by inhibiting the TLR4/MyD88/NF-κB pathway and reducing production of inflammatory factors, to achieve the effect of treating PCOS-IR. The above experimental results show that Huatan Tongmai Yin can improve intestinal flora and polycystic ovary syndrome by upregulating the expression of RFRP-3, inhibiting GnRH and regulating the "microbiome-gut-HPO" axis.

Conclusion

Huatan Tongmai Yin has obvious therapeutic effects on insulin-resistant polycystic ovary syndrome. It can up-regulate the expression of RFRP-3 to inhibit the TLR4/MyD88/NF-κB pathway, interfering with the "microbiome-gut-HPO" axis by improving inflammation and insulin resistance, thereby regulating LH and FSH levels. However, given that RFRP-3 can regulate the release of pituitary hormones through multiple pathways and considering the involvement of various brain-gut peptides in the brain-gut axis, this study did not fully elucidate its exact mechanism.

In addition, the study lacked cellular-level studies and had limited discussion of genetics. Therefore, it is necessary to conduct further research to explore the mechanism of Huatan Tongmai Yin in PCOS-IR, in order to provide new insights into PCOS-IR treatment. By studying the therapeutic effect of Huatan Tongmai Decoction on PCOS-IR, we can explore

Table 9. Serum inflammatory factor levels of rats in each group (n = 13, $\bar{x}\pm s$)

Group/Indicator	CRP (μg/L)	IL-1β (ng/L)	IL-6 (pg/mL)	TNF-α (ng/L)
Control group	2430.52±113.9^{d}	42.94±1.82^{d}	125.08±6.42^{d}	405.99±17.62^{d}
Model group	3161.39±114.81^{a}	57.76±2.41^{a}	162.26±6.19^{a}	556.83±20.63^{a}
RFRP-3 overexpression group	2561.03±162.46^{c}	45.55±2.25^{c}	134.32±6.61^{c}	431.89±19.06^{c}
Virus empty group	3054.26±123.5^{b}	54.09±2.10^{b}	156.65±6.22^{b}	533.46±20.91^{b}

Note: CRP, C-reactive protein; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6. Superscripts with different letters indicate significant differences ($P<0.05$).

Table 10. Serum LPS, TLR4, and NF-κB levels of rats in each group (n = 13, $\bar{x}\pm s$)

Group/Indicator	TLR4 (ng/mL)	LPS (ng/L)	NF-κB (ng/L)
Control group	31.06±1.87^{b}	110.94±5.13^{c}	785.36±32.48^{b}
Model group	35.46±1.39^{a}	144.18±6.56^{a}	850.25±33.55^{a}
RFRP-3 overexpression group	31.04±1.47^{b}	118.82±4.9^{b}	803.4±46.66^{b}
Virus empty group	35.39±1.82^{a}	141.37±5.4^{a}	837.77±43.51^{a}

Note: LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; NF-κB, nuclear factor κB. Superscripts with different letters indicate significant differences ($P<0.05$).

