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Research Paper:

Altered Testicular Histomorphometric and Antioxidant Levels Following *In vivo* Bisphenol-A Administration

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ABSTRACT

Background: Bisphenol-A (BPA) is a pervasive environmental toxin that is used in the production processes of many consumables and equipment that are in daily application. The aim of this study was to determine the effects of BPA on the structural and functional integrity of the reproductive system in male Wistar rats and its interaction with melatonin.

Methods: Adult female rats in pro-estrus phases were mated with adult male rats and the conception determined. The male pups were divided into two groups of A and B. These groups were further subdivided into six subgroups each. They were administered varying low doses of BPA (25 or 50mg/kg) and melatonin (10mg/kg) at neonatal and adolescent ages. The testes, epididymis and blood samples were collected for histological, semen and biochemical investigations, respectively.

Results: The results show that BPA caused histological alterations, reduced quality and quantity of sperm cells, and induced oxidative stress at birth and adolescence.

Conclusion: Bisphenol A exposure, even at low dose, is toxic to the male reproductive system, and melatonin administration did not significantly improve the alterations caused by the BPA.

Keywords: Adolescence, Bisphenol A, Histology, Melatonin, Neonate

Introduction

Bisphenol-A, 2,2-bis-4-hydroxyphenyl propane (BPA), is an endocrine disruptor which mimics the actions of endogenous estrogen. This compound is an environmental contaminant widely used as monomers to manufacture polycarbonate plastics and epoxy resins i.e., plastics that line food and drink containers, thermal receipts, and medical devices, such as dental sealants. The hydrolysis of BPA monomers from these products causes its release into the environment, due to high temperature at either acidic or basic condition [1]. Although the quantity of BPA released is typically very low but the long-term health risks after prolonged exposure to BPA are significant. Over the past decade, studies on the effects of Endocrine Disrupting Chemicals (EDCs) on the reproductive functions of animals have raised health concerns. In response to these concerns, the World Health Organization (WHO) has several publications, including the recent State of the Science of endocrine disrupting chemicals in 2012 [2]. Similarly, it has been adopted that EDCs were to be in-

cluded in emanating issue under Strategic Approach to International Chemicals Management (SAICM) [3].

Laboratory studies have shown that fetal and neonatal exposure to relatively low doses of BPA may result in reproductive and developmental disorders, including impaired sexual differentiation in the brain [3, 4]. The accumulation of BPA deposits also has clinical implications on the male reproductive system since exposure to its low doses during prenatal life has been shown to affect spermatogenesis in the offspring of male mice [5]. The involvement of BPA has also been reported in cardiovascular disorders, type 2 diabetes and liver enzyme abnormalities in humans in a representative sample of US population [6]. Sex specific changes in the function of infant's hypothalamo-pituitary-adrenal axis, which may culminate in anxiety or depression-like behaviors in offspring, can be associated with prenatal exposure to BPA [7].

Melatonin plays a role in the regulation of circadian rhythms, such as sleep-wake, neuroendocrine and body temperature cycles [8, 9]. Recent reports have associated melatonin with beneficial properties other than sleep-wake regulation. These include anti-oxidative, anti-cancer, hepatoprotective and neuroprotective properties [10-13]. There are numerous publications and research findings to suggest that BPA has toxic and endocrine activities [14]. However, these findings have been controversial at times, creating discrepancies in terms of the reported effects at the suggested doses used in those studies. This has culminated in controversies on the BPA safety and the effects on the normal homeostasis of reproduction.

As the world nations develop and urbanize, the demands for the production and use of BPA also grows. Currently, the application of BPA is growing in foods and beverages packaging, electronic and medical equipment, paper coatings, among others. In this context, there is an upsurge in the possible health threats posed by BPA, as an EDC, since it may be found in our environment, foods, and many publicly consumed products. Therefore, this study aimed at investigating the potential protective effect of melatonin on the BPA-induced testicular lesions in male Wistar rats.

Materials and Methods

Ethical clearance: The animals used in this study were handled and treated humanely, in compliance with the internationally accepted guidelines for the care and use of laboratory animals. The study's protocol was approved by the Ethics and Review Committee (ERC) of the University of Ilorin, Nigeria. The ethical approval to conduct this study was received from the University of Ilorin's Ethics and Research Review Committee (Code: UERC/ASN/2018/1471; Protocol ID: UERC/BMS/112). Specifically, the study was carried out in the animal house of the College of Health Sciences, University of Ilorin between January and October 2019.

