

The Therapeutic Effect of Shugan Xiehuo Formula in Female Rat Model with Central Precocious Puberty

Weiping Yin

Nanjing University of Chinese Medicine

Songmei Li

Yunnan Provincial Hospital of Chinese Medicine

Kun Zhang

Yunnan University of Chinese Medicine

Ying Xu

Yunnan University of Chinese Medicine

Dianfei Ma

Yunnan Provincial Hospital of Chinese Medicine

Tingting Shi

Yunnan Provincial Hospital of Chinese Medicine

Lei Xiong (✉ gZR2018@126.com)

Nanjing University of Chinese Medicine

Jie Xia

Yunnan Provincial Hospital of Chinese Medicine

Research

Keywords: CPP, SXF, follicles maturation, uterine wall thickening, GnRH, GnRHR

Posted Date: June 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-37959/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Central precocious puberty (CPP) severely affected children's physical and mental health, which needs to be treated promptly and effectively.

Objective: To research the therapeutic effect of Shugan Xiehuo Formula (SXF) on CPP.

Methods: CPP female rat model was established and treated by leuprolide and different dose of SXF. Sex organs volume and index were measured. The ovary and uterus was experienced HE staining. Concentration of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL) and estradiol (E2) in peripheral blood was determined. Expression of gonadotropin-releasing hormone (GnRH), gonadotropin-releasing hormone receptor (GnRHR), estrogen receptor alpha (ER α) and G protein-coupled receptor 30 (GPR30) in hypophysis was investigated by qRT-PCR and Western Blot. GnRH expression in hypothalamus and GnRHR expression in ovary was detected by immunohistochemistry.

Results: SXF reduced the volume of bilateral ovaries, uterus, hypothalamus and hypophysis in CPP female rat, and diminished the index of ovary, uterus, hypothalamus and hypophysis in CPP female rat ($P < 0.05$ or $P < 0.01$). SXF treatment inhibited follicles maturation and uterine wall thickening in CPP female rat. SXF declined FSH, LH, PRL and E2 concentration in peripheral blood in CPP female rat ($P < 0.01$ or $P < 0.001$). SXF suppressed the expression of GnRH, GnRHR, ER α and GPR30 in hypophysis, the expression of GnRH in hypothalamus and GnRHR in ovary of CPP female rat ($P < 0.05$, $P < 0.01$ or $P < 0.001$).

Conclusions: SXF had effective therapeutic effects on CPP female rat, which was worthy of promotion clinically.

Background

Central precocious puberty (CPP) is caused by premature activation of the hypothalamic-pituitary-gonad (HPG) axis, which ultimately leads to the premature development of secondary sexual characteristics in children [1–3]. According to report, 1 out of every 5000 to 10,000 children develops CPP, and in general, the incidence of CPP in girls is much higher than that in boys [4]. If CPP is not properly treated, it will affect the growth of children and lead to a shorter height than normal adults. CPP results in a series of psychological and physical problems in children suffering from this annoying disease, which is deeply concerned by parents and society.

In recent decades, the main treatment strategy of CPP is the application of gonadotropin-releasing hormone analogues (GnRHa) [5, 6]. This treatment method has been shown to effectively inhibit the hypothalamo-pituitary-gonadal axis, thereby achieving the treatment aims [7]. Unfortunately, although GnRHa has found to be conductive in the treatment of CPP, the benefits of GnRHa for the growth of CPP children are still uncertain because of the lack of sufficient randomized controlled studies in clinical. In addition, the relatively expensive price of GnRHa has also caused a certain degree of financial burden on patients' families. Recently, a common Chinese medicine formula, Shugan Xiehuo Formula (SXF), was

found to have therapeutic effects on CCP in children. Researchers revealed that the total effective rate of SXF to female patients with CPP was as high as 88.89%. Moreover, SXF treatment significantly diminished the volume of ovarian as well as the level of sex hormones [8]. In the female rat model with CPP, the intervention of SXF reduced gonadotropin-releasing hormone (GnRH) mRNA expression in hypothalamus and gonadotropin-releasing hormone receptor (GnRHR) mRNA expression in hypophysis. High expression of GnRH and GnRHR was the important indicators of the activation of HPG axis. Thus, SXF was considered to treat CPP by suppressing the HPG axis activation [9]. However, more evidence should be presented to support the application of SXF in the clinical treatment of CPP.

In this research, the female rat model with CPP was established via subcutaneous injection of N-methyl-DL-aspartic acid (NMA). SXF with different dose was used to treat CPP female rat in order to explore the therapeutic effect of SXF on CPP. This study will provide more reliable theoretical basis for the treatment of CPP with SXF.

Materials & Methods

Ingredients of SXF

Every 100 g of SXF was composed of the following ingredients: Chinese Thorowax Root (10 g), Angelica sinensis (10 g), White paeony root (10 g), Poriacocos Wolf (10 g), Rhizomaatractylodismacrocephalae (10 g), Peppermint (10 g), Herbaecliptae (10 g), Ligustrumlucidum (10 g), Spica prunellae (10 g), Radix glycyrrhizaepreparata (10 g). All Chinese medicine ingredients were purchased from JiangyinTianjiang Pharmaceutical Co., Ltd. (Jiangsu, China). SXF was diluted in distilled water to a concentration of 2 g/mL and then placed in a 4°C refrigerator for storage.

Animals

A total of 60 female Sprague-Dawley (SD) rats (71.15 ± 8.22 g) were commercially provided by Shanghai Laboratory Animal Co. Ltd. (Shanghai, China). Rats were kept separately in cages in an SPF laboratory animal room at (22 ± 1) °C and (55 ± 5)% humidity with free access to water and food. The light/dark cycle was 12 h. All animal experiments in this study have been approved by the Animal Ethics Committee (No.: R-082018033).

Construction and treatment of female rat model with CPP

Rats were randomly divided into 6 groups (10 rats per group) and named as follows: Control group, CPP group, CPP-LP group, CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group. Rats of Control group were injected subcutaneously with 0.9% sodium chloride injection (0.2 mL/time) at 14:00 and 16:00 daily. Notably, for rats of the other 5 groups, they were subjected to the construction of CPP model through subcutaneous injection of NMA (40 mg/kg) (Solarbio, Beijing, China) at 14:00 and 16:00 daily. Meanwhile, at 9:00 every day, rats in Control group and CPP group were given gavage of 0.9% sodium chloride injection (1.0 mL per rats). However, rats in CPP-LP group were injected intramuscularly with

leuprolide (LP) acetate microspheres (100 µg/kg). At the same time point, rats of CPP-LD-SXF group, CPP-MD-SXF group and CPP-HD-SXF group were subjected to SXF gavage with a dose of 2.13 g/kg, 4.25 g/kg and 8.50 g/kg, respectively.