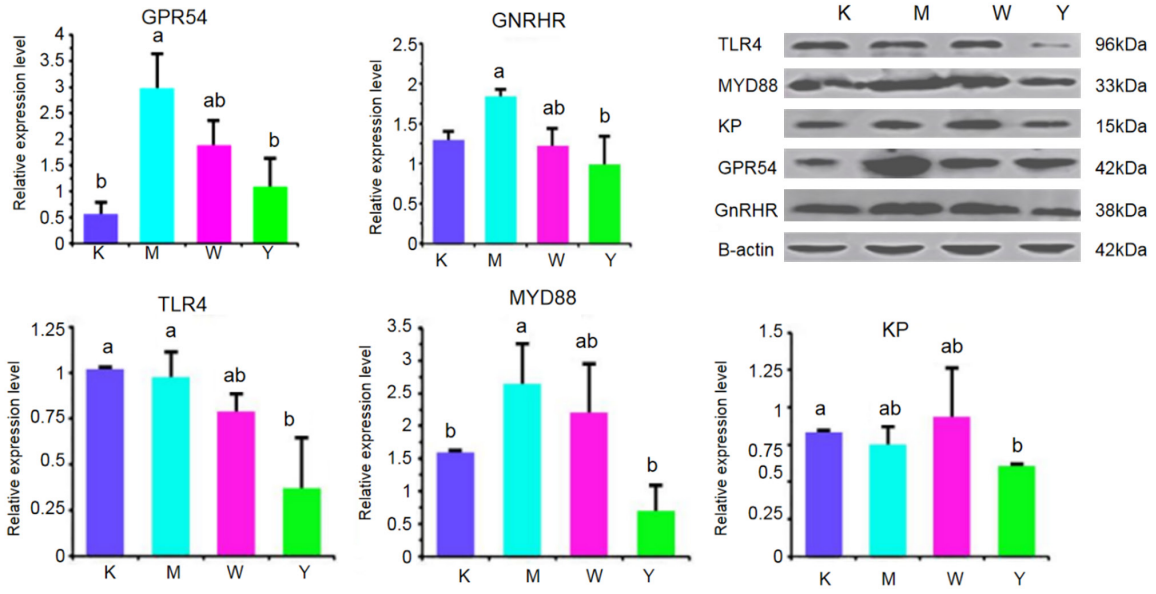

Figure 12. Protein expression of GNRHR, KP, GRP54, TLR4, and MYD88 in each group. Note: K, control group; M, model group; Y, RFRP-3 overexpression group; W, empty virus group. Superscripts with different letters in the same row indicate significant differences ($P<0.05$).

the role of RFRP-3-mediated "microbiome-intestinal-HPO" axis in the pathogenesis of PCOS-IR and supplement our understanding of PCOS-IR as well as identify new therapeutic targets.

In addition, due to the multi-target characteristics of Huatan Tongmai Yin, its mechanism of action on the brain-gut axis, HPO axis and regulation of intestinal flora is worth exploring. These findings are valuable for clinical treatment of patients and provide a wider range of therapeutic possibilities.

Acknowledgements

We gratefully acknowledge Guangxi University of Chinese Medicine for providing the necessary equipment for this study. This study was supported by the National Natural Science Foundation of China (No. 82160919).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hanmei Lin, Department of Gynaecology, The First Affiliated Hospital of Guangxi University of Chinese Medicine, No. 89-9 Dongge Road, Qingxiu District, Nanning 530022, Guangxi, China. Tel: +86-0771-5603921; E-mail: Linhanmei0806@126.com

References

[1] Safiri S, Noori M, Nejadghaderi SA, Karamzad N, Carson-Chahhoud K, Sullman MJM, Collins GS, Kolahi AA and Avery J. Prevalence, incidence and years lived with disability due to polycystic ovary syndrome in 204 countries and territories, 1990-2019. Hum Reprod 2022; 37: 1919-1931.

[2] Zhao H, Zhang JQ, Cheng XY, Nie XZ and He B. Insulin resistance in polycystic ovary syndrome across various tissues: an updated review of pathogenesis, evaluation, and treatment. J Ovarian Res 2023; 16: 9.

[3] Zhonghua Kou Qiang Yi Xue Za Zhi. 2009 Aug; 44(8). Zhonghua Kou Qiang Yi Xue Za Zhi 2009; 44: 511-2.

[4] Cheng L, Yang S, Si L, Wei M, Guo S, Chen Z, Wang S and Qiao Y. Direct effect of RFRP-3 microinjection into the lateral ventricle on the hypothalamic kisspeptin neurons in ovariectomized estrogen-primed rats. Exp Ther Med 2022; 23: 24.

[5] He F and Li Y. The gut microbial composition in polycystic ovary syndrome with insulin resistance: findings from a normal-weight population. J Ovarian Res 2021; 14: 50.

[6] Asadi A, Shadab Mehr N, Mohamadi MH, Shokri F, Heidary M, Sadeghifard N and Khoshnood S. Obesity and gut-microbiota-brain axis: a narrative review. J Clin Lab Anal 2022; 36: e24420.

[7] Ahmed H, Leyrolle Q, Koistinen V, Kärkkäinen O, Layé S, Delzenne N and Hanhineva K. Microbiota-derived metabolites as drivers of gut-brain communication. Gut Microbes 2022; 14: 2102878.

[8] Qi X, Yun C, Sun L, Xia J, Wu Q, Wang Y, Wang L, Zhang Y, Liang X, Wang L, Gonzalez F, Patterson AD, Liu H, Mu L, Zhou Z, Zhao Y, Li R, Liu P, Zhong C, Pang Y, Jiang C and Qiao J. Gut microbiota-bile acid-interleukin-22 axis orchestrates polycystic ovary syndrome. Nat Med 2019; 25: 1225-1233.

[9] Shen HR, Xu X, Ye D and Li XL. Berberine improves the symptoms of DHEA-induced PCOS rats by regulating Gut microbiotas and metabolites. Gynecol Obstet Invest 2021; 86: 388-397.

