

Quantitative proteomic analysis of mouse testis uncovers cellular pathways associated with bisphenol A (BPA)-induced male infertility

Jian Jia^{1,*}, Hang Xu^{1,*}, Changbo Chen¹, Xiaoqian Zhang², Xuexue Zhang³, Wei Li⁴ and Jing Ma¹

¹ Department of Traditional Chinese Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi, China

² College of Basic Medicine, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China

³ First College of Clinical Medicine, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China

⁴ Department of Histology and Embryology, The Fourth Military Medical University, Xi'an, Shaanxi, China

Abstract. Quantitative proteomic analysis was performed using iTRAQ to explore the potential regulation of differentially expressed proteins (DEPs) by bisphenol A (BPA) in murine testis. BPA was intraperitoneally injected into mice at a dose of 100 mg/kg body weight for 7 consecutive days. After BPA treatment, the histopathology changes of testis were examined. The circulating levels of testosterone (T) and estradiol (E₂) were determined. iTRAQ was used to assess the expression levels of DEPs and to reveal potential interactions between different DEPs. Results showed that BPA caused histological damage in testicular tissues. The levels of T and E₂ were affected by BPA exposure. The abundances of orosomucoid 1 (Orm1), haptoglobin (Hp), and insulin-like 3 (Insl3) were significantly lower in BPA-treated mice than those in control mice. The expression changes in the above-mentioned proteins were further validated at the protein level using Western blot analysis. We concluded that BPA affects histological morphology of testis and sex hormone productions. The regulation of key proteins (such as Orm1, Hp and Insl3) may reflect that these proteins may serve as important factors in male reproductive disorders caused by BPA, and these proteins are probably biomarkers for infertility caused by endocrine disrupting chemicals.

Key words: Bisphenol A — Testis — Infertility — Proteomics — Isobaric tags for relative and absolute quantitation

Introduction

Infertility has been recognized as a public health issue by WHO (Boivin et al. 2007) due to male reproductive dysfunction in approximately 50% of the affected couples (Anawalt 2013). Endocrine-disrupting chemicals (EDCs)

have estrogenic effects, and may account for the increase in male reproductive disorders (Sharpe and Skakkebaek 1993). These EDCs are found in various materials such as additives, plastic products, building materials, personal care products and so on. Exposure to EDCs, even at low levels, leads to abnormality in health, including male and female reproductive disorders, breast development and cancer, prostate cancer, neuroendocrine issues, thyroid problems, metabolism and obesity, and cardiovascular endocrine issues (Gore et al. 2015). EDCs may contribute to male reproductive dysfunction by interfering with spermatogenesis, a process that is highly dependent on sex hormones (Dohle et al. 2003; Sweeney et al. 2015; WHO and UNEP, 2013).

Bisphenol A (BPA), namely 2,2-bis (4-hydroxyphenyl) propane, is one of the highest volume of EDCs produced worldwide (Dohle et al. 2003; Sweeney et al. 2015; WHO and UNEP, 2013). It is frequently used to produce plas-

* These authors equally participated in this manuscript.

Correspondence to: Jing Ma, Department of Traditional Chinese Medicine, Xijing Hospital, The Fourth Military Medical University, No.169 West Changle Road, Xincheng District, Xi'an, Shaanxi 710032, China

E-mail: jingma@fmmu.edu.cn

Wei Li, Department of Histology and Embryology, The Fourth Military Medical University, No.169 West Changle Road, Xincheng District, Xi'an, Shaanxi 710032, China

E-mail: liweipepeyato@163.com

tics that serves as line food or drink containers, goods of common use, thermal receipts, and medical devices such as dental sealants (Chianese et al. 2018). It is well known that BPA can be detected in many human tissues, such as serum, urine, sweat, milk and placenta (Chianese et al. 2018). In general, the daily safe intake of BPA in human should be less than 1 $\mu\text{g}/\text{kg}$ per day (Kang et al. 2006). The estrogenic and/or anti-androgenic properties of the chemically stable compound can impair testosterone production during male development, and therefore influence prostate and testis function in addition to spermatogenesis (Yeung et al. 2011). BPA induces disruption in meiotic progression during spermatogenesis (Liu et al. 2013), causes reduction in chromosome crossover (Vrooman et al. 2015), augments oxidative stress and apoptosis in mouse testis (Kaur et al. 2018). It is reported that exposure to xenoestrogens such as BPA could increase genital-tract abnormalities and increase incidence of infertility in Europe and the U.S. during the last 50 years (Vandenberg et al. 2007; Calafat et al. 2008). The BPA detection is associated with a higher level of circulating estradiol and a lower level of testosterone (Lan et al. 2017). The decreased sexual function in men is linked to the exposure to BPA in a dose-dependent manner (Rochester 2013). Furthermore, BPA is toxic to Leydig cells (LCs), especially on proliferation and motility (Chen et al. 2016), as well as testosterone production (Gonçalves et al. 2018). BPA causes damage to testicular tissue both *in vivo* and *in vitro*.

The technology of isobaric tags for relative and absolute quantitation (iTRAQ) has been applied to numerous experiments with the feature of measuring eight samples at one experiment with high precision. Nowadays, it is widely used in quantitative proteomics and has achieved good results. However, the current proteomics researches in male infertility are based on serum and spermatogenic cells, and few studies have been conducted on the proteomics of testis tissues of male infertility using iTRAQ technique.

In this study we employed iTRAQ technique to explore the potential regulation of proteins by BPA in testicular tissues of infertile men. The aim was to evaluate the action of BPA on testis tissues and explore the mechanism of infertility caused by EDCs.

Materials and Methods

Animals and treatments

Sixteen 6-week-old Kunming male mice (20 ± 3 g) were obtained from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All the experimental protocols have been approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and in compliance with the National Institutes of

Health (NIH) Guide for the Care and Use of Laboratory Animals. They were housed in air-conditioned animal quarters in a constant 12-hour light/dark cycle at $25 \pm 2^\circ\text{C}$ temperature and $50 \pm 10\%$ air humidity, with free access to food and water. BPA (CAS number 80-05-7, $\geq 99\%$) was purchased from Sigma-Aldrich and the dose was chosen according to previous reports (Li et al. 2014; Tomza-Marciniak et al. 2018; Zhang et al. 2013). It was dissolved in corn oil (CAS number 8001-30-7, Sigma, MO, USA) and stored at 4°C . The mice were randomly divided into Control group and treatment group ($n = 8/\text{group}$). The mice in treatment group were injected intraperitoneally with BPA at 100 mg/kg body weight for 7 consecutive days (BPA group). In contrast, mice in Control group were injected intraperitoneally with corn oil at the same equal volume. After treatment, the mice were sacrificed to collect testis tissues and serum. To evaluate pathology, the left testis tissues were fixed with 4% paraformaldehyde overnight and then were subjected to routine hematoxylin and eosin (H&E) staining. The right testis tissues were stored at -80°C immediately after collection. The ophthalmic artery blood was immediately collected, and was centrifuged for serum preparation at $2,000 \times g$ for 15 min to assess the serum levels of sex hormones.