Experimental materials: Bisphenol-A and sesame seed oil were purchased from Sigma® (CAS –No: 80-05-7; Germany) while absolute ethanol and melatonin were obtained from a standard laboratory supplier in Ilorin, Nigeria.

Initial animal grouping: Twenty adult female rats weighing 150±10g and 10 adult male rats weighing 200±20g were purchased from a licensed animal breeding farm in Oyo State, Nigeria. Adult female rats in proestrus cycle were housed overnight with male rats of proven fertility. The rats were mated at a ratio of two females to one male rat. The day on which the sperm was found in the vagina was designated as the gestation day 1. Each pregnant rat was isolated in a cage until the parturition day. The rats were kept under controlled environmental condition at 22±2°C, under 14hr of light (06:00 a.m. to 20:00 p.m.) and had access to standard rat food and clean water ad libitum.

Experimental procedures: After parturition, a total of 72 male pups, were broadly divided into 2 Groups of A and B (N=36 in each group), which were further subdivided into six subgroups I to VI, consisting of 6 rats in each:

Group A were given daily subcutaneous injections of the assigned compounds, using insulin syringes, starting from the post-natal day zero for four consecutive days.

Group B received daily gavage administration of the assigned compounds, starting on day 19 of life for 49 consecutive days.

Treatment groups: There were six sub-groups I to VI (N=6, each) within the main groups A and B, each was assigned to the individual treatment plan as follows:

Subgroup I was the control group and received distilled water.

Subgroup II received 10 mg/kg melatonin.

Subgroup III received 25 mg/kg BPA.

Subgroup IV received 25 mg/kg BPA plus 10 mg/kg melatonin.

Subgroup V received 50 mg/kg BPA.

Subgroup VI received 50 mg/kg BPA plus 10 mg/kg melatonin.

All of the groups were kept on their assigned daily treatment until day 80, before they were sacrificed [13]. The recommended Lowest Observed Adverse Effect Level (LOAEL) of BPA reported by previous animal studies has been 50mg/kg [14-18]. However, even at this LOAEL, the safety of BPA is still uncertain. Hence, this study considered the use of the LOAEL as a guide and selected a lower dose (25mg/kg BPA) to observe the potential adverse effects.

Sample collection & analyses: Before sacrificing the animals, they were administered 20mg/kg body weight ketamine intraperitoneally. For oxidative stress markers assay, the blood samples were taken from the apex of the rats' heart, using a 5ml syringe. The blood samples were centrifuged at 3000rpm for 15 minutes to separate the serum. The testes were identified and excised followed by the removal of the cauda epididymis for seminal fluid analysis. The testes samples were also used for histological examinations after being fixed in Bouin's solution. The sections were embedded in paraffin wax and stained according to Canene-Adams' method [19-21]. Additional sections were stained with Hematoxylin and Eosin, using the method described by Fischer, et al. [22]. The epididymis samples were immersed in normal saline for the evaluation of the sperm count, motility and morphology. The sperms were counted, using a hemocytometer and further evaluated via the improved Neubauer chamber (Depth 0.1 mm; LABART, Munich, Germany).

Histomorphometry: The histomorphometry analysis of the seminiferous tubule, lumen and germinal epithelium diameters were performed, using Image J software (National Institute of Mental Health, Bethesda, MD, USA) and the photomicrographs of the testes prepared on an Olympus microscope (SC50) at 40x magnification.

Biochemical assays: The blood samples were centrifuged at 3000 rpm for 15 minutes and the sera were used for the Malondialdehyde (MDA), Glutathione Peroxidase (GPx), Catalase (CAT) and Superoxide Dismutase (SOD) assays. The oxidative stress markers were assessed by an enzyme-linked immunosorbent assay kit (IB79174, IBL-America, Minneapolis, MN, USA) [21].

Data analyses: The statistical analysis of the data was performed, using two-way Analysis of Variance (ANOVA) and Tukey's (HSD) multiple comparison test on GraphPad Prism software, version 6 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the Means±Standard Error of the Means (SEM). The statistical significance level was set at $P<0.05$.

