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Fetal exposure to endocrine disrupting-bisphenol A (BPA) alters testicular fatty acid metabolism in the adult offspring: relevance to sperm maturation and quality

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Abstract

Daily exposure to endocrine-disrupting substances like bisphenols can affect reproductive functions due to their pseudo-estrogenic and/or anti-androgenic effects. Testicular lipids contain high levels of polyunsaturated fatty acids (PUFA) necessary for sperm maturity, motility, and spermatogenesis. Whether prenatal exposure to bisphenols alters testicular fatty acid metabolism in the offspring is unknown. The effects of in-utero bisphenol exposure on the lipid metabolism of adult offspring testis (90d) were examined. Pregnant Wistar rats were exposed to BPA and BPS (0.0, 0.4, 4.0, 40.0 µg/kg bw/d) by oral gavage from gestational day 4 to 21. Despite increased body and testis weight, the fetal BPA exposure did not affect total testicular cholesterol, triglyceride levels, and plasma fatty acid composition in offspring. Lipogenesis was upregulated as evidenced by increased SCD-1, SCD-2, and expression of lipid storage (ADRP) and trafficking protein (FABP4). The arachidonic acid, 20:4 n-6 (ARA) and docosapentaenoic acid, 22:5 n-6 (DPA) levels were significantly decreased ($p<0.05$) in the BPA-exposed testis,

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while BPS exposure had no effects. The expression of PPAR-signaling proteins (PPARα and PPARγ) was reduced in the testis, which is important for sperm energy dissipation. BPA exposure reduced the expression of CATSPER2 in adult offspring testis. The endogenous conversion of linoleic acid,18:2n-6 (LA), to ARA was impaired, as evidenced by a reduced ARA/LA ratio and decreased FADS1 expression. The reduced ARA might have impacted steroidogenesis. Scattered and decreased signals of FADS1 suggest its disrupted activities and reduced expression in these testes. The BPA exposure on the testis and its impacts on endogenous lipid metabolism might be underlying factors that dysregulate sperm maturation and quality.

Keywords: Testis; PUFA; BPA; Male fertility; Desaturase; PPAR;

Abbreviations:
ACSBG2, Acyl Co-A synthetase bubblegum family member 2; ADIPOQ, Adiponectin; ADRP, Adipose related differentiation protein; ARA (20:4 n-6), Arachidonic acid ; BPA, Bisphenol A; BPS, Bisphenol S; CATSPER, Sperm cation channel-like proteins ; COX2, Cyclooxygenase-2; CTCF, Corrected total cell fluorescence ; DEGS1, Delta 4 desaturase sphingolipid 1; DHA (22:6 n-3), Docosahexaenoic acid ; DPA (22:5 n-6), Docosapentaenoic acid; DTA (22:4 n-6), Docasotetraenoic acid ; ELOVL2, Elongation of very long chain fatty acids- like 2; ELOVL5, Elongation of very long chain fatty acids-like 5; FABP, Fatty acid-binding protein ; FADS1, Fatty acid desaturase 1/ delta 5-desturase; FADS2, Fatty acid desaturase 2/ delta 6-desaturase; FASN, Fatty acid synthase; gD, Gestational day ; HFD, High-fat diet; IGF-1, Insulin like growth factor-1; LA (18:2), Linoleic acid; LC, Long chain ; LD, Lipid droplets ; LEP, Leptin; LIPE, Hormone sensitive lipase; LPL, Lipoprotein lipase; MUFA, Monounsaturated fatty acid; OA (18:1), Oleic acid; PG, Prostaglandin; PPAR, peroxisome proliferator activated receptor; PUFA, Polyunsaturated fatty acids; RIPA, Radio immunoprecipitation buffer; SCD1, Stearoyl Coenzyme-A desaturase 1; SCD2, Stearoyl Coenzyme-A desaturase 2; SFA, Saturated fatty acids; SLC25A20, Mitochondria fatty acid transporter; TDI, Tolerable daily intake; VLC-PUFA, Very long chain- PUFA;

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Highlights

- In utero BPA exposure not BPS affected testicular long-chain n-6 PUFA metabolism in offspring
- Fetal exposure to BPA alters delta-9-desaturase indices in the adult testis
- BPA exposure reduced fatty acid desaturase1 expression in offspring testis
- Fetal BPA exposure dysregulated lipogenesis in offspring testis

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1. Introduction

Global decline in fertility rate and increasing male infertility could originate from the fetus. Testicular dysgenesis syndrome is suspected due to adverse environmental exposure to fetal life gonadal programming (Skakkebaek et al. 2016). Exposure to endocrine disrupting chemicals (EDC) like bisphenols is inevitable in our everyday life, even microscopic, through various modes, including dermal exposure, leaching from food-containing surfaces, microplastic contaminants from marine sourced foods, and recycling of non-degradable residual pollutants. Although bisphenol A (BPA) is detected in maternal and fetal tissues (Lee et al. 2018), the fetus is not equipped with BPA-antagonizing machinery, making it vulnerable to exposure during the developmental window, which could have long-lasting programming on the functions of organs. BPA binds to estrogen-related receptor gamma with high affinity and suppresses androgen receptors in the testis, increasing the risk for male infertility (Adegoke et al. 2020). Emerging data suggest a closer alignment between urinary BPA levels and reproductive malfunctions in males (Radwan et al. 2018). The in-utero BPA exposure alters the functioning of the male reproductive system in the offspring (Molangiri et al. 2022), affects the testicular physiology (Frungieri et al. 2015), disrupting its bioenergetics capacity (Chianese et al. 2018), and metabolism (Dunder et al. 2018). Testicular metabolism is tightly regulated by each cell type performed within its microenvironment. A detailed understanding of the potential risk associated with fetal exposure to BPA on testicular physiology could help identify underlying causes of declining male fertility.

Lipids are essential in sperm membrane formation since their compositional changes during testis maturity determine sperm motility and affect spermatogenesis (Beckman et al. 1978). Spermatogenesis could also be influenced by an interplay of hormone-disrupting substances like bisphenols and membrane lipid biosynthesis of sperm since exposure to BPA mimics high-fat diet (HFD)-induced metabolic changes in the offspring (García-Arevalo et al. 2014). Exposed to pollutant mixture containing BPA, exhibited dysregulated lipid homeostasis

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with increased hepatic triglyceride and a few similarities with HFD-induced changes, particularly with lipid metabolism (Labaronne et al. 2017), indicating the modulation of the endocrine-metabolic axis by BPA. Since the structural requirement of maturing testis demands an increased lipid biosynthesis, their turnover increases during development. Spermatids account for more than 60% of the total testis volume in rats, with fatty acid concentrations similar in both testis and spermatids. The fatty acid composition determines the performance not only of sperm maturity but also of acrosomal reaction and sperm-oocyte fusion (Collodel et al. 2020). Fatty acid composition in the testis is associated with male fertility (Aksoy et al. 2006; Craig et al. 2019), affecting sperm maturity due to the non-availability of the substrates to the testis (Collodel et al. 2020). Understanding the impacts on testis fatty acid metabolism from exposure to bisphenols could help identify a potential cause of male fertility. Even though fetal BPA exposure to fatty acid composition in the liver and adipose (Dunder et al. 2018) is reported, in-utero exposure to BPA and its substitute BPS on testicular lipid metabolism in adult offspring is not known.

In addition to the liver and other fatty acid-synthesizing tissues, the testis is the site for the active biosynthesis of polyunsaturated fatty acids (PUFAs). The activities of desaturases and elongases regulate it. The testicular fatty acids profile affects their cells and influences sperm maturity, capacitation, acrosome reactions, and sperm-oocyte fusion, contributing to male fertility (Collodel et al. 2020). The metabolism of PUFAs harnesses energy for spermatogenesis and epididymal sperm maturation. The fatty acids, especially long chain (LC) PUFAs, play a vital role in spermatogenesis, and the disruption in fatty acid composition produces infertile sperm, affecting sperm maturation and motility. Increased MUFAs and decreased PUFAs in spermatozoa are correlated with men's infertility (Aksoy et al. 2006). Very long-chain PUFAs in the testis positively connect with sperm quality and are involved with improved sperm quality (Craig et al. 2019).

PUFAs influence male reproductive functions in several ways. They are the precursors of prostaglandin (PG) synthesis and modulate the expression of key enzymes involved in steroid

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and fat metabolism of the testis. Spermatozoa need plasma membrane PUFA enrichment to support the membrane fluidity during fertilization. But it also makes spermatozoa susceptible to attack by reactive oxygen species. BPA exposure promotes oxidative stress in the testis, thereby establishing a potential association with reduced fertility. Eicosanoid biosynthesis involves testicular development by supporting PG synthesis, which is needed for germ cell development and steroidogenesis. Arachidonic acid, 20:4 n-6 (ARA), is a precursor for PG-2 series during testicular steroidogenesis in spermatogenesis (Tran-Guzman and Culty 2022). Although the involvement of PGs is well established in female reproduction, their role in male fertility was later evidenced by dysregulated cyclooxygenase-2 (COX2) expression in infertile subjects (Matzkin et al. 2010).

Fatty acid composition is influenced by diet and endogenous desaturation activities in the testis. The LCPUFAs, such as docosapentaenoic acid (DPA, 22:5, n-6) and docosahexaenoic acid (DHA, 22:6, n-3) are essential for sperm quality. Local accumulation of these LCPUFAs in spermatids supports spermatogenesis (Shishikura et al. 2019). The fatty acid composition of testicular spermatozoa determines sperm characteristics (Andersen et al. 2016). LCPUFAs are synthesized locally in Sertoli cells from their precursor, uptaking, and transported to the testis germ cell. Sertoli cells contain much higher fatty acid elongation and desaturation activities than germ cells (Retterstøl et al. 2001). Unlike other PUFA-rich tissue, fatty acids are continuously utilized in the testis as spermatozoa are transported to the epididymis. All the major desaturases, such as FADS1, FADS2, SCD1 and SCD2, are localized in the Sertoli cells of the rat testis (Saether et al. 2003). The activity of desaturases is involved in the endogenous biosynthesis of monounsaturated fatty acids (MUFAs), PUFAs, and LCPUFAs from their precursors by SCDs, FADSs, and ELOVLs enzymes. Despite evidence that suggests SCDs (Dunder et al. 2018; Saether et al. 2007) and FADSs are altered in male fertility and spermatogenesis (Collodel et al. 2020), prenatal bisphenol exposure to testicular PUFA metabolism and its relation with FADS activities in the developmentally exposed offspring is elusive.

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Spermatogenesis is an energy-intensive process transforming round spermatids into elongated spermatozoa for fertile sperm production. Site-specific metabolic fuel utilization supports reproductive energy requirements during spermatogenesis. Peroxisome proliferator-activated receptor gamma (PPARγ) is a master regulatory switch for the energy metabolism of fats, predominantly expressed in germ cells and Sertoli cells of the testis, involved in regulating the expression of lipid metabolic genes by forming PPARγ-RXRα heterodimer in Sertoli cells (Thomas et al. 2011), and its signalling contributes a role in energy metabolism in the testis. Studies reported that lipid metabolic genes in the testis regulates male fertility (Thomas et al. 2011) and plays a role in male reproduction by binding to DNA promoters of the genes for fatty acid metabolism (Olia Bagheri et al. 2021). Despite these data, the mechanism by which PPARs regulate energy metabolism in the testis during endocrine disruption is unclear.

Fatty acid-binding proteins (FABPs) involve intracellular fatty acid trafficking and metabolism to support the synthesis and remodelling of membrane lipids in the testis (Oresti et al. 2013). FABP9 is expressed in the interstitial tissue of the testis, a major component of the rat sperm perinuclear theca. It plays a protective role against the oxidation of sperm fatty acids, and sperm function. Since expressions of FABPs are involved in the testicular lipid metabolism and functionally involved in the final stages of germ cell differentiation in the testis, it needs to be investigated.

Excess adiposity affects the fatty acid composition of spermatozoa via regulating testis fatty acid metabolism (Andersen et al. 2016). Fetal BPA exposure results in elevated triglycerides, adiposity, and adipogenic gene expression in developmentally exposed rodent offspring (Dunder et al. 2018; Lejonklou et al. 2017). However, studies investigating the fetal programming of testicular fatty acid metabolism and its functions following developmental exposure to BPA are limited.

Our recent data demonstrated that prenatal exposure to bisphenols (BPA and BPS) significantly increased body weight, testis weight, and altered testosterone levels, androgen,

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estrogen receptor expression, hyperplasia, inflammation, and oxidative stress in the offspring testis (Molangiri et al. 2022). We hypothesize that lipogenesis in the testis may augment hyperplasia and increase vacuolization. Assessing the effects of in-utero BPA exposure on the fatty acids profile of the offspring testis could help identify the risk of exposing lifestyle factors to reproductive functions. Therefore, the effects of in-utero bisphenol exposure on testicular fatty acid metabolism of the adult offspring were investigated. The study conducted in rodents may represent a model for understanding lipid metabolism in the human testis, particularly when fetal gonadal development is exposed to EDC. The study reports the impact of bisphenol exposure on the total PUFA composition, the metabolic pathway of LCPUFAs, localization, and expression of active desaturase, PPARs, and fatty acid binding proteins in adult offspring testis exposed to bisphenols prenatally.

2. Materials and Methods

2.1 Animal experiments

Three-month-old females (n=40) and males (n=20) Wistar rats were obtained from the animal facility of the National Institute of Nutrition, India. Ethical clearance was obtained from Institutional Animal Ethical Committee (#ICMR-NIN/IAEC/02/008/2019). Rats were housed in pairs per cage at a constantly regulated temperature (22 ± 2°C) and light-controlled housing conditions (~12:12 h light/dark cycles). Rats were allowed to acclimate for a week and provided a chow diet and water ad libitum throughout the study. The fertile estrous cycle was determined by methylene blue staining of vaginal smears. Rats were kept for mating overnight (M: F = 2:1) during their fertile period. The presence of vaginal plugs confirmed mating. After mating, males were removed, and the day was considered a gestational day (gD) 0. Pregnant female rats were randomly divided into seven groups. Bisphenols (BPA #CAS 80-05-7, BPS #CAS 80-09-1, Sigma Aldrich) were dissolved in extra virgin olive oil to get the desired concentrations of 0.4, 4.0, and 40.0 μg/kg body weight per day. The control group was administered only extra virgin

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olive oil. Pregnant rats were administered bisphenols via oral gavage from gD 4 to 21 as described previously (Molangiri et al. 2022).

2.2 Collection of blood and testis

Blood was collected in EDTA-coated tubes from adult rats (90 d) by puncturing the retro-orbital plexus using a sterile capillary tube. Plasma was separated and stored at -80° C for further analysis. Male adult rats were euthanized by carbon dioxide asphyxiation. The testis was removed and washed in sterile PBS to remove traces of blood. Testis was stored in Bouin solution and snap-frozen in liquid nitrogen.

