Indirubin-3’-oxime promotes the efficacy of GnRHa in obese-induced central precocious puberty and maintains normal bone growth and body weight via the ERK-Sp1-KISS-1/GPR54 axis

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ABSTRACT
Central precocious puberty (CPP) is a widespread developmental abnormality. The application of gonadotropin-releasing hormone agonist (GnRH) is widely useful for the medical therapy of CPP. This study aimed to investigate the combination effect and mechanism of indirubin-3’-oxime (I3O), an active ingredient analogue of traditional Chinese medicine, and GnRHa on the progression of CPP. First, female C57BL/6 mice were fed with a high-fat diet (HFD) for the induction of precocious puberty and treated with GnRhA and I3O alone or in combination. Development of sexual maturation, bone growth and obesity were determined by vaginal opening detection, H&E staining and ELISA. The protein and mRNA expression levels of related genes were evaluated via western blotting, immunohistochemical method and RT-qPCR. Subsequently, tBHQ, an inhibitor of ERK, was applied to verify whether the mechanism of I3O was associated with this signaling. The results showed that the treatment of I3O alone or in combination with GnRHa could alleviate the HFD-induced earlier vaginal opening and serum levels of the gonadal hormone in mice. And, I3O could significantly eliminate the role of growth deceleration of GnRHa in bone development and reversed the side effect of GnRHa on body weight. More importantly, we found that I3O decreased the expression of KISS-1 and GPR54 by suppressing the phosphorylation of ERK1/2 and Sp1 in the hypothalamus in mice. In summary, these data indicated that I3O could promote the efficacy of GnRHa in HFD-induced precocious puberty, and maintain bone growth and body weight in mice via the ERK-Sp1-KISS-1/GPR54 axis.

Keywords: Indirubin-3’-oxime, central precocious puberty, GnRHa, ERK, Sp1

Introduction
Precocious puberty is a common developmental abnormality of the pediatric endocrine system and is characterized by the early appearance of puberty including attainment of secondary sexual characteristics and reproductive capacity before 8 years of age in girls and before 9 years of age in boys (1). Precocious puberty can be subdivided into central precocious puberty (CPP) and peripheral precocious puberty (PPP) from the perspective of etiology, of which CPP is caused by the activation of the hypothalamic-pituitary-gonadal (HPG) axis in advance and the release of excessive gonadotropin-releasing hormone (GnRH), and is mostly idiopathic (2). And idiopathic CPP is more common in girls than in boys. CPP can result in early epiphyseal closure because of the advancement of bone age in children, leading to decreased adult final height, as well as a series of psychological problems, including depression and anxiety (3).

The medical therapy of gonadotropin-releasing hormone analogue (GnRHa) is widespread for the care of sex hormone-dependent diseases including CPP, and is the clinical gold-standard treatment for CPP (4, 5). Studies have shown that GnRHa treatment can effectively alleviate symptoms of precocity in children and improve final adult height (6, 7). However, the height of some children may not achieve the desired final height after GnRHa treatment, which was closely related to the inappropriate deceleration of growth (4, 8-10). Therefore, GnRHa and growth hormone (GH) combination therapy is usually used to promote bone growth and maturation in clinical practice, but the inconvenient application and high price of GH limited its use in patients (11). Moreover, GnRHa treatment may have a side effect on body mass index (12). And there was a gradual increase in obesity during GnRHa treatment (13, 14). The reason for weight gain may be related to the expression of the GnRH receptor on the surface of adipocytes, which promotes the proliferation of adipocytes and the accumulation of lipid droplets (15). Therefore, it is of great clinical significance to explore and discover new adjuvant therapy drugs for overcoming the growth deceleration and fat accumulation induced by GnRHa therapy in children with CPP.

Indirubin-3’-monoxime (I3O) is an analogue of indirubin which is an active ingredient of Danggui Longhui Wan, the traditional Chinese herbal mixture used for the anticancer treatment of chronic leukemia (16, 17). Recently, increasing studies revealed obesity in children as a high risk for central precocious puberty via inducing the premature activation of the HPG axis (18, 19). I3O was reported to restore bone loss and inhibit adipocyte differentiation in high-fat diet-induced obese mice (20, 21). During the process of sexual development, kisseptin-1 (encoded by the KISS-1 gene) secreted by Kisspeptin neurons binds to the...
receptor GPR54 expressed in GnRH neurons to stimulate the secretion of GnRH, as well as the secretion of lutetinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland, among which, KISS-1 gene transcription was reported to be regulated by the transcription factor Sp1 (22, 23). In recent years, I3O gene was reported to prevent neuronal apoptosis and inhibit the migration and invasion of pancreatic cancer cells by inhibiting ERK signaling (24, 25). And ERK MAPK is required for the activation of Sp1 activity (26).

