Chinese Herbs Antagonize The Effects of Bisphenol A On Kiss1 Expression Through Epigenetic Regulation In The Promoter Region

Wenke Dong
Children's Hospital of Fudan University
Jingwei He
Children's Hospital of Fudan University
Junqi Wang
Ruijin Hospital affiliated to Shanghai Jiao Tong University Medical School
Wen Sun
Children's Hospital of Fudan University
Yanyan Sun
Children's Hospital of Fudan University
Jian Yu (yuj@shmu.edu.cn)
Children's Hospital of Fudan University https://orcid.org/0000-0002-1077-0397

Research

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Abstract

Background: Bisphenol A (BPA), an environmental endocrine disruptor, is involved in precocious puberty. Chinese Herbs for Nourishing Yin and Purging Fire (NYPF) have been effective in delaying early onset of puberty. However, the underline mechanism is unknown. As epigenetics plays an important role in advanced puberty caused by extraneous factors. We intend to investigate the epigenetic impact of BPA and NYPF herbs on Kiss1 gene, a vital gene driving puberty onset, in hypothalamic neuron cells.

Methods: GT1-7 cells derived from GnRH neurons were used in the study. A series of concentration of BPA and serum containing NYPF herbs were prepared in advance. GT1-7 cells were administrated with BPA or vehicle first, followed by NYPF herbs or normal saline (NS) for 24 hours. Afterward, quantification of Kiss1 expression was performed through Realtime-PCR and Weston-blot methods. In addition, epigenetic modifications at Kiss1 promoter were examined through Bisulphite Sequencing (BSP) and Chromatin immunoprecipitation combined real-time PCR（Chip-qPCR）assays.

Results: Compared to control group, GT1-7 cells with BPA 10mg/l administration presented lower methylation status (61.25% Vs. 76.56%) concomitantly with enhanced abundance of mixed-lineage leukemia 1(MLL1) (1.05%±0.15 Vs. 0.27%±0.01) and Tri-Methyl-Histone 3 at lysine 4 (H3K4me3) (0.77% ±0.51 Vs. 0.02%±0.04) at Kiss1 promoter, leading to elevated expression of Kiss1. On the contrary, TCM for NYPF promoted increased DNA methylation (83.75% Vs. 72.5%), accompanied with less deposition of MLL1/H3K4me3 (MLL1: 0.14%±0.02 Vs. 0.97%±0.09; H3K4me3: 0.01%±0.00 Vs. 0.57%±0.30) at Kiss1 promoter than BPA 10mg/l group, which resulted in markedly inhibition of Kiss1 expression.

Conclusions: BPA may induce elevated expression of Kiss1 through influencing DNA methylation and histone modification at Kiss1 promoter. And NYPF herbs reverse the adverse effects of BPA on Kiss1 gene through epigenetic modification as well.

Introduction

Precocious puberty is defined as early emergence of secondary sexual characteristics in girls (before 8 years old) and boys (before 9 years old). Global trends with advanced puberty, as well as early menarche for girls, within the recent hundred years have been reported. Recent researches implicates that both environmental factors and nutritional status are involved in the process of puberty onset[1].

Environmental endocrine-disrupting chemicals (EDCS), including Bisphenol A (BPA) and its analogs and phthalates, have attracted attention due to their role in induction of precocious puberty [2]. BPA is mainly used to synthesize polycarbonate or epoxy resin and widely exists in the environment. It is usually detected in children's blood or urine samples, even in maternal amniotic fluid[3, 4]. Studies have shown that BPA is an estrogenic compound and induces precocious puberty in female pups[5, 6]. Epidemiological researchers also find that the content of BPA in serum or urine increases in precocious puberty girls [7, 8],

further supporting the theory that BPA disrupts the regular process of puberty in both children and animal pups.

Traditional Chinese Medicine (TCM) is an effective treatment for precocious puberty in China. According to TCM theory, precocious puberty is generally caused by changes of Yin Deficiency and Fire Excess, or Pathogenic Fire generated by Liver Depression, and Phlegm-Dampness Stagnation. Therefore, the therapeutic principle is nourishing Yin and purging Fire. We have explored for 40 years, in TCM clinical practice, a Nourishing Yin and Purging Fire formula capable of effectively delaying advanced onset of puberty. Preclinical experiments have shown that this NYPF herbs delay puberty process by regulation of Kiss1[9], mTOR [10]and lin28/let7 [11] expressions.