Morphological examination

Upon harvest, testes were immediately fixed in 4% polyformaldehyde overnight at room temperature. Paraffin-embedded testes tissue samples were generated and then cut into 5- μm -thick sections. These sections were then stained with H&E, and were observed under a light microscope (Carl Zeiss, ImagerM1, DE) at $200\times$ and $400\times$ magnification.

Enzyme-linked immunosorbent assay (ELISA)

Two ELISA kits (Cloud-Clone Corp, TX, USA) were used to determine the levels of testosterone (T, CEA458Ge) and estradiol (E_2 , CEA461Ge), as per the manufacturer's instructions. After reconstitute the standard with standard diluent, samples and standards were added into 96-well strip plate. Once reagents were added, the plate was incubated for 30 min at 37°C . The results were assessed on a microplate absorbance reader (Tecan sunrise, Austria) at 450 nm measurement. Average the duplicate readings for each standard, control and samples. Create a standard curve with the log of concentration on the y-axis and absorbance on the x-axis. Based on this standard curve, the data were calculated. All tests were repeated three times.

Proteome sample preparation and LC-MS/MS analysis

Lysis buffer (8 M Urea, 40 mM Tris-HCl or TEAB with 1 mM PMSF, 2 mM EDTA and 10 mM DTT, pH 8.5) was used to

extract proteins of 16 testis tissues. After centrifugation with $25,000 \times g$ at 4°C for 20 min, the supernatant was transferred into a new tube, reduced with 10 mM dithiothreitol (DTT) at 56°C for 60 min, alkylated by 55 mM iodoacetamide (IAM) in the dark at room temperature for 45 min, and quantified by Bradford after centrifugation ($25,000 \times g$, 4°C , 20 min). After Bradford assay, $30 \mu\text{g}$ proteins were mixed with loading buffer in a centrifuge tube and heated at 95°C for 5 min. Subsequently, the supernatant with proteins was centrifuged at $25000 \times g$ for 5 min and loaded to sample holes in 12% polyacrylamide gel with the running of SDS-PAGE in constant voltage at 120 V for 120 min. Once it was finished, the gel was stained with Coomassie Blue for 120 min, added destaining solution (40% ethanol and 10% acetic acid), and put on a shaker with an exchange of destaining solution for 3~5 times, 30 min each time. The gel lanes were then cut into multiple bands based on molecular weight and protein abundance in the specific region. Each gel band was digested with Trypsin Gold (Promega, Madison, WI, USA) before LC-MS/MS analysis (liquid chromatography linked with tandem mass spectrometry). After peptide labeling and fractionation, each fraction was resuspended in loading buffer (2% acetonitrile, 0.1% formic acid) and centrifuged at $20,000 \times g$ for 10 min. The supernatant was loaded on UHPLC (UltiMate 3000, Thermo Scientific, USA) equipped with a trap and an analytical column. The peptides separated from nanoHPLC were subjected into the tandem mass spectrometry Q Exactive HF X (Thermo Fisher Scientific, San Jose, CA) for data-dependent acquisition (DDA) detection by nano-electrospray ionization. The parameters for MS analysis are as follows: electrospray voltage, 2.0 kV; precursor scan range, 350–1500 m/z at a resolution of 60,000 in Orbitrap; MS/MS fragment scan range, $>100 \text{ m/z}$ at a resolution of 15,000 in HCD mode; normalized collision energy setting, 30%; dynamic exclusion time, 30 s; automatic gain control (AGC) for full MS target and MS2 target, 3×10^6 and 1×10^5 , respectively; number of MS/MS scans following one MS scan, 20 most abundant precursor ions above a threshold ion count of 10,000.

MS/MS data analysis

The raw MS/MS data were converted into MGF format through a thermo scientific tool Proteome Discoverer, and the exported MGF files were searched through Mascot (v2.3.02) in this project against the selected database (Uniprot-Mus musculus). The labeled peptides with isobaric tags were quantitatively analyzed by IQuant, a tool which combines multiple search engine results based on isobaric tags (Wen et al. 2014). The false discovery rate (FDR) at 1% was estimated by Picked protein FDR strategy to control the rate of false-positive at protein level after protein inference (protein-level FDR ≤ 0.01) (Savitski et al. 2015).

Bioinformatic analysis

We explored the functions of DEPs through Gene Ontology (GO) database (<https://www.ebi.ac.uk/ols/ontologies/go>) based on Uniprot database (<https://www.uniprot.org/>). Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.kegg.jp/>) was used to study the relationships among different pathways. STRING (<https://string-db.org/>) was performed to link DEPs in protein-protein interactions (PPI) to illustrate the interaction of DEPs.

Western blotting

Testicular tissues were stored at -80°C before operation. After total proteins were extracted by RIPA Lysis Buffer (P0013B, Beyotime, China), protein concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). $30 \mu\text{g}$ of samples were separated by 10% SDS/PAGE and transferred to PVDF (polyvinylidene fluoride) membranes (Merck Millipore, Taunton, MA, USA). The membranes were then blocked with 5% skim milk dissolved in TBS-T for 2 h, and then incubated with primary antibodies overnight at 4°C : anti-Insl3 (1:1000, Abcam, Cambridge, UK), anti-Hp (1:1000, Proteintech, CA, USA), anti-Orm1 (1:1000, Proteintech, CA, USA) and anti-GAPDH (1:1000, St John's Laboratory, CA, USA). Then, the blots were incubated with a peroxidase-conjugated secondary antibody (SA-10011, BioCytoSci, TX, USA) at room temperature for 2 h, and were visualized with ECL-Plus reagent (IC-5009, BioCytoSci, TX, USA). The optical density of the target band was analyzed by Image Pro Plus software (6.0, Media Cybernetics, MD).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS (version 19.0, IBM, USA) and Prism softwares (version 7.00, GraphPad, CA, USA), and were presented as mean \pm S.D. Significant differences between means were determined using Duncan and Tukey's honestly significant difference test; $p < 0.05$ was considered as significant.

