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Restorative effects of melatonin on bisphenol a-induced interference of gene expression in hypothalamic pituitary axis following early exposure

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***Abstract*---**Background: Bisphenol-A is a standard monomer used industrially in manufacturing plastics and epoxy resins, and it is widely used in food preservation and packaging. There is a global

increase in its use due to increased demand and the growth in world population. Bisphenol A is an endocrine-disrupting chemical miming the endogenous estradiol hormone. However, due to its exposure to the environment, food and other consumables, its effects on reproductive health have been a growing area of interest. Melatonin regulates sleep-wake cycles and plays essential physiological roles in the body through its antioxidative, anti-cancer and neuroprotective properties. This research aims to ascertain the impact of Bisphenol A on the hypothalamic-pituitary-ovarian axis and determine melatonin's function on possible BPA-induced effects. Methods: Six adult male Wistar rats and 12 adult female Wistar rats of proven fertility were bred and organized into groups. Litters were divided into seven groups, each comprising six rats. These animals were subjected to subcutaneous injections of high and low doses of bisphenol A from postnatal days 0-3, then oral melatonin. The rats were allowed to mature into full-grown adults and euthanized at 120 ±4 days. The serum and hypothalamic-pituitary-ovarian tissues were collected for various assays, histology and genetic studies. Results: Compared to the control groups, groups administered varying doses of bisphenol A showed significant overexpression of estrogen and androgen receptors. Administration of Melatonin showed some reversal and reparative effects on BPA-induced damage of the hypothalamic pituitary ovarian axis. Conclusion: Elevated estrogen receptor levels induced by Bisphenol A altered receptor function, ultimately impairing hormonal cascades that regulate reproductive functions. Melatonin showed some promising reparative effects.

***Keywords*---**Bisphenol-A, Melatonin, endocrine disruptor, estrogen, Androgen, Receptors.

1. Introduction

The hypothalamic-pituitary-gonadal axis is an inter-communicating set of tissues that plays a vital role in regulating body systems, especially the reproductive system. GnRH-expressing neurons in the hypothalamus secrete the gonadotropin-releasing hormone, which is released in pulses stimulating the pituitary gland to secrete Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) by binding to the Gonadotropin-releasing hormone receptor (GnRHR). The action of LH and FSH on the gonads includes the production of estrogen and testosterone (Chen *et al.*, 2021).

Bisphenol-A, otherwise written as 2,2-bis (4-hydroxyphenyl) propane (BPA), is an endocrine-disrupting chemical (EDC) and environmental contaminant that can impersonate the activities of endogenous estrogen hormones. BPA is used extensively as a monomer in manufacturing epoxy resins and polycarbonate plastics for medical devices such as dental sealants, thermal receipts and plastics employed in food and drink containers (Almeida and Almeida-gonz, 2018). In high temperatures and acidic or basic conditions, BPA monomers are hydrolyzed from these products and released into the environment (Rubin, 2011). The

consumption of BPA globally attained 5.5 million metric tons in 2011 and was present in 95% of urine samples in the United States (Cao et al., 2018). While the amount of BPA released is small, the chronic health hazards associated with long-term exposure are remarkable (Hengstler et al., 2010). As countries develop and urbanize, BPA production demands have also increased use in food, beverages, electronic equipment, medical equipment, and paper coatings packages. However, there is a rising concern about the potential health hazards EDCs (including BPA) may cause since they are present in our environment, food, and other products consumed daily (Tempfer et al., 2000). In the last decade, there has been an increasing focus and concern on the impact of EDCs on animals' reproductive functions. The World Health Organization (WHO) published the State of the Science on EDCs to address these concerns in 2012. In addition, EDCs were included in issues raised under the Strategic Approach to International Chemicals Management [SAICM] (Gore et al., 2014). Studies have indicated that minimal exposure to BPA during fetal or neonatal stages could lead to developmental and reproductive effects, which include disruption of sexual differentiation in the brain (Rubin et al., 2006). Giesbrecht *et al.* also reported sex-specific changes in hypothalamic–pituitary–adrenal (HPA) axis function in infants following prenatal BPA exposure in their human study (Giesbrecht et al., 2017).

Melatonin regulates circadian rhythms, including body temperature regulation, neuroendocrine control, and sleep-wake cycles (Liu et al., 2016; Ekmekcioglu, 2006). Recent findings indicate that melatonin possesses beneficial properties beyond sleep-wake cycle regulation, such as neuroprotective, antioxidative, hepatoprotective, and anti-cancer properties (Eid et al., 2015). Also, some studies have documented the physiological roles of melatonin in reparative functions in the body, its antioxidative effect, and therapeutic roles for reproductive damage (Mayo et al., 2016)

BPA disrupts the endocrine milieu, thereby disrupting the regular hormonal milieu, which may also contribute to infertility of unknown origin if exposed continuously. Furthermore, the crucial and vulnerable periods of EDC susceptibility are during intrauterine life, infancy, childhood and puberty (Huo et al., 2015); they are known as the period of reproductive formation. Ziv-Gal and Flaws further stated that BPA might disrupt oestrous cyclicity and implantation. They also noted that further studies are required to identify potential toxicity to the reproductive system at earlier stages (Ziy-gal and Flaws, 2017). For BPA, the recommended Lowest Observed Adverse Effect Level (LOAEL) is 50 mg/kg in animal studies (Fao and W.H.O.E, 2010; Peretz et al., 2007; Richter et al., 2008; Vandenberg et al., 2013; States et al., 2007). Despite this LOAEL, there are still uncertainties about BPA safety in this dose. Therefore, this study used two doses: the 50 mg/kg recommended LOAEL for animals and 25 mg/kg, which is a lower dose than the LOAEL, at postnatal day (PND) 0-3 to detect the effects even with a reduced dose following early neonatal exposure to BPA on reproductive maturity.