GnRH neurons in the hypothalamus, dynamically synthesizing gonadotropin releasing hormone (GnRH), are considered as puberty control center. GnRH, coded by GnRH1, is regulated by activatory and inhibitory neurotransmitter inputs. Before puberty initiation, it is mainly controlled by inhibitory neurotransmitters and only a small amount of GnRH is synthesized. As the age of puberty is approaching, activatory synaptic inputs become gradually dominant, leading to GnRH expression increased[12]. Kisspeptin, coded by Kiss1 gene, has been confirmed as one potent molecular to stimulate GnRH1 transcription. Additionally, Kiss1 neurons integrate nutrition and environmental signals and project onto GnRH neurons. Hence, Kiss1 gene plays an important role in environmental-mediated control of sexual development [13].

Epigenetic mechanisms, including DNA methylation, histone modification and miRNA and lincRNA regulation, have been demonstrated to play crucial roles in early onset of puberty induced by environmental factors[12]. Previous studies suggests that BPA exposure during early developmental period of animal pups alters expressions of DNA methyltransferase and histone post-translational regulatory enzymes[14]. Studies on TCM also found that herbal monomers or compounds are also involved in regulation of epigenetic related enzymes[15, 16]. However, the epigenetic mechanisms underlying BPA and TCM's involvement in adolescence remain unknown.

GT1-7 cells are derived from GnRH neurons and express both Kiss1 and GnRH1 genes. In this study, the cells were chosen to explore the relationship between BPA/ NYPF herbs and Kiss1/GnRH1 expressions, and furthermore the underline epigenetic mechanisms.

Materials And Methods

GT1-7 cells

GT1-7 cell line was provided by Pediatrics Laboratory of Ruijin Hospital in Shanghai. All interventions were conducted as the cells were at the logarithmic growth phase.

BPA Preparation

First, a series of concentration of BPA solutions (500ng/l, 50ug/l, 500ug/l, 5mg/l and 10mg/l) were prepared by dissolving different dose of BPA in an equal volume of phosphate buffered saline (PBS). Then, BPA-containing PBS diluted in cell medium, depending on the group designation, was used for an additional culture period of 24 hours.

NYPF herbs

The NYPF herbs are an original prescription from TCM Department, Children's Hospital of Fudan University. In this study, particle prescription of NYPF herbs was used. It is composed of nine herbal concentrate-granules, namely, Rehmannia glutinosa libosch root (Sheng-Di-Huang 5g), Scrophularia buergeriana (Xuan-Shen 3g), Anemarrhena asphodeloides (Zhi-Mu 3g), Cortex phellodendri (Huang-Bai 3g), Alisma plantago-aquatica L. var. orientale Sam (Ze-Xie 3g), Carapax et Plastrum Testudinis (Zhi-Gui-Ban 2g), Hordeum vulgare L (Mai-Ya 6g), Asparagus cochinchinensis (Tian-Dong 3g) and Roast Radix Glycyrrhizae (Zhi-Gan-Cao 2g), provided by PuraPharm Corporation (Nanning, Guangxi, China). The particle mixture was dissolved in distilled water at a final concentration of 0.4g/ml. The dose for animal administration was 1.0g/100g.d, which was the equivalent dosage for the treatment of precocious puberty in clinic[17].

Animals

Pregnant SD rats were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd (Certification number: SCXK (Zhe) 2019-0001) and raised in the Laboratory Animal Center at Fudan University Shanghai Medical College, with controlled photoperiod (12/12 h light/dark cycle) and temperature (23–25 °C), meeting Specified Pathogen Free standards. All the animals had ad libitum access to tap water and pelleted chow. All the animals and experimental procedures were approved by the research ethics board in accordance with established ethical guidelines.

Pharmaceutic Serum

The preparation methods of Pharmaceutic Serum were based on previous research [18] with a little modification. The brief protocol was as follows. Female rats at postnatal 21 (P21) were randomly divided into TCM group or normal saline (NS) group (N=10 each). They were continuously given TCM for NYPF or equal volume of NS as designated by gavage twice a day, respectively, from P21 to P25. On the morning of P25, one hour after the last administration, rats were sacrificed and blood samples were drawn from abdominal aorta. The serum was then separated by centrifugation and inactivated through heating at 56℃ for 30 min followed by aseptic filtration. Finally, the samples were stored at -80℃ for later use.

GT1-7 cells were intervened first by BPA, followed by serum containing TCM or NS at the final concentration of 10% (vol/vol). Then the cells were proceeded to culture for an additional 24 hours before harvesting.