[10] Shen C, Li H, Xiao M, Jiang X, Jin J, Zhou J, Xiong B, Chen Y and Zhao M. Study on the mechanism of the Chinese herbal pair Banxia-Chenpi in ameliorating polycystic ovary syndrome based on the CYP17A1 gene. J Ethnopharmacol 2025; 344: 119503.

[11] Jung W, Choi H, Kim J, Kim J, Kim W, Nurkolis F and Kim B. Effects of natural products on polycystic ovary syndrome: from traditional medicine to modern drug discovery. Heliyon 2023; 9: e20889.

[12] Zheng R, Shen H, Li J, Zhao J, Lu L, Hu M, Lin Z, Ma H, Tan H, Hu M and Li J. Qi Gong Wan ameliorates adipocyte hypertrophy and inflammation in adipose tissue in a PCOS mouse model through the Nrf2/HO-1/Cyp1b1 pathway: integrating network pharmacology and experimental validation in vivo. J Ethnopharmacol 2023; 301: 115824.

[13] Jin J, Hu QY, Xu WW, Zhu WJ, Liu B, Liu J, Wang W and Zhou HF. Tanshinone IIA attenuates estradiol-induced polycystic ovarian syndrome in mice by ameliorating FSHR expression in the ovary. Exp Ther Med 2019; 17: 3501-3508.

[14] Zhang Y, Cao S, Liang JX, Hu SH, Guo XF, Chunjing S and Ge LN. Effects of Zishen Yutai pills combined with metformin on women with polycystic ovary syndrome undergoing in vitro fertilization. Medicine (Baltimore) 2024; 103: e39030.

[15] Gao H, Qian B, Ni Y, Sun L and Fu J. Research progress in the pathogenesis of polycystic ovary syndrome. Sichuan Da Xue Xue Bao Yi Xue Ban 2024; 55: 1049-1054.

[16] Raul Schmidt A, Ignacio Felipe Inserra P, Andres Cortasa S, Proietto S, Fidel V, Halperin J, Daniel Vitullo A and Berta Dorfman V. Distribution of kisspeptin system and its relation with gonadotropin-releasing hormone in the hypothalamus of the South American plains vizcacha, Lagostomus maximus. Gen Comp Endocrinol 2022; 317: 113974.

[17] Tuo W, Wang S, Shi Y, Cao W, Liu Y, Su Y, Xiu M and He J. Angelica sinensis polysaccharide extends lifespan and ameliorates aging-related diseases via insulin and TOR signaling pathways, and antioxidant ability in Drosophila. Int J Biol Macromol 2023; 241: 124639.

[18] Xu D, Lu M, Liu Y, Chen W, Yang X, Xu M, Zhou H, Wei X, Zhu Y and Song Q. An analysis of the clinical medication rules of traditional Chinese medicine for polycystic ovary syndrome based on data mining. Evid Based Complement Alternat Med 2023; 2023: 6198001.

[19] Rocha DHA, Seca AML and Pinto DCGA. Seaweed secondary metabolites in vitro and in vivo anticancer activity. Mar Drugs 2018; 16: 410.

[20] Laniewski P, Ilhan ZE and Herbst-Kralovetz MM. The microbiome and gynaecological can-

