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Research Article

Bisphenol F induced alteration in testicular p53 and localization: Implications for cauda epididymal sperm characteristics and morphology

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Abstract

Bisphenol F (BPF), a structural analogue of Bisphenol A (BPA), is widely used in the production of plastics and epoxy resins. Emerging evidence suggests that BPF may disrupt endocrine function and impair fertility. This study investigates the effects of BPF on sperm characteristics, morphology, and germ cell viability, with a focus on its potential mechanisms of action in male reproductive toxicity. Wistar rats were randomly assigned to five groups (n=5/group): control (Group I), BPF-treated groups (100 (Group II), 500 (Group III), and 1000 (Group IVa) µg/kg/day for 45 days), and a recovery group (IVb) (1000 µg/kg/day for 45 days followed by a 45-day recovery). Post-treatment, sperm parameters were assessed, and immunohistochemical analysis of p53 expression in testicular tissue was performed. BPF exposure led to significant, dose-dependent declines in sperm count, motility, and viability, accompanied by increased morphological abnormalities, particularly in the sperm head region. Irregularities in the acrosomal system, plasma membrane, and perforatorium suggested oxidative stress during epididymal transit. A 5–8% reduction in sperm viability and a 2–15% increase in abnormalities were observed across dose groups. Elevated p53 expression in testicular tissues indicated germ cell apoptosis and impaired spermiogenesis. Recovery period showed significant resumption, indicative of temporary damage to testicular function. In conclusion, BPF exerts direct, dose-dependent toxicity on male reproductive function by promoting oxidative stress and upregulating p53-mediated germ cell elimination, ultimately contributing to teratozoospermia and reduced fertility potential.

Keywords: Bisphenol F, Spermatogenesis, P53, Sperm morphology

INTRODUCTION

Bisphenols are a class of chemicals that have gained prominence due to their critical role in the production of plastics, epoxy resins, and a wide range of consumer products. The most widely used bisphenol is bisphenol A (BPA). However, recent confirmatory reports highlighting its harmful effects on the endocrine system have prompted the search for suitable alternatives.¹ Although bisphenols were first synthesized in 1891, their widespread industrial use was firmly established by the mid-20th century. The commercial production of BPA began in the 1950s, primarily for the manufacture of epoxy resins and polycarbonates. The need for alternatives to BPA initiated an active search for compounds with similar physicochemical properties but without the associated adverse effects. The most promising candidates emerged from within the bisphenol group itself, among which bisphenol S (BPS) and bisphenol F (BPF) were actively adopted by industries.

The initial industrial use of BPF dates back to the 1980s and 1990s. Owing to its structural similarity to BPA, BPF was similarly employed in the production of

epoxy resins, polycarbonates, and protective coatings.² However, the very structural features that made BPF a suitable substitute for BPA have also subjected it to scientific scrutiny for potential health and environmental risks.³ Like BPA, BPF is classified as an endocrine-disrupting chemical and is thus expected to contribute to reproductive dysfunction, metabolic disorders, and developmental anomalies.⁴ Notably, BPF has been detected in human urine samples, thereby reinforcing concerns regarding its bioavailability and potential health implications.⁵⁻⁷

Toxic compounds present in the environment can significantly impair sperm quality, reduce testosterone levels, and diminish fertility outcomes. A review by Wong and Cheng⁸ reported that both high levels of acute and low levels of chronic exposure to environmental toxicants can alter specific metabolic activities and cellular signalling pathways, thereby disrupting fundamental cellular functions. Similarly, the reproductive systems of both sexes are substantially affected by environmental toxicants.⁹ In males, such exposures are particularly associated with compromised

fertility and are frequently implicated in testicular atrophy.¹⁰⁻¹¹

Exposure to bisphenols during critical windows of development has been linked to numerous adverse effects on the male reproductive system. These include alterations in sex hormone levels, impaired sperm parameters, and feminization of male fetuses.¹² Previous studies have demonstrated that bisphenols interfere with steroidogenesis, disrupt germ cell proliferation, and impair spermatogenesis.¹³⁻¹⁴ Additionally, bisphenols may hinder the sperm maturation process by impairing capacitation during their transit through the epididymis.¹⁵ These disruptions during epididymal transit are associated with reduced sperm motility and compromised acrosomal integrity.¹⁶ While most investigations have focused on BPA, relatively fewer studies have addressed its analogues, highlighting the need for further research. Thus, understanding sperm morphology during epididymal transit remains of considerable importance.