The vaginal opening of rats in each group was observed at 8:30 every day. When the vaginal opening of rats in CPP group was observed to be open, the injection of NMA was stopped. At the same time, rats of Control group were stopped the injection of 0.9% sodium chloride injection. Meanwhile, the injection of NMA for rats of CPP-LP group, CPP-LD-SXF group, CPP-MD-SXF group and CPP-HD-SXF group was also stopped. In CPP group, rats with open vaginal opening were subjected to vaginal smears. The vaginal smears were observed under a microscope in order to determine the sexual cycle of the rats. Then rats of CPP group were sacrificed at the first pre-estrus stage, and rats in the other 5 groups were also sacrificed at the same time. It should be noted that, before being sacrificed, the body weight was measured and the peripheral blood was obtained. After being sacrificed, the bilateral ovaries, uterus, hypophysis and hypothalamus of each rat was obtained, weighted and stored at -80°C.

Determination of estrogen concentration in peripheral blood

The peripheral blood of each rat was obtained and concentrated for 5 min at 3000 × g, 4°C to collect the serum. The concentration of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL) and estradiol (E2) in the serum was determined by radioimmunoassay testing kits (Hengyuan biotechnology Co., Ltd., Shanghai, China).

Measurement of sex organ index

Before being sacrificed, the body weight of each rat was measured. The weight of ovary, uterus, hypophysis and hypothalamus was also measured after rat being sacrificed. The ratio of ovary/body weight, uterus/body weight, hypophysis/body weight and hypothalamus/body weight was subsequently calculated.

Hematoxylin-eosin (HE) staining

The ovary and uterus of rats were fixed with 4% paraformaldehyde for 24 h at 4°C. Gradient alcohol was used to dehydrate. After treatment with xylene, the ovary and uterus was embedded in the paraffin, followed by being prepared into sections with a thickness of 5 µm. Sections were dried in an oven at 45°C. Thereafter, xylene was used to dewax the sections and gradient alcohol was applied to hydrate the sections. The sections were stained with hematoxylin and eosin subsequently. After being dehydrated with alcohol and transparentized by xylene, sections were sealed in neutral resin and placed under a microscope to observe the growth of follicles and thickness of the uterine wall.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Sex hormone receptor related genes expression in hypophysis, including GnRH, GnRHR, estrogen receptor alpha (ERα) and G protein-coupled receptor 30 (GPR30), was investigated by qRT-PCR. In general,

hypophysis tissues were lysed on ice with the addition of TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA) to extract total RNA. PrimeScriptTMRT kit (TaKaRa, Shiga, Japan) was used for the reverse transcription reaction with 5 µg total RNA sample to synthesis cDNA template. Thereafter, a total of 2 µL cDNA template was subjected to the PCR amplification reaction in a 20 µL reaction system. The amplification reaction conditions were 40 cycles of 95°C for 30s, 95°C for 15s and 62 °C for 20 s. β-actin was used as the control. Primers were as follows: GnRH, forward: 5'-CCGCTGTTGTTCTGTTGACT-3', reverse: 5'-GCAGATCCCTAAGAGGTGAA-3'. GnRHR, forward: 5'-TCACTCAGCCCTTAGCTGTCC-3', reverse: 5'-GAAGGCTTCATGCCACCATTG-3'. ERα, forward: 5'-TCAGGCTACCATTACGGAGT-3', reverse: 5'-CGCTTGCTTCAACATTCT-3'. GPR30, forward: 5'-TCATTTCTGCCATGCACCCA-3', reverse: 5'-GTGGACAGGGTGTCTGATGT-3'. β-actin, forward: 5'-GGAGATTACTGCCCTGGCTCCTA-3', reverse: 5'-GACTCATCGTACTCCTGCTTGCTG-3'. At last, 2^{-ΔΔCt} method was used for the calculation of relative mRNA expression.

Western Blot

Hypophysis tissues were ground into powder in liquid nitrogen. Cell lysate was added into the hypophysis tissues powder to collect total proteins on ice. The concentration of total proteins was detected using BCA assay kit (Pierce Chemical Company, Rockford, IL, USA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins in each sample. After being transferred onto a polyvinylidene fluoride (PVDF) membrane, total proteins were experienced blocking with 5% skim milk for 1 h. Rabbit anti-GnRH, anti-GnRHR, anti-ERα and anti-GPR30 primary antibodies (1:1000, Cell Signaling, Danvers, MA, USA) were used to incubate the membrane at 4°C overnight. The membrane was then washed with Tris-buffered saline/0.1% Tween (TBST) for 3 times with 10 min per time. Thereafter, horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:5000, Solarbio, Beijing, China) was added onto the membrane for 1 h incubation at room temperature. The membrane was washed with TBST for 3 times again. The proteins bands were visualized using enhanced chemiluminescence (ECL) reagent. β-actin was used as the control.

Immunohistochemistry

The expression of GnRH in the hypothalamus and GnRHR in the ovary was detected by immunohistochemistry. In short, the hypothalamus and ovary were subjected to fix with 4% paraformaldehyde and then were prepared into sections with a thickness of 5 µm. The sections were blocked with 5% bovine serum albumin for 1 h. Rabbit anti-GnRH and anti-GnRHR (1:250, Lichen Biotechnology Co., Ltd., Shanghai, China) was used to treat the sections overnight at 4 °C. Subsequently, biotin-labeled anti-rabbit IgG antibody (1: 200, Solarbio, Beijing, China) was added onto the sections for 2 h incubation at room temperature. Sections were then stained with 3, 3'-diaminobenzidine (DAB) and counterstained with hematoxylin. After being dehydrated and transparentized, the sections were sealed in neutral resin and observed under a microscope. Brown-yellow particles were considered to be positive GnRH and GnRHR expression signals.

Statistical analysis

In this research, all data were expressed as mean \pm standard deviation based on three independent repeated trials. SPSS 19 software (SPSS Inc., Chicago, IL, USA) was used to process the data. GraphPad Prism (version 5, La Jolla, CA, USA) was used for the making of statistical graphs. Student's t-test was responsible for the comparison between two groups. One-way analysis of variance was applied for the comparison among at least three groups. $P < 0.05$ indicated the statistically significant differences.