2.3 Total fatty acid composition

Plasma and testis from adult (90d) rats were used to estimate total fatty acid composition. As performed before, the lipids were isolated from plasma and testis tissue (~100mg) (Srinivas et al. 2021). The total lipid fraction was methylated by incubating with sulfuric acid (2%) containing methanol and butylated hydroxytoluene (10 mg/l). After cooling the fraction with petroleum ether, fatty acid methyl esters were drawn out. Gas chromatography (Perkin Elmer Clarus 680) connected with a silica capillary column (30 m × 0.25 mm × 0.2 µm, Supelco, USA) analyzed the plasma and testis fatty acid composition. The fatty acid standards based on recognized retention times were employed to identify the unknown samples (Nuchek, USA).

2.4 Triglyceride and cholesterol assays

Using a blade homogenizer, testis tissue (50-100 mg) was homogenized in 5% aqueous Nonidet P40 (NP-40). The homogenate was heated at 80°C in a water bath to solubilize the lipids. Testicular triglycerides and cholesterol were assayed using an enzymatic kit (Biosystems, Spain).

2.5 Real-time-polymerase chain reaction (RT-qPCR)

Total RNA isolation was carried out using TRIzol reagent (Cat# T9424, Sigma). Briefly, testis tissue (50 mg) was homogenized in TRIzol solution using zirconia beads (Cat#11079124zx, Biospec) in a mini bead beater (Biospec). The addition of chloroform

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achieved phase separation. The upper aqueous phase was separated, and RNA was precipitated using chilled isopropanol. The RNA pellet was dissolved in sterile nuclease-free water. Total RNA was purified from genomic DNA by employing a DNase I kit (Cat#AMPD1, Sigma). Subsequently, Nanodrop (Thermo scientific ND1000) was employed to assess the quality and quantity of RNA. Next, total RNA (1μg) was converted to cDNA using a kit (Cat #1708891 Biorad). Finally, qRT-PCR was performed in Stratagene Mx3005p qPCR system (Agilent Technologies) using TB green pre-mix Ex-Taq II (Cat#RR208, Takara) and KiCqStart SYBR green primers (Sigma Merck, **Supplementary Table 1**). Obtained Ct values were used for relative quantification of mRNA expression using the ddCt method. The expression of β-actin mRNA was used as endogenous control.

2.6 Immunoblotting

Testis lysates were prepared by homogenizing tissue using liquid nitrogen in RIPA buffer containing protease inhibitor cocktail. The supernatant was collected, and the protein was quantified using the BCA method (Pierce™ BCA protein assay kit). An equal quantity of protein was loaded in each well and resolved onto 12% SDS-PAGE gel. After resolving, proteins were transferred onto a nitrocellulose membrane (#1620112, Bio-Rad) using a wet transfer system. The membrane was blocked with 5% skim milk powder in TBS to avoid non-specific antibody binding. The blots were incubated with primary antibody overnight using corresponding dilutions (**Supplementary Table 2**). Blots were washed thrice with TBST, and incubated HRP conjugated secondary antibody was for 90 mins at room temperature. Immunoblot signals were captured in the gel imaging system after adding the ECL substrate. The band intensity was quantified as a measure of protein expression using Image J software (NIH, USA).

2.7 Immunofluorescence

Testis tissues were collected from adult rats (90 d) and fixed in Bouins solution overnight. The fixed tissues were embedded in the paraffin, and 4 μm sections were obtained with the help of a microtome. Paraffin sections were mounted onto pre-coated slides. Slides

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were heated at 60°C for deparaffinization, followed by a couple of xylene washes. Sections were rehydrated using varying ethanol concentrations and washed with distilled water. Antigen retrieval was achieved by boiling the slides in a microwave oven for 5 mins using citrate buffer (pH 6.0). Slides were washed with PBS and blocked with 3% horse serum in a moist, humidified chamber. Primary antibody was added and incubated at 4°C overnight. Slides were washed with PBS twice and incubated with Alexa fluor conjugated secondary antibody (#A11034, ThermoFischer) for 1 h, then mounted with fluoroshield in DAPI medium (#F6057, Sigma). Images were captured using a confocal microscope (DMi8) with SP8 infrared lasers (Leica microsystems).

2.8 Statistical analysis

Data normally distributed were analyzed by one-way ANOVA followed by post-hoc Sidak's or Tukey's multiple comparison tests using Graph pad Prism v.8. Data were also calculated by paired t-test to compare two groups. Results are calculated from the data derived from repeated independent experiments mentioned in the text or figure legend. Statistical significance was considered when $p<0.05$. All experimental data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1 In utero, BPA exposure affects long-chain fatty acid composition in the testis independent of plasma

Whether lipogenesis and /or changes in fatty acid composition in testes are probable underlying reasons for testicular hyperplasia observed previously was examined by measuring their levels in plasma and testis. Plasma total fatty acid composition of the adult offspring did not report any significant changes in the saturated fatty acids (SFA), monounsaturated fatty acid (MUFA), PUFA, and LCPUFA levels across the experimental groups involved with various concentrations (0.0, 0.4, 4.0, and 40.0 μg/kg bw/d) of bisphenols (BPA and BPS; **Table 1**) exposed during gestation. In contrast, the total fatty acid composition was significantly altered in the offspring's testes which were exposed to BPA (4.0 μg/kg bw/d) in utero (**Table 2**). The

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concentration of SFAs (palmitic acid and stearic acid) were decreased ($p<0.05$), while MUFAs (palmitoleic acid, oleic acid,) and PUFA (linoleic acid) were increased ($p<0.05$) in BPA (4.0 μg/kg) exposed testis. While in control testis, LA was converted efficiently to produce ARA and DPA and increased their levels by ~2.9 and ~3.2 folds, but their levels were decreased by ~5.8 and ~9.6 folds, respectively in BPA-exposed testis (**Table 2)**.

The stearoyl-coenzyme A desaturases (SCD) or delta-9-desaturase are involved in the biosynthesis of MUFA from SFAs by introducing a double bond. Therefore SCD indexes (SCD-16 and SCD-18) were estimated by product/precursor ratios of fatty acids. The increased SCD-16 and SCD-18 in BPA-exposed testis (**Table 2**) reflect a higher SCD-1 activity. Increased SCD indexes indicate that fetal BPA exposure might have programmed the testicular lipid homeostasis, increasing lipogenesis in the adult offspring testis. Overall, fetal exposure to BPA affects the testicular lipid metabolism of adult offspring by modulating SCD-1 activities.

3.2 LCPUFAs were decreased in BPA-exposed testis, while BPS exposure had no effects

The LCPUFAs such as arachidonic acid, 20:4n-6 (ARA), adrenic acid (C22:4 n-6), and docosapentaenoic acid, C22:5 n-6 (DPA) were significantly decreased by ~3.4, ~2.0 and ~6.3 folds ($p<0.05$). In contrast, the levels of oleic acid, C18:1 (OA), and linoleic acid, C18:2 (LA) were significantly increased ($p<0.05$) by ~2.4 and ~4.9 folds respectively only in BPA-exposed offspring (**Table 2**). However, the level of docosahexaenoic acid, C22:6 n-3 (DHA) was unaffected in testis among these groups. Whether increased lipid levels in these offspring were due to an increase in total cholesterol and triglycerides, their levels were measured in the plasma and testes of the adult offspring. In utero, BPA exposure did not alter the triglycerides and total cholesterol in the plasma and testes of 90 d adult offspring (**Table 3**). Collectively, these data indicate that maternal exposure to BPA, not BPS during gestation, increases testis fatty acid composition and lipogenesis in the rats at their age of 90 days.

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3.3 Expression of fatty acid storage, lipogenesis and sperm motility mediator was affected in the testis due to BPA exposure

To understand the mechanism of disrupted lipid composition in offspring testes, the expression of proteins associated with fatty acid storage, trafficking, metabolisms such as ADRP, FABP4, FABP5, FABP7, and FABP3, were measured in the testis homogenate obtained from 90 d male rat offspring (**Fig.1A-C**). Expression of ADRP, and FABP4, were significantly increased by ~5.6 and ~5.3 folds ($p<0.05$) in BPA-exposed testis (**Fig.1A and B**), while the expression of FABP5 and FABP7 was not detected in the testis (data not presented). In contrast, expression of FABP3 was significantly decreased by ~1.7 folds in prenatally BPA-exposed adult offspring testis (**Fig.1C**). Taken together, these data suggest that BPA exposure during pregnancy profoundly affected the offspring's testicular expression of lipid storage and trafficking proteins in their adult life.

To examine the mechanism associated with lipogenesis, the mRNA expression of genes that regulate lipogenesis and steroidogenesis was investigated in the adult testis. In utero, BPA exposure significantly upregulated hormone-sensitive lipase (LIPE) expression by ~1.35 folds ($p<0.05$). In contrast, the expression of lipoprotein lipase (LPL) was decreased ($p>0.05$) in the offspring testis (**Fig.2**). Expression of testis-specific fatty acid binding protein 9 (FABP9) and cyclooxygenase 2 (COX2) mRNAs were significantly downregulated ($p<0.05$) in rat testis exposed to BPA (**Fig.2**). The expression of genes involved in lipogenesis such as fatty acid synthases (FASN), mitochondrial fatty acid transporter (SLC25A20) remained unchanged in testis when compared to control (data not presented). The expression of growth and signalling molecules such as insulin growth factor-1 (IGF-1), leptin (LEP), and adiponectin (ADIPOQ) was decreased marginally ($p>0.05$) in BPA-exposed rat testis (data not presented).

Testicular lipid turnover is critically involved in successful spermatogenesis. The expression of nuclear receptor proteins such as PPARα and PPARγ associated with fatty acid metabolism and energy homeostasis in spermatogenesis, therefore, measured in the testis

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homogenate prepared from 90d male rat offspring. Expression of PPARγ and PPARα were significantly decreased by ~1.6 and ~1.9 folds ($p<0.05$) in BPA-exposed testis (**Fig.3A-B**) as compared to control rats. Thus, BPA exposure in utero significantly affected the expression of PPAR-signalling proteins in the testis, which may regulate sperm energy dissipation. In addition, BPA exposure to dam during pregnancy significantly downregulated CATSPER2 mRNA expression ($p<0.05$), but the expression of CATSPER1 had no significant effects as compared to the control (**Fig.4**).

3.4 Stearoyl-CoA desaturase activities, and fatty acid desaturase & elongase expression were altered in adult testis due to in-utero BPA exposure

Since LCPUFA biosynthesis was severely affected in testes, the expression of fatty acid desaturase 1 (FADS1 or delta-5 desaturase), fatty acid desaturase 2 (FADS2 or delta-6 desaturase), delta 4 desaturase sphingolipid 1 (DEGS1), elongation of very long chain fatty acids-like 2 (ELOVL2), elongation of very long chain fatty acids-like 5 (ELOVL5), stearoyl-coenzyme A desaturase 2 (SCD2), and stearoyl-coenzyme A desaturase 1 (SCD1) were screened quantitatively. The mRNA expression of fatty acid desaturase 1 (FADS1) was significantly downregulated ($p<0.05$) in the testis of BPA (4.0 μg/kg) exposed rats compared to the control (**Fig.5**). The expression of other desaturases, such as DEGS1, SCD1, and SCD2 was also decreased ($p>0.05$) in the testes of these rats. However, the mRNA levels of FADS2 and ELOVL2, ELOVL5 remained unaltered due to BPA exposure (data not presented).

Further, the expression of enzymes involved in the PUFA biosynthesis, such as FADS1 and ELOVL2 proteins, were measured in the testis homogenate prepared from 90d male rat offspring. In the testes, the expression of FADS1 was significantly decreased by ~4.3 folds ($p<0.05$, **Fig.6A**), while ELOVL2 expression was increased by ~1.4 folds ($p<0.05$, **Fig.6B**) in BPA-exposed rats. BPA exposure in utero significantly lowered the FADS1 expression with

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compensatory expression of ELOVL2, indicating a disruption in the desaturation and elongation activities associated with LCPUFA biosynthesis in these adult offspring testes.

3.5 FADS1 proteins are dispersed and scattered in the seminiferous tubules of the testis due to BPA exposure

The localization and expression of FADS1 were examined by immunofluorescence in the offspring testes, which were gestationally exposed to BPA (4.0 μg/kg). Green dots indicate the expression and localization of FADS1 protein in the testicular tissue obtained from control and BPA-exposed rats (**Fig.7A**). The normalized corrected total cell fluorescence (CTCF) of FADS1 was significantly decreased in the BPA exposed (4.0 μg/kg) testis compared to control (control vs. BPA: 35.42 ± 2.49 vs. 11.69 ± 0.67, $p<0.001$, **Fig.7B**). The FADS1 signal (green) was observed predominantly around the lumen of the seminiferous tubule, which is rich with elongated spermatids and Sertoli cells. Scattered and decreased signals of FADS1 concludes disrupted activities and reduced expression of the fatty acid desaturase 1 enzyme in the adult rat testes that were gestationally exposed to BPA.

4. Discussion

For the first time, this study reports that TDI level (4.0μg/kg bw/d) of prenatal BPA exposure affected lipogenesis, stearoyl-CoA desaturase (delta-9-desaturase) index, levels of MUFAs and n-6 LCPUFAs, and fatty acid desaturase 1 (FADS1 or delta-5 desaturase) expression in the adult offspring testis, which in turn may be responsible for dysregulation of sperm maturity. The PUFA composition of the whole testis showed a reduced proportion of n-6 LCPUFAs in the BPA-exposed testis than controls, where ARA (20:4 n-6) and DPA (22:5 n-6) levels were dramatically decreased. The lower content of these n-6 LCPUFAs can reduce the membrane fluidity (Wathes et al. 2007) in the BPA-exposed offspring testis and, thus, consequently, affect the maturity of the sperm. Localization and reduced expression of FADS1

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in the adult testes indicates that gestationally, BPA exposure affected fatty acid desaturase machinery in these rats.

Decreased levels of n-6 LCPUFA may affect spermatogenesis by reducing prostaglandin (PG) production. The cyclooxygenase (COX) enzyme regulates gonadal development via PG in the testis (Tran-Guzman and Culty 2022). ARA, liberated from phospholipids, is rapidly converted to PGs by COX enzymes and thus stimulates testicular steroidogenesis during spermatogenesis. Decreased availability of ARA in BPA-exposed offspring testis (**Table 2**) can affect fertilization due to reduced production of PGE2, which plays a major role in sperm survival and maturity. Suppressed testicular PGE2 production by COX2-specific inhibitor (Winnall et al. 2007) suggested upregulated expression of COX2, and IL6 observed previously (Molangiri et al. 2022) results in testicular inflammation in BPA-exposed testis. In addition to COX2's roles in regulating inflammation, it also involves in steroidogenesis and steroid hormone-regulated physiological processes in the testis (Frungieri et al. 2015). The decreased COX2 expression in prenatal BPA-exposed offspring testis (**Fig.2**) could impact PG synthesis in the testis. Although PGE2 level was not measured in the present study, their production might have been altered due to decreased COX2 expression in BPA-exposed adult testis.

