Troxerutin protects against DHT-induced polycystic ovary syndrome in rats

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Abstract

The exact pathogenesis of polycystic ovary syndrome (PCOS), the most common neuroendocrine disorder in women of reproductive age, has not been fully elucidated. Recent studies suggested that chronic inflammation and neurotransmitter disorder involved in the progress of PCOS. Troxerutin, a natural flavonoid, was reported to possess neuroprotective effect in several disease models by inhibiting inflammation or enhancing neurotrophic factor. In this study, we investigated the possible protective effect and mechanism of troxerutin in a dihydrotestosterone (DHT)-induced rat model of PCOS. The PCOS rat models were treated with troxerutin at a dose of 150mg/kg or 300mg/kg for up to 4 weeks. Results showed that 300mg/kg troxerutin significantly decreased the body weight gain and improved the pathological changes of ovary induced by DHT. Meanwhile, the elevated gonadotrophin-releasing hormone (GnRH), gonadotrophin and testosterone in the serum of PCOS rats were reduced with the treatment of troxerutin. The expression of kisspeptin and NKB in arcuate nucleus and their receptors kiss1r and NK3r in GnRH positive neurons of median eminence were markedly decreased in troxerutin-treated rats. Of note, the GnRH inhibitory regulator GABA and stimulatory regulator glutamate were also restored to the normal level by troxerutin. The present study indicated that troxerutin may exhibit a protective effect in PCOS rat model via regulating neurotransmitter release.

Introduction

Polycystic ovary syndrome (PCOS) is a reproductive endocrinopathy with the prevalence estimated to be 6% ~ 20%(depending on the different diagnostic criteria used), making it the most common endocrine condition in women of reproductive age [1]. The diagnostic features of PCOS include androgen excess, ovulatory dysfunction and polycystic ovaries[1]. Obesity is present in 30–60% of PCOS patients, depending on the country of origin[2, 3]. The most consistent biochemical abnormality in women with PCOS is hyper-secretion of androgen, elevated serum luteinizing hormone (LH) levels and low to normal serum follicle stimulating hormone (FSH) levels[4, 5]. Animal models that reflect PCOS features are crucial resources to investigate this syndrome. A chronically 5α-dihydrotestosterone (DHT)-treated rat model closely mimics the human PCOS phenotype and is a suitable model for investigations about PCOS.

The hypothalamus-pituitary-gonad (HPG) axis plays a central role in the exquisite neuroendocrine regulation of reproduction. Hypothalamic secretion of gonadotrophin-releasing hormone (GnRH), has been robustly established as the key pathway that controls reproductive function[6]. Axons of GnRH neurons protrude into the median eminence and release GnRH, which via hypophyseal portal system, enters anterior pituitary. Anterior pituitary, the primary target of GnRH, responds to stimulation by increasing the secretion of LH and FSH, which in turn lead to steroid production from the ovaries and stimulate follicle genesis and ovulation[7]. Thus, GnRH is known as the master hormone and provides the final common output of the network that regulates reproductive function.

GnRH is influenced by extrinsic factors such as neurotransmitters and neuropeptides, any alteration of the GnRH regulatory neurotransmitters, such as inhibitory gamma-aminobutyric acid(GABA) and stimulatory glutamate(Glu), may result in reproductive endocrine dysfunction[8]. Actually, the chronic anovulation characteristic of PCOS is attributed to increased central GnRH drive and resulting gonadotrophin aberrations, which likely result from the cumulative effect of altered GnRH stimulatory and inhibitory neurotransmitter in hypothalamus and pituitary gland[9]. Kisspeptin and subsequently discovered neurkinin B(NKB), two novel GnRH regulatory neuropeptides proved to be essential for normal GnRH secretion in humans, have come under intense spotlight in the last decade[10, 11]. The related discovery of kisspeptin-/ neurokinin B-/ dynorphin-(KNDy) pathway has further strengthened the understanding of GnRH secretion modulation[12]. KNDy neurons residing in the arcuate nucleus region of rodents co-express kisspeptin, NKB and DYN[12, 13]. NKB and kisspeptin derived from KNDy stimulate the release of GnRH by binding Kisspeptin 1 receptor (Kiss1r)and neurokinin 3 receptor(NK3r) expressed on GnRH neurons[14, 15].

Troxerutin, a trihydroxyethylated derivative of the naturally occurring plant flavonoid rutin, has been identified in tea, coffee, cereals, and a variety of fruits and vegetables. It has been reported that troxerutin possesses important biological activity such as anti-oxidant and anti-inflammatory effects and exerts beneficial effects on animal models of different central nervous system diseases [16, 17]. Troxertuin attenuated cognitive deficit and oxidative stress in mouse brain through decreasing reactive oxygen species[18],inhibited hippocampal neuron apoptosis in a rat model of Alzheimer's disease[19]. Since the hypothalamus plays an important role in reproduction, we supposed that troxerutin may have a protective effect against reproductive neuroendocrine dysfuction in PCOS. This study aimed to address this issue and preliminarily investigate the potential mechanism underlying its action.

Materials And Methods

Animals and troxerutin administration

Postnatal 14 days female SD rats were purchased from the Breeder center of Rodents (Jinan, China).The rats were housed under controlled lighting (12 h light, 12 h darkness, lights were turned on at 08:00 and turned off at 20:00) and temperature (24℃) with free access to food and water for a week of acclimatization. All experimental protocols used in this study were approved by the Animal Care and Ethical Committee of Xuzhou medical University. Postnatal 21 days rats weighing approximately 50–55 g, were randomly divided into four groups: sham group (each rat was implanted with a tube without DHT), PCOS group (each rat was implanted with a silastic tube filled with 5α- DHT at dose of 7.5 mg under anesthesia), PCOS + 150 mg/kg troxerutin group and PCOS + 300 mg/kg troxerutin group (PCOS rat with intraperitoneal injection of 150 mg/kg or 300 mg/kg troxerutin daily during the 4 weeks after implantation). DHT was procured from sigma, St. Louis, Mo and troxerutin (purity > 99%) was from Baoji Fangsheng Biotechnology Co., Ltd, Baoji, China. The rats were housed individually after surgery.

Blood And Tissue Sampling

At the terminal of the study, rats were decapitated and trunk blood was collected and centrifuged at 3000 rpm for 15 minutes. Plasma was separated and stored at -80℃ until analyzed for biochemical and hormonal analysis. Ovaries were cleaned in saline and managed fat free. Ovaries were fixed in 10% buffered formalin for 48 hours and paraffin-embedded. Paraffin-embedded tissue sections were de-waxed, and stained with hematoxylin and eosin (H&E). The section with the largest area was chosen for analysis to observe the follicles and corpora lutea in ovaries under microscope.

