BISPHENOL-A AND MELATONIN EXPOSURE ALTERED HISTOMORPHOLOGICAL AND REPRODUCTIVE GENETIC BIOMARKERS OF HYPOTHALAMO-PITUITARY-OVARIAN AXIS IN WISTAR RATS

KADIR, Eniola Risikat

(08/68LD003)

APRIL, 2021

BISPHENOL-A AND MELATONIN EXPOSURE ALTERED HISTOMORPHOLOGICAL AND REPRODUCTIVE GENETIC BIOMARKERS OF HYPOTHALAMO-PITUITARY-OVARIAN AXIS IN WISTAR RATS

KADIR, Risikat Eniola

(08/68LD003)

MB;BS (ABU) 2005, M.Sc. (ILORIN) 2010, FMCOG (NPMCN) 2016

A THESIS SUBMITTED TO THE

DEPARTMENT OF ANATOMY, FACULTY OF BASIC MEDICAL SCIENCES, UNIVERSITY OF ILORIN, ILORIN, NIGERIA,

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN

ANATOMY

APRIL, 2021

CERTIFICATION

This is to certify that this thesis has been read and approved as meeting the requirements of the Department of Anatomy, University of Ilorin, Ilorin, Nigeria, for the Award of Doctor of Philosophy (Ph.D.) degree in Anatomy.

_____ _____

Prof. M.S. Ajao Date
(Supervisor)

_____ _____

Dr. G.O. Omotoso Date
(Head of Department)

_____ _____

Dr. Misturah Y. Adana Date
(Postgraduate Coordinator)

_____ _____

(Internal/External Examiner) Date

_____ _____

(External Examiner) Date

DEDICATION

This work is dedicated to my family for all their sacrifices in pursuing my career.

DECLARATION

I, KADIR Eniola Risikat, a Ph.D. student in the Department of Anatomy, University of Ilorin, Ilorin, hereby declare that this thesis entitled “Bisphenol-A and Melatonin Exposure Altered Histomorphological and Reproductive Genetic Biomarkers of Hypothalamo-Pituitary-Ovarian Axis In Wistar Rats”, submitted by me is based on my actual and original work. Any materials obtained from other sources or work done by any other persons or institutions have been duly acknowledged. In addition, the research has been approved by the University of Ilorin Ethical Review Committee.

---------- ----------

KADIR Eniola Risikat (08/68LD003) Date

ACKNOWLEDGEMENTS

I thank Almighty Allah, The Most Beneficient, The Most Merciful, for making it possible to complete this programme and obtain another degree.

I would like to express my sincere gratitude to my supervisor, Professor M.S. Ajao, for his invaluable guidance, contributions and encouragement throughout the course of this study. This has been an inspiring experience for me.

I equally want to appreciate the Head of Department, Dr. G.O. Omotoso for his numerous advise, encouragement and contributions in completing this study as well as in my carreer progression. Special thanks goes to Dr O.J. Olajide, for his assistance in facilitating the genetic studies component of this research work in Italy. I also want to thank Dr A. Imam and Dr M.Y. Adana for their guide during the writing of this thesis. My sincere appreciation goes to other lecturers of the Department of Anatomy, University of Ilorin, for their constant drills in improving the outcome of the work.

A special appreciation goes to Dr. O. Akinola of Department of Pharmacology and Therapeutics, University of Ilorin, for his constant presence and guide throughout the conduct of this research. His numerous linkages to laboratories in lendingequipments and procurement of materials for carrying out this project are all well appreciated. My sincere appreciation also goes to Dr. O.O. Folaranmiand MrsAkanbi-Ola, both from Department of Pathology, University of Ilorin and Mr I.A. Lawal of Al-Hikmah University, for their assistance in the laboratory work.

I also want to say a very big thank you to Olivia, Tomiwa, Taiye, Abideen, Mercy, Adeola, Inioluwa, Tolani, Omoyemi, Hamza, Anuoluwapo,Bala, Lukman and Malik for all their

support and assistance during the laboratory work and thesis proofreading. You are all highly appreciated.

To my mother and siblings, thank you for your constant encouragement. A special tribute to my late father who died in the course of this work, may the Almighty grant you eternal rest. And to my spouse,Dotunand our boys (Nazif, Al-Amin and Fawaz), I really appreciate your love, care, patience and sacrifices, especially for all those days I leave home for the laboratory forgetting tocare and more. You are all awesome.

Kadir E.R.

April, 2021

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND ACRONYMS

AMH	Antimullerian Hormone
AR	Androgen receptor
BPA	Bisphenol-A
CDC	Centre for Disease Control and Prevention
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstibostrol
DHT	Dihydrotestosterone
EDCs	Endocrine Disrupting Chemicals
EPA	Environmental Protection Agency
FAO	Food and AgricultureOrganisation
FDA	Food and Drug Administration
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin Releasing Hormone
GnRHR	Gonadotrophin Releasing Hormonr Receptor
GPx	Glutathione Peroxidase
HPG	Hypothalamo-pituitary-gonadal
IGF	Insulin like growth factor
LH	Luteinizing Hormone
LOAEL	Lowest Observed Adverse Effect Level
NMDA	N-methyl-D-Aspartate
NOAEL	No Observed Adverse Effect Level
NOS	Nitric Oxide Synthase
PBBs	Polybrominated biphenyls
PC	Polycarbonate plastics

PCBs	Polychlorinated Biphenyls
PVC	Polyvinyl chloride
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
UDP	Uridine diphosphate glucuronic acid
UGT	Uridine diphosphate glucuronyltranferase
UNEP	United Nations Environment
WHO	World Health Organisation

ABSTRACT

Bisphenol-A (BPA) is an endocrine-disrupting chemical which mimics the actions of endogenous oestrogen. It is widely used as monomer in the production of polycarbonate plastics, epoxy resins, food and drink containerset.c. Endocrine disruptors affect the reproductive system, especially in females, thereby increasing the burden of infertility. The study was designed to elucidate a potential ameliorative role of melatonin on possible effects of BPA on the ovarian structural integrity and reserve as it relates to female fertility. The specific objectives of the study were to determine the effects of BPA on (i) reproductive hormones produced by the Hypothalamo-Pituitary-Ovarian (HPO) axis; (ii) ovarian oxidative stress (iii) histomorphological differentiation of the ovaries; (iv) genes of the hypothalamus and pituitary gland that regulate reproduction; and (v) the effects of melatonin in the HPO axis of BPA exposed Wistar rats.

Eighty-four female pups were divided into two main groups A and B of 42 rats each. Each group was further subdivided into seven, with each sub-group consisting of 6 rats. Sub-groups in A were administered subcutaneous injection of BPA from post-natal days (PND) 0 – 3. Group B rats had oral administration of BPA from PND 19 – 68. The subgroups were administered as follows: I – normal saline control, II – vehicle control, III -10 mg/kg body weight (bw) melatonin, IV – 25 mg/kg bw BPA, V – 25 mg/kg bw BPA + 10 mg/kg bw melatonin, VI – 50 mg/kg bw BPA, VII – 50 mg/kg bw BPA + 10 mg/kg bw melatonin. Upon completion of substance administration, the animals were left until 120 ± 4 days for sacrifice in their proestrous phase of cycle. Blood samples were collected by cardiac puncture for hormonal analysis. The hypothalamus and pituitary were excised for genetic studies while the ovarian tissues were excised for histological processing and oxidative studies. Data were analysed using one-way analysis of variance and p values < 0.05 were considered as significant.

The findings of the study were that:

i. bisphenol-A increased follicle stimulating hormone (252.1±47.55ng/ml) and luteinising hormone levels (823.5±103.5ng/ml) concentrations above the controls (55.77±9.35ng/ml and 215.6±43.63ng/ml respectively);
ii. bisphenol-A increased superoxide dismutase (458.8 ± 51.69U/mg protein) and nitric oxide synthase level (10.26±2.06U/mg protein) as compared to the control (101.0±39.70U/mg protein and 0.93±0.22U/mg protein respectively);
iii. there were histologically reduced follicular size and deformed shapes and decreased follicle number (3.0 ±0.58) in BPA exposed rats in comparison to the control group (5.5 ± 0.29);
iv. there was overexpression of KiSS-1 receptors (4.80±0.87) and Gonadotropin releasing hormone mRNA (79.57±13.89) in BPA treated in comparison to the controls (1.00±0.05 and 1.05±0.24 respectively); and
v. melatonin had no observable ameliorative influence on the effects observed in the BPA-treated groups.

The study concluded that BPA adversely affected the functional and structural integrities of female rat reproductive tissues with distortion of the reproductive regulating genes, and melatonin had no observed ameliorative effects. It is recommended that BPA should be used with caution in order to mitigate its contribution to reproductive distortions.

CHAPTER ONE: INTRODUCTION

1.1 Background of the Study

Conception should be a natural part of life that should occur spontaneously. Approximately 15 - 25% of couples within the reproductive age are struggling to conceive, and require medical attention to achieve this and only about 1 – 2% of couples are sterile (Agarwal, Virk, Ong, & du Plessis, 2014).Infertility is defined as the inability of a couple to conceive after one or more years of regular unprotected sexual intercourse with an adult of the opposite sex. Infertility is classified as primary when the couple have never been able to conceive while it is secondary if there has been previous conception but are presently unable to conceive. Infertility occurs due to an abnormality in a part(s) of the hypothalamo-pituitary-gonadal axis. It constitutes a medical as well as social problem since the dawn of humanity. Recent researches on the pathophysiology of infertility have identified the overproduction of Reactive Oxygen Species (ROSs) as an important factor in infertility(Agarwal *et al*., 2014; Wagner*et al.,* 2018) as well as abnormalities with female endocrinology.

In the last decade, studies regarding the effects of Endocrine Disrupting Chemicals (EDCs) on the reproductive functions of animals have raised concern(Maria*et al.,* 2014). In response to these concerns, the World Health Organisation (WHO) had several publications which include the State of the Science of endocrine disrupting chemicals in 2012. Subsequently, it was adopted that EDCs was to be included in emerging issue under Strategic Approach to International Chemicals Management [SAICM],(WHO, 2015).

Endocrine Disrupting Chemicals are agents that can interfere with the endocrine system in mammals (Adewale *et al.*, 2009).Endocrine-disrupting compound was defined by the United States Environmental Protection Agency (USEPA) as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural

blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Diamanti-Kandarakis *et al*., 2009). These chemicals interfere with the endocrine system by mimicking or antagonising endogenous steroid hormones (Wisniewski *et al.*, 2015).The group of molecules identified as endocrine disruptors are highly heterogeneous and include synthetic chemicals used as industrial solvents/lubricants and their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol-A (BPA)], plasticizers [phthalates], pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides [vinclozolin], and pharmaceutical agents [diethylstilboestrol (DES)].

Endocrine disrupting chemical of concern in this study is bisphenol-A ((2,2-bis(4-hydroxyphenyl) propane; BPA). Bisphenol-A is produced as a single molecule, which becomes polycarbonates when in polymerised form (Robertson and Farrelly, 2015). Bisphenol-A is a chemical that is used primarily as a monomer in the production of polycarbonate plastics (PC) and epoxy resins, that are generally used for drinking bottles and food coolers (Peretz, *et al*., 2014). It is also used in polyester, polysulfone and polyacrylate resins, as well as in flame retardants. This chemical is mostly man-made and found in various materials, hence it is a synthetic chemical compound that is produced in billions of pounds annually (Preethi et al., 2014). Exposure to Bisphenol-A is often via ingestion of food, dust and water, inhalation of gases and particles in the air, as well as through the skin. Aggregated exposure reflects the summated exposure through all routes, which also include toys, diet, dust, cosmetics, thermal paper, etc. Uncertainty in the exposure estimates for non-dietary sources also contributes, which adds to the dietary exposure values.

Bisphenol-A interferes with hormonal action, hence tagged as an endocrine disruptor (Peretz, *et al*., 2014). Li *et al*., in 2015 has shown that BPA can bind to the hydrophobic pocket of

some nuclear receptors, and that it has stable interaction with the nuclear receptors by mimicking the behavior of the natural hormone oestradiol. These binding has also been shown to be mainly driven by van der Waals and hydrogen bond interactions. However if this affects reproduction in a significant manner is a question to be explored.

Bisphenol-A is a high production synthetic chemical compound, that is used in the production of many consumables and equipments that are of daily consumption and use by man as linings of water bottles, food containers, dental sealants, etc(FAO/WHO, 2010). BPA is a recognised endocrine disruptor, and has been shown to interfere with oestrogen as well as androgen production in animals (N'Tumba-Byn *et al*., 2012; Wisniewski *et al*., 2015). They are suspected to be associated with altered reproductive function in males and females, as well as being associated with increased incidence of breast cancer, abnormal growth pattern, neurodevelopmental delays in children, etc(Schierow and Lister, 2010; Preethi *et al*., 2014).

Disruption of the endocrine functions as well as alteration in reproductive functions may ultimately culminate in interference with fertility. Infertility being 8-15% in prevalence, could be due to male or female factor. Wo*et al*., in 2019reports a prevalence of 58.9% in females compared to males contributing 40.6%, while 42.4% and 35.5% in females and males respectively were documented in an African study (Elhussein*et al.*, 2019; Wo *et al*., 2019)

In couples less than 5years of marriage, female factor has been found to be the commonest contributing factor to infertility, with females contributing 46% compared to males of 20% (Deshpande, 2019). These goes to show that women contribute more to infertility, and 46.6% of this has been attributed to being caused by endocrine factor, being the commonest aetiology for female factor infertility.Therefore, this study aims at determining the effects of endocrine disruption caused by bisphenol-A in the female wistar rats.

Melatonin is a natural hormone produced by the pineal gland in the body, and it is used as a dietary supplement.(Naufel*et al.*, 2019). It is best known for its effectiveness as a sleep aid and regulating the sleep wake cycle (Ferracioli-Oda*et al.*, 2013). However, recent data are documenting the role of melatonin and its metabolites as endogenous free radical scavengers, with an impressive ability to control oxidative damage (Reiter *et al.*, 2016). Its antioxidation role has been documented in the central nervous system, cardiovascular diseases, etc(Naseem and Parvez, 2014).Melatonin has been known to be a better free radical scavenger than most known other agents (Ajao *et al.*, 2011). Therefore, these antioxidant properties of melatonin is intended to also be explored in this study, against BPA, which has also been implicated in the generation of free radicals and may consequently lead to reproductive toxicities(Acaroz *et al.*, 2019; Anet*et al.*, 2019).

