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Retrieval action of zinc and folic acid for the restoration of normal reproductive function in bisphenol-A exposed male albino mice

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Abstract: Bisphenol-A (BPA) has become a great concern due to its toxic effects. The present study investigated the retrieval action of zinc (Zn) and folic acid (FA) supplementation against BPA-induced reproductive toxicities in male albino mice. A total of seventy-five 25–28 day-old mice were divided into five equal groups (group A–E, 15 mice in each group). The mice were given normal rations (control, group A) or administered with daily doses of BPA at 50 mg/kg body weight (b.w.) (group B–E). The mice from groups C, D and E were supplemented with Zn (10 mg/kg b.w.), FA (3 mg/kg b.w.) and both in the feed, respectively, daily for 12 weeks. Blood samples were collected, and the sera were separated for biochemical and hormonal analyses. The standard method was followed to test the sperm motility and sperm count. The testis samples were processed for a routine histopathological study using haematoxylin and eosin staining. The sperm counts, motility, and serum testosterone significantly declined in the BPA-exposed animals, but dramatically increased after the Zn and FA supplementation. There was significant degeneration of the seminiferous tubules in the testes of the BPA-exposed mice, which was recovered moderately by the Zn and FA supplementation. The study shows the retrieval action of zinc and folic acid in the restoration of normal reproductive function in bisphenol-A exposed male mice.

Keywords: BPA; biochemical; histopathology; hormone; reproduction

Environmental toxicants pose a significant threat to the health and reproduction of humans and animals (Rattan and Flaws 2019). Bisphenol-A [2, 2-*bis* (4-hydroxyphenyl) propane] (BPA) is one of the most synthesised chemical compounds produced globally (Vandenberg et al. 2013a) which is largely used in the production of polycarbonate plastics, epoxy saps, paints and dental materials (Rahman et al. 2021a). BPA may enter the food chain via food and water contamination, posing a number

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of health risks (Bjornsdotter et al. 2017; Rahman et al. 2021a). Biologically, BPA is an endocrine-disturbing substance with estrogenic and thyroid chemical-like effects (Khalil et al. 2014; La Merrill et al. 2020), which interferes with various endocrine signalling pathways (Vandenberg et al. 2013b; La Merrill et al. 2020). BPA exposure to adults may lead to reproductive damage, which often pass down to the next generation (Skinner et al. 2013). Research shows a positive relationship between BPA exposure and increased risk of reproductive dysfunction (Khalil et al. 2014). Paternal BPA exposure disrupted spermatogenesis by decreasing the size and number of testicular seminiferous epithelial cells, which eventually led to a decline in the total sperm count (Rahman et al. 2020).

Zinc is essential for cell growth and differentiation. Zinc deficiency is associated with hypogonadism in animals and humans (Prasad 2009). The zinc-deficient animals show decreased basal testosterone levels and reduced testicular weights and other androgen sensitive organs in comparison to the healthy control animals (McClain et al. 1984). In humans, a zinc-deficient diet reduces libido, serum testosterone concentrations, and sperm counts significantly (Zago and Oteiza 2001). Zinc is essential in spermatogenesis as a part of steroid receptors and metalloenzymes associated with DNA transcription (Abbasi et al. 1976). Zinc reduces the oxidative stress by supplying the metallothionein (Ruttkay-Nedecky et al. 2013) as well as by decreasing the tumour necrosis factor (TNF) secretion.

Folic acid is a synthetic form of folate (vitamin B9) that can be found in a variety of foods. Folate significantly contributes to the formation of red blood cells and also supports brain and spinal cord developments. The sperm are very sensitive to oxidative damage, which involve chromatin modification, peroxidation of the sperm membranes, impaired motility, and increased apoptosis (Aitken et al. 2014). Folic acid provides the carbon for the DNA synthesis and methylation which is vital to spermatogenesis as well as removing free radicals (Forges et al. 2007). Folic acid depends on zinc for proper functionality and bioavailability as well as having a synergistic action with zinc (Wong et al. 2002). The combined treatment of folic acid and zinc for 26 weeks enhanced the total sperm count in infertile and sub-fertile males (Azizollahi et al. 2013), and demonstrated a 74% rise in the total sperm count.