Results

Histology changes

The histopathological image/observation of testis tissues is shown in Figure 1. Figure 1A and C showed the normal development and morphology of testes in control mice, the structure of seminiferous tubules was normal and the spermatogenic cells were tightly connected and organized. In BPA group (Fig. 1B and D), however, the mount of Leydig

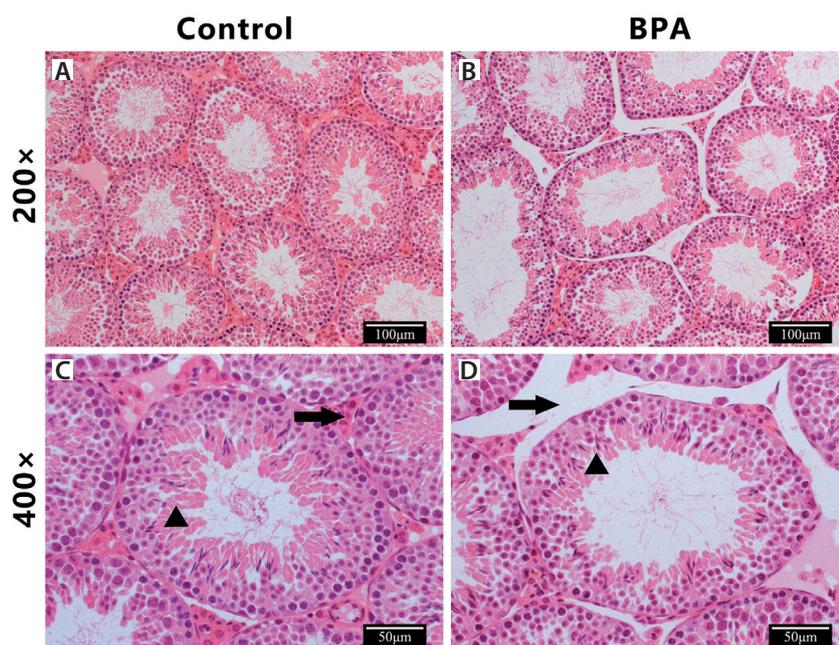


Figure 1. Histopathological changes of testis tissues: Control group (A, C) and BPA group (B, D). Arrow head refers to enlarged glandular cavity and decreased Leydig cells. Triangle refers to the distribution of sperm cells. Magnification $\times 200$ (A, B); $\times 400$ (C, D).

cells decreased and most seminiferous tubules contained less spermatogenic cells. The glandular cavity in BPA group was slightly enlarged and increased compared to that in Control group. The morphology of Leydig and spermatogenic cells was abnormal. The layers of sperm cells were reduced, the array of sperm cells was disturbed, and the seminiferous tubules exhibited shrinkage and vacuolation.

Effects of the sex hormone secretion by BPA

Figure 2 showed the effects of the hormone secretion on the serums of mice using ELISA Kits. The testosterone level was slightly decreased in the BPA group but there was no significant difference between two groups (Fig. 2A). However, the estradiol level was significantly enhanced after BPA treatment (Fig. 2B), with the significant decrease of sex hormone ratio (T/E_2) in BPA group (Fig. 2C). BPA affected hormone secretion and disrupted the homeostasis of sex hormones in testis. Compared to androgen, BPA demonstrated more obvious effect on the estrogen production.

Identification of DEPs after BPA treatment

Totally 931866 spectrums were generated, 20728 peptides and 5192 proteins were identified with 1% FDR. Among them 101 were up-regulated and 86 were down-regulated with foldChange > 1.2 while 10 were up-regulated and 9 were down-regulated with foldChange > 1.5 . Table 1 shows DEPs in BPA group with foldChange > 1.5 compared with Control group.

GO and pathway analyses of DEPs

Gene Ontology (GO) is consisted of three parts, including cellular component, molecular function and biological process. Figure 3A showed the results of GO enrichment analysis between BPA and Control groups. GO is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species. GO enrichment analysis showed the GO terms in which the DEPs were enriched in all candidate proteins, which represents

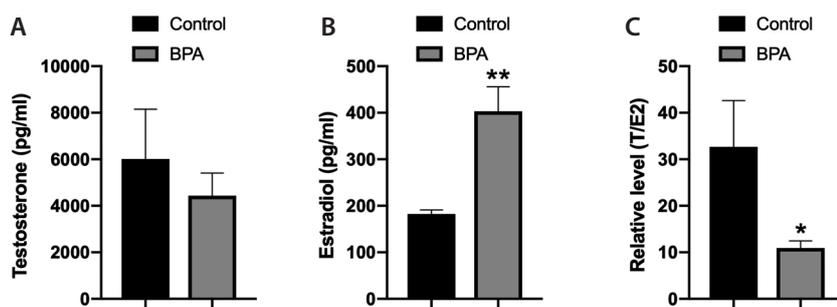


Figure 2. Effects of BPA treatment on sex hormone secretion levels. **A.** Testosterone production in serum ($p > 0.05$). **B.** Estradiol production in serum. **C.** The relative ratio of sex hormone (T/E_2). * $p < 0.05$, ** $p < 0.001$ compared with the Control group. BPA, bisphenol A. T, testosterone. E_2 , estradiol.

Table 1. DEPs in BPA group compared with Control group (Fold-Change >1.5 or <0.67)

Protein ID	Protein	Fold-Change	p value ($\times 10^{-5}$)
Up-regulated			
sp P98203 ARVC_MOUSE	Arvcf	2.40	2.98
sp O55137 ACOT1_MOUSE	Acot1	1.78	0.104
sp Q3TCJ8 CCD69_MOUSE	Ccdc69	1.77	348.7
sp P61148 FGF1_MOUSE	Fgf1	1.70	147
sp P12710 FABPL_MOUSE	Fabp1	1.69	36.8
sp Q8BQZ4 RLGPB_MOUSE	Ralgapb	1.59	2471
sp Q7TPM3 TRI17_MOUSE	Trim17	1.57	53.28
sp Q80U22 RUSC2_MOUSE	Rusc2	1.56	1098
sp Q5NCY3 CB5D1_MOUSE	Cyb5d1	1.55	429.1
sp Q5ND34 WDR81_MOUSE	Wdr81	1.50	345.5
Down-regulated			
sp Q8VIM9 IRGQ_MOUSE	Irgq	0.48	38.77
sp Q8BND4 INT6L_MOUSE	Ints6l	0.52	0.0913
sp Q5XG69 F169A_MOUSE	Fam169a	0.52	40.35
sp P33622 APOC3_MOUSE	Apoc3	0.53	0.683
sp P02088 HBB1_MOUSE	Hbb-bt	0.62	75.99
sp Q9QZ85 IIGP1_MOUSE	Iigp1	0.63	25.16
sp Q60590 A1AG1_MOUSE	Orm1	0.64	0.00514
sp Q61646 HPT_MOUSE	Hp	0.65	100.4
sp Q6Q2Z6 ACOT5_MOUSE	Acot5	0.65	891.5

FoldChange, the fold values of DEPs in BPA group compared with Control group; DEPs, differentially expressed proteins; BPA, bisphenol A.

the important or typical biology functions in the current study. "Reproduction" term in biological process was useful to explore DEPs of infertility caused by BPA. Thus, Table 2 demonstrated the DEPs of reproduction term.