Body Weight, Biochemical And Western Blot Analysis

Body weight was measured every week after implantation for a total of 4 weeks.

The serum concentration of GnRH, LH, FSH and testosterone were measured via Enzyme Linked Immuno Sorbent Assay (ELISA) with the help of commercial kits (ELISA kit, CUSABIO, Inc, Wuhan, China)and the procedure was followed as given in the kit catalog. The concentration of GnRH, LH, FSH and testosterone was estimated by the standard curve.

To identify the hypothalamic GnRH status, regions of hypothalamus pituitary were homogenized to obtain protein samples. Then the protein (25 μg) was separated by SDS-PAGE and transferred to membranes. The membranes were incubated with mouse monoclonal anti-GnRH antibody (1:1000, MAB5456-C, Millipore, Billerica, MA) and mouse anti-GAPDH antibody (1:20000, Proteintech, Chicago, USA). After being washed with TBST three times, the membranes were incubated with IRDye-labeled secondary antibodies in TBST for 2 h. The bands on the membrane were scanned with an Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA) and the density of the bands was analyzed with ImageJ software.

Chromatographic Analysis

All standard biomarkers used for identification purpose in chromatographic studies were procured from Zhongke Co, Ltd, Beijing, China. High-performance liquid chromatography (HPLC)was performed on a Waters e2695 system(Waters, USA)equipped with a hypersil ODS column(Elite, Dalian, China). The mobile phase A was a mix of natrium aceticum(pH4.8),water and tetrahydrofuran with the ratio of 410:85:5, and mobile phase B was pure acetonitrile. Flow rate was 1 ml/min. The column temperature was set at 30℃. Samples were detected at the wavelength of 265 nm. Injection volume was 20 μL.

Immunofluorescent Staining

After intracardiac perfusion with normal saline followed by fixation with 4% cold paraformaldehyde (PFA), the rat brains were separated and postfixed in 4% PFA for 6 h at 4 °C. Then the brains were

incubated in 30% sucrose- 100 mM sodium phosphate buffer (pH 7.4) for 48 h at 4 °C. Coronal sections (20 μm) were made from the bregma anterior-posterior + 1.0 to − 1.0.

For immunofluorescence, the primary antibodies rabbit anti-NKB (1:1000, Novus biologicals, NB300-201), rabbit anti-kisspeptin (1:1000, H-048-56, Phoenix Pharmaceuticals), rabbit anti-Kiss-1r (1:500, AKR-001, Alomone Labs), rabbit anti-NK3r(1:500, abx217136, Abbexa) and mouse anti-GnRH (1:1000, MAB5456-C, Millipore, Billerica, MA) were used. After incubating with the antibodies for 24 h at 4 °C, the sections were washed with PBS and then were treated with goat anti-mouse IgG (H + L) Alexa Fluor ®555 or 488 - conjugated or goat anti-rabbit IgG (H + L) Alexa Fluor ®488 (Invitrogen, Eugene, OR, USA) secondary antibodies. According to the manufacturer's instructions, DAPI (Beyotime Biotechnology, Shanghai, China) was used to label nucleus. For negative controls, sections were incubated with PBS instead of the primary antibodies. Fluorescence images were captured using a Zeiss Axioskop 40 microscope(Carl Zeiss, Oberkochen, Germany). For quantitative analysis of the NKB and kisspeptin positive cells in the arcuate nucleu(ARC), integral optical density(IOD) and a region of interest were measured by Image-Pro Plus 6.0 software using the following equation: ΣIOD/ΣArea. The numbers of GnRH/kiss1r and GnRH/NK3r double-labeled positive cells were determined in an area of 350 μmx350 μm within the median eminence (ME).

Statistical analysis

All statistical analyses were performed with SPSS software (version 16.0), and the data were analyzed using the Student's t-test or one-way Analysis of Variance (ANOVA). The data were expressed as the mean ± s.e.m. Statistical significance was set at $P < 0.05$ for all tests.

Results

Troxerutin reduced obesity and improved abnormal ovarian morphology in PCOS rats

Given that obesity is a major clinical feature of PCOS patients, we first investigated whether troxerutin administration has any influence on body weight in DHT-induced PCOS rat models. The time course of troxerutin administration and evaluation of phenotypes were illustrated (Fig. 1). Body weight for individual animal was weighed weekly after DHT implantation for up to 4 weeks. Body weight at baseline and 1st week showed no significant difference among all groups. DHT-treated rats showed significantly higher body weight at 2nd ($P < 0.01$), 3rd($P < 0.01$) and 4th ($P < 0.001$) week than the age-matched sham rats which denoted normal body weight, indicating that PCOS group have gained more body weight, hence a PCOS-like obesity feature. In comparison to PCOS group, treatment with troxerutin 150 mg/kg did not induce any significant changes. Nevertheless, the body weight of PCOS + 300 mg/kg troxerutin group were markedly reduced as compared to that of PCOS group at 2nd ($P < 0.05$),3rd($P < 0.01$) and 4th ($P < 0.05$)week, suggesting that troxerutin alleviated obesity in PCOS. Since ovarian histological change is another feature of PCOS, we also investigated whether troxerutin improved histological structure in rat

ovaries. As demonstrated in Fig. 1b, normal ovarian histological features were observed in sham sections, which generally exhibited corpora lutea and follicles in various stages of development. In ovarian sections of PCOS rats, microscopic examination confirmed the absence of corpora lutea and the presence of cystic follicles with saccular dilation. As compared to the multi-cystic follicles in ovaries of PCOS rats, the number of cystic follicles in troxerutin groups showed tendency toward decreasing. These findings suggest that troxerutin is a positive regulator of obesity and abnormal ovarian histological structure in PCOS rats.

Troxerutin reversed the abnormal serum levels of gonadotrophin and testosterone in PCOS rats

Serum LH, FSH and testosterone levels were measured at the fourth week by ELISA (Fig. 2). PCOS rats showed a significant increase in serum LH and testosterone levels compared to sham rats ($P < 0.001$; $P < 0.05$), which was in accordance with the characteristic LH and testosterone elevation in patients with PCOS. Though there was no significant difference in FSH levels among all four groups, administration of troxerutin to rats for 4 weeks significantly reversed the elevated serum LH (300 mg/kg; $P < 0.01$) and testosterone levels (both 150 mg/kg and 300 mg/kg; $P < 0.05$), suggesting that troxerutin has a beneficial effect on the aberrant gonadotrophin and testosterone in PCOS rats.