1.2 Statement of the Problem

Bisphenol-A is one of the most highly produced industrial chemicals globally, withover 2 billion pounds used in the production of epoxy resins and polycarbonate plastics every year.(Preethi et al., 2014)These plastic products are inevitably used in food and drink packaging, thereby causing unintended direct ingestion. The United States Environmental Protection Agency (2012) acknowledges BPA is a reproductive, developmental, and systemic toxicant based on animal studies and acknowledges the interaction with oestrogen receptors, but questions the potential impact on humans and the environment.(Health & Metz, 2016)

As countries develop and urbanise, production demands for use of bisphenol-A has also increased. Therefore, there is increase use for food and beverages packaging, electronic equipments, medical equipments, paper coatings, etc.

There are paucity of literature on the endotoxicity of BPA, especially now that its uses are in increase by humans, and in this case mostly women, coupled with increases in rise in infertility especially female factors. It is very important to look into its activities as it affects the endocrine pathways and reflects on infertility.

1.3 Justification for the Study

In July 2013, France banned BPA in almost all materials with food contact. Belgium and Switzerland also announced plans to ban BPA in packaging foods intended for children younger than 3 years of age (Health and Metz, 2016). However, the Polycarbonate/BPA Global Group of the ACC provided their support for BPA as a safe product outlining many uses of this safe consumer product (ACC, ThePolycarbonate/BPA Global Group, 2009).(Health & Metz, 2016) These divergent views and reports across nations provide a niche for further study, in order to add to the debate on the safety or otherwise of BPA. In addition, there have been no provision of legislation from the Nigerian government regarding the use of BPA.

BPA being an endocrine disruptor is associated with distortion of normal hormonal milieu and may contribute to unexplained infertility through chronic exposure. In addition, the critical and sensitive windows of susceptibility to EDCs is majorly during intrauterine life, infancy, childhood and puberty (Huo et al., 2015), which are the formative reproductive period. Thus, the examination of the effects of BPA on reproductive functions and the search for a possible efficacy (through the use of melatonin, a known efficacious antioxidant) against its unintentional poisoning is very important, especially as this may affect fertility, and endocrine factors in females being the commonest underlying aetiology of infertility.

1.4 Broad Aim of the Study

The broad aim of this study was to evaluate the effects of bisphenol-A on the hypothalamo-pituitary-ovarian axis in female Wistar rats and the efficacy of melatonin on BPA effects.

1.5 Objectives of the Study

The specific objectives of the study were to investigate the effects ofbisphenol-A and melatonin on:

1. some reproductive hormones (Follicle Stimulating Hormone, Luteinising Hormone, oestradiol, progesterone, Anti-Mullerian Hormone and testosterone) in the exposed rats;
2. oxido-nitrosative stress and metabolic enzymes (Superoxide dismutase, Glutathione peroxidase, Nitric oxide synthase and Uridyl diphosphateglucuronosyl transferase) in the ovary of exposed rats;
3. some genes of the hypothalamus (nuclear receptors, androgen receptor, antimullerian mRNA and KiSS1 receptor), and pituitary gland (gonadotropin mRNA and gonadotropin releasing hormone receptor) in the exposed rats; and
4. the ovarian histoarchitecture of the exposed rats.

1.6 Research Questions

1. What quantitative and histological alterations does BPA have on the reproductive system of female rats exposed for acute and chronic periods?

2. What effects does melatonin have on the effects observed with BPA exposure

1.7 Hypothesis

Null (H_O)

- Low dose Bisphenol-A has no effect(s) on the hypothalamo-pituitary-ovarian axis of Wistar rats.
- Melatonin has no effect against bisphenol-A effectson some endocrine markers of the HPO axis in Wistar rats

Alternate(H_1)

- Low dose Bisphenol-A has effect(s) on the hypothalamo-pituitary-ovarian axis of Wistar rats.
- Melatonin is effective against bisphenol-A effects on some endocrine markers of the HPO axis in Wistar rats

1.8 Scope of the Study

This study specifically assessed some reproductive hormones (FSH, LH, oestrogen, progesterone, AMH, testosterone) in the serum,antioxidants (SOD, GPx, NO)in the ovary, histomophological changes in the ovary and genes regulating reproduction in the hypothalamus and pituitary (KiSS1, GnRH, AR and Nuclear receptors). These assessments were done via hormonal assay, antioxidant assay, histological and quantitative Polymerase Chain Reaction (qPCR) techniques.

1.9 Significance of the Study

This study will contribute to resolving the safety concerns in the use of BPA, and more specifically on the effects on the female reproductive system. It most likely will provide the antidotal novelty of melatonin against any of the reproductive toxicity by BPA.

1.10 Expectations of the Study

It was expected that this study will reveal the changes in the biochemical, oxidative, histological and genetic effects of the HPO axis following exposure of female wistar rats to BPA for both acute and chronic periods. In addition, it is expected that melatonin will mitigate some/all of the adverse effects that may be induced by BPA administration.

CHAPTER TWO: REVIEW OF RELATED LITERATURE

2.1 Endocrine Disrupting Chemicals

Endocrine Disrupting chemicals (EDCs) were recently defined by the Endocrine Society, which is one of the largest international group of scientists and physicians that work and practice in the field of endocrinology as 'exogenous agents that interferes with synthesis, secretion, transport, metabolism, binding action or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process' (Diamanti-Kandarakis *et al.*, 2009). They are also defined as 'exogenous (non-natural) chemical, or mixture of chemicals, that interfere with any aspect of hormone action'(Gore*etal.*, 2014). An endocrine disruptor is regarded as an exogenous substance or a mixture of chemicals that alters the function(s) of the endocrine system, thereby causing adverse health effects in those organisms, their progeny, or (sub)populations (Preethi et al., 2014). EDCs occur indifferent forms as found naturally as phytoestrogens, as industrial substances and as plasticizers(Diamanti-Kandarakis *et al.*, 2009).

Endocrine disrupting chemicals mechanism of disruption of the endocrine system is thought to be by mimicking or blocking a natural hormone(Gore *et al.*, 2014). To be a hormone mimic, an EDC "tricks" the hormone's receptor into believing that the EDC is the hormone, which then inappropriately activates the receptor, thereby triggering the processes normally activated only by that natural hormone. On the other hand, it can bind to a hormone's receptor, hence blocking that receptor which cannot be activated, even if the natural hormone is present (Manibusan and Touart, 2017).

Every endocrine hormone has a unique chemical composition and shape, with each also having a corresponding receptor(s) localised on target cells. The lock and key theory of hormone mechanism of action, which describes "A key is specific to a lock", can be likened

to the way the hormones shape (like a key) is complementary to the receptors shape (like a lock). The response of a given tissue or organ to a hormone is determined by the presence of receptors on target cells and receptor activation by hormone binding. Many factors are put into consideration for the hormone to be able to activate its receptor. Such factors include how much hormone is synthesised and released by the endocrine gland, how it is transported through the circulation, how much reaches the target organ, how potently is it and for how long the hormone can activate its receptor (Street *et al*., 2018).

2.1.1 Historical Perspectives of Endocrine Disrupting Chemicals

Generally, there has been an exponential increment in the number, and quantity of made synthetics, some of which have been discharged (deliberately or not) into the earth. This compound unrest has irreversibly changed biological systems in a way that has impactly affected natural life and human wellbeing. Rachel Carson's book Silent Spring, publishedby Houghton Mifflin Company in 1962, was the primary open admonition that natural sullying, specifically the pesticide DDT, may be liable for the diminished quantities of winged animals because of regenerative disappointment brought about by this and other dangerous synthetic substances(Gore *et al.*, 2014).

It is presently well-acknowledged that a few synthetic substances and pharmaceuticals can cross the placenta. However, prior to now, it was believed that the placenta went about as a boundary, shielding the developing embryo from being exposed to any form of harmful substances. Two deplorable clinical occasions changed and eventually refuted this viewpoint. The first was the acknowledgment that pregnant women offered thalidomide to mitigate sickness during the principal trimester in some cases brought forth babies with serious birth defects. Clearly, the embryo was helpless against pharmaceuticals given to the mother. The second leap forward disclosure was that of diesthylstilbestrol given to pregnant women to

deflect premature delivery. Diethylstilboestrol (DES) is comparable in its properties to characteristic oestrogen hormones. Women who had been exposed to DES in the uterus frequently had reproductive tract contortions and some created uncommon conceptive tumors in adolescence that were ordinarily just observed in postmenopausal women.

2.1.2 Sources of Endocrine Disrupting Chemicals

Endocrine disrupting chemicals can be gotten when some natural contaminants cooperate with hormones and may apply antagonistic results because of their activities. There are more than 85,000 made synthetic chemicals, of which thousands might be EDCs(Gore *et al.*, 2014)

Table 2.1: A shortlist of representative EDCs and their applications.

EDCs	Application
Dichlorodiphenyltrichloroethane(DDT), chlorpyrifos, atrazine, 2,4-D, glyphosate	Pesticides
Lead, phthalates, cadmium	Children's products (baby bottles, children's toys)
Bisphenol-A (BPA), phthalates, phenol	Food contact materials
Brominated flame retardants, PCBs	Electronics and Building materials
Phthalates	Personal care products, medical tubing
Triclosan	Antibacterials
Perfluorochemicals	Textiles, clothing

Abbreviations: BPA: bisphenol-A; 2,4-D: 2,4-dichlorophenoxyacetic acid; DDT: dichlorodiphenyltrichloroethane; and PCBs: polychlorinated biphenyls

(Gore *et al.*, 2014)

Individuals interact with EDCs by an assortment of courses, including utilisation of nourishment and water, through the skin, by inhalation, and by transfer from the mother to foetusthrough the placenta (vertical transmission) or mother to the newborn child (by means of lactation) if a woman has EDCs in her body.

2.1.3 Recent Discussions and Controversies about EDCs

Evidence has risen that EDCs can create unfavourable impacts, even at low doses that were initially assumed safe. However, systemic surveys and examination concentrating on human investigations have shown conflicting outcomes. Epidemiological investigations have indicated methodological impediments, including the unusual net impacts of blends, non-monotonic portion reaction connections, and low dependability of exposure assessment(Lee, 2018).

Recently, organisations such as the Endocrine Society, World Health Organization, (WHO) and United Nations Environment Programme (UNEP), have issued official reports describing the possible health threats posed by EDCs. They focused on exposure during critical periods of development which includes the foetal, neonatal and adolescent periods and concluded that exposure to EDCs is related to a multitude of diseases, including impaired reproduction, neurodevelopment, thyroid function, and metabolism, as well as increases in hormone-sensitive cancers(Gao *et al.*, 2015; Wang*et al.*, 2017). Thus, these reports called for urgent action to regulate EDC usage in most of the manufacturing companies, in order to enhance human and environmental safety. Debates have however continued between researchers who supported the conclusions of the report and those who opposed it after the release of the WHO/UNEP report in 2012. Although the proponents are often accused of being associated with or funded by chemical industries, some of their arguments deserve sober discussion from the scientific community(Lee, 2018).One criticism is the lack of evidence of the harm of

EDCs to humans, despite the persuasive evidence from *in vitro* and *in vivo* studies (Lee, 2018).

In contrast to the conclusion of official reports on EDCs, numerous ongoing foundational audits or meta-examinations concentrating on epidemiological investigations, particularly of fleeting EDCs, appear to help the restriction. For instance, reviews of the effects of bisphenol-A (BPA) in humans revealed conflicting outcomes as for pubertal improvement and diabetes. Comparative conclusions have been reached to on phthalate and obesity, triclosan and adverse health effects, and developmental exposure to numerous EDCs and male reproductive disorders(Lee, 2018).

2.1.4 Controversies on Effects ofEDCs on Human Body

Investigation of the human data by itself, while producing concerns, has so far neglected to give firm proof of direct causal relationship between low-level (i.e. levels estimated in the overall public) exposure to chemical with EDCs and adverse health results. It is difficult to look at and coordinate outcomes from various human investigations, since data are frequently gathered at various timespans, utilising diverse exploratory structures and under various exposure conditions. Regular exposure data are totally deficient. Of specific concern is theabsence of exposure data during basic times of improvement that influence later working in grown-up life. Moreover, the focuses and potencies of endogenous hormones and phytoestrogens are commonly higher than those of exogenous synthetic concoctions. In spite of these difficulties, exposure to EDCs has been proposed to assume a job in adverse health results, and concerns remain. The accompanying models outline these worries (FAO/WHO,2010).

On Fertility

Various investigations report a decrease (since the 1930s) in human sperm quality in a fewnations.(Merzenich, Zeeb, & Blettner, 2010) There obviously are significant varieties in sperm tally, both inside and betweennations, however there are no firm data that straightforwardly tended to the conceivable circumstances and logical results connection between declining sperm quality and exposure to EDCs.(Halder, Jain, & Kumar, 2014) Studies to date have been retrospective. A few meta-investigations of existingexaminations arrived at various resolutions, and the issue remains unclear. Regardless of whether there has been decay in semen quality, this may not really be because of endocrine disturbance. Accessible human and test creature studies exhibit that elevated level of exposureto certain ecological chemical can disable ripeness and increase the pace of unconstrained fetus removal, yet the relationship to endocrine interruption stays theoretical. Declining sex proportions (less males) have been recorded in various districts and nations, and unidentified outer influences may be related with such changes, however the mechanism(s) is obscure. Transient increments in the recurrence of advancement variations from the norm of the male conceptive tract, especially cryptorchidism and hypospadias, have been accounted for,however the job of exposure to EDCs is misty. Trial data show that various synthetic substancescan upset advancement of the male regenerative tract by means of endocrine components(FAO/WHO, 2010).

2.2 Bisphenol-A

2.2.1 General Introduction on Bisphenol-A

Bisphenol-A (BPA) is a monomer utilised fundamentally in the production of polycarbonate (PC) plastics and epoxy resins. It is a plastic ingredient produced in huge amounts for use principally in the generation of polycarbonate plastics and epoxy resins, for example, utilised in a wide assortment of consumer items (Preethi et al., 2014). BPA is a small (228 Da)

particle which is utilised as a monomer in polymerisation reaction to produce polycarbonate plastics. It is a beginning material for the synthesis of plastics, just as some polysulfones and certain niche materials. BPA-based plastic is clear and tough, and is made into an assortment of regular consumer products, for example, plastic jugs including water bottles, athletic gear, compact discs and digital video discs(FAO/WHO, 2010).