Real-Time PCR (RT-PCR) Analysis

The mRNA levels of Kiss1 and GnRH1 were detected by RT-PCR. Total RNA was extracted using RNAiso Plus reagent (9109, TAKARA, Japan) and quantified by ultraviolet spectrophotometry (Nanodrop2000, Thermo-Scientific). Then 1ug of RNA per sample was transcribed into cDNA using reverse transcriptase kit (RR036A, Takara, Japan). Each tube for RT-PCR contained 2ul of cDNA, 1.6ul of primers (0.8ul each), and TB Green® Premix Ex Taq™ II reagent (RR820B, TAKARA, Japan) in a final volume of 20ul. And PCR amplified conditions were as follows: a cycle of 30s at 95°C for pre-denaturation and followed by 40 cycles for denaturation at 95°C for 15s and annealing at 60°C for 60s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, using the $2^{-\Delta\Delta Ct}$ method to calculate the relative mRNA levels. All the primers used in the assay (shown in Table 1) were designed by Primer3 software and synthesized by Shanghai Sangon Biotech Inc. (Shanghai, China).

Western Blot Analysis

Kiss1 and GnRH1 proteins were quantitatively analyzed by WB. Total protein was obtained using RIPA lysis buffer (Medium 20115ES60, YEASEN, Shanghai, China). BCA protein detection kit (20201ES76, YEASEN, Shanghai, China) was used to test protein concentration. Afterward, 40–60ug of total protein were separated by 15% SDS-PAGE, and it then was transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were locked with 5% (wt./vol) skimmed milk for 2 hours and probed with primary antibody overnight at 4 ℃. Next, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:4,000; CST, New York) for 2 hours. Subsequently, the greyscale images were obtained and quantified using the Image J software (NIH). The primary antibodies used in the assay contained rabbit polyclonal anti-Kisspeptin antibody (ab19028; 1:200 dilution; Abcam, Los Angeles, CA), anti-GnRH1 antibody (abs138073, 1:1,000 dilution, Absin, Shanghai, China), mouse monoclonal anti-GAPDH (T0004, Affinity, Jiangsu, China) or rat polyclonal anti-Tubulin antibody (30302ES20, YEASEN, Shanghai, China).

Bisulfite DNA Sequencing PCR (BSP)

The DNA methylation levels at Kiss1 promoter were tested by BSP. Genomic DNA was extracted using a genomic DNA extraction kit (DP304, Tiangen, Beijing, China). First, the genomic DNA was modified using Methylamp DNA Modification Kit (P-1001-1, EPIGENTEK, Farmingdale, USA). Afterward, a total of 20ul mixture, including 2ul of sulfite-modified DNA, 2ul of primers (1ul each), 2ul of 10X buffer, 2.7ul of Mg+, 9.8ul of ddH2O, 1.7ul of dNTP and 0.1ul of enzyme, was prepared for PCR reaction. PCR fragments were confirmed by agarose gel electrophoresis and they were then cloned to pGEM-T Vector (A1360, PROMEGA, USA). Subsequently their identity was verified by Sanger sequencing by Shanghai BioSune Co., LTD (Shanghai, China). The sequence was blasted online using PubMed comparison software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, methylation rate was determined using the formula: methylated CpG/ (methylated + unmethylated CpG) *100%. BSP primers were designed using

MethPrimer software (http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi) and shown in Table1.

Chromatin Immunoprecipitation Assay

To assess the content of MLL1 and H3K4me3 at the promoter region of Kiss1, we performed Chip assays using chromatin extracted from each group according to the instructions of Magna ChIP® A/G Chromatin Immunoprecipitation Kit (17-10085, Millipore, USA). The brief protocols were as follows: cell samples counting $2*10^6$ each group were washed once in ice-cold phosphate-buffered saline (PBS) containing a protease inhibitor cocktail II. Thereafter, cells were crosslinked by exposing to 1% formaldehyde for 10 min at room temperature. After two additional washing steps the samples were lysed with cell and nuclear lysis buffer, and sonicated for 30 s by 10 times to yield chromatin fragments of 200~500 base pairs (bp) using the Ultrasonic Processor (Bioruptor Pico, Diagenode, Belgium) at 4℃. Size fragmentation was confirmed by agarose gel electrophoresis. The sonicated chromatin was clarified by centrifugation at 12,000g for 10 min at 4℃, then take 50ul of supernatant into a clean EP tube, brought up to 500ul in chip dilution buffer for each reaction. Input sample with 50ul was removed from each tube of chromatin in advance and the remains were incubated with 5ug of MLL1(05-765, Millpore, USA) or H3K4me3(9727, CST, USA) antibodies and with 25μl of protein A or G beads solution (Dynabeads) for 4 hours with rotation at 4°C. Four hours later the beads were washed first with 0.5 ml low-salt wash buffer, followed by high-salt wash buffer, LiCl buffer and finally with TE buffer. Thereafter, the complexes were eluted with 100μl of chip elution buffer containing proteinase K at 62°C for 2 hours with rocking. To reverse the crosslinking reaction the samples were incubated at 95°C for 10 min. Then DNA for qPCR analysis was recovered using ChIP DNA Clean & Concentrator columns. All the reagents mentioned above were included in the Magna ChIP Kit.

