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Original Article

Effects of *pterostilbene* on treating hyperprolactinemia and related mechanisms

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Abstract: Hyperprolactinemia (HPRL) frequently causes primary menopause and reproductive disorders. Pterostilbene is known to have anti-inflammation and modulation on cell apoptosis. However, its role in treating HPRL and potential mechanisms remain unclear yet. Healthy female virgin SD rats were randomly assigned into control, HPRL model group, bromocriptine treatment group, and low (20 mg/kg) and high (40 mg/kg) pterostilbene treatment groups. All groups except control ones received metoclopramide hydrochloride injection for generating HPRL model. Uterus and ovarian index in all animals were monitored. Prolactin (PRL), estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) were quantified by ELISA. Caspase 3 activity was assayed, with real time PCR measuring Bcl-2 and Bax mRNA levels. HPRL rats had lower uterus and ovarian index, accompanied with elevated PRL, caspase 3 activity, Bax expression, and decreased FSH, LH, E2 and Bcl-2 expression as compared to control group ($p<0.05$). Pterostilbene treatment significantly increased uterus and ovarian index, FSH, LH, E2 and Bcl-2 expression, and decreased PRL, caspase 3 activity and Bax expression as compared to control group ($p<0.05$). 40 mg/kg pterostilbene had similar efficacy as those of bromocriptine. Pterostilbene exerts its function in the treatment of HPRL via modulating apoptosis-anti-apoptosis homeostasis, inhibiting serum PRL level, and regulating secretion of gonadotropin hormones.

Keywords: Hyperprolactinemia, pterostilbene, prolactin, cell apoptosis

Introduction

Hyperprolactinemia (HPRL) is a functional disorder in hypothalamus-pituitary-gonad axis that can be caused by both endogenous and exogenous factors. HRPL includes both organic lesion and dysfunction [1, 2]. With more than 0.4% incidence among the whole population, HPRL often leads to primary menopause and reproductive disorder in females [3, 4]. It thus severely affects people's health and life quality, making it a major health concern worldwide [5, 6]. HPRL can be caused by stress factors such as hypoglycemia, surgery and trauma, or physiological conditions including feeding, sleeping and mental disorders, as well as diseases affecting pituitary, hypothalamus, hypothyroidism, and liver/kidney dysfunction [7, 8]. Clinical treatment against HPRL includes medicine, surgery or radiotherapy. The main medication currently used is bromocriptine, which, however, has certain side effects due to long treatment period, worse compliance, both of which compromise efficacy, thus aggravating economic and mental burdens of patients and their families [9]. Therefore, development of novel drug candidates is of critical importance for treating HPRL.

As one homologous derivative of resveratrol, pterostilbene is a non-flavonoid polyphenol compound. It is enriched in plant products such as grape, nuts, strawberry, propolis and guanxi dragon's blood, as one important ingredient in traditional Chinese medicine [10, 11]. Similar to those of resveratrol, pterostilbene also had anti-fungal, anti-proliferation, preventing oxidative stress response, anti-inflammation and decreasing blood lipid functions [12]. It has been found to have significant efficacy in treat-

Table 1. Primer sequence

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
Bcl-2	AGGGAGATCTCTAGGAATAAC	CCAGGGTCTGGCGTGTCCAGTA
Bax	TCCAGGGAGTATCCAGGGA	ATGTCCAGGTGGTCTGT

Table 2. Uterus and ovarian indexes of rats

Group	Uterus index (mg/g)	Ovarian index (mg/g)
Control	1.91±0.26	0.43±0.06
Model	1.21±0.32*	0.28±0.03*
Bromocriptine	1.82±0.41#	0.40±0.07#
Pterostilbene (20 mg/kg)	1.43±0.73*,#	0.33±0.02*,#
Pterostilbene (40 mg/kg)	1.78±0.35#	0.39±0.06#

Note: *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

ing Alzheimer's disease, cardiovascular disease, brain injury, tumor and hypercholesterolemia [13, 14]. The role of pterostilbene in treating HPRL and related mechanisms, however, remain unknown yet. This study thus treated HPRL model rats with pterostilbene, in order to illustrate the function and mechanism of pterostilbene on HPRL.