Oxidative stress (OS) occurs due to an imbalance between the levels of antioxidants and oxidants (free radicals or reactive species), resulting in a continuous increase in reactive nitrogen and oxygen species (RNS and ROS, respectively), generated as by-products of cellular metabolism (Bratovcic, 2020). Superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase are the

essential antioxidants of the defence system that remove free radicals that are produced during metabolic reactions (Sathiya et al., 2015). Nitric oxide is a free radical that mediates physiological functions. A study by Tempfer *et al.* shows that application of Nitric oxide synthase inhibitors locally, such as NG-methyl-L-arginine and aminoguanidine, can prevent in vivo ovulation in rats, and a reversal effect is observed in vivo studies when nitric oxide generators, such as sodium nitroprusside, was added (Tempfer et al., 2000)

Uridine diphosphate glucuronosyl transferases (UDP-GT) are proteins in the endoplasmic reticulum membrane and are primarily an isoenzyme present in the ovaries of rats (Lee et al., 2019). Glucuronide is the primary metabolite of BPA in rats, and large quantities of unmodified and hydroxylated BPA are detected in feces following oral ingestion of 14C-labelled bisphenol-A. Investigations have also shown monoglucuronide to be the major metabolite of BPA in rats and humans (Aboul Ezz et al., 2015). Metabolic conjugation of BPA in rats has been recommended to be, for the most part, catalyzed by UDP-GT. UDP-GT is critical in metabolizing and removing xenobiotics in the gonads and liver. Inadequate clearance of toxins in ovarian cells leads to increased xenobiotics in the ovary, thereby impairing the secondary and tertiary follicles and preventing ovulation, resulting in subfertility in the reproductive ages (Hoyer and Keating, 2023).

The hypothalamus contains brain circuits that regulate the activity of GnRH neurons that regulate reproduction: the androgen receptors, nuclear receptors, GnRH mRNA and the Kisspeptin mRNA. Kisspeptins-54 (KP-54) are peptide results of the KiSS 1 gene, constituting part of the control of the hypothalamic-pituitary-gonadal path (Branavan et al., 2019), prominently expressed in the hypothalamus's arcuate nucleus and the anteroventral periventricular nucleus (ARC and AVPV, respectively). The proximity of kisspeptin neurons to GnRH neurons and the expression of kisspeptin receptors by GnRH neurons result in the production of GnRH in both in-vivo and in vitro studies, ultimately leading to the regulation of ovarian activities (Skorupskaite et al., 2014). Subcutaneous infusion of kisspeptin to infertile women with hypothalamic amenorrhoea resulted in increased plasma gonadotrophs, and pulsatile administration of kisspeptin led to continued gonadotropin secretion (Trevisan et al., 2018). In addition, the GnRH neurons also express the receptor antimullerian hormone receptor-2 (AMHR2). AMRH2 receptor is activated by antimullerian hormone, which then triggers the production of gonadotropins (Barbotin et al., 2019; Kereilwe and Kadokawa, 2020).

Given the known endocrine disrupting effect of BPA, especially at the critical and delicate period of greatest vulnerability to EDCs occurring mainly during in-utero, infancy, early childhood till puberty (Huo et al., 2015), we hypothesized that BPA at the recommended LOAEL in animals would not negatively affect neonatal-exposed Wistar rats. In addition, our second hypothesis was that melatonin would not have an effect following BPA exposure, which was aimed at elucidating the effects of melatonin on the hypothalamic-pituitary-ovarian axis in neonatal Wistar rats exposed to BPA for short periods. Thus, this study will further add to the literature aiming to resolve the controversies surrounding the health safety effects of BPA and the possible role of melatonin in this regard. The scope of this study was limited to female Wistar rats, and hypothalamic-pituitary-ovarian (HPO)

organs were analyzed using RNA studies, histology, and biochemical analysis of the HPO axis.

2. Materials and Methods

The study was conducted in two phase: Phase I involved breeding the animals to obtain the second-generation neonatal Wistar rats. Phase II involved administering varying doses of BPA and melatonin to these second generation female animals for the experiment, then were allowed to grow into adulthood. The rats were then sacrificed at proestrus phase of the cycle for analysis. Blood was aspirated from the apex of the heart for hormonal analysis using Enzyme-Linked Immunosorbent Assay (ELISA) technique with ELISA kits. The whole brain was excised, and the hypothalamus and pituitary were taken and fixed in RNA later for PCR quantification of proteins. The ovaries were harvested for oxidative markers and histology.

The data were analyzed using GraphPad Prism. 9.0 Software (San Diego, CA, USA). The Shapiro–Wilk test was used to determine if the data was normally distributed. Further analysis was then with parametric tests, analysis of variance (ANOVA) then Tukey's post-hoc analysis. Data are presented as means ± SEM (standard error of the mean). Statistical significance was taken as less than 0.05 ($p<0.05$).

The animals were humanely treated, following the standard guidelines for the use and care of experimental animals. The research obtained protocol approval from the Ethical Review Committee (UERC/ASN/2018/1154), University of Ilorin, Ilorin, Nigeria. This research was conducted in the Animal Holding of the College of Health Sciences, University of Ilorin.

Experimental design, Breeding and Grouping of animals

Bisphenol A and sesame seed oil vehicle were procured from Sigma® (CAS –No: 80-05-7) in Germany. Also, absolute ethanol and melatonin were purchased from the central research laboratory in Ilorin. For this experiment, we analyzed the HPO axis.

To justify our hypotheses, we analyzed the hypothalamus and pituitary and assessed some proteins that regulate reproduction. We went further by assaying antioxidants and tissue histology for the ovarian tissues. In addition, a serum analysis of hormones secreted in the HPO axis regulating reproduction was assayed.

Animals used for the experiment were procured from a neighboring State in Nigeria. Fourteen mature female Wistar rats weighing 150 ± 10 g and seven mature male Wistar rats weighing 200 ± 20 g, bred in the Oyo farm holding, were purchased. The animals were maintained in a clean and habitable environment, with a regular day and night schedule. They had access to water liberally and chow rat-mouse diet freely. They were acclimatized for seven days in our College of Health Sciences Animal Holding.