To the best of our knowledge, no experiment has been carried out to elucidate the beneficial actions of zinc and folic acid on the reproductive physiology of BPA-exposed animals using an experimental model. Therefore, the current study was performed to assess the preventive action of zinc and folic acid supplementation on BPA-induced reproductive damage in male mice.

MATERIAL AND METHODS

Ethical declaration

The animal experimentation was approved by the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Mymensingh 2202 [Reference: AWEEC/BAU/2021(18)].

Animals and housing conditions

A total of 75 male Swiss Albino mice, aged 3.5–4 weeks and weighing 25 ± 1.92 g, were used in the current research. The animals were bred and reared under standard housing conditions in the animal facility at the Department of Physiology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202. All the animals had access to food and water *ad libitum*.

Experimental approach

The mice were randomly assigned to 5 groups (n = 15). The control mice (group A) received normal mice rations. The mice in group B were treated with bisphenol-A (BPA) at 50 mg/kg body weight (b.w.) daily. The BPA dose was selected according to the study by Rahman et al. (2021b) who said that environmental exposure to BPA during pregnancy affects the fertility in future generations in both humans and the other animals. Group C received daily dose of BPA at 50 mg/kg b.w. with zinc sulfate at 10 mg/kg b.w. supplementation. The mice in group D received daily doses of BPA at 50 mg/kg b.w. with folic acid at 3 mg/kg b.w. supplementation. The group E mice received BPA at 50 mg/kg b.w. supplemented with both zinc sulfate at 10 mg/kg and folic acid at 3 mg/kg b.w. daily. The experiment was conducted for 12 weeks.

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Serum collection

About 1.5 ml of blood was collected from each mouse directly from the heart using a vacutainer and the blood was allowed to clot. The serum was separated and cleared by centrifuging at 3 000 rpm for 10 minutes.

Hormonal assay

The serum testosterone was determined by using a Testosterone Radioimmunoassay Kit (Berthold, Bad Wildbad, Germany) at the Institute of Nuclear Medicine & Allied Sciences (INMAS; Mymensingh Medical College, Mymensingh, Bangladesh) following the standard protocol.

Analysis of sperm physiological parameters and testis weight

At the end of the experimental period (12 weeks), the mice were euthanised. The testes were collected in physiological saline, soaked into chromatographic paper and weighed using an electronic balance. The epididymis was collected to test the sperm motility and concentration as previously described (Saalu et al. 2010). Briefly, a small amount of the diluted suspension was transferred on a pre-warmed slide and a cover slip was placed on it. The sperm motility was calculated in percentage (%). The epididymal sperm collection and sperm counting were performed according to Del Val and Robledano (2013). For that, the cauda epididymis of the mice was collected in a Petri dish and macerated using scissors. Four millilitres (4 ml) of the pre-warmed (37 °C) physiological saline were placed into a test tube and the minced epididymis was transferred into the tube. The sperm were then allowed to release for 5–10 minutes. Finally, the sperm were counted using Neubauer's chamber under a high-power objective of the microscope.

Histopathological studies and microscopy

The testes from each group of mice were collected after the complete removal of blood following perfusion with phosphate buffered saline. Pieces of the testes were collected in 10% neutral buffered formalin. The fixed tissue samples were processed, sectioned and stained with routine haematoxylin and eosin stain as per the standard protocol (Luna 1968). The slides were examined under a photomicroscope (ZEISS Primo Star; Carl Zeiss AG, Göttingen, Germany).

