# Oral Bisphenol-A Regulates The Development and Function of Reproductive System through Differential Expression of METTL3

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**Research**

**Keywords:** BPA, hyperandrogenism, METTL3, ovary, uterus

**Posted Date:** May 13th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-28387/v1

# Abstract

Endocrine-disrupting chemicals (EDCs), which have a profound impact on the reproductive system, can cause endocrine and reproductive disorders, such as polycystic ovary syndrome (PCOS). Bisphenol-A (BPA) is a common endocrine disruptor which can affect the function of the reproductive system. However, the mechanism by which this molecule disrupts normal reproduction is still unclear. In this study, we hypothesized that oral BPA would have a effect on the structure and function of the reproductive system in adolescent female rats, and we would explore a kind of feasible mechanism for this effect. 4-week-old Sprague-Dawley (SD) rats were intragastrically treated for 10 weeks, which were divided into blank group (n = 8), control group (soybean oil, n = 8), three BPA-treatment groups (0.5 mg/kg BPA + soybean oil, 5 mg/kg BPA + soybean oil, 50 mg/kg BPA + soybean oil, n = 8). The results showed that 0.5 mg/kg oral BPA increased the coefficient of uteri, and oral BPA increased the length of uteri in rats without causing hyperandrogenism and ovarian polycystic changes. Oral BPA disturbed the expression of CYP17A1, CYP11A1 and METTL3 in ovaries. Our results suggested that oral BPA might partially interfere uterine morphology and the level of androgen synthetases with RNA methylation by disturbing the expression level of METTL3. RNA methylation might be a new way to explain the interference mechanism of BPA.


# Introduction

Endocrine-disrupting chemicals (EDCs) are a group of exogenous substances which can interfere with the normal biological processes such as synthesis, secretion, transport and metabolism of natural hormones in an organism. EDCs can impact the functioning of nervous, immune and reproductive systems, resulting in endocrine diseases including polycystic ovary syndrome (PCOS). PCOS is a common endocrine disorder in women of childbearing age, characterized by sparse ovulation or anovulation, hyperandrogenism, and polycystic changes of the ovaries. In recent years, researchers have tried to explore the etiology of PCOS from genetic, metabolic, environmental, and inflammatory perspectives [1]. Hyperandrogenism is one of the cardinal features of PCOS. Both clinical and laboratory studies have revealed that the over production of androgen is correlated to the expression of enzymes which involved in androgen synthesis (following called androgen synthetases) in the ovary [2, 3], such as StAR, CYP11, CYP17 [4], HSD3B [5], etc. It has been found that CYP11A1(tttta)n repeat polymorphism may be a potential molecular marker of PCOS risk [6, 7].

Being a very representative EDC on endocrine disorders, Bisphenol-A (BPA) was first exploited as a synthetic estrogen. This substance is also commonly used to harden plastics. BPA in the environment can enter the human body through the mouth, skin, respiratory tract, etc. The bound free BPA can be metabolized by glucuronic acid or sulfonic acid in the liver and excreted via the kidney [8]. BPA exposure can affect the brain [9], heart [10], liver [11], kidney [12], adipose tissue, breast [13] and the reproductive system [14, 15] at lower relative concentrations, resulting in, for example, obesity, diabetes [16], PCOS [17]. Furthermore, substantial BPA-induced reproductive toxicity has been observed. BPA is found to be able to change the phenotypes of progeny by stably altering the parental epigenome [18, 19]. Perinatal BPA

exposure can alter the function of reproductive endocrine system by hypomethylating some imprinting genes during oocyte maturation or decreasing the expression of the estrogen receptor ER at both the mRNA and protein levels [20]. The direct toxicity of BPA is manifested in the disruption of decidualization in vitro [21], enhanced expression of estrogen receptors-α (ER-α) and oxidative stress in embryonic stem cells [22], and promotion of apoptosis [23].