Result

SXF reduced the volume of sex organs in female rat model with CPP

After being sacrificed, the bilateral ovaries, uterus, hypothalamus and hypophysis of rats in each group was obtained and photographed. As shown in Figure 1A B, relative to Control group, rats of CPP group had obviously larger volume of bilateral ovaries, uterus, hypothalamus and hypophysis. Interestingly, in comparison with CPP group, the volume of bilateral ovaries, uterus, hypothalamus and hypophysis of rats in CPP-LP group, CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group was all reduced. Among the three groups treated with different SXF concentrations, rats in CPP-HD-SXF group exhibited the smallest volume of bilateral ovaries, uterus, hypothalamus and hypophysis.

SXF reduced sex organ index in female rat model with CPP

The sex organ index of rats was monitored, including ovary index, uterus index, hypothalamus index and hypophysis index. The ovary index of rats in CPP group was seriously elevated when compared with Control group ($P < 0.05$). However, relative to CPP group, rats of CPP-LP group exhibited much lower ovary index ($P < 0.05$). Compared with CPP-LP group, the ovary index of rats in CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group was not obviously changed (Figure 2A). Furthermore, much higher uterus index was found in rats of CPP group when compared with Control group ($P < 0.05$). In comparison with CPP group, the uterus index of rats in CPP-LP group was markedly reduced ($P < 0.01$). The uterus index difference between CPP-LP group and CPP-HD-SXF group was not statistically significant (Figure 2B). In terms of hypothalamus index, rats of CPP group present much higher hypothalamus index than Control group ($P < 0.05$). Conversely, the hypothalamus index of rats in CPP-LP group was obviously lower than that in CPP group ($P < 0.05$). No significant change in hypothalamus index was observed in CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group when compared with CPP-LP group (Figure 2C). Moreover, the hypophysis index of rats in CPP group was significantly increased when relative to Control group ($P < 0.01$). On the contrary, rats of CPP-LP group exhibited remarkably decreased hypophysis index when compared with CPP group ($P < 0.01$). In comparison with CPP group, the hypophysis index in rats of CPP-HD-SXF group and CPP-MD-SXF group was not obviously changed (Figure 2D).

SXF inhibited maturation of follicles and thickening of the uterine wall in female rat model with CPP

The ovary and uterus of rats were experienced HE staining to observed the growth of follicles and thickness of the uterine wall. For rats of Control group, the follicles were mainly primordial follicles, primary follicles and a small number of secondary follicles. Tertiary follicles were not found in the ovary of rats in Control group. However, multiple secondary follicles and tertiary follicles were observed in the ovary of rats in CPP group. Relative to CPP group, the number of secondary follicles and tertiary follicles were decreased in rats of CPP-LP group, CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group. Obviously, CPP-HD-SXF group exhibited less secondary follicles and tertiary follicles than CPP-MD-SXF group and CPP-LD-SXF group (Figure 3A). In addition, compared with Control group, the uterine wall of rats in CPP group was obviously thickened. In comparison with CPP group, the uterine wall thickness of rats in CPP-LP group, CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group was obviously diminished. The uterine wall thickness of rats in CPP-HD-SXF group was much thinner than that of CPP-MD-SXF group and CPP-LD-SXF group (Figure 3B).

SXF declined estrogen concentration in peripheral blood in female rat model with CPP

The concentration of important estrogen in the peripheral blood of rats was detected. Rats of CPP group showed much increased FSH concentration than that of Control group ($P < 0.01$). The FSH concentration was significantly decreased in rats of CPP-LP group when compared with CPP group ($P < 0.01$). Relative to CPP-LP group, the FSH concentration in rats of CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group was not obviously changed (Figure 4A). In addition, the LH concentration of rats in CPP group was distinctly higher than that of Control group ($P < 0.01$). However, relative to the LH concentration of rats in CPP group, it was seriously reduced in CPP-LP group ($P < 0.001$). Compared with CPP-LP group, the change in LH concentration was not obvious in CPP-HD-SXF group and CPP-MD-SXF group (Figure 4B). Detection of PRL concentration showed that, compared with Control group, remarkably higher PRL concentration was found in rats of CPP group ($P < 0.001$). In comparison with CPP group, the PRL concentration was dramatically diminished in rats of CPP-LP group ($P < 0.01$). Relative to CPP-LP group, obviously changes in PRL concentration was not observed in rats of CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group (Figure 4C). Moreover, the E2 concentration was much higher in rats of CPP group when relative to Control group ($P < 0.01$). Oppositely, prominently lower E2 concentration was observed in rats of CPP-LP group when compared with CPP group ($P < 0.01$). No significant statistical difference in E2 concentration was observed in CPP-HD-SXF group and CPP-MD-SXF group when relative to CPP-LP group (Figure 4D).

SXF suppressed the expression of sex hormone receptor related genes in female rat model with CPP

The expression of sex hormone receptor related genes in hypophysis was explored. qRT-PCR results indicated that, rats of CPP group had distinctly higher mRNA expression of GnRH, GnRHR, ER α and GPR30 than that of Control group ($P < 0.01$ or $P < 0.001$). When relative to CPP group, the GnRH, GnRHR, ER α and GPR30 mRNA expression was markedly reduced in rats of CPP-LP group ($P < 0.05$ or $P < 0.01$). In comparison with CPP-LP group, the change in the GnRH, GnRHR and ER α mRNA expression was not obvious in CPP-HD-SXF group and CPP-MD-SXF group. Meanwhile, no statistically significant difference

was found in the GPR30 mRNA expression between CPP-LP group and CPP-HD-SXF group (Figure 5A). According to Western blot, obviously increased GnRH, GnRHR, ER α and GPR30 proteins expression was found in rats of CPP group when compared to Control group. However, relative to CPP group, the GnRH, GnRHR, ER α and GPR30 proteins expression was all seriously reduced in CPP-LP group. When compared to CPP-LP group, not obvious change was found in GnRH, GnRHR and GPR30 proteins expression in rats of CPP-HD-SXF group and CPP-MD-SXF group. At the same time, the ER α expression was not obvious changed in rats of CPP-HD-SXF group when compared with CPP-LP group (Figure 5B).