Real-time PCR Detection of Chromatin Immunoprecipitated DNA

The 5'-flanking regions of Kiss1 gene between nt -2000 and nt +1, using transcriptional start site (TSS) as reference point, were supposed as promoter region. The putative promoter fragments were predicted online using the website (https://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl) and qualified by RT-PCR using Chromatin Immunoprecipitated (IP) samples. Each reaction system was comprised of 2uL of IP or input sample, 2ul of primers (1ul each) and TB Green® Premix Ex Taq™ II reagent (Tli RNaseH Plus) (RR820B, Takara, Japan) in a final volume 20 ul. Data are expressed as % of IP signal/input signal.

Statistical analysis

All the data were presented as mean ± standard error (SEM) and statistical analyses were performed using SPSS 17.0 (IBM Corporation, Armonk, NY, USA). One-way ANOVA was used to make comparisons among multiple groups. The data were first subjected to a normality and an equal variance test. If the variance was homogeneous, the LSD tests were used. If not, the Dunnett T3 tests were conducted. In addition, comparison of proportion analysis was evaluated by Chi-square test. Values were considered to be significantly different as $P < 0.05$.

Table1.Primers used in the study

Gene	accession	primers	sequence	amplicon
Kiss1	NM_178260.3	Kiss1-F-q-PCR	CCCAGGAACTCGTTAATGCC	94bp
		Kiss1-R-q-PCR	CATGGCGACGACCTACGA◦	
GnRH1	NM_008145.3	GnRH1-F-q-PCR	CTGATGGCCGGCATTCTACT	136bp
		GnRH1-R-q-PCR	CCTCCTTGCCCATCTCTTGG	
Kiss1	NC_000067.7	Kiss1-F-methylation	AGTTGGTGATATTAAGAAAAAGTAGGG	393bp
		Kiss1-R-methylation	AAAAAACTCATAAAAATAACCCC	
Kiss1	NC_000067.7	Kiss1-F-Chip-qPCR	GCCATTAATAGCTCCCCAAGC	117bp
		Kiss1-R-Chip-qPCR	ACCAGCTGCTATCTATGCCTG	

Results

Effects of BPA on Kiss1 and GnRH1 expressions

Compared to control group, GT1-7 cells with BPA 10mg/l administration presented increased mRNA levels of Kiss1 and GnRH1 ($P < 0.05$), as well as protein expressions (Fig1). In addition, GnRH1 mRNA levels also elevated in BPA 500ug/l group than control group.

Effects of TCM on Kiss1 and GnRH1 expressions

TCM for NYPF inhibited the overexpression of Kiss1 and GnRH1 induced by BPA exposure. As shown in Fig2, NYPF herbs presented inhibiting effects on both mRNA and protein levels of these two genes.

Effects of BPA/TCM on DNA methylation at Kiss1 promoter

The fragments between nucleotide (nt) -2000 and nt +1 in the 5'-flanking regions of Kiss1 gene were sequenced. Methylation differences were detected in the fragment between nt -547 and nt -155 among the four groups. As shown in Fig 3, compared to control group, the levels of DNA methylation decreased with BPA 10mg/l administration. Conversely, the methylation levels elevated in TCM mediated group than NS group. Furthermore, there were significant differences in methylation incidence at sequences ranged from nt -436 to nt -377, containing four methylation sites of CpG2/3/4/5, between BPA and control group (37.5% Vs. 62.5% $P<0.05$). The methylation rate of CpG3 (nt -427) was significantly different between TCM and NS group (100% Vs. 50% $P<0.05$).

Effects of BPA/TCM on Histone modification at Kiss1 promoter

Chip-qPCR analysis were targeted at the DNA fraction between nucleotide (nt)-2068 and +1 using IP samples incubated with H3K4me3 or MLL1 antibodies. As shown in Fig 4, the content of MLL1 and H3K4me3 elevated simultaneously at the fragments between nt -657 and nt -540. Once administration of Chinese herbs, both H3K4me3 and MLL1 deposition decreased significantly at the same region. In addition, similar changes of H3K4me3 deposition were found in the following fragments (nt -2068 to -1948, nt -865 to -736). However, there was not alteration of MLL1 content at the two fragments.