Polycarbonate applications incorporate enormous returnable, refillable water bottles and food service items, for example, sports bottles, child bottles, pitchers, tumblers, home food container and flatware. Epoxy applications incorporate defensive coatings for the insides and outsides of food and drink containers just as dental materials. Bisphenol-A subsidiaries are utilised, to a restricted degree, as added substances for polyvinyl chloride (PVC). BPA is also present in recycled and thermal paper(FAO/WHO, 2010).

Bisphenol-A is a xenoestrogen, exhibiting oestrogen-imitating, hormone-like properties that raise worry about its suitability in some consumer products and food containers (Steinmetz *et al*., 2015).

2.2.2 Sources of Bisphenol-A

Bisphenol-A is one of the most profoundly produced modern synthetic substances internationally. More than 2 billion pounds of BPA are utilised in the generation of epoxy resins and polycarbonate plastics consistently. These plastic items are regularly utilised in food and drink packaging making an immediate ingestion introduction pathway. The resins are utilised as veneers for metal jars, bottle tops, and ground water channels (Preethi et al., 2014). The manufacturing procedure of BPA includes the combination and condensation of one piece of $(CH3)_2CO$ with two pieces of phenol (within the sight of an impetus and advertiser) under states of high temperatures and low pH, trailed by decontamination of the item. It is for the most part accepted that shopper presentation to BPA happens basically by

means of food in contact with BPA-containing materials, for example, polycarbonate infant bottles, table product and food holders just as food and refreshment jars fixed with epoxy resins. As of late, it has additionally been indicated that BPA can be moved to the skin from specific kinds of warm printing paper, for example, a few sorts of clerk's receipts(Preethi et al., 2014).

Expert bodies, for example, WHO, FAO had a specialist meeting and they considered BPA fixations from the aftereffect of the gathering was from food overviews and BPA movement from food contact and dental materials. BPA fixations in air, residue and water were additionally considered (FAO/WHO, 2010). The expert meeting noticed that by a long shot, most of concentrates on BPA focuses detailed from food studies, were from food and refreshments in epoxy-covered jars and, to a minor degree, glass compartments with covered metal tops. In addition, most of concentrates on BPA fixations in food because of relocation from food contact materials included polycarbonate (PC) baby bolstering bottles. A couple of concentrates on BPA fixations in paper were accessible.

BPA focuses in food from food survey information were separated by food type and age: newborn child equation and bosom milk (0–6 months), infant and baby food (1 - 6 years) and grown-up food. Most accessible information are for noting (aglycone) BPA. Notwithstanding, at times (for example for bosom milk), one might want to utilize absolute convergences of BPA (for example free in addition to conjugated BPA) for presentation evaluation.

For breast milk, three examinations speaking to in excess of 200 examples by and large gave all out BPA levels beneath 8 µg/l.Be that as it may, two of the investigations were viewed as of flawed utility due to their analytical shortcomings.For canned fluid infant formula, six investigations speaking to in excess of 50 examples gave free BPA levels underneath 10 µg/l

as devoured. The investigations are essentially from North America(FAO/WHO, 2010). One of the investigations was viewed as faulty as far as technique approval.

For toddler food, one examination in North America, speaking to around 100 examples, gave free BPA levels of around 1 µg/kg at the mean. Another investigation found no recognisable BPA, however the breaking point of discovery of the technique utilised was moderately high. For the movement of BPA from PC, most pessimistic scenario sensible uses were characterised. For the utilisation of child bottles, the most dire outcome imaginable was characterised as filling the jug with bubbling water, including milk equation and leaving the container to chill off. On account of PC silverware, the most dire outcome imaginable was spoken to by a 30 min contact time at 95 °C. In light of the huge dissemination of accessible test outcomes, a most extreme movement was chosen for the two circumstances for use in the presentation evaluation.

Data exist on the levels of BPA in faucet water and filtered water. Since the fixations differ generally, a most extreme convergence of BPA in water was chosen for use in the introduction appraisal. The concentration of BPA in air and residue are broadly appropriated, and two papers show that there is no distinction between centralisations of BPA in indoor and open air. Distributed evaluations of introduction to BPA from air and residue were utilised in the presentation appraisal.

Barely any examinations on BPA in paper bundling, paper treatment, water and warm paper were accessible. BPA levels were higher in reused paper than in virgin paper. Extra examinations on BPA relocation from paper bundling to food are required (FAO/WHO, 2010).

Figure 2.1: Sources of Bisphenol-A (Horton, *et al.*, 2017)

2.2.3 Chemical Structure of Bisphenol-A

Common Name: Bisphenol-A (BPA)

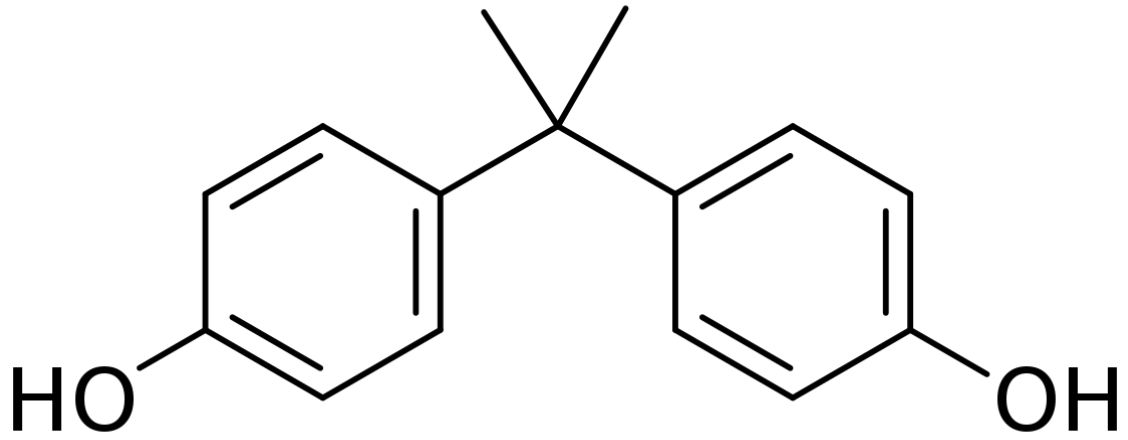


Figure 2.2: The chemical structure of Bisphenol-A (Varshney and Nalvarte, 2017)

Chemical structure of Bisphenol-A

Chemical Name: 4,4'-(propane-2, 2'-diyl)diphenol

Molecular Formula: $C_{15}H_{16}O_2$

Molar mass: 228.291 g·mol^{-1}

Appearance: White solid

Density: 1.20 g/cm^3

Melting point: 158 to 159 °C (316 to 318 °F; 431 to 432 K)

Boiling point: 220 °C (428 °F; 493 K) 4 mmHg

Solubility in water: 120–300 ppm (21.5 °C)

Vapour pressure: 5×10^{-6} Pa (25 °C)

(Monti., 2013)

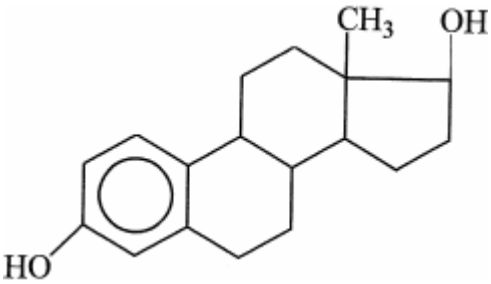


Figure 2.3: The chemical structure of oestradiol(Kuhl, 2005)

Chemical Name: (17β)-estra-1,3,5(10)-triene-3,17-diol

Chemical Class: Oestradiol Congeners

Molecular Formular: $C_{18}H_{24}O_2$

Molecular Weight: 272.38 g/mol

Appearance: white to off-white crystalline powder, has no smell and does not dissolve readily in water, but is very soluble in organic solvents such as ethanol, DMSO, and dimethylformamide (DMF)

Melting Point: 173-179 C

Water Solubility: practically insoluble (3.6 mg/l)

Vapour Pressure: 1.26E-08 (mmHg at 25C)

(Kuhl., 2005)

2.2.4 Mode of entry ofBisphenol-A into the body

The most well-known way that individuals are exposed to BPA is through food and drinksstored in compartments containing the substance. Cleaning polycarbonate bottles with cruel cleansers, or utilising them to store hot or acidic nourishments, can build your presentation to BPA. Polycarbonate bottles that ended up shady through long haul use may contribute more significant levels of BPA to fluids put away in them. The follow measure of BPA that mightbe taken in through the ordinary day by day diet is underneath a level that could cause health effect. When ingested, BPA is assimilated through the intestinal divider. The vast majority of the BPA that is taken is changed over in the digestive tract to an idle sort of sugar with no known organic action. Any follow measure of BPA, that is the remaining parts is then changed overin the liver to the equivalent latent substance before entering the circulation system. The idle sugar-compound is eliminated through the urine (Dose and Daily, 2015).

2.2.5 Health Effects of Bisphenol-A

Various effects of BPA in animals have been widely researched and target organsdistinguished in rehearsed portions in animal studies which incorporated digestive tract, liverand kidney. Be that as it may, the impacts of most concern have been those identified withthe hormonal action of BPA and conceivably related consequences for physical, neurologicaland social advancement. The discussion about the human health effect impacts of BPAexposure is restricted by an absence of epidemiological information. Presently, there isn'tadequate measurable capacity to recognise low portion impacts or decide all the health effectresults of exposure to BPA in people. Regular exposure is viewed as consistent after sometime, with the complete portion evaluated by the combined introduction, which is determinedas the result of fixation whenever. In any case, if introduction isn't

consistent after some time,a similar all out aggregate exposure conveyed in various examples, may deliver distinctive natural impacts(Preethi et al., 2014).

There is concern that BPA is perceived to cross the human placenta,along these linesuncovering the baby, the creating hatchling is more powerless than the grown-up living beingexposed to oestrogenic synthetic substances and it can possibly meddle with the steroid-subordinateassociation of the neurochemical and neuroendocrine frameworks, especially those that areoestrogen delicate. It is in creating cerebrum; modifications in the oestrogenic milieu impactdifferent parts of cell separation, including neuritis augmentation and expanding, synapticarrangement, articulation of synapses, cell demise as well as endurance. It was discoveredthat no relationship between the mothers urinary BPA fixations and newborn child neuro conductassessed at 5 weeks of age. These human data, albeit conflicting, demonstrate that prenatalBPA-exposure may influence child behaviours in a sex-subordinate way. Be that as it may,they are not viewed as satisfactory (Balakrishnan *et al*., 2010).s

Relationship between mothers' urinary BPA focuses during pregnancy and on edge,burdensome and hyperactive practices at 2 and 3 years old, which were more articulated for young ladies than for boys. It was accounted for that diminished on edge/discouraged and forceful practices in 3-multi year old young ladies yet expanded animosity and sincerely receptive practices in 3-multi year old young men with higher pre-birth exposure to BPA (estimated in mothers' urine during pregnancy) (Rochester, 2013).

Just a couple of studies have taken a look at relationship between bisphenol-A exposureand disorders of reproduction or developmental effects in humans. The studies on humanshave taken a gender at the connection between urine or blood concentration of aggregate or free bisphenol-A and a variety of health effect estimates, which included:

1. Certain hormones that helps to regulate reproduction markers of Deoxyribo-Nucleic-Acid (DNA) damage.
2. Miscarriage
3. Defects in fetuses
4. Fertility and obesity in women
5. Effects on the tissue that lines the uterus (“endometrium”)
6. Polycystic ovary syndrome, and birth outcomes and length of gestation.

Several studies in humans report a link between BPA exposure and effects on thereproductive system in adult women, e.g. endometrial hyperplasia, recurrent miscarriages and polycysticovary syndrome (Hengstler *et al*., 2011).

The effects of BPA on the cerebrum and conduct are thought to be credited to its oestrogen receptor(ER)- interceded activity. However, it is not clear how its low potency could represent the solid effects that are seen in numerous tissues after exposure to generally low portions. BPA exposure (10–1000nmol/l for 30 minutes) quickly advances dynamic changes in dendritic morphology through the ER-intervened pathway by attending phosphorylation of N-methyl-D-aspartate (NMDA) receptor subunit NR2B. There is additional proof that adjustments in quality articulation in utero endure into adulthood and henceforth conceivably include epigenetic component. Ishido and Suzuki, in 2010,found a portion subordinate (0-100 μmol/l) inhibitory effect of BPA on neural stem cell migration and proliferation (Preethi et al., 2014).

Various ecological chemicals can meddle with complex endocrine flagging pathways and cause adverse chemical effects. It is not amazing that PCBs adjust hormonal levels and they are known to influence pretty much parts of the endocrine framework. Exposure to PCBs during improvement changes grown-up conceptive and other explicitly dimorphic practices.

Perinatal exposure to BPA has been linked to some changes. For instance, neonatal exposure to A1254 decreases sexual receptivity (lordosis remainder), while A1221 has no effect. Be that as it may, when treatment incorporates pre-birth and postnatal exposure, A1254 diminishes sexual inspiration and A1221 diminishes sexual receptivity (Preethi et al., 2014)

One of the new rising effects or method of action for EDCs is that exposure to EDCs may assume a job in stoutness. Changed parameters of adipocyte science that outcome in terminal rebuilding or increase of fat mass prompting corpulence would be a decent affirmation of a connection among EDCs and weight. Be that as it may, to affirm the theory about EDCs and their effect on the advancement of corpulence, a fitting atomic objective influencing guideline of fat physiology must be found. Utilising breast, subcutaneous and instinctive adipose tissue explants just as disengaged develop adipocytes from more than 20 patients, it detailed that BPA at 1 and 10 nM focuses restrains adiponectin discharge (Preethi et al., 2014).

The metabolic effects of BPA are fundamentally essential to perceive the full range of its activities on human fat. The repeated administration of BPA has toxic effects on the liver in mice and it has been seen in past investigations with a most reduced watched antagonistic effect level (LOAEL) of 120 mg/kg bw/day, proposing that liver lethality is at any rate as touchy an endpoint for BPA as conceptive and formative effects. Bisphenol-A aggregation in rodent fat tissue was low in one examination, yet high in another (Hengstler *et al*., 2011).

