ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 1/2023
183–190
https://doi.org/10.18388/abp.2020_6450

Regular paper

Timosaponin AIII attenuates precocious puberty in mice through downregulating the hypothalamic-pituitary-gonadal axis

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Precocious puberty (PP) has increasingly become a social concern. This study aimed to investigate the effect of timosaponin AIII (TAIII) on the precocious puberty and its possible mechanisms in mice. Four groups of mice consisting of controls that received saline or TAIII, a model that received leptin to induce precocious puberty (PP), and leptin+TAIII (the leptin model treated with TAIII) were used to determine the effect of TAIII on PP. Pathological and cytological examinations were conducted to investigate the signs and onset of PP and the development of reproductive organs. The level of serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) were determined using enzyme-linked immunosorbent assay (ELISA). The expression of genes related to the hypothalamic-pituitary-gonadal axis (HPGA) was assessed using qRT-PCR and Western blotting. Bone mineral density (BMD) was determined using high resolution peripheral quantitative computed tomography. In mice treated with leptin, earlier vaginal opening and estrus were observed, as well as the increased ovarian and uterine weight, total uterine cross-sectional size, number of corpora lutea, and elevated serum sex hormone levels and HPGA expression. On the other hand, TAIII treatment delayed the vaginal opening and vaginal estrus to 32.1 and 37.5 days after birth, and delayed the development of reproductive organs, leading to significantly smaller uterus and ovary size, less corpora lutea and low BMD ($P<0.05$). In addition, the serum levels of LH, FSH and E2 were significantly reduced ($P<0.05$) and so was the expression of HPGA and leptin genes ($P<0.05$). Our experimental data demonstrated that TAIII has activity against leptin-induced PP activity and may attenuate PP by reducing reproductive hormones and deactivating the hypothalamic-pituitary-gonadal axis through downregulating leptin expression.

Keywords: precocious puberty; herbal medicine; triterpenoid; serum hormone; gene expression

Received: 05 August, 2022; revised: 30 December, 2022; accepted: 16 January, 2023; available on-line: 16 March, 2023

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Abbreviations: ANOVA, analysis of variance; CPP, central PP; E2, estradiol; ELISA, enzyme-linked immunosorbent assay; FSH, follicle stimulating hormone; GRP54, G protein-coupled receptor 54; HE, hematoxylin-eosin; HPGA, hypothalamic-pituitary-gonadal axis; KISS1, KiSSpeptin-1; LH, luteinizing hormone; PCR, polymerase chain reaction; PP, precocious puberty; PVDF, polyvinylidene fluoride; qRT-PCR, real-time quantitative reverse transcription PCR; TAIII, timosaponin AIII; VE, vaginal estrus; VO, vaginal opening

INTRODUCTION

Precocious puberty (PP) is a growth and developmental disorder that manifests as early onset of secondary sexual characteristics, occurring before the age of 8 and 9 years in girls and boys (Bradley *et al*., 2020; Yang *et al*., 2021). The incidence of precocious puberty is growing and the age of puberty onset gradually decreases, largely due to the change of lifestyles (Kim *et al*., 2015). Clinical signs such as premature breast and pubic hair development, together with determination of bone age, pelvic echography and hormone tests are among the routine procedures to diagnose central (gonadotropin-releasing hormone (GnRH)-dependent) PP (CPP) and peripheral (GnRH-independent) PP (PPP) (Antoniazzi & Zamboni, 2004; Neely & Crossen, 2014). Due to the early onset of puberty, children with PP develop earlier and grow shorter, due to shorter time of bone growth and subsequently shorter stature (Censani *et al*., 2019) because the activation of the hypothalamic-pituitary-gonadal axis (HPGA) results in the release of gonadotropins that induces the development of puberty and promotes bone maturation (Cheuiche *et al*., 2021). In addition, PP can cause a series of psychological and physiological problems, which may also be associated with diseases, obesity, overweight and other environmental factors (Chae *et al*., 2021; Sitruk-Ware *et al*., 1986). At the molecular level, PP has been found to up- and down-regulate hundreds of proteins that potentially impact numerous metabolic pathways (Wang *et al*., 2021). In recent years, the age of PP onset trends to decrease and PP has become an important social issue, particularly for girls, who have the condition 10 times more often than boys, and their families (Cesario & Hughes, 2007).