Similarly, testicular dysfunction due to BPA exposure has been widely documented. Critical factors contributing to impaired testicular function include hormonal imbalances, disrupted cellular growth, and germ cell apoptosis. These effects have been observed in BPA-exposed animal models.¹⁷⁻¹⁸ In the context of germ cell apoptosis, the tumour suppressor protein p53 plays a central role by eliminating damaged or defective germ cells. Furthermore, p53 is essential in regulating spermatogenesis, including the proliferation of spermatogonial stem cells, spontaneous DNA repair, and the removal of defective germ cells.¹⁹ Dysregulation of p53 expression or activity can impair spermatogenesis and lead to male infertility. A study by Dairkee *et al.*²⁰ reported that BPA inactivates the p53 signalling axis in non-malignant human breast epithelial cells.

Although numerous studies have been conducted on BPA, there is comparatively limited research on BPF and other structural analogues. Therefore, the present study aims to investigate sperm morphological alterations in the cauda epididymis and the expression of p53 in testicular cells following BPF exposure in Wistar rats. Additionally, the study explores the relationship between p53 subcellular localization in testicular tissue and morphological changes in cauda epididymal sperm.

MATERIALS AND METHODS

Chemical compound

Analytical grade Bisphenol F (BPF), also known as Bis(4-hydroxyphenyl)methane or 4,4'-Methylene-diphenol ($\text{CH}_2(\text{C}_6\text{H}_4\text{OH})_2$), CAS No. 620-92-8, was obtained from Sigma Aldrich-Merck (NJ, USA).

Animal model and ethical clearance

Male Wistar albino rats (*Rattus norvegicus*), aged three months and weighing between 150–200 g, were used in this study. All animals were housed under veterinary supervision at the Department of Zoology's animal facility, University of Rajasthan, Jaipur. The rats were kept in polypropylene cages measuring 43×27×15

cm, with drinking water available *ad libitum* and maintained on a 12-hour light/12-hour dark cycle.

Prior approval for the experimental procedures was obtained from the Institutional Animal Ethics Committee (IAEC). All experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).²¹

Experimental design

Animals were randomly assigned to groups, each consisting of five rats, except for Group IV, which included ten rats. Group I served as the vehicle-treated control. Based on the administered doses BPF, the treatment groups were designated as follows: Group II received 100 µg/kg body weight/day, Group III received 500 µg/kg body weight/day, and Group IVa received 1000 µg/kg body weight/day. Group IV was subdivided into two sets of five rats each: Group IVa underwent active treatment with BPF, while Group IVb, the self-recovery subgroup, was observed for an additional 45 days following BPF withdrawal to assess potential recovery.

BPF treatment was administered continuously for 45 days. On the 46th day, animals were euthanized in accordance with CPCSEA guidelines. The euthinization of Group IVb was carried out on the 91st day of experiment. BPF was dissolved in distilled water to prepare the required concentrations and was administered orally using a stainless-steel gavage needle (2" length, 18 gauge). All dosing regimens were adapted from previously published studies^(22,5) and complied with the OECD guidelines for the testing of chemicals.²³

Sperm characteristics

Sperm parameters, including sperm count, motility, viability, and abnormalities, were assessed following the guidelines of the WHO laboratory manual.²⁴ For sample collection, a portion of the cauda epididymis was excised and gently macerated in 1 ml of normal saline (pH 7.0).

Sperm morphology evaluation by SEM

Spermatozoa were collected from the cauda epididymis and washed twice in phosphate buffer (pH 7.0) by gentle stirring, followed by centrifugation at 1500 × g for 15 minutes at 4°C. The sperm pellet was fixed in 2.5% glutaraldehyde for 30 minutes at 4°C, then washed three times with phosphate buffer and once with distilled water. Samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%), and a thin smear of spermatozoa was prepared on clean glass slides, air-dried, and mounted on SEM stubs using silver paint. The samples were sputter-coated with a 350 Å layer of gold and examined under a scanning electron microscope.