Discussion

There is not dose-effect relationship between BPA exposure and precocious puberty. In-vitro trials performed on embryonic hypothalamus cells from mice have shown that a BPA dosage of 20nM-20uM doesn't have a significant effect on GnRH1 mRNA expression. However, an increase to 200uM can significantly inhibit its expression[19]. Additionally, studies on the rhesus monkey hypothalamus establish that 10nM BPA inhibits the expressions of both Kiss1 and GnRH1[20]. Another study on hard-bone fish shows that 10ug/l of BPA can significantly increase the expression of Kiss1 and GnRH1 genes[21]. Our study performed on GT1-7 cells have verified that 10mg/l of BPA, equivalent to 440uM, significantly enhances expressions of Kiss1 and GnRH1.

Epigenetic modification is regarded as an important way of environmental factors, drugs and food affecting human growth and development. DNA methylation is a common manner of epigenetic modification. It has been demonstrated that DNA hyper-methylation is associated with gene silencing, while hypo-methylation is associated with gene activation. This is concurrent with prior findings that hyper-methylation of Kiss1's CpG island near the TSS results in decrease in mRNA and protein levels of the Kiss1 gene in human tumor cell lines [22]. The expressions of Kiss1 are significantly increased when Azacytidine, one demethylated drug, is administrated, suggesting that DNA methylation affects the expression of Kiss1[23]. However, the CpG island is not present in the rodent Kiss1 gene, hinting at racial differences in its regulation[23]. In our study, although CpG island doesn't exist near the TSS of Kiss1 gene in mouse GT1-7 cells, DNA methylation incidence at the fragments between nt -547 to nt -155 is verified to associate with Kiss1 expression. 10mg/l of BPA promotes DNA hypo-methylation, contributing to expression of gene. However, the NYPF reverses DNA hypo-methylation induced by BPA, which subsequently leads to repression of Kiss1 expression. Therefore, our study suggests that the DNA methylation between nt -547 to nt -155 near TSS, especially at the range from nt -436 to nt -377 (the fragment of CpG2/3/4/5), may be a target of BPA and TCM for Kiss1 regulation in GT1-7 cells.

Chromatin histone modification is another major manner of epigenetic modification. Previous research identifies MLL1 and MLL3 as central components of an activating epigenetic machinery that dynamically regulate Kiss1 gene in rat KNDy neurons[24]. Preceding puberty, MLL1 changes the chromatin configuration at the promoters of Kiss1 from repressive to permissive by H3K4me2/3 deposits[24]. In our study, we also have found that high levels of H3K4me3 and MLL1 deposition at chromatin region from nt -657 to nt -540 near TSS is accompanied by enhanced Kiss1 gene expression. As promoters of active

genes display high levels of active histone maker H3K4me3 and MLL1[24], suggesting the fragment behaves as a promoter domain. It is reported that BPA involves in ovarian developmental process through modulation of DNA methyltransferase, TET enzyme, and histone modification enzymes[25, 26]. Our study also manifests that BPA enhances gene expression through modification of MLL1 and H3K4me3.

This experiment indicates both DNA methylation and histone modification are two epigenetics mechanisms involved in regulation of Kiss1 expressions by TCM and BPA. It might not only represent the epigenetic mechanisms through which BPA induces precocious puberty but also be a viable indicator of the beneficial therapeutic role of TCM.

This experiment was carried out in-vitro, and further studies in-vivo are needed to clarify the relationship between BPA / TCM and precocious puberty.

Conclusions

In summary, our study shows that BPA promotes Kiss1 expression through Epigenetic regulation of Kiss1 promoter, represents a crucial step in BPA-induced precocious puberty mechanisms. Meanwhile, the implementation of the Traditional Chinese Medicine for NYPF recovered the poised promoter status, in consequence, reversed the adverse effects of BPA, therefore, establishing the epigenetic mechanisms of TCM therapy in precocious puberty.

Abbreviations

TCM	Traditional Chinese Medicine
NYPF	Nourishing Yin and Purging Fire
CpG	Cytosine Phosphate Guanine
TSS	Transcription Start Site
BPA	Bisphenol A
MLL1	mixed-lineage leukemia 1
H3K4me3	Tri-Methyl-Histone 3 at lysine 4
mRNA	Messenger Ribonucleic acid
DNA	Deoxyribonucleic acid
BSP	Bisulphite Sequencing
Chip-Qpcr	Chromatin immunoprecipitation combined Real-time PCR

nt nucleotide

Declarations

Acknowledgements

Not applicable.

Authors' contributions

WKD and JWH equally contributed to the work; JY, YYS and WS designed and conceived the study; WKD, JWH and JQW carried out the experiments and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethic Committee of Children's Hospital of Fudan University (Approval No. [2019]134, date of approval by ethic committee: 2019/02/27), and the experimental procedures were conducted in accordance with the ethical standards of the Chinese Association of Accreditation of Laboratory Animal Care.