Clinical data demonstrate increased levels of BPA in patients with PCOS [24], and the levels can reach those in males [25]. PCOS induced by BPA exposure is also associated with hyperandrogenism and hyperinsulinism. BPA not only stimulates ovarian production of testosterone but also inhibits the activity of testosterone hydroxylase which helps maintain the testosterone concentration [26]; BPA-treated mice develop ovarian polycystic changes and luteal loss [27, 28], unbalanced expression between androgen synthesis-related enzymes and androgen receptors in the ovary, and abnormal expression of steroidogenic genes [29]. Following transient exposure to BPA before puberty, rats experience ovarian weight loss and follicle number decline [30]. Direct cellular exposure to BPA induces an increase in the percentage of germ cells and a decrease in the percentage of primordial follicles [31]. Several experiments have found that BPA exposure which exceeded the maximum safe dose would present direct toxicity by damaging structure or function on the reproductive system, but the dose below the maximum produced chronic toxicity [32]. Whether the chronic toxicity is dominant depends on the factors such as species, sex, age, concentration and contact method, etc. Meanwhile, below the maximum safe dose, the interference of BPA exposure is more visible at a lower dose [33].

It is well established that epigenetic modification can regulate mRNA and protein expression without altering the DNA sequence. Several excellent reviews have alluded to the possible correlation of the hypomethylation status of gene promoter regions, such as EPHX1, to PCOS [34]. N6-methyladenosine (m6A), formed with methyltransferase, demethylase and YTHDF proteins [35], is the most abundant regulator for the post-transcriptional modification of mammalian mRNA which is reversible. By using methylated RNA immunoprecipitation (MeRIP), the m6A residues are revealed to be located within a 100–200 nt-long transcript region. A strong correlation between the m6A and obesity, self-renewal and metastasis of stem cells [36], cell senescence [37], apoptosis [38] and metabolic regulation of cancer, for example, gastric cancer [39] and colorectal cancer [40], has been reported. The interaction of RNA and protein in vivo affected by m6A has been intensively investigated, affecting the structure, maturation and stability of mRNA [41]. METTL3, highly conserved in most eukaryotes, is a major factor in abnormal m6A repair and cell self-renewal. Previous studies have indicated that increased levels of METTL3 and m6A are caused by the Wnt and PI3K-Akt pathway in gastric cancer [39], which is a detrimental factor in the prognosis of patients [42]. Furthermore, negative correlation between wide distribution of m6A and gene expression has been confirmed in chicken embryos, which coincided with the increase in m6A methylation peaks and their transcript levels during follicle selection [43], suggesting that m6A is critical in the occurrence of life. It is noteworthy that METTL3 can also suppress cell apoptosis by promoting IL-1β production [44] and inhibit proliferation, migration and invasion of cancer cells through the p38/ERK pathway [40]. However, the biological functions of METTL3 await more thorough investigation.

The effect of BPA on reproductive system has not been properly addressed in adolescent female rats. Moreover, RNA epigenetic modification has great impact in reproductive disorders [45]. Therefore, we underwent to further determine the effect of BPA on reproduction from an epigenetic modification perspective using PCOS as a model system to explore the mechanism of BPA-induced hyperandrogenism in adolescent female rats.

## Methods

# Animals

Forty female experimental Sprague-Dawley (SD) rats, 3-week-old, weight ~ 50 g, were obtained from Qinglongshan, Inc., Nanjing, China. The animals were raised in SPF environment (Jiangsu Provincial Key Laboratory of Molecular Medicine), with temperature 22 ± 1 °C, relative humidity 50 ± 1%, light/dark cycle 12/12 hours, providing free access to food and water.

After one-week adaptive feeding, the animals were randomly divided into 5 groups: blank group (n = 8), control group (n = 8), three BPA-treatment groups (n = 8). The blank group was not treated, and the control group was gavaged with soybean oil. According to the previous studies, the lowest concentration of systemic toxicity caused by BPA is 500 mg/ (kg · d), *p.o.* [30], and the lowest concentration causing adverse reactions in the reproductive system is 50 mg/ (kg · d), *p.o.* [17]. We set 50 mg/ kg as lowest observed adverse effect level for SD rats[46]. BPA (obtained from Tianjin Guangfu Fine Chemicals Institute) was dissolved in absolute ethanol in a ratio of 1: 1, configuring 4% solution after mixing it with soybean oil (obtained from Zhejiang Tianyushan medicinal oil limited Company). Set the BPA concentration gradient for three BPA-treatment groups with 0.5, 5 and 50 mg/ (kg · d) (following called Group BPA0.5, Group BPA5, Group BPA50). Measure the volume of BPA solution for each rat according its weight, and gavage with 16-gauge needle daily at 16:00–18:00. The gavage treatment lasted 10 weeks. Record the body weight and fur color daily. The rats were injected with PMSG two days before the anatomical operation, and they were deeply anesthetized by 5% chloral hydrate with 0.07 mL per 10 g weight, *i.p.*, after fasting overnight. The blood was collected from inferior vena cava, and livers, bilateral ovaries and uteri were dissected and weighted. The weight of tissues was divided by the weight of rats to calculate the tissue coefficient to reflect the weight of uteri and ovaries. Ovaries were fixed in 4% paraformaldehyde for 24 h at 4 °C, and then embedded in paraffin. The rest of the tissues were frozen in − 80 °C for further Western blotting.