Localization and expression of p53 in testicular cells

Tissue sections were prepared for immunohistochemical staining by graded rehydration, followed by a 10-minute wash in wash buffer. Endogenous peroxidase activity was quenched using a

peroxidase blocking reagent for 5 minutes. The slides were then incubated with a primary antibody specific to p53 (Invitrogen, MD, USA). After primary antibody incubation, the slides were thoroughly rinsed with PBS and treated with Histostain-Plus HRP and a secondary antibody (diluted 60:40) from ThermoFisher Scientific (MA, USA). The slides were left at room temperature for 30 minutes to complete the secondary antibody reaction.

Following this, the DAB (3,3'-diaminobenzidine) substrate was applied for 30 seconds to visualize antibody binding. The tissue sections were then examined under a microscope. Subsequently, the sections were counterstained with haematoxylin, dehydrated through a graded series of alcohols, and cleared in xylene. A drop of DPX mounting medium was added, and coverslips were placed.

The slides were evaluated based on the intensity of brown staining, indicating the presence of p53-positive cells. Immunolabeling was assessed by examining the bluish-brown coloration in the tissue. The number of immunohistochemically (IHC) positive cells was quantified using ImageJ software (NIH, USA) by analysing 10 randomly selected fields per testicular cross-section. Cell counts were normalized to a standard area of 1.0 mm².

Statistical analysis

All numerical values were expressed as mean \pm standard error (SE). The level of significance for variance was evaluated in comparison to the sham control group. Student's t-test (MS Excel, SV, USA) was applied for all paired data. One-way ANOVA (MINITAB, USA), followed by Tukey's multiple comparison test, was used to analyse non-parametric data. Statistical significance was assessed at confidence intervals (CIs) of 95%, 99%, and 99.99%.

RESULTS

Cauda epididymal sperm characteristics

Exposure to BPF resulted in significant impairments in multiple sperm parameters, as evidenced by marked reductions in sperm count, motility, and viability, along with increased morphological abnormalities. Sperm counts in cauda epididymal samples showed a significant decline in Groups II, III, and IVa ($p < 0.05$), with mean values of 40.98 ± 0.96 , 41.65 ± 1.21 , and 38.72 ± 1.29 million/mL, respectively, compared to 45.25 ± 1.32 million/mL in the control group (Group I). Following a 45-day self-recovery period (Group IVb), a partial restoration in sperm count was observed, reaching 42.84 ± 0.89 million/mL (Figure 1A). Sperm motility was also significantly affected, with highly pronounced reductions in Groups III, IV, and IVb ($p < 0.01$), where motility dropped to $39.60 \pm 2.95\%$, $35.18 \pm 2.49\%$, and $38.32 \pm 1.60\%$, respectively, compared to $67.03 \pm 1.66\%$ in the control (Figure 1B). Group II showed a moderate but significant decrease in motility ($55.56 \pm 3.70\%$; $p < 0.05$), approximately 12% lower than the control.

Similarly, sperm viability was significantly reduced in all treated groups. While the control group exhibited a viability rate of $85.85 \pm 1.19\%$, Groups II, III, and IVa ($p < 0.05$) showed viability rates of $81.51 \pm 0.65\%$, $78.93 \pm 1.84\%$, and $78.15 \pm 1.87\%$, respectively. Group IVb, assessed after the 45-day recovery period, continued to show reduced viability at $80.37 \pm 0.87\%$, reflecting an incomplete recovery (Figure 1C). Furthermore, a substantial increase in sperm abnormalities was recorded in Groups III ($38.89 \pm 1.23\%$) and IVa ($39.46 \pm 1.21\%$) ($p < 0.01$), representing an 8–14% increase compared to the control group, which exhibited $24.50 \pm 0.90\%$ abnormal sperm. Although Group IVb also displayed a significant elevation in abnormal sperm forms ($30.76 \pm 1.09\%$), the increase was less pronounced ($<6\%$) relative to other treated groups, again suggesting partial but incomplete recovery from BPF-induced effects (Figure 1D).