The study was conducted in two phase: Phase I involved breeding the animals to obtain the second-generation neonatal Wistar rats. Phase II involved administering varying doses of BPA and melatonin to these second generation female animals for the experiment.

Following acclimatization, the females had vaginal smears in the morning between 7 a.m. and 9 a.m. Female rats in the proestrus phase of the estrus cycle cohabited overnight with adult Wistar rats whose fertility had been proven, in a ratio of one male to one female rat. The exposed females then had another vaginal smear the following morning. Detection of sperm cells in the vaginal smear was taken as Gestational Day 1 (GD 1). Once pregnancy was confirmed, the pregnant rat was kept in a different wire gauze cage to stay until parturition, with 12 pregnant females being used for the study. These constituted the first generation of animals.

Following parturition, the pups were examined, and the females selected for the study were randomly assigned into seven groups, having 6 litres in each group. These were the second-generation Wistar rats that were used for the study. They had daily subcutaneous injections from postnatal day 0 for four days (PND 0 - 3). The seven groups had the following order of administration: I – Control group administered Normal saline; II – vehicle control group was administered sesame oil and ethanol (vehicle); III - melatonin only (10 mg/kg) (Juma'a et al., 2009) IV – 25 mg /kg BPA; V – 25 mg/kg BPA + melatonin (10 mg/kg); VI – 50 mg/kg BPA; and VII – 50 mg/kg BPA + melatonin (10 mg/kg). These doses were based on animal studies' recommended LOAEL of BPA (Fao and W.H.O.E, 2010; Peretz et al., 2007; Richter et al., 2008; Vandenberg et al., 2013; States et al., 2007). All administrations were done in the morning between 7a.m and 9a.m of each day, and all the groups were allowed to mature till adulthood day 120 ± 4 days, when they were euthanized in the morning, between 7 a.m. and 9 a.m (Peretz et al., 2014).

Sample collection and processing

After administration of BPA and melatonin treatments and following the maturation of the animals till day 120 ± 4 days, they were euthanized by administration of 20 mg/kg body weight of ketamine intramuscularly (Eshar et al., 2019). A midline longitudinal abdominal incision collected the ovaries on both sides. Then this incision was extended to the diaphragm, then the thoracic cage and the heart was seen beating, through which a 5 mL needle and syringe were inserted into the apex to collect the blood sample of the Wistar rats. Following this, a midline longitudinal scalp incision was made with the lateral reflection of the scalp; the skull was promptly exposed with tissue forceps, and then the brain was extracted. The hypothalamus, situated alongside the pituitary gland at the floor of the sella turcica, was pinpointed, collected, and placed into cryovials preloaded with RNA later and kept frozen in liquid nitrogen tanks. The harvested brains were then utilized for genetic (RNA) studies.

The harvested female gonads were used for follicular count on histology (the left gonads), which was fixed for 48 hours in 4% paraformaldehyde, while the right ovary of each animal was homogenized in 0.25 M ice-cold phosphate buffer

followed by centrifugation of homogenates for enzyme studies. The ovarian tissue was stored in paraffin wax embedded sections according to Canene-Adams (Canene-Adams, 2013) and Haematoxylin and Eosin staining by the protocols outlined by Andrew and Kenneth, 2014). The various follicles (primary, secondary, preantral, antral, and corpora lutea were identified, counted, and then recorded. Three sections per ovary were counted using ImageJ software.

Hormonal Assay

Whole blood was collected from the apex of the animal's heart using 5 mL needles and syringes for hormonal analysis. The sample was spun in a centrifuge to obtain the serum at 3000 rpm for 15 minutes. Plasma LH, FSH, estrogen, progesterone, testosterone, and anti-Mullerian hormone concentration measurements were assayed using the Enzyme-Linked Immunosorbent Assay (ELISA) technique with ELISA kits purchased from Monobind Inc., Lake Forest, USA, following the manufacturer's guide.

Biochemical assay

Nitric oxide levels were assessed following the method outlined by Moneim, 2012, which involved measuring the levels in an acidic environment, where nitrite, upon formation, was used to create nitrous acid, which reacted with sulfanilamide to form a product. This resulting product was then coupled with N-(1-naphthyl) ethylenediamine to give an azo dye displaying a vibrant reddish-purple color, quantifiable at 540 nm. Glutathione peroxidase (GPx) activity was determined using the method outlined by Paglia and Valentine, 1967. A unit of GPx activity was defined as the enzyme quantity necessary to catalyze the oxidation of 1 nmol NADPH per minute at 25 °C. The assessment of Superoxide dismutase (SOD) was carried out following the method detailed by Nishikimi *et al.*, 1972. The SOD enzyme's capability to impede the phenazine methosulfate-mediated reduction of nitro-blue tetrazolium dye was measured by monitoring absorbance at 560 nm over 5 min at 25 °C.

Quantitative real-time polymerase chain reaction procedure

Euro Gold Tri-Fast solution (Euro Clone) was used to prepare the RNA. The tissue was pulverized using a tissue homogenizer, followed by DNase treatment on the extracted RNA samples to eliminate DNA contamination from the total RNA prepared. Purification (through acid phenol-chloroform), precipitation, and suspension of the RNA in distilled water (dH2O) were then carried out.

The reverse transcriptase enzyme (Invitrogen) was used for the reverse transcription of total RNA, using M-MLV reverse transcriptase (Invitrogen), which involved retrotranscription of 1 µg of total RNA in quantifying mRNA expression in experiments. The samples were gently mixed by repetitive pipetting up and down and then incubated at 37 °C. The M-MLV reverse transcriptase was rendered inactive by heating at 70 °C for 15 minutes. The complementary DNA was stored at -20 °C.