The regenerative toxicity investigations of bisphenol-A include:

1. Assessment of fertility
2. Sperm counts
3. Oestrous cycling

4. Growth or cellular damage in reproductive tissues.

Regenerative wellbeing ultimately relies upon the correct association and capacity of the hypothalamo-pituitary-gonadal (HPG) axis, a neuroendocrine framework including the nerve center, the pituitary organ, lying just underneath the cerebrum; and the gonads (ovaries in females, testicles in males). Bisphenol-A exposure can upset pubertal planning and bargain the ability to keep up a customary ovulatory cycle in rodents and these imperfections result from the unusual association of the hypothalamic pituitary-gonadal axis, the essential neuroendocrine pathway that controls regenerative capacity. Bisphenol-A has likewise been found to actuate apoptosis and cell capture in refined ovarian granulosa cells, proposing that BPA may likewise affect the adult ovary (Preethi et al., 2014).

2.2.6 Reproductive and Developmental Toxicity of Bisphenol-A

In the course of last several decades, there have been exploratory examinations on the potential conceptive and formative danger of BPA in research facility and residential animal species, the vast greater part of the investigations being directed with rodents and mice. Many of the failure portions examines that controlled BPA orally, the most important course of introduction, were directed utilising research facility rodents. These examinations have been explored as of late by a few administrative bodies, and most have distinguished an oral conceptive and formative NOAEL of 50 mg/kg bw every day(Richter *et al.,* 2008;FAO/WHO, 2010; States *et al.,* 2010; Vandenberg *et al.,* 2013; Peretz *et al.,* 2014).

2.2.7 Roles of Regulatory Bodies on Bisphenol-A

The Food and Drug Agency National Center for Toxicological Research is finding a way to lessen human introduction to BPA in the food supply by supporting the industry' activities to quit delivering BPA-containing child jugs and infant feeding cups, by encouraging the advancement of safe alternatives, more oversight of BPA, and looking for open remark in

regards to BPA (Health and Metz, 2016). The U.S. EPA (2012) recognises BPA is a conceptive, formative, and fundamental toxicant dependent on animal studies and recognises the collaboration with oestrogen receptors, however questions the potential effect on people and the earth. In 2010, in light of expanding concerns, the EPA discharged a compound activity plan with respect to BPA dangers and introduction data. In view of the EPA's appraisal, most human presentation to BPA originates from food and drink bundling; under 5% of the BPA delivered is utilised in food bundling(Health and Metz, 2016). The EPA has included BPA on the Concern List under the Toxic Substances Control Act (TSCA), yet is not starting administrative activity under TSCA. Under this standard, the EPA, as a team with the FDA, Centers for Disease Control and Prevention (CDC), and National Institute of Environmental Health Sciences(NIEHS) is to test or screen landfills, fabricating plants, and other comparable areas for groundwater and drinking water pollution to decide potential exposures (Environmental Protection Agency, 2015). In July 2013, France prohibited BPA in practically all materials with food contact. Belgium and Switzerland likewise reported designs to boycott BPA in bundling foods expected for children younger than 3 years old (Salmon, 2013).

The Polycarbonate/BPA Global Group of the ACC gave their help to BPA as a sheltered item laying out numerous employments of this protected purchaser item (ACC, The Polycarbonate/BPA Global Group, 2009). In particular, the ACC records that the FDA denied the 2012 resident appeal from the Natural Resources Defense Council, which mentioned the FDA deny the utilisation of BPA in human food and food bundling. Likewise recorded are proclamations from the U.S. Department of Health and Human Services (DHHS) and the FDA that BPA has not been demonstrated to be a health hazard for children or adults, however they do take note of "some concern," which the FDA will proceed to address and screen(Health and Metz, 2016).

Another country’s reactions to the ACC's fact sheet is Health Canada, which expresses a few examinations do give proof to worry during growth and early postnatal life; explicitly, neural improvement could be unfavorably influenced by BPA (ACC, The Polycarbonate/BPA Global Group, 2009). The ACC cases support from the European Food Safety Authority and Australia that BPA introduces no significant health dangers and questions all cases that BPA causes malignancy and coronary illness, aggregates in the body, or can be consumed through the skin (Health and Metz, 2016).

2.3 Roles of Hormones on the Reproductive System and Fertility

2.3.1 Hypothalamo-pituitary-gonadal Axis

The hypothalamo-pituitary-gonadal (HPG) axis is constrained by a negative criticism circle. In the healthy cerebrum, the nerve center discharges gonadotropin releasing hormone (GnRH) into the middle prominence, and afterward GnRH is moved by means of the hypophyseal portal system to the anterior pituitary where it follows up on its receptor (GnRHr).Signaling from GnRHr prompts the creation and emission of the gonadotropins, including luteinising hormone (LH) and follicle-stimulating hormone (FSH). When the gonadotropins are emitted into the circulatory system, LH follows up on its receptor in the gonads, which thusly invigorates the release of the sex steroids, androgens and oestrogens. These sex steroids complete a negative input circle by repressing the release of GnRH. Peripheral hormone levels have for some time been involved in changes of conduct. Investigations of the modulatory impacts of hormones on perception have prompted the disclosure that hormone receptors are communicated in the CNS. Critically, a significant number of these hormone receptors are available in zones of the cerebrum related with learning and memory, for example, the hippocampus. Significant to HPG pivot brokenness is that the pace of hormone blend differs for a mind-blowing duration (Gillies and Mcarthur, 2010). With age, androgen creation diminishes bit by bit in men, while there is a sudden

reduction in gonadal emission of oestrogens in women (Gillies and Mcarthur, 2010). Moreover, the dysregulation of the HPG axis brought about by menopause might be because of a decreased capacity of oestrogens to restrain the nerve center (Gonzalez*et al.*, 2011). Interestingly, notwithstanding the negative input oestrogens have on the HPG pivot, oestrogens can likewise deliver positive feedback, which is vital for fertility.

2.3.2 Oestrogens and Hypothalamo-pituitary-gonadal Axis

Onwards from puberty, oestradiol is produced during the follicular period of the ovarian cycle by the ovarian follicles. Enrolled overwhelming follicles produce oestradiol and inhibin which stifle the FSH emission from the adenohypophysis. Low oestrogen levels decrease the terminating level of GnRH neurons in the pre-ovulatory-flood focus.However significant levels of oestradiol which would happen during the mid-to late follicular stage causes an increase inrelease of GnRH by the pre-ovulatory-flood focus (Cortès*et al.*, 2018).

Aromatisation is the last advance in oestrogen development. This response is catalysed by the P450 mono-oxygenase protein complex that is available in the smooth endoplasmic reticulum and capacities as a demethylase. In three consecutive hydroxylating responses, oestrone and oestradiol are framed from their mandatory antecedents androstenedione and testosterone, respectively. The last hydroxylating step in aromatisation doesn't require enzymatic activity and is not product sensitive(Spiering, 2019).

The essential wellsprings of oestradiol in women are the theca and granulosa cells of the ovaries and the luteinised subordinates of these cells. As per the hypothesis of oestrogen union, the theca cells emit androgens that diffuse to the granulosa cells to be aromatised to oestrogens. There is, however, proof that both of these sorts of cells might have the option to shape the two androgens and oestrogens(Spiering, 2019).Oestrone and oestriol are basically

framed in the liver from oestradiol. Aromatase movement has likewise been identified in muscle, fat, sensory tissue, and the Leydig cells of the testicles.

Puberty in girls is initiated by low-amplitude nocturnal pulses of gonadotropin that raise serum oestradiol fixations to 15 - 35 pg per milliliter (55to 128 pmol per liter). During menstrual cycles, oestradiol production changes consistently, with the most elevated rates and serum focuses in the preovulatory stage. Oestradiol production and serum fixations are most reduced premenstrually. In the perimenopausal period, depletion of ovarian follicles prompts an unfaltering decrease in ovarian oestradiol generation, despite the fact that serum oestradiol focuses shift extensively.

Oestrogen maintains auxiliary sexual attributes and applies feedback activity on the hypothalamo-pituitary unit to influence the synchronised preovulatory arrival of gonadotropins. Oestrogen improves "self-priming" effect of GnRH on the pituitary, with the goal that when oestrogen levels are high, the underlying pulses of GnRH prime pituitary reaction to resulting GnRH pulses are low. Oestrogen additionally potentiates pituitary responsiveness to GnRH by expanding the quantity of GnRH receptors through direct incitement of protein union required for receptor development. Oestrogensynergises with FSH at the ovarian level to build the quantity of FSH receptors per granulosa cell. Oestrogens advance improvement of female auxiliary sexual qualities: sexual drive, thelarche (breast advancement), adrenarche (axillary and pubic hair advancement) and partly, the menstrual cycle changes in the endometrium and furthermore changes in sexual drive during menopause through its different subtypes: oestrone, oestradiol and oestriol.

Oestrogens invigorate an expansion in both the size of myometrial and endometrial cells (hypertrophy), just as increment in their number (hyperplasia), setting up the endometrium for implantation of the incipient organism which is the vital occasion separating the fertile

and nonfertile ovulatory cycle. Embryonic implantation is sustained by the endometrium experiencing explicit development, proliferation, and differentiation occasions, including secretory change of the glandular components, oedema arrangement, vascular expansion and decidualisation of the stroma, intervened to some extent by oestrogens.

Bisphenol-A declines aromatase (CYP19A1) articulation and oestradiol production in human granulosa cells. BPA restrains oestradiol amalgamation by diminishing the steroidogenic intense administrative protein (Star), 3β-hydroxysteroid dehydrogenase (Hsd3b1) and 17α-hydroxylase (Cyp17a1) in mice and rats(Spiering, 2019).

2.3.3 Progesteroneand Hypothalamo-pituitary-gonadal Axis

Progesterone is fundamental for the regulation of normal female reproductive capacities. The major physiological activities of progesterone include those in the uterus and ovary (induction of ovulation, facilitation of implantation, and support of early pregnancy); in the mammary gland (lobular-alveolar development in anticipation of milk emission);in the cerebrum (neurobehavioral articulation related with sexual responsiveness); andin the bone (counteractive action of bone misfortune).

It is a 21-carbon steroid which is an antecedent particle for steroids biosynthesis. Progesterone is essentially produced by the granulosa-lutein cells of the corpus luteum (CL) during the luteal period of the menstrual cycle and the syncytiotrophoblast of the placenta during pregnancy. However, zona fasiculata and zona reticularis of the adrenal cortex produce few measures of progesterone which is less than 1mg/day.

Progesterone secretion is negligible during the follicular stage, yet there is a little yet huge ascent in its level at the hour of inception of the gonadotropin surge (Kim, 2008). In low concentration, it facilitates LH release which is being accepted to be conceivably required for

the full articulation of the gonadotropins. Progesterone secretion is predominant in the luteal stage, bringing about a significant reduction in GnRH/LH pulse recurrence all through the luteal stage.

In primates, luteinisation and follicular rupture happen 36–38 hours after the beginning of midcycle gonadotropin surge. During this preovulatory stage, granulosa cells experience changes in light of the ovulatory boost that outcome in terminally separated luteal cells. While separating (luteinising) granulosa cells emit enormous amounts of progesterone.

The preovulatory surge of gonadotropins enacts a course of proteolytic catalysts bringing about the rupture of the follicular wall and the release of a fertilisable ovum during ovulation. Several lines of evidence support a role for progesterone in the induction of proteolytic activity in the preovulatory follicle of primate and nonprimate species. Levels of mRNAs for network metalloproteinases-1 (MMP-1) and its tissue inhibitor metalloproteinases-1 (TIMP-1) expanded significantly inside 12h of gonadotrophin upgrade and were upregulated by progesterone. Additionally, restraint of progesterone combination or blocking progesterone activity with RU486 diminished MMP movement in the rat and ewe. An administrative role for progesterone in the enactment of other ovulation-related proteases, such as plasminogen-activator, has been recommended also, on the grounds that organisation of a specific progesterone receptor adversarybrought about lower plasminogen-activator movement levels. Changes in proliferative exercises of the glandular epithelium and stromal components of the human endometrium connect to the coursing levels of oestrogens and progesterone. During oestrogen-dominant follicular stage, cell poliferations happen in both epithelial and stromal cells. This is trailed by a decrease in expansion in the principal half of the secretory, progesterone commanded period of the cycle. In the late luteal stage, while proliferative action stays low in the epithelium, a second pinnacle of expansion, reliable with decidual

changes, is found in the stromal components. Oestrogen stimulates epithelial cell poliferation, while progesterone restricts the mitotic effects of oestrogen and represses poliferation. In Progesterone Knock Out (PRKO) mice, removal of both PR-A and PR-B isoforms brought about an imprint hyperplasia in the endometrial epithelium because of unopposed proliferative oestrogen activity. However, in a PR-A knock-out mice (PRAKO), in which the expression of the PR-A isoform is specifically removed, the PR-B isoform capacities to intercede as opposed to restrain cell poliferations. This addition of PR-B-subordinate proliferative endless supply of PR-A shows that PR-A is vital not exclusively to restrict oestrogen-incited poliferations, yet in addition required to restrain expansions prompted by progesterone acting through the PR-B proteins (Al-Asmakh, 2007).

Bisphenol-A expanded articulation of steroidogenic intense administrative protein (StAR), cholesterol side-chain cleavage catalyst and 3î²-hydroxysteroid dehydrogenase in granulosa cells.However, BPA anticipated the basal and the FSH-instigated progesterone generation. Bisphenol-A made sequestration of cholesterol the perinuclear territory, as obvious by the Filipin recoloring. It diminished mRNA articulation of ATP binding cassette transporter-A1 (Abca1) and expanded degree of sterol administrative component restricting protein 1. Expansion of exogenous cell-porous cholesterol reestablished the effect of BPA on Abca1 and Star mRNA articulation and somewhat switched BPA's effect on progesterone production(Al-Asmakh, 2007).

2.3.4 Follicle Stimulating Hormoneand Hypothalamo-pituitary-gonadal axis

The GnRH cause the release of the hormones FSH and LH. In females, FSH animates improvement of egg cells, called ova, which are found in structures called follicles. Follicle cells produce the hormone inhibin, which restrains FSH production.