Long-acting gonadotropin-releasing hormone analogs have revolutionized the treatment of CPP, resulting in the stabilization of pubertal progression, a reduced growth velocity, and a decreased bone age advancement (Cheuiche *et al*., 2021). However, questions remain regarding their optimal use in CPP and other conditions. Although most cases of precocious sexual maturation are gonadotropin-dependent and currently assumed to be idiopathic, there are mutations in genes involved in pubertal development, such as MKRN3 and DLK1, which may require different treatment options. Additional studies are needed to address key areas related to the psychosocial effects of CPP and their alterations due to the use of gonadotropin-releasing hormone analogs (Carel *et al*., 2009).

In East Asia, herbal medicines are used for the treatment of PP (Liu *et al*., 2016). Traditional Chinese medicine Zhimu is made from the roots of *Anemar-*

rhena asphodeloides and is an important member of the herbal medicines that have activity against PP. For example, Zhibaidihuang decoction made with *A. asphodeloides*, *Rehmannia glutinosa*, *Cornus officinalis*, peony bark, yam, *Poria cocos* and *Alisma orientalis* was found to alleviate PP by reducing the levels of serum hormone FSH, LH and E_2 (Xu & Qiu, 2007; Yu *et al.*, 2014). Sarsasapogenin, a steroidal sapogenin, is a main active ingredient in Zhimu (Bao *et al.*, 2007) and was found to have anti-PP activity through inhibiting the HPGA (Hu *et al.*, 2020a). In addition, pharmacological studies have shown that sarsasapogenin also has activities against thrombosis, Alzheimer's disease, tumor, inflammation, and depression (Han *et al.*, 2018; Wang *et al.*, 2014). Timosaponin AIII (TAIII), a triterpenoid, is also a main ingredient in *A. asphodeloides* extract with a number of pharmacological activities (Lin *et al.*, 2020). However, little is known about its activity against PP although decoctions made with *A. asphodeloides* are shown to be effective to attenuate PP (Xu & Qiu, 2007; Yu *et al.*, 2014).

The aim of this study was to investigate the therapeutic effect of TAIII on PP and its possible mechanisms by measuring serum sex hormone levels and expression of KISS1/GPR54 genes in mouse model of PP.

MATERIALS AND METHODS

Animals and treatments

Female C57BL mice, female, aged 2 weeks and weighing 12±1.1g, were purchased from the Laboratory Animal Center, Yanjiang Biotech, Shanghai, China. The animals were housed in groups of three in plastic box cages kept in pathogen-free animal rooms at 25°C and 42% humidity with 12-h light (06:00–18:00) and dark (18:00–06:00) cycles and *ad libitum* access to chow and drinking water. At the completion of experiments, mice were euthanized by carbon dioxide asphyxiation and tissues were isolated for subsequent analysis. Carbon dioxide was supplied at a flow rate of 20% of the cage volume per minute (5L/min). The death after exposure to carbon dioxide was confirmed after careful assessment of cardiac arrest. The study was carried out in compliance with the ARRIVE guidelines. The animal experimental protocols procedures were approved by the Institutional Animal Care and Use Committee of Beijing University of Chinese Medicine Shenzhen Hospital, Shenzhen, China.

The animals were randomly divided into controls (saline and TAIII), model (leptin), and TAIII (leptin+TAIII) groups with 16 mice in each group. At the age of 20 days, the mice were subcutaneously injected daily with leptin at 2 μg/g body weight (cat. no. 177404-21-6, Guangjian Pharmaceuticals, Shenzhen, China) in 100 μl saline with 0.1% DMSO i.p. at 09:00–10:00 hours during the light cycle as described previously (Ahima *et al.*, 1997) (model) and additionally TAIII at 10 μg (100 μl) per g body weight (cat.no. HY-N0810, MedChemExpress LLC, Shanghai, China) was administered to mice by gavage (TAIII group). Before use, TAIII was dissolved in 0.1% DMSO to 10 μg/μl to keep as a stock solution and stored at −20°C. Controls were injected with 100 μl saline or TAIII containing 0.1% DMSO. From the age of 25 days, the vaginal opening (VO) was checked daily. For the mice with VO, vaginal smear was checked every morning and the onset of vaginal estrus (VE) was determined as described previously (Nelson *et al.*, 1990).

Detection of serum hormone levels

Blood samples were collected from the abdominal aorta, and centrifuged at 4°C to obtain serum, which was stored at –20°C before analysis. Serum LH (ab72838, 1:2000), FSH (ab21011, 1:2000) and E_2 (ab100969, 1:2000) levels were determined using ELISA kits according to the supplier's instructions (abcam, Shanghai, China) with a Accuris SmartReader 96 (Labrepco, PA, USA). All assessments were repeated three times.