Statistical analysis

All the data were entered and stored in Microsoft Excel 2007 (Microsoft, USA). Then, they were transferred to the software GraphPad Prism v5.0 (GraphPad Software Inc., USA) for analysis using a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison test. A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Effects of zinc and folic acid supplementation on the functionality of the testes of mice following the BPA toxicity

The effects of the Zn and FA supplementation on the reproductive function of male mice following the BPA toxicity were analysed. To this end, mice exposed to BPA were kept untreated or treated with Zn or FA or both Zn and FA supplementation. Testis weight was recorded at necropsy. The study revealed that the testis weight was 152.5 ± 0.13 mg in group A (control) which significantly decreased in the mice treated with BPA (group B, 118 ± 0.21 mg) (Figure 1A). Whereas the BPA-treated mice supplemented with Zn (group C, 246.5 ± 3.46 mg), FA (group D, 187.5 ± 0.01 mg) and the Zn and FA combination (group E, 204.5 ± 0.38 mg) showed significantly higher testicular weight when compared to the BPA-treated mice alone. Of note, the testicular weight of the BPA-treated mice fed with Zn or FA or both was significantly higher than the untreated control mice ($P \leq 0.001$). Beside this, the sperm physiological properties, like the sperm concentration and motility, were also recorded. Similar to the testicular weight, the BPA-exposed mice showed a lower sperm concentration and motility in comparison to the control mice, which was restored after the Zn and FA supplementation (Figure 1B,C). For example, the percent of motile sperm in the cauda

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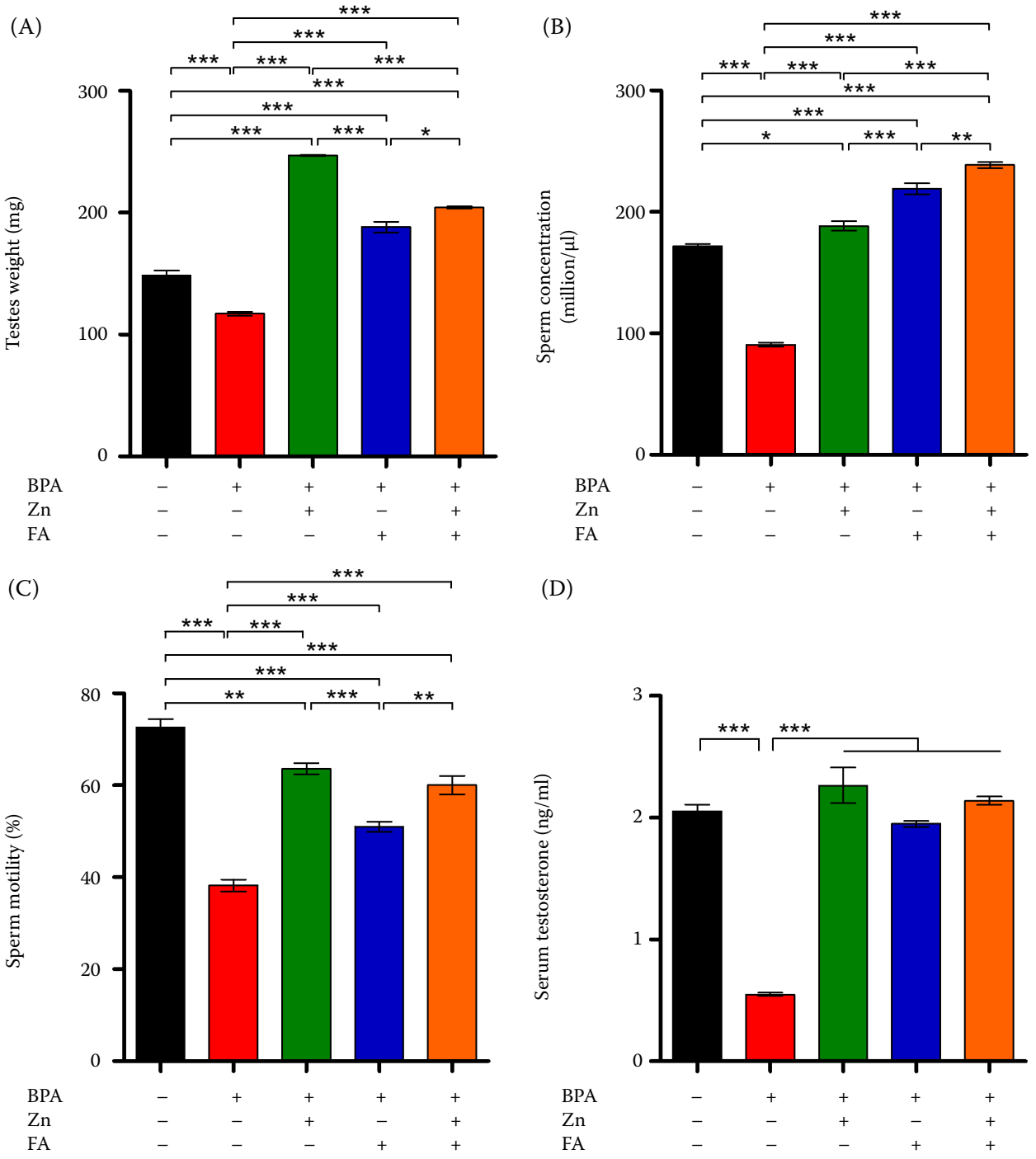