1300

NanoDrop™ 1000 spectrophotometer was used to measure cDNA and RNA concentrations. Nucleic acid quality was noted via absorbance ratios at 260 nm/280 nm and 260 nm/230 nm for cDNA and RNA, respectively. Quantifying the relative amount of the transcript of a particular gene was done using a qRT-PCR. Then, amplification and generating melting curves for the amplicons were performed using a two-step technique with Sybr green supermix (Biorad). The melting curve data was verified by running the PCR product on 2% agarose gel. The primer's efficacy was checked in reactions with six consecutive 1:10 dilutions of complementary DNA that served as a template; then, a calibration curve was plotted.

Primers sequence (rattus novergicus)

	Primers	**Sequence**	**Expected Product (bp)**
1	**Nuclear receptor 1I3 (NR1I3, CAR)**		
	RT-mCAR-DIR	GCCATGGCTCTCTTCTCTCC	160
	RT-mCAR-REV	CTAGCAGGCCCATCAGCTTT	
2	**Androgen receptor (Ar)**		
	RT-mAr-DIR	CAGGGACCACGTTTTACCCA	229
	RT-mAr-REV	TTTCCGGAGACGACACGATG	
3	**Anti-mullerian hormone receptor (Amhr)**		
	mAmh-DIR	CTGGGAGCAAGCCCTGTTAG	180
	mAmh-REV	GGTTGAAGGGTTAGGGCGAG	
4	**KISS1 receptor (Kiss1r)**		
	mKiss1r-DIR	GCTAGTCGGGAACTCACTGG	120
	mKiss1r-REV	ACGCAGCACAGAAGGAAAGT	
5	**Gonadotropin-releasing hormone 1 (Gnrh1)**		
	mGnrh1-DIR	TGGTATCCCTTTGGCTTTCAC A	192
	mGnrh1-REV	GATCCTCCTCCTTGCCCATC	
6	**Gonadotropin releasing hormone receptor (Gnrhr)**		
	mGnrhr-DIR	GCCTCAGCCTTGTCTCATGT	140
	mGnrhr-REV	TATGTTGGGCTTTCCCGGTC	

Statistical methods and analysis

The data were analyzed using GraphPad Prism. 9.0 Software (San Diego, CA, USA). The Shapiro–Wilk test was used to determine if the data was normally distributed. Further analysis was then with parametric tests, analysis of variance (ANOVA) then Tukey's post-hoc analysis. Data are presented as means ± SEM (standard error of the mean). Statistical significance was taken as less than 0.05 ($p<0.05$)

3. Results

Hormonal analysis

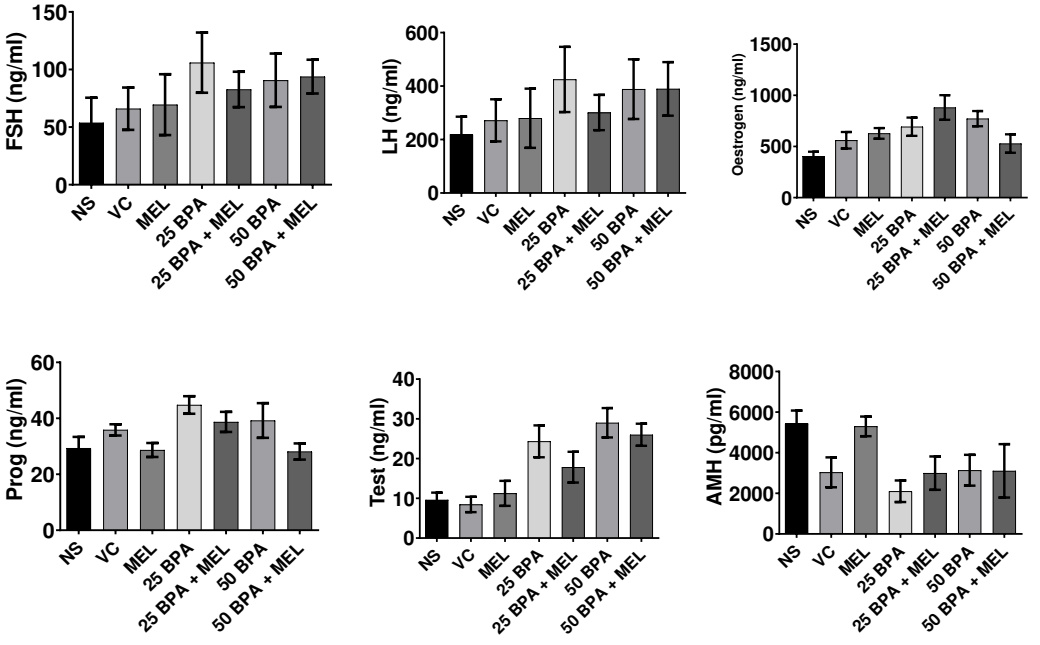

Fig 1: Hormonal effects following BPA administration in newborn Wistar rats

Follicle-stimulating hormone, LH, and testosterone levels were increased in the experimental groups administered BPA compared to the vehicle control groups. However, these increases were not statistically significant. Anti-mullerian hormone (AMH), an indicator of ovarian reserve level, decreased in the experimental groups with BPA administered all through compared to the control groups, which was similarly not statistically significant.