Results

The results demonstrated that BPA caused histological alterations, reduced quality and quantity of sperm cells and induced oxidative stress at birth and at adolescence. Melatonin administration did not appreciably improve the alterations caused by BPA (Figures 1 and 2 and Tables 1, 2 and 3).

Effects on testicular microstructures: As seen in Figure 1, exposure to BPA was associated with decreased intercellular space, distortion of the sperm cells lumen, with marked disruptions of the epithelial lining of sperm cells, especially in groups exposed to 50mg/kg BPA.

Effects on serum reproductive hormones: As shown in Figure 2, the BPA exposure was associated with increased LH levels in comparison to the FSH levels as well as increase in testosterone levels. Exposure to melatonin did not show any significant reversal in the BPA-induced hormonal disruptions.

Effects on testicular histomorphometry: As reflected in Table 1, there was a decrease in the geminal epithelium diameter in the BPA exposed group compared to the controls and melatonin exposed groups. A similar decrease was also observed in the seminiferous tubule diameters in the group exposed to BPA only.

Effects on seminal fluid analysis: There was marked disruptions of the seminal fluid parameters, manifested as significant reductions in all analyzed parameters, such as total sperm counts, morphology and motility (Table 2).

Effects on testicular oxidative stress markers: The BPA exposure was associated with significant alterations in the oxidative stress markers. There was a significant increase in malondialdehyde but reductions in glutathione peroxidase and catalase in the BPA exposed groups compared to the controls (Table 3).

Discussion

The histological observations corroborated those of previous reports [22, 23]. Bisphenol A exerted unfavor-