Materials and methods

Animals

A total of 50 healthy virgin SD rats (aged 2 month, SPF grade, body weight 250±20 g) were purchased from Laboratory Animal Center of Shandong university and were kept in an SPF grade facility with fixed temperature (21±1°C) and humidity (50~70%) with 12/12 light/dark cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Weifang People's Hospital.

Reagents and equipment

Pterostilbene (Fuji, Japan); Bromocriptine (Novartis, Swiss); Metoclopramide hydrochloride (Laien Pharma, China); Sodium pentobarbital and lidocaine (Zhaohui Pharma, China); Caspase 3 activity assay kit (Jiancheng, China). ELISA kits for prolactin (PRL), estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH) were purchased from R&D (US). Surgical microscope was purchased from Suzhou Medical Instrument (China). RNA extraction kit and reverse transcription kit were ordered from Axygen (US). ABI 7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (US). Microplate reader was a product of BD (US). Animal ventilator was purchased from Shanghai Medical Instrument (China). Other reagents were provided by Sangon (China).

Animal grouping and model

After 2-week normal feeding, 50 rats were randomly assigned into five groups (N=10 each): control group, model group, bromocriptine treatment group and high/low dosage pterostilbene groups; All groups except control ones received metoclopramide hydrochloride injection (50 mg/kg daily for 10 consecutive days) for generating HPRL model. Bromocriptine was given by gavage at 1 mg/kg daily for 4 weeks. In pterostilbene groups, 20 mg/kg or 40 mg/kg drugs were given by gavage daily for 4 weeks.

Samples collection

Blood samples were collected from rat abdominal aorta via negative pressure tubes at 24 h after the last drug delivery and at the end of model preparation. Blood samples were incubated at room temperature for 30 min. After blood clotting, it was centrifuged at 3600 rpm under 4°C for 10 min. The supernatant was saved and frozen at -20°C for further use. Ovary and uterus tissues were detached, weighted and frozen at -80°C fridge.

Ovarian and uterus index

Separated ovarian and uterus tissues were weighted and calculated for respective indexes, which were defined as the ratio of tissue weight to total body mass.

ELISA for measurement of serum PRL and gonadotropin

Serum samples were frozen at -80°C for further use. ELISA kits were used to test serum PRL, E2, FSH and LH levels following manual instructions of test kits. Optical density (OD) values of each well at 450 nm wavelength were mea-

Table 3. Serum PRL and sex hormone levels

Group	PRL (ng/ml)	E2 (pg/ml)	LH (mIU/ml)	FSH (mIU/ml)
Control	12.31±3.17	75.46±12.52	6.15±0.81	5.97±0.66
Model	21.21±4.38*	31.53±15.09*	3.92±0.45*	3.16±0.21*
Bromocriptine	13.81±3.45#	71.41±16.37#	5.97±0.41#	4.77±0.41*,#
Pterostilbene (20 mg/kg)	17.47±5.72*,#	46.53±15.42*,#	4.37±0.51*,#	3.69±0.58*,#
Pterostilbene (40 mg/kg)	14.18±4.55#	68.39±17.26#	5.91±0.87#	4.56±0.81*,#