Biochemical analysis

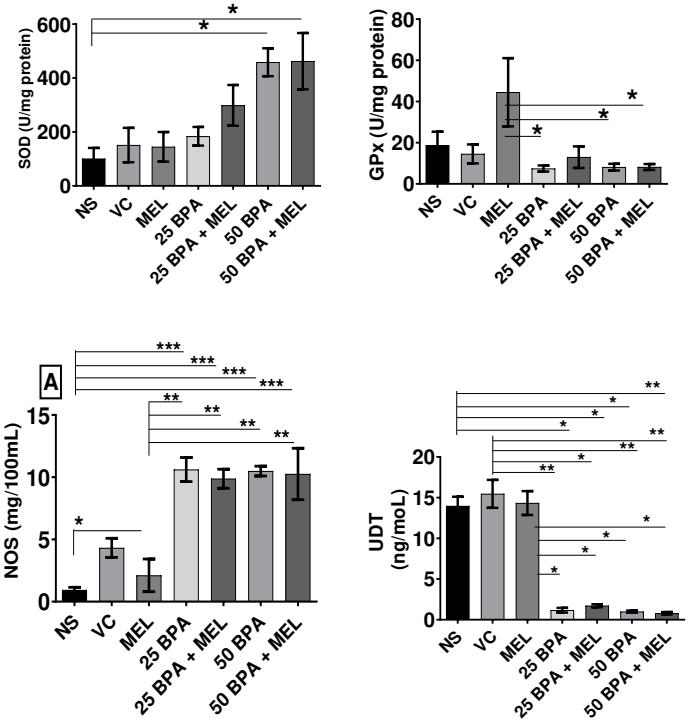

Fig 2: Levels of oxidative and metabolic enzymes of the ovarian tissue in newborn Wistar rats
*: P < 0.05; **: P < 0.01; ***: P < 0.001

There were significantly decreased levels of SOD and GPx as well as Uridyl diphosphate (UDP, a metabolic enzyme in the ovary) in the experimental groups administered BPA all through. At the same time, the level of Inos increased significantly. Melatonin showed some appreciable effects observed in the BPA-administered groups.

Histology

Decreased corpus luteum count in all experimental groups administered bisphenol-A, and appreciable differences indicated some structural restoration in the melatonin groups compared to the bisphenol-A-only groups. See Figure 3 below.

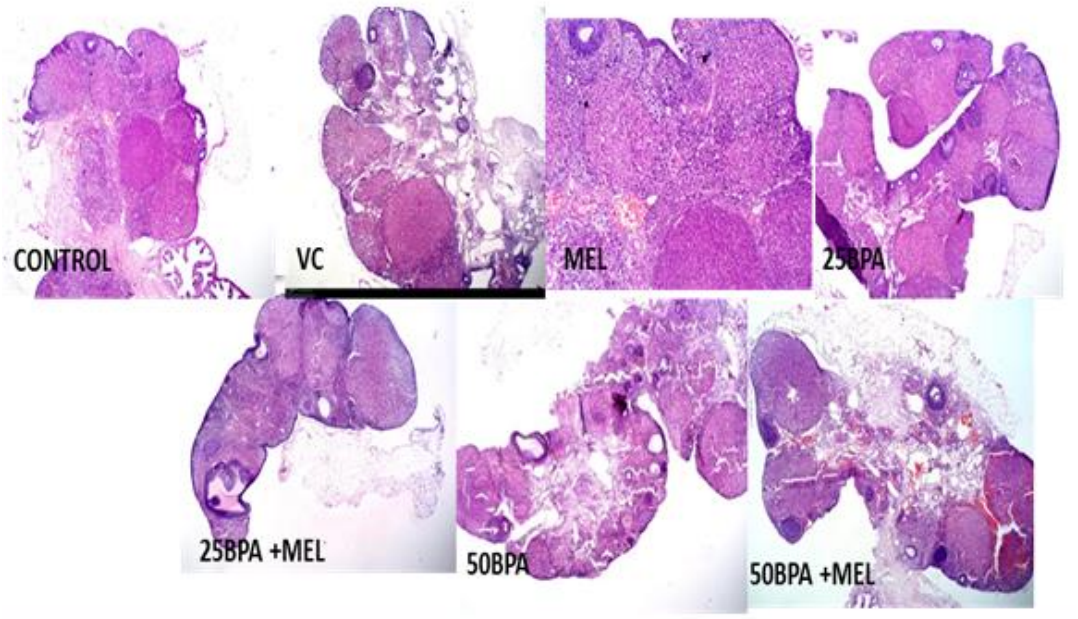

Fig 3a: Corpus luteum count in female Wistar rats administered Bisphenol A and Melatonin at low doses. *+ P≤0.05. * statistical significant different compared to control, + - compared to vehicle control. CP – corpus luteum

Corpus luteum count

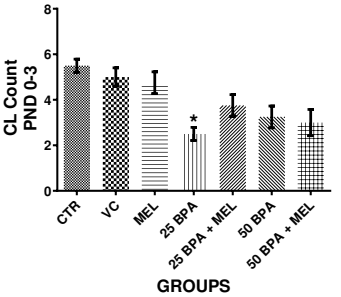

Fig 3b: Corpus luteum count in female Wistar rats administered Bisphenol A and Melatonin at varying low doses from H&E. *: P < 0.05 compared to control