Consent to publish

The authors give full consent to publish.

Competing interests

The authors declared no competing interests.

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Availability of data and materials

The data used to support the current study are available from the corresponding author on reasonable request.

Footnotes

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Figures

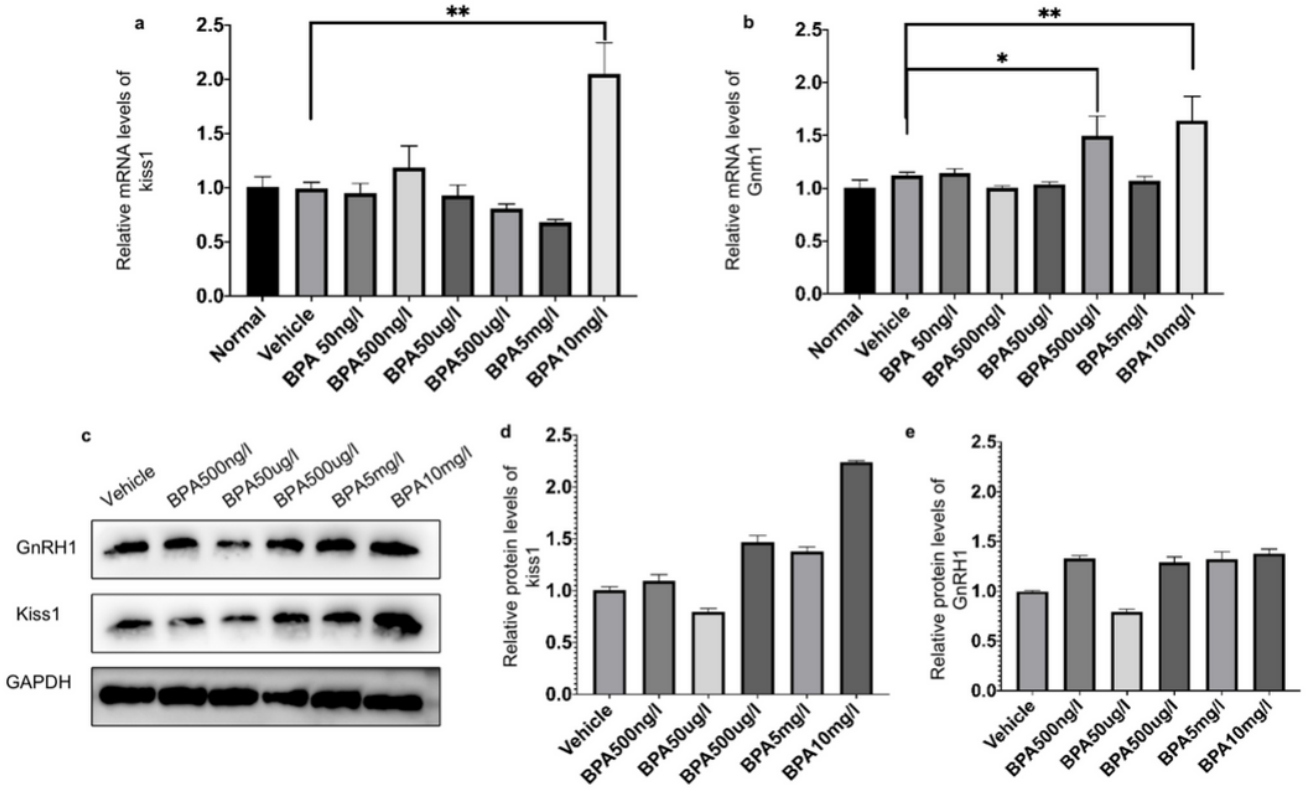

Figure 1

The effects of BPA on Kiss1 and GnRH1 expressions. a Relative mRNA levels of Kiss1 in different dose of BPA administration. b Relative mRNA levels of GnRH1 in each group. c Examples of original western blotting bands showing expressions of Kiss1 and GnRh1. d Relative protein levels of Kiss1. e Relative protein levels of GnRH1. The data were presented as mean ± SEM (n = 3 in each group). **P < 0.01, *P < 0.05.