# Estrus Cycle

After 6-week gavaging, vaginal smear was taken from 7:30 to 8:00 every day when reproductive system of rats has been already mature, without stopping gavaging. Moisten the dedicated cotton swab for gynecological examination with normal saline, and choose epithelial cells from 1/3 lower segment of vagina and apply them on the slides. The cells were immersed in 1% toluidine blue dye solution for 30

minutes, and the back of the slide was rinsed with running water. After drying, the estrus cycle was described by cell type and morphology observed under the microscope (Leica Microsystems, Germany).

# Serum Testosterone Level

Serum and red blood cells were separated after collecting the blood of rats with a coagulation tube, and the supernatant serum was taken after centrifugation. The serum testosterone level was measured according to the competition principle of the enzyme-linked immunosorbent assay (ELISA), using the Rat Testosterone ELISA Kit (obtained from Elabscience Biotechnology) to measure the optical density (OD value) of each well.

# Hematoxylin And Eosin (he) Staining

The 4 μm-thick paraffin sections of the ovarian tissue were stained by hematoxylin and eosin after dewaxing. The sections were sealed by neutral gum after drying and observed under the optical microscope. Count the number of follicles on 8 consecutive sections and calculate the average.

# Western Blotting

Proteins of ovaries and livers were extracted using RIPA lysis buffer (Beyotime), determining concentration of protein by BCA working solution (Thermo Fisher). Proteins were electrophoresed on a 10% SDS-polyacrylamide gel for 130 minutes, and then transferred onto a PVDF membrane (Merck Millipore) at 350 mA with the electrophoresis & trans-membrane system of Bio-Rad (Bio-Rad, USA) for 70 minutes. In this study, the target bands were sequentially incubated in primary antibodies, namely rabbit anti-Androgen receptor (1:500, Abcam, Cambridge, UK), rabbit anti-HSD3B1 (1:1,000, Bioss, Beijing, China), rabbit anti-StAR (1:500, Bioss), rabbit anti-METTL3 (1:1,000, Abcam), rabbit anti-CYP11A1 (1:1,000, Bioworld, Minnesota, USA), rabbit anti-CYP17A1 (1:500, Abcam), rabbit anti-GAPDH (1:5,000, Bioworld), rabbit anti-Estrogen receptor (1:1,000, Abcam). After incubating with secondary antibody (Goat-anti-rabbit IgG (H + L) HRP) (Bioworld, USA), the target bands were analyzed with protein imprinted image collecting by the HRP-ECL chemiluminescence.

# Data analysis

The target bands from Western blotting were analyzed using Image J on gray values. Data were expressed using mean ± standard deviation (S.D.) or standard error of the mean (s.e.m.) from at least three independent experiments. Differences were analyzed using Graphpad prism 6.07 (Graphpad Inc.; La Jolla, USA), and statistical significance was determined using one-way analysis of variance (ANOVA). In this paper, statistical significance was set at a $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

# Results

### Oral administration with 0.5 mg/ kg BPA disrupts the morphology and weight of uterus.

To confirm whether BPA could stimulate unusual fat accumulation and growth by disrupting hormone balance, we recorded the body length, weight and fur color of rats daily during a 10-week-gavage treatment. No obvious differences were seen between BPA-treatment groups and control groups (Fig. 1a). Moreover, no weight differences between the blank group and the control group were observed, suggesting no discernable effect of soybean oil as a vehicle. This observation was consistent with no overwhelming liver anatomical changes (e.g., steatosis). There is no obvious differences on ovarian coefficient between control group and BPA-treated groups. Notably, a robust difference was observed between the BPA0.5 group and the BPA50 group (Fig. 1b). The uteri of rats after treatment with the BPA0.5 group are thicker in appearance, with colorless clear liquid inside (Fig. 1c). We next measured the length of uterine horn, and found extreme elongation after treatment with BPA, especially with a 0.5 mg/kg dose (Fig. 1d). We also observed increased uterus coefficient after treatment with BPA (Fig. 1e). The morphology of ovaries and corpus luteums of rats remained unchanged after treatment with BPA based on HE staining (Fig. 1f). To further examine whether follicular maturation was arrested at a certain stage, the number of follicles were progressively counted. Surprisingly, follicle numbers did not differ between treatment and control (Fig. 1g).