KEGG pathway is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks. Proteins usually interact with each other to play roles in certain biological functions. Figure 3B showed the effect of pathway enrichment analysis of DEPs based on KEGG database. The main pathways ($p < 0.05$) included ribosome, complement and coagulation cascades, malaria, antigen processing and presentation, leishmaniasis, ribosome biogenesis in eukaryotes, biosynthesis of unsaturated fatty acids, African trypanosomiasis, fatty acid elongation, Th1 and Th2 cell differentiation, inflammatory bowel disease, and systemic lupus erythematosus.

PPI of the DEPs

STRING (<https://string-db.org/>) is a database of known and predicted PPI. PPI is constructed to show the interaction of DEPs between the two groups (Fig. 4), the foldChange was

more than 1.2 in Figure 4A when it was more than 1.5 in Figure 4B. The minimum required interaction score was 0.4 (medium confidence) and the disconnected proteins in the network were hidden. In Figure 4B, 19 proteins were assigned to this network, among which 7 were identified as node proteins, including Fabp1, Apoc3, Hp, Orm1, Hbb-bt, Acot1, Acot5.

Validation of the proteins highly related to reproduction

We selected three proteins to verify the accuracy of proteomic analysis. Haptoglobin (Hp) and orosomucoid 1 (Orm1) were chosen from PPI because both of them are nodes. Insulin-like 3 (Insl3) was highly expressed in testis tissues rather than other tissues, and it was obviously expressed in reproduction term of GO analysis. These three proteins are very likely to be involved in reproduction process. Western blot was conducted to verify these three DEPs (Fig. 5). The levels of Insl3, Orm1 and Hp were decreased in the BPA group compared with Control group. The data were consistent with iTRAQ analysis, suggesting that our Proteomic data were reliable.

Discussion

With the rapid development of industry and economy, the deterioration of environment results in a large number of

Table 2. DEPs of GO reproduction term

Protein ID	Protein	Fold-Change	p value ($\times 10^{-5}$)
Up-regulated			
sp Q8C4X1 CENPX_MOUSE	Cenpx	1.23	1722
sp P58742 AAAS_MOUSE	Aaas	1.24	218.9
sp P49891 ST1E1_MOUSE	Sult1e1	1.38	5.62
sp Q8BWP5 TTPA_MOUSE	Ttpa	1.32	39.07
sp O55047 TLK2_MOUSE	Tlk2	1.41	1.06
sp Q8BMD2 DZIP1_MOUSE	Dzip1	1.35	2.5
sp Q8VCB1 NDC1_MOUSE	Ndc1	1.24	99.52
sp P35235 PTN11_MOUSE	Ptpn11	1.22	23.04
sp Q9Z0E1 M1AP_MOUSE	M1ap	1.28	10.76
sp P42232 STA5B_MOUSE	Stat5b	1.26	306.4
sp Q5F2C3 MEIKN_MOUSE	Meikin	1.26	341.5
Down-regulated			
sp Q9JKL5 CHP3_MOUSE	Tesc	0.83	203.7
sp Q8CJ14 H1FNT_MOUSE	H1fnt	0.80	432.4
sp O09107 INSL3_MOUSE	Insl3	0.79	339.4
sp Q61646 HPT_MOUSE	Hp	0.65	100.4

FoldChange, the fold values of DEPs in BPA group compared with Control group; DEPs, differentially expressed proteins; BPA, bisphenol A; GO, Gene Ontology database.