Taken together, we intended to explore whether I3O in combination with GnRHa would further suppress precocious sexual maturation and whether I3O could play a role in bone growth and obesity during GnRHa treatment in this study. In addition, we also wondered whether the underlying mechanism involved is related to the ERK-Sp1-KISS-1/GPR54 axis.

Materials and Methods

Animal model

In the experiments, female C57BL/6 mice, aging 21-day-old, were used and were purchased from HFK Biotechnology Co., Ltd (Beijing, China). The feeding conditions are: the temperature of the animal room is 22-23°C and the relative humidity is 45-55%. All animals were used in accordance with the requirements of the Animal Experimentation Ethics Committee of Shandong University.

Design 1: To investigate the therapeutic effects of different drugs on central precocious puberty.

Female mice were fed with a chow diet (fat: 6.2%, carbohydrate: 35.6%, protein: 20.8%, and the calorific value: 17.6 KJ/g), labeled as the chow diet (CHD) group (n=7). The central precocious puberty model was established by induction of a high-fat diet (fat: 60%, carbohydrate: 20%, protein: 20%, and the calorific value: 22.0 KJ/g) in C57BL/6 mice, labeled as the HFD group (n=28). Mice in HFD group were randomly divided into four groups: while modeling, seven mice in the HFD group were given saline intraperitoneally, labeled as the HFD + saline group; seven mice were given I3O (1 mg/kg, Med Chem Express, China) intraperitoneally once every two days, labeled as the HFD + I3O group; seven mice were given HFD + GnRHa (2.5 mg/kg, Med Chem Express, China) intraperitoneally once every two days, labeled as the HFD + GnRHa group; seven mice were given I3O (1 mg/kg, Med Chem Express, China) intraperitoneally once every two days, labeled as the HFD + I3O group.

Design 2: To investigate the pharmacological mechanism of I3O in the treatment of central precocious puberty.

During the process of a high-fat diet feeding, seven mice aged three weeks were pre-injected subcutaneously with t-Butyhydroquinone (50 mg/kg, tBHQ, ERK activator, Med Chem Express), followed by the intraperitoneal injection of I3O (1 mg/kg) once every two days, labeled as the HFD + tBHQ group. tBHQ alone injection for HFD mice was used as the positive control.

All mice were euthanized at 35 days, the day on which all mice reached puberty. The serum and the related tissues including the hypothalamus were collected for the subsequent experiments.

Observation of the vaginal opening condition

Mice were kept in place, and the vulva was exposed by lifting the tail. Observe the color of the mucous membrane, the opening and the turgidity of the vulva. The vulva mucous membrane of the proestrus female mice is light red and slightly moist, vaginal opening is not open, slightly swollen around. Female mice in estrus have swollen and moist vulvae, open vaginal openings, and excessive mucous secretion.

Western blotting assay

Minced tissues were subjected to extract protein using RIPA solution supplemented with a protease inhibitor cocktail and protein phosphatase inhibitor cocktail. The protein concentration was quantified using a BCA protein quantitation kit (Thermo Fisher Scientific, USA). The 30 µg of proteins were separated via 10–12% SDS-PAGE and then transferred to nitrocellulose (NC) membranes. After blocking, the membranes were incubated with the following primary antibodies against GnRH (1:1000, Abcam, UK), Kisspeptin-1 (1:800, Proteintech, USA), GPR54 (1:2500, Poteintechn, total-Sp1 (1:900, ABclonal, China), phospho-Sp1 (1:1200, ABclonal), total-ERK1/2 (1:1500, ABclonal), phospho-ERK1/2 (1:1500, ABclonal), MMP-13 (1:1200, Abcam), Col-X (1:1500, ABclonal), Col-II (1:1200, Thermo Fisher Scientific) and β-tubulin (1:4000, ABclonal) overnight at 4 °C. Finally, the membranes were incubated with HRP-labeled secondary antibodies (1:5000, ZSGB-BIO, China). The protein band was visualized and measured with an ECL kit (Merck Millipore, USA). ImageJ software was applied for the analysis of band intensity.