Furthermore, GnRH expression in hypothalamus and GnRHR expression in ovary was explored by immunohistochemistry. As shown in Figure 5C D, rats of CPP group exhibited more positive signals of GnRH and GnRHR proteins than that of Control group. Conversely, when compared with CPP group, the positive signals of GnRH and GnRHR proteins were significantly declined in rats of CPP-LP group. Unobvious change was occurred in the positive signals of GnRH and GnRHR proteins in rats of CPP-HD-SXF group, CPP-MD-SXF group and CPP-LD-SXF group when relative to CPP-LP group.

Discussion

It is well known that the onset of puberty begins with the increase of GnRH released by the hypothalamus, which in turn leads to the activation of the HPG axis [10]. CPP is also caused by the early activation of the HPG axis, which results in the early development of uterus, ovary and early appearance of the corpus luteum. This process eventually causes the occurrence of CPP [2, 11]. CPP seriously affects the physical development and mental health of children, but also increases the risk of metabolic diseases related to CPP [12]. Therefore, the implementation of effective CPP treatment is very crucial. This article investigated that SXF possessed effective therapeutic effect for the treatment of female rat model with CPP.

The function of NMA to promote precocious puberty in female rat has been confirmed in previous studies, and NMA with a dose of 40 mg/kg was found to be effective to establish female rat model with precocious puberty [13, 14]. Thus, in this research, female rat model with CPP was successfully established by subcutaneous injection of NMA (40 mg/kg). LP is a kind of GnRH analogue, which is commonly used in clinical treatment of childhood CPP in the clinic [15, 16]. Research had shown that LP with a dose of 100 μ g/kg could control the development of gonads in rats to the greatest extent [17]. In this study, CPP female rats treated by LP were thus served as the positive control. We noticed that, similar to LP, SXF possessed effective therapeutic effect for female rat with CPP, including inhibiting follicular development and thickening of the uterine wall, reducing sex organs volume and index, suppressing hormone levels and expression of sex hormone receptor-related genes. These evidences clearly revealed the therapeutic effect of SXF on CPP.

In CPP female rat model, the increased expression of GnRH was observed in the hypothalamus and hypophysis. At the same time, the elevated expression of GnRHR in hypophysis and ovary was also detected. GnRH is the key to regulating reproductive function [18]. The concentration of GnRH is difficult

to detect in the blood, and thus the detection of GnRH expression in the hypothalamus is commonly used [11]. Researchers have reported that the elevated secretion of GnRH in the hypothalamus can combine with GnRHR in the hypophysis, thereby promoting the secretion of LH and FSH. Thereafter, LH and FSH further stimulate the synthesis of E2 and PRL by acting on the gonads, which ultimately promotes the development of sex organs, such as the development of the uterus and ovaries as well as the formation and maturation of follicles [19]. Thus, the detection of LH and FSH levels in the blood is important indicators for the clinical evaluation of the therapeutic effect of CPP. Results from this paper indicated that SXF could effectively reduce the expression of GnRH, GnRHR and estrogen concentration (including FSH, LH, PRL and E2) in peripheral blood in female rat model with CPP. As a result, sex organs volume and index, the thickening of the uterine wall and the maturation of the follicles were subsequently suppressed. Therefore, SXF might achieve therapeutic effects on CPP through inhibiting the GnRH expression in the hypothalamus and GnRHR expression in the hypophysis. Then this further suppressed the secretion of estrogen, thereby inhibiting the development of sex organs and delaying the activation of the HPG axis.

This study also monitored that in female rat model with CPP, the expression of ER α and GPR30 in hypophysis was aberrantly elevated. Interestingly, the treatment with SXF prominently reduced the expression level of ER α and GPR30 in hypophysis in CPP female rat model. Estrogen is a crucial factor which triggers CPP and the onset of puberty. ER α and GPR30 are two kind of estrogen receptor which can mediate the expression of estrogen. The increased expression of ER α and GPR30 can enhance the sensitivity of estrogen receptor, which is closely related to the etiology of CPP [20–22]. It was also reported that GPR30 could modulate the homeostasis of ER α [23]. Data from this research firstly illustrated that SXF could suppress the expression of ER α and GPR30 in hypophysis in CPP female rat model.

Conclusions

Collectively, this article discovered that SXF has effective therapeutic effects on female rat model with CPP. SXF reduced estrogen concentration, including FSH, LH, PRL and E2, but also suppressed expression of sex hormone receptor related genes such as GnRH, GnRHR, ER α and GPR30. These evidences clearly revealed the therapeutic effect of SXF on CPP. Therefore, SXF should be promoted in clinical treatment of CPP.

Abbreviations

CPP: Central precocious puberty

SXF: Shugan Xiehuo Formula

FSH: Follicle stimulating hormone

LH: Luteinizing hormone

PRL: Prolactin

E2: Estradiol

GnRH: Gonadotropin-releasing hormone

GnRHR: Gonadotropin-releasing hormone receptor

ER α : Estrogen receptor alpha

GPR30: G protein-coupled receptor 30

HPG: Hypothalamic-pituitary-gonad

GnRHa: Gonadotropin-releasing hormone analogues

NMA: N-methyl-DL-aspartic acid

HE: Hematoxylin-eosin

qRT-PCR: Quantitative real-time polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

PVDF: Polyvinylidene fluoride

TBST: Tris-buffered saline/0.1% Tween

ECL: Enhanced chemiluminescence

DAB: 3, 3-diaminobenzidine

Declarations

Acknowledgements

Not applicable.

Authors' contributions

WY, LX and JX designed the research. SL, KZ, YX, DM and TS performed the experiments. SL and JX analyzed data. WY and LX wrote the manuscript with contributions from all authors. All authors read and approved the final manuscript.

Funding

This study was supported by 2018 National Natural Science Foundation of China (Grant No. : 81860869)

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental protocols and animal treatment procedures are approved by the Animal Experiment Ethics Committee of Yunnan University of Chinese Medicine (Pemit Number: R-082018033).