We observed that testicular MUFA levels (palmitoleic acid and oleic acid) were significantly increased in BPA-exposed offspring. A decreased level of oleic acid is associated with normal sperm maturation in rats (Davis et al. 1966), indicating dysregulated sperm maturity in the offspring. The optimal levels of MUFA in the testis also demonstrate success in spermatogenesis since its level correlated negatively with sperm concentration, maturity, regular morphology, vitality, and fertility index and positively with sperm necrosis (Collodel et al. 2020). MUFAs serve as unsaturated fatty acid substrate pools that convert to 20- and 22-carbon n-9 PUFAs, further desaturating and elongating in the testis. The elongation capacities for unsaturated fatty acids in the testis are high. Excess accumulation of MUFAs in BPA-exposed

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testis indicates an inefficient conversion of these unsaturated fatty acids into PUFAs that might have impacted testis maturity due to an inadequate supply of LCPUFAs.

Like BPA-exposed testis in this study, a higher level of palmitoleic acid was associated with the increased fat synthesis in BPA-exposed rat adipose (Dunder et al. 2018), and plasma level of palmitoleic acid was correlated with abdominal adiposity in humans (Paillard et al. 2008), indicating BPA's role in modulating fat metabolism in offspring testis. Again, high SCD (stearoyl-CoA desaturase or delta-9-desaturase) activity is associated with increased fatty acid synthesis, decreased fatty acid oxidation (Matsui et al. 2012), and carries a higher risk of developing metabolic diseases (Warensjö et al. 2006). Since SCD-1 and SCD-2 activities were not induced in response to lower PUFA content of the mature testis (Saether et al. 2007), implicates that induction of these lipogenic enzymes probably occurred due to endocrine or other disorders in the offspring rats.

Estrogen mimics of BPA might affect spermatogenesis in several ways, including changing fatty acid composition in the testis due to increased lipogenesis, as observed in our study. Our data suggest that the balance between testicular fatty acid metabolism (utilization and synthesis) was disrupted in BPA-exposed rats. BPA, an estrogen-mimicking steroid, can easily diffuse into the nucleus across the cell membrane and modulate the transcription of genes (Basak et al. 2018). Therefore, BPA exposure can affect the lipogenic regulation of adult offspring testis due to fetal epigenetic aberration of an endocrine-metabolic switch of the male gonads.

Testicular metabolism observed in BPA-exposed rats exhibits similarities to its obesogenic effects demonstrated by disrupting metabolic activity and making the body prone to overweight and obesity. Excess BMI regulates fatty acid metabolism in the testis (Andersen et al. 2016). Despite the changes in body weight and adiposity (Molangiri et al. 2022), lipid profiles such as triglyceride and total cholesterol levels did not change in BPA and BPS-exposed offspring in plasma and testis (**Table 3**). Thus, in utero, BPA exposure meditates subtle tissue-

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specific metabolic changes in regulating the endocrine-metabolic axis of the male reproductive system.

The composition of n-6 LCPUFAs (ARA and DPA) in the testis was decreased in BPA-exposed rats (**Table 2**). In contrast, increased DPA is associated with normal sperm maturation in rats (Davis et al. 1966) and may be responsible for dysregulated sperm maturity in the offspring exposed to BPA but not with BPS. Moreover, majorities of the LCPUFAs generally decreased except DPA and DTA (22:4 n-6), which remained constant to support the synthesis of very long-chain PUFA during rodent sperm maturity. The rat testicular cells contain high amounts of DPA (Retterstøl et al. 1998), whereas human testicular cells contain high amounts of DHA. Thus, DPA may perform an equivalent role in rodent testis as DHA does in humans (Beckman et al. 1978). A decreased DPA level indicates its effects on membrane fluidity and structural development of spermatids in the BPA-exposed testis. The decreased DHA and PUFA in spermatozoa are correlated with men's infertility (Aksoy et al. 2006), indicating potential risks of offspring for infertility due to in-utero BPA exposure. Decreased DPA could impair sperm quality since reduced LCPUFAs containing spermatids showed attenuated sperm numbers associated with infertility index in male mice (Shishikura et al. 2019). A DPA-rich lipid moves to surround the spermatid heads when these are embedded in the Sertoli cytoplasm of a healthy mature rat testis (Collodel et al. 2020).

Several enzymes are involved in LCPUFA biosynthesis by desaturation and elongation activities, such as FADS1, FADS2, ELOVL2, ELOVL5, and others. In addition, SCD1, SCD2, FASN, and Acyl Co-A synthetase 6 are also involved in maintaining fatty acid homeostasis in the testis. Despite higher levels of LA which is the precursor of n-6 LCPUFAs, the ARA and DPA levels were decreased, indicating that the desaturation and elongation of fatty acids were inhibited by BPA exposure. The increased LA and decreased ARA in BPA-exposed testis indicate that both FADS1 and FADS2 are likely to be affected. Again, due to reduced ARA and DTA, the DPA was synthesized much lower in BPA-exposed rat testis. Although FADS2 is

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considered the rate-limiting step as it acts twice in the LCPUFA synthesis pathway, FADS2 mRNA expression did not change in BPA-exposed testis (**Fig.5**) in our study. FADS2 is predominantly expressed in Leydig cells, while FADS1 is expressed in Leydig, Sertoli, and Germ cells of the testis (Gautier et al. 2020). A higher signal of FADS1 (**Fig.7**) in the regions around seminiferous tubules where Sertoli and Germ cells are predominant indicates FADS1 could be the predominant desaturase type in the testis of BPA-exposed rats.

Besides the liver, brain, and adipose, the mammalian testes are also involved in PUFA metabolism. The testis and epididymis also perform specific spermatogenesis roles by modulating LCPUFA metabolism. Viable sperms have a high level of LCPUFAs in their membrane, which maintains sperm fluidity and moves around with a greater velocity. Adequate LCPUFAs are ensured by liver metabolism involving desaturase and elongase enzymes. **Table 2** suggests that the whole testis is involved in differential LCPUFA synthesis between control and BPA-exposed rats. While LA, the precursor n-6 PUFAs, intermediator's metabolites like MUFAs (OA) were formed in excess, n-6 LCPUFAs contents were reduced in the testes of the BPA-exposed group.

FADS1 metabolites (such as ARA and their metabolites) are active in Sertoli cells, helping elongate spermatids. FADS1 is involved in the initial step of LCPUFA synthesis, i.e., ARA, by introducing double bonds to LA. Decreased expression of FADS1 in the ARA metabolic pathway of BPA-exposed testis may result in abnormal sperm maturation as the latter depends on this metabolite. The BPA exposure impairs the processes associated with spermatogenesis by modulating fatty acid metabolism in the testis, and the changes were closely similar to HFD-mediated effects (Luo et al. 2020).

The immunolocalization of FADS1 underlines their active metabolism in the seminiferous tubule. FADS1 is regulated by transcription factors controlling fatty acid metabolism and lipogenic gene regulation by producing PUFAs. FADS1 is typically detected in elongated spermatids and epididymal cells in animals (Gautier et al. 2020). A decreased FADS1

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expression in BPA-exposed testis failed to add adequate n-6 LCPUFA, such as ARA, during the fusion of the sperm membrane, which might affect sperm competency of the testis. Since ARA is secreted by Sertoli cells in rat testis by an FSH-dependent pathway (Paillamanque et al. 2017), the estrogen mimic BPA might have dysregulated ARA secretion in their testicular cells in an FSH-dependent manner.

PPARα and PPARβ are expressed in interstitial, germ, and Sertoli cells, while PPARγ expression is specific to Sertoli cells (Liu et al. 2015). The testicular Sertoli cells play a vital role in spermatogenesis by supporting germ-cell differentiation, meiosis, and elongation of spermatozoa. A decreased expression of PPARα and PPARγ in BPA-exposed testis (**Fig.3**) indicates that tissue collected in our analysis contains both cell types. Loss of PPARγ in mice oocytes results in impaired fertility (Cui et al. 2002), while it is adequately expressed in human spermatozoa to maintain energy homeostasis in the testis (Aquila et al. 2006). BPA exposure could affect sperm energy metabolism due to reduced expression of PPARs. Activation of PPARγ signalling promotes the transcriptional activation of lipid metabolic genes and regulates the metabolic energy demands in Sertoli cells. Therefore, decreased expression of PPARγ in BPA-exposed testis could compromise energy utilization, affect germ cell differentiation, meiosis, spermatids elongation, and dysregulate normal spermatogenesis. In addition, decreased PPARγ expression could affect fertilization in BPA-exposed testis since increased PPARγ expression in ejaculated spermatozoa improves sperm physiology, capacitation, metabolism, and viability (Aquila et al. 2006).

The involvement of testicular cells with the available LCPUFAs depending on the expression of the PPARs (Echeverría et al. 2016). The skewed LCPUFA composition and altered expression of lipid metabolic genes in our study could be mediated by the BPA-activated PPARγ receptor in the testis (Gao et al. 2020). BPA could impact testicular fatty acid metabolism by dysregulating the expression of PPARs. Impaired spermatogenesis is associated with reduced expression of lipid metabolic genes regulated by PPARγ (Olia Bagheri et al. 2021). Prenatal BPA

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exposure downregulated the fatty acid metabolic genes expression such as FADS1, and SCD1 in this study, suggesting PPARγ mediated regulation of lipid metabolic genes in the offspring testis.

PPAR signalling affects sperm energy capacitation and motility and thus attracts targets for male infertility. The mechanism involving BPA-induced changes in testicular fatty acid composition and PPAR signalling with sperm energy metabolism is not understood yet. The reduced expression of PPARγ and PPARα could be due to the lower availability of ARA and DPA in the testis. Reduced expression of FADS1 in BPA-exposed rat testis might be mediated by decreased expression of PPARα in producing lower levels of LCPUFAs observed in our study.

Endothelial lipase or hormone-sensitive lipase (LIPE) and lipoprotein lipase (LPL) are expressed in normal testis but perform distinct biological roles. Increased LIPE expression could be responsible for increased testosterone production (Nielsen et al. 2010) in BPA-exposed testis, as we observed previously (Molangiri et al. 2022). The LIPE-knockout mice showed increased expression of FADS1, SCD-1 and decreased expression of ELOVL2 in the testis (Casado et al. 2013). However, in the present study, BPA-exposed testis exhibited opposite changes in the expression of FADS1, SCD-1, and ELOVL2, along with a decreased synthesis of n-6 LCPUFAs indicating compensatory involvement of LIPE in the mobilization of selected fatty acids to the testis (Raclot et al. 2001). ELOVL2 controls the level of n-6 LCPUFA (>25 carbon) in the testis, a prerequisite for male fertility and sperm maturation in mice testis (Zadravec et al. 2011). Increased ELOVL2 expression in BPA-exposed testis (**Fig.6B**) could compensate for reduced FADS1 expression and protect the complete arrest of spermatogenesis. ACSBG2 (acyl Co-A synthetase bubblegum family member 2) catalyzes the activation of long-chain fatty acids (LCFAs) to their acyl-CoA form for membrane synthesis of cellular lipids and beta-oxidation. As sperm formation requires LCFAs and VLCFAs for membrane biogenesis (Kihara 2012), decreased trend in ACSBG2 expression in our study (**Fig.2**) may impair fatty acyl CoA activation in spermatogenesis (Fraisl et al. 2006).

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Fatty acid-binding protein 9 (FABP9) is required in sperm acrosomal vesicle assembly and its formation. Its absence is associated with increased sperm head abnormalities (Selvaraj et al. 2010). In rodents, FABP9 (PERF15) is expressed during spermatogenesis in testicular cells. During spermatid development, FABP9 protein progressively accumulates on the acrosome membrane, facilitating rodent spermiogenesis (Korley et al. 1997). Downregulated expression of FABP9 transcript in the BPA-exposed testis (**Fig.2**) could affect spermatogenesis as FABP9 expression is critically required for germ cell maturation from spermatocytes to spermatids (Oresti et al. 2013). Moreover, decreased FABP9 expression could impair the structural integrity and stability of the acrosomal membrane in the BPA-exposed offspring testis (Oko and Morales 1994).

FABP4 regulates lipid trafficking and is involved in lipogenesis. BPA-activated PPARγ disturbs lipid metabolism by modulating the expression of FABP4 (Gao et al. 2020). Mutant mice (MM-1a) produced round-shaped spermatids but lacked elongated spermatids and exhibited upregulated FABP4 expression in the testis (Yamane et al. 2015). Thus, BPA-induced FABP4 expression in the testis could affect spermatid formation by modulating lipid metabolism. A decreased FABP3 (**Fig.1C**) could affect the differentiation of interstitial cells in the testis and delay the physiological maturity of germ cells (Oresti et al. 2013). The prenatal BPA exposure could have affected the differentiation of testicular cells due to decreased expression of FABP3 and PPARα (Zhuang et al. 2021)

Testicular cells produce neutral lipids, store them in lipid droplets (LD) and maintain lipid homeostasis by regulating the expression of LD proteins. LDs control the regulation of lipid storage and oxidation in Sertoli cells. ADRP (PLIN2), is expressed during testis development in rodents and helps maintain scrotal temperature for effective spermatogenesis. Increased ADRP (**Fig.1A**) could promote heat stress and temperature imbalance in BPA-exposed testis, suppressing spermatogenesis since ADRP expression is upregulated after scrotum heat treatment in mice (Liu et al. 2012). Sperm cation channel-like proteins (CATSPER) are

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exclusively expressed in the testis, where its expression remained maximal at the adult testis and thus plays important roles in sperm functions. Decreased CATSPER2 mRNA expression in BPA-exposed offspring testis (**Fig. 4**) could affect sperm functions since the level of CATSPER2 mRNA expression was significantly downregulated in varicocele-induced rats (Soleimani et al. 2018). Moreover, CATSPER2 transcript level was upregulated in the high-motile spermatozoa than in the low-motile fraction in humans (Li et al. 2007), indicating that in utero BPA exposure might have reduced sperm motility by lowering the expression of CATSPER2 in adult offspring testis.