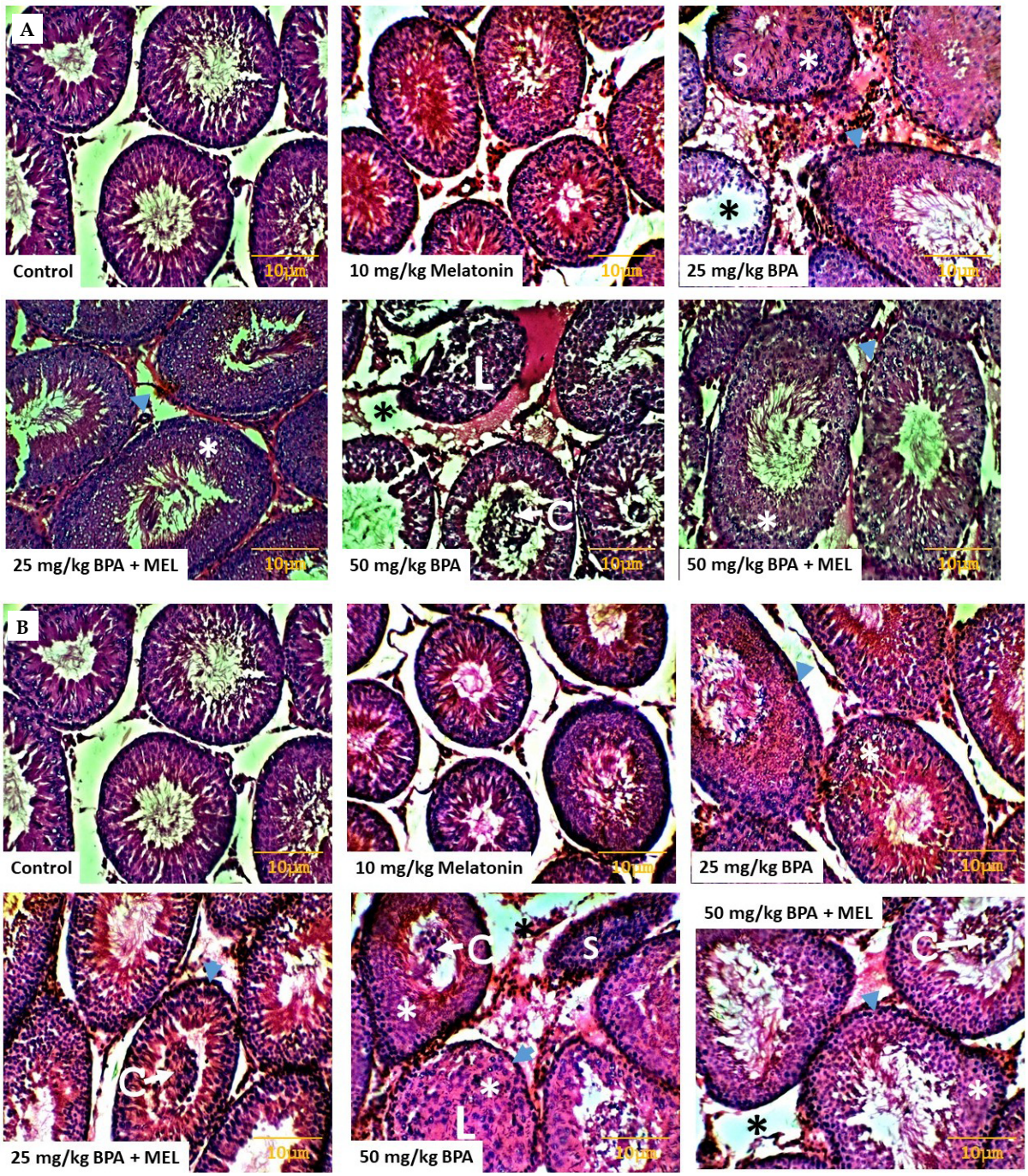

Figure 1. Photomicrograph of testicular microstructure of animals exposed to varying low doses of Bisphenol-A (BPA) and Melatonin (MEL)

Neonatal age Day 0 (Panel A) and adolescent Day 19 (Panel B). Magnification - x100. Stain: Hematoxylin & Eosin (H & E). Degeneration of spermatogenic cells and Leydig cells within the seminiferous tubule and interstitium respectively (*), slowed or no spermatogenesis progression (L), structural alteration of the basement membrane (arrow head), presence of spermatogenic cells in the seminiferous tubule lumen (C), seminiferous tubule displacement and shrinkage (S).

able effects on the testicular microstructures of the treated animals, resulting in reduced sperm counts. These effects are normally worse in animals exposed to BPA at adolescence than those exposed to it at birth [24-26]. Our observations were aided by the histomorphometry whereby noticeable but not significant alterations were observed in the seminiferous tubule structures in rats treated with BPA. The diameters of the germinal epithelia were affected severely in animals treated with varying doses of BPA only, especially those exposed during their

Table 1. Histomorphometric analysis

Groups		A	B
Seminiferous Tubule Diameter (STD) (*10^3 µm)	Control	2.94±0.27	2.94±0.17
	10 mg/kg Melatonin	2.99±0.24	2.59±0.06
	25 mg/kg BPA	3.41±0.17	2.74±0.18
	25 mg/kg BPA + Melatonin	3.09±0.28	3.10±0.05
	50 mg/kg BPA	2.48±0.12	2.42±0.07
	50 mg/kg BPA + Melatonin	3.17±0.03	2.60±0.04
Lumen Diameter (LD) (*10^3 µm)	Control	0.94±0.19	0.94±0.19
	10 mg/kg Melatonin	1.20±0.10	0.79±0.07
	25 mg/kg BPA	1.24±0.24	1.11±0.13
	25 mg/kg BPA + Melatonin	1.31±0.16	1.54±0.14
	50 mg/kg BPA	1.22±0.13	0.92±0.15
	50 mg/kg BPA + Melatonin	1.31±0.13	1.12±0.12
Germinal Epithelium Diameter (GED) (*10^3 µm)	Control	0.74±0.11	0.74±0.11
	10 mg/kg Melatonin	1.02±0.08	0.96±0.06
	25 mg/kg BPA	0.76±0.05	0.63±0.05$^+$
	25 mg/kg BPA + Melatonin	0.73±0.08	0.74±0.05
	50 mg/kg BPA	0.56±0.03$^+$	0.59±0.04$^+$
	50 mg/kg BPA + Melatonin	0.79±0.06	0.78±0.05

Histomorphometry of testes of animals exposed to varying low doses of Bisphenol-A (BPA) and Melatonin at

A: Neonatal age and B: adolescent. Data are shown as Mean±SEM; *P values significant at <0.05 compared to control group; $^+$P values significant at <0.05 compared to melatonin group.

adolescence. This may have been caused by the apoptosis of spermatogenic cells in the seminiferous tubules. Melatonin was unable to effectively palliate the debilitating effects of BPA on the testicular microstructures in the treated rats.