Note: *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

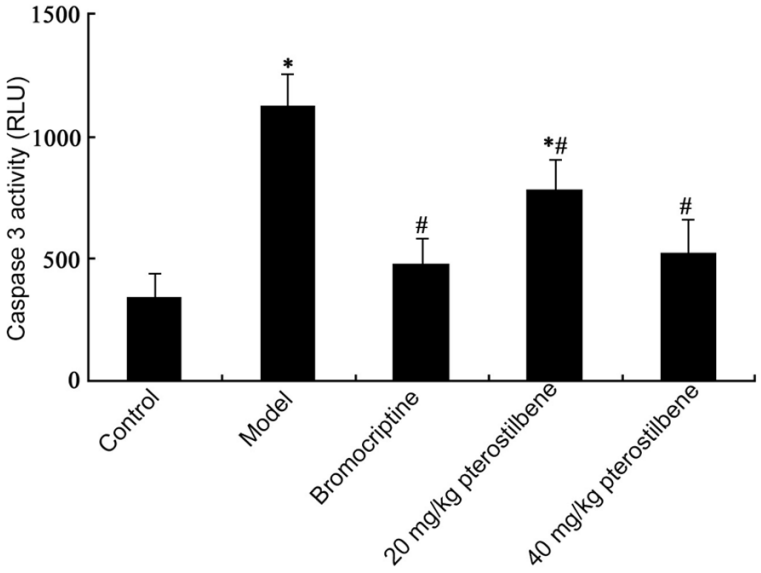

Figure 1. Caspase 3 activity in uterus tissues. Uterus tissues from different group were firstly digested in trypsin followed and then lysed by tissue lysis buffer. After that, 2 mM Ac-DEVD-pNA was then added to detect optical density (OD) value at 405 nm wave length, reflecting active level of caspase 3. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

sured by a microplate reader within 15 min of quenching buffer. Linear regression function was deduced based on the concentration of standard samples and respective OD values. Sample concentration was then calculated based on OD values and regression model.

Caspase 3 activity assay

The activity of caspase 3 in uterus and ovarian of rats was measured according to the manual instruction of test kit. In brief, cells were firstly digested in trypsin by 600 g centrifugation at 4°C for 5 min. Supernatant was discarded, with the addition of tissue lysis buffer for 15-min iced treatment. The mixture was then centrifuged at 20,000 g for 5 min at 4°C. 2 mM Ac-DEVD-pNA was then added to detect optical density (OD) value at 405 nm wave length, reflecting active level of caspase 3.

Real-time PCR to detect Bcl-2 and Bax mRNA levels

Under sterilized condition, Trizol reagent was used to extract mRNA from ovarian and uterus tissues of rats. cDNA was then synthesized based on respective primers (**Table 1**). Target gene expression was detected by real-time PCR under the following conditions: (for Bcl-2) 52°C 1 min, followed by 35 cycles each containing 90°C 30 s, 58°C 50 s and 72°C 35 s; (for Bax) 55°C 1 min, followed by 35 cycles each containing 90°C 30 s, 62°C 50 s and 72°C 35 s. Data were collected by ABI 7700 Fast fluorescent quantitative PCR cycler and were calculated for CT values based on GAPDH house-keeping gene and respective standard curves. Quantitative analysis was performed by $2^{-\Delta Ct}$ approach.

Statistical analysis

SPSS16.0 software was used to process all collected data, which were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare means across groups. Between-group-comparison was performed in LSD test. A statistical significance was defined when $p < 0.05$.

Results

Ovarian and uterus indexes of all rats

We analyzed the alternation of uterus and ovarian indexes in all groups and found significantly decreased indexes in HPRL model rats gener-

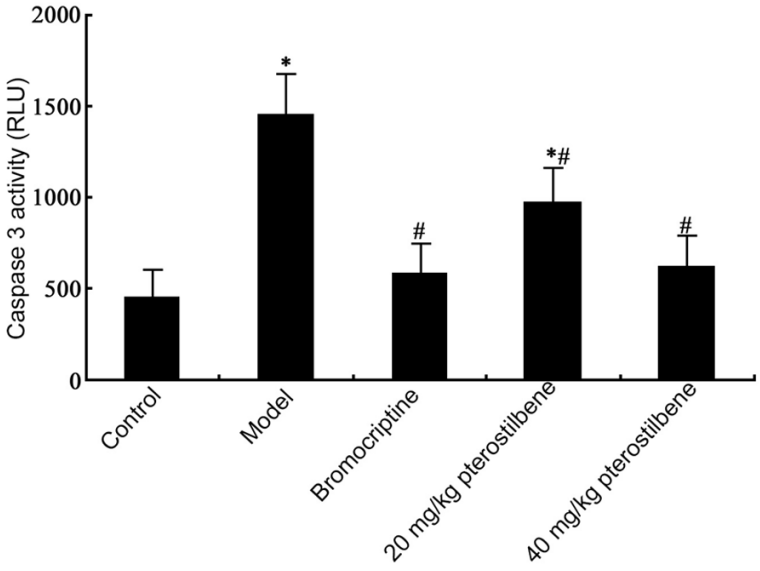

Figure 2. Caspase 3 activity in ovarian tissues. Ovarian tissues from different group were firstly digested in trypsin followed and then lysed by tissue lysis buffer. After that, 2 mM Ac-DEVD-pNA was then added to detect optical density (OD) value at 405 nm wave length, reflecting active level of caspase 3. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