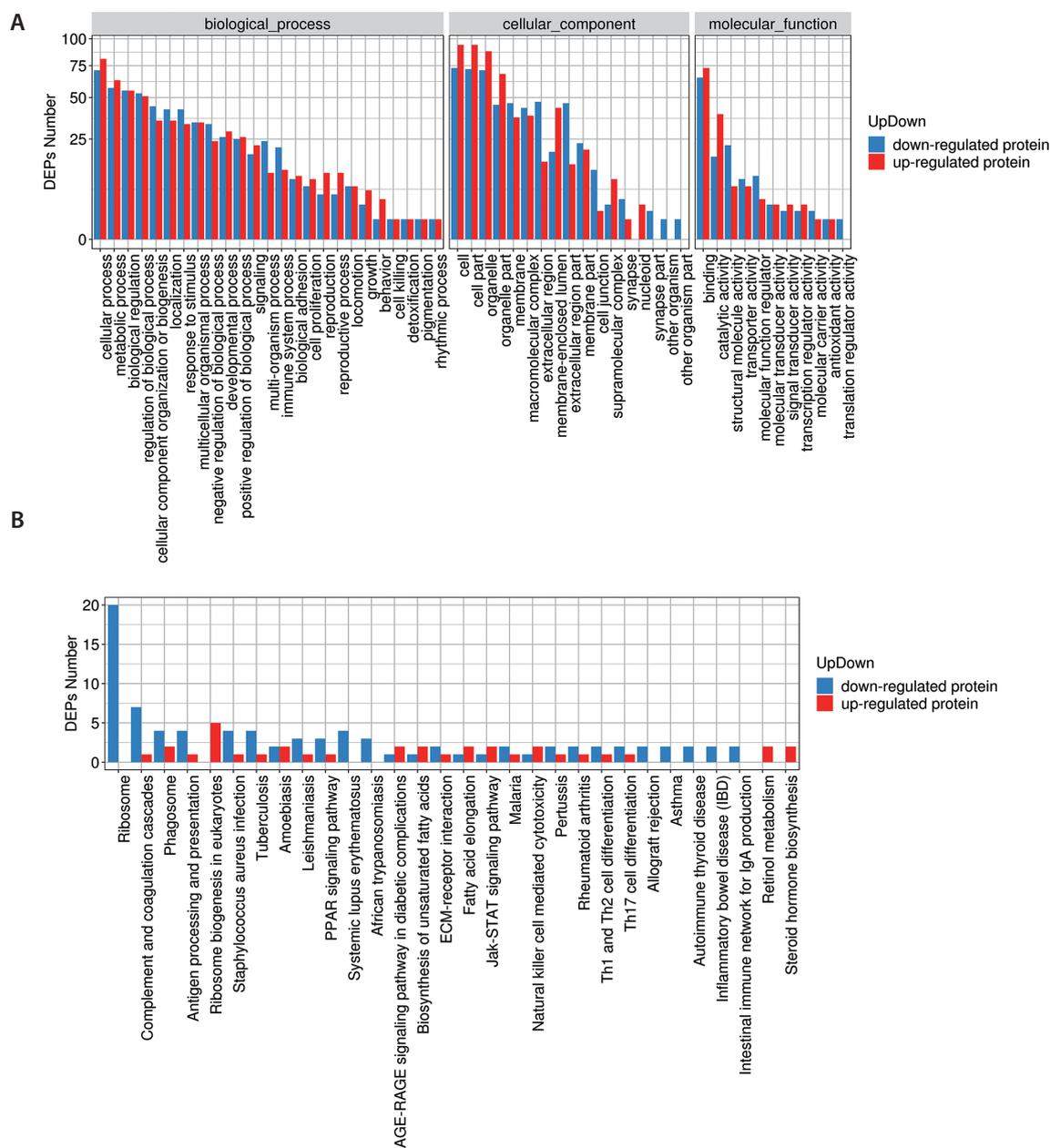


Figure 3. Gene Ontology (GO) enrichment (A) and Pathway enrichment (B) analysis for BPA vs. Control group. X-axis displays GO or pathway terms, and y-axis displays protein count. Red column, up-regulated proteins; blue column, down-regulated proteins. (See online version for color figure).

reproductive health problems, including the sexual precocity. More people are concerned about the effects of environmental hormones on human body. EDCs are related to xenoestrogens, antiestrogens, antiandrogens, disruption of thyroid function, disruption of corticoid function and other metabolic effects (Vidaeff and Sever 2005; De Coster and van Larebeke 2012). They exist everywhere in our daily life, and we can not avoid them. BPA, as one of classic EDCs, impairs

the functions of testicular cells in men as well as reduces sperm counts in a rodent model and a human epidemiology study (Richter et al. 2007; Meeker et al. 2010).

Previous studies have focused on the proteomics of semen and Leydig cells (Chen et al. 2016; Rahman et al. 2018), while our research focuses on the whole testis tissue. The results from protein screening analyses have shown that many proteins are involved in reproductive health. We

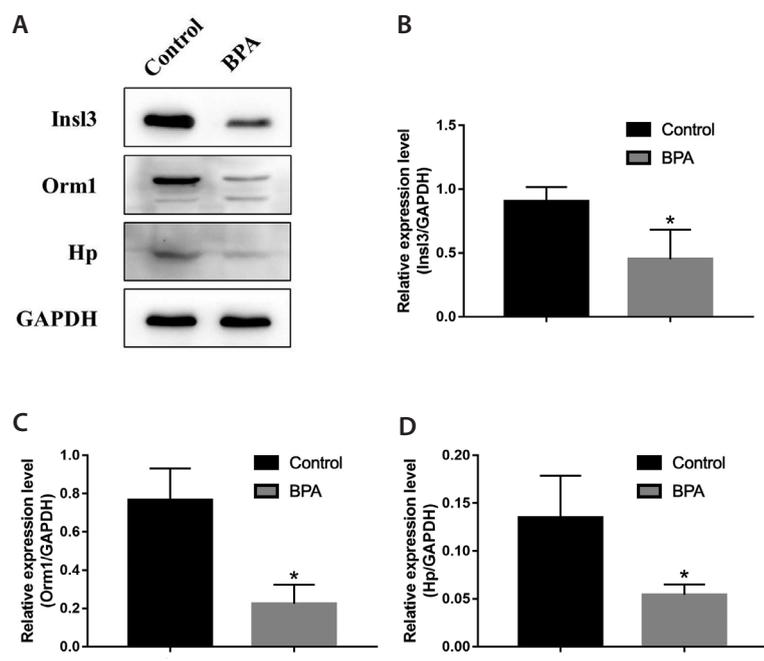


Figure 5. Validation of proteins related to reproduction. Protein expression level was detected by Western blotting (A). GAPDH was used as the internal loading control. The results shown below the gel indicated the quantification results normalized against the internal control. The protein expression of InsI3 (B), Orm1 (C) and Hp (D). * $p < 0.05$ compared with the Control group. BPA, bisphenol A.

chose three distinct proteins for validation because they were selected from the PPI nodes and GO enrichment. The result shows that their expression levels are significantly changed. In recent studies, the reproductive effects of BPA were mainly reflected on cytotoxic effect, apoptosis (Li et al. 2009; Qian et al. 2014), cell viability and follicle growth inhibition. Consistently in our study, histopathological observation of testis demonstrates that BPA significantly decreased spermatogenic cells in seminiferous tubules and large vacuolization could be observed within seminiferous tubules. Furthermore, the production of sex hormone secretion is affected by BPA. GO and Pathway enrichments show different aspects of DEPs function, especially reproduction-related process. PPI was helpful to find protein nodes which are worth studying.

Three potential proteins were selected based on aforementioned bioinformatic analysis. Hp, as a Sertoli and germ cell product in testes, adds a new member to the growing family of metal transporters in the testis that are likely to play important roles in iron metabolism in the testis (O'Bryan et al. 1997). It is reported that Hp phenotype affects maternal fertility, and the effects of smoke on fertility depends on the Hp phenotype (Bottini et al. 2002). Additionally, women with mechanical factor infertility were more likely ($p = 0.042$) to have the Hp 1-1/2-1 phenotypes than the Hp2-2 phenotype (Weiss et al. 2013). HP has been implicated in reproductive function through its effect on inflammation, the immune system, and angiogenesis making it a protein of great interest to reproductive biologists (Bottini et al. 1999; Ueda et al. 2001). Hp α and β peptides