Troxerutin reduced the elevated GnRH levels in PCOS rats

The origin of LH and FSH alteration often times lies at hypothalamic GnRH level, which plays a pivotal role in stimulating pituitary release of gonadotrophin, thus we further detected the serum and hypothalamic-pituitary status of GnRH. As shown in Fig. 3a, PCOS rats demonstrated significantly increased serum levels of GnRH as compared to sham rats($P < 0.001$). Troxerutin 300 mg/kg treatment exhibited significant decrease in serum GnRH level compared to PCOS group($P < 0.001$). Similar to the Elisa data, the western blotting results also showed increased GnRH in hypothalamic-pituitary of the PCOS rats ($P < 0.01$), however, troxerutin 300 mg/kg markedly decreased the level of GnRH compared to the PCOS rats($P < 0.05$; Fig. 3b).

Troxerutin altered GnRH regulatory neurotransmitters in the hypothalamus of PCOS rats

GnRH could be influenced by regulatory neurotransmitters such as the major inhibitory GABA and stimulatory Glu. As depicted in Fig. 4, neurotransmitter levels in the hypothalamus showed significant difference among certain groups. To be specific, PCOS rats exhibited markedly low GABA ($P < 0.01$) and high Glu ($P < 0.01$) compared to sham rats. Although no significant difference was observed between troxerutin 150 kg/mg group and PCOS group, administration of troxerutin 300 kg/mg to rats for 4 weeks successfully reversed the notably low GABA($P < 0.05$) and high Glu ($P < 0.01$) levels in the hypothalamus compared to PCOS group.

Troxerutin reduced the expression of Kisspepetin1/Kiss1r and Neurokinin B/NK3r in the hypothalamus

Kisspeptin and NKB secreted from the KNDy neurons in ARC, are considered to be novel GnRH stimulatory neurotransmitters and stimulate expression of kisspeptin receptor (kiss1r) and neurokinin B receptor (NK3r) of GnRH neurons in the median eminence (ME). Here, we carried out immunofluorescence staining to observe whether troxerutin administration alters the expression of kisspeptin/ kiss1r and NKB/ NK3r in their respective regions. The IOD of positive cells in the ARC were markedly increased in PCOS rats compared to sham rats ($P < 0.001$; $P < 0.001$), troxerutin 300 mg/kg treatment significantly reversed the increase of kisspeptin and NKB IOD induced by DHT ($P < 0.01$; $P < 0.01$). Meanwhile, the changes of the expression of kiss1r and NK3r in ME were also observed. The numbers of GnRH/kissr1 and GnRH/NK3r double-labeled positive cells increased in the PCOS group compared to the sham group ($P < 0.001$; $P < 0.01$), however, they were significantly decreased in the 300 mg/kg troxerutin-treated rat ($P < 0.01$; $P < 0.01$) .

Discussion

PCOS is the most common endocrine condition affecting approximately 20% of reproductive -aged women. Though some ovulation induction agents, such as letrozole, clomiphene citrate and metformin, have been used to improve reproductive outcomes in clinic, the side effects of these drugs should not be ignored[20, 21]. Thus, numerous studies attempted to guide the development of new and effective therapies for PCOS. Recently, treatment based on traditional Chinese medicine and food ingredients, such as AF extract, soy isoflavones and crocetin, provided a novel therapeutic way for PCOS [22–24]. All these observations suggested rooms for improvement in PCOS therapies. Possession of beneficial effects on animal models of different central nervous system diseases made troxerutin, a rutin derivative, an attractive therapeutic method for us to investigate its possible effect on PCOS. Specifically, troxerutin inhibited cyclin-dependent kinase 1 expression, enhanced type 1 protein phosphatase α dephosphorylation and abolished MEK/ERK1/2/C/EBP β activation, which subsequently reversed the memory impairment in the DA-treated mice[25]. The neuroprotective potential of troxerutin in 6-OHDA rat model of Parkinson's disease was through mitigation of apoptosis ,astrogliosis, oxidative stress and part of its effect was dependent on PI3K/ERβ signaling[26]. Troxerutin and cerebroprotein hydrolysate injection acted as a neuroprotective agent against cerebral ischemia/reperfusion injury via anti-inflammation, anti-apoptosis and blood-brain barrier maintenance[27]. In the present study, we evaluated the possible effect of chronic administration of troxerutin in a DHT-induced PCOS rat model, and two important findings were revealed. First, administering troxerutin 300 mg/kg exerted a beneficial effect of reducing body weight, the elevated levels of LH, testosterone and GnRH in PCOS rats and its effect was superior to troxerutin 150 mg/kg. Second, the potential mechanism behind the observed effect on overweight, polycystic ovaries and endocrine aberration involved altering GABA, glutamate, kisspeptin/kiss1r and NKB/NK3r in the hypothalamic pituitary region. To the best of our knowledge, this is the first study to investigate the possible effect of troxerutin on PCOS using a rat model.

GnRH pulses stimulate the synthesis and secretion of LH and FSH from anterior pituitary. It is well known that although produced in the same cell named gonadotroph, LH and FSH synthesis is regulated by different frequency of GnRH pulses, with LH favored by fast pulse frequencies(> 1 pulse per hour) and FSH favored by slow pulse frequencies(< 1 pulse per 2–3 hour). As for PCOS, a neuroendocrine hallmark is persistent and rapid GnRH pulses, which favor pituitary synthesis of LH and contribute to the increased LH levels[28]. LH is a stimulus for androgen synthesis, so the increased LH drive in turn caused the elevated androgens [29, 30]. Therefore, in terms of biochemical indicators, the most important and characteristic abnormality referring to PCOS is elevated LH, subsequently elevated testosterone and low to normal FSH levels in serum[4, 31]. Consistent with previous studies, PCOS rats displayed similar biochemical abnormality including markedly increased LH, concomitantly increased testosterone levels and non-significant FSH levels as compared to sham rats. Next, we observed the serum LH, testosterone and FSH levels in PCOS and troxerutin groups in order to determine whether troxerutin treatment has any therapeutic effect on PCOS. As the results revealed, troxerutin treatment indeed inhibited the elevated LH and testosterone levels without significant influence of FSH level. In addition, we further estimated the circulating and hypothalamic status of GnRH, which were both significantly increased in PCOS rats. However, there was a significant troxerutin-caused (300 mg/kg) decline in serum and hypothalamic GnRH. In regard to the serum hormone levels in rats, many previous studies have reported that LH were generally in the range of 1.5 ~ 3 mIU/ml[32, 33], FSH levels varied from 8mIU/ml to 20mIU/ml [22, 34]and testosterone varied from 0.2 to 10 ng/ml[32, 34–37], which were approximately consistent with the corresponding results in the present study. Conclusively, troxerutin treatment 300 kg/mg to rats for up to 4 weeks inhibited the hyperactive GnRH/LH system in PCOS rats.