Quantitative RT-PCR

The application of TRizol (Invitrogen, USA) was for the isolation of total RNA from the hypothalamus tissues. And Hieff® AdvanceFast 1st Strand cDNA Synthesis Kit (YEASEN, China) was used to reverse-transcribe RNA. The qPCR was conducted to assess the expression of RNA using Hieff® qPCR SYBR Green Master Mix (YEASEN) on Roche LightCycler96 (Roche, Germany). The experiment was carried out three times for each sample. The primer sequences were listed as follows:

KISS-1, Forward, 5′-GCTCCGTCCAACGCCTT-CAGG-3′,

Reverse, 5′-CCGACCAGCCAAAGCAACAT-3′;

GPR54, Forward, 5′- GCTCCGTCCAACGCTT-CAGG-3′,

Reverse, 5′-CCGACCAGCCAAAGCAACAT-3′;

GAPDH, Forward, 5′-TGGCCTTCCGTGTTCCTAC-3′,

Reverse, 5′-GAGTTGCTGTGAGTGCG-3′.

GAPDH was used as the internal control for KISS-1 and GPR54. The fold change of gene expression was analyzed using the 2-ΔΔCt method.

Enzyme-linked-immunosorbent assay (ELISA)

The level of hormones and fat-related factors in the serum were evaluated using an ELISA kit according to the manufacturer’s instructions. ELISA kits examining the serum levels of mouse GnRH, LH, FSH, Estradiol (E2), leptin, insulin-like growth factor (IGF-1) and free fatty acids were obtained from Elabscience Biotechnology Co., Ltd. (China). ELISA kit detecting the levels of Triglycerides, and cholesterol was purchased from Cloud-clone Co., Ltd. (China). The absorbance was evaluated with a microplate reader.
Hematoxylin & Eosin (H&E) staining
The bone tissues and liver tissues were excised, and fixed with 4% paraformaldehyde (PFA). In addition, for bone tissues, decalcification was performed using 0.5M EDTA (Sigma-Aldrich, USA) at 4°C. After dewaxing and rehydration, the paraffin sections with 5-μm thickness were stained with hematoxylin and eosin staining solution. The change in morphometric features of the pulmonary artery was observed under an optical microscope (Olympus, Japan).

For image acquisition analysis, H&E-stained sections were measured for growth plate cartilage height using Image J image analysis software; for measuring growth plate height, three different fields of view were taken for each section and three images were acquired.

Immunohistochemical staining
4% PFA-fixed and paraffin-embedded sections with 5-μm thickness were deparaffinized and rehydrated using xylene and graded ethanol, followed by retrieving the antigen in a boiled Citrate Antigen Retrieval solution. After cooling, 3% H2O2 was used to eliminate endogenous peroxidase interference. After blocking with 5% goat serum for 60 minutes, the slides were incubated with the primary antibody against MMP-13 (1:500, Abcam), Col-X (1:500, Abcam), Col-II (1:500, Thermo Fisher Scientific) overnight at 4°C. After washing thrice, the sections were probed with a biotin-labeled secondary antibody for 1h. Finally, HRP-conjugated streptomycin solution was used to incubate the slides at room temperature, and followed by a reaction with a DAB kit (Beyotime, China). Observe the results under the light microscope (Olympus).

Statistical analysis
All data were calculated as mean ± standard deviation (SD) analyzed by GraphPad Prism software version 8. Student’s t-test was executed for the statistical analyses between the two groups. One-way ANOVA with Bonferroni’s posthoc test was for statistical differences among multiple groups. When a value of P was less than 0.05, the difference was deemed statistical significance.