Follicle Stimulating Hormone (FSH) that is produced in the anterior pituitary gland stimulates the Sertoli cells within the seminiferous tubules to synthesize Androgen Bbinding Protein (ABP) [27] and the testicular fluid needed for spermatogenesis. Luteinizing Hormone (LH) acts on the interstitial Leydig cells to secrete testosterone that triggers spermatogenesis [28]. Testosterone also maintains the libido in male animals and humans [24] for that matter.

Based on the current study findings, FSH secretion from the pituitary was affected compared to the controls. The FSH secretion reduced in all BPA treated animals across the developmental stages. This may be due to the toxic effects of BPA on the structural and functional integrity of the hypothalamus in the treated rats. Also, in the experimental group treated with melatonin only, especially in those at adolescence, showed an insignificant reduction in the serum FSH concentrations. This may imply that melatonin altered the FSH expression.

The serum LH concentrations increased in the experimental animals exposed to BPA during their neonatal stage while those exposed to BPA during adolescence showed an insignificant reduction in LH levels in groups treated with BPA only. The testosterone concentrations reduced insignificantly in the experimental animals treat-

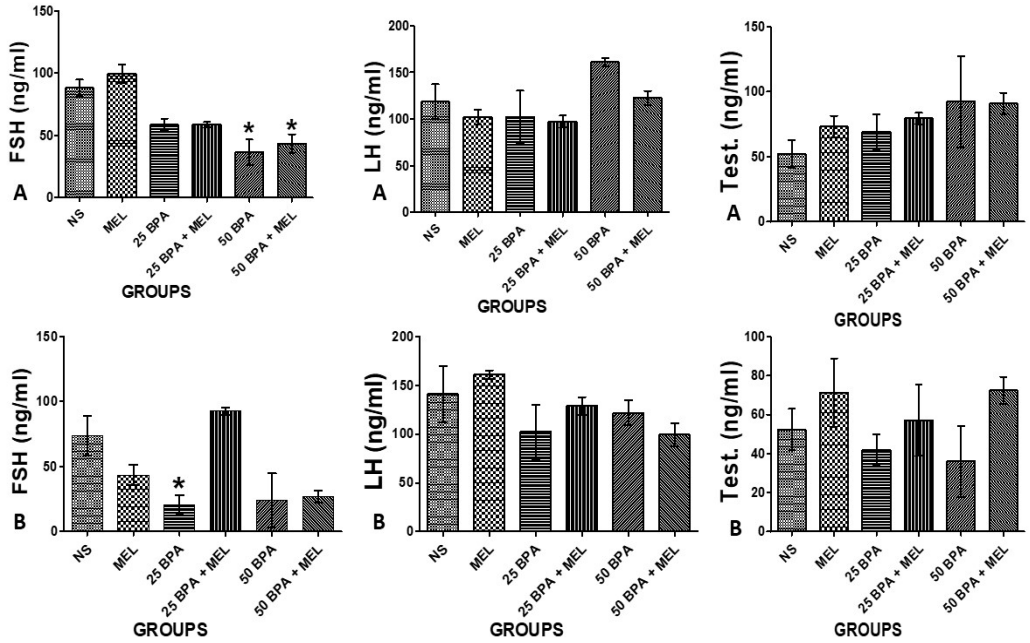

Figure 2. Hormonal profile in animals exposed to varying low doses of Bisphenol-A (BPA) and Melatonin at Neonatal age (PND 0 – 3) and adolescent (Day 19)

A and B : Follicle Stimulating Hormone (FSH) concentration, Testosterone concentration (Test), Luteinizing hormone concentration (LH). Data are shown as Mean±SEM. *P values significant at <0.05 compared to control group.

ed with Bisphenol-A (PBA) during the developmental stage. These findings negate those of previous studies [29-32] that reported that low doses of BPA increased the production of FSH and LH in male rats but decreased the levels significantly at 200mg/kg, indicative of the dose-response relationships [25]. Liang, et al. have also reported a direct association between BPA treatment and increased gonadotropic hormones expression in men [31].