### Oral administration with BPA extensively disrupts the estrus cycle.

We next investigated the functional impact of BPA on the physiology of the ovary and uterus. Rats' vaginal smears were prepared 6 weeks after BPA treatment. We saw more irregular estrus cycles in BPA treatment groups than the control group. Interestingly, the intervals of diestrus and proestrus in a 10-day period was recorded. Shorter durations of proestrus after 5 mg/kg and 50 mg/kg treatments was recorded (Fig. 2a). However, durations of diestrus was not significantly affected by BPA treatment (Fig. 2b). Taken together, our data demonstrated that oral administration with BPA disrupted the normal patterns of vaginal epithelial cell growth in adolescent female rats.

### Oral administration with 0.5 mg/ kg BPA suppresses the expression of METTL3 without modulating hyperandrogenism.

We next asked whether the hormone level was regulated by oral administration of BPA. Modest changes in rat serum testosterone levels were observed after treatment with BPA. However, lower levels in the BPA50 group were observed compared with the BPA0.5 group (Fig. 3a). As regulation of hormonal responses can also be mediated by receptor modulation, we also measured the expression levels of androgen receptors and estrogen receptors in liver tissues. No statistically significant differences were observed (Fig. 3b, c, d). Taken together, our data suggested that oral BPA exposure did not seem to induce hyperandrogenism.

It has been previously reported that levels of androgen synthetases were modulated by intraperitoneal injection of BPA [26]. We were interested in understanding whether oral BPA exposure could also regulate androgen synthetase production. We found that oral BPA treatment induced higher expression levels of CYP17A1, CYP11A1 and StAR (Fig. 3e, f, g, h, i, j). Interestingly, we observed different levels of expression of METTL3 following treatment with oral BPA at different concentrations (Fig. 3e). The expression of METTL3 was progressively augmented with increasing concentrations of BPA (Fig. 3k).

# Discussion

As a type of EDCs, BPA exerts agonistic or antagonistic effects depending on binding to different estrogen receptors in the nucleus [13]. It stimulates ovarian production of testosterone and inhibits the testosterone hydroxylase activity to increase testosterone levels. Several attempts have been made to show that the effect of low-dose BPA on female rats is more obvious than with a high concentration [26, 33]. A strong relationship between BPA and obesity has been reported in the literatures, suggesting that early adipogenesis under exposure to BPA could be interfered with by regulating hypertrophy of fat cells and overexpression of fat genes [8, 47]. Nevertheless, the growth of rats in our study was normal without remarkable variation among different groups after a 10-week gavaging treatment, and no abnormal adipose tissues were found during the dissection. This result was in accord with studies on liver tissues without steatosis, suggesting that adolescent female rats had a stronger ability to metabolize exogenous BPA to avoid serious systemic toxicity than those young rats following exposure to BPA before they were born.

In our results, the tissue coefficient of uteri following treatment with BPA at 0.5 mg/kg substantially changed, and abnormal morphological changes of uteri were also recorded. Our findings supported differential effects following exposure using different concentrations of BPA, accompanied with the proliferation of uterine epithelial fibroblasts [14]. The state of endometrium and subsequent pregnancy preparation would be affected by changes in uterine morphology, which accounts for the mechanism of inference of uterus development by BPA. Other studies have pointed that irregular estrus cycles in adult rats are caused by BPA exposure, resulting in higher rate of rutting [48, 49]. We observed that a more irregular estrus cycle following BPA treatment, especially in proestrus period.

Previous clinical and cellular studies have reported that BPA could induce polycystic changes in the ovary[27] and reduce ovarian reserve function of PCOS patients[50]. Considerable research efforts have been devoted to hypomethylation of several imprinted genes caused by BPA-exposure during oocyte maturation. BPA can also accelerated the conversion from primordial follicles to primary follicles, and promote the degradation of primordial follicle pools [31], which provides evidences for ovarian ovulation interfered with by BPA. Our results showed integral follicles and corpus luteums without polycystic changes in ovarian sections in this study. The reason for failing to develop the polycystic model could be complained with the first-pass effect of gavaging [51, 52], which resulted in lower-dose BPA in rat blood because of its metabolism in the liver. Overall, the toxicity of oral BPA was not as strong as

intraperitoneal injection, because of the higher threshold toxicity level through the oral route to induce ovarian polycystic changes.