Scanning electron micrograph of cauda epididymal sperm

Control cauda epididymal sperm exhibited normal morphology, characterized by an intact acrosomal system with a distinct perforatorium, a well-connected head-midpiece junction at the neck region, and an uncoiled tail free of bends or twists (Figure 2A). In contrast, sperm abnormalities were markedly increased in groups treated with BPF regardless of doses, predominantly affecting the head and tail regions. Ultrastructural analysis revealed tail twisting caused by plasma membrane constriction and occasional cytoplasmic droplets (Figure 2B). Swelling of the plasma membrane was frequently observed at the neck and flagellar regions, indicating localized dilation. Head abnormalities included irregular acrosomal systems and perforatoria, as well as flattened apical heads in some BPF-treated sperm (Figures 2C-D). Irregularities in the plasma membrane were also noted in the mid-piece and neck, often localized to the mitochondrial sheath. Severe head damage was evident in some sperm, presenting as abnormal head cap contours and microcephalic-like deformities, accompanied by disrupted plasma membranes and cytoplasmic dilation at the acrosomal site (Figure 2E).

Evaluation of p53 expression in testicular tissues

The p53 plays a critical role in regulating cell division by suppressing uncontrolled cellular proliferation and inducing apoptosis in non-transformed cells. Expression of p53 was minimal in Groups I and II (Figure 3A-B). In Group III, p53 positivity was primarily localized to spermatocytes (Figure 3C). Notably, Group IVa exhibited markedly elevated p53 expression, with strong localization in both spermatocytes and spermatids (Figure 3D). Following a 45-day recovery period (Group IVb), p53 expression was substantially diminished, although sporadic positivity persisted in some spermatids (Figure 3E). It was profoundly clear that increase in dose of BPF directly impacted expression of p53 in testicular tissues eliminating large scale of germ cells thus substantially reducing the sperm production.