Figure 1. Effects of the zinc and folic acid supplementation on the restoration of the male reproductive function of BPA-exposed mice

The mice exposed to BPA were kept untreated (control) or treated with Zn, FA or both Zn and FA and (A) the testicular weight, (B) sperm concentration, (C) sperm motility and (D) serum testosterone levels were measured. Data indicate the mean ± SEM of 3–5 replicates; One-way ANOVA with Bonferroni multiple comparison test was performed

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

epididymis decreased significantly ($P \leq 0.001$) in the BPA-exposed mice (37.75 ± 0.23%) when compared to the control mice (72.75 ± 0.11%) (Figure 1C). However, the Zn (63.25 ± 0.25%), FA (51.25 ± 0.29%) and combined Zn and FA supplementation (61.5 ± 0.19%) produced sperm with higher ($P \leq 0.001$)

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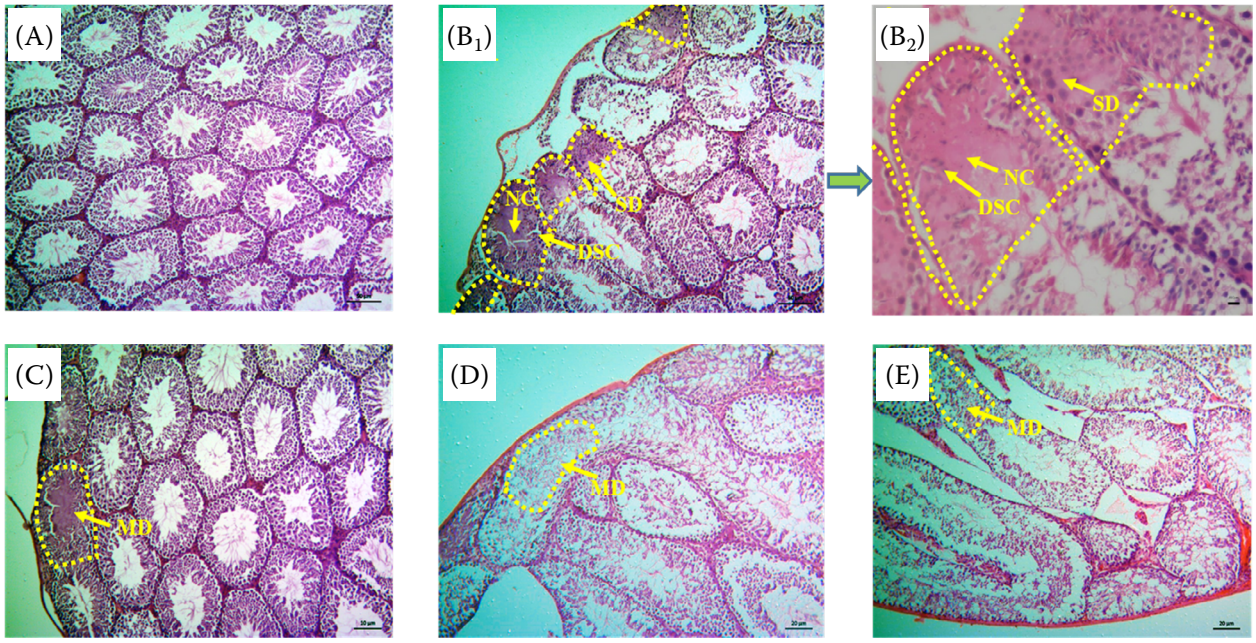