as well as Hp proteins have been identified in the follicular fluid aspirated during *in vitro* fertilization (IVF) (Angelucci et al. 2006; Schweigert et al. 2006). Orosomucoid 1 (Orm1), which is also called α -1-acid glycoprotein (AGP), is one of the acute-phase proteins. It is mainly synthesized by the liver and then is secreted to almost the entire body. Many biological properties of Orm have already been discussed in the literatures including protective effect against neonatal sepsis, interaction with phospholipid membranes, histamine binding capacity, and inhibition of platelet aggregation, neutrophil chemotaxis, superoxide production, and lymphocyte proliferation (Kremer et al. 1988). In addition, Orm has a more meaningful role here: acting as an acute-phase reactant. High dose BPA injection induces systemic injury, which causes a drastic change in the hepatic production of many plasma proteins. In our study, Orm1 was down-regulated probably because chronic hepatitis, liver cirrhosis, or acute liver failure happened. Furthermore, Orm has the ability to bind and carry numerous basic and neutral drugs (Paxton 1983), especially the neutral drug steroids (Ganguly et al. 1967). BPA is one of the EDCs and acts like estrogen (Milligan et al. 1998; Rubin 2011). Its extensive integration with Orm may have led to the down-regulation of Orm1. It is reported that Orm in the oviducts additively suppresses sperm phagocytosis by polymorphonuclear neutrophils, which indicates that locally produced Orm may be involved in protecting sperm from phagocytosis by polymorphonuclear neutrophils in the bovine oviduct (Liu et al. 2014). InsI3 was first discovered as a unique and testis-specific gene transcript sequence

independently in pigs (Adham et al. 1993) and mice (Pusch et al. 1996), respectively. Insl3 was originally referred to as Ley-IL (Leydig-insulin-like) or RLF (Relaxin-like factor). It is a major secretory product of the testicular Leydig cells in fetus and adult men. In rodent models, reduction in fetal Insl3 expression is an early marker of the testicular dysgenesis syndrome. Insl3 is also involved in adult traits, such as spermatogenesis and bone metabolism, and its expression declines consistently with age in adult men (Ivell and Anand-Ivell 2009). Insl3 is a member of the insulin-like group of peptide hormones. The functions of Insl3 such as testicular descent, lactation, pregnancy and birth, all of which are specific to mammalian viviparity and internal fertilization, have caused these molecules to be termed 'neohormones' which consists of other peptides such as hCG and oxytocin belong (Anand-Ivell et al. 2013). Furthermore, the Insl3/Rxfp2 system plays an integral role in promoting the production of the essential steroid precursor androstenedione through stimulation of the enzyme 17 α -hydroxylase (Ivell and Anand-Ivell 2018). It is reported that the significantly monotonic trends in semen volume, sperm concentration and motility were associated with increasing quartiles of Insl3 ($p < 0.001$) (Chang et al. 2017). Additionally, Insl3 correlated negatively with BPA is proved and providing indirect evidence for an impact of EDCs on fetal Leydig cell Insl3 production (Chevalier et al. 2015), and diethylhexyl phthalate (DEHP) also down-regulated Insl3 mRNA expression (Song et al. 2008). Estrogens, acting through the estrogen receptor α (ER α), have been shown to repress expression of the gene encoding Insl3, a small peptide produced by testicular Leydig cells that is essential for normal testis descent. Estradiol (E₂) could repress Insl3 mRNA levels in MA-10 cells, a Leydig cell line model (Lague and Tremblay 2009).

It is reported that BPA could affect histological morphology of testis and sex hormone productions (Li et al. 2009; Nakamura et al. 2010; Ibrahim et al. 2016). The estrogenic and anti-androgenic properties of BPA can also decrease testosterone production and increase estradiol production in our study. This may be due to the effects of BPA on Leydig cells. Testicular tissue contains a variety of proteins. The dose of BPA up to 100 mg/kg for 1 week causes acute testicular injury, which leads to changes in multiple pathways. Our experiment is the first to study the proteomics of testis tissues and provides a basis for clinic treatment.

In conclusion, this study provided the first evidence that infertility of BPA-caused mice might be involved in acute stimulation, inflammation, immunoreaction and production and metabolism of hormones in the testes. These reactions often occur simultaneously. Hp, Orm1 and Insl3 could act as potential targets for the therapeutic consideration in male infertility caused by BPA.

Acknowledgments. This study was supported by National Natural Science Foundation of China (No. 81573851) and Shaanxi International Cooperation and Communication Program (2016kw-012).

Conflict of interest. The authors declare that there are no conflicts of interest.

References

- Adham IM, Burkhardt E, Benahmed M, Engel W (1993): Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. *J. Biol. Chem.* **268**, 26668-26672
- Anand-Ivell R, Dai Y, Ivell R (2013): Neohormones as biomarkers of reproductive health. *Fertil. Steril.* **99**, 1153-1160
<https://doi.org/10.1016/j.fertnstert.2012.12.023>
- Anawalt BD (2013): Approach to male infertility and induction of spermatogenesis. *J. Clin. Endocrinol. Metab.* **98**, 3532-3542
<https://doi.org/10.1210/jc.2012-2400>
- Angelucci S, Ciavardelli D, Di Giuseppe F, Eleuterio E, Sulpizio M, Tiboni GM, Giampietro F, Palumbo P, Di Ilio C (2006): Proteome analysis of human follicular fluid. *Biochim. Biophys. Acta* **1764**, 1775-1785
<https://doi.org/10.1016/j.bbapap.2006.09.001>
- Boivin J, Bunting L, Collins JA, Nygren KG (2007): International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum. Reprod.* **22**, 1506-1512
<https://doi.org/10.1093/humrep/dem046>
- Bottini N, Gimelfarb A, Gloria-Bottini F, La Torre M, Lucarelli P, Lucarini N (1999): Haptoglobin genotype and natural fertility in humans. *Fertil. Steril.* **72**, 293-296
[https://doi.org/10.1016/S0015-0282\(99\)00210-1](https://doi.org/10.1016/S0015-0282(99)00210-1)
- Bottini N, Magrini A, Macmurray J, Cosmi E, Nicotra M, Gloria-Bottini F, Bergamaschi A (2002): Smoking, haptoglobin and fertility in humans. *Tob. Induc. Dis.* **1**, 3-6
<https://doi.org/10.1186/1617-9625-1-1-3>
- Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL (2008): Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. *Environ. Health Perspect.* **116**, 39-44
<https://doi.org/10.1289/ehp.10753>
- Chang WH, Wu MH, Pan HA, Guo PL, Lee CC (2017): Semen quality and insulin-like factor 3: Associations with urinary and seminal levels of phthalate metabolites in adult males. *Chemosphere* **173**, 594-602
<https://doi.org/10.1016/j.chemosphere.2017.01.056>
- Chen ZJ, Zhang KS, Ge LC, Liu H, Chen LK, Du J, Wang HS (2016): Signals involved in the effects of bisphenol A (BPA) on proliferation and motility of Leydig cells: a comparative proteomic analysis. *Toxicol. Res. (Camb)*. **5**, 1573-1584
<https://doi.org/10.1039/C6TX00258G>
- Chevalier N, Brucker-Davis F, Lahlou N, Coquillard P, Pugeat M, Pacini P, Panaia-Ferrari P, Wagner-Mahler K, Fenichel P (2015): A negative correlation between insulin-like peptide 3 and bisphenol A in human cord blood suggests an effect of endocrine disruptors on testicular descent during fetal development. *Hum. Reprod.* **30**, 447-453