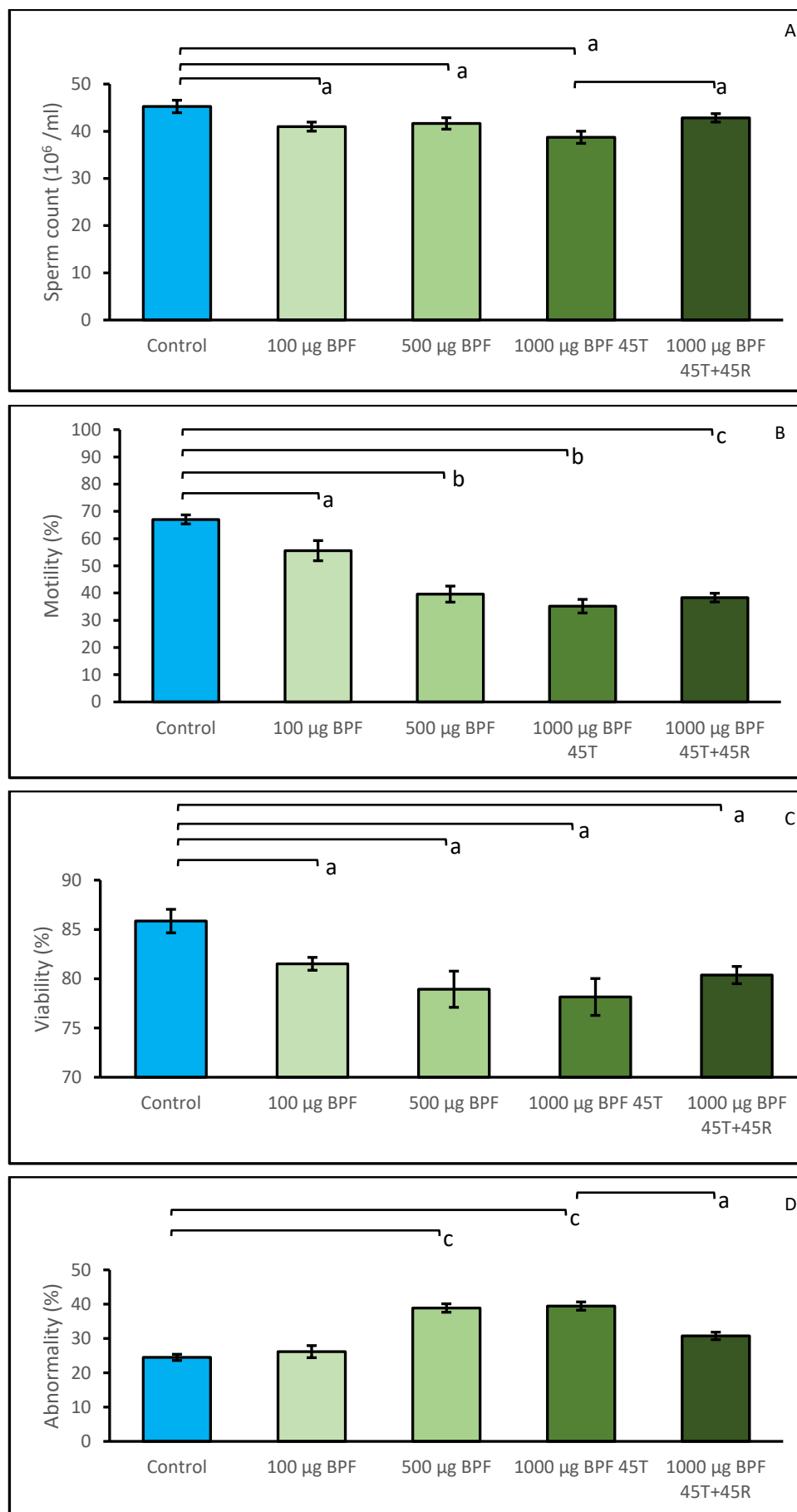


Figure 1: Cauda epididymal sperm quality in groups of rats exposed to BPF with doses 100, 500 and 1000 µg/kg body weight/day. The variations were compared against control except for count and motility where Group IVa (1000 µg/kg body weight/day for 45 days) was compared against Group IVb (1000 µg/kg body weight/day for 45 days and later allowed to recover for another 45 days). a. $p < 0.05$, b. $p < 0.01$, and c. $p < 0.001$.

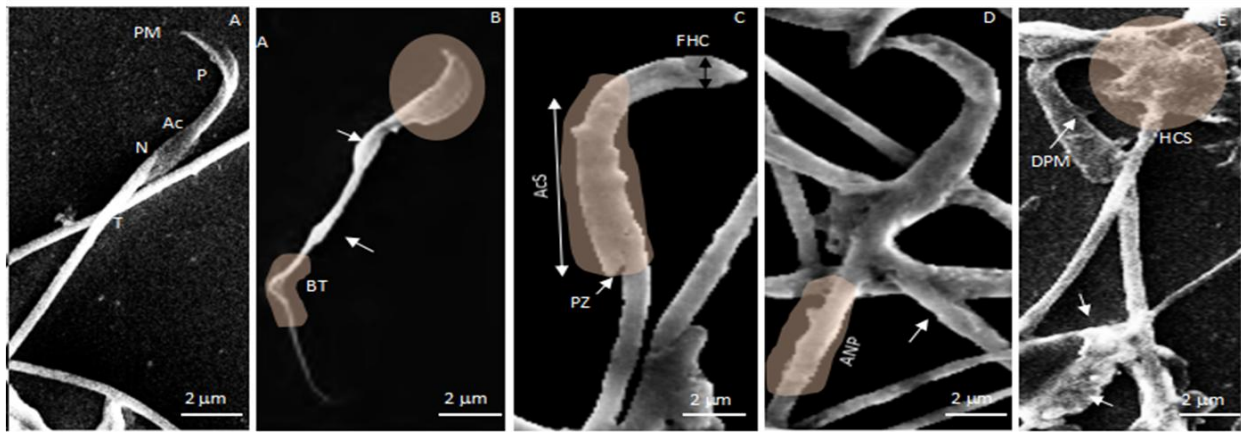


Figure 2: Scanning electron micrograph of cauda epididymal sperm exposed to various doses of BPF, showed variable types of damages including bent tail (BT), distorted head cap system (HCS), irregular acrosomal system (Ac) and perforatorium (P), flattened head cap (FHC) and damaged plasma membrane (DPM). Appearances of morphological abnormalities were more common in Group IVa comparing to other groups, however, other test groups also showed distinctly higher number of these damages comparing to control. Image A shows control morphology, whereas, B-E images show types of exclusive deformities present in the BPF exposed groups. PZ: Perifossal zone; PM: Plasma membrane; N: Nucleus; T: Tail; AcS: Acrosomal system; ANP: Abnormal neck-piece. Shading of abnormal area has been applied with 58% transparency to the original images.