Cellular studies have also revealed that BPA exerted anti-androgenic effects and increased activity of brain by stimulating ovarian production of testosterone, altering the splicing form of androgen receptor and increasing the thyroxin effect in the brain, which ultimately leads to hyperandrogenism [47]. In our study, there were no obvious differences of serum testosterone levels between the control group and the BPA-treatment groups, which indicated that oral BPA failed to cause hyperandrogenism. However, 50 mg/ kg BPA showed a stronger capacity in metabolizing androgen than 0.5 mg/ kg BPA (Fig. 3a). The expression levels of proteins in the ovary and liver represented the ability of production and metabolism of androgen. The relative levels of CYP17A1, CYP11A1 and StAR in ovaries showed the differences between control group and BPA-treatment groups without inducing hyperandrogenism. Meanwhile, no differences were found on the levels of androgen receptor and estrogen receptor in the liver. Although BPA is reported to be capable of causing hepatocyte degeneration and necrosis by altering oxidative stress and affecting liver enzymes [53], our results failed to support any disorders in the liver.

Interestingly, we found remarkable differences on the levels of METTL3 in BPA-treatment groups by Western blotting, which showed a relatively lower level in 0.5 mg/ kg BPA and a higher level in 5 mg/ kg and 50 mg/ kg BPA ($p < 0.01$). Several excellent reviews have described METTL3 as one of the most critical methyltransferases in RNA methylation, suggesting that its downregulation reflects internal changes in the m6A system. On the basis of our study, it could be concluded that these results might explain the interference mechanism of RNA involved in the reproductive system, although our results failed to directly confirm the effect of BPA on RNA methylation modification. The self-regulation of RNA methylation can stabilize the balance of methylation modification by various approaches such as methylation, demethylation and reduction of the demethylase activity.

Several questions still need to be addressed appropriately. For example, why does a lower level of serum testosterone and a higher level of METTL3 occurs simultaneously following treatment with BPA at 50 mg/kg? Moreover, in order to gain more insight of the estrus cycle, will a shortened time interval before collecting vaginal smears reveal the duration of each period? All these questions require further experimental verification and explanation on the levels of m6A, relevant proteins and hormones.

# Conclusion

After gavage-treatment on adolescent female rats with different concentrations of BPA, our study demonstrated that oral BPA below the lowest observed adverse effect level failed to form ovarian polycystic changes or hyperandrogenism. On the contrary, 0.5 mg/kg oral BPA interfered with abnormal uterine coefficient and the uterine morphology. BPA also disrupted the expression levels of CYP17A1, CYP11A1, StAR and METTL3 in ovaries. Our study provided the evidence on the differential expression of METTL3 by oral BPA in a dose-dependent manner for the first time. It suggested that the interference of BPA with the reproductive system in adolescent female rats could be explained in part by RNA

methylation. Our data provide an alternative interpretation about the mechanisms that account for the effect of EDCs on the reproductive system.

# Abbreviations

PCOS Polycystic ovary syndrome

BPA Bisphenol-A

SD Sprague-Dawley

EDCs Endocrine-disrupting chemicals

ER-α Estrogen receptors-α

m6A N6-methyladenosine

MeRIP Methylated RNA immunoprecipitation

ELISA Enzyme-linked immunosorbent assay

OD Optical density

HE Hematoxylin and eosin

METTL3 Methyltransferase like protein 3

CYP17A1 Cytochrome P450 17A1

CYP11A1 Cytochrome P450 11A1

StAR Steroidogemc acuteregulatory protein

HSD3B1 3β- hydroxysteroid dehydrogenase

RIPA Radio immunoprecipitation assay

SDS Sodium dodecyl sulfate

BCA Bicinchoninic acid

PVDF Polyvinylidene fluoride

IL-1β Interleukin-1β

HRP-ECL Horseradish peroxidase-electrochemiluminescence

YTHDF YTH domain family proteins

EPHX1 Epoxide hydrolases 1

PMSG Pregnant mare serum gonadotropin

# Declarations


## Funding

This work was supported by the National Natural Science Foundation of China [grant numbers 81971346, 81771539; and the Foundation of State Key Laboratory of Analytical Chemistry for Life Science [grant number 5431ZZXM1603].


## Competing interests

The authors declare that there are no competing interests associated with the manuscript.