- <https://doi.org/10.1093/humrep/deu340>
- Chianese R, Viggiano A, Urbanek K, Cappetta D, Troisi J, Scafuro M, Guida M, Esposito G, Ciuffreda LP, Rossi F, et al. (2018): Chronic exposure to low dose of bisphenol A impacts on the first round of spermatogenesis via SIRT1 modulation. *Sci. Rep.* **8**, 2961
<https://doi.org/10.1038/s41598-018-21076-8>
- De Coster S, van Larebeke N (2012): Endocrine-disrupting chemicals: associated disorders and mechanisms of action. *J. Environ. Public Health* **2012**, 713696
<https://doi.org/10.1155/2012/713696>
- Dohle GR, Smit M, Weber RF (2003): Androgens and male fertility. *World J. Urol.* **21**, 341-345
<https://doi.org/10.1007/s00345-003-0365-9>
- Ganguly M, Carnighan RH, Westphal U (1967): Steroid-protein interactions. XIV. Interaction between human alpha 1-acid glycoprotein and progesterone. *Biochemistry* **6**, 2803-2814
<https://doi.org/10.1021/bi00861a022>
- Gonçalves GD, Semperebon SC, Biazzi BI, Mantovani MS, Fernandes GSA (2018): Bisphenol A reduces testosterone production in TM3 Leydig cells independently of its effects on cell death and mitochondrial membrane potential. *Reprod. Toxicol.* **76**, 26-34
<https://doi.org/10.1016/j.reprotox.2017.12.002>
- Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, Toppari J, Zoeller RT (2015): Executive summary to EDC-2: the endocrine society's second scientific statement on endocrine-disrupting chemicals. *Endocr. Rev.* **36**, 593-602
<https://doi.org/10.1210/er.2015-1093>
- Ibrahim MA, Elbakry RH, Bayomy NA (2016): Effect of bisphenol A on morphology, apoptosis and proliferation in the resting mammary gland of the adult albino rat. *Int. J. Exp. Pathol.* **97**, 27-36
<https://doi.org/10.1111/iep.12164>
- Ivell R, Anand-Ivell R (2009): Biology of insulin-like factor 3 in human reproduction. *Hum. Reprod. Update* **15**, 463-476
<https://doi.org/10.1093/humupd/dmp011>
- Ivell R, Anand-Ivell R (2018): Insulin-like peptide 3 (INSL3) is a major regulator of female reproductive physiology. *Hum. Reprod. Update.* **24**, 639-651
<https://doi.org/10.1093/humupd/dmy029>
- Kang JH, Kondo F, Katayama Y (2006): Human exposure to bisphenol A. *Toxicology* **226**, 79-89
<https://doi.org/10.1016/j.tox.2006.06.009>
- Kaur S, Saluja M, Bansal MP (2018): Bisphenol A induced oxidative stress and apoptosis in mice testes: Modulation by selenium. *Andrologia* **50**
<https://doi.org/10.1111/and.12834>
- Kremer JM, Wilting J, Janssen LH (1988): Drug binding to human alpha-1-acid glycoprotein in health and disease. *Pharmacol. Rev.* **40**, 1-47
- Lague E, Tremblay JJ (2009): Estradiol represses insulin-like 3 expression and promoter activity in MA-10 Leydig cells. *Toxicology* **258**, 101-105
<https://doi.org/10.1016/j.tox.2009.01.013>
- Lan HC, Wu KY, Lin IW, Yang ZJ, Chang AA, Hu MC (2017): Bisphenol A disrupts steroidogenesis and induces a sex hormone imbalance through c-Jun phosphorylation in Leydig cells. *Chemosphere* **185**, 237-246
<https://doi.org/10.1016/j.chemosphere.2017.07.004>
- Li Y, Zhang W, Liu J, Wang W, Li H, Zhu J, Weng S, Xiao S, Wu T (2014): Prepubertal bisphenol A exposure interferes with ovarian follicle development and its relevant gene expression. *Reprod. Toxicol.* **44**, 33-40
<https://doi.org/10.1016/j.reprotox.2013.09.002>
- Li YJ, Song TB, Cai YY, Zhou JS, Song X, Zhao X, Wu XL (2009): Bisphenol A exposure induces apoptosis and upregulation of Fas/FasL and caspase-3 expression in the testes of mice. *Toxicol. Sci.* **108**, 427-436
<https://doi.org/10.1093/toxsci/kfp024>
- Liu C, Duan W, Li R, Xu S, Zhang L, Chen C, He M, Lu Y, Wu H, Pi H, et al. (2013): Exposure to bisphenol A disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity. *Cell Death Dis.* **4**, e676
<https://doi.org/10.1038/cddis.2013.203>
- Liu J, Marey MA, Kowsar R, Hambruch N, Shimizu T, Haneda S, Matsui M, Sasaki M, Hayakawa H, Pfarrer C, Miyamoto A (2014): An acute-phase protein as a regulator of sperm survival in the bovine oviduct: alpha 1-acid-glycoprotein impairs neutrophil phagocytosis of sperm in vitro. *J. Reprod. Dev.* **60**, 342-348
<https://doi.org/10.1262/jrd.2014-049>
- Meeker JD, Ehrlich S, Toth TL, Wright DL, Calafat AM, Trisini AT, Ye X, Hauser R (2010): Semen quality and sperm DNA damage in relation to urinary bisphenol A among men from an infertility clinic. *Reprod. Toxicol.* **30**, 532-539
<https://doi.org/10.1016/j.reprotox.2010.07.005>
- Milligan SR, Balasubramanian AV, Kalita JC (1998): Relative potency of xenobiotic estrogens in an acute in vivo mammalian assay. *Environ. Health Perspect.* **106**, 23-26
<https://doi.org/10.1289/ehp.9810623>
- Nakamura D, Yanagiba Y, Duan Z, Ito Y, Okamura A, Asaeda N, Tagawa Y, Li C, Taya K, Zhang SY, et al. (2010): Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. *Toxicol. Lett.* **194**, 16-25
<https://doi.org/10.1016/j.toxlet.2010.02.002>
- O'Bryan MK, Grima J, Mruk D, Cheng CY (1997): Haptoglobin is a Sertoli cell product in the rat seminiferous epithelium: its purification and regulation. *J. Androl.* **18**, 637-645
- Paxton JW (1983): Alpha 1-acid glycoprotein and binding of basic drugs. *Methods Find Exp. Clin. Pharmacol.* **5**, 635-648
- Pusch W, Balvers M, Ivell R (1996): Molecular cloning and expression of the relaxin-like factor from the mouse testis. *Endocrinology* **137**, 3009-3013
<https://doi.org/10.1210/endo.137.7.8770925>
- Qian W, Zhu J, Mao C, Liu J, Wang Y, Wang Q, Liu Y, Gao R, Xiao H, Wang J (2014): Involvement of CaM-CaMKII-ERK in bisphenol A-induced Sertoli cell apoptosis. *Toxicology* **324**, 27-34
<https://doi.org/10.1016/j.tox.2014.06.001>
- Rahman MS, Kwon WS, Ryu DY, Khatun A, Karmakar PC, Ryu BY, Pang MG (2018): Functional and proteomic alterations of F1 capacitated spermatozoa of adult mice following gestational exposure to bisphenol A. *J. Proteome Res.* **17**, 524-535
<https://doi.org/10.1021/acs.jproteome.7b00668>
- Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, Vom SF