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author details

¹Department of Pediatrics, Nanjing University of Chinese Medicine, Nanjing 210023, Jiangsu Province, China

²Department of Pediatrics, Yunnan University of Chinese Medicine, Kunming 650500, Yunnan Province, China

³Department of Pharmacy, Yunnan Provincial Hospital of Traditional Chinese Medicine, Kunming 650021, Yunnan Province, China

⁴Department of Internal Medicine, Yunnan University of Chinese Medicine, Kunming 650500, Yunnan Province, China

References

1. Chen M, Eugster EA. Central Precocious Puberty: Update on Diagnosis and Treatment. *Paediatr Drugs*. 2015;17(4):273–81.
2. Aguirre RS, Eugster EA, Central Precocious Puberty: From Genetics to Treatment. *Best Practice & Research Clinical Endocrinology & Metabolism*, 2018. 32(4).
3. Sang LH, et al. Increased final adult height by gonadotropin-releasing hormone agonist in girls with idiopathic central precocious puberty. *Plos One*. 2018;13(8):e0201906.
4. Eugster EA. Treatment of Central Precocious Puberty. *J Endocr Soc*. 2019;3(5):965–72.
5. Arcari AJ, et al., One-year treatment with gonadotropin-releasing hormone analogues does not affect body mass index, insulin sensitivity or lipid profile in girls with central precocious puberty. *Journal of*

- Pediatric Endocrinology & Metabolism, 2019.
6. Bereket A. A Critical Appraisal of the Effect of Gonadotropin-Releasing Hormon Analog Treatment on Adult Height of Girls with Central Precocious Puberty. *Journal of Clinical Research in Pediatric Endocrinology*, 2018: p. 33–48.
 7. Bereket A. A Critical Appraisal of the Effect of Gonadotropin-Releasing Hormon Analog Treatment on Adult Height of Girls with Central Precocious Puberty. *J Clin Res Pediatr Endocrinol*. 2017;9(Suppl 2):33–48.
 8. Weiping Y, Jie X, Yan S, et al. Clinical Study on Shugan Xiehuo Recipe in Treating 36 Cases of Idiopathic Central Sexual Precocity in Girls[J]. *Hebei Traditional Chinese Medicine*. 2015;37(7):997–8.
 9. Weiping Y, et al., Effect of Shuganxiehuo recipe on expressions of gonad,estrogen,GnRH and pituitary GnRH-R in the hypothalamus of female precocious rats. *pharmacology and clinics of chinese materia medica*, 2018.
 10. Mayer, et al., Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nature Neuroscience*, 2011.
 11. Karolina S, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. *Human Reproduction Update*, 2014(4): p. 4.
 12. He Y, et al., Precocious Puberty and the Lin28/Let7 Pathway: The Therapeutic Effect of the Nourishing “Yin” and Purging “Fire” Traditional Chinese Medicine Mixture in a Rat Model. *Evidence Based Complementary & Alternative Medicine*, 2018. 2018: p. 1–15.
 13. Zhou Q, et al., Expression of melatonin receptor in the hypothalamus and pituitary of female rats with precocious puberty. *Acta Universitatis Medicinalis Nanjing*, 2013.
 14. Cheng M, et al. [Experimental study on therapeutic effect of Dabuyin Wan on true precocious puberty in female rats]. *China Journal of Chinese Materia Medica*. 2013;38(3):386–90.
 15. José, et al., Neurological improvement in patients with chronic spinal cord injury treated with leuprolide acetate, an agonist of GnRH. *Acta Neurobiologiae Experimentalis*, 2018.
 16. Flavia, et al., Gonadotropin and Estradiol Levels after Leuprolide Stimulation Tests in Brazilian Girls with Precocious Puberty. *Journal of Pediatric & Adolescent Gynecology*, 2015.
 17. Periti DP, Mazzei T, Mini E. Clinical Pharmacokinetics of Depot Leuprorelin. *Clin Pharmacokinet*. 2002;41(7):485–504.
 18. Larco DO, et al. Abstract 944: The effects of GnRH-(1–5) on endometrial cancer cell lines. *Can Res*. 2016;76(14 Supplement):944–4.
 19. Gianetti E, Seminara S. Kisspeptin and KISS1R: a critical pathway in the reproductive system. *Reproduction*. 2008;136(3):295–301.
 20. H., W.H., et al., 505 - Activation of G Protein-Coupled Receptor 30 (GPR30), a Novel Estrogen Receptor, by the Potent GPR30-Selective Agonist G-1 Greatly Enhances Cholesterol Cholelithogenesis in Female Mice. *Gastroenterology*, 2018. 154(6): p. S-1093-S-1094.

21. Martin SG, et al. Low expression of G protein-coupled oestrogen receptor 1 (GPER) is associated with adverse survival of breast cancer patients. *Oncotarget*. 2018;9(40):25946–56.
22. Hae, et al., Estrogen receptor α gene analysis in girls with central precocious puberty. *Journal of Pediatric Endocrinology & Metabolism Jpem*, 2013.
23. Ariazi E, et al., GPR30 modulates estrogen-stimulated proliferation of breast and endometrial cancer cells by regulating estrogen receptor alpha homeostasis. *Cancer Research*, 2008. 68.