Regulation of the reproductive system is a procedure that requires the activity of hormones from the pituitary organ, the adrenal cortex, and the gonads. During puberty in both males and females, the nerve center produces gonadotropin-releasing hormone (GnRH), which invigorates the generation and arrival of follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary organ. These hormones direct the gonads (testes in males and ovaries in females) and in this manner are called gonadotropins. In both males and females, FSH invigorates gamete generation and LH animates production of hormones by the gonads. An expansion in gonad hormone levels restrains GnRH generation through a negative feedback circle(Abdalla and Thum, 2006).

Follicle stimulating hormone, is a hormone that has an immediate bearing on the menstrual cycle and ovulation. At the point when FSH levels are excessively low (as might be found in women with hypothalamic or pituitary pathologies), or excessively high (as might be found in women encountering premature menopause), it can complicate capacity to end up pregnant. A "normal" FSH level for a woman wanting to conceive is normally underneath 10mIU/ml in the follicular stage. The degree of FSH the body produces correlate to the ovarian reserve (the quality and amount of the rest of the eggs). Therefore, realising the FSH level can be useful in predicting fertility. At the point when the egg amount and quality starts to wane, the body attempts to remunerate by producing more FSH trying to help invigorate ovarian capacity. Therefore, more significant levels of FSH may deduce that menopause is drawing closer. Low FSH levels can likewise affect fertility, bringing about a suspension of reproductive cycles(Raju *et al.*, 2013).

2.3.5 Luteinising Hormoneand the Hypothalamo-pituitary-gonadal axis

There is no uncertainty that morphological and practical changes happening inside the follicle during the pre-ovulatory period are of essential significance for definite maturation of the

ovum. It has however become progressively clear that hormonal occasions before the mid-cycle LH top, explicitly the hesitancy of LH and FSH during the early follicular stage, may essentially effect the improvement of the oocyte and decide its ensuing meiotic capability and fertilisation ability. The effect of pituitary FSH on control of ovarian capacity during the follicular stage is undoubted. Follicle-stimulating hormone assumes a significant role in female propagation by stimulating gonadal differentiation and development by means of its demonstration particle on the granulosa cell in the ovary. Concentrates in rats show that during the follicular stage, FSH controls major morphological and cell occasions.For example, procurement of the antral pit, induction or actuation of catalysts engaged with biosynthesis, acceptance of LH receptors on granulosa cells, and initiation of the aromatase system. These effects of FSH empower the follicle to deliver oestrogen, to ovulate, and luteinise in light of the LH flood. The overall significance of LH in the follicular stage and its role in the incitement of the follicle is unsure, and the ideal measure of LH or the proportion of FSH:LH in the stimulating medications utilised is an issue that goes back to the beginning of gonadotropin therapy(Raju *et al.*, 2013).

The secretion of LH at midcycle brings about finishing of the principal meiotic division, and roughly 24 to 36 hours after the fact ovulation happens and a developed oocyte is discharged from the follicle. There are animal categories to animal groups distinction in the interim between consummation of the principal meiotic division of oocytes and their treatment. This interim speaks to a period during which conditions for creating a normal embryo are optimal(Robker, Hennebold, & Russell, 2018).

The hypothesis that the two gonadotropins are required for complete incitement of follicular maturation goes back several decades. Fevold was the first to bring it up in 1941 that treatment with profoundly pure FSH (pFSH) expanded ovarian development and follicle

improvement in juvenile hypophysectomized rats without invigorating the release of oestrogen. After a while,Gonzales and Kretser, in 1989 showed in hypophysectomised rats that FSH brought about just follicular development and that entire pituitary extract, with assumed LH action, was important to create uterine development demonstrating follicular generation of oestrogen. It was exhibited that albeit both theca and granulosa cells had the ability to incorporate steroids in vitro, the production of oestradiol by granulosa cells was particularly improved by the expansion of theca cells(Urrego *et al*., 2015).

Recently, the requirement for LH during the hour of follicular development has been addressed on the grounds that in monkeys rendered hypogonadotropic by the organisation of GnRH antagonists, that infusing expanded portions of pure FSH without including LH caused various follicular improvement, and ovulation could obviously be actuated by the organisation of human chorionic gonadotrophin (hCG)(Raju *et al.,* 2013).

As circling oestradiol levels increase during the follicular stage, gonadotropin fixations decline. In menopausal women or women who have experienced ovariectomy, in whom oestradiol emission is inadequate, supported increments in LH and FSH discharge happen. In these conditions, the organisation of physiologic dosages of oestradiol brings about a fast and supported abatement in LH and FSH to levels proportional to those seen during the menstrual cycle (Requena*et al.,* 2014). The oestradiol negative feedback circle acts to diminish LH emission quickly, for the most part by controlling the sufficiency of the LH pulse. As the follicular stage advances, LH pulse quantity decreases. Luteinising hormone pulse recurrence during the follicular stage (at 60–100-minute intervals) approximates that seen at menopause or after ovariectomy, recommending that oestradiol doesn't especially influence LH pulse recurrence (Requena *et al.,* 2014). At higher physiologic focuses, oestradiol additionally applies a different stimulatory (positive feedback loop) effect on gonadotropin discharge.

Progesterone, at high fixations, for example, those seen during the luteal period of the cycle, likewise applies an inhibitory effect on gonadotropin emission. Rather than oestradiol, progesterone influences principally LH pulse recurrence. It is accepted that the decrease in FSH after its top in the early follicular period of the typical cycle results from a negative feedback activity of inhibin B at the pituitary level. This activity has been demonstrated uniquely under test conditions, be that as it may, for instance after antioestrogen treatment in the midfollicular stage (Andersen, 2017). At menopause or in untimely ovarian disappointment, information show a diminished discharge of inhibin with regenerative maturation, proposing that inhibin B negative feedback might be a significant factor controlling the early monotropic increment in FSH. In perspective on these input circles, it isnot astonishing that the attributes of pulsatile LH emission shift extracommonly with the phase of the menstrual cycle. During the oestrogenic stage or follicular stage, pulses of high recurrence however of low sufficiency are seen, while during the progesterone stage or luteal stage, there is a dynamic decrease in the recurrence of the LH pulse, with pulse interims happening like clockwork or more before the finish of the luteal stage. This diminished heart pulse recurrence is joined by a noteworthy increment in pulse increases(Taraborrelli, 2015).

2.3.6 Testosteroneand the hypothalamo-pituitary-gonadal axis

The hypothalamus sends a signal to the pituitary gland to discharge gonadotropic substances (follicle stimulating hormone and luteinising hormone). Luteinising hormone stimulates testosterone production. In the event that an excessive amount of testosterone is delivered, the nerve center cautions the pituitary organ to make less LH, which regulates the testes and ovariesin diminishing testosterone levels. The majority of secreted testosterone in females, especially that from the ovaries are converted to oestradiol. The fetal testes secretes testosterone and anti-mullerian hormone (AMH) at the eighth week of gestation (Grinspon*et al.*, 2018).

2.3.7 Antimullerian Hormoneand Hypothalamo-pituitary-gonadal Axis

Anti-mullerian hormone was at first discovered in view of its role in Mullerian pipe relapse during male foetal development. In females, AMH is produced postnatally by granulosa cells; levels bit by bit increment, with pinnacle levels corresponding with peak fertility periodin the mid-20s, and decrease from that point getting to become imperceptible at time of practical menopause. Anti-mullerian hormone is liable for the relapse of the mullerian conduits that in the female embryo separate into the oviducts, the uterus and the upper segment of the vagina.

In infant females, ovaries contain primodial follicle pool containing 1-2million of oocytes captured in the diplotene phases of meiotic prophase 1 and encompassed by smoothed pre-granulosa cells. These primordial follicles remain in their captured state for a considerable length of time till the beginning of puberty. Anti-mullerian hormone is expressed in granulosa cells of developing follicles and keeps on being available all through the reproductive ages. In young women, AMH flowing qualities are practically imperceptible during childbirth with a slight increment inside the primary years old, before puberty (Moini *et al*., 2019).

Anti-Mullerian hormone is one of the normally present hormones in the female body that can give a sign of richness potential. Anti-Mullerian hormone is a substance which creating egg sacs (ovarian follicles) discharge, and its levels are distinguishable by the basic methods for an AMH blood test. The higher the quantity of eggs staying in the ovaries, the higher the degree of AMH which will appear in the circulation system. Accordingly, a low level is viewed as an indication of a low ovarian reserve, for example hardly any outstanding follicles. This would be typical for a woman who is moving toward menopause. By a similar token, a woman who has polycystic ovarian disorder, described by the nearness of numerous little ovarian follicles, would commonly be required to show elevated levels of AMH. Along these lines AMH plasma levels mirror the nonstop development of little follicles, thus mirror

the size of the rest of the follicle pool, therefore speaking to be a valuable marker of ovarian reserve. Anti-Mullerian hormone is especially valuable as an endocrine marker for evaluating the age-related decay of the ovarian pool and subsequently its capacity to anticipate future reproductive life expectancy. It is especially valuable, in spite of the fact that not a total analytic arrangement, since an AMH test can be done whenever of the month to month cycle, and regardless of whether the woman concerned is on hormonal contraceptives. Regularly, the sitting tight time for the consequences of different sorts of hormonal tests may incorporate the hang tight for the correct point in themonth to month cycle for the test to be completed, this is not an issue with AMH(Moini *et al*., 2019).

2.4 Roles of Antioxidants on Reproductive System and Fertility

Reactive oxygen species (ROS) are oxygen inferred atoms, which are shaped as intermediary products and are a class of powerful oxidants in the human body. ROS incorporate superoxide anion ($O^{-}2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). High-effect condition is a consistent wellspring of ROS through in vivo components, for example, electron spillage during biologic oxidations, and by physical enactment of oxygen by outer specialists, as well as light for ultraviolet daylight. ROS are portrayed by their capacity to respond with any atom they come in contact and alter it oxidatively. The change may bring about basic and useful adjustments and hinder numerous cell forms.

Antioxidants are atoms with low sub-atomic weight that can assault free radicals and kill them by giving an electron hence lessening their ability to harm (Mulla*et al.*, 2018). These association can securely stop a progression of harming responses that can influence significant particles. The human body is worked to make its own antioxidant and a few antioxidant chemicals are known. Glutathione is viewed as the central characteristic antioxidant, as it is viable in the detoxifying component and keeping up the cell redox state

by keeping up the essential harmony among ROS and antioxidants (Mulla *et al.,* 2018). What's more, its essence is basic for oocyte development. Different antioxidant system parts are additionally critical, in this way a shortage of any of the segment may harm the capacity of the entire system.

The antioxidants are said to have been overpowered by the free radicals. This happens when the generation of free radicals surpasses the level which the body's regular antioxidant guard components can adapt to; therefore making a cell oxidative condition which triggers the oxidation of basic biomolecules like DNA, protein and lipids, prompting different infection conditions. The interchange between the trio of free extreme antioxidants, and infections is significant in looking after wellbeing, maturing and age-related ailment (Ighodaro andAkinloye,2018). Contribution of exogenous antioxidants to human wellbeing has additionally been advertised. Indeed, it has been recommended that diminished introduction to free radicals and expanded admission of antioxidant rich nourishments or antioxidant enhancements will upgrade the body's capability to limit the danger of free radical related medical issues (Ighodaroand Akinloye, 2018).

Antioxidants, for example, polyphenols, ascorbic corrosive, alpha-lipoic corrosive, thioredoxin, glutathione, melatonin, coenzyme Q, beta carotenoids, alpha-tocopherols just as antioxidant catalysts including superoxide dismutase, catalase, glutathione peroxidases, glutathione reductases and glutathione transferases have been generally examined for the counteractive action and treatment of infections coming about because of oxidative damage (Allocati, Masulli, Di Ilio, & Federici, 2018).

2.4.1 Antioxidants of Study

Superoxide dismutase (SOD) represents a group of enzymes that utilises as cofactor copper and zinc, or manganese, iron, or nickel ions (Cristiana*et al.*,2014). There are three significant

groups of superoxide dismutase, contingent upon the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which binds either iron or manganese), and the Ni type, which binds nickel (just in prokaryotes). SOD1 is situated in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. The first is a dimer (comprises of two units), while the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, and SOD2, the mitochondrial enzyme, has manganese in its reactive site (Cristiana *et al.*, 2014).

SOD enzyme families are available in various disorders and ovaries of various mammals. The location of SOD in the human body was first dictated by utilising immunohistochemistry (Wang*et al.*,2017). Neither SOD1 nor SOD2 has been seen in primordial and essential follicles. SOD1 starts to show up in theca cells after the development of the antral cavity. SOD1 cannot be detected in granulosa cells until follicles enter the dominant follicle stage. High expression levels of both SOD1 and SOD2 have been detected in luteinised granulosa and theca cells.

Biochemically, a pinnacle of aggregate SOD activity shows up during the pro-oestrus stage, which includes the most minimal degree of superoxide radicals contrasted and other oestrous stages (Jiwakanon, Persson, Kaeoket, & Dalin, 2005). Both the sum and activity of the three SOD isoforms in follicular liquid are more prominent in little and medium follicles than in enormous antral follicles, and these discoveries have been evaluated and checked in various animals (Combelles, Holick, Paolella, Walker, & Wu, 2010). However, no changes in SOD have been seen in granulosa cells as to the size of the follicles. An oocyte in the preovulatory follicle obtains developmental fitness and a functioning metabolism, and during this procedure, a lot of ROS can be produced; along these lines, SOD is required to neutralise O_2•− in the cytoplasm of oocytes and subsequently, SOD must be kept up at a specific concentration and activity level inside the follicles to ensure a balance between O_2•− and

H_2O_2 for typical cell function (Wang *et al*., 2017). Decisively, a specific measure of SOD not just guarantees a utilitarian concentration of ROS for ovulation, yet in addition shields oocytes from oxidative stress.

Glutathione peroxidase (GPx) is an antioxidant enzyme class with the ability to scavenge free radicals. This is thus counteracting lipid peroxidation and maintain intracellular homeostasis just as redox balance. Glutathione Peroxidase (GPx) is a significant intracellular enzyme that breakdown hydrogen peroxides (H_2O_2) to water; and lipid peroxides to their comparing alcohols fundamentally in the mitochondria and sometimes in the cytosol (Ighodaro andAkinloye, 2018). Most times, its activity relies upon a micronutrient cofactor known as selenium. Therefore, GPx is frequently alluded to as a selenocysteine peroxidase. The enzyme assumes an increasingly critical role of hindering lipid peroxidation procedure, and consequently shields cells from oxidative stress.