## Ethics approval and consent to participate

All procedures of the animal experiments were approved by the Animal Research Committee of Nanjing University Medicine School.

## Consent for publication

Not applicable

## Consent to participate

All procedures of the animal experiments were approved by the Animal Research Committee of Nanjing University Medicine School.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article.


## Acknowledgments

The authors are grateful to the help of Professor Xiang Zou for language.


## Author's contributions

ZZ, ZY, ZZF, WHW, YGJ and WY contributed to study design, analysis, method investigation and experiment performance. ZZ and WY wrote the manuscript. ZZ, ZY, ZZF participated in the animal experiments and interpretation of the data. ZZ, ZY, ZZF and WY contributed to data acquisition and statistical analysis. All authors read and approved the final manuscript.

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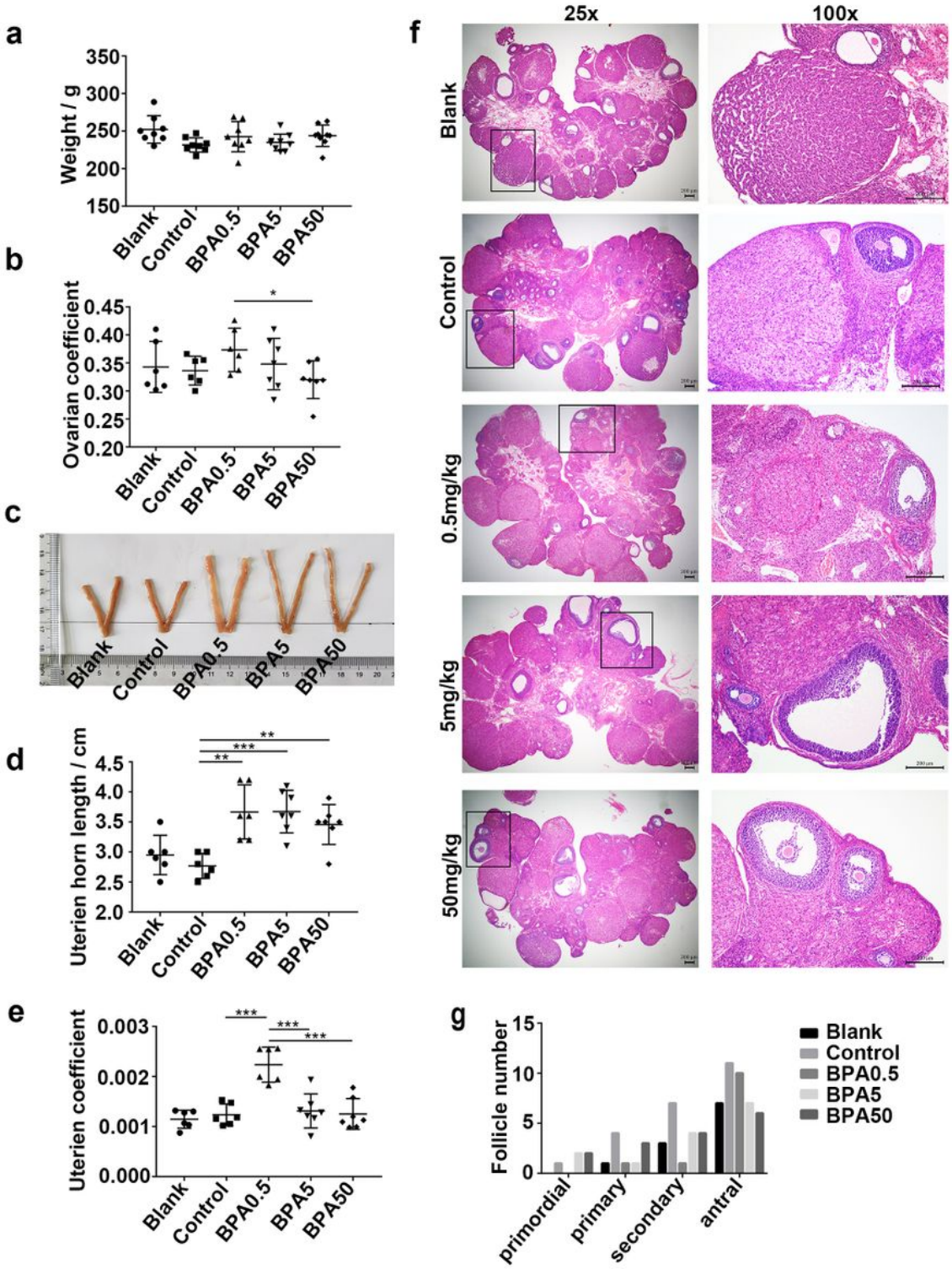
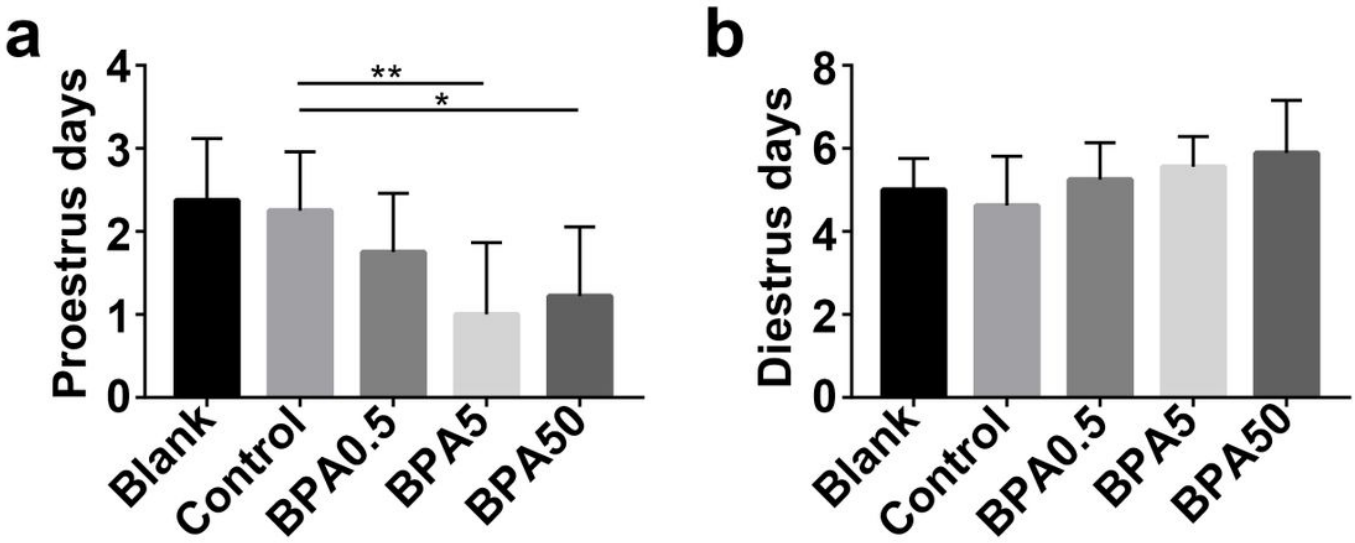
# Figures