In animals exposed to BPA, either at birth or adolescence, we observed a marked reduction in the concentration, form and mobility of the sperms. This reduction was more pronounced in animals exposed to BPA at adolescence [4, 22]. The low impact of BPA in animals exposed at the neonatal age might be due to the development of immune system alleviating the toxicity of BPA in animals at birth. The innate immune system combated the adverse effect of BPA to some degrees on the sperms, which was reflected by the insignificant rise in the sperm counts, morphology and mobility compared to those exposed to PBA at adolescence. The evaluation of spermatogenesis and transition in the epididymis is most reliably done by the sperm count. In this context, our findings may suggest that the reduced sperm count may be linked to infertility in the male rats.

The reduction in the FSH concentrations in the experimental animals may reflect negatively on spermatogenesis, such that less Androgen Binding Protein (ABP) and testicular fluid would be produced by the Sertoli cells. The testosterone produced by the Leydig cells binds with ABP to drive spermatogenic cell proliferation, and reduction in ABP expression may have impacted negatively on the rate of spermatogenesis in the experimental animals.

Scientists have postulated that one of the mechanisms of BPA action is via oxidative stress i.e., production of more Reactive Oxygen Species (ROS) than the cells can metabolize [27]. Our findings revealed that reduced activities of glutathione peroxidase and catalase were due to their reduced concentrations in the serum of rats exposed at birth and adolescence. The high malondialdehyde concentrations in all BPA treated animals, whether exposed at birth or adolescence, indicate that lipid peroxidation occurred, as evident by the observed alterations in the basement membranes of the testicular seminiferous tubules. The increased concentration of SOD, especially in the 50mg/kg BPA group, implies that there was increased level of the oxidant (superoxide anion), which was dismutated by SOD.

Previous reports have suggested that melatonin has varying degrees of mitigating properties, such as neuroprotective, anti-oxidative and anti-cancer properties in experimental animals [11, 12]. We administered melatonin (10mg/kg) concurrently with varying low doses of

Table 3. Oxidative stress markers

Groups		A	B
Malondialdehyde (U/mg protein)	Control	0.19±0.02	0.19±0.02
	10 mg/kg Melatonin	0.20±0.05	0.21±0.02
	25 mg/kg BPA	1.27±0.02*+	0.99±0.04*+
	25 mg/kg BPA + Melatonin	0.43±0.14	0.59±0.11*+
	50 mg/kg BPA	1.28±0.03*+	0.98±0.06*+
	50 mg/kg BPA + Melatonin	0.55±0.02*+	0.64±0.06*+
Glutathione peroxidase (U/mg protein)	Control	28.1±5.98	28.1±5.98
	10 mg/kg Melatonin	12.56±5.17*	46.8±7.80
	25 mg/kg BPA	22.03±4.45	15.9±6.27+
	25 mg/kg BPA + Melatonin	16.83±2.82*	9.0±2.08+
	50 mg/kg BPA	26.81±9.37	9.76±1.96+
	50 mg/kg BPA + Melatonin	27.48±6.34	16.4±8.47+
Catalase (U/mg protein)	Control	140.7±16.69	140.7±16.69
	10 mg/kg Melatonin	147.9±5.469	121.3±11.06
	25 mg/kg BPA	114.0±5.719	74.41±2.59*+
	25 mg/kg BPA + Melatonin	138.1±6.767	69.16±7.16*+
	50 mg/kg BPA	89.54±3.732*+	60.04±3.98*+
	50 mg/kg BPA + Melatonin	93.07±6.430*+	67.67±3.68*+
Superoxide dismutase (U/mg protein)	Control	293.2±43.42	293.2±43.42
	10 mg/kg Melatonin	373.1±71.81	257.0±12.04
	25 mg/kg BPA	137.6±7.22	709.0±13.22
	25 mg/kg BPA + Melatonin	310.9±80.28	273.7±55.64
	50 mg/kg BPA	373.1±71.81	640.4±10.97
	50 mg/kg BPA + Melatonin	242.5±51.91	580.4±20.73

Antioxidant levels in animals exposed to varying low doses of Bisphenol-A (BPA) and Melatonin at A - Neonatal age and B – adolescent. Data are shown as Mean±SEM; *P values significant at <0.05 compared to control group; +P-values significant at <0.05 compared to melatonin group.