Figures

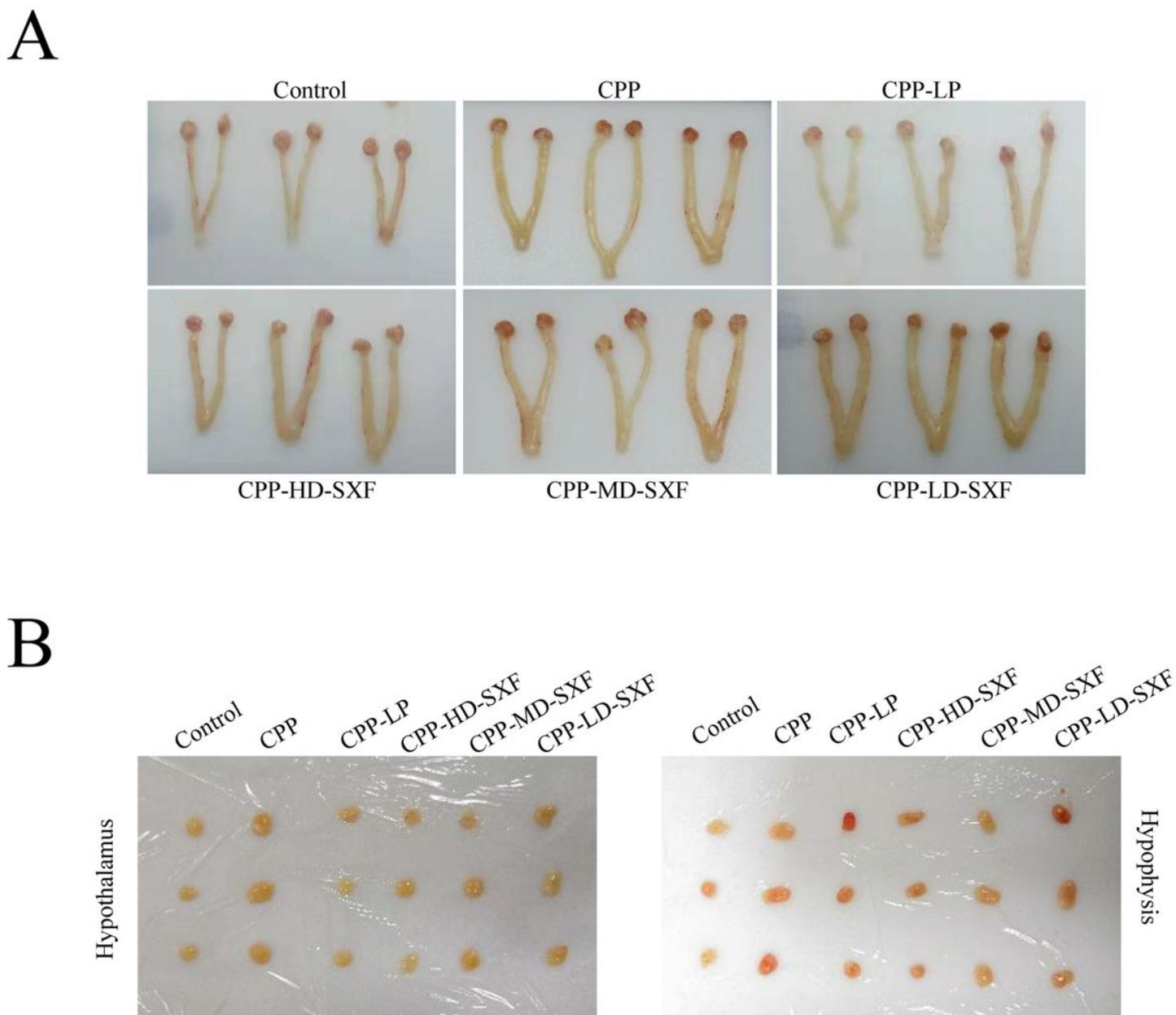


Figure 1

SXF reduced the volume of sex organs. (A) The bilateral ovaries and uterus of female rat model with CPP was obtained and photographed after being sacrificed. (B) The hypothalamus and hypophysis of female rat model with CPP was obtained and photographed after being sacrificed.

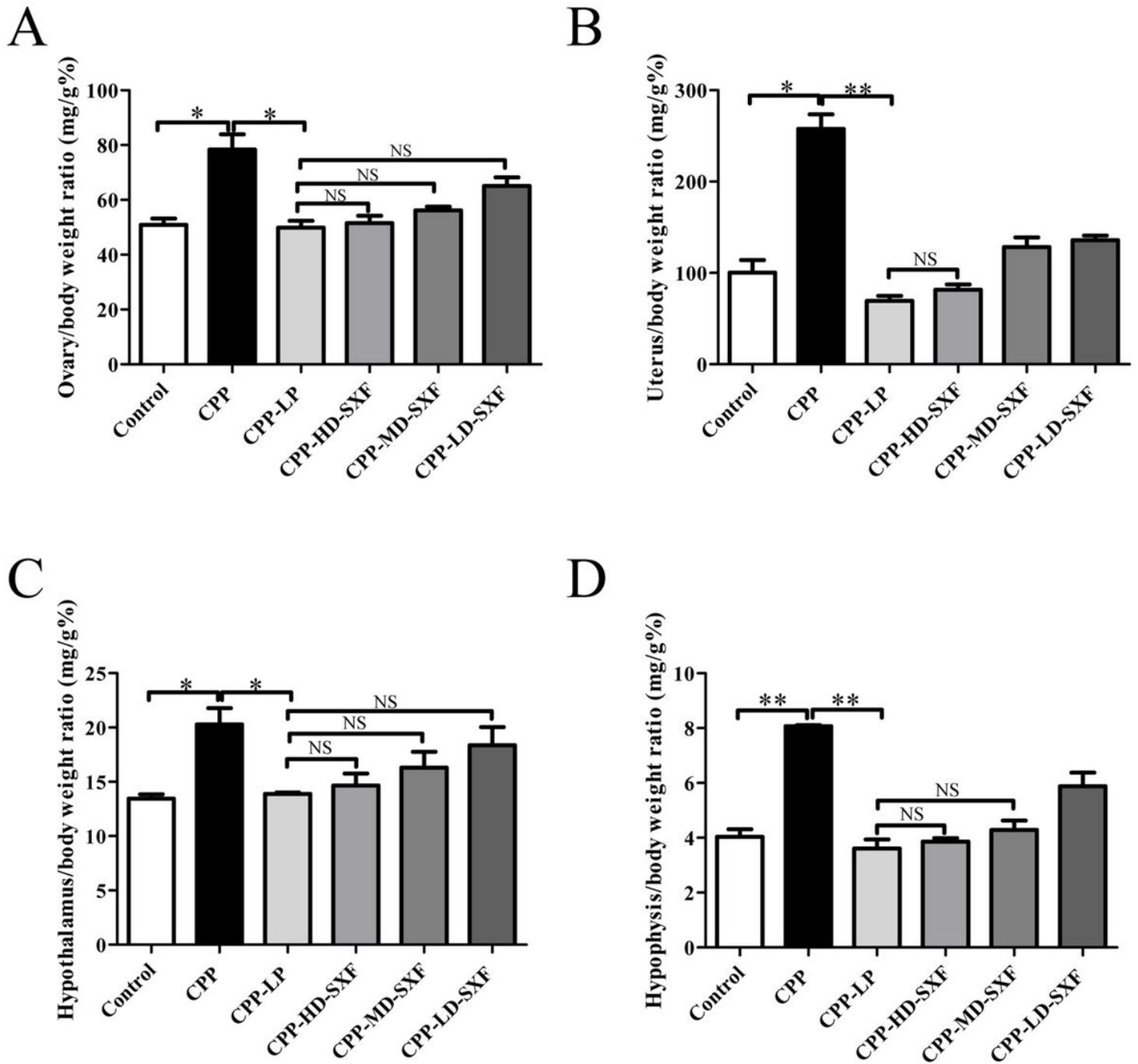
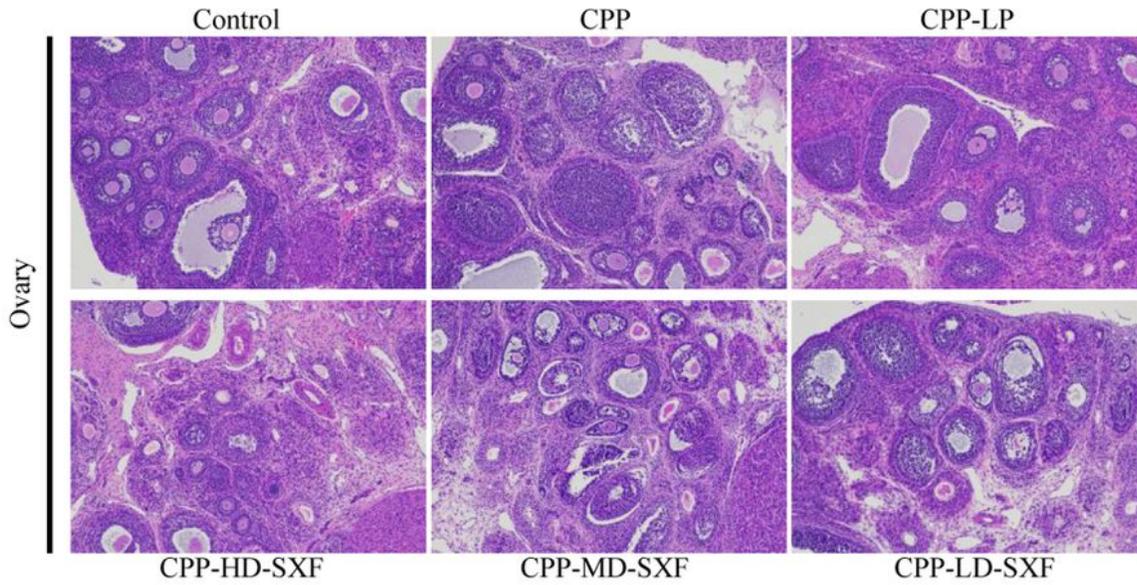