Compelling evidence provided the strong support for brain being the culprit in PCOS[38, 39]. The secretion of GnRH from the brain is itself regulated by numerous upstream factors, such regulators include GABA, norepinephrine, dopamine, serotonin and glutamate ,amongst others[40, 41]. While specific impairments in the brain were difficult to assess in humans, rat models greatly facilitated the identification of difference underpinning the hyperactive HPG axis in PCOS. Therefore, the representative GnRH regulators of inhibitory GABA and stimulatory Glu were observed in the present study. In comparison to sham rats, PCOS rats showed a turnover of regulatory neurotransmitters with a significantly decreased GABA and increased Glu, which were both reversed by administering troxerutin 300 mg/kg to rats for up to 4 weeks. As far as the hypothalamic neurotransmitter levels were concerned, previous studies reported that hypothalamic GABA was in range of 12–20 μM/g and hypothalamic Glu was in range of 22–45 μM/g in rats ,each approximately consistent with our findings after unit conversion. To date, one arcuate nucleus population of particular recent interest has been the kisspeptin/neurokinin B/dynorphin expressing 'KNDy' neurons ,which synthesized and released GnRH stimulatory neurotransmitters kisspeptin and NKB. Protrusions of GnRH neurons in the ME express their receptors kiss1r and NK3r. In the present study, troxerutin decreased the expression of kisspeptin1 and NKB in the arcuate nucleus, and also the receptors kiss1r and NK3r in GnRH positive neurons of the ME. These results indicated that the protection of troxerutin against PCOS partially was due to its ability to regulate hypothalamic GABA, Glu, kisspeptin/kiss1r and NKB/NK3r. Although it remains determined whether this troxerutin-caused reversal

is permanent, the present study highlighted its therapeutic potential for PCOS. More in depth research will be necessary to determine whether troxerutin targeted at hypothalamic neurotransmitters alone is a promising therapeutic approach for the treatment of PCOS.

Declarations

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Authors' Contributions

Zixuan Gao and Xiaochen Ma performed the experiments, collected and analyzed the data and prepared the manuscript. Yuhang Ge, Lei Wang and Ping Fu performed the experiments and provided the data. Zhian Liu, Ruiqin Yao, Xiaonan Yan designed the study, provided financial support and edited the manuscript. All the authors approved the final approval of the manuscript.

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Availability of data and materials

All the data is contained in the manuscript.

Ethics approval

All animal experiments were approved by the Institutional Animal Care Committee of Xuzhou Medical University and in strict accordance with the Guidelines for the Care and Use of Laboratory Animals(National Research Council of People's Republic of China,2010)

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest to declare. The funders had no role in the study design; in the collection, analyses or interpretation of data; in the manuscript and decision to publish the results.

References

1. Goodarzi, M. O.; Azziz, R., Diagnosis, epidemiology, and genetics of the polycystic ovary syndrome. *Best practice & research. Clinical endocrinology & metabolism* **2006,** 20, (2), 193-205.
2. Hahn, S.; Tan, S.; Elsenbruch, S.; Quadbeck, B.; Herrmann, B. L.; Mann, K.; Janssen, O. E., Clinical and biochemical characterization of women with polycystic ovary syndrome in North Rhine-Westphalia. *Horm Metab Res* **2005,** 37, (7), 438-444.
3. Carmina, E.; Legro, R. S.; Stamets, K.; Lowell, J.; Lobo, R. A., Difference in body weight between American and Italian women with polycystic ovary syndrome: influence of the diet. *Hum Reprod* **2003,** 18, (11), 2289-2293.
4. Zumoff, B.; Freeman, R.; Coupey, S.; Saenger, P.; Markowitz, M.; Kream, J., A chronobiologic abnormality in luteinizing hormone secretion in teenage girls with the polycystic-ovary syndrome. *N Engl J Med* **1983,** 309, (20), 1206-1209.
5. Gilling-Smith, C.; Story, H.; Rogers, V.; Franks, S., Evidence for a primary abnormality of thecal cell steroidogenesis in the polycystic ovary syndrome. *Clin Endocrinol (Oxf)* **1997,** 47, (1), 93-99.
6. Tsutsumi, R.; Webster, N. J. G., GnRH pulsatility, the pituitary response and reproductive dysfunction. *Endocr J* **2009,** 56, (6), 729-737.
7. Kaiser, U. B.; Conn, P. M.; Chin, W. W., Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. *Endocr Rev* **1997,** 18, (1), 46-70.
8. Smith, M. J.; Jennes, L., Neural signals that regulate GnRH neurones directly during the oestrous cycle. *Reproduction* **2001,** 122, (1), 1-10.
9. Chaudhari, N.; Dawalbhakta, M.; Nampoothiri, L., GnRH dysregulation in polycystic ovarian syndrome (PCOS) is a manifestation of an altered neurotransmitter profile. *Reprod Biol Endocrinol* **2018,** 16, (1), 37-37.
10. de Roux, N.; Genin, E.; Carel, J.-C.; Matsuda, F.; Chaussain, J.-L.; Milgrom, E., Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* **2003,** 100, (19), 10972-10976.
11. Topaloglu, A. K.; Reimann, F.; Guclu, M.; Yalin, A. S.; Kotan, L. D.; Porter, K. M.; Serin, A.; Mungan, N. O.; Cook, J. R.; Imamoglu, S.; Akalin, N. S.; Yuksel, B.; O'Rahilly, S.; Semple, R. K., TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nature genetics* **2009,** 41, (3), 354-358.
12. Goodman, R. L.; Lehman, M. N.; Smith, J. T.; Coolen, L. M.; de Oliveira, C. V. R.; Jafarzadehshirazi, M. R.; Pereira, A.; Iqbal, J.; Caraty, A.; Ciofi, P.; Clarke, I. J., Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* **2007,** 148, (12), 5752-5760.
13. Foradori, C. D.; Amstalden, M.; Goodman, R. L.; Lehman, M. N., Colocalisation of dynorphin a and neurokinin B immunoreactivity in the arcuate nucleus and median eminence of the sheep. *J Neuroendocrinol* **2006,** 18, (7), 534-541.