The study is limited to the fact that PUFA composition in the testis was based on total fatty acid rather than phospholipid composition. Again, VLC-PUFAs (C ≥ 22) could not be measured in testis due to the unavailability of the standards. Despite changes in the SCD or delta-9-desaturase index, its protein expression could not be measured. While the earlier report suggests that BPA delivery in pregnancy altered fatty acid composition at a concentration of 0.5μg/kg bw/d (Dunder et al. 2018), however, BPA (0.4μg/kg bw/d) with a closely similar concentration in our study did not affect the fatty acid composition of the testis and plasma. The discrepancies could be due to the higher potency of BPA when dissolved in ethanol than olive oil and ad libitum intake of drinking water than controlled gavage delivery used in the present study. Despite these limitations, these data highlight that a disruption in the tightly regulated testicular metabolism due to in-utero BPA exposure affected lipogenesis, LCPUFA composition, and desaturase expression in the adult offspring testis, which may affect spermatogenesis associated with bioenergetics, sperm motility, and its function. Earlier, we reported that fetal BP exposure-induced metabolic changes resulted in ROS overproduction and oxidative stress in the testis (Molangiri et al. 2022). Excess ROS produced in the testis can damage sperm DNA, and sperm lipids and contribute to decreased sperm quality. The desaturase expression is unaffected in rat testis even during essential fatty acid deficiency (Saether et al. 2007), but in-utero BPA

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exposure results in a change of FADS1 expression in offspring testis, indicating its greater disruption than the insults from dietary deficiency.

In summary, LA level is increased relatively higher in BPA-exposed testis than in control rats. Still, their utilization was less in these testes, indicating reduced metabolism and increased lipogenesis as measured by increased SCD-1 and SCD-2, FABP4, and ADRP expression in the testis. Despite higher MUFAs in BPA-exposed testis, the endogenous conversion to ARA was reduced due to decreased desaturase activities in these testes. Decreased FADS1 and PPARγ expression and reduced ARA production in BPA-exposed testis suggest that FADS1 expression is regulated by dysregulated expression of transcription factors, PPARγ, and desaturation of fatty acids. Compared to control testis, BPA-exposed rats exhibited a dispersed FADS1 signal around ST cells, reduced PPARγ expression, decreased n-6 LCPUFA, and FADS1 expression levels. The desaturation /elongation rate of PUFAs is differed by species, sex, endocrine status, and diet but is not reported due to BPA exposure in utero. This first report suggests that, in utero, BPA exposure can affect FADS1 expression in the offspring of the mature testis. The Sertoli cells were possibly affected more than germ cells of the testis because Sertoli cells are more active in LCPUFA metabolism, richer in polyunsaturated fatty acids (PUFAs), and correlate a higher expression of Δ5- (FADS1) and Δ6-desaturase (FADS2). The reduced ARA could have produced fewer metabolites supporting steroidogenesis in BPA-exposed testis.

5. Conclusions

In-utero BPA exposure could disrupt the fetus's gonadal programming via dysregulated fatty acid metabolism in the offspring testis. This supports the notion that disrupted testicular development in fetuses could arise due to adverse environmental exposure. Prenatal BPA exposure decreased n-6 LCPUFAs in the testis by modulating the endogenous metabolism of these fatty acids aided with concomitant reduction of expression of FADS1, which may affect sperm maturity and quality. Thus, fetal BPA exposure reduces testicular long-chain fatty acid availability in adult testis, which may ultimately affect spermatogenesis and pose a risk for male

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infertility. The development disturbance of the gonads due to prenatal BPA exposure could be linked with fertility impairment due to aberrant changes in the LCPUFA composition of the offspring testis. Thus, lifestyle choices and ubiquitous chemical exposure may affect the reproductive health of adults due to adverse effects on fetal development.

Male fertility is declining globally. In addition to depleted LCPUFAs in the diet, daily exposure to endocrine-disrupting substances could negatively affect spermatogenesis at metabolic and gonadal levels. Testicular dysgenesis syndrome causally originates from adverse environmental exposure, which may disrupt fetal gonadal development and programming due to BPA exposure. Furthermore, the interplay of bisphenol exposure and endogenous lipid metabolism of the testis could dysregulate functions of male reproductive systems. However, further studies are required to examine the endocrine-metabolic cross-talk to the early life exposure of this most abundant plasticizer on the male reproductive function in humans.

CRediT authorship contribution statement

Saikanth Varma : Investigation, Experiment, Methodology, Data curation. Archana Molangiri : Investigation, Experiment. Suryam Reddy Kona : Experiment. Ahamed Ibrahim : Comments, Writing- review & editing; Asim K Duttaroy : Critical comments, Writing-review & editing. Sanjay Basak : Conceptualization, Project administration, Supervision, Drafting, Writing-review & editing, Interpretation, Funding acquisition.

Declaration of Competing Interest

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The fund sponsored the work (No.R.12020/02/2018-HR) received from the Department of Health Research, Ministry of Health and Family Welfare, Government of India. Archana Molangiri received a NIN-SRF fellowship, and Saikanth Varma received an ICMR-JRF fellowship from Govt. of India. We are grateful to Srinivas for his assistance in tissue sectioning for histology.

Data availbality

Data will be made available on request.

Figure Legends

Fig. 1. Expression of fatty acid storage and trafficking proteins in the adult offspring testis exposed to BPA in utero. Immunoblots of (A) adipose differentiation-related protein (ADRP), (B) fatty acid binding protein 4 (FABP4), and (C) fatty acid binding protein 3 (FABP3) are derived from the testis homogenate of 90d rat. Blots show the expression of these proteins. Respective quantitative bars indicate changes in protein levels expressed as the relative band

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density after normalized with β actin protein expression. Data represent mean ± SEM of three independent experiments (n=4 rats/group). Individual p-values are indexed in figures. * p< 0.05, ** p< 0.005 vs. control (Student's t-test).

Fig. 2. The mRNA expression of genes regulates lipogenesis, growth and signalling molecules of steroidogenesis in adult rat testis whose mothers were exposed to BPA during pregnancy. The expression of hormone-sensitive lipase (LIPE), lipoprotein lipase (LPL), fatty acid binding protein 9 (FABP9), cyclooxygenase-2 (COX2), acyl-CoA synthetase bubblegum family member 2 (ACSBG2) levels were measured in the offspring (90d) rat testis using RT-qPCR as described in the method. Values are represented as means ± SEM (n=5-6 rats/group). Individual p-values are indexed in figures. *p< 0.05 vs. control (Student's t-test).

Fig. 3. Expression of fatty acid-regulated transcription factors in the adult rat testis exposed to BPA in utero. Immunoblots of (A) peroxisome proliferator-activated receptor gamma (PPARγ) and (B) peroxisome proliferator-activated receptor alpha (PPARα) is performed with the testis homogenate of 90d rats. Blots show the expression of these proteins and respective quantitative changes in protein levels expressed as the relative band density after normalized with β actin protein expression. Data represent mean ± SEM of three independent experiments (n=3 rats/group). Individual p-values are indexed in figures. *** p< 0.0005 vs. control (Student's t-test).

Fig.4. The mRNA expression of genes involved in sperm motility. CATSPER1 and CATSPER2 were measured in rat testis tissue using RT-qPCR after normalizing their expression with the expression of endogenous control β-actin. Values are represented as means ± SEM (n=4). Values are expressed as means ± SEM (n=5-6 rats/group). Individual p-values are indexed in figures. *p< 0.05 vs. control (Student's t-test).

Fig.5. The mRNA expression of genes involved in the long chain-PUFA synthesis (desaturation and elongation) was measured in the testis of 3-month-old rats exposed to BPA in utero. The

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expression of fatty acid desaturase 1 (FADS1 or delta-5 desaturase), fatty acid desaturase 2 (FADS2 or delta-6 desaturase), delta 4 desaturase sphingolipid 1 (DEGS1), elongation of very long chain fatty acids-like 2 (ELOVL2), elongation of very long chain fatty acids-like 5 (ELOVL5), stearoyl-coenzyme A desaturase 2 (SCD2), and stearoyl-coenzyme A desaturase 1 (SCD1) levels were measured using RT-qPCR as described in the method. Values are represented as means ± SEM (n=5-6 rats/group). Individual p-values are indexed in figures. *p< 0.05 vs. control (Student's t-test).

Fig. 6. Expression of fatty acid desaturase and elongase in the offspring (90d) testis exposed to BPA in utero. Immunoblots of (A) fatty acid desaturase 1 (FADS1) and (B) elongation of very long chain fatty acids-like 2 (ELOVL2) were performed in testis homogenate of 90d rats. Blots show the expression of these proteins and respective quantitatively expressed normalized proteins. Data represent mean ± SEM of three independent experiments (n=3 rats/group). Individual p-values are indexed in figures. *** p< 0.0005 vs. control (Student's t-test).

Fig. 7. Localization and expression of FADS1 protein by immunofluorescence in the testis of adult Wistar rats that were gestationally exposed to BPA (4.0 μg/kg bw/d). Testis was fixed in bouin's solution, dehydrated, and paraffinized. Tissue sections were rehydrated and stained with rabbit anti-FADS1 primary antibody followed by Alexa fluor 488 goat anti-rabbit secondary antibody. (A) Immunofluorescence staining of testis tissue sections was performed with Alexa Fluor 488 tagged-FADS1 antibody (green) and nuclei with DAPI (blue). Images were captured with a confocal laser scanning microscope (Leica microsystems, Germany) at 63 X magnification using an oil immersion objective. (B) The total fluorescence of the images was quantified using ImageJ software (NIH, USA). Corrected total cell fluorescence (CTCF) was calculated after measuring the fluorescence [CTCF= integrated density – (area of selected cell x mean fluorescence of background readings)] and expressed in arbitrary units. Data are expressed as

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means ± SEM (n=3). Statistical significance was considered when the p-value < 0.05 vs. control (Student's t-test).

References

Adegoke, E.O.; Rahman, M.S.; Pang, M.G. Bisphenols Threaten Male Reproductive Health via Testicular Cells. Front Endocrinol (Lausanne) 2020;11:624

Aksoy, Y.; Aksoy, H.; Altinkaynak, K.; Aydin, H.R.; Ozkan, A. Sperm fatty acid composition in subfertile men. Prostaglandins Leukot Essent Fatty Acids 2006;75:75-79

Andersen, J.M.; Rønning, P.O.; Herning, H.; Bekken, S.D.; Haugen, T.B.; Witczak, O. Fatty acid composition of spermatozoa is associated with BMI and with semen quality. Andrology 2016;4:857-865

Aquila, S.; Bonofiglio, D.; Gentile, M.; Middea, E.; Gabriele, S.; Belmonte, M.; Catalano, S.; Pellegrino, M.; Andò, S. Peroxisome proliferator-activated receptor (PPAR)gamma is expressed by human spermatozoa: its potential role on the sperm physiology. J Cell Physiol 2006;209:977-986

Basak, S.; Srinivas, V.; Duttaroy, A.K. Bisphenol-A impairs cellular function and alters DNA methylation of stress pathway genes in first trimester trophoblast cells. Reproductive toxicology 2018;82:72-79

Beckman, J.K.; Gray, M.E.; Coniglio, J.G. The lipid composition of isolated rat spermatids and spermatocytes. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 1978;530:367-374

Casado, M.E.; Pastor, O.; Mariscal, P.; Canfrán-Duque, A.; Martínez-Botas, J.; Kraemer, F.B.; Lasunción, M.A.; Martín-Hidalgo, A.; Busto, R. Hormone-sensitive lipase deficiency disturbs the fatty acid composition of mouse testis. Prostaglandins Leukot Essent Fatty Acids 2013;88:227-233

Chianese, R.; Viggiano, A.; Urbanek, K.; Cappetta, D.; Troisi, J.; Scafuro, M.; Guida, M.; Esposito, G.; Ciuffreda, L.P.; Rossi, F.; Berrino, L.; Fasano, S.; Pierantoni, R.; De Angelis, A.; Meccariello, R. Chronic exposure to low dose of bisphenol A impacts on the first round of spermatogenesis via SIRT1 modulation. Scientific reports 2018;8:2961

Collodel, G.; Castellini, C.; Lee, J.C.; Signorini, C. Relevance of Fatty Acids to Sperm Maturation and Quality. Oxidative medicine and cellular longevity 2020;2020:7038124

Craig, L.B.; Brush, R.S.; Sullivan, M.T.; Zavy, M.T.; Agbaga, M.P.; Anderson, R.E. Decreased very long chain polyunsaturated fatty acids in sperm correlates with sperm quantity and quality. J Assist Reprod Genet 2019;36:1379-1385

Cui, Y.; Miyoshi, K.; Claudio, E.; Siebenlist, U.K.; Gonzalez, F.J.; Flaws, J.; Wagner, K.U.; Hennighausen, L. Loss of the peroxisome proliferation-activated receptor gamma (PPARgamma) does not affect mammary development and propensity for tumor formation but leads to reduced fertility. J Biol Chem 2002;277:17830-17835

Davis, J.T.; Bridges, R.B.; Coniglio, J.G. Changes in lipid composition of the maturing rat testis. The Biochemical journal 1966;98:342-346

Dunder, L.; Halin Lejonklou, M.; Lind, L.; Risérus, U.; Lind, P.M. Low-dose developmental bisphenol A exposure alters fatty acid metabolism in Fischer 344 rat offspring. Environ Res 2018;166:117-129

Echeverría, F.; Ortiz, M.; Valenzuela, R.; Videla, L.A. Long-chain polyunsaturated fatty acids regulation of PPARs, signaling: Relationship to tissue development and aging. Prostaglandins, Leukotrienes and Essential Fatty Acids 2016;114:28-34

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Fraisl, P.; Tanaka, H.; Forss-Petter, S.; Lassmann, H.; Nishimune, Y.; Berger, J. A novel mammalian bubblegum-related acyl-CoA synthetase restricted to testes and possibly involved in spermatogenesis. Archives of biochemistry and biophysics 2006;451:23-33

Frungieri, M.B.; Calandra, R.S.; Mayerhofer, A.; Matzkin, M.E. Cyclooxygenase and prostaglandins in somatic cell populations of the testis. Reproduction 2015;149:R169-180

Gao, P.; Wang, L.; Yang, N.; Wen, J.; Zhao, M.; Su, G.; Zhang, J.; Weng, D. Peroxisome proliferator-activated receptor gamma (PPARγ) activation and metabolism disturbance induced by bisphenol A and its replacement analog bisphenol S using in vitro macrophages and in vivo mouse models. Environment international 2020;134:105328

García-Arevalo, M.; Alonso-Magdalena, P.; Rebelo Dos Santos, J.; Quesada, I.; Carneiro, E.M.; Nadal, A. Exposure to bisphenol-A during pregnancy partially mimics the effects of a high-fat diet altering glucose homeostasis and gene expression in adult male mice. PLoS One 2014;9:e100214