Pathological examination

The uterus and ovary samples were immediately isolated after the last blood samples were taken. The uterus and ovaries were isolated, weighed and fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE). To measure the size of uterus and ovary, the sections were pictured at 40× magnification, the edge of uterine was traced and grided with 100×100 point-grid using CAST software (v.2.0). The numbers of points falling on the cross section of the whole uterus and endometrium were counted and used to calculate the area through the built-in area conversion system in the software.

Bone densitometry

The tibias of the right hind limb were isolated and fixed in in 70% ethanol after removing the surrounding tissue. The tibial samples were longitudinally scanned using high resolution peripheral quantitative computed tomography (HR-pQCT) to display the morphology of the proximal tibia. HR-pQCT scans were performed 3.0 mm and 12.0 mm below the epiphysis line for determination of the cancellous and compact bone density.

qRT-PCR

Total RNA was extracted using Beyozol reagent (cat. no. R0011, Beyotime, Beijing China) from the hypothalamus tissues isolated from the brain according to the supplier's instructions and quantified suing a UV spectrophotometer. The RNA was converted to cDNA using BeyoRT first strand cDNA kit (cat. no. D7166, Beyotime). Real-time PCR amplification and quantification were carried out using BeyoFast SYBR Green One-Step qRT-PCR Kit (cat. no. D7268s, Beyotime) on the ProFlex™ PCR Systems (Thermo Fisher, USA). The cycle parameters consisted of initial denaturation at 96°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 15 s, and extension at 60°C for 60 s. β-actin gene was used as the internal standard, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of genes (Livak & Schmittgen, 2001). Primers for the PCR were synthesized at Huada Gene Inc., Shanghai and the sequences were:

KISS1
forward, 5'-GATGTCTGCGACCTGAGTCCC,
reverse, 5'-AGGCATTAACGAGTTCCTGGG;
GPR54
forward, 5'-GCGGCCACAGATGTCACTTT and
reverse, 5'-AGGTGGGCAGCGGATAGA;
GnRH
forward, 5'-GGAGCTCTGGAACGTCTGATT,
reverse, 5'-CAGCGTCAATGTCACACTCG,
gonadotropin releasing hormone receptor (GnRHR)
forward, 5'-CAGGACCCACGCAAACTACA,
reverse, 5'-GGGAGTCCAGCAGATGACAA,
leptin
forward, 5'-GCTGTGCCCATCCAAAAAGTCC,

Table 1. Weight and size of the ovaries and uterus and number of corpora lutea in mice treated with leptin and TAIII.

Group	No. animals	Ovarian weight (mg)	Uterine weights (mg)	Total uterine cross--sectional area (mm^2)	Endometrial cross--sectional area (mm^2)	No. corpora lutea
Control (Saline)	6	15.8±1.21	25.8±2.91	0.45±0.05	0.20±0.02	0
TAIII	6	15.2±1.11	25.5±2.11	0.48±0.06	0.20±0.02	0
Leptin	6	22.4±2.21*	40.3±4.99*	1.05±0.08*	0.55±0.03*	5.48±0.75*
Leptin+TAIII	6	18.8±1.81#	27.8±2.01#	0.65±0.06#	0.35±0.04#	1.28±0.25#

Data were presented as mean±standard deviation. *and #P<0.05 compared to controls (saline and TAIII) and leptin treatment, respectively, using one-way ANOVA.

reverse, 5'-CCCAGGAATGAAGTCCAAACCG and
β-actin
forward, 5'-CATTGCTGACAGGATGCAGAAGG,
reverse, 5'-TGCTGGAAGGTGGACAGTGAGG.

All reactions were repeated three times.

Western blot

Western blot analysis was performed to assess the expression of the KISS1 and GRP54 genes. The brain tissues were lysed in RIPA buffer (Thermo Fisher Scientific, USA) with protease inhibitors according to the supplier's instructions. Proteins in the lysates were quantitated using BAC protein quantification kit (Thermo Fisher Scientific) according to manufacturer's instructions. After boiling at 100°C for 5 min, 50 μg denatured proteins were subjected to each lane of 12% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. The KISS1 and GRP54 proteins were detected by incubating the membranes with antibodies against KISS1 (ab226786, 1:1500, Abcam, US), GRP54 (ab108606, 1500, Abcam), GnRH (ab281844, 1:1500, Abcam), GnRHR (ab183079, 1:1500, Abcam), leptin (ab219260, 1:1200, Abcam) and β-actin (as internal reference, ab179467, 1:1500, Abcam) and horseradish peroxidase conjugated IgG (H + L) (ZB-2301, 1:2000, ZSbio, Beijing). The immunoreactive bands were visualized using chemiluminescent substrate (ab5801, Abcam) in the dark according to the supplier's protocols and quantified using Quantity one (v4.62) analysis software (General Electric, UK).