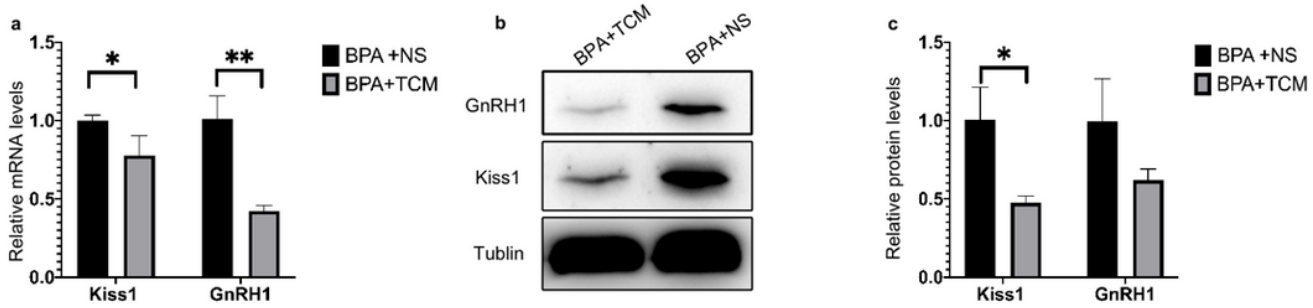

Figure 2

The effects of TCM on Kiss1 and GnRH1 expressions. a Relative mRNA levels between TCM treated group and control group. b Examples of original Western blotting bands showing expressions of Kiss1 and GnRh1. c Relative protein levels of Kiss1 and GnRH1.The data are presented as mean ± SEM (n = 3 in each group). **P < 0.01, *P < 0.05.