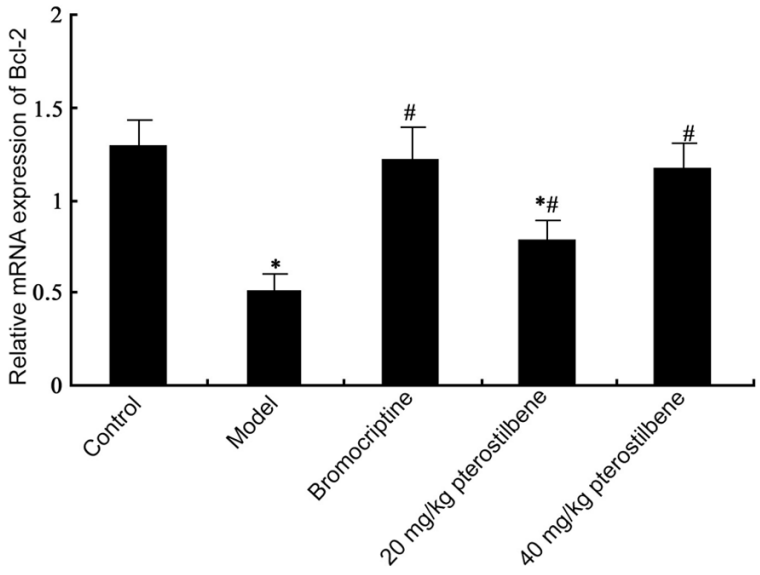

Figure 3. Bcl-2 mRNA levels in uterus tissues. Total RNA was isolated from uterus tissues followed by measurement of Bcl-2 mRNA level by RT-qPCR. *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

ated by metoclopramide hydrochloride injection ($p < 0.05$ compared to control group). After treated with pterostilbene, however, both uterus and ovarian indexes were significantly elevated ($p < 0.05$ compared to model group). With elevated dosage of pterostilbene, such effect became more obvious as similar to those in bromocriptine (**Table 2**).

Serum PRL and sex hormone levels

Serum PRL, E2, FSH and LH levels in all rats were observed. As shown by results, HPRL model rats had significantly elevated PRL levels ($p < 0.05$ compared to control group), suggesting successful establishment of HPRL model. After pterostilbene treatment, serum PRL level was significantly decreased ($p < 0.05$ compared to model group), suggesting treatment effect of pterostilbene on HPRL. Further analysis regarding E2, FSH and LH levels revealed their reduction in model rats and recovery after pterostilbene treatment. With higher dosage, hormone levels mimicked those in bromocriptine treatment group, which were used as one positive control cohort (**Table 3**).

Caspase 3 activity in uterus and ovarian tissues

Caspase 3 activity assay kit revealed alternations of caspase 3 activity in uterus and ovarian tissues from all groups of rats. Results showed significantly elevated caspase 3 activity in HPRL model rats ($p < 0.05$ compared to control group). After pterostilbene treatment, however, caspase 3 activity was significantly decreased ($p < 0.05$ compared to model group). With higher dosage, such effects became more obvious as those in bromocriptine group (**Figures 1**, **2**).