## Figure 1

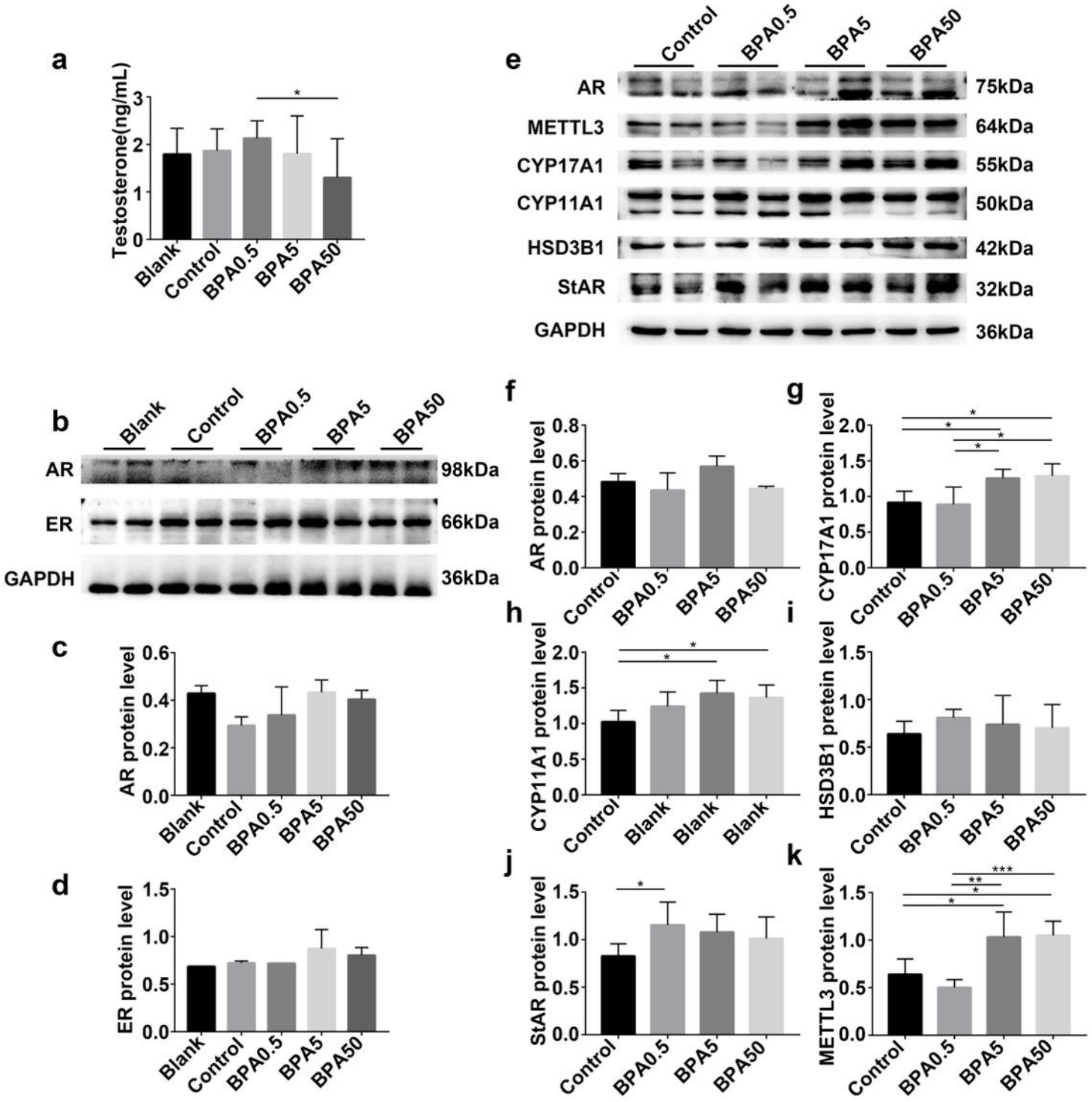
0.05 mg/ kg Bisphenol-A (BPA) disrupts the morphology of uterine tissues. Rats received daily gavaging with BPA for 10 consecutive weeks. a Rat body weights were recorded at the last treatment day. b Ovaries were harvested and weighed to calculate the ovarian coefficient. * P< 0.05. c Uterine tissues were cleared of surrounding connective tissues to contrast the morphology of uterus. d The actual length of uterine horn was measured. ** P < 0.01, *** P < 0.001. e Uteri were harvested and weighed to calculate the

coefficient. *** P < 0.001. f Paraffin sections of the ovarian tissues were stained by hematoxylin and eosin (HE) followed by microscopic examination at 25 x; the insets show 100 x. g Follicle numbers were enumerated at various developmental stages.




## Figure 2

Oral Bisphenol-A (BPA) disrupts the estrus cycle. Rats received daily gavaging with BPA for 10 consecutive weeks. Daily vaginal smears were taken for 10 days since week 6 after the BPA treatment and were immersed in a 1% toluidine blue solution to reveal the estrus cycle. a Variation in proestrus periods during a 10-day estrus cycle was recorded. * P < 0.05, ** P < 0.01. b Variation in diestrus periods during a 10-day estrus cycle was recorded.




## Figure 3

Bisphenol-A (BPA) disrupts the expression of androgen synthetases and METTL3 without modulating hyperandrogenism. Rats received daily gavaging with BPA for 10 consecutive weeks. a Serum was collected from inferior vena cava. The levels of serum testosterone were measured by enzyme-linked immunosorbent assay (ELISA). * $P < 0.05$. b to d Total proteins of liver were extracted using an RIPA lysis buffer. Androgen receptor and estrogen receptor molecules in livers were detected using Western blotting

(b). The relative levels of androgen receptor (c) and estrogen receptor (d) were quantified. e to k Total ovarian proteins were extracted using an RIPA lysis buffer. The androgen synthetases and METTL3 were revealed using Western blotting (e), and relative levels of androgen receptor (f), CYP17A1 (g), CYP11A1 (h), HSD3B1 (i), StAR (j), METTL3 (k) were quantified. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

## Supplementary Files

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