The clinical significance of GPx has been underlined by various investigations. Noblanc *et al*., 2011hypothesised that people with lower GPx action are inclined to debilitated antioxidant insurance, which prompts oxidative harm to layer unsaturated fatty acids and useful proteins, and by surmising, neurotoxic harm.Sarıkaya and Doğan in 2020had theorised that GPx inadequacy directly actuates an expansion in vascular oxidative stress, with attendant endothelial dysfunction.

Nitric oxide (NO) is produced from L-arginine by means of nitric oxide synthase (NOS). It requires oxygen and various cofactors, for example, nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), calmodulin, and calcium, bringing about the production of NO just as a by-product known as L-citrulline (Mulgund*et al.,* 2015). There are three types of NOS that apply their effect through protein interactions and catalyse the previously mentioned response: (1) endothelial

NOS (eNOS), (2) inducible NOS (iNOS), and (3) neuronal NOS (nNOS). Each isoform has a reductase space that contains a compound known as tetrahydrobiopterin (BH4), which is basic for the proficient generation of NO (Mulgund *et al.*, 2015).

The degrees of NOS and NO are likewise connected with the degrees of the reproductive hormones oestrogen and progesterone. In an investigation on endometriosis-related infertility, fasting blood tests obtained from women with this condition showed a positive correlation between the degrees of oestrogen and progesterone and eNOS protein levels (Bednarek-Tupikowska, Tworowska-Bardzińska, & Tupikowski, 2008). In particular, NO enacts cyclooxygenase-2 (COX-2), which thus expands the degrees of prostaglandin E2, in this way causing the degrees of aromatase, a chemical essential for oestrogen production. The resultant oestrogen height invigorates further eNOS quality articulation in a positive input circle (Mulgund *et al.*, 2015).

2.5 Role of Metabolic Enzyme Uridine DiphosphateGlucoronosyl Transferaseon Fertility

Conjugation with glucuronic acid is the significant fate of numerous lipophilic compounds in animal cells. This procedure is interceded by the uridine diphosphate (UDP) glucuronosyltransferase (UGT) multigene family. Uridine diphosphate glucuronosyltransferases are found principally in the mammalian liver and catalyse the transfer of the glucuronic acid moiety of UDP glucuronic acid (UDPGA) to a wide scope of acceptor molecules (Lee *et al.*, 2014). The sugar acid can be coupled to the substrate through the - O.R, - SR, N.R'R" or - C.R groups framing a P-D-glucopyranosiduronic acid or glucuronide. Substrates for glucuronidation are normally little hydrophobic particles that are named aglycones lacking starch(Lee *et al.*, 2014).

Glucuronosyl transferases are a group of enzymes that catalyse the exchange of glucuronic acid from uridine diphosphate (UDP)- glucuronic acid to acceptor molecules containing hydroxyl, phenol, carboxylic acid, thiol, or amine groups (Ouzzine*et al.,* 2014). Dynamic investigations have demonstrated that these proteins pursue an arbitrary successive component. Glucuronosyl transferases are situated in the endoplasmic reticulum, and their biosynthesis can be instigated by various medications and xenobiotics. A few types of glucuronosyl transferases are coordinately incited with cytochromes P450. Conjugation with glucuronic acid makes lipophilic particles considerably more water-solvent and promptly excretable, and consequently is viewed as a detoxication pathway. Nonetheless, acyl glucuronides can experience basic improvements and respond with nucleophilic bunches in cells (Ouzzine *et al.,* 2014). Along these lines glucuronidation of certain particles may speak to bioactivation. For instance, the acyl glucuronide of the hypolipidemic tranquilise clofibrate acylates cell macromolecules. Be that as it may, the toxicological noteworthiness of this response is vague.

Uridine diphosphate glucuronosyltransferase (UGT) is a significant phase II drug-metabolism enzyme superfamily engaged with biotransformation of xenobiotics in humans by catalysing the exchange of glucuronic acid to hydroxyl, carboxyl, or amine bunch compounds (Liu *et al.,* 2016). UGT1A1 and UGT1A9 are two individuals from this superfamily and catalyse the metabolism of numerous endogenous substances and medications. For instance, bilirubin and oestradiol, buprenorphine, acetaminophen, quercetin, etoposide, SN-383 are known as substrates of UGT1A1, while 4-hydroxy oestrone, acetylenic oestrone and retinoic acid, propofol, acetaminophen, retinoic acid, mycophenolic acid4, and quercetin are accounted for as substrates of UGT1A9(Liu *et al.,* 2016).

2.5.1 Uridine Diphosphateand the Ovary

It is realised that xenobiotic-metabolising enzymes can be instigated by endogenous compounds, for example, hormones, including gonadotropic hormones. Prior investigations uncovered that UGT and sulphotransferase activities in rat ovary are managed during the oestrous cycle and by the gonadotropins FSH and LH. Another xenobiotic-conjugating compound that has its degree of articulation directed by hormones is glutathione transferase. This chemical is up-managed in the liver of pregnant mare's serum gonadotropin (PMSG)hyperstimulated female rats, just as during sexual development of female rats. It is directed by different hormones too, incorporating adrenocorticotropic hormone in the adrenal organ and thyroid hormones and testosterone in the liver.

UGT1A6 is evidently the main isoenzyme of UGT communicated in rat ovary. Steroids assume a significant role in ovarian capacity and it is conceivable that different isoenzymes of UGT, for example steroid UGTs, are also present, yet at extensively lower levels. In fact, two UGT groups have been identified in human ovary(Lee *et al.*, 2014). Changes in the activity of this enzyme on treatment with PMSG reflect up-regulation of the measure of protein and, clearly, of the relating mRNA. Regulation of these progressions includes LH, hCG and progersterone(Poulsen *et al.*, 2020). A comparative relationship among action and the measure of protein was found in the ovaries of youthful rats, animals in the preovulatory and corpus luteal stages and developed animals. However, the action of PMSG-hyperstimulated rats didn't increase as much as the measure of protein, in correlation with the other four groups. This may demonstrate the presence of another ovarian UGT structure. UGT is a basic film protein, with its dynamic site situated towards the lumen of the endoplasmic reticulum. It is imperative to have the right lipid condition for maximal explicit action of this chemical(Lee *et al.*, 2014).

The localisation of UGT in the ovarian cells is still unclear, but the key cells for the metabolism of 1-naphthol in the ovary are the theca and stroma cells which also have the highest production rate of progesteroneand are the only ovarian cells that express the LH receptors in immature animals(Becedas, Lundgren, & Pierre, 1998).

UGT is vital in the clearance of xenobiotics in the ovary and liver. It is still unclear if the phenol- UGT isoenzyme acts an endogenic role in ovulation and/or the luteinising phase.Insufficient synthesis of UGT can cause accumulation of xenobiotics in the ovarian cells. This might lead to depletion in the ovarian structure hereby leading to ovarian failure. Also, impairments to the larger follicles would avert ovulation which will ultimately cause infertility during reproductive stages (Hoyer and Keating, 2014).

Alternatively, proper functioning of the UGT would facilitate proper ovarian function by clearing ovarian toxins hereby enhancing ovarian function which will have a positive effect on fertility.

2.5.2 Uridine diphosphoGlucuronysyltransferase (UDP-GT)and Bisphenol-A

The significant metabolite of BPA in rats is glucuronide, and extensive measures of unaltered BPA and hydroxylated BPA are identified in excrement following oral organisation of 14C-named BPA. As of late, a few examinations have indicated that BPA monoglucuronide is the dominating in vivo metabolite in rats, and in people (Aboul Ezz, Khadrawy, & Mourad, 2015). Glucuronidation of BPA by rat liver microsomes has been recommended to be for the most part catalysed by UDP-glucuronosyltransferase (UGT) isoform UGT2B1. Then again, Yoshihara *et al*., 2004detailed that 3-hydroxy (BPA catechol) and BPA uquinone are detected as cytochrome P450 (CYP) subordinate metabolites in liver S9 divisions from rats, mice,

monkeys, and people. In this manner, drug-metabolising enzymes, for example, CYP and UGT are connected with the metabolism and toxicity of BPA in mammals.

Uridine glucuronosyl transferases are typical membrane proteins of the endoplasmic reticulum and the atomic envelope. They have been characterised into two families (UGT1 and UGT2) in view of amino acid succession similitudes (Zbucka-Kretowska *etal.,* 2018). Each UGT shows extranormal substrate and disorder specificbinds, and its activity or articulation is affected by hereditary and ecological components. UGT1A6 is communicated in the liver, kidney, cerebrum, bile duct, stomach, and colon in people (Zbucka-Kretowska *et al.,* 2018). The outflow of UGT1A6 has been demonstrated to be directed by aryl hydrocarbon receptor agonists, for example, 2,3,7,8-tetrachlorodibenzo-p-dioxin or potentially antioxidant type inducers, for example, t-butylhydroquinone in mammalian cell lines.Accordingly, UGT1A6 is viewed as a significant key protein in natural effects. In spite of the fact that BPA is broadly utilised in the compound business, little is thought about the communication of BPA with drug metabolising enzymes in humans.

2.6 Melatonin

Melatonin is a pineal hormone that assumes significant role in circadian rhythm. Melatonin is found in plants and animals just as different life forms. Pinealocytes function as neuroendocrine transducers to secret melatonin during the dark period of light/dim cycle and, therefore, melatonin is frequently called the hormone of darkness. Past its main effects in light-dim cycle, melatonin has neuroprotective, anti-inflammatory, and antioxidant properties(Fernando and Rombauts, 2014).

2.6.1 Biology of Melatonin

Melatonin (N-acetyl-5-methoxytryptamine), was isolated in 1958 by Aaron Lerner as a neuro-hormone primarily incorporated and secreted from the pineal organ(Karasek*et al.,*

2006). Melatonin is a methoxynidole subordinate delivered dominatingly by the pineal organ.It is also produced by extrapineal organs such as the retina, harderian organ, gastrointestinal tract, cerebrum, eye, lungs, skin, kidney, thyroid, thymus, pancreas, resistant system, reproductive system (Fernando and Rombauts, 2014). It is synthesised by pinealocytes which is the primary wellspring of melatonin in blood and its essentially emitted at night. The pace of melatonin combination relies upon the activity of its antecedents, serotonin, arylalkylamine N-acetyltransferase (AANAT), and tryptophan hydroxylase (TPH) in a circadian and regular way (Fernando and Rombauts, 2014).

Melatonin activity is mediated by two high affinity melatonin receptors, MT1 and MT2 having a place with the superfamily of G-protein-coupled receptors (GPCR) through atomic receptors RZR/ROR (Eller *et al.,* 2014). These receptors are found in the suprachiasmatic cores (SCN) of the hypothalamus of the mammalian cells, arrange the combination of melatonin in the pineal organ, and furthermore take part in a few neuroendocrine and physiological procedures. Melatonin receptors are generally dispersed in CNS, some other organs such as CVS, liver, gall bladder, skin e.t.c.CytochromeP450 processes melatonin to 6-hydroxymelatonin, N-acetylserotonin. It additionally has an affinity for a third melatonin receptor, (MT3) indistinguishable from the cytosolic chemical, quinone reductase 2(QR2). One non-receptor-interceded activity of melatonin have been detected. One of them is its free radical rummaging and antioxidant limit (Eller *et al.,* 2014).

It has additionally been exhibited that melatonin controls both the electron transport chain and oxidative phosphorylation by keeping up ATP level in normal cells and furthermore during maturation. Melatonin is a wide range antioxidant and flexible when contrasted with its normally happening sub-atomic analogs, ramelteon and agomelatine.

2.6.2 Functions of Melatonin

Melatonin might be utilised for the management of sleep disorder, for example, sleep deprivation and the confusion emerging from night function(Urrestarazu and Iriarte, 2016). The benefits of melatonin in connection to other sleep-inducing agents are the non-appearance of after-effect the following morning, the non-attendance of withdrawal side effects and the absence of addiction (Urrestarazu and Iriarte, 2016). In individuals who are visually impaired, the administration of melatonin may help in the synchronisation of biological rhythms to the earth (Andrews *et al.,* 2019).

Melatonin might be utilised effectively for the counteractive action and treatment of fly slack. Stream slack is regularly seen in transoceanic explorers crossing a few time zones. It emerges because of absence of synchronisation of the endogenous mood of the life form with the light–dull cycle of the earth in the goal territory. The effect of oral organisation of melatonin on stream slack after transoceanic flights crossing a few time zones was assessed. Melatonin is a natural marker associated with characterising the circadian typology of every person(Andrews *et al.,* 2019).

Melatonin is a very potent free extreme beneficiary and a general antioxidant. As an antioxidant, melatonin binds strongly the lethal hydroxyl and hyper oxide radicals. The antioxidant properties of melatonin have been demonstrated in homogenised disorders and in living life forms (Reiter *et al.,* 2016). The antioxidant activity of melatonin is applied both in its original form and through its metabolites. The property of melatonin to act as an antioxidant in different forms makes it amazingly viable, even at a low concentration, in the security of living life forms from oxidative pressure(Reiter *et al.,* 2016). In concurrence with melatonin's defensive capacity, significant measures of melatonin have been distinguished in disorders and organs presented to unfriendly ecological assaults, for example, the skin and

the gut, and in organs with high oxygen utilisation such as the mind, melatonin generation being expanded by operators inciting low-force pressure, such as, practice in people (Erland and Saxena, 2017). Intense oxidative stress brings about an intense reduction in flowing melatonin levels because of its utilisation, as it is known to be a self-destructive antioxidant, being expended during its antioxidant activity(Kurutas, 2016).