Gautier, C.; Scarlet, D.; Ertl, R.; Walter, I.; Wulf, M.; Nagel, C.; Aurich, J.; Aurich, C. Expression of enzymes involved in polyunsaturated fatty acid synthesis in the stallion testis and epididymis. Reprod Fertil Dev 2020;32:851-861

Kihara, A. Very long-chain fatty acids: elongation, physiology and related disorders. The Journal of Biochemistry 2012;152:387-395

Korley, R.; Pouresmaeili, F.; Oko, R. Analysis of the protein composition of the mouse sperm perinuclear theca and characterization of its major protein constituent. Biol Reprod 1997;57:1426-1432

Labaronne, E.; Pinteur, C.; Vega, N.; Pesenti, S.; Julien, B.; Meugnier-Fouilloux, E.; Vidal, H.; Naville, D.; Le Magueresse-Battistoni, B. Low-dose pollutant mixture triggers metabolic disturbances in female mice leading to common and specific features as compared to a high-fat diet. J Nutr Biochem 2017;45:83-93

Lee, J.; Choi, K.; Park, J.; Moon, H.-B.; Choi, G.; Lee, J.J.; Suh, E.; Kim, H.-J.; Eun, S.-H.; Kim, G.-H.; Cho, G.J.; Kim, S.K.; Kim, S.; Kim, S.Y.; Kim, S.; Eom, S.; Choi, S.; Kim, Y.D.; Kim, S. Bisphenol A distribution in serum, urine, placenta, breast milk, and umbilical cord serum in a birth panel of mother–neonate pairs. Science of The Total Environment 2018;626:1494-1501

Lejonklou, M.H.; Dunder, L.; Bladin, E.; Pettersson, V.; Ronn, M.; Lind, L.; Walden, T.B.; Lind, P.M. Effects of Low-Dose Developmental Bisphenol A Exposure on Metabolic Parameters and Gene Expression in Male and Female Fischer 344 Rat Offspring. Environ Health Perspect 2017;125:067018

Li, H.G.; Ding, X.F.; Liao, A.H.; Kong, X.B.; Xiong, C.L. Expression of CatSper family transcripts in the mouse testis during post-natal development and human ejaculated spermatozoa: relationship to sperm motility. Mol Hum Reprod 2007;13:299-306

Liu, L.L.; Xian, H.; Cao, J.C.; Zhang, C.; Zhang, Y.H.; Chen, M.M.; Qian, Y.; Jiang, M. Peroxisome proliferator-activated receptor gamma signaling in human sperm physiology. Asian journal of andrology 2015;17:942-947

Liu, M.; Qi, L.; Zeng, Y.; Yang, Y.; Bi, Y.; Shi, X.; Zhu, H.; Zhou, Z.; Sha, J. Transient scrotal hyperthermia induces lipid droplet accumulation and reveals a different ADFP expression pattern between the testes and liver in mice. PLoS One 2012;7:e45694

Luo, D.; Zhang, M.; Su, X.; Liu, L.; Zhou, X.; Zhang, X.; Zheng, D.; Yu, C.; Guan, Q. High fat diet impairs spermatogenesis by regulating glucose and lipid metabolism in Sertoli cells. Life sciences 2020;257:118028

Matsui, H.; Yokoyama, T.; Sekiguchi, K.; Iijima, D.; Sunaga, H.; Maniwa, M.; Ueno, M.; Iso, T.; Arai, M.; Kurabayashi, M. Stearoyl-CoA Desaturase-1 (SCD1) Augments Saturated Fatty Acid-Induced Lipid Accumulation and Inhibits Apoptosis in Cardiac Myocytes. PLOS ONE 2012;7:e33283

bioRxiv preprint doi: https://doi.org/10.1101/2023.01.18.524658; this version posted January 20, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Matzkin, M.E.; Mayerhofer, A.; Rossi, S.P.; Gonzalez, B.; Gonzalez, C.R.; Gonzalez-Calvar, S.I.; Terradas, C.; Ponzio, R.; Puigdomenech, E.; Levalle, O.; Calandra, R.S.; Frungieri, M.B. Cyclooxygenase-2 in testes of infertile men: evidence for the induction of prostaglandin synthesis by interleukin-1β. Fertil Steril 2010;94:1933-1936

Molangiri, A.; Varma, S.; Satyavani, M.; Kambham, S.; Duttaroy, A.K.; Basak, S. Prenatal exposure to bisphenol S and bisphenol A differentially affects male reproductive system in the adult offspring. Food and Chemical Toxicology 2022;167:113292

Nielsen, J.E.; Lindegaard, M.L.; Friis-Hansen, L.; Almstrup, K.; Leffers, H.; Nielsen, L.B.; Rajpert-De Meyts, E. Lipoprotein lipase and endothelial lipase in human testis and in germ cell neoplasms. International journal of andrology 2010;33:e207-215

Oko, R.; Morales, C.R. A novel testicular protein, with sequence similarities to a family of lipid binding proteins, is a major component of the rat sperm perinuclear theca. Dev Biol 1994;166:235-245

Olia Bagheri, F.; Alizadeh, A.; Sadighi Gilani, M.A.; Shahhoseini, M. Role of peroxisome proliferator-activated receptor gamma (PPARγ) in the regulation of fatty acid metabolism related gene expressions in testis of men with impaired spermatogenesis. Reproductive Biology 2021;21:100543

Oresti, G.M.; García-López, J.; Aveldaño, M.I.; Del Mazo, J. Cell-type-specific regulation of genes involved in testicular lipid metabolism: fatty acid-binding proteins, diacylglycerol acyltransferases, and perilipin 2. Reproduction 2013;146:471-480

Paillamanque, J.; Sanchez-Tusie, A.; Carmona, E.M.; Treviño, C.L.; Sandoval, C.; Nualart, F.; Osses, N.; Reyes, J.G. Arachidonic acid triggers [Ca2+]i increases in rat round spermatids by a likely GPR activation, ERK signalling and ER/acidic compartments Ca2+ release. PLoS One 2017;12:e0172128

Paillard, F.; Catheline, D.; Duff, F.L.; Bouriel, M.; Deugnier, Y.; Pouchard, M.; Daubert, J.C.; Legrand, P. Plasma palmitoleic acid, a product of stearoyl-coA desaturase activity, is an independent marker of triglyceridemia and abdominal adiposity. Nutrition, metabolism, and cardiovascular diseases : NMCD 2008;18:436-440

Raclot, T.; Holm, C.; Langin, D. Fatty acid specificity of hormone-sensitive lipase. Implication in the selective hydrolysis of triacylglycerols. J Lipid Res 2001;42:2049-2057

Radwan, M.; Wielgomas, B.; Dziewirska, E.; Radwan, P.; Kałużny, P.; Klimowska, A.; Hanke, W.; Jurewicz, J. Urinary Bisphenol A Levels and Male Fertility. American journal of men's health 2018;12:2144-2151

Retterstøl, K.; Haugen, T.B.; Woldseth, B.; Christophersen, B.O. A comparative study of the metabolism of n-9, n-6 and n-3 fatty acids in testicular cells from immature rat. Biochim Biophys Acta 1998;1392:59-72

Retterstøl, K.; Tran, T.N.; Haugen, T.B.; Christophersen, B.O. Metabolism of very long chain polyunsaturated fatty acids in isolated rat germ cells. Lipids 2001;36:601-606

Saether, T.; Tran, T.N.; Rootwelt, H.; Christophersen, B.O.; Haugen, T.B. Expression and regulation of delta5-desaturase, delta6-desaturase, stearoyl-coenzyme A (CoA) desaturase 1, and stearoyl-CoA desaturase 2 in rat testis. Biol Reprod 2003;69:117-124

Saether, T.; Tran, T.N.; Rootwelt, H.; Grav, H.J.; Christophersen, B.O.; Haugen, T.B. Essential fatty acid deficiency induces fatty acid desaturase expression in rat epididymis, but not in testis. Reproduction 2007;133:467-477

Selvaraj, V.; Asano, A.; Page, J.L.; Nelson, J.L.; Kothapalli, K.S.D.; Foster, J.A.; Brenna, J.T.; Weiss, R.S.; Travis, A.J. Mice lacking FABP9/PERF15 develop sperm head abnormalities but are fertile. Dev Biol 2010;348:177-189

Shishikura, K.; Kuroha, S.; Matsueda, S.; Iseki, H.; Matsui, T.; Inoue, A.; Arita, M. Acyl-CoA synthetase 6 regulates long-chain polyunsaturated fatty acid composition of membrane phospholipids in spermatids and supports normal spermatogenic processes in mice. The FASEB Journal 2019;33:14194-14203

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Skakkebaek, N.E.; Rajpert-De Meyts, E.; Buck Louis, G.M.; Toppari, J.; Andersson, A.M.; Eisenberg, M.L.; Jensen, T.K.; Jørgensen, N.; Swan, S.H.; Sapra, K.J.; Ziebe, S.; Priskorn, L.; Juul, A. Male Reproductive Disorders and Fertility Trends: Influences of Environment and Genetic Susceptibility. Physiological reviews 2016;96:55-97

Soleimani, M.Z.; Jalali Mashayekhi, F.; Mousavi Hasanzade, M.; Baazm, M. Alteration in CatSper1 and 2 genes expression, sperm parameters and testis histology in varicocelized rats. Int J Reprod Biomed 2018;16:183-190

Srinivas, V.; Molangiri, A.; Mallepogu, A.; Kona, S.R.; Ibrahim, A.; Duttaroy, A.K.; Basak, S. Maternal n-3 PUFA deficiency alters uterine artery remodeling and placental epigenome in the mice. J Nutr Biochem 2021;96:108784-108796

Thomas, K.; Sung, D.Y.; Chen, X.; Thompson, W.; Chen, Y.E.; McCarrey, J.; Walker, W.; Griswold, M. Developmental patterns of PPAR and RXR gene expression during spermatogenesis. Frontiers in bioscience (Elite edition) 2011;3:1209-1220

Tran-Guzman, A.; Culty, M. Eicosanoid Biosynthesis in Male Reproductive Development: Effects of Perinatal Exposure to NSAIDs and Analgesic Drugs. Frontiers in toxicology 2022;4:842565

Warensjö, E.; Ohrvall, M.; Vessby, B. Fatty acid composition and estimated desaturase activities are associated with obesity and lifestyle variables in men and women. Nutrition, metabolism, and cardiovascular diseases : NMCD 2006;16:128-136

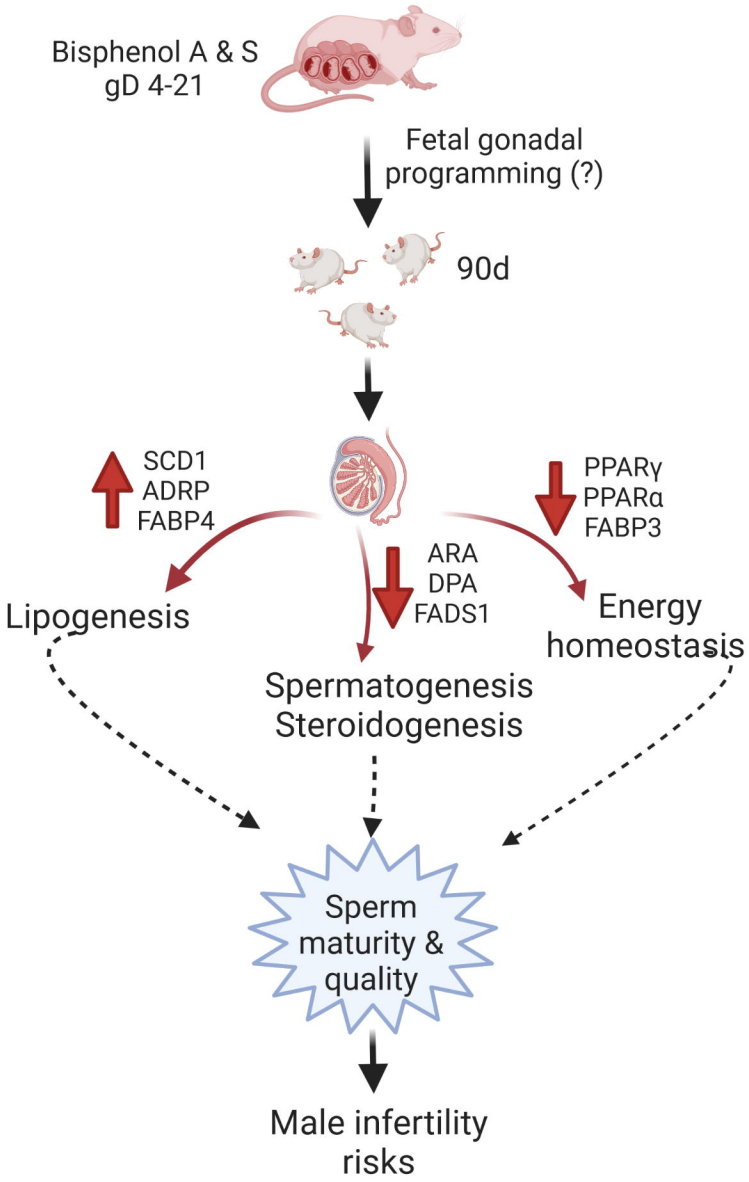
Wathes, D.C.; Abayasekara, D.R.; Aitken, R.J. Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 2007;77:190-201

Winnall, W.R.; Ali, U.; O'Bryan, M.K.; Hirst, J.J.; Whiley, P.A.F.; Muir, J.A.; Hedger, M.P. Constitutive Expression of Prostaglandin-Endoperoxide Synthase 2 by Somatic and Spermatogenic Cells Is Responsible for Prostaglandin E2 Production in the Adult Rat Testis1. Biology of Reproduction 2007;76:759-768

Yamane, T.; Shimizu, T.; Takahashi-Niki, K.; Takekoshi, Y.; Iguchi-Ariga, S.M.M.; Ariga, H. Deficiency of spermatogenesis and reduced expression of spermatogenesis-related genes in prefoldin 5-mutant mice. Biochemistry and Biophysics Reports 2015;1:52-61

Zadravec, D.; Tvrdik, P.; Guillou, H.; Haslam, R.; Kobayashi, T.; Napier, J.A.; Capecchi, M.R.; Jacobsson, A. ELOVL2 controls the level of n-6 28:5 and 30:5 fatty acids in testis, a prerequisite for male fertility and sperm maturation in mice. J Lipid Res 2011;52:245-255

Zhuang, L.; Mao, Y.; Liu, Z.; Li, C.; Jin, Q.; Lu, L.; Tao, R.; Yan, X.; Chen, K. FABP3 Deficiency Exacerbates Metabolic Derangement in Cardiac Hypertrophy and Heart Failure via PPARα Pathway. Frontiers in Cardiovascular Medicine 2021;8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Spermatogenesis
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Male infertility
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Fig.1