BPA to the experimental rats. At this dosage, melatonin was unable to effectively counter the adverse effects of BPA on the male reproductive system. This was evident as the slight improvements seen in the histological photomicrographs, semen evaluation and concentrations of the oxidative stress markers in groups concurrently treated with BPA and Melatonin. Melatonin at the dose tested had more noticeable ameliorating effects on animals treated with 25mg/kg BPA than those with 50mg/kg.

BPA exerted its toxic effects on the reproductive system of the experimental animals irrespective of the age and dosage. It is likely that BPA was not easily cleared from

Table 2. Semen quantity and quality evaluation

Groups		A	B
Total Sperm count (*106 ml)	Control	105.0±9.57	105.0±9.57
	10 mg/kg Melatonin	110.0±577	115.0±5.00
	25 mg/kg BPA	47.5±6.29*+	45.0±13.23*+
	25 mg/kg BPA + Melatonin	65.0±5.40*+	60.0±2.04*+
	50 mg/kg BPA	75.0±5.00+	31.0±13.03*+
	50 mg/kg BPA + Melatonin	70.0±10.8*+	26.3±7.47*+
Sperm morphology (%)	Control	88.8±1.25	88.8±1.25
	10 mg/kg Melatonin	95.0±1.78	92.5±1.44
	25 mg/kg BPA	60.0±3.54*+	63.8±3.75*+
	25 mg/kg BPA + Melatonin	65.0±2.04*+	55.0±5.00*+
	50 mg/kg BPA	65.0±2.89*+	40.0±5.77*+
	50 mg/kg BPA + Melatonin	66.3±2.39*+	45.0±2.89*+
Sperm motility (%)	Control	81.3±4.27	81.3±4.27
	10 mg/kg Melatonin	93.8±1.25	91.0±3.72
	25 mg/kg BPA	42.5±7.50*+	38.8±5.15*+
	25 mg/kg BPA + Melatonin	53.8±4.27+	55.0±6.46*+
	50 mg/kg BPA	32.5±2.50*+	40.0±2.04*+
	50 mg/kg BPA + Melatonin	43.8±14.34*+	50.0±4.08*+

Semen quality and quantity evaluation of animals exposed to varying low doses of Bisphenol-A (BPA) and Melatonin at A - Neonatal age and B – adolescent. Data are shown as Mean±SEM. *P-values significant at <0.05 compared to control group; +P values significant at <0.05 compared to melatonin group.

the biological system or that BPA had a longer period of interaction with the biological systems in the Wistar rats.

Conclusions

Our findings have been able to demonstrate that BPA exposure resulted in histological alterations, reduction in both sperm quality and quantity as well as induction of oxidative stress, at lower than recommended dosage. Therefore, we conclude that exposure to BPA, even at lower dose than the benchmark is toxic to the male reproductive system of Wistar rats, and that melatonin did not significantly reverse the BPA-induced toxicity.

Limitations of the study: Lack of funding and access to electron microscope needed for viewing the ultrastructure of the testicular tissue was a limitation of this study.

Recommendations for future research: There is a need for further research to come up with alternatives to Bisphenol-A use in domestic products. There needs to be further enforcements of policies that are governing the production and marketing of BPA-containing products to protect the populace from the adverse effects of BPA.

Ethical Considerations

Compliance with ethical guidelines

Ethical approval to conduct this study was received from the University of Ilorin Ethics and Research Review Committee (Protocol Approval Cole: UERC/ASN/2018/1471; Protocol I.D. Code: UERC/BMS/112).

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Author's contributions

Conceptualisation: Eniola Risikat Kadir; Methodology: Eniola Risikat Kadir, Lekan Sheriff Ojulari, Gabriel Olaiya Omotoso; Data collection, investigation: Eniola Risikat Kadir, Taiye Abdullah Gegele, Lukman Sulu-Gambari, Ismail Adetayo Lawal; Resources and funding, writing, review, editing: All authors. Supervision:Gabriel Olaiya Omotoso.

Conflict of interest

The authors declared no conflict of interest.

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