It has numerous utilisations in restorative practice among which is its remedial effects have been accounted for in a few disorder, for example, certain tumors; cardiovascular ailmentsjust as in mental disorder (Reiter *et al.*, 2016). Neurological and neuropsychological inabilities brought about by mind wounds are a significant general health concern. Accordingly, diminishing deficiencies after a stroke is a significant disorder. In this line, various late investigations have announced the significant role of melatonin in neuroprotection in animal models of stroke(Andrabi, Parvez, & Tabassum, 2015).Organisation of melatonin after a trial stroke in animals diminishes localised necrosis volume(Naseem and Parvez, 2014). Such a defensive effect can be seen in both dark and white disorder, melatonin lessens likewise fiery reaction, cerebral oedema formation and blood-brain barrier permeability(Moyosore*et al*, 2011; Naseem and Parvez, 2014). Its neuroprotective action in animal models of ischemic stroke, just as its absence of genuine harmfulness recommends that melatonin could be utilised for human stroke treatment later on (Sadanandan *et al.*, 2020).

2.6.3 Melatonin and BPA/EDCS

Gustavo *et al*., in 2017analysed the effect of long haul melatonin treatment on the ovaries. They have detailed that melatonin improves ovarian capacity by expanding the quantity of auxiliary and graaffian follicles as well as corpus luteum and furthermore it shields oocytes and granulosa cells from nicotine harm. Additionally, the introduction of male rats to BPA have affirmed these endocrine upsetting exercises as indicated by certain investigations

(Woalder, 2017). For instance, dosages underneath the present most minimal watched unfriendly effect level (LOAEL <50 mg/kg) for BPA were related with diminished sperm tallies, debilitated sperm motility, sperm DNA harm and diminished testosterone levels.

2.6.4 Melatonin and Fertility

Melatonin is a naturally occurring compound found in animals, plants, and organisms. A few investigations revealed that melatonin assumes significant role in proliferation and maturation. There is a relationship between endogenous melatonin level and beginning of puberty(Boafo *et al.,* 2019). The pineal organ is huge in children however recoils at puberty, so it assumes significant role in sexual improvement. There is immediate connection between melatonin secretion and menstrual cycle. Melatonin reaches a peak level during the late luteal phase and its nadir during ovulation(Boafo *et al.,* 2019). Melatonin levels decline during maturation and achieves least levels at menopause. Female fertility tops at around age 25 years and decreases quickly after age 35 years(Song *et al*., 2016). Ovarian maturation is joined by a huge decrease in telomere length, ovarian follicle pool and oocyte reserve, just as an expanded number of low-quality oocytes not able for further improvement (Butts *et al*., 2009). An investigation was directed which proposed that melatonin improves the solid follicle number, telomerase action and telomere length, just as the oocyte quality and amount, demonstrating that it fights off the ovarian maturation. The entrenched worldview of generation in well evolved animals holds that females are brought into the world with a fixed number of oocytes which persistently decrease until few or none remain. The outcomes from the investigation recommend that maturing ovaries display decreased numbers, just as diminished amount and nature of oocytes. The gainful effects of melatonin on ovarian maturation may add to the eased back loss of non-sustainable female germ cells(Song *et al.*, 2016).

2.7 Oestrus Cycle in Rats

The reproductive cycle of female rats is called oestrous cycle and is portrayed as proestrus, oestrus, metestrus (or diestrus I) and diestrus (or diestrus II). Ovulation happens from the earliest starting point of proestrus to the end of oestrus. From the beginning of sexual development up to the age of a year, the mean cycle length in the female rat is 4 days and this short cycle length makes the rat a perfect animal for examination of changes happening during the reproductive cycle(Cora*et al.,* 2015). The cycle has also been described as ranging from 4 – 5 days. The oestrous cycle is an intermittent procedure that depicts changes in reproductive hormone levels brought about by ovarian activity affected by pituitary hormones. Changes in reproductive hormone levels further lead to auxiliary changes in the propagation tract netfunction. The oestrous cycles are portrayed by morphological changes in ovaries, the uterus and the vaginal cells (Rina *et al.,* 2017). and during this period, the vaginal mucosa experiences huge basic changes and is influenced by hormones, for example, FSH (stimulates the development and improvement of ovarian follicles), LH (manages oestrogen and progesterone hormones)(Rina *et al.,* 2017). Vaginal smear is used in determining the oestrous cycle stage.

During the oestrusphase, prolactin, LH and FSH stay low and increases toward the evening of the proestrus stage. Progesterone secretion also increases during metestrus and diestrus with a decrease a while later. At that point the progesterone worth ascents to arrive at its second top towards the finish of proestrus. During proestrus, oestrogen level increases and ovarian follicles develop quickly. The portrayal of each stage depends on the extent among three kinds of cells seen in the vaginal smear; epithelial cells, cornified cells and leukocytes (Cora *et al.,* 2015).

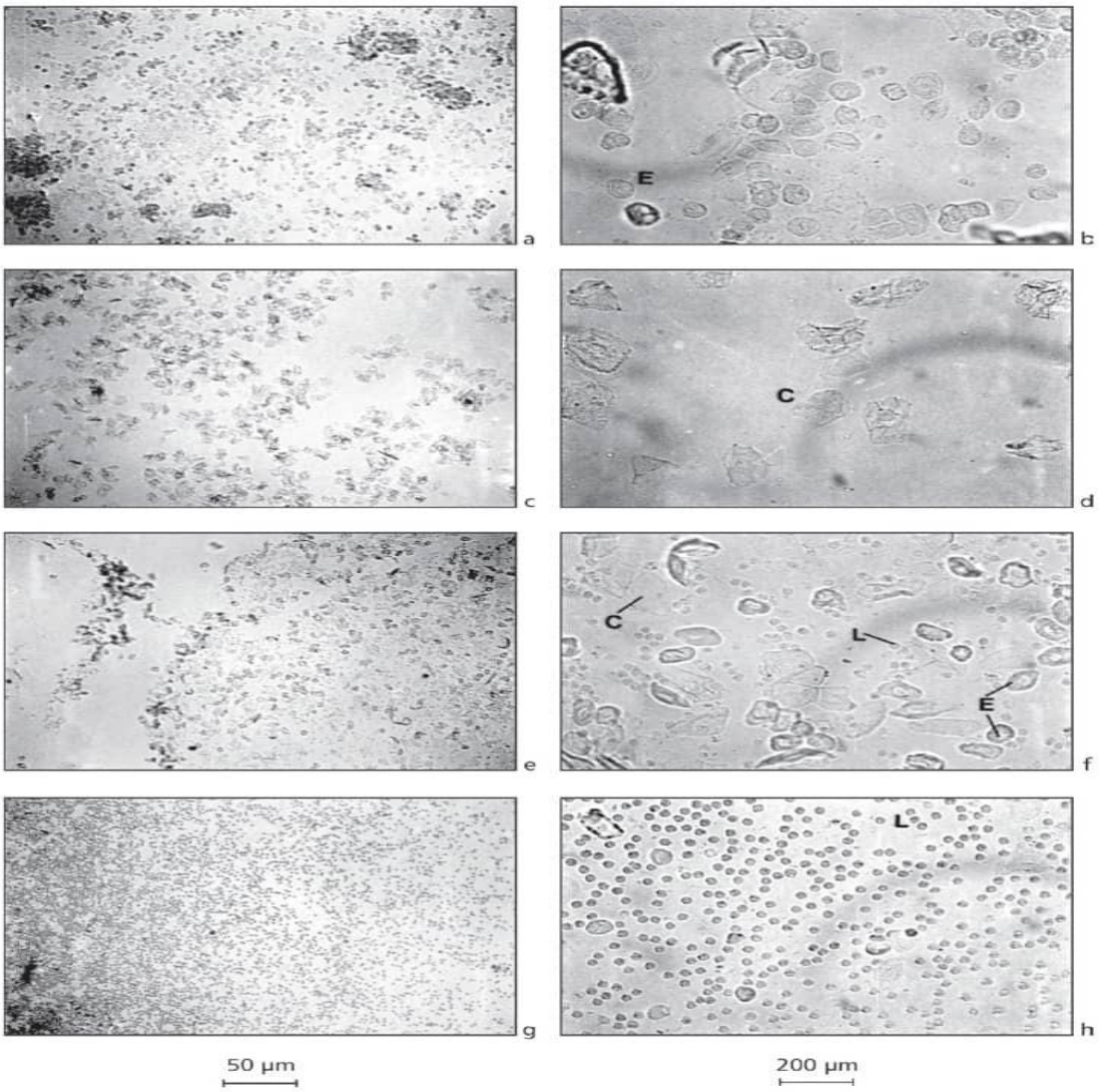
The proestrus period of the oestrous cycle compares to the human follicular period of the menstrual cycle. During proestrus stage, vaginal smear contains many nucleated epithelial

cells and a few leukocytes. FSH and LH fixations will in general be higher during proestrus(Ajayi and Akhigbe, 2020). This phase lasts for about 12 – 14 hours. The leukocytes are small and round cells while the epithelial cells show a superficial mucoid layer. The cornified cells appear as a result of progressively acidophilic nucleated epithelial cells.

During oestrus stage, there is the described cornification of the cells and the loss of leukocytes. In this phase, most of the cells are cornified cells. This phase last about 25 – 27 hours. The cornified cells are irregular anucleated cells. The cells appear as a result of detachment of the cornified epithelium. Papinal vaginas are portrayed by the practically select discovery of unpredictably molded squamous epithelial cells frequently in clusters. There is consistent ascent in levels of the progesterone hormone (Rina *et al.,* 2017).

Over the span of the metestrus, the sloughed cornified coating and mucosal invasion by leucocytes happens, cell types present in the vaginal swab during this divided stage, the cornified epithelial cells and the little dull leukocyte. At this stage of the cycle, there is complete detachment of the cornified epithelium which leaves few cornified cells and basophilic cells. The infiltrating leukocytes also appear as small round cells. LH concentrations will in general be higher during proestrus and metestrus. The progesterone level first ascents before a sharp decrease happens (Aritonang *et al*., 2017). This phase lasts for about 6 - 8 hours.

In the diestrus stage, the substance of the vagina consistently lacks cornified cells and leukocytes prevail in the smear. This diestrous phase is mostly dominated by the leukocytes. It lasts for about 53 – 57 hours. The end of this phase is characterised by the presence of epithelial cells proliferation. The recurrence of the cornified epithelial cells diminishes and the nucleated epithelial cells start to be detected just before the progression to the proestrus.



(Marcondes and Bianchi, 2002)

Figure 2.4: Vaginal smear (unstained) depicting phases of the oestrous cycle in rats. a, b (proestrus); c,d (oestrus); e, f (metestrus); g, h (diestrus).

2.8 Receptors and Proteins of Hypothalamo-pituitary Organ that Regulate Reproduction

2.8.1 Androgen Receptors

The androgen receptor (AR) is a piece of the steroid hormone receptor group of atoms, which likewise incorporates the progesterone, oestrogen, mineralocorticoid, retinoic acid, thyroid, and nutrient D receptors. The AR is a transcription factor, fundamentally liable for interceding the physiologic effects of androgens through official of the androgen–AR complex to explicit DNA target arrangements and initiating or smothering the translation of target genes (Bleach and McIlroy, 2018).

Androgen receptor, which has been restricted to the long arm of the X chromosome (at Xq11-12), is an individual from the atomic receptor superfamily and goes about as a ligand-inducible transcription factor to modulate expression of target genes(Bleach and McIlroy, 2018). The binding of testosterone or its metabolite 5-dihydroxytestosterone (DHT) to AR induces receptor dimerisation, facilitating the capacity of AR to bind to its related reaction components and enroll coregulators to advance the declaration of target genes (Bleach and McIlroy, 2018).

In females, androgens are produced principally in the ovaries and adrenal organs. In the ovary, testosterone is integrated by theca cells in response to LH. It is for the most part accepted that androgens straight-forwardly effect ovarian capacity through cooperation with AR during early follicular improvement yet fill in as forerunners for the amalgamation of oestrogens during late preovulatory development (Franks and Hardy, 2018).

In different mammalian species, the AR is expressed in different ovarian cell types, including theca cells, granulosa cells, stroma, and oocytes. Introduction of nonhuman primates and women to high serum androgens brought about advancement of enormous ovaries with expanded quantibinds of antral follicles (Franks and Hardy, 2018). Both testosterone and DHT advance mouse follicular development in-vitro because the AR is communicated overwhelmingly in granulosa cells of developing ovarian follicles.It is accepted that androgen applies direct actions on these cells. Hence, a considerable lot of the differentiating actions of FSH on granulosa cells, including cholesterol metabolism, progesterone emission, articulation of steroidogenic proteins, and induction of aromatase action, were expanded by AR agonists (Laird *et al*., 2017).

It has been accounted for that androgen treatment up-regulated the outflow of ovarian FSH receptor in different species just as that of insulin-like growth factor (IGF)- I and IGF-I receptor (IGF-IR) in granulosa cells and oocytes of rhesus monkeys (Mazerbourg, Monget, Mazerbourg, & Monget, 2018). Furthermore, it is also stated that DHT improved expansion of porcine cumulus-oocyte edifices in little antral follicles stimulated by IGF-I and in enormous antral follicles invigorated by FSH, recommending that the AR may function at various phases of follicular advancement. Androgen has been proposed to advance atresia during cyclic enrollment. This idea is upheld by the perception of elevated levels of atretic follicles in the ovaries of oestrogen receptor beta (ERb) knockout mice with overexpression of the AR in granulosa cells and rebuilding of sound late antral follicles and corpora lutea in these mice after treatment with the antiandrogen hydroxyflutamide (HF).

2.8.2 Nuclear Receptors

The nuclear receptor (NR) superfamily establishes a group of 48 translation factors in people, which incorporates the receptors for steroid hormones, thyroid hormone, lipophilic nutrients,

and cholesterol metabolites (Duntasand Brenta, 2018). Around half of NRs are named vagrant receptors since they don't have all around portrayed ligands. The NRs direct a wide scope of physiologic and formative procedures, and for all intents and purposes all the NRs that have distinguished ligands are well-described concentrations for the improvement of medications to treat various ailments including malignant growth, diabetes, atherosclerosis, irritation, and endocrine/reproductive disordere.t.c.(Duntas and Brenta, 2018).

In contrast to most intercellular ambassadors, the ligands can cross the plasma film and legitimately communicate with atomic receptors inside the cell as opposed to acting by means of cell surface receptors (Sever and Glass, 2013). When enacted, nuclear receptors legitimately direct translation of genes that control a wide assortment of natural procedures, including cell poliferation, advancement, metabolism, and propagation. Albeit atomic receptors basically function as transcription factors, some have likewise been found to direct cell functions inside the cytoplasm. For instance, oestrogens act through the oestrogen receptor in the cytoplasm of endothelial cells to quickly initiate flagging pathways that control vascular tone and endothelial cell activity (Sandoo, Zanten, Metsios, Carroll, & Kitas, 2010).