Results
GnRHa combined with I3O treatment alleviated the symptoms of central precocious puberty in high-fat diet-induced mice
To evaluate the effect of I3O on the process of pubertal development and the treatment process of GnRHa, HFD-induced mice were injected alone with I3O (1 mg/kg) or combined with and GnRHa (2.5 mg/kg). We monitored the opening time of the vagina in mice and observed the delayed vaginal opening time after I3O treatment for mice in the HFD + I3O group compared with the HFD + saline group, and the suppressive effect of I3O is slightly weaker than GnRHa treatment (Figure 1A, B). And the time of vaginal opening of mice in the HFD + GnRHa + I3O group was similar to that of mice in the CHD group, indicating the therapeutic effect of I3O for sexual precocity (Figure 1A, B). Next, the finding was verified by the decreased protein level of GnRHa in the hypothalamus and serum level of GnRHa in HFD + I3O group and HFD + I3O + GnRHa group compared with HFD + saline group (P<0.001, Figure 1C, D). Furthermore, we measured the serum level of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E2) via ELISA kit, and we found that the serum level of LH, FSH and E2 was remarkably elevated in the HFD + saline group compared with the CHD group, I3O and GnRHa alone or combination treatment significantly reversed the increased level of sex-related hormones induced by high-fat diet (P<0.001, Figure 1D). The above data showed that I3O treatment could suppress sexual precocity caused by a high-fat diet in female mice, and I3O promoted the therapeutic effect of GnRHa treatment on CPP in mice.

GnRHa combined with I3O treatment maintained normal bone growth
Next, to observe whether I3O treatment influenced the growth deceleration effect of GnRHa on bone development, we carried out H&E staining to measure the width change of growth plate tissues. The result showed a dramatical reduction in the width of growth plate, the width of proliferation zone and the width of the hypertrophic zone of mice in the HFD + saline group compared with that in the CHD group (P<0.05, Figures 2A). I3O treatment significantly increased the width of growth plate, the width of proliferation zone and the width of the hypertrophic zone of HFD mice, and inhibited remarkably the growth deceleration effect of the single GnRHa treatment on the width of the growth plate, the width of proliferation zone and the width of hypertrophic zone (P<0.001, Figure 2A). Immunohistochemical staining demonstrated that the expressions of Col-II, MMP-13 and Col-X in tissues of the growth plate were decreased in HFD + GnRHa group compared with HFD + saline group, but the expressed levels of Col-II, MMP-13 and Col-X were higher in HFD + GnRHa + I3O group than HFD + GnRHa group (Figure 2B). As shown by Figures 2C and 2D, the promotive effect of I3O on the protein expression of Col-II, MMP-13 and Col-X in growth plate tissues of mice induced high-fat diet and I3O could suppress the decreased protein levels of MMP-13, Col-X and Col-II caused by GnRHa treatment. These results demonstrated that I3O could promote the development of growth plates and efficiently suppress the GnRHa treatment-mediated growth deceleration in HFD-induced mice.

Figure 1. Single I3O and combination with GnRHa treatment all alleviated the sexual precocity induced by a high-fat diet in mice. (A) The percent of Vaginal opening in mice. (B) Vaginal opening days in mice. (C) The protein level of GnRHa was detected by Western blot. (D) Serum levels of GnRHa, LH and FSH were detected by ELISA. *P<0.05, **P<0.01, ***P<0.001.
Obesity from the perspective of sex hormones. I3O treat the effect of GnRHa on bone growth and the side effect on mechanism of action of inhibiting the growth deceleration symptoms of central precocious puberty, and searched the lease, which can explain the reason why I3O relieved the Here, we explored the mechanism of I3O on GnRH re...t of HFD-induced obesity in mice by inhibiting the ERK-Sp1-KISS-1/GPR54 axis I3O alleviated HFD-induced precocious puberty in mice by inhibiting the ERK-Sp1-KISS-1/GPR54 axis. And single I3O treatment could reduce the fat of subcutis, the fat of viscera, and the gonadal fat of mice in HFD + I3O group compared with HFD + saline group, and GnRHa treatment induced the increase of the fat of subcutis, the fat of viscera, and gonadal fat of high-fat diet-treated mice were significantly inhibited by I3O (P<0.01, Figure 3B). The same results were also seen in the weight of the ovary and uterus of mice (Figure 3C). In addition, we collected the serum samples of mice to detect the level of triglycerides, cholesterol and free fatty acids via ELISA assay and observed that there were higher levels of triglycerides, cholesterol and free fatty acids in the HFD + saline group and highest in HFD + GnRHa group, but these elevated serum levels of the fat-related metabolites were significantly reduced by I3O treatment (P<0.001, Figure 3D). Furthermore, the serum level of leptin, secreted by adipocyte, and IGF-1 was lowest in the CHD group, lower in HFD + GnRHa + I3O group, and the levels were decreased sequentially from HFD + saline group to HFD + GnRHa group (Figure 3E). These results revealed the effective treatment of I3O on obesity induced by HFD and the side effect of GnRHa treatment on body weight.