cer development, prevention and therapy. Nat Rev Urol 2020; 17: 232-250.
[21] Zeng B, Lai Z, Sun L, Zhang Z, Yang J, Li Z, Lin J and Zhang Z. Structural and functional profiles of the gut microbial community in polycystic ovary syndrome with insulin resistance (IR-PCOS): a pilot study. Res Microbiol 2019; 170: 43-52.
[22] Zhao H, Chen R, Zheng D, Xiong F, Jia F, Liu J, Zhang L, Zhang N, Zhu S, Liu Y, Zhao L and Liu X. Modified Banxia Xiexin Decoction ameliorates polycystic ovarian syndrome with insulin resistance by regulating intestinal microbiota. Front Cell Infect Microbiol 2022; 12: 854796.
[23] Zhu Y, Li Y, Liu M, Hu X and Zhu H. Guizhi Fuling Wan, Chinese herbal medicine, ameliorates insulin sensitivity in PCOS model rats with insulin resistance via remodeling intestinal homeostasis. Front Endocrinol (Lausanne) 2020; 11: 575.
[24] Ma L, Ma Y and Liu Y. β-sitosterol protects against food allergic response in BALB/c mice by regulating the intestinal barrier function and reconstructing the gut microbiota structure. Food Funct 2023; 14: 4456-4469.
[25] Chen C, Shen JL, Liang CS, Sun ZC and Jiang HF. First discovery of beta-sitosterol as a novel antiviral agent against white spot syndrome virus. Int J Mol Sci 2022; 23: 10448.
[26] Xu J, Liu C, Shi K, Sun X, Song C, Xu K and Liu Y. Atractyloside-A ameliorates spleen deficiency diarrhea by interfering with TLR4/MyD88/NF-κB signaling activation and regulating intestinal flora homeostasis. Int Immunopharmacol 2022; 107: 108679.
[27] Wang Z, Wu W, Kim MS and Cai D. GnRH pulse frequency and irregularity play a role in male aging. Nature Aging 2021; 1: 904-918.
[28] Scheithauer TPM, Rampanelli E, Nieuwdorp M, Vallance BA, Verchere CB, van Raalte DH and Herrema H. Gut microbiota as a trigger for metabolic inflammation in obesity and type 2 diabetes. Front Immunol 2020; 11: 571731.
[29] Wang T, Yan H, Lu Y, Li X, Wang X, Shan Y, Yi Y, Liu B, Zhou Y and Lü X. Anti-obesity effect of Lactobacillus rhamnosus LS-8 and Lactobacillus crustorum MN047 on high-fat and high-fructose diet mice base on inflammatory response alleviation and gut microbiota regulation. Eur J Nutr 2020; 59: 2709-2728.
[30] Yurtdas G and Akdevelioglu Y. A new approach to polycystic ovary syndrome: the gut microbiota. J Am Coll Nutr 2020; 39: 371-382.
[31] Hersoug LG, Moller P and Loft S. Gut microbiota-derived lipopolysaccharide uptake and trafficking to adipose tissue: implications for inflammation and obesity. Obes Rev 2016; 17: 297-312.
[32] Nehir Aytan A, Bastu E, Demiral I, Bulut H, Dogan M and Buyru F. Relationship between hyperandrogenism, obesity, inflammation and polycystic ovary syndrome. Gynecol Endocrinol 2016; 32: 709-713.
[33] Gonzalez F, Considine RV, Abdelhadi OA and Acton AJ. Saturated fat ingestion promotes lipopolysaccharide-mediated inflammation and insulin resistance in polycystic ovary syndrome. J Clin Endocrinol Metab 2019; 104: 934-946.
[34] Wang B, Hao M, Yang Q, Li J and Guo Y. Follicular fluid soluble receptor for advanced glycation endproducts (sRAGE): a potential protective role in polycystic ovary syndrome. J Assist Reprod Genet 2016; 33: 959-65.
[35] Huang Y, Cheng Y, Zhang M, Xia Y, Chen X, Xian Y, Lin D, Xie S and Guo X. Oxidative stress and inflammatory markers in ovarian follicular fluid of women with diminished ovarian reserve during in vitro fertilization. J Ovarian Res 2023; 16: 206.
[36] Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, Fujii R, Watanabe T, Kikuchi K, Terao Y, Yano T, Yamamoto T, Kawamata Y, Habata Y, Asada M, Kitada C, Kurokawa T, Onda H, Nishimura O, Tanaka M, Ibata Y and Fujino M. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. Nat Cell Biol 2000; 2: 703-708.
[37] Ubuka T and Tsutsui K. Reproductive neuroendocrinology of mammalian gonadotropin-inhibitory hormone. Reprod Med Biol 2019; 18: 225-233.
[38] Chaudhari NK and Nampoothiri LP. Neurotransmitter alteration in a testosterone propionate-induced polycystic ovarian syndrome rat model. Horm Mol Biol Clin Investig 2017; 29: 71-77.
[39] Hu KL, Chang HM, Li R, Yu Y and Qiao J. Regulation of LH secretion by RFRP-3 - from the hypothalamus to the pituitary. Front Neuroendocrinol 2019; 52: 12-21.
[40] Peragine DE, Pokarowski M, Mendoza-Viveros L, Swift-Gallant A, Cheng HM, Bentley GE and Holmes MM. RFamide-related peptide-3 (RFRP-3) suppresses sexual maturation in a eusocial mammal. Proc Natl Acad Sci U S A 2017; 114: 1207-1212.
[41] Xiong X, Hu Y, Pan B, Zhu Y, Fei X, Yang Q, Xie Y, Xiong Y, Lan D, Fu W and Li J. RFRP-3 influences apoptosis and steroidogenesis of yak cumulus cells and compromises oocyte meiotic maturation and subsequent developmental competence. Int J Mol Sci 2023; 24: 7000.
[42] Rahdar P, Khazali H, Hosseini A and Raeisi A. The impact of morphine on reproductive activity in male rats is regulated by rf-amid-related peptide-3 and substance P adjusting hypothalamic kisspeptin expression. J Mol Neurosci 2019; 69: 456-469.