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Protocol status: Working
We use this protocol and it's working

Exposure to Bisphenol-A and Melatonin altered the Expression of Estrogen and Androgen Receptors in Neonatal Female rats

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ABSTRACT

This protocol details about grouping and breeding of animals, sample collection and processing, hormonal assay, biochemical assay and quantitative real-time polymerase chain reaction procedure.

ATTACHMENTS

py6hccsw7.pdf

GUIDELINES

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

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Grouping and breeding of animals

1

Note

- Procure bisphenol A and sesame seed oil from Sigma (CAS –No: 80-05-7), Germany, also, purchase absolute ethanol and melatonin from a standard laboratory in Ilorin.
- Purchase the animals from an animal breeding farm in Oyo State, Nigeria for the experiment. Mature female rats weighing 150 ± 10g and male rats weighing 200g ± 20g. Maintain animals in a controlled environment, with a 12-hour day and night schedule. They have access to water and chow rat-mouse diet freely.

Cage adult male wistar rats of proven fertility overnight with females in proestrous phase of the cycle.

2 Gestational Day 1 (GD 1) is the day detect sperm cells in the vagina. Once pregnancy is confirmed, isolate the pregnant rat in a cage to stay until parturition, with a total of 12 pregnant females being used for the study.

3 After parturition, recognise and group the female litters . These are the second-generation Wistar rats. They have daily subcutaneous injections from post-natal day 0 for four days (PND 0 - 3). The seven groups are administered as follows:

- I – Normal saline
- II – sesame oil and ethanol (vehicle)
- III - melatonin only (10mg/kg)
- IV – 25mg/kg BPA
- V – 25mg/kg BPA + melatonin (10mg/kg)
- VI –50mg/kg BPA
- VII – 50mg/kg BPA + melatonin (10mg/kg).

4 Perform all administrations in the morning, and allow all the groups to mature till adulthood day 120 ± 3 days, when they are sacrificed in the morning, between 7 a.m. and 9 a.m.

Sample collection and processing

5 After administration of BPA and melatonin treatments, the animals are left until day 120 ± 4 days. They are euthanised by administration of 20 mg/kg body weight of ketamine intramuscularly.

6 Following the incision and lateral reflection of the scalp, expose the skull promptly with tissue forceps to extract the entire brain and pinpoint, collect, and place the hypothalamus, situated alongside the pituitary gland at the floor of the sella turcica) into cryovials preloaded with RNA, then they are subsequently kept frozen in liquid nitrogen tanks.

7 The harvested brains are the nutilized for genetic (RNA) studies. Additionally, collect the female gonads; the left ovary are for follicular count on histology, which is fixed for 48 hours in 4% paraformaldehyde, and the right ovary of each animal is homogenized in [M] 0.25 Molarity (M) ice-cold phosphate buffer followed by centrifugation of homogenates for enzyme studies.

8 Perform staining of the ovarian tissues in paraffin wax embedded sections according to Canene-Adams, 2013 and Haematoxylin and Eosin staining by the protocols outlined by Fischer et al., 2008. The various follicles (primary, secondary, preantral, antral, and corpora lutea are identified, counted then recorded.

Hormonal Assay

15m

9 Obtain the blood samples from the apex of the heart for hormonal analysis using 5 mL needles and syringes.

10 Centrifugation for 3000 rpm, 00:15:00 is used to separate the serum. 15m

11 Assay plasma luteinizing hormone, follicle stimulating hormone, oestrogen, progesterone, testosterone, and Anti-Mullerian Hormone concentration measurements using the Enzyme-Linked Immunosorbent Assay

(ELISA) technique and purchase ELISA kits from Monobind Inc. Lake Forest, USA, following manufacturer's guide.

Biochemical assay

5m

12

Note

Assess Nitric oxide levels following the method outlined by Montgomery and Dymock in 1961.

This involved measuring the levels in an acidic environment, where use nitrite, upon formation, to create nitrous acid, which react with sulfanilamide to form a product. This resulting product is then coupled with N-(1-naphthyl) ethylenediamine, to give an azo dye displaying a vibrant reddish-purple color, quantifiable at 540 nm.

13

Note

Determine the activity of Glutathione peroxidase (GPx) using the method outlined by Paglia and Valentine, 1967.

A unit of GPx activity is defined as the enzyme quantity necessary to catalyze the oxidation of [M] 1 nanomolar (nM) NADPH per minute at 25 °C.

14

5m

Note

The assessment of Superoxide dismutase (SOD) is carried out following the method detailed by Nishikimi et al. in 1972.

The SOD enzyme's capability to impede the phenazine methosulfate-mediated reduction of nitro-blue tetrazolium dye is measured by monitoring absorbance at 560 nm over a 00:05:00 period at 25 °C.

Quantitative real-time polymerase chain reaction procedure

15m

15 Euro Gold Tri-Fast solution (Euro Clone) is used to prepare the RNA.

15.1 The tissue is then pulverized using a tissue homogenizer, followed by DNase treatment on the extraction samples of total RNA, such that DNA contamination from the total RNA prepared could be eliminated.

15.2 Purification (through acid phenol-chloroform), precipitation, and suspension of the RNA in distilled water (dH_2O) are then carried out.

16 The reverse transriptase enzyme (Invitrogen) is used for reverse transcription of total RNA, using M-MLV reverse transcriptase (Invitrogen), which involves retrotranscription of 1 µg of total RNA to quantify mRNA expression in experiments.

17 A gently mixing of the samples through up and down pipetting and then incubate at 37 °C . M-MLV R 15m
is inactivated for 00:15:00 at 70 °C . Keep cDNA at -20 °C .

18 NanoDrop™ 1000 spectrophotometer is used to measure cDNA and RNA concentrations.

19 Nucleic acid quality is noted via absorbance ratios at 260 nm/280 nm and 260 nm/230 nm.

20 Measurement of the relative amounts of the transcript of a specific gene is performed using a qRT-PCR, via a two-step procedure using:

- Sybr green supermix (Biorad)- the amplification reaction and generation of melting curves of the amplicons subsequently.
- Verification of the melting curve data is running through the PCR product on 2% agarose gel.

21 The efficiency of the Primer is tested in reactions with six serial 1:10 dilutions of cDNA as a template to perform a calibration curve.

	A	B	C	D	E
		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	TGGTATCCCTTTGGCTTTCACA	192	
		mGnrh1- REV	GATCCTCCTCCTTGCCCATC		
	6	Gonadotropin releasing hormone receptor (Gnrhr)			
		mGnrhr-DIR	GCCTCAGCCTTGTCTCAT	140	

	A	B	C	D	E
			GT		
		mGnrhr-REV	TATGTTGGGCTTTCCCGGTC		

Data analysis

22

Collect the data and analyze by two-way analysis of variance (ANOVA) and subsequently subjecte to Tukey's (HSD) test of multiple comparison using GraphPad Prism v.6 (GraphPad Software, Inc., La Jolla, CA, USA).

- Express data as means ± SEM (standard error of mean).
- Statistical significance is taken as P value less than 0.05 ($p<0.05$).