Figure 2

SXF reduced sex organ index. (A) SXF decreased ovary index in female rat model with CPP. (B) SXF reduced uterus index in female rat model with CPP. (C) SXF declined oviduct index in female rat model with CPP. * $P < 0.05$. ** $P < 0.01$. NS indicated that there was no significant statistical difference between two groups.

A



B

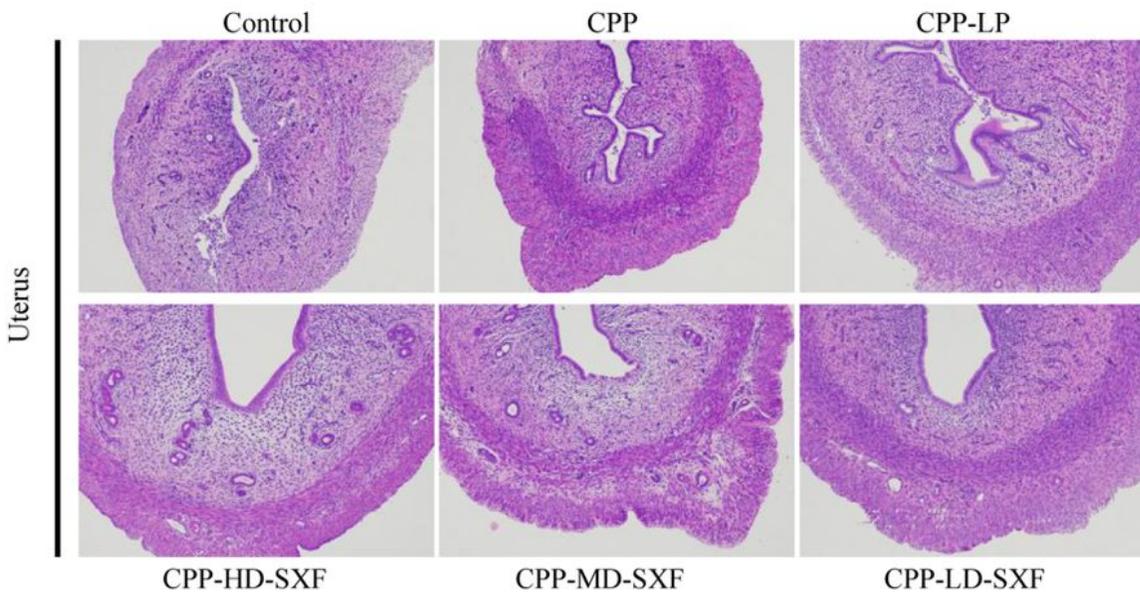


Figure 3

SXF inhibited maturation of follicles and thickening of the uterine wall. (A) SXF inhibited maturation of follicles in female rat model with CPP. (B) SXF inhibited thickening of the uterine wall in female rat model with CPP.