I3O alleviated HFD-induced precocious puberty in mice by inhibiting the ERK-Sp1-KISS-1/GPR54 axis in the hypothalamus

Excessive estrogen secretion in children with central precocity affects bone growth and promotes obesity. Here, we explored the mechanism of I3O on GnRH release, which can explain the reason why I3O relieved the symptoms of central precocious puberty, and searched the mechanism of action of inhibiting the growth deceleration effect of GnRHa on bone growth and the side effect on obesity from the perspective of sex hormones. I3O treatment was found to inhibit GnRH secretion in a mouse model of CPP induced by a high-fat diet, which may attribute to the attenuation of upstream signaling to some extent in GnRH neurons. Therefore, western blotting assay and RT-qPCR were carried out and the data revealed that I3O could inhibit the protein and mRNA level of Kisspeptin-1 and GPR54, which may be involved in its mechanism of action on sexual precocity development (Figure 4A, B). Studies have demonstrated that Sp1, as a transcription factor, is a key modulator of KISS-1 gene transcription (22, 23). And activation of the ERK pathway was necessary for the phosphorylation of Sp1 (26). Therefore, we speculated that I3O may regulate the phosphorylation of the ERK-Sp1 axis to affect the expression of kisspeptin-1 and GPR54 in the hypothalamus. Herein, Western blotting assay was performed to evaluate the changes of total and phosphorylated levels of ERK1/2 and Sp1 in the hypothalamus to verify the above speculation. And we found that I3O could inhibit the phosphorylation level of ERK1/2 and Sp1, indicating and verifying that I3O could reduce the expression of the downstream gene KISS-1 by suppressing the phosphorylation level of the transcription factor Sp1, and this process may be mediated by inhibition of the phosphorylation of ERK (Figure 4C). Next, to observe and verify that I3O suppressed the phosphorylation of Sp1 through reducing the activation of ERK, t-Butylhydroquinone (tBHQ, ERK activator) was injected subcutaneously 2 hours before I3O treatment in high-fat diet-induced mice for rescue experiment (Figure 4D). And we found that the suppressive effect of I3O treatment on protein and mRNA expression of GnRH, Kisspeptin-1 and GPR54 in serum and hypothalamus was all restored by tBHQ in HFD mice (Figure 4E-4H).

To measure and verify whether I3O played a role in CPP model mice, we monitored the vaginal opening time of mice, and we found that tBHQ treatment significantly reversed the inhibitory effect of I3O treatment on the vaginal opening time of HFD mice (Figure 4I). More importantly, I3O induced the decrease of body mass and the increased expression of MMP-13 and Col-X in growth plate tissues of high-fat diet-treated mice was inhibited by tBHQ, indicating that I3O could delay the process of sexual maturation and suppress increased body mass and promote bone

![Figure 2. I3O treatment suppressed the effect of GnRHa on bone growth deceleration in mice.](Image 45x640 to 283x795)

![Figure 3. I3O treatment reduced fat accumulation in mice.](Image 313x112 to 551x276)
growth-related protein expression via inhibiting ERK/Sp1 signaling (Figure 4J, 4K). The above experimental data indicated that I3O could inhibit the phosphorylation of Sp1 and the transcription level of the downstream target gene KISS-1 by inhibiting ERK phosphorylation, thus reducing the expression levels of Kisspeptin-1, its receptors GPR54 and GnRH, and relieving the central precocious puberty in mice induced by a high-fat diet.

Discussion

Puberty is a process in which adolescents acquire secondary sexual characteristics, accelerated physical growth and the development and maturation of the reproductive system (27). In the endocrine system, sex hormone is a kind of hormone that affects the growth and development of the human body and maintains sexual function. The secretion of sex hormones is regulated by HPG axis, and GnRH secreted by hypothalamic arcuate nucleus nerve cells combines with the receptors so that the pituitary gland is stimulated and periodically secretes gonadotropin, including LH and FSH, both of which promote the levels of sex hormones through acting on the gonads and affect the growth and development of mammary gland, adrenal cortex and bone (28). Central precocious puberty is caused by the premature activation of the HPG axis and the release of excessive GnRH. Several pieces of research have demonstrated that early puberty is often related to obesity, and obesity is a risk inducer for CPP (29, 30). Therefore, in this study, central precocious puberty model mice were established by the induction of a high-fat diet and verified by the increased serum levels of GnRH, LH and FSH, consistent with the results of previous studies (31).