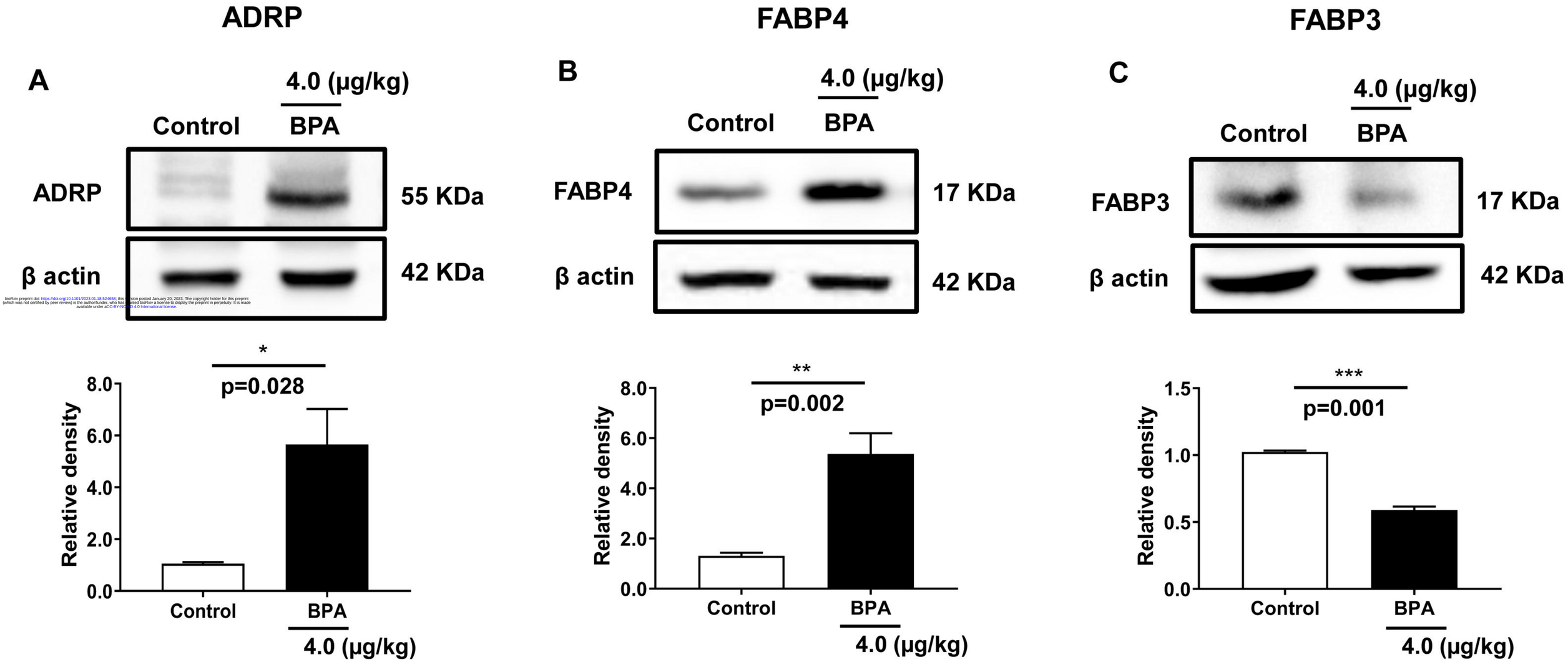

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Fig.2

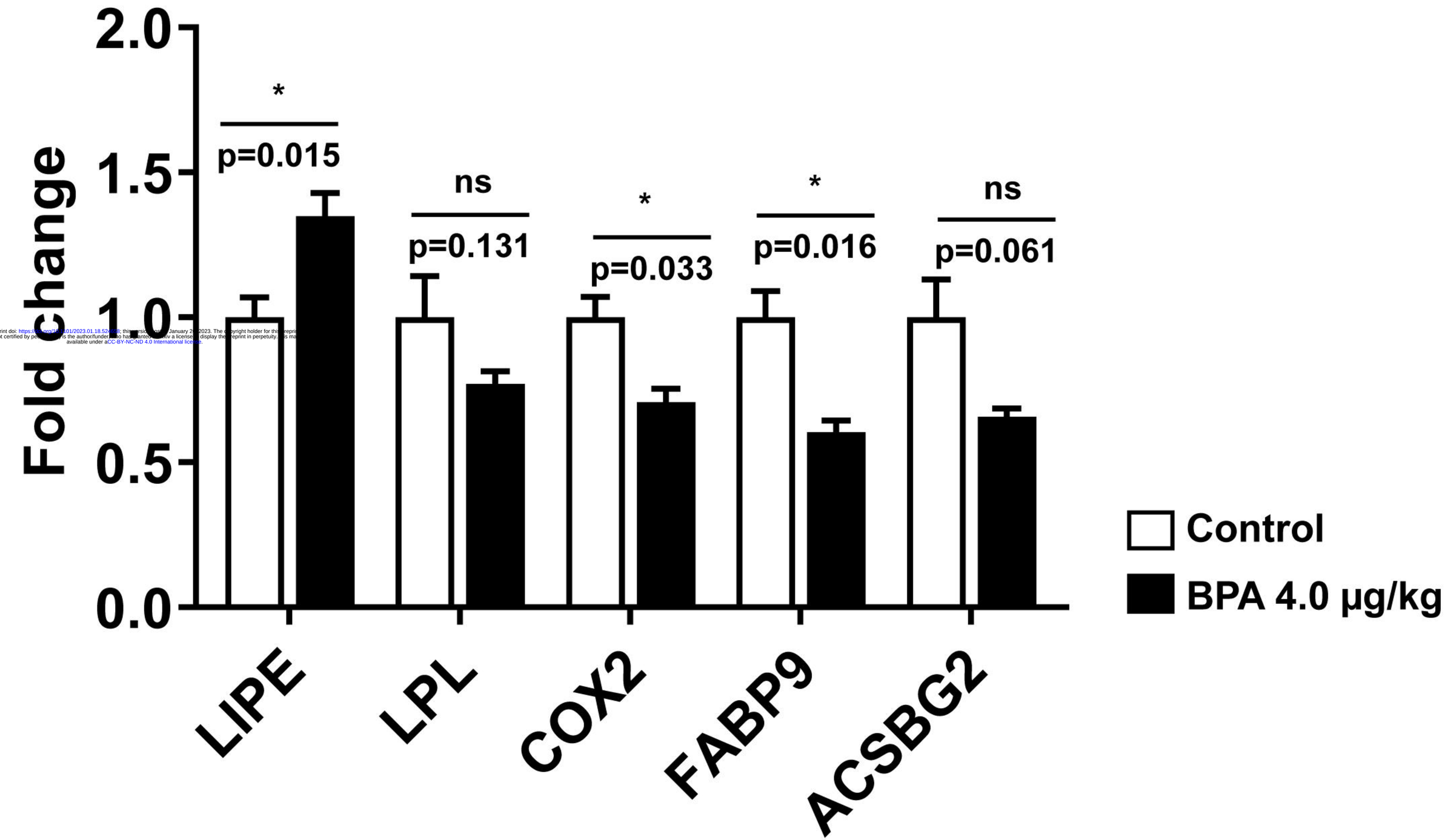

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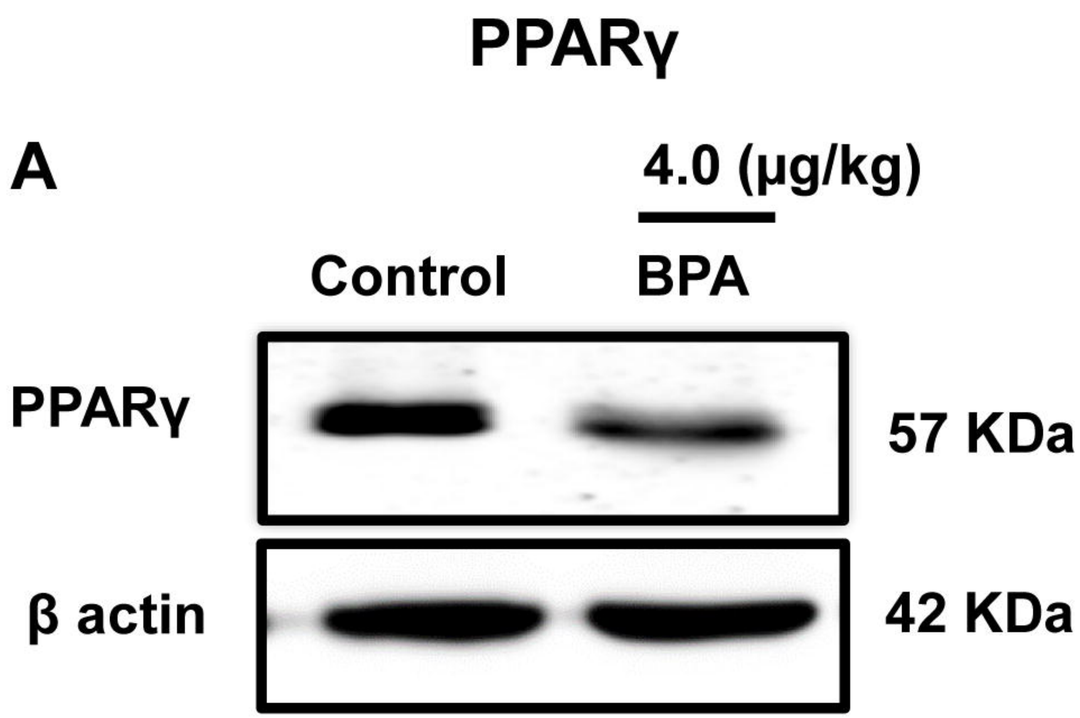
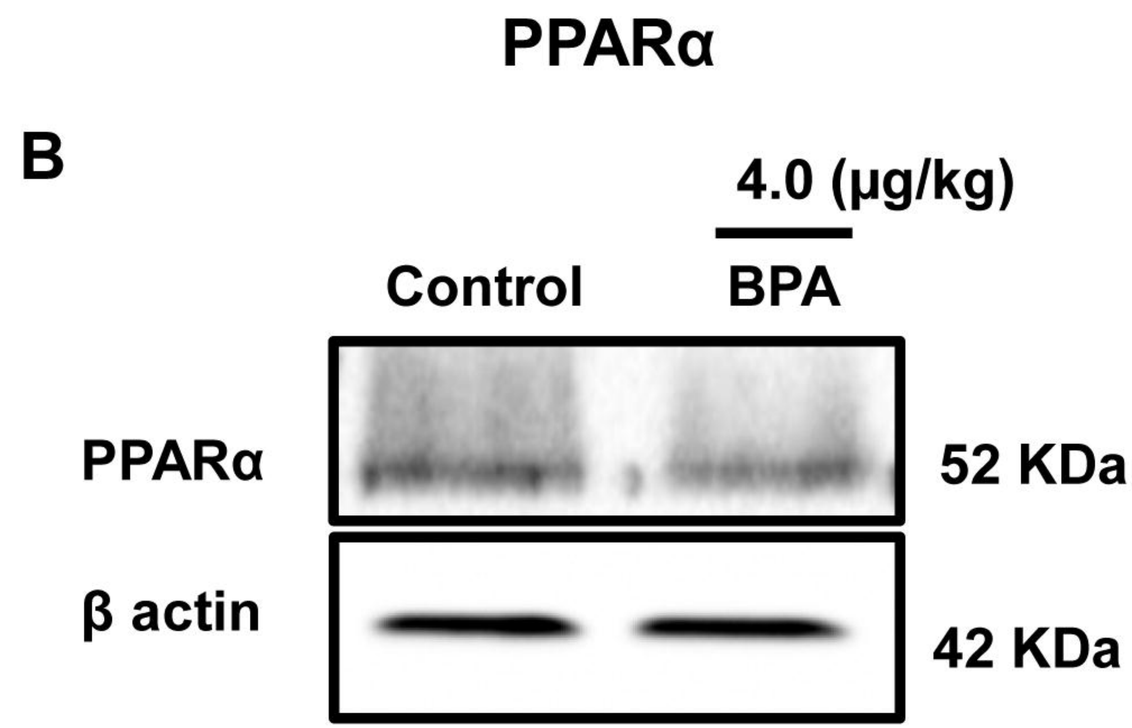
Fig.3





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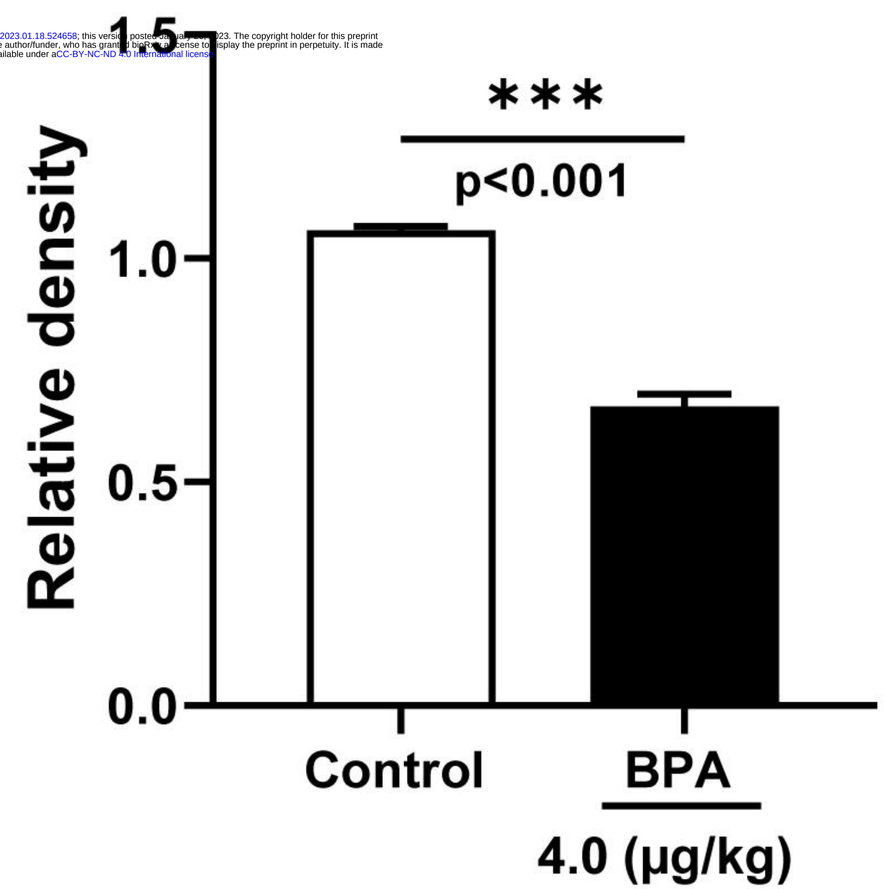