Statistical analysis

Microsoft Excel (IBM, USA) with statistical add-in was used for statistical analysis of experimental data. The measurement data were compared among and between groups were conducted using one-way analysis of variance (AVONA) and *t*-test, respectively, and the data were expressed as mean ± standard deviation (m ± S.D.). P<0.05 was regarded as statistically significant.

RESULTS

TAIII postpones VO and VE

From day 25, all mice were checked for VO. For controls (saline or TAIII containing 0.1% DMSO-treated mice), VO occurred similarly at 32.4±1.96 and 32.6±1.86 days after birth. In leptin-treated mice, VO occurred at 29.47±1.26 days, which was significantly earlier than in controls (P<0.05). The VO occurred at 32.1.8±2.26 days in mice receiving leptin+TAIII, which was significantly later than for those receiving leptin treatment alone (P<0.05), suggesting that TAIII postpones leptin-induced PP. Similarly, VE occurred earlier in leptin-treated mice (34.8±2.74) as compared with controls (38.0±2.61 and 37.5±2.22) and was delayed in leptin+TAIII-treated mice (37.5±2.47) as compared with leptin-treated mice (Fig. 1). Leptin+TAIII-treated mice looked and behaved normally as compared with saline-, TAIII- and leptin-treated mice, during the experimental period.

TAIII delays sexual development

No difference was observed between the controls (saline or TAIII alone) in sexual development. Compared with the controls, leptin facilitated the development of ovaries and uterus, resulting in increased weight and size of these organs (P<0.05, Table 1). However, treatment with TAIII hindered this effect, as the sizes of ovaries and uterus were significantly reduced (P<0.05, Table 1), with lower ovarian weight (18.8 *vs* 22.4mg), uterine weight (27.8 *vs* 40.3mg), smaller total uterine cross-sectional area (0.65 *vs* 1.05 mm^2) and smaller endometrial cross-sectional area (0.35 *vs* 0.55 mm^2) as compared with leptin-treated mice (P<0.01, Table 1), although they were still larger than in the controls. Leptin treatment also resulted in the early development of corpora lutea, while leptin+TAIII treatments significantly reduced the number of corpora lutea as compared to the leptin-treated mice (P<0.01, Table 1).

TAIII reduces BMD

Bone development was similar between the controls (saline or TAIII alone) (Table 2). After leptin treatment, mice had significantly higher BMD, TBD and CBD as compared with controls (Table 2). TAIII treatment sig-

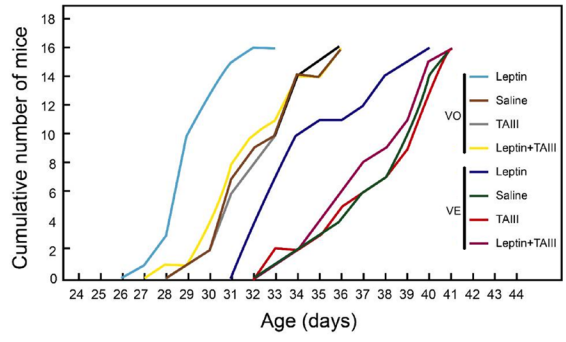

Figure 1. Onset of puberty in leptin-, saline- and TAIII-treated female mice.
The mice were weaned at 21 d, housed in groups of three under 12 h light (06:00–18:00) and dark (18:00–06:00) cycles, and allowed ad libitum access to chow and water. They were injected with recombinant mouse leptin, 2 μg/g body weight, saline vehicle, 100 μl i.p., or leptin plus TAIII, 10 μg/g body weight once a day at 10:00–11:00 hours until day 42. n=15 per group.

Table 2. Bone mineral density, trabecular bone density and cortical bone density in mice treated with leptin and TAIII.