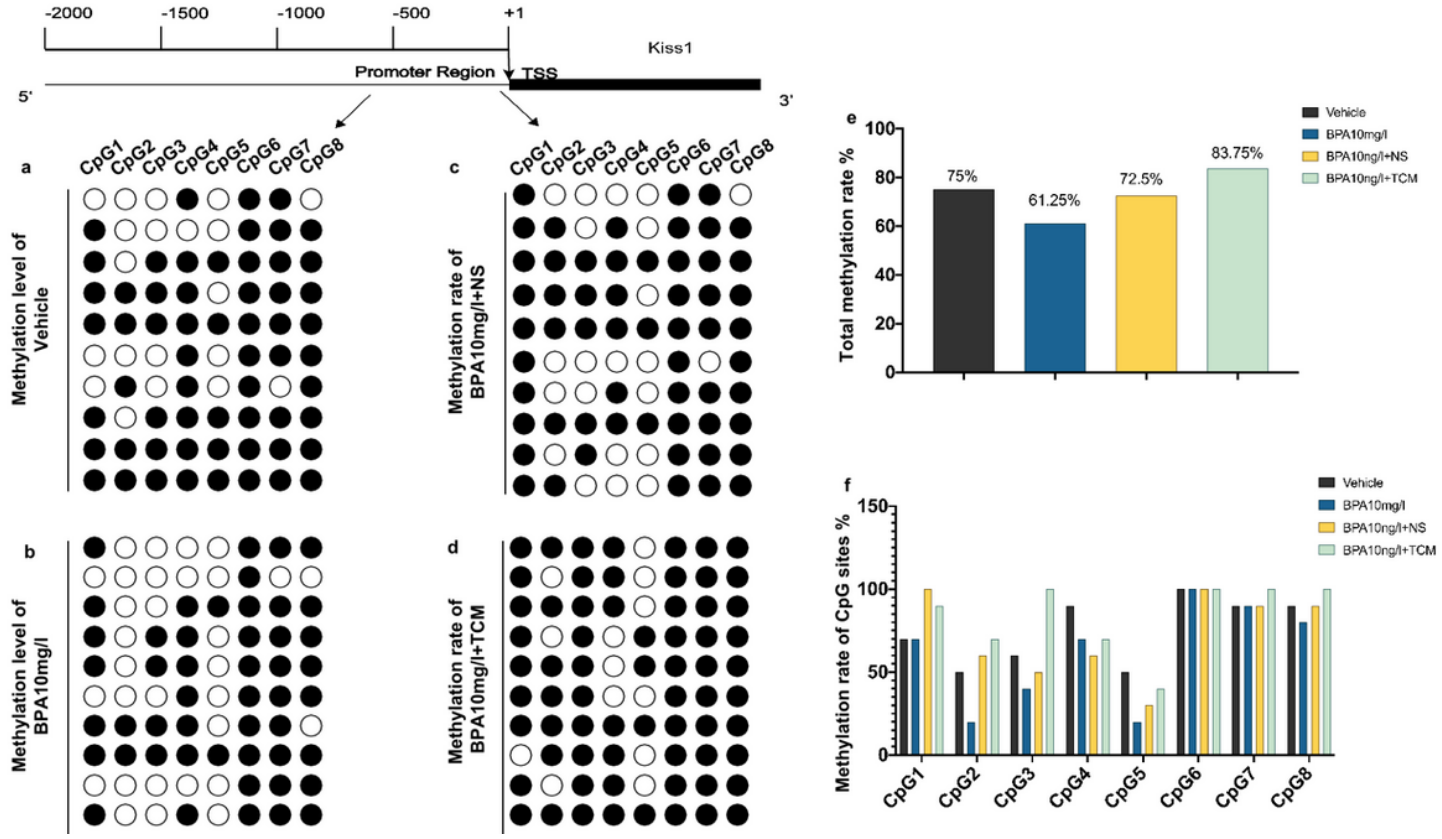

Figure 3

The effects of BPA and TCM of NYPF on methylation incidence at Kiss1 promoter. a DNA methylation status at Kiss1 promoter in vehicle group. b DNA methylation status at Kiss1 promoter with 10mg/l BPA administration. c DNA methylation status at Kiss1 promoter in BPA combined NS intervention group. d DNA methylation status at Kiss1 promoter in BPA combined TCM mediated group. e Total methylation levels at the region between nt -547 and nt -155 near TSS in each group. f The methylation levels at each CpG site in four groups. All the data was presented as percentages.

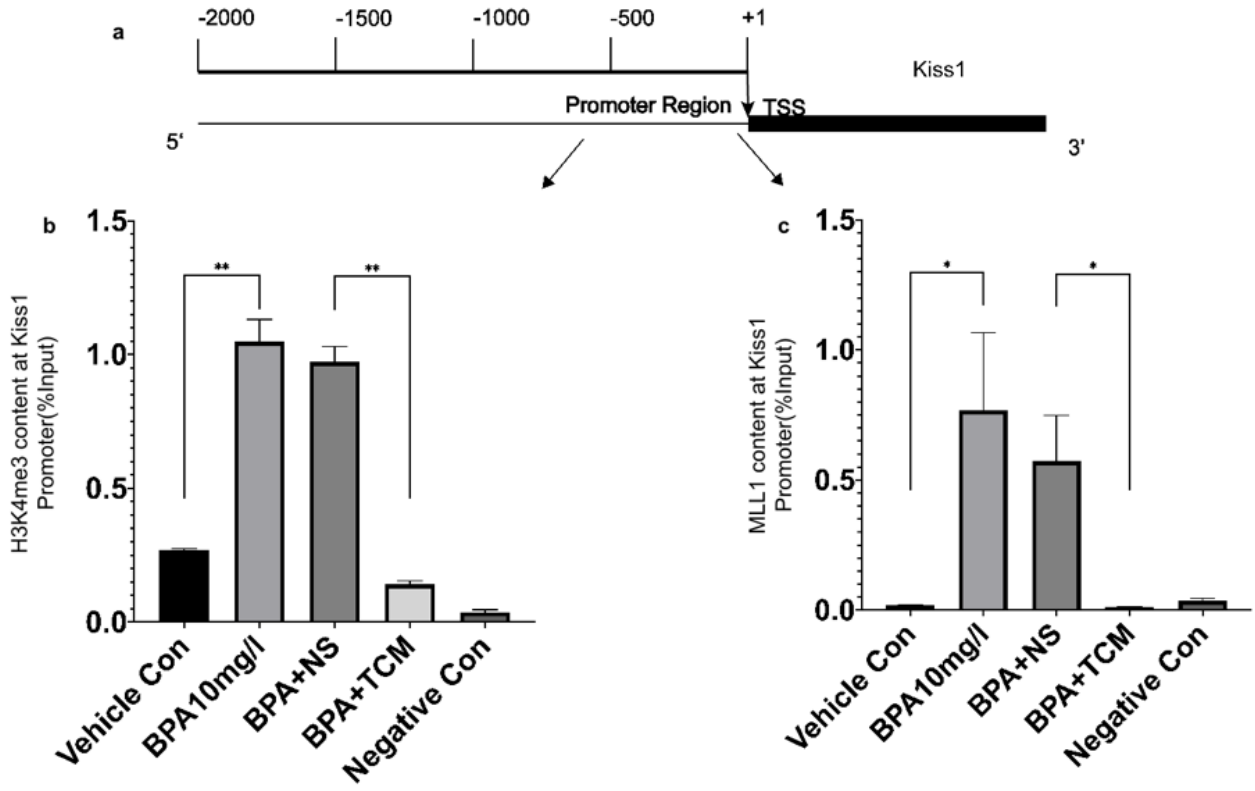

Figure 4

The effects of BPA and TCM for NYPF on histone modification at Kiss1 promoter. a A genomic region upstream of TSS was subjected to ChIP-qPCR assay. The fragments between nt -657 and nt -540 near TSS behaved as Kiss1 promoter domain as H3K4me3 and MLL1 were enriched in this region. b The levels of H3K4me3 deposition in each group. c The levels of MLL1 deposition in each group. All the data was presented as percentages. **P < 0. 01. *P < 0.05.