Histomorphometry

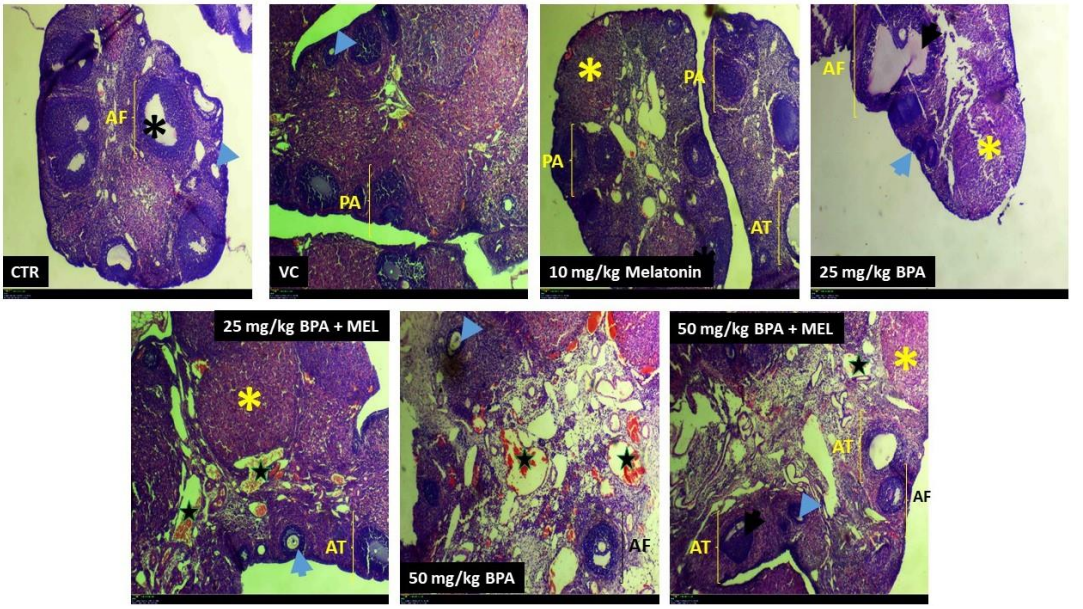

Fig 4a: Photomicrograph showing the rate of folliculogenesis in female animal rats administered Bisphenol A and Melatonin at varying low doses at neonatal age**. * -** corpus luteum**, arrowhead (blue) –** primary follicle**, AT –** follicular atresia**, PA,** preantral follicle, **star** – blood vessels, **fd** – graffian follicle degeneration, arrow head (black) – distorted antral follicle.

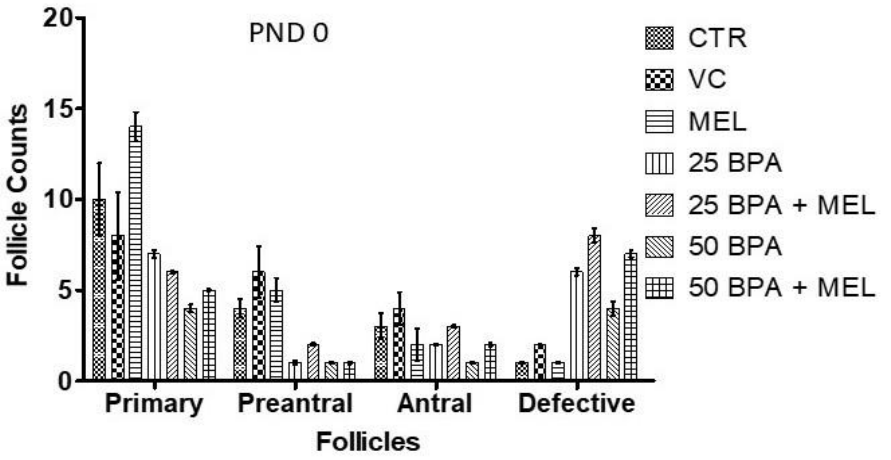

Fig 4b: Follicular counting in female animal rats at neonatal age administered different low Bisphenol A and Melatonin doses.

qPCR

The experimental groups administered BPA significantly overexpressed estrogen, androgen, and Kiss 1 (GPR54) receptors compared to the control groups. Similarly, the expression of AMH mRNA was increased. These are shown in Table 1. qPCR showing underexpression of Gonadotropin-releasing hormone receptor (GnRHr) while GnRH mRNA level was increased in the BPA only administered groups.

Table 1: Hypothalamic and pituitary expression of mRNA and receptors in animals administered different low doses of BPA and Melatonin at neonatal age.

Groups	**AR**	**ER**	**GPR54**	**AMH mRNA**	**GnRH mRNA**	**GnRH**
Control	1.01±0.10	1.01±0.09	1.00±0.05	1.01±0.07	1.05±0.24	1.12±0.39
Vehicle control	2.06±0.48	0.58±0.13	0.81±0.04	1.19±0.30	1.29±0.41	1.04±0.35
10 mg/kg melatonin	0.57±0.26	0.95±0.27	0.50±0.06	5.42±0.82	1.32±0.16	1.20±0.43
25 mg/kg BPA	1.75±0.01	6.64±0.19*#+	1.51±0.59	14.39±0.00*#	1.61±0.24	1.86±0.75
25 mg/kg BPA + 10 ml/kg Melatonin	1.16±0.28	4.40±1.37*#+	1.68±0.75	10.68±0.47	1.18±0.24	1.12±0.35
50 mg/kg BPA	4.77±0.17*#	3.16±0.08	3.78±0.55*#	42.17±135*#+	35.55±2.13	3.26±1.78
50 mg/kg BPA + 10 ml/kg Melatonin	2.83±0.54*#	1.19±0.22	2.55±0.14#	19.16±6.19*#+	24.68±22.51	0.75±0.36

AMH – Anti-mullerian hormone, GnRH – Gonadotropin-releasing hormone, ER – Estrogen receptors, AR – Androgen receptors, GPR54 – KiSS1 receptor, mRNA – messenger ribonucleic acid
*#+ P≤0.05 ***** - compared to control; **#** - compared to vehicle control; **+** compared to melatonin.

4. Discussion

The neonatal stage of development represents a stage where neonates undergo developmental programming, during which exposure to environmental stressors can alter regular programming that may evoke detrimental effects in adulthood. Like other endocrine-disrupting chemicals (EDCs), our study has shown that BPA can trigger estrogen signalling pathways. It has been implicated in causing damaging effects on the reproductive systems via endocrine disruption, oxidative stress, and genomic damage. BPA toxicity occurs through lipid peroxidation, increased reactive oxygen species (ROS) and the generation of free radicals, causing oxidative stress.