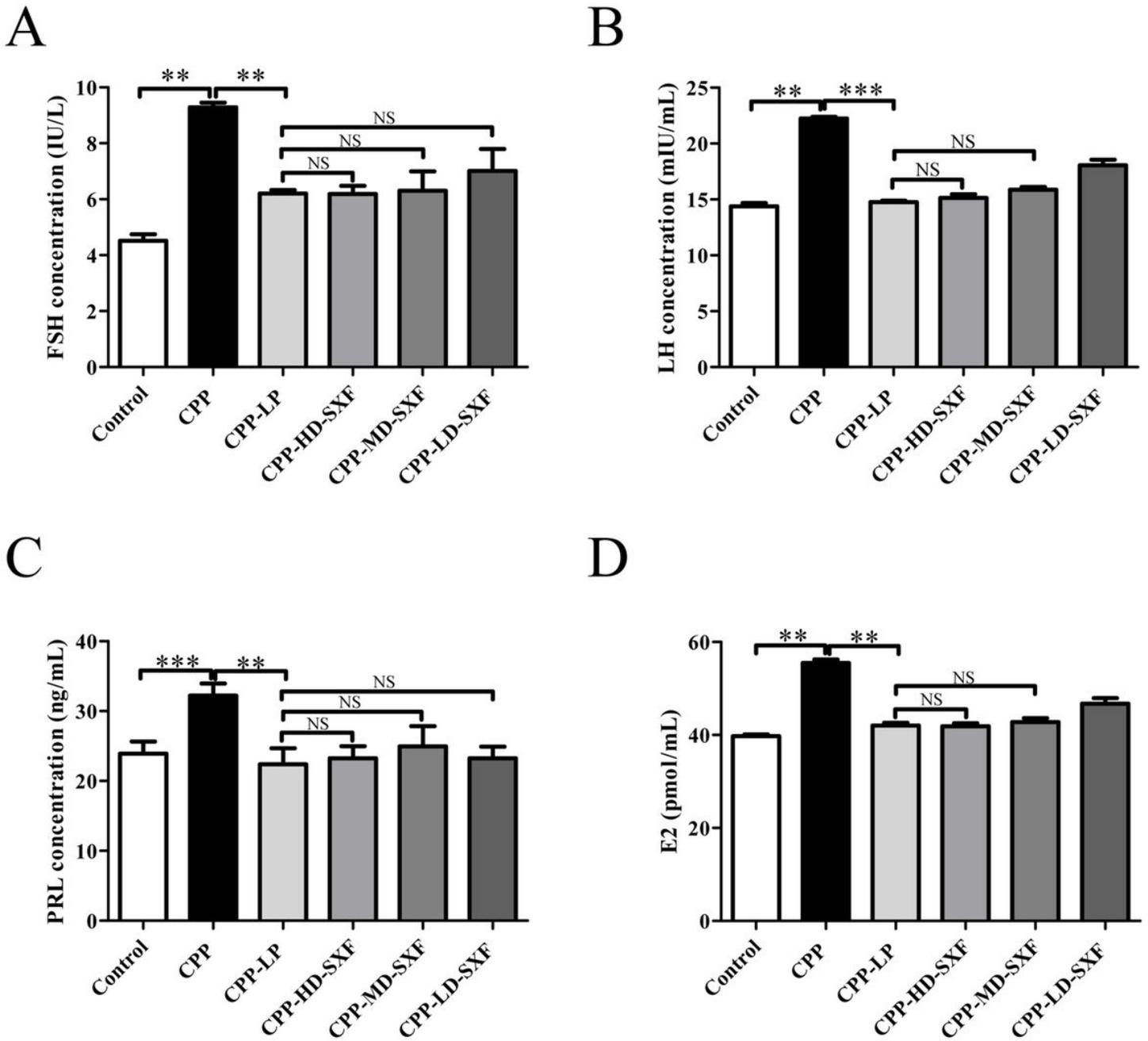
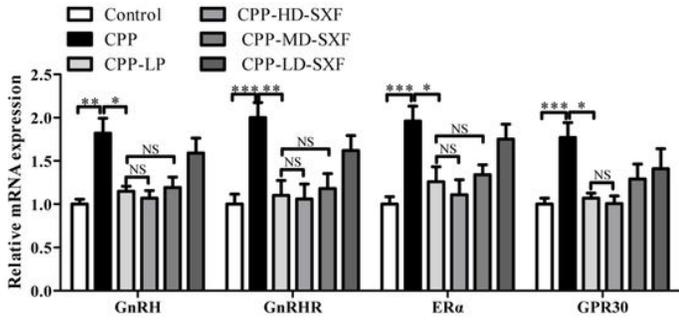


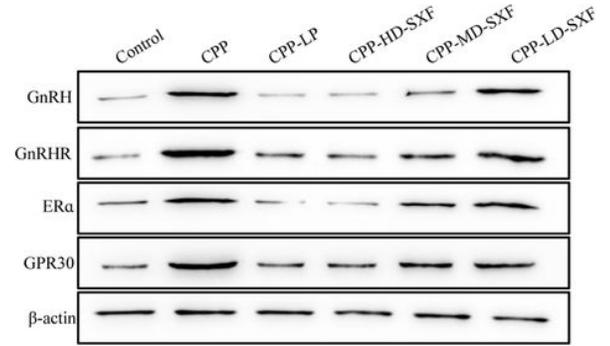
Figure 4

SXF declined estrogen concentration in peripheral blood. (A) SXF decreased FSH concentration in the peripheral blood of female ratmodel with CPP. (B) SXF reduced LH concentration in the peripheral blood of female ratmodel with CPP. (C) SXF declined PRL concentration in the peripheral blood of female ratmodel with CPP. (D) SXF diminished E2 concentration in the peripheral blood of female ratmodel with CPP. ** $P < 0.01$. *** $P < 0.001$. NS indicated that there was no significant statistical difference between two groups.

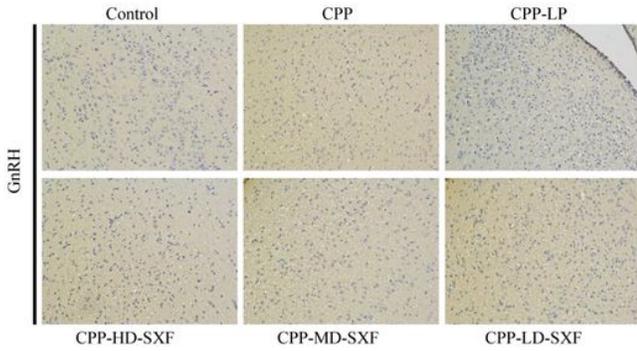
A



B



C



D

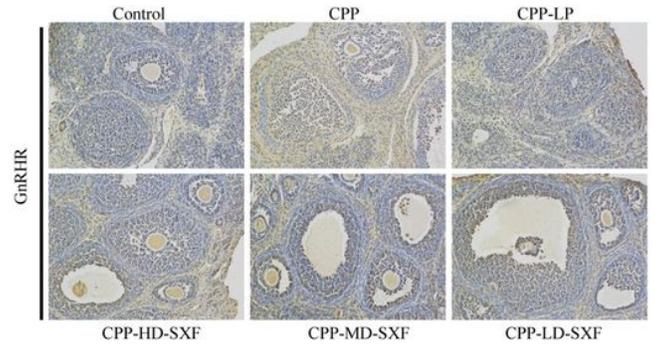


Figure 5

SXF suppressed the expression of sex hormone receptor related genes. (A) SXF suppressed GnRH, GnRHR, ERα and GPR30 mRNA expression in hypophysis in female rat model with CPP. (B) SXF reduced GnRH, GnRHR, ERα and GPR30 proteins expression in hypophysis in female rat model with CPP. (C) SXF reduced the positive signals of GnRH protein in hypothalamus according to immunohistochemistry. (D) Immunohistochemistry indicated that SXF decreased the positive signals of GnRHR protein in ovary. * P < 0.05. ** P < 0.01. *** P < 0.001. NS indicated that there was no significant statistical difference between two groups.