Figure 2. Effects of the zinc and folic acid supplementation on the histoarchitecture of the testes of BPA-treated mice
(A) Section of the testis of a control mouse showing normal architecture. (B_1–B_2) Section of the testis of BPA-exposed mouse showing severe degeneration (SD) and necrotic changes (NC) in the seminiferous tubules (dotted area) and disruption of the spermatogonial cells (DSC). Sections of the testes of mice fed with (C) Zn, (D) FA and (E) both Zn and FA showing mild degeneration (MD) (dotted area) in the seminiferous tubules. Haematoxylin and eosin stain, bar = 20 µm (A, B_1, C, D, E) and bar = 400 µm (B_2) indicate the magnification

motility than the BPA-exposed mice (Figure 1C). The results of the hormonal assay (testosterone) showed that the BPA treatment (0.54 ± 0.01 ng/ml) significantly reduced the serum testosterone level ($P \leq 0.001$) when compared to the control mice (2.05 ± 0.02 ng/ml) (Figure 1D). However, the serum testosterone level was maintained following the Zn (2.23 ± 0.12 ng/ml), FA (1.94 ± 0.003 ng/ml), and combined Zn and FA (2.11 ± 0.001 ng/ml) supplementation (Figure 1D).

Effects of zinc and folic acid supplementation on the histoarchitecture of the testes of the mice following the BPA toxicity

The histopathological changes in the testes of the mice from the five different groups were examined and are presented in Figure 2. The testes of the BPA treated mice displayed disorganisation and degeneration of the seminiferous tubules and arrest of spermatogenesis at different stages with severe necrosis of the spermatogonial cells (Figure 2B) when compared to the normal architecture of the testes in the control mice (Figure 2A). However, the Zn, the FA or combination of the Zn and FA supplementation moderately restored the histoarchitectures of the testes (Figure 2C–E). Of note, the restoration rate was higher in the mice receiving the FA (group D) and the Zn and FA combination (group E) supplementation than the mice receiving only the Zn supplement (group C). Taken together, the BPA-exposure produced significant tissue damage in the gonads of the male mice which was improved moderately following the Zn and FA supplementation.

DISCUSSION

The effects of the zinc (Zn) and folic acid (FA) supplementation on the functioning and histoarchitecture of mice testes after exposure to BPA were investigated. In the testicular tissues of mice, the BPA exposure caused considerable toxicity. The Zn and FA supplementation significantly improved the function and histoarchitecture of the testes of the BPA-exposed mice.

Testis weight is an important reproductive trait in males because of its direct connection with the spermatogenic ability (Harcourt et al. 1981).

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Abnormalities in the sperm count, morphology, and motility are among the useful markers to study the fertility potential of an individual (Zribi et al. 2011). In this study, the BPA exposure significantly reduced the weight of testes in the mice. However, the Zn and FA supplementation maintained the testicular weight of the BPA exposed mice, indicating the therapeutic potential of these two nutrients in treating BPA toxicity. A significant reduction in the testis and epididymal weights was recorded in BPA fed adult male Wistar rats along with high ventral prostate weight (Chitra et al. 2003). Others have revealed that BPA causes a decrease in the body and testis weights gain (Nakamura et al. 2010).