Group	No. animals	Bone mineral density (mg/cm²)	Trabecular bone density (mg/cm²)	Cortical bone density (mg/cm²)
Control (saline)	6	340.5±2.21	180.5±1.24	540.5±4.14
Control (TAIII)	6	342.5±2.21	183.5±1.54	537.5±3.24
Leptin	6	410.5±3.23*	230.1±1.94*	680.5±5.04*
Leptin+TAIII	6	380.2±1.11#	210.8±2.11#	650.2±3.31

Data were presented as mean ± standard deviation. *and #$P<0.05$ compared to controls (saline and TAIII) and leptin treatment, respectively, using one-way ANOVA.

Table 3. Serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E_2) levels in mice treated with leptin and TAIII.

Group	No. animals	LH (ng/ml)	FSH (ng/ml)	E_2 (pg/ml)
Control (saline)	6	5.8±0.91	22.4±3.91	25.2±2.6
TAIII	6	5.7±0.61	21.4±3.56	24.7±2.4
Leptin	6	12.4±1.21*	39.3±4.99*	55.7±7.6*
Leptin+TAIII	6	7.8±0.81#	27.8±3.01#	±4.3 #

Data were presented as mean ± standard deviation. *and #$P<0.05$ compared to controls (saline and TAIII) and leptin treatment, respectively, using one-way ANOVA.

nificantly decreased BMD, TBD but not CBD in leptin-treated mice (Table 2).

TAIII reduces LH, FSH and E_2 levels

Next, we investigated the changes of several serum hormones that are related to PP development. The results showed that LH, FSH and E_2 levels were similar between controls (saline or TAIII alone) (Table 3). However, compared with the controls, the hormone levels were increased significantly after leptin treatment ($P<0.01$, Table 3). On other hand, TAIII administration reduced the levels of these hormones significantly as compared with leptin-treated mice ($P<0.01$, Table 3), although they were still higher than the controls.

TAIII downregulates the expression of KISS1, GPR54, GnRH and GnRHR

qRT-PCR and Western blot analyses showed that the expression of KISS1, GPR54, GnRH and GnRHR at both mRNA and protein levels was significantly upregulated in leptin treated mice (Fig. 2) but not in TAIII treated mice, as compared with control mice (saline-treated). Leptin+TAIII treatment significantly downregulated the expression of the four genes compared to leptin treatment (Fig. 2).

TAIII downregulates the expression of leptin

Since leptin is involved in GnRH secretion from hypothalamic neurons (Burcelin *et al.*, 2003), we assessed leptin gene expression in brain tissue. qRT-PCR and Western blot analysis showed that both mRNA and protein levels of leptin were not affected by either TAIII or leptin treatment, as compared with saline treatment (Fig. 3). However, both mRNA and protein levels of leptin were significantly reduced in leptin-treated mice after TAIII treatment (Fig. 3) the control, TAIII- or leptin-alone treated mice.

DISCUSSION

PP is a developmental disorder in children caused by early activation and hyperfunction of the HPGA axis. Our experimental data showed that leptin could induce PP in mice, manifesting with earlier VO and VE and increased BMD. TAIII delayed leptin-induced sexual development acceleration, as evidenced by reduced sex hormone production and decreased expression of the genes encoding HPGA components: KISS1, GPR54, GnRH and GnRHR. TAIII also reduced BMD, TBD, and leptin expression in the leptin-treated mice. These findings indicate that TAIII is one of the active ingredients in *A. asphodeloides* preparations that confers anti-PP activity.

Puberty is a complex developmental process, which is affected by a variety of genetic and endocrine factors. To investigate the therapeutic effect of TAIII, we constructed murine model of PP using leptin and measured the progress of sexual maturation using several indexes such as VO, VE and BMD. VO and VE are external signs of the initiation of female sexual development, and are also used as the criteria for judging the onset of puberty (Zhou & Li, 2014). After leptin treatment, VO and VE were accelerated, and BMD was increased, as compared with the controls (saline or TAIII). Furthermore, pathological and biochemical analysis showed that the ovarian and uterus development was enhanced and serum hormone levels were increased in the leptin-treated mice as compared with control, confirming that the leptin treatment induced PP in mice. This is consistent with the results obtained in a previous study showing that leptin could facilitate the onset of puberty in female mice (Ahima *et al.*, 1997). On other hand, TAIII treatment delayed VO and VE, reduced BMD and delayed the sexual organ developments in the leptin-treated mice, indicating that TAIII attenuates leptin-induced PP. The crude preparations of *A. asphodeloides* are used as herbal medicine for PP (Xu & Qiu, 2007; Zhou & Li, 2014), it would be interesting to investigate whether this purified compound from *A. asphodeloides* is also able to alleviate PP in humans. Recent study shows that leptin communicates metabolic information with the brain neurons that control reproduction using GABAergic circuitry, resulting in releasing of GnRH from hypothalamic neurons (Burcelin *et al.*, 2003) and puberty onset (Egan *et al.*, 2017). In rats, the model of PP is established using da-