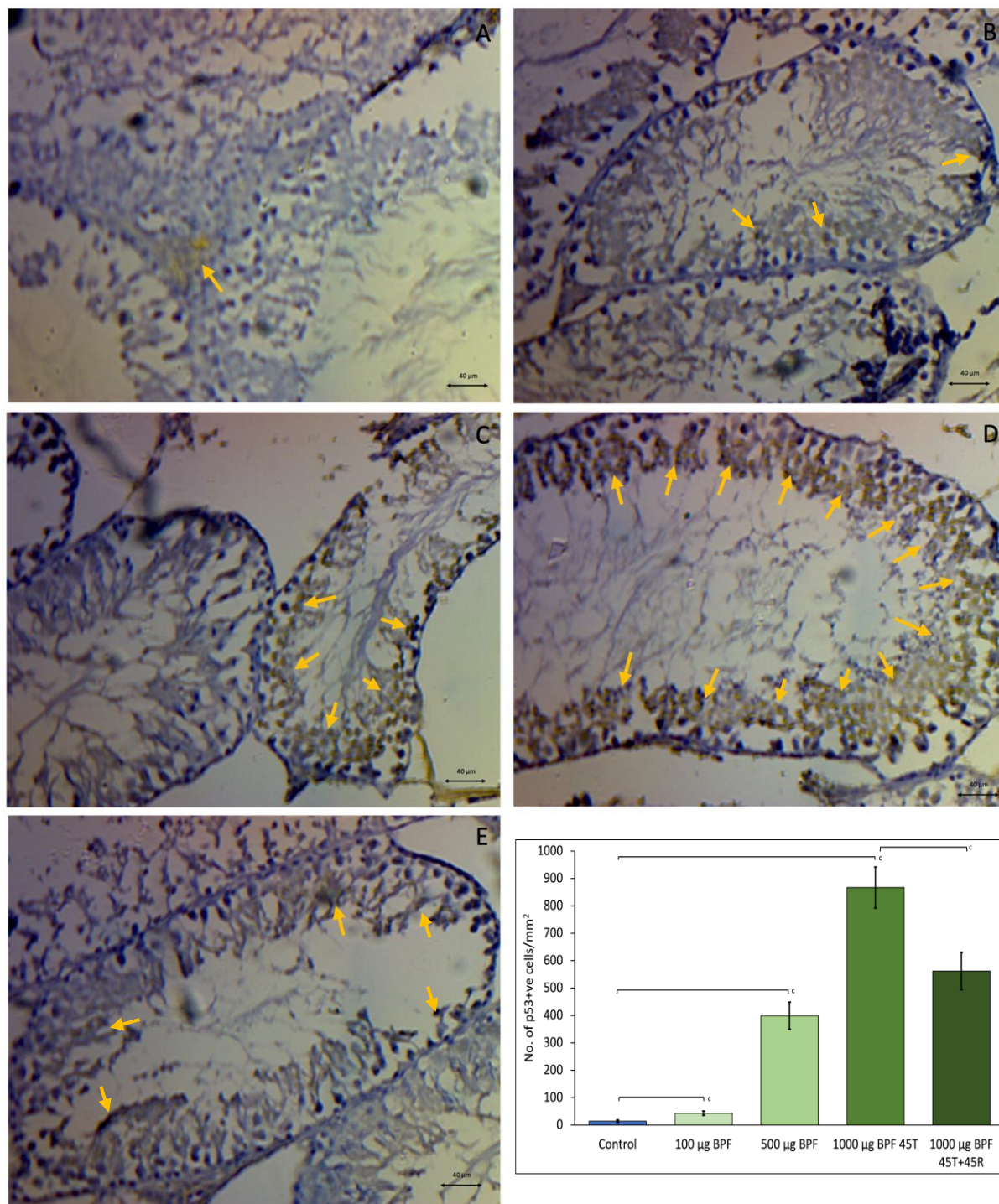


Figure 3: Limited number of p53 positive germ cells were visible in Group I (A) and II (B). While number of positive cells increased substantially in Group III (C) and IVa (D). Number of p53 positive cells in Group IVa was significantly higher when compared with other test groups. Spermatocytes and spermatids were more positive cells in Group IVa (shown with yellow pointer). Withdrawal from BPF exposure (Group IVb) (E) revealed significant reduction in p53 expression in testicular cells comparing to Group IVa. Graph shows the number of p53 positive cells counted per mm² in treatment groups where individual test groups were compared against control, except for the Group IVb (1000 µg/kg body weight/day for 45 days and later allowed to recover for another 45 days) which was compared against Group IVa (1000 µg/kg body weight/day for 45 days). c. $p < 0.001$