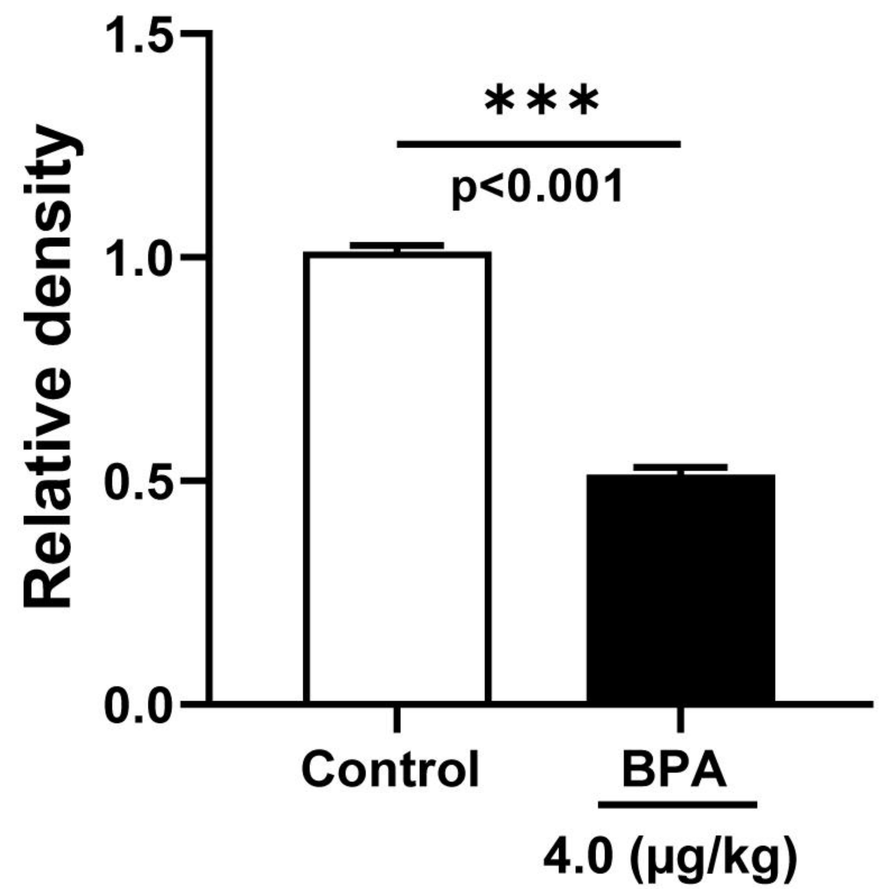

Fig.4

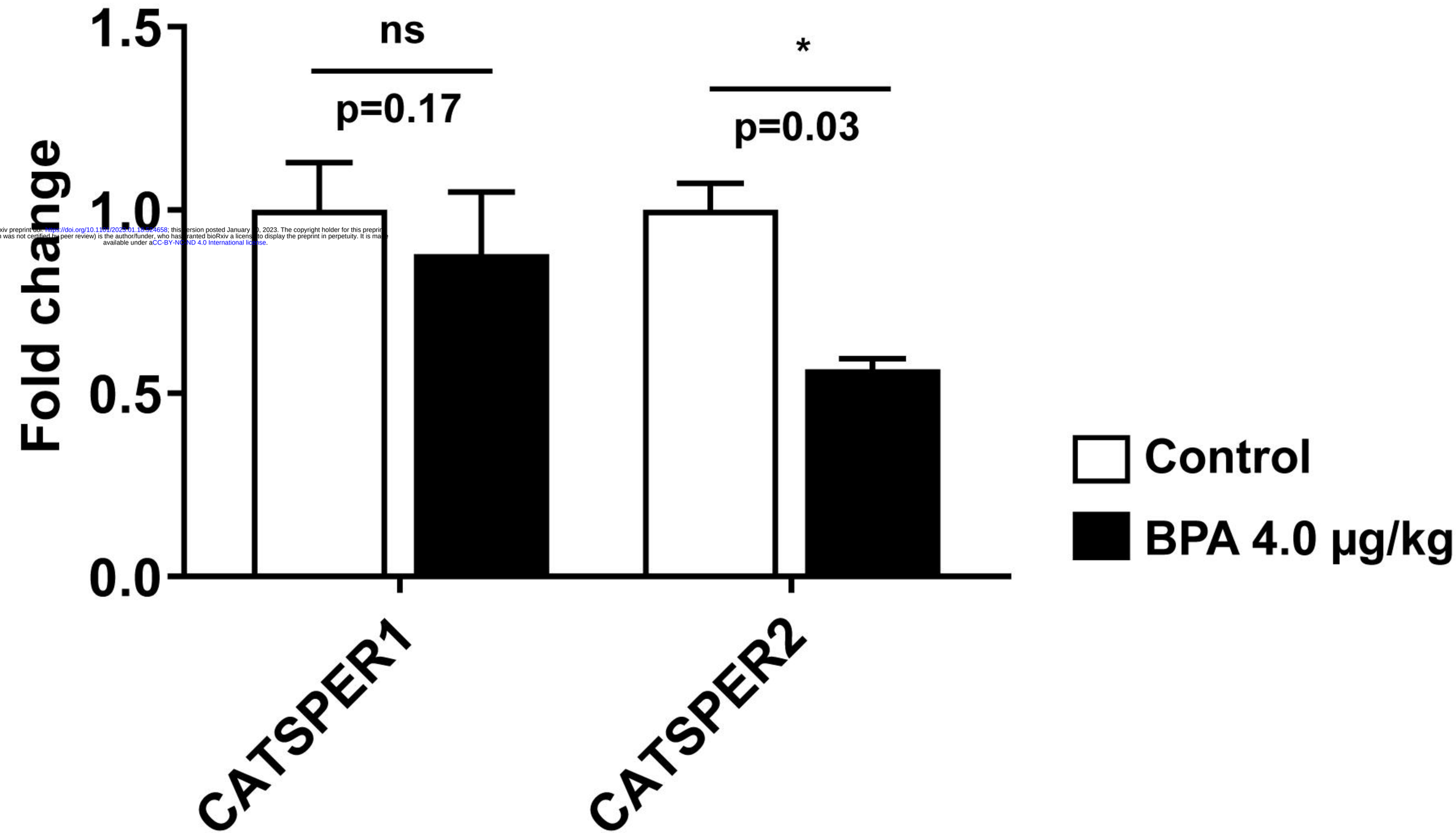

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Fig.5

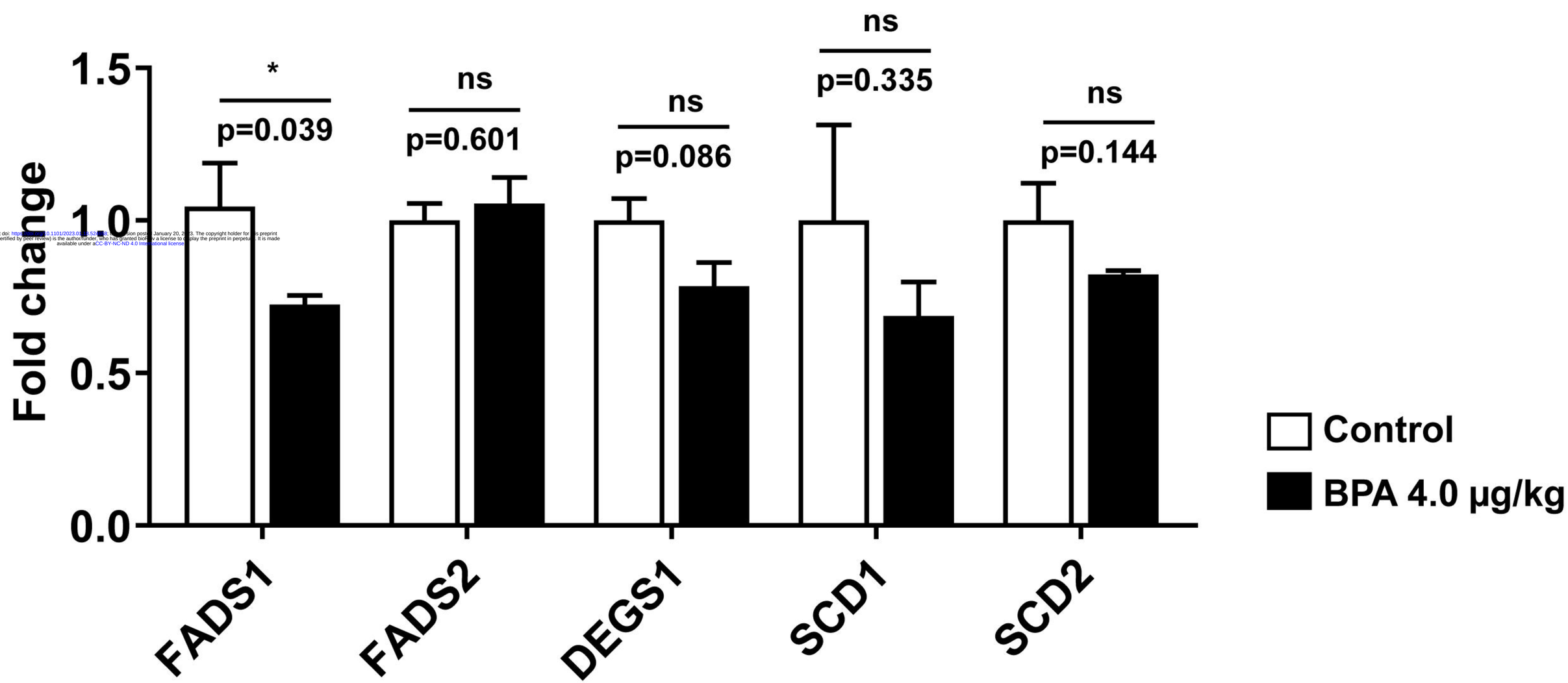

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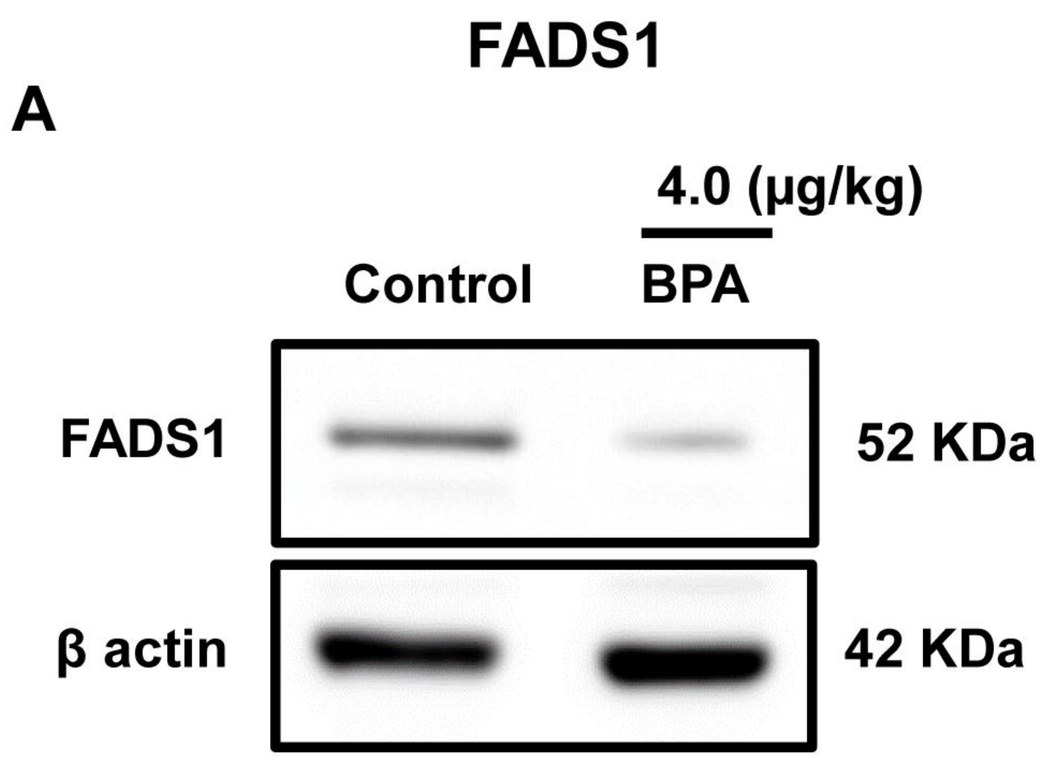
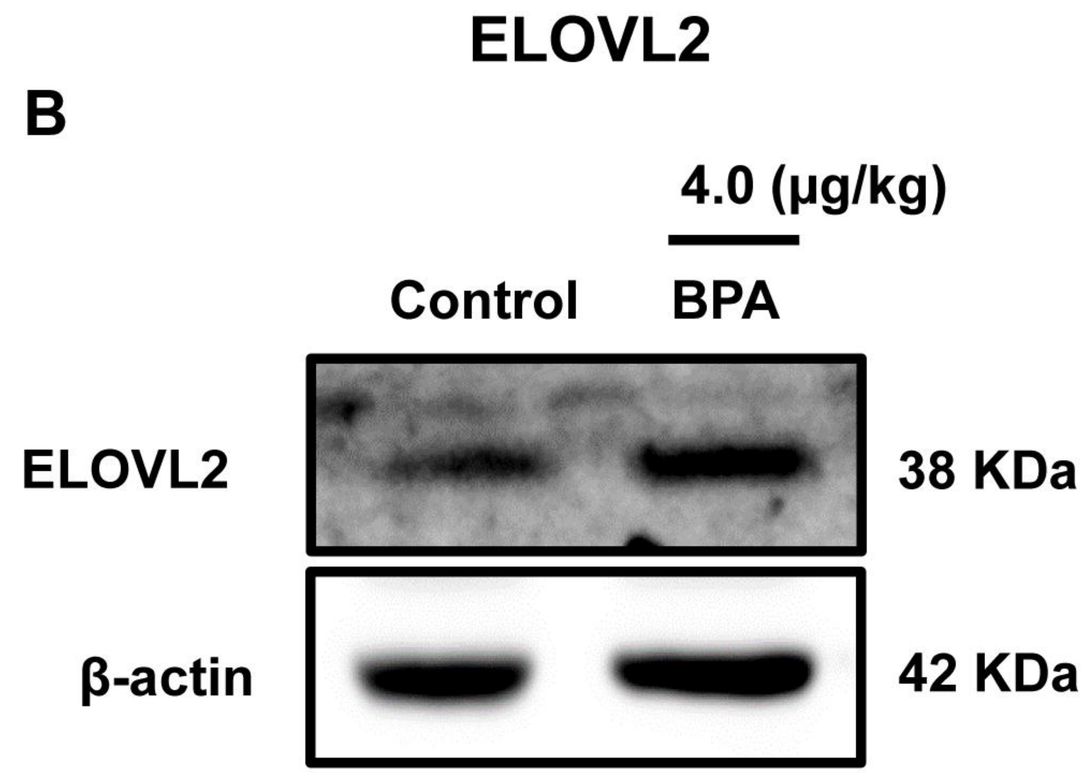
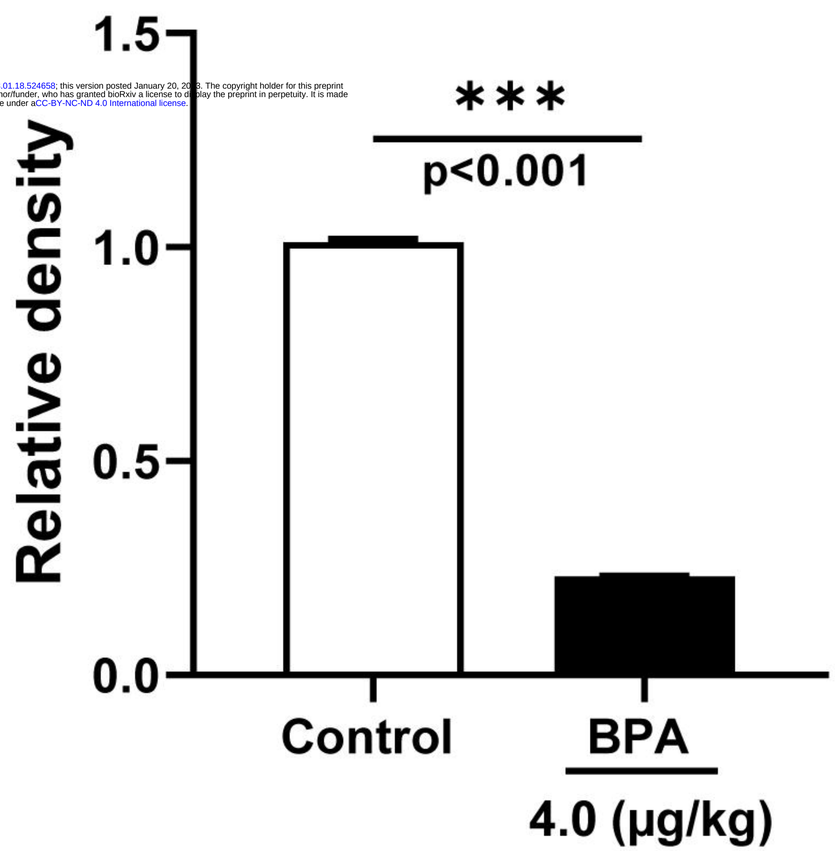
Fig.6