- (2007): In vivo effects of bisphenol A in laboratory rodent studies. *Reprod. Toxicol.* **24**, 199-224
<https://doi.org/10.1016/j.reprotox.2007.06.004>
- Rochester JR (2013): Bisphenol A and human health: a review of the literature. *Reprod. Toxicol.* **42**, 132-155
<https://doi.org/10.1016/j.reprotox.2013.08.008>
- Rubin BS (2011): Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. *J. Steroid Biochem. Mol. Biol.* **127**, 27-34
<https://doi.org/10.1016/j.jsbmb.2011.05.002>
- Savitski MM, Wilhelm M, Hahne H, Kuster B, Bantscheff M (2015): A scalable approach for protein false discovery rate estimation in large proteomic data sets. *Mol. Cell. Proteomics* **14**, 2394-2404
<https://doi.org/10.1074/mcp.M114.046995>
- Schweigert FJ, Gericke B, Wolfram W, Kaisers U, Dudenhausen JW (2006): Peptide and protein profiles in serum and follicular fluid of women undergoing IVF. *Hum. Reprod.* **21**, 2960-2968
<https://doi.org/10.1093/humrep/del257>
- Sharpe RM, Skakkebaek NE (1993): Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* **341**, 1392-1395
[https://doi.org/10.1016/0140-6736\(93\)90953-E](https://doi.org/10.1016/0140-6736(93)90953-E)
- Song XF, Wei GH, Liu X, Zhang DY, Chen X, Deng YJ (2008): Effects of diethylhexyl phthalate (DEHP) on INSL3 mRNA expression by Leydig cells derived from mouse embryos and in newborn mice. *J. Int. Med. Res.* **36**, 512-521
<https://doi.org/10.1177/147323000803600316>
- Sweeney MF, Hasan N, Soto AM, Sonnenschein C (2015): Environmental endocrine disruptors: Effects on the human male reproductive system. *Rev. Endocr. Metab. Disord.* **16**, 341-357
<https://doi.org/10.1007/s11154-016-9337-4>
- Tomza-Marciniak A, Stepkowska P, Kuba J, Pilarczyk B (2018): Effect of bisphenol A on reproductive processes: A review of in vitro, in vivo and epidemiological studies. *J. Appl. Toxicol.* **38**, 51-80
<https://doi.org/10.1002/jat.3480>
- Ueda K, Yamamasu S, Nakamura Y, Honda K, Ozaki K, Fujino Y, Ishiko O, Ogita S (2001): Involvement of phenotypes and serum levels of haptoglobin in the outcome of in vitro fertilization and embryo transfer. *Gynecol. Obstet. Invest.* **51**, 219-222
<https://doi.org/10.1159/000058053>
- Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007): Human exposure to bisphenol A (BPA). *Reprod. Toxicol.* **24**, 139-177
<https://doi.org/10.1016/j.reprotox.2007.07.010>
- Vidaeff AC, Sever LE (2005): In utero exposure to environmental estrogens and male reproductive health: a systematic review of biological and epidemiologic evidence. *Reprod. Toxicol.* **20**, 5-20
<https://doi.org/10.1016/j.reprotox.2004.12.015>
- Vrooman LA, Oatley JM, Griswold JE, Hassold TJ, Hunt PA (2015): Estrogenic exposure alters the spermatogonial stem cells in the developing testis, permanently reducing crossover levels in the adult. *PLoS Genet.* **11**, e1004949
<https://doi.org/10.1371/journal.pgen.1004949>
- Weiss A, Ozeri D, Beck-Fruchter R, Lavee M, Geslevich Y, Levy AP (2013): Haptoglobin phenotypes and in vitro fertilization treatment outcomes. *Syst. Biol. Reprod. Med.* **59**, 281-284
<https://doi.org/10.3109/19396368.2013.794874>
- Wen B, Zhou R, Feng Q, Wang Q, Wang J, Liu S (2014): IQuant: an automated pipeline for quantitative proteomics based upon isobaric tags. *Proteomics* **14**, 2280-2285
<https://doi.org/10.1002/pmic.201300361>
- WHO, UNEP (2013): State of the science of endocrine disrupting chemicals - 2012. World Health Organization
- Yeung BH, Wan HT, Law AY, Wong CK (2011): Endocrine disrupting chemicals: Multiple effects on testicular signaling and spermatogenesis. *Spermatogenesis* **1**, 231-239
<https://doi.org/10.4161/spmg.1.3.18019>
- Zhang C, Wang A, Sun X, Li X, Zhao X, Li S, Ma A (2013): Protective effects of lycium barbarum polysaccharides on testis spermatogenic injury induced by bisphenol A in mice. *Evid. Based Complement. Alternat. Med.* **2013**, 690808
<https://doi.org/10.1155/2013/690808>

Received: December 20, 2019

Final version accepted: April 2, 2020