14. Messager, S.; Chatzidaki, E. E.; Ma, D.; Hendrick, A. G.; Zahn, D.; Dixon, J.; Thresher, R. R.; Malinge, I.; Lomet, D.; Carlton, M. B. L.; Colledge, W. H.; Caraty, A.; Aparicio, S. A. J. R., Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci U S A* **2005,** 102, (5), 1761-1766.
15. Krajewski, S. J.; Anderson, M. J.; Iles-Shih, L.; Chen, K. J.; Urbanski, H. F.; Rance, N. E., Morphologic evidence that neurokinin B modulates gonadotropin-releasing hormone secretion via neurokinin 3 receptors in the rat median eminence. *J Comp Neurol* **2005,** 489, (3), 372-386.
16. Panat, N. A.; Maurya, D. K.; Ghaskadbi, S. S.; Sandur, S. K., Troxerutin, a plant flavonoid, protects cells against oxidative stress-induced cell death through radical scavenging mechanism. *Food Chem* **2016,** 194, 32-45.
17. Zhang, Z.-f.; Fan, S.-h.; Zheng, Y.-l.; Lu, J.; Wu, D.-m.; Shan, Q.; Hu, B., Troxerutin protects the mouse liver against oxidative stress-mediated injury induced by D-galactose. *J Agric Food Chem* **2009,** 57, (17), 7731-7736.
18. Lu, J.; Wu, D.-m.; Zheng, Z.-h.; Zheng, Y.-l.; Hu, B.; Zhang, Z.-f., Troxerutin protects against high cholesterol-induced cognitive deficits in mice. *Brain* **2011,** 134, (Pt 3), 783-797.
19. Farajdokht, F.; Amani, M.; Mirzaei Bavil, F.; Alihemmati, A.; Mohaddes, G.; Babri, S., Troxerutin protects hippocampal neurons against amyloid beta-induced oxidative stress and apoptosis. *EXCLI J* **2017,** 16, 1081-1089.
20. Palomba, S.; Falbo, A.; Zullo, F., Management strategies for ovulation induction in women with polycystic ovary syndrome and known clomifene citrate resistance. *Curr Opin Obstet Gynecol* **2009,** 21, (6), 465-473.
21. Misso, M. L.; Teede, H. J.; Hart, R.; Wong, J.; Rombauts, L.; Melder, A. M.; Norman, R. J.; Costello, M. F., Status of clomiphene citrate and metformin for infertility in PCOS. *Trends Endocrinol Metab* **2012,** 23, (10), 533-543.
22. Lee, Y. H.; Yang, H.; Lee, S. R.; Kwon, S. W.; Hong, E. J.; Lee, H. W., Welsh Onion Root (Allium fistulosum) Restores Ovarian Functions from Letrozole Induced-Polycystic Ovary Syndrome. *Nutrients* **2018,** 10, (10).
23. Rajan, R. K.; M, S. S.; Balaji, B., Soy isoflavones exert beneficial effects on letrozole-induced rat polycystic ovary syndrome (PCOS) model through anti-androgenic mechanism. *Pharmaceutical biology* **2017,** 55, (1), 242-251.
24. Hu, Q.; Jin, J.; Zhou, H.; Yu, D.; Qian, W.; Zhong, Y.; Zhang, J.; Tang, C.; Liu, P.; Zhou, Y.; Wang, X.; Sheng, L., Crocetin attenuates DHT-induced polycystic ovary syndrome in mice via revising kisspeptin neurons. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **2018,** 107, 1363-1369.
25. Lu, J.; Wu, D. M.; Zheng, Y. L.; Hu, B.; Cheng, W.; Zhang, Z. F.; Li, M. Q., Troxerutin counteracts domoic acid-induced memory deficits in mice by inhibiting CCAAT/enhancer binding protein beta-mediated inflammatory response and oxidative stress. *Journal of immunology* **2013,** 190, (7), 3466-79.

26. Baluchnejadmojarad, T.; Jamali-Raeufy, N.; Zabihnejad, S.; Rabiee, N.; Roghani, M., Troxerutin exerts neuroprotection in 6-hydroxydopamine lesion rat model of Parkinson's disease: Possible involvement of PI3K/ERbeta signaling. *European journal of pharmacology* **2017,** 801, 72-78.
27. Zhao, H.; Liu, Y.; Zeng, J.; Li, D.; Zhang, W.; Huang, Y., Troxerutin and Cerebroprotein Hydrolysate Injection Protects Neurovascular Units from Oxygen-Glucose Deprivation and Reoxygenation-Induced Injury In Vitro. *Evidence-based complementary and alternative medicine : eCAM* **2018,** 2018, 9859672.
28. Taylor, A. E.; McCourt, B.; Martin, K. A.; Anderson, E. J.; Adams, J. M.; Schoenfeld, D.; Hall, J. E., Determinants of abnormal gonadotropin secretion in clinically defined women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **1997,** 82, (7), 2248-2256.
29. Sagnella, F.; Apa, R.; Guido, M.; Villa, P.; Spadoni, V.; Miceli, F.; Lanzone, A., Suppression and recovery of gonadotropin and steroid secretion by a gonadotropin-releasing hormone receptor antagonist in healthy women with normal ovulation versus women with polycystic ovary syndrome in the early follicular phase. *Fertil Steril* **2009,** 91, (5), 1857-1863.
30. Ertunc, D.; Tok, E. C.; Savas, A.; Ozturk, I.; Dilek, S., Gonadotropin-releasing hormone antagonist use in controlled ovarian stimulation and intrauterine insemination cycles in women with polycystic ovary syndrome. *Fertil Steril* **2010,** 93, (4), 1179-1184.
31. Kazer, R. R.; Kessel, B.; Yen, S. S., Circulating luteinizing hormone pulse frequency in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **1987,** 65, (2), 233-236.
32. Pyun, B. J.; Yang, H.; Sohn, E.; Yu, S. Y.; Lee, D.; Jung, D. H.; Ko, B. S.; Lee, H. W., Tetragonia tetragonioides (Pall.) Kuntze Regulates Androgen Production in a Letrozole-Induced Polycystic Ovary Syndrome Model. *Molecules* **2018,** 23, (5).
33. Jahan, S.; Abid, A.; Khalid, S.; Afsar, T.; Qurat Ul, A.; Shaheen, G.; Almajwal, A.; Razak, S., Therapeutic potentials of Quercetin in management of polycystic ovarian syndrome using Letrozole induced rat model: a histological and a biochemical study. *Journal of ovarian research* **2018,** 11, (1), 26.
34. Zhao, H.; Zhou, D.; Chen, Y.; Liu, D.; Chu, S.; Zhang, S., Beneficial effects of Heqi san on rat model of polycystic ovary syndrome through the PI3K/AKT pathway. *Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences* **2017,** 25, (1), 21.
35. Kabiri, N.; Tabandeh, M. R.; Tabatabaie, S. R., Beneficial effects of pioglitazone and metformin in murine model of polycystic ovaries via improvement of chemerin gene up-regulation. *Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences* **2014,** 22, 39.
36. Karateke, A.; Dokuyucu, R.; Dogan, H.; Ozgur, T.; Tas, Z. A.; Tutuk, O.; Agturk, G.; Tumer, C., Investigation of Therapeutic Effects of Erdosteine on Polycystic Ovary Syndrome in a Rat Model. *Medical principles and practice : international journal of the Kuwait University, Health Science Centre* **2018,** 27, (6), 515-522.
37. Chaudhari, N. K.; Nampoothiri, L. P., Neurotransmitter alteration in a testosterone propionate-induced polycystic ovarian syndrome rat model. *Hormone molecular biology and clinical investigation* **2017,** 29, (2), 71-77.