The histopathological investigation of the testicular tissue of the BPA-exposed mice also displayed disorganisation and deformity of the seminiferous tubules and arrested spermatogenesis at different stages with necrosis and vacuolation of the spermatogonial cells. However, the distorted morphology was moderately restored in the Zn and FA supplemented mice. BPA is known to cause atrophy and damage to the seminiferous tubule, germinal cell debris and congestion and degeneration of spermatocytes (Aydogan et al. 2010). BPA exposure for 56 days in mice showed disrupted spermatogenesis, deformed basal lamina in the seminiferous tubules and tight junctions in the Sertoli cells (Tian et al. 2017). Severe degeneration of the acrosome and spermatid nucleus along with abnormal ectoplasmic specialisation in BPA-supplemented rat testis were also observed (Liu et al. 2013a). BPA induced seminiferous tubule degeneration, necrosis, wide interstitial tissues, desquamation of germinal cells and the deceleration of spermatogenesis (Takahashi and Oishi 2001).

A similar result was found while analysing the sperm motility and sperm concentration. We found that the BPA exposure significantly decreased the sperm counts and motility, but the Zn and FA supplementation maintained the sperm motility and sperm concentration in the BPA exposed mice. Poor sperm integrity was examined in mice supplemented with 2 000 µg/kg BPA (Lombo et al. 2019). The oral administration of BPA (2–200 ng/kg b.w.) for six days showed a decrease in the sperm count and affected testicular weight and structure in adult male rats (Chitra et al. 2003).

Testosterone is fundamental for keeping up with spermatogenesis and male fertility, yet BPA disturbs the spermatogenesis (Sofikitis et al. 2008). We observed that the serum testosterone concentration was significantly reduced in the BPA-exposed mice. Kamel et al. (2018) studied BPA-exposed rats with various concentrations and duration and found a decrease in the body and testicular weight and the testosterone level. Similarly, Liu et al. (2013b) stated that BPA supplementation (5 mg/kg for 6 consecutive days and 200 µg/kg/day, orally for 60 days) decreases the sperm motility, sperm counts, spermiation, and induces DNA damage in rats. Our findings are compatible with these earlier findings. However, the FA and Zn supplementation maintained a normal serum testosterone level in the BPA-exposed mice. Low Zn levels decrease the sperm motility, increase the sperm head and tail abnormalities and impair the process of spermatogenesis by testicular Zip6 and Zip10 depletion. A dietary Zn deficiency for 4 weeks leads to body weight reduction, growth retardation, and marked depletion in the serum testosterone level (Omu et al. 2015). Moreover, Zn is also involved in functions that are crucial for sperm physiology. Zinc is well known to provide sperm membrane integrity, to increase the sperm motility and to control the spiral movements of the sperm tail (Tuncer et al. 2011). Similarly, Shalaby et al. (2010) found that FA supplementation significantly increases the serum testosterone concentration. A reduction in the FA level in seminal plasma is associated with low sperm production and an increased incidence of sperm DNA damage (Boxmeer et al. 2009). FA treatments reduced the plasma and testis malondialdehyde (MDA) concentrations, and increased the sperm count and motility in cases in hypothyroid rats with testicular dysfunction (Ibrahim et al. 2011).

In conclusion, it could be stated that Zn and FA supplementation in the meal might greatly reduce the harmful effects of BPA exposure on the testes of male albino mice. As a result, Zn and FA supplementation may be an effective strategy to prevent BPA-induced testicular injury. However, more research is needed to determine the precise mechanism in which Zn and FA reduce the testicular injury in BPA-exposed mice.

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Conflict of interest

The authors declare no conflict of interest.

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