Bcl-2 mRNA expression levels

Real-time PCR was used to detect the expression level of Bcl-2 mRNA in both uterus and ovarian tissues in all rats. Results showed significantly decreased Bcl-2 levels in HPRL model rats ($p < 0.05$ compared to control group). After pterostilbene treatment, however, Bcl-2 mRNA

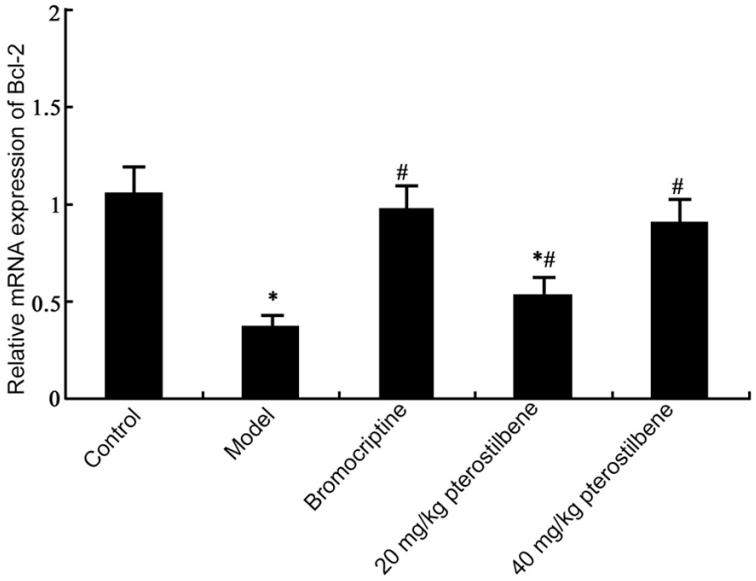

Figure 4. Bcl-2 mRNA levels in ovarian tissues. Total RNA was isolated from ovarian tissues followed by measurement of Bcl-2 mRNA level by RT-qPCR *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

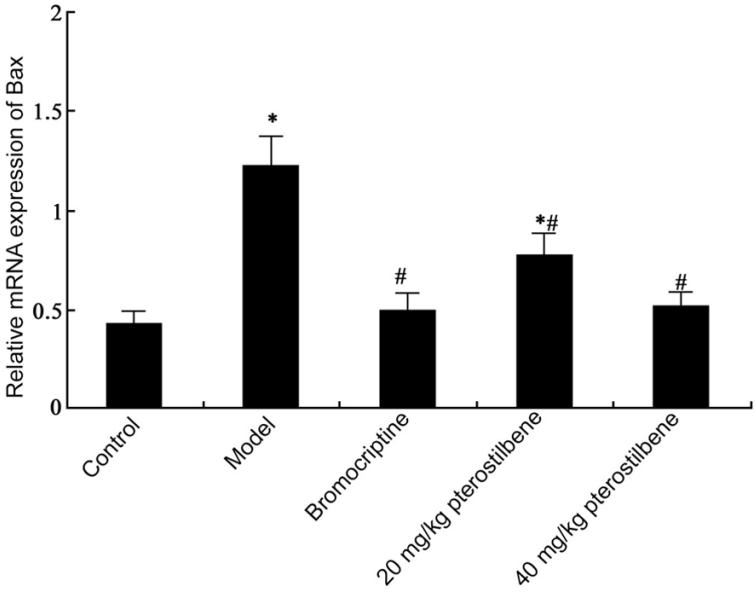

Figure 5. Bax mRNA levels in uterus tissues. Total RNA was isolated from uterus tissues followed by measurement of Bax mRNA level by RT-qPCR *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

was significantly up-regulated ($p < 0.05$ compared to model group). With higher dosage, such effects became more clear as those in bromocriptine group (**Figures 3**, **4**).

Bax mRNA expression in uterus and ovarian tissues

We further quantified the expression level of Bax mRNA in both uterus and ovarian tissues in all rats. Results showed opposite trends: Bax level was significantly enhanced in HPRL model rats ($p < 0.05$ compared to control group). After pterostilbene treatment, however, Bax mRNA was significantly down-regulated ($p < 0.05$ compared to model group). With higher dosage, such effects became more obvious as those in bromocriptine group (**Figures 5**, **6**).

Discussion

PRL is secreted in anterior pituitary and is under the modulation of both releasing and inhibiting factors from hypothalamus. PRL has certain circadian rhythm and pulse of release patterns. It is metabolized mainly in liver and kidney. Besides the stimulation of lactation, PRL can also regulate functions of uterus, ovary and body immune system [15, 16]. Under various physiological, pathological and pharmaceutical factors both exogenously and endogenously, the imbalance of PRL releasing inhibitor (such as dopamine) and stimulating factor (5-HT) can cause the over-production of PRL, leading to HPRL syndrome [17]. Current medications against HPRL require long treatment period, higher cost and induces several side effects, largely impeding the promotion in clinics [18].