38. Moore, A. M.; Campbell, R. E., Polycystic ovary syndrome: Understanding the role of the brain. *Frontiers in neuroendocrinology* **2017,** 46, 1-14.
39. Coutinho, E. A.; Kauffman, A. S., The Role of the Brain in the Pathogenesis and Physiology of Polycystic Ovary Syndrome (PCOS). *Medical sciences* **2019,** 7, (8).
40. Shi, X.; Zhang, L.; Fu, S.; Li, N., Co-involvement of psychological and neurological abnormalities in infertility with polycystic ovarian syndrome. *Arch Gynecol Obstet* **2011,** 284, (3), 773-778.
41. Watanabe, M.; Fukuda, A.; Nabekura, J., The role of GABA in the regulation of GnRH neurons. *Frontiers in neuroscience* **2014,** 8, 387.

Figures

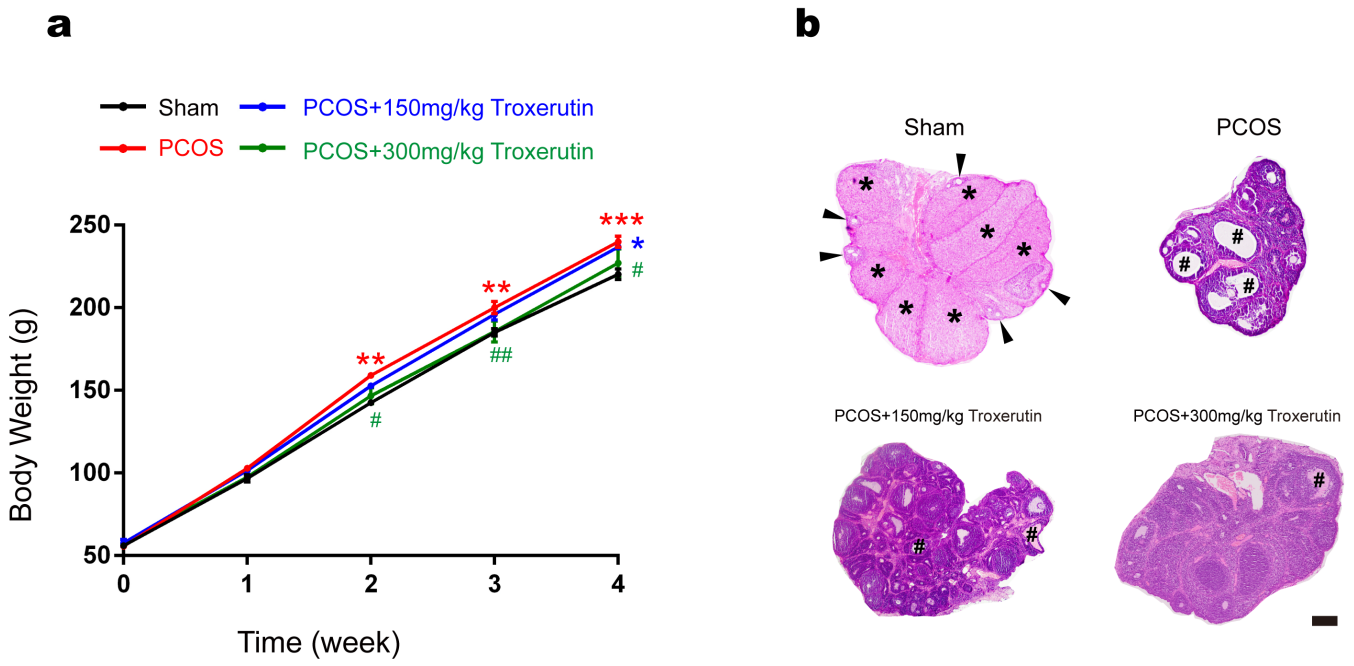

Figure 1

Troxerutin reduced obesity and improved abnormal ovarian morphology in PCOS rats (a) Body weight (b) Hematoxylin and eosin staining of representative ovaries. For a, P values were determined by one-way ANOVA with Turkey's multiple comparison test and data were presented as means±s.e.m. n=12 rats per group. *P<0.05, **P<0.01, ***P<0.001 vs. sham group; #P<0.05, ##P<0.01 vs. PCOS group. For b, follicles in various stages of development were indicated by black triangles, corpora lutea were indicated by asterisks, while the cystic follicles were indicated by hashtags. Scale bar: 200μm. Images were representative of three independent experiments with similar results.