We have analyzed the hypothalamic pituitary ovarian axis to determine the path of BPA-induced damage. We also explored the roles of melatonin as an intervention model, an antioxidant with varied functions that modified these

distortions induced by BPA. Melatonin (MEL) is a well-established antioxidant with a high free radical scavenging property. It can cross biological barriers, hence preventing oxidative damage by agitating antioxidant enzymes through inhibiting pro-oxidative enzymes, preventing lipid peroxidation and preventing cell membrane destruction. Thus, this study examined the effects of low-dose bisphenol A at 25 and 50 mg/kg, based on the recommended LOAEL (Fao and W.H.O.E, 2010; Peretz et al., 2007; Richter et al., 2008; Vandenberg et al., 2013; States et al., 2007), and Melatonin (10 mg/kg) (Juma'a et al., 2009) on hormonal, biochemical, and expression of genes related to female reproduction in neonatal Wistar rats.

To begin, we examined the effects of BPA on plasma levels of reproductive hormones, FSH, LH, testosterone, estrogen, progesterone, and AMH. We observed some increased hormonal levels of FSH and LH and decreased AMH levels in the groups treated with BPA compared to the vehicle control (VC) group, though not statistically significant. The alterations observed in plasma hormone levels in response to BPA exposure, such as increased FSH, LH, estrogen, and testosterone levels, reflect the intricate disruption of the hypothalamic-pituitary-ovarian (HPO) axis. BPA's ability to mimic or interfere with hormonal actions manifests in the increased levels of FSH and LH, essential for ovarian function, potentially indicating an overstimulation of the HPO axis (Gamez et al., 2014; Mukhopadhyay et al., 2022). Animal studies have shown that low-dose BPA increases the release of FSH and LH from the pituitary gland in female rats, with the effect being more pronounced on LH than on FSH. In adult female rats and mice, exposure to oral administration of low-dose BPA was associated with increased serum FSH and LH levels during the proestrus phase (Juan et al., 2016). A possible mechanism by which this occurs is that BPA selectively stimulates AVPV-kisspeptin neurons in the hypothalamus, as shown by our finding of elevated kisspeptin receptors. Our result shows that administration of BPA led to an increase in AVPV-Kiss1 mRNA and kisspeptin protein levels in a dose-dependent manner, triggering a rapid activation of the HPO axis. This enhanced AVPV-kisspeptin expression and BPA's impact on ERα-mediated gene expression ultimately resulted in heightened FSH and LH secretion. This finding is corroborated by Gracelli *et al.*, 2020. We observed decreased serum levels of AMH hormone, which can be attributed to the suppressive effect of BPA on AMH (Saleh and Favetta, 2019). The increase in testosterone and estrogen levels observed post-BPA exposure can be attributed to the elevated gonadotropins, which can also increase hormones produced in the gonads and alter the expression of the androgen receptors as shown in the qPCR results, as similarly documented by Gamez and Matuszczak (Gamez et al., 2014; Matuszczak et al., 2019).

We sought to determine if there was a role of melatonin in hormonal alterations induced by BPA by administering melatonin to some experimental groups, which was shown to have some reversal effects, as seen in Fig 1. This is similar to our previous study, where the effects of MEL were examined against BPA, and melatonin showed a mild reversal effect (Kadir et al., 2021). This can be ascribed to the regulatory role of melatonin in the neuroendocrine axis. Melatonin has been reported in various experimental studies to have antioxidant properties, scavenging free radicals and returning the redox balance to normal basal levels,

as shown in a recent systematic review and meta-analysis (Ebrahimi et al., 2021; Pena-corona et al., 2023). However, its limited effectiveness in the current investigation might be attributed to shorter treatment durations, echoing the need for prolonged administration to manifest its protective potential optimally.

We further explored the effects of BPA on the oxidative markers. The presence of elevated levels of SOD and GPx after exposure to BPA suggests the activation of an adaptive mechanism aimed at mitigating the augmented oxidative stress. This observation is consistent with other researches that emphasize the involvement of these enzymes in counteracting oxidative damage caused by environmental pollutants (Kadir et al., 2021; Sabry et al., 2021). The observed elevation in levels of iNOS suggests a possible augmentation in reactive nitrogen species, potentially contributing to damage from oxidation. This phenomenon has been shown in some research studies investigating oxidative stress generated by toxins (Amjad et al., 2020; Meli et al., 2020; Nayak et al., 2022).

Furthermore, the significant reduction in UDP-GT concentrations implies a disruption in ovarian metabolic pathways caused by exposure to BPA. Mukhopadhyay *et al*. documented that exposure to BPA can disrupt ovarian metabolic pathways, potentially resulting in the development of polycystic ovarian syndrome (PCOS) (Mukhopadhay et al., 2022; Prabhu et al., 2023) UDP-GT plays a crucial role in several metabolic pathways, and its depletion can indicate compromised metabolic activity, which has implications for cellular activities critical for reproductive health (Dae-Gwan, 2015). In addition, UDP-GT is involved in the metabolism of hormones, including estrogen and progesterone, which are crucial in regulating various reproductive processes, whose depletion can impact hormone levels and activities influencing reproductive outcomes (Seppen, 2012)
Interestingly, we observed melatonin showing restoration of the assayed oxidative makers, SOD, GPx, and iNOS. Prior studies have shown the effectiveness of melatonin in mitigating oxidative stress in several experimental settings, indicating its potential to alleviate oxidative damage caused by toxins or environmental stressors (Chitimus et al., 2020; Zarezadeh et al., 2022). Moreover, the capacity of melatonin to alleviate the disturbance in UDP-GT levels implies a possible protective effect in the maintenance of metabolic processes in the ovaries, which contribute to the preservation of cellular well-being and operational efficiency (Tan et al., 2013). The results of this study together emphasize the potential of melatonin as a preventive agent against the biochemical disturbances caused by exposure to BPA.