DISCUSSION

The bisphenol analogues were expected to show similar biological and metabolic trends once ingested due to chemical and structural similarities and identical properties. Nonetheless, use of BPF and BPS gained momentum due to their low interference in metabolic

functions comparing to BPA.²⁵⁻²⁶ Therefore, the present replacement of BPA was merely to minimize side-effects and not to eliminate the risk. At least two decades long extensive research on BPA since 2007 to 2025 provided extensive information on its potential adverse health outcomes that led to a stringent evaluation of all

bisphenol-based analogues to counter or limit its exposure and related health adversities. The present study was also an attempt to evaluate BPF for its potential role in low sperm quality and post-maturation damages.

The present study revealed exclusive dose-based alteration in sperm quality, and sperm morphology. Results clearly showed that the higher the dose of BPF, the higher abnormality in the cauda epididymal sperms. Likewise, other parameters of sperm characteristics also revealed dose-based decline such as; motility and viability. However, sperm count was relatively separated in trend while it did show significant decline following 45 days exposure, nonetheless, the dose-based deviation was remarkably low. Previous studies have consistently reported interference in spermatogenesis by BPF exposure due to structural damages in testis.²⁷⁻²⁹ Which indicate that storage of sperm in the cauda epididymis was remained competitive despite significant loss of spermatogenesis. Nevertheless, reduction in motility and viability, and extensive increase in sperm abnormalities indicate substantial role of BPF during capacitation in epididymis. It was however, important to note that withdrawal from treatment of BPF profoundly resumed the sperm characteristics. A clear significant increase in sperm count following withdrawal period was evidence of self-recovery. Similarly, marginal increase in motility, viability, and decline in abnormality following recovery period indicate that damages posed by BPF are recoverable and not permanent.

Ultrastructure of cauda epididymal sperm revealed typical abnormalities that appear to be during spermatogenesis or epididymal sperm maturation. Damages such as head tail separation and irregular plasma membrane is indicative of high oxidative stress,³⁰ which is one of the key maker responses of bisphenols.³¹ A study by Ullah et al.³² reported that BPF can disrupt normal function of the epididymis affecting the overall quality of sperm. The present study recorded abundance in number of head related damages including irregular acrosomal system, abnormal perforatorium, flattened head cap, and distorted head cap system. Previous reports suggest that damages in sperm heads are associated with both spermiogenesis and sperm maturation. For example, Ball and Peters³³ reported that morphological alteration sperm head associated with chromatin instability and lack of protamine. Likewise, Jakubik-Uljasz et al.³⁴ reported that sperm morphogenesis failure could be the most promising reason behind head defects in sperms. It is important to note that induced apoptosis may also cause teratozoospermia, previous studies have reported bisphenols induce abortive apoptosis in germ cells through high oxidative stress.³⁵⁻³⁶

The present study reported direct role of BPF exposure with p53 expression in the testicular tissues. A clear increase in number of p53 positive spermatogonial and spermatocytes indicated large scale elimination of germ during spermatogenesis. It also explained the typical increase in the sperm head related damages in the

cauda epididymis. A study by Zhou et al.³⁷ reported that BPF exposure in mouse TM3 Leydig cells increased expression of p53 causing resultants increase in apoptosis. Similarly, Odetayo et al.²⁹ reported that BPF induces gonadal toxicity by interfering in p53 and BCL2 signalling pattern, thus, apoptosis in germ cells becomes inevitable. The present study indicated that withdrawal from the BPF exposure significantly lowered the expression of p53 in germ cells which further supports the claim that BPF is directly involved in the p53 signalling cascade.

CONCLUSION

The present study affirmatively showed direct involvement of BPF in the epididymal sperm maturation process, which was evident from resultant sperm parameters. Increase in the sperm abnormalities was indicative of morphological failure during spermatogenesis and maturity during epididymal transit. High expression of p53 confirmed low sperm viability and increase in sperm motility. The typical damages in the sperm head were also indicative of induced oxidative stress, which was supported by various previous studies. Finally, the current study concludes that BPF does interfere with p53 leading large scale elimination of germ cells during spermatogenesis leading to low sperm count and quality.

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Conflict of interest: There is no conflict of interest.

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