In the clinic, GnRHa is widely used in the treatment of children with CPP. However, the effect of GnRHa on final height remains not necessarily well (8). Here, we explored and found the suppressive effect of I3O, the analogue of indirubin of Chinese traditional medicine, on the advanced vaginal opening time and increased gonadotropin levels in CPP mice induced by a high-fat diet for the first time. In high-fat diet-induced obese mice, I3O reversed bone loss and fat gain (20). In this study, we observed that I3O could promote the increase of growth plate width to enhance bone development and suppress the body mass in mice induced high-fat diet, this finding was consistent with the previous report (20). Additionally, GnRHa was found to result in the growth deceleration of bone development and promoted the body weight verified by the decreased growth plate width, the decreased expression of MMP-13, Coll-X and Col-II, the slightly increased body mass and weight of fat in each organ during treatment, but these effects could be inverted by I3O treatment in HFD mice.

Kisspeptin plays a centrally functional role in regulating the secretion of GnRH and peripheral factors, including adipokines, that affect puberty timing. The high-fat diet was reported to impair follicular development by regulating the kisspeptin/GPR54 axis expressed in ovarian of offspring (32). And accumulating shreds of evidence showed that KISS-1 gene (coded kisspeptin-1) expressed in kisspeptin neurons exerts the role by binding its receptor GPR54 expressed in GnRH neurons to stimulate the secretion of GnRH (33). Among them, the KISS-1 gene was regulated by Sp1, a transcription factor that was affected by the ERK pathway (22, 26). In this study, we revealed that I3O alleviated the sexual precocity induced by a high-fat diet in mice by inhibiting the ERK-Sp1-KISS-1/GPR54 axis in the hypothalamus at the first time verified by the lessened expression of phosphor-ERK and phosphor-Sp1 under I3O treatment and the tBHQ-reversed the suppressive effect of I3O on the phosphorylation level of Sp1. However, in recent years, I3O was reported to activate Wnt/β-catenin signaling and impair adipocyte differentiation to maintain a healthy weight (21). It is well known that adipocyte differentiation plays an important role in the occurrence of obesity (34, 35). And it has been found that I3O stimulated chondrocyte maturation and longitudinal bone growth by activating the Wnt/β-catenin pathway (36). Based on these studies, we will explore and find the related mechanism of I3O treatment on body mass and bone growth from the perspective of adipocyte and chondrocyte of high-fat diet-induced mice in future work.

In conclusion, our study revealed that I3O could alleviate the symptoms of central precocious puberty induced by a high-fat diet and promote bone growth to increase final height, as well as reduce the side effects of GnRH on weight gain. The mechanism of I3O involved the regulation of the ERK-Sp1-KISS-1/GPR54 axis in the hypothalamus. Our present research exerted a new understanding viewpoint on the drug therapy for obesity-induced central

Figure 4. I3O inhibited the phosphorylation of ERK1/2 and Sp1 to reduce the expression of KISS-1 and GPR54 in the hypothalamus. (A) The expression levels of kisspeptin-1 and GPR54 were detected by Western blot. (B) The mRNA level of kisspeptin-1 and GPR54 was detected by RT-qPCR. (C) The total protein and phosphorylation levels of Sp1 and ERK1/2 were detected by Western blot. (D) The total protein and phosphorylation levels of Sp1 and ERK1/2 were detected by Western blot. (E) Serum level of GnRH was detected by ELISA. (F) The mRNA levels of kisspeptin-1 and GPR54 were detected by RT-qPCR. (G, H) The protein levels of GnRH, kisspeptin-1 and GPR54 were detected by Western blot. (I) The percent and days of the vaginal opening in mice. (J) Body mass of mice. (K) The protein levels of MMP-13 and Col-X were detected by IHC staining in the hypertrophic zone of growth plate tissues. *P<0.05, **P<0.01, ***P<0.001, ns no significance.
precocious puberty in children.

**Author contributions**

Yibiao Wang conceived and designed the project. Chunjie Jiang carried out the experiments and acquired the data. Chunjie Jiang and Lu Qi analyzed the data. Chunjie Jiang drafted the manuscript. Lu Qi and Jiang Xue critically revised the manuscript. All authors gave final approval of the submitted and published versions.

**Availability of data and materials**

The datasets used in this present study are available from the corresponding author upon reasonable request.

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**Conflict of Interests**

The authors have declared no conflict of interests exists.

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