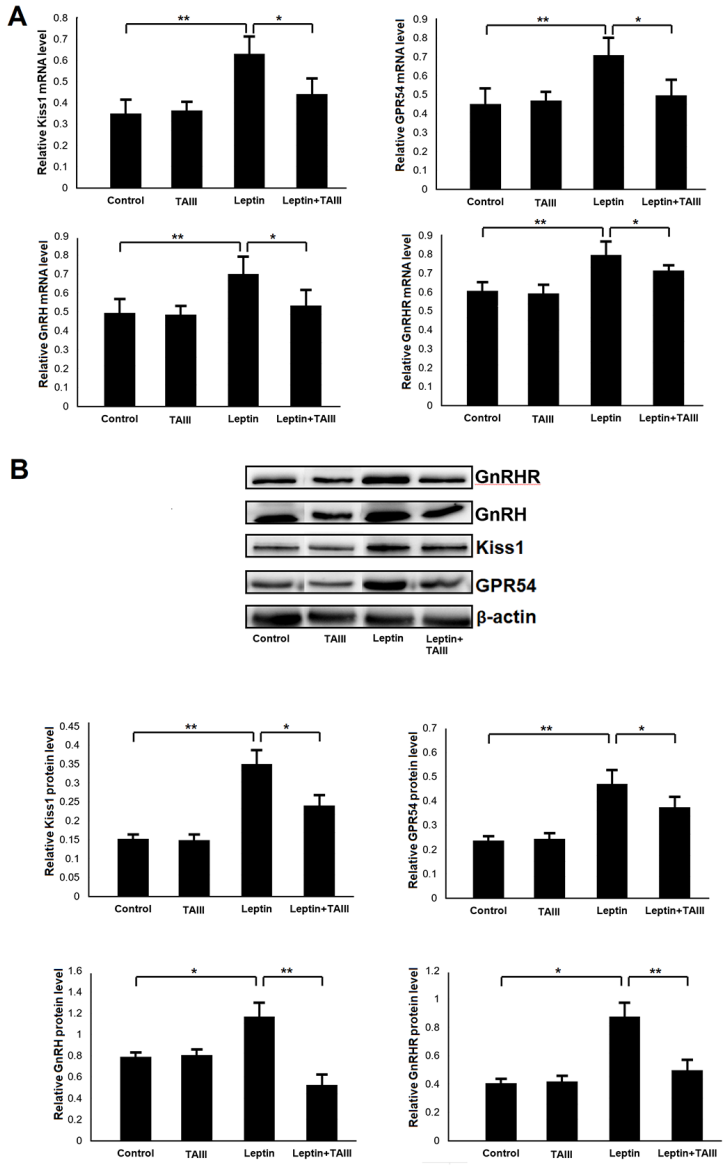

Figure 2. mRNA and protein expression of KISS1 and GPR54 after leptin and TAIII treatment.
qRT-PCR and Western blot analyses were performed using mRNA and protein extracted from the issues of mice treated with leptin and TAIII for 15 days. (**A**) Relative mRNA levels, (**B**) upper panel: representative Western blots, lower panel: relative protein levels. All assays were triplicated. One-way ANOVA was performed to compare the difference among the groups. *, **P<0.05 and <0.01, respectively.

nazol, a drug that can rapidly activate the HPGA leading to increased expression of KISS1 and GRP45 and onset of PP (Park *et al*., 2021).

To further investigate the mechanisms underlying TAIII-mediated anti-PP activity, we profiled the changes of several serum hormones that are related to PP. In females, LH and FSH are synthesized in the anterior pituitary gland and the levels of LH and FSH exhibit rhythmic changes throughout the menstrual or estrous cycle and have an important influence on development of puberty, gonads, and reproductive function. Premature secretion of LH and FSH leads to the early gonad activation and development of secondary sexual traits (Coss, 2018). E_2 is a steroid hormone secreted in the ovaries and is involved in the regulation of the estrous and menstrual reproductive cycles in female (Park *et al*., 2006; Siemieniuch *et al*., 2010). E_2 receptors are distributed in the uterus, breast and ovary. They function to promote the maturation of sexual organs (Hewitt & Korach, 2018). Earlier secretion of E_2 and other sex hormones accelerate bone maturation, resulting in shortened bone growth cycle and reduced bone mineral density (Khosla & Monroe, 2018; Manolagas *et al*., 2002). Therefore, LH, FSH, E_2 and GnRH stimulation test have been used as intuitive clinical indexes to diagnose PP (Yeh *et al*., 2021).