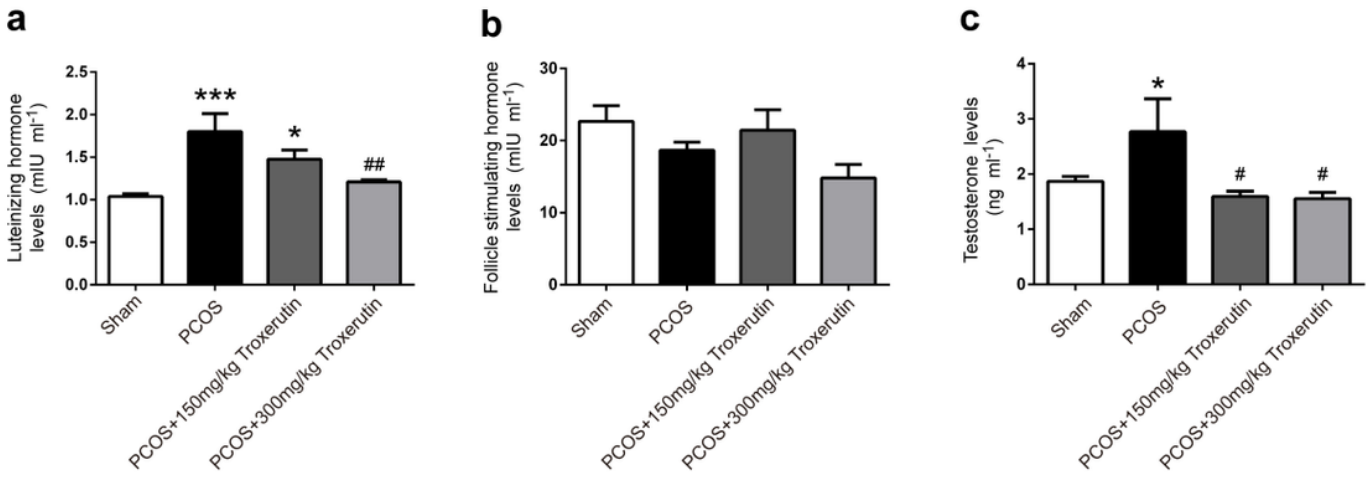

Figure 2

Troxerutin reversed the abnormal serum levels of gonadotrophin and testosterone in PCOS rats (a) Serum LH levels (b) Serum FSH levels (c) Serum testosterone levels. P values were determined by one-way ANOVA with Turkey's multiple comparison test and data were presented as means±s.e.m. n=9 rats per group. *P<0.05, ***P<0.001 vs.sham group; #P<0.05, ##P<0.01 vs. PCOS group.

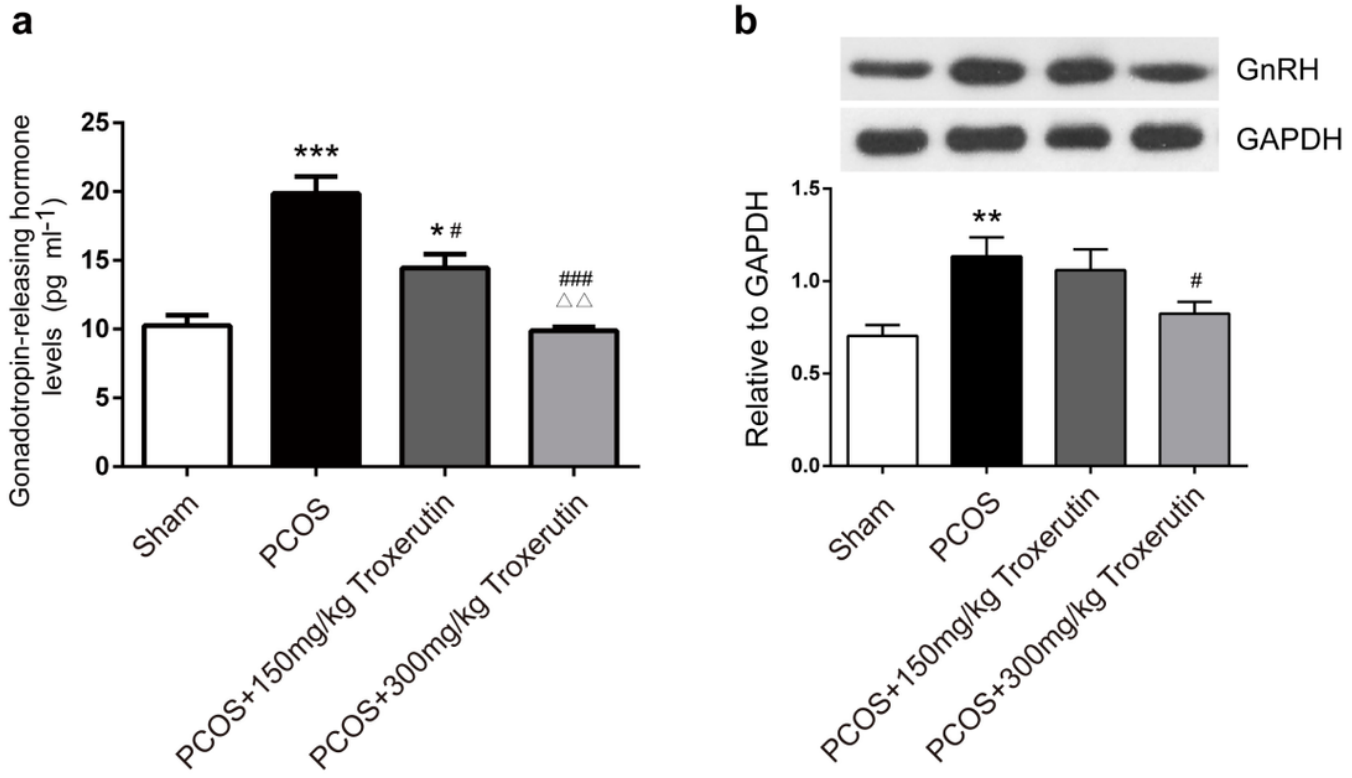

Figure 3

Troxerutin reduced the elevated GnRH levels in PCOS rats (a) Serum levels of GnRH (b) Hypothalamic status of GnRH. P values were determined by one-way ANOVA with Turkey's multiple comparison test and data were presented as means±s.e.m. n=9 rats per group.*P<0.05, **P<0.01, ***P<0.001vs. sham group; #P<0.05, ##P<0.01 vs. PCOS group; △△P<0.01 vs. PCOS+150mg/kg troxerutin group.