Pterostilbene can significantly clear active oxygen and modulate body oxidation-reduction balance via anti-oxidation pathway, thus participating in the regulation of apoptosis-anti-apoptosis, further exerting certain roles in multiple diseases including ischemia-reperfusion injury, inflammation and tumors. *In vivo* study showed that pterostilbene can reduce the injury of kidney, testis, skeletal muscle, ovary, bone marrow and brain tissues [19, 20]. This study applied intra-

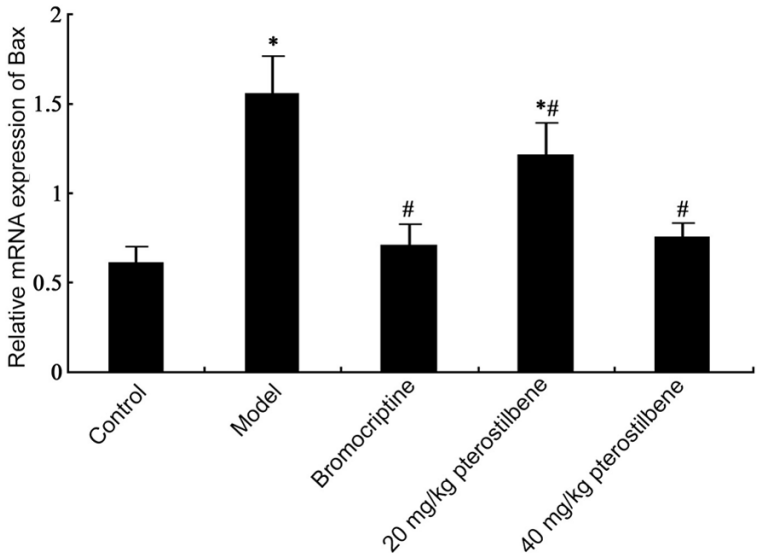

Figure 6. Bax mRNA levels in ovarian tissues. Total RNA was isolated from ovarian tissues followed by measurement of Bax mRNA level by RT-qPCR *, $p < 0.05$ compared to control group; #, $p < 0.05$ compared to model group.

peritoneal injection of metoclopramide hydrochloride, which can block dopamine receptor in hypothalamus, thus facilitating the secretion of PRL for establishing HPRL model, in an attempt to investigate the functional mechanism of pterostilbene on the treatment of HPRL. Results showed significantly decreased ovarian and uterus indexes in model rat, which were all recovered after pterostilbene treatment. With higher drug dosage, more obvious effects were observed which was similar to the bromocriptine treatment group. HPRL could decrease the secretion of sex hormones including E2, FSH and LH via short feedback loop as a result of enhanced PRL secretion, thus affecting follicle development. The intact function of follicle is the basis for pregnancy by timely ovulation. High level of PRL affects follicle development, leading to decreased quality of eggs, compromising fecundability, causing problems including menopause, galactorrhea and menoxenia [21]. Treatment with pterostilbene could reduce PRL secretion and enhance sex hormone secretion, further improving follicle development. Further mechanism study confirmed that pterostilbene can decrease caspase 3 activity, Bax expression and increase Bcl-2 expression. Caspase 3 is one factor inducing apoptotic activity, while apoptotic protein Bax and anti-apoptotic protein Bcl-2 had antagonistic effects. When Bax expression is enhanced, Bcl-2 expression was decreased to initiate apoptotic signal and vice versa to inhibit cell apoptosis [22, 23]. These results showed the treatment efficacy of pterostilbene on HPRL was via modulating apoptosis/anti-apoptosis balance.

In summary, pterostilbene can exert treatment effects on HPRL via mediating ovarian and uterus tissue apoptosis/anti-apoptosis balance, inhibiting serum PRL level and mediating reproductive hormone secretion. This study provided theoretical grounds and evidences for optimizing treatment strategy for HRP in clinics.

Disclosure of conflict of interest

None.

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