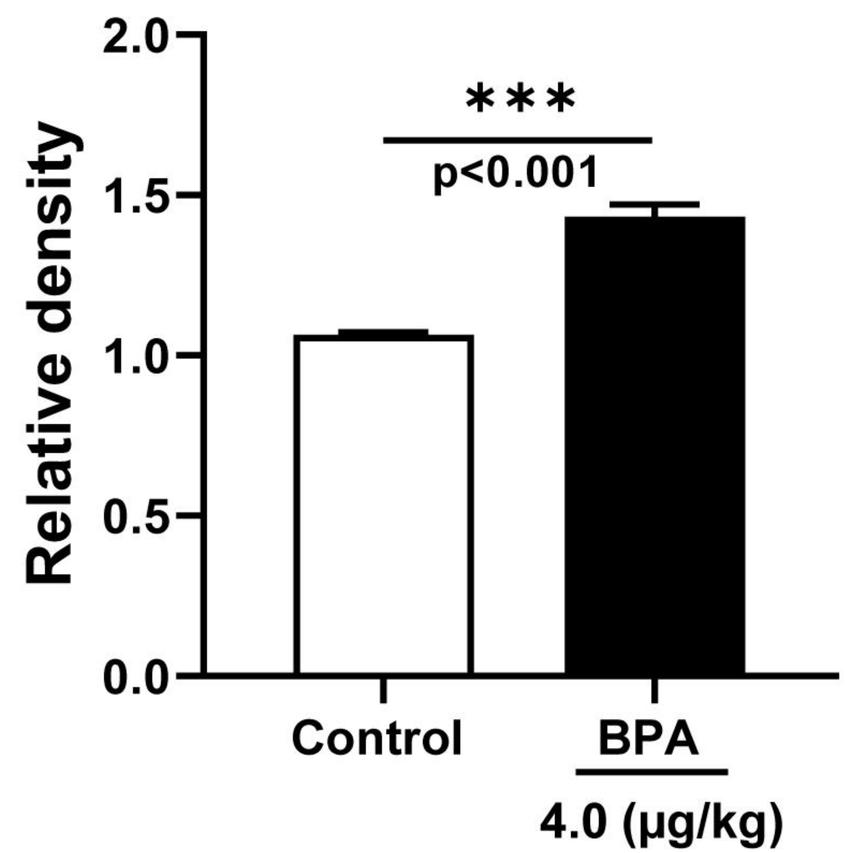


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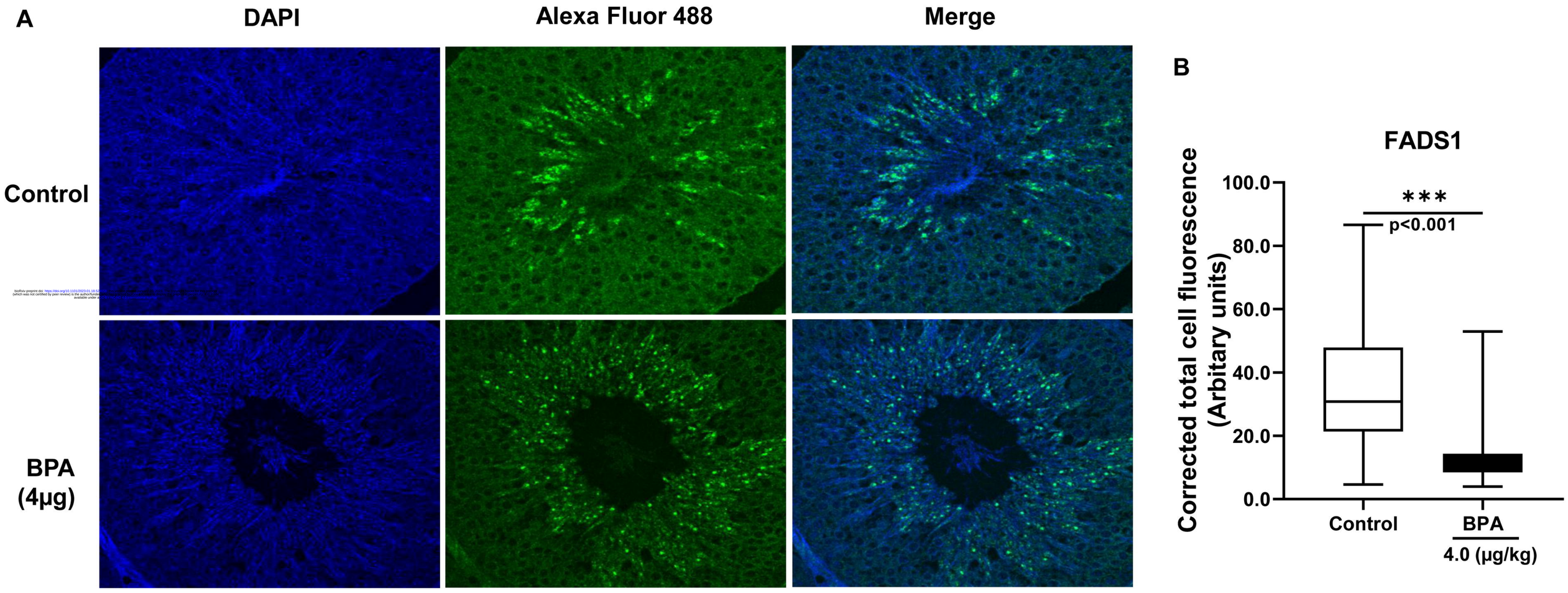
Fig.7


A
DAPI
Alexa Fluor 488
Merge
Control
BPA
(4μg)
B
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Corrected total cell fluorescence
(Arbitary units)
100.0
80.0
60.0
40.0
20.0
0.0
***
p<0.001
Control
BPA
4.0 (μg/kg)

Table. 1. Plasma total fatty acid composition of the adult offspring (90d) born to dam exposed to bisphenols during gestation (gD4 to gD21) [#]

	Experimental groups (µg/kg bw /d)						
Fatty acids (nmol %)	**Control (0.0)**	**BPA (0.4)**	**BPS (0.4)**	**BPA (4.0)**	**BPS (4.0)**	**BPA (40.0)**	**BPS (40.0)**
C16:0 (Palmitic acid)	24.14±0.08[a]	23.29±0.29[a]	23.66±0.66[a]	25.31±1.03[a]	24.09±0.22[a]	25.95±0.35[a]	25.12±0.11[a]
C16:1 (Palmitoleic acid)	2.02±0.47[a]	1.69±0.41[a]	1.32±0.11[a]	1.59±0.41[a]	1.23±0.03[a]	1.72±0.31[a]	1.33±0.04[a]
C18:1 (Stearic acid)	11.65±0.48[a]	11.27±0.46[a]	10.65±0.41[a]	14.59±1.95[a]	11.47±0.45[a]	11.42±0.18[a]	11.81±0.03[a]
C18:1 (Oleic acid)	15.46±0.04[a]	16.17±1.22[a]	16.22±0.21[a]	13.81±0.33[a]	13.86±0.58[a]	14.50±0.14[a]	14.13±0.61[a]
C18:2 n-6 (Linoleic acid)	23.99±0.98[a]	25.21±0.57[a]	26.32±0.80[a]	22.13±1.42[a]	23.98±0.19[a]	22.76±0.09[a]	22.94±0.22[a]
C20:4 n-6 (Arachidonic acid)	17.82±0.61[a]	18.63±2.51[a]	18.13±0.51[a]	19.37±1.73[a]	22.43±0.28[a]	19.86±0.46[a]	21.32±0.81[a]
C22:4 n-6 (Docosatetraenoic acid)	0.10±0.06[a]	0.15±0.08[a]	0.26±0.05[a]	0.07±0.02[a]	0.20±0.05[a]	0.18±0.01[a]	0.22±0.03[a]
C22:5 n-6 (Docosapentaenoic acid)	0.26±0.06[a]	0.38±0.06[a]	0.39±0.07[a]	0.19±0.03[a]	0.29±0.04[a]	0.40±0.03[a]	0.37±0.03[a]
C22:6 n-3 (Docosahexaenoic acid)	0.55±0.12[a]	0.82±0.07[ab]	0.97±0.10[b]	0.69±0.13[a]	0.93±0.03[a]	0.82±0.01[a]	0.83±0.07[a]

[#] Data were analysed with one-way ANOVA with post-hoc Sidak's multiple comparison test. Values are represented as means ± SEM (n=3). The experimental group with unlike letters is significantly different at $p<0.05$ vs control.

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Table. 2. Testis total fatty acid composition of adult male offspring (90 d) born to dam those exposed to bisphenols during gestation (gD4 to gD21) [#]

Fatty acids (nmol %)	Experimental groups (µg/kg bw /d)				
	Control (0.0)	**BPA (0.4)**	**BPS (0.4)**	**BPA (4.0)**	**BPS (4.0)**
C16:0 (Palmitic acid)	38.34±0.26[a]	40.42±0.26[a]	38.91±0.43[a]	**22.31±1.88[b]**	38.73±0.80[a]
C16:1 (Palmitoleic acid)	1.37±0.08[a]	1.03±0.25[a]	1.15±0.12[a]	**9.35±0.76[b]**	1.46±0.32[a]
C18:0 (Stearic acid)	8.41±0.23[a]	8.11±0.26[a]	8.24±0.38[a]	**3.11±0.12[b]**	8.11±0.27[a]
C18:1 (Oleic acid)	12.20±0.36[a]	10.55±0.07[a]	11.19±0.42[a]	**29.39±0.86[b]**	12.44±0.66[a]
C18:2 n-6 (Linoleic acid)	5.03±0.16[a]	4.08±0.22[a]	4.70±0.52[a]	**24.75±1.02[b]**	5.30±0.95[a]
C20:4 n-6 (Arachidonic acid)	14.64±0.44[a]	13.96±0.10[a]	14.47±0.23[a]	**4.24±0.40[b]**	14.20±0.71[a]
C22:4 n-6 (Docosatetraenoic acid)	1.84±0.01[a]	1.84±0.03[a]	1.81±0.12[a]	**0.89±0.08[b]**	1.81±0.23[a]
C22:5 n-6 (Docosapentaenoic acid)	16.14±0.61[a]	17.84±0.31[a]	17.97±0.36[a]	**2.56±0.64[b]**	16.55±0.37[a]
C22:6 n-3 (Docosahexaenoic acid)	0.23±0.03[a]	0.26±0.02[a]	0.31±0.01[a]	0.24±0.07[a]	0.29±0.01[a]
SCD-16 (C16:1/C16:0)	0.03±0.00[a]	0.02±0.00[a]	0.02±0.00[a]	**0.43±0.06[b]**	0.03±0.00[a]
SCD-18 (C18:1/C18:0)	1.45±0.06[a]	1.30±0.03[a]	1.36±0.09[a]	**9.42±0.57[b]**	1.54±0.13[a]
Desaturation index (C20:4 n-6/C18:2 n-6)	2.92±0.15[a]	3.45±0.17[a]	3.19±0.34[a]	**0.17±0.02[b]**	2.97±0.55[a]

[#] Data were analysed by one-way ANOVA with post-hoc Sidak's multiple comparison test. Values are represented as means ± SEM (n=4). The experimental groups with unlike letters are significantly different ($p<0.05$ vs control).

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Table. 3. Triglycerides and cholesterol contents of adult rat testis (90 d) exposed to BPA from gD 4 to gD 21

	Experimental groups (µg/kg bw /d)		
Lipid content	**Control (0.0)**	**BPA (4.0)**	**p value**
Plasma (mmol/L)			
Triglycerides, TG	1.59 ± 0.03	1.53 ± 0.03	0.27
Total cholesterol, TC	2.90 ± 0.02	2.71 ± 0.08	0.08
Testis (mg/g tissue)			
Triglycerides, TG	4.05 ± 0.05	3.04 ± 0.57	0.34
Total cholesterol, TC	1.00 ± 0.10	1.02 ± 0.12	0.91

Data were analysed with unpaired t-test. Values are represented as means ± SEM. Plasma (n=-8) and testis (n=3-5). $p<0.05$ was considered as statistically significant vs control.