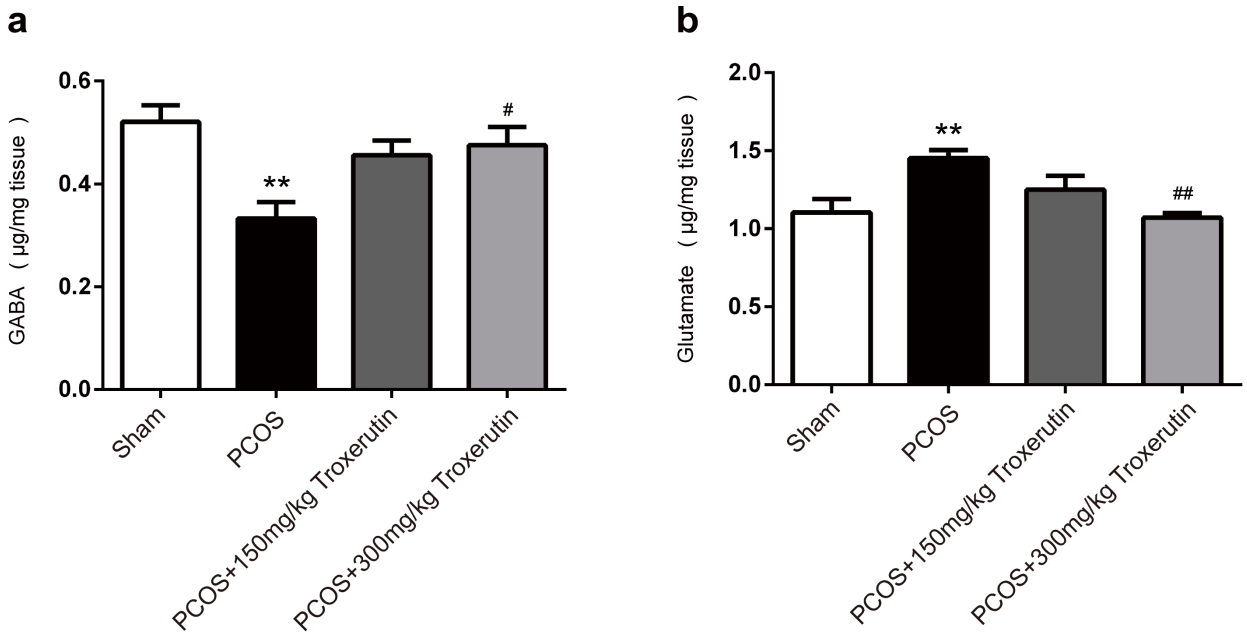

Figure 4

Troxerutin altered GnRH regulatory neurotransmitters in the hypothalamus of PCOS rats (a) Hypothalamic levels of GnRH inhibitory GABA (b) Hypothalamic levels of GnRH stimulatory glutamate. P values were determined by one-way ANOVA with Turkey's multiple comparison test and data were presented as means±s.e.m. n=9 rats per group. **P<0.01vs. sham group; #P<0.05, ##P<0.01 vs. PCOS group.

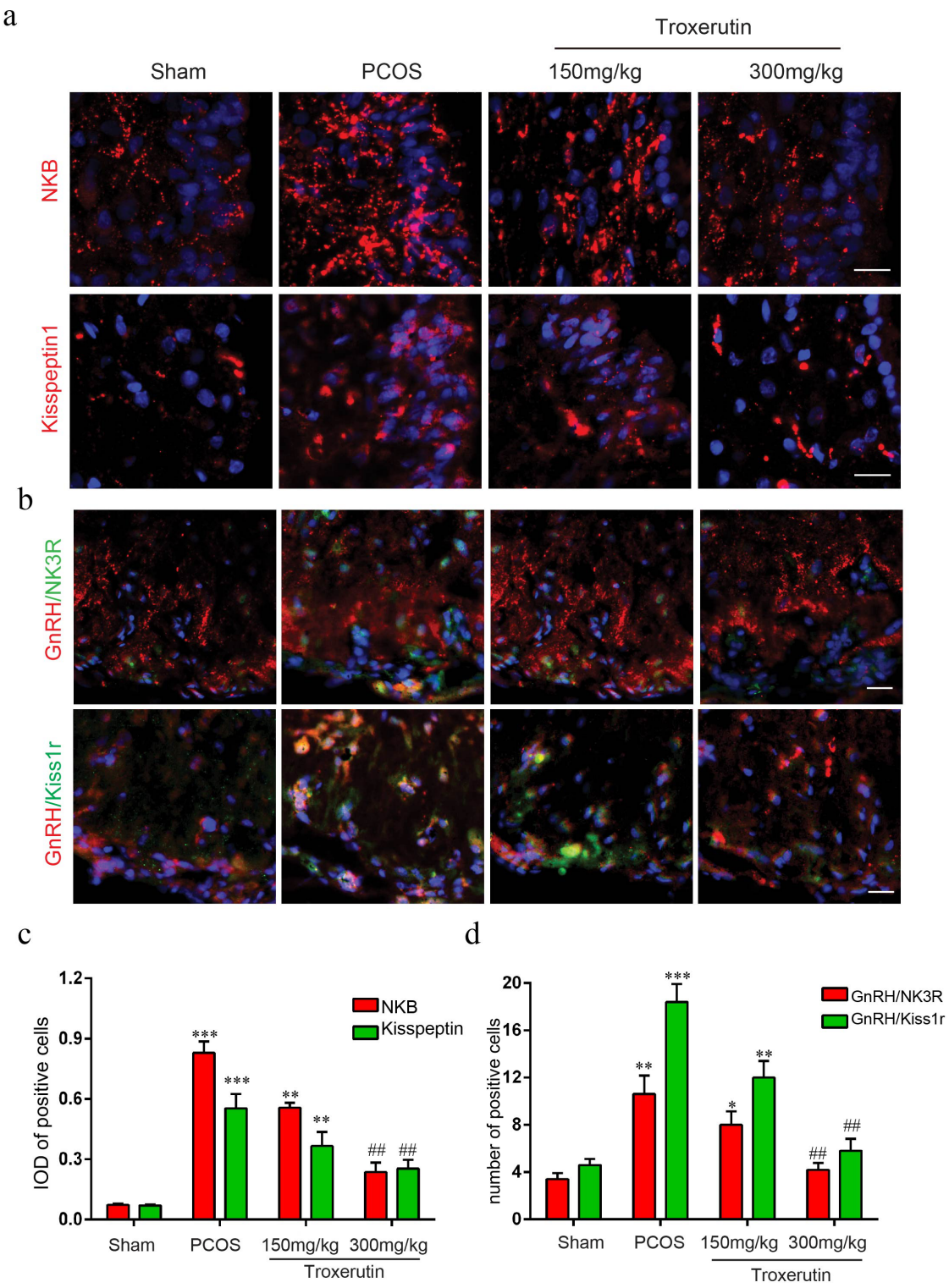

Figure 5

Troxerutin reduced the expression of Kisspepetin1/Kiss1r and Neurokinin B/NK3r in the hypothalamus (a) and (b) Immunofluorescence photomicrograph showing that the expression of Kisspeptin1 and NKB in the arcuate nucleus (ARC) and Kiss1r, NK3r in GnRH positive neurons in the median eminence (ME). Scale bar, 50μm. (c) and (d) Quantitative analysis integral optical density (IOD) of Kisspeptin1 and NKB in the ARC and the number of Kiss1r/ GnRH and NK3r/GnRH positive cells in the ME. P values were determined

by one-way ANOVA with Turkey's multiple comparison test and data were presented as means±s.e.m. n=6 rats per group. **P<0.01, ***P<0.001vs. sham group; #P<0.05, ##P<0.01 vs. PCOS group.