Having observed the findings from the biochemical analysis, we looked at the ovarian cytoarchitecture. Oogenesis is a specific process that entails the development and maturation of oocytes in the ovary. This process is an interplay of proteins, ovarian steroids, and genes. The harmful effects of BPA on meiosis and maturation of oocytes were documented by Lowther et al., similar to our finding of lower follicular counts in BPA-exposed animals. Researchers also noted a significant decrease in antral follicle count (AFC) with increasing BPA concentration in urine samples, thus suggesting exposure to BPA can have adverse effects on ovarian functions and capabilities (Lowther et al., 2014; Huo et al., 2015)

Similarly, research indicates that BPA can potentially alter the expression of genes associated with the growth and maturation of ovarian follicles, thereby influencing their structural integrity and overall health (Veiga-Lopez et al., 2013; Cariati et al., 2019). Our findings support this data, demonstrating that exposure to varying doses of BPA disrupted the growth and proliferation of the ovarian follicles, as shown in the cytoarchitecture and histomorphometry of the ovaries. In addition, BPA exposure caused elevated levels of reactive oxygen species, resulting in oxidative damage to ovarian tissue and compromising the integrity and viability of follicles, a finding corroborated by Meli and Sun (Meli et al., 2020; Sun et al., 2019) Melatonin showed some reversal effects on histological alterations by better preserving the histology features compared to the BPA-only groups, similar to some studies documenting that melatonin may not directly affect follicular counts (Tamura et al., 2012; Zheng et al., 2021)

We further looked into the outcomes of BPA on some hypothalamus and pituitary proteins to understand the BPA-induced distortions, which we believed the complex interactions within the endocrine system might be better understood by examining the gene expression responses triggered by exposure to BPA. A significant event preceding puberty's onset is the activation of GnRH neurons, which entails kisspeptin and GRR54 signalling (kiss1-derived peptide receptor), activating the hypothalamus's GnRH neurons. Our findings show that doses of BPA cause upregulation of the GPR54 receptor, which is in tandem with a study by Johnson *et al.*, where they reported upward expression of estrogen receptors and kiss1 genes in BPA-exposed animals (Johnson et al., 2018; Faheem et al., 2021). However, the precise mechanism by which BPA upregulates the GPR54 receptor is yet to be fully elucidated. The G protein receptor, Gonadotropin Releasing Hormone Receptor (GnRHR), is located mainly on pituitary gonadotrophs and is concerned with triggering the effects of hypothalamic GnRH. Receptor activation leads to gonadotrophin release by the anterior pituitary gland (Livadas and Chrousos, 2019). The overexpression of the GnRH mRNA and hormone in BPA exposed translated into higher gonadotrophs being released, which distorts the usual functional hormonal milieu for regular reproductive activities. Distortions in the rhythmic secretion patterns of GnRH and the subsequent release of gonadotropins have been linked to reproductive disorders such as amenorrhoea and polycystic ovarian syndrome.

The increased expression of estrogen receptors following exposure to BPA indicates a compensatory response aimed at mitigating the effects of this endocrine disruptor. Androgen receptor expression was noted to be in a dose-dependent order. Thus leading to higher testosterone levels, a finding corroborated by Galloway (Galloway et al., 2010). The increase in serum testosterone can also be attributed to stimulation of the ovaries by androgens, leading to more of its production. BPA's impact on the expression of hormonal receptors, specifically estrogen and androgen receptors, could worsen the disruption of the hormonal balance in the ovary (Vandenberg et al., 2013). The expression of mRNA associated with AMH, a key regulator of ovarian function and follicular development, was significantly elevated in the groups exposed to BPA. The observed upregulation can be attributed to a possible decrease in the ovarian reserve and follicular maturation, corroborated by the histological findings. BPA has been associated with negative impacts on AMH expression and follicular

development, subsequently impacting the health and maturation of ovarian follicles (Saleh and Favetta, 2019). The change in AMH expression suggests that there may be long-term effects on the ovarian reserve and follicle health.

The complex connection between gene expression and functional outcomes necessitates a detailed interpretation. Previous studies have identified a discrepancy between increased gene expression and potential disruption of receptor function about the impact of BPA. These studies have shown that higher transcription levels do not necessarily enhance receptor activity (Fahrenkopf and Wagner, 2020; Sonavane, 2022). The discrepancy may arise from the complex interference of BPA with receptor binding and downstream hormonal signalling, which could result in modified cellular responses despite increased gene expression. Additionally, it is essential to note that the increased presence of estrogen receptors does not necessarily correspond to improved physiological functioning. BPA can competitively bind to estrogen receptors, which may lead to receptor dysfunction and sensitivity changes, thereby impacting normal hormone responses (Acconcia et al., 2015; Takayanagi et al., 2006; Wetherill et al., 2007). Our findings have shown that exposure of newborns to BPA can alter developmental programming and subsequent reproductive capabilities in Wistar rats, thereby having the potential to cause long-term effects on reproductive health during adolescence and adulthood.

5. Conclusions

This study shows the impact of bisphenol-A administration on the hypothalamic pituitary ovarian axis. Exposure to BPA in the critical period of development results in alterations of protein expressions in the hypothalamus, alterations in the pituitary and ovary's reproductive hormones, and distortions in ovarian folliculogenesis and oxidative markers. We have also strengthened the observation of various studies on the beneficial effect of melatonin on oxidative stress and reproductive functions via restoration of some BPA-induced damage of the HPO axis. Early life exposure to BPA has far-reaching reproductive consequences in adulthood and should be avoided in all ways.

Ethical statement
The research obtained protocol approval from the Ethical Review Committee, University of Ilorin, Nigeria. The approval number is UERC/ASN/2018/1154. This research complied with the ARRIVE guidelines and was conducted in the Animal Holding of the University College of Health Sciences.

Conflict of interest
The authors declare no conflict of interest.

Availability of Data and Materials
All data generated or analysed during this study are included in this published article.

Author Contributions
ERK: Conceptualisation of work, practicals/research proper, laboratory analysis, writing

ADY and AI: Laboratory analysis, Drafting of manuscript and Revision of manuscript
OJO and IAL: Laboratory analysis and Histological analysis
ALO and MOH: Laboratory analysis and manuscript writing
LSO: Research practical and writing, MSA: Supervision and article review

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