Our data showed that LH, FSH and E_2 were increased following leptin treatment as compared to the controls, which is consistent with the symptoms of PP, and the increases observed in other PP models (Park *et al*., 2021). However, after TAIII treatment, the levels of LH, FSH and E_2 in leptin-treated mice were decreased significantly, suggesting that TAIII is able to modulate the synthesis and/or secretion of these hormones, although the exact mechanisms of this modulation remain unclear. In parallel with the hormone levels, the signs of sexual development in leptin-treated mice, such as ovarian and uterus weight and size, and corpora lutea number, were reduced after TAIII treatment, further conforming that TAIII has activity against puberty onset. While hormones have been shown to play important role in sexual (Park *et*

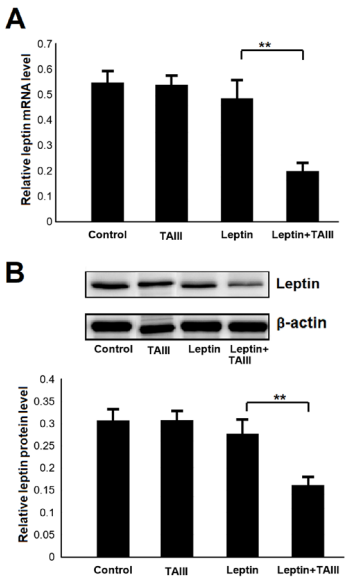

Figure 3. Leptin mRNA and protein levels in mice treated with leptin and TAIII.
qRT-PCR and Western blot analyses were performed using mRNA and protein extracted from the issues of mice treated with leptin and TAIII for 15 days. (**A**) Relative mRNA levels, (**B**) upper panel: representative Western blots, lower panel: relative protein levels. All assays were triplicated. One-way ANOVA was performed to compare the difference among the groups. $^{**}P<0.01$.

al., 2006; Siemieniuch *et al*., 2010; Miyakoshi, 2004) and bone development (Miyakoshi, 2004; Olney, 2003), the causal relationship between the reduced hormone levels, reproductive organ development and bone development needs to be further investigated to better understand the model of action of TAIII.

The hypothalamus secretes GnRH, which triggers the anterior pituitary to release FSH and LH that act on the gonads (ovaries) to secrete E2, forming the HPGA axis (Lockett *et al*., 1977). KISS1 was discovered as a novel human malignant melanoma metastasis-suppressor gene in melanoma cells on chromosome 6 (Lee *et al*., 1996). KISS1 expression is significantly upregulated in pubertal rats (Kuohung & Kaiser, 2006) and may be induced by estrogenic mycotoxin (Yang *et al*., 2016). GPR54 is a member of G protein-coupled receptors in the rhodopsin family and is expressed in brain regions (pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, frontal cortex, and striatum), as well as in peripheral regions (liver and intestine) (Lee *et al*., 1999). Although the actual role of the KISS-1/GPR54 system in the timing of puberty onset remains unexplored, the activation of the gonadotrophic axis at puberty represents the final point of a complex sex developmental cascade for reproductive capacity. KISS1 and its receptor GPR54 gene are important for puberty onset. The levels of KISS1 and GPR54 mRNA in the thalamus are elevated at puberty onset and they could stimulate GnRH neurons to trigger various pathways engaged in the onset of puberty (Kuohung & Kaiser, 2006). In PP rat, serum sex hormone concentrations are closely related to GnRH mRNA levels, while the synthesis and release of GnRH and GnRHR are regulated by KISS-1/GPR54. Early activation of KiSS1/GPR54 could activate the production of GnRH in the neurons, and subsequently the HPGA axis, resulting in accelerated sexual development (Dedes, 2012; Song *et al*., 2017). Our data showed that mRNA and protein expression of KISS1 and GPR54, as well as GnRH and GnRHR, in serum, was significantly upregulated after the leptin treatment, which was reversed by concomitant TAIII treatment. In rat model of PP, increased KISS1 and GPR54 expression was also observed following danazol treatment. However, GPR54 expression was not affected by sarsasapogenin, another active ingredient from *A. asphodeloides*, although it has anti-PP activity (Hu *et al*., 2020b). Apparently, the molecular mechanism of action of sarsasapogenin is different from the mechanism of action of TAIII, which downregulated KISS1 and GPR54 expression elevated by leptin treatment, suggesting that TAIII may exert anti-PP activity through the KISS1/GPR54 system.

As an adipocyte-derived hormone, leptin stimulates the secretion of gonadotropins from the pituitary and hence play important roles in pubertal development and maintenance of reproductive function (Dehpour *et al*., 1976; Tezuka *et al*., 2002). It regulates development and is a permissive factor for the initiation of human puberty, but not the only stimulator of puberty initiation (Terasawa & Fernandez, 2001). The difference in body fat quantity can affect leptin concentration to regulate the expression of genes related to obesity (Hu *et al*., 2001). Through informing brain centers about the amount of fat stored in the body, leptin exerts various regulatory functions especially associated with energy intake and metabolism, including controlling the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Roubos *et al*., 2012). Our analysis showed that after TAIII treatment, leptin mRNA and protein levels were reduced in the brain of leptin-treated mice. This might generate signals leading to reduced HPGA activities, including decreased KiSS1, GPR54**,** GnRH and GnRHR expression, and consequently reduced serum hormone levels, reduced BMD, and postponed PP (Antoniazzi & Zamboni, 2004). However, the impact of TAIII on leptin level in the blood was not assessed and should be investigated further. It is also noted that leptin protein level in the brain remained unchanged in leptin-treated mice compared to untreated mice. The exact reasons for this are unclear. It is likely that the subcutaneously injected leptin might need leptin receptor as a transporter to enter the brain via choroid plexus (Lynn *et al*., 1996) due to the blood-brain barrier, or it might be diluted/partially degraded after injection. While leptin treatment was reported to cause PP (Ahima *et al*., 1997), which is confirmed in the present study, no increase in brain leptin level observed in the leptin-treated mice suggests that leptin may signal outside of the brain to regulate gene expression in the brain indirectly.

Our assessment showed that endogenous leptin expression was only reduced in leptin and TAIII-treated animals, but not in control, TAIII, or leptin treated animals. There might be several mechanisms for this. TAIII might be bound/altered/activated by the injected leptin to downregulate the expression of leptin in the brain. Since the concentration of injected leptin was likely much higher than endogenous leptin in the blood (Odle *et al*., 2014), TAIII could act as concentration-dependent antagonist of leptin to inhibit the expression of leptin, resulting in differential outcomes in in leptin-treated animals and untreated animals. This hypothesis should be further tested to verify the relationship between TAIII concentration and leptin expression level. For example, ligand-binding assays could be used to investigate the molecular interaction between TAIII and leptin to better explain the results observed in the present study.

Although TAIII has been shown to have multiple pharmacological targets, including VEGFR, XIAP,

BMI1, mTOR, NF-κB, COX-2 and MMPs, the way it impacts gene expression of these targets is still unclear (Lin *et al.*, 2020). It is possible that TAIII may bind to regulatory sequences or targeting microRNAs that subsequently impact the expression of the target genes. Ligand binding assays, immunoprecipitation, compound-target binding assays and *in silico* predictions of herbal compound target may be designed to gain insight on the physical interactions between the molecule and cellular targets for better exploration of its anti-PP activity.

Taken together, our work demonstrated that TAIII is an active ingredient of *A. asphodeloides* preparation that counteracts PP. It downregulates the expression of KISS1, GPR54, GnRH, GnRHR and leptin, reduces the levels of serum LH, FSH, E_2, and delays BMD and sexual development in leptin-induced PP murine models. However, since this is a single dose study with model animals, the optimal efficacy and dose for PP control have not been determined. The safety profiles of this compound need to be determined and the therapeutic effects need to be further verified in other animal models and eventually in human subjects in order to develop it into a clinically applicable agent. Meanwhile, chemical modifications or screening of analogs may be carried to improve the efficacy and safety of this compound for clinical use.

Declarations

Ethics approval: the Institutional Animal Care and Use Committee of Beijing University of Chinese Medicine Shenzhen Hospital, Shenzhen, China.

Patient consent for publication: n/a.

Availability of data and material: The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: This study did not receive any specific fund from public and private sector.

Authors' contributions: LZ and XY designed the study. LZ, YR, QW and XY collected the data and performed analysis. LZ, DL, WZ, CL, and XY drafted the manuscript. All authors read and approved the final version of manuscript.

Acknowledgements: none

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