An assortment of embed studies uncover that steroid hormones act in the cerebrum to effect reproductive conduct. The ovarian steroid receptors oestrogen receptors (ER)and progesterone receptors (PR) are found in various hypothalamic mind areas, including the mPOA, arcuate core, and ventromedial core (VMN) just as numerous extrahypothalamic locales, including the hippocampus, cortex, amygdala, and midbrain focal dark. Neural ER are basic for the statement of female reproductive conduct in rats. The VMN contains a high thickness of ER and gives off an impression of being the most delicate site for oestrogen-subordinate reproductive practices(Thornton, 2013).

The oestrogen receptor (ER) assumes a significant role in intervening oestrogen activity on target disorders. Two subtypes of ER are known, ER-alpha encoded by the ESR1 quality on chromosome 6 and ER-beta encoded by the ESR2 quality on chromosome 14. ER-alpha, the main distinguished and the most copious, is found in all human reproductive disorders. Its part in proliferation has been clarified by thinks about on male and female alpha-ER knockout (ERKO) mice that demonstrated total infertileness(Sever and Glass, 2013). The female mice were infertile since they were an ovulatory, had adjusted pituitary gonadotropin fixations and weakened uterine reaction to oestrogen, though the nonattendance of ER-alpha in the alpha-ERKO male mice brought about seriously hindered spermatogenesis and sperm production (Sever and Glass, 2013).

2.8.3 Gonadotrophin mRNA

Gonadotropin releasing hormone (GnRH) is secreted from the hypothalamus and advances the synthesis and release of the gonadotropins, follicle stimulating hormone (FSH), and luteinising hormone (LH), from the anterior pituitary gland (Glanowska *et al.,* 2014).

GnRH is a decapeptide and has numerous isoforms crosswise over species in both vertebrates and invertebrates animals, including GnRH-I and GnRH-II. However, its hypothalamic combination, GnRH-I mRNA, protein and its receptors are additionally communicated in numerous non-hypothalamic disorders, including the pituitary organ, cow-like uterus and oviducts, gonads of people, non-human primates, rats and non-mammalian vertebrates (Glanowska *et al.,* 2014).

2.8.4 Gonadotrophin Receptor

The gonadotropin-releasing hormone receptor (GnRHR), otherwise called Luteinising hormone releasing hormone receptor (LHRHR), is an individual from the seven-transmembrane, G-protein coupled receptor (GPCR) family. The human GnRH receptor

comes up short on a cytoplasmic carboxy-terminal tail however has amino acid succession themes normal for rhodopsin-like, class A, G protein-coupled receptors (GPCR) sign to intracellular flagging pathways by examining the structures of related GPCRs that have been solidified and consolidating this with biochemical investigations (Flanagan*et al.*,2017).

Based on conserved amino acid sequence features, the GnRH receptor is a class A GPCR. Class A is the largest and best-contemplated class of GPCR proteins and incorporates rhodopsin, adrenergic and other monoamine synapse receptors and numerous peptide and protein-binding receptors. The membrane-spanning segments of GPCRs are most conserved (Parrill and Bautista, 2011).

GnRH peptides are evident contender for extraovarian stimulators of primate sexual conduct. Since they likewise go about as strong neuroendocrine controllers of ovarian capacity. GnRHs can conceivably drive ovarian effects on female sexual conduct. The basic mammalian structure, GnRH I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2), is believed to be principally secreted by the hypothalamus to stimulate FSH and LH secreted from pituitary gonadotropes that regulate the ovarian or menstrual cycle. Conversely, GnRH II, developmentally monitored from hard fish to people (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2), seems to function more as a synapse than a hypothalamic neurosecretory item. Both GnRH peptides can stimulate female sexual conduct in an assortment of vertebrate animal types, however, GnRH II has gotten later intrigue (Maggi *et al.*, 2016).

Despite the fact that GnRH II regulation of primate sexual conduct has up to this point been just a matter of hypothesis, such a role is bolstered by neural limitation of GnRH II and its exceptionally explicit receptors in cerebrum regions associated with the outflow of sexual conduct in both regular marmosets and rhesus macaques, including the preoptic zone and ventro and dorsomedial cores. At present, two particular GnRH receptors (type I and type II)

are contender to intervene GnRH II activity. Type I receptors, which are found in all mammals are likewise equipped for restricting GnRH II with high liking and might have the option to differentially react to the two types of GnRH. Interestingly, type II receptors, have high ligand selectivity for GnRH II and are unlikely mediators of GnRH I effects (Flanagan *et al.,* 2017).

2.8.5 KiSS1 mRNA

KiSS1 mRNA is a metastasis suppressor gene situated on chromosome 1q32. It is 6151 base combines long, having four exons with the principal exon not interpreted while exons 2 and 3 are coding and was first detected by (West, Vojta, Welch, & Weissman, 1998). Kisspeptins are peptide results of KiSS1 quality, partaking in controlling the hypothalamic-pituitary-gonadal pivot, HPG(Branavan *et al.,* 2019). They are named a RF amide peptide family for example neuroactive peptides with genes Arg-Phe-NH2 theme. Kisspeptins act by means of the G-protein coupled receptor,GPR54 demonstrating that the C-terminal decapeptide shared by all kisspeptins is the base amino acid grouping compulsory both for official and enactment of the receptor. GPR54 is an individual from the rhodopsin group of G-protein-coupled receptors, fundamentally like the galanin receptor(McCallum *et al.,* 2016).

Central expression of kisspeptin and its receptor have been exhibited in two significant neuronal populaces inside the hypothalamus of rats: in the arcuate core (ARC) and anteroventral periventricular core (AVPV). In people and primates, kisspeptin mRNA is prevalently communicated inside the infundibular core (likeness the ARC in a specific order of warm blooded animals)(McCallum *et al.,* 2016).

Close juxtaposition of kisspeptin neurons with GnRH neurons and articulation of kisspeptin receptor by GnRH neurons lead to GnRH secretion in both *in vivo* and *in vitro* investigations, with the effect restrained by the organisation of GnRH antagonists. Focal and

otherorganisation of kisspeptin prompted a checked increment in flowing luteinisinghormone levels in both animal and human examinations just as a lesser increment in FSH, with the effect canceled in GPR54-/ - mice. The effect of kisspeptin on gonadotropin secretion is probably going to be expected to kisspepetin incitement of GnRH secretion into the gateway dissemination, which thusly leads to the release of LH and FSH from the gonadotrophs of the anterior pituitary organ (Skorupskaite, George, & Anderson, 2014).

Kisspeptin could likewise be a mediator between leptin (a feeding hormone) and fertility (GnRH) since anorexic females in the pubertal stage neglect to create auxiliary sexual genes, prompting essential amenorrhoea and GnRH neurons express kisspeptin receptors. Intravenous infusion of kisspeptin to sound male subjects, brought about a huge increment in plasma LH, FSH, testosterone; subcutaneous infusion of kisspeptin to solid pre-menopausal female subjects, evokes a stamped ascend in LH, which is progressively articulated in the pre-ovulatory period of the menstrual cycle. Subcutaneous infusion (twice every day) to infertile women with practical hypothalamic amenorrhoea because of low body weight, viably stimulated an ascent in plasma gonadotrophs, with the effect most stamped following the main infusion and altogether lessening following two weeks of treatment while fortnightly organisation of kisspeptin brings about a continued gonadotropin reaction to kisspeptin. Women with hypothalamic amenorrhoea had a fourfold more noteworthy LH reaction to infused kisspeptin than solid female subjects examined in the follicular stage, proposing an upgraded reactiveness to kisspeptin in women with hypothalamic amenorrhea or expanded pituitary affectability with the effects of GnRH. Kisspeptin was at first known as metastin, thought about a metastasis silencer, because of its ability to restrain cell intrusion, modifying cell motility or potentially bond, which are properties fundamental in fetus implantation as it is a basic advance for propagation achievement. The nidation depends on trophoblast intrusion of the uterine extracellular lattice. Kisspeptin together with other

calming cytokines, especially TNFα, compel trophoblast attack and may assume a role in the hour of conveyance, balancing the trophoblast apoptosis(Trevisan *et al.,* 2018).

In women, there is proof that kisspeptin levels increase every trimester during pregnancy and it could arrive at a 200-overlay more significant level in the third trimester when contrasted with non-pregnant women. Kisspepin levels underneath 1630 pmol/L during the principal trimester were viewed as an unnatural birth cycle biomarker, since women who endure miscarriages show kisspeptin levels 60% lower than unaffected pregnancies. Low degrees of kisspeptin in the subsequent trimester (16-20 weeks) were additionally connected with intrauterine development limitation, while low levels in late pregnancy were related with preeclampsia(Trevisan *et al.,* 2018).

2.8.6 Antimullerian Hormone and Hypothalamus

The control of reproduction in vertebrates is ultimately dependent upon neural circuits in the brain. Brain circuits within the hypothalamus in synergy dictate the proper functioning of GnRH neurons, which are the final output to the control of gonadotropin secretion. To achieve this, GnRH neurons release GnRH peptide in a pulsatile manner into the hypophyseal portal system. Antimullerian hormone has been noted to act via the hypothalamo-pituitary gonadal axis, in addition to previously known action on the ovary. Different chemosignals affect the GnRH neuron migration. Recent studies are documenting that GnRH neurons produce AMH from in -utero in mice as well as in human foetuses. The receptor AMHR2 is also expressed in the GnRH neurons. Antimullerian hormone activates the AMHR2, which then stimulates synthesis of gonadotropins(Barbotin and Giacobini, 2019; Kereilwe and Kadokawa, 2020).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Ethical Clearance

Ethical clearance was obtained for this study from the University of Ilorin Ethical Review Committee with approval number UERC/ASN/2018/1154 on 15th August 2018, through the Faculty of Basic Medical Sciences. The experiment was conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals', as well as strictly adhering to the institutional and animal care guidelines(Book, 2011).

3.2 Procurement of Chemicals

Bisphenol-A was procured from Sigma Aldrich® (CAS –No: 80-05-7), Germany, as well as Sesame oil that was used in subcutaneous injection. The corn oil and ethanol were procured from a standard certified laboratory in Nigeria.

3.3 Animal Procurement, Housing and Care

Forty-two adult female wistar rats (Rattus Norvegicus) and 14 adult male rats, all weighing between 160 – 200g were procured from a certified commercial breeder. They were housed in the animal house of College of Health Sciences, University of Ilorin, Ilorin. The animals were acclimatised for two weeks while being fed with standard rat pellets and given clean water liberally. They were kept at room temperature. The animals were housed in wooden cages, which were cleaned daily.

3.3.1 Procedure for Vaginal Smears

Vaginal smears were done to determine the phase of cycle the rats were, which guided when to introduce the males, as well as determine if mating had occurred for dating pregnancy. Following the acclimatisation, the female rats had vaginal smears in the early hours of the morning between 7a.m – 9a.m daily(Cora *et al.,* 2015). Animals in the proestrus phase were housed with males, and a repeat vaginal smear was done the following morning to examine for presence of vaginal mucus plug and sperm cells, which if positive, then it is termed as Day 0 of conception.

3.3.2 Animal Grouping

The female pups were identified, then housed with their mothers and randomly assigned into groups for the study. There were 14 groupsas shown in the table overleaf. Group A represent the newborn group whose administration started on day of birth while group B were bred till day 19 before administration began.

It has been recommended that the Lowest Observed Adverse Effect Level (LOAEL) in animal studies is 50mg/kg (Richter *et al.*, 2008;FAO/WHO, 2010; States *et al.*, 2010; Vandenberg *et al.,* 2013; Peretz *et al.,* 2014). However, even at this LOAEL, uncertainties as to the safety of bisphenol-A still abound. Therefore, the recommended LOAEL for animals was used, and a lower dose of 25mg/kg to observe for any possible adverse effects even with a lower dose was also used.

Table 3.1: Varying doses of Bispheol-A and melatonin administered to Newborns on days 0-3 (Group A)

Subgroups	Chemical/Drug/Route	Duration
I – Negative control (6 rats)	Normal saline 0.2ml/10g bw/day	4 days
II – Vehicle control (6 rats)	Ethanol hydroxide + sesame oil subcut 0.2ml/10g bw/day	4days
III – Positive control (6 rats)	Melatonin 10mg/kg bw/day orally to the mothers	4days
IV –BPA (6 rats)	25mg/kg bw /day subcut	4 days
V –BPA + melatonin (6 rats)	25mg/kgbw/day subcut + 10mg melatonin	4 days
VI – BPA (6 rats)	50mg/kg bw/day subcut	4 days
VII – BPA + melatonin (6 rats)	50mg/kg bw/day + 10mg melatonin	4 days

Table 3.2: Varying doses of Bispheol-A and melatonin administered to on days 19-68 (Group B; childhood/pubertal)

Subgroups	Chemical/Treatment/Route	Duration
I Negative control (6 rats)	Normal saline 1ml/100g bw/day	7 weeks
II Vehicle control (6 rats)	Ethanol hydroxide (EtOH) + Sesame oil – 1ml/100g bw/day/oral	7 weeks
III Positive control (6 rats)	Melatonin 10mg/kg bw/day	7 weeks
IV- BPA (6 rats)	25mg/kg bw/day	7 weeks
V- BPA + melatonin (6 rats)	25mg/kg bw/day + 10mg melatonin	7 weeks
VI – BPA (6 rats)	50mg/kg bw/day	7 weeks
VII - BPA + melatonin (6 rats)	50mg/kg bw/day + 10mg melatonin	7 weeks

Following completion of administration as outlined in the table above, the rats were bred till 120 days (± 4days) when they were anaesthesised, then sacrificed (at their proestrus phase).

3.4 Animal Sacrifice, Blood and Tissue Collection

Following the administration from day 118, vaginal smears were commenced for each study group of wistar rats. Those in the proestrus were then euthanised using 20mg/kg ketamine intramuscularly. They were dissected at the midline abdominally extending to the thorax using dissecting forceps, and access was gained to the heart and the abdomen.The thorax and abdomen were reflected laterally with a pair of dissecting forceps and scissors, then 5ml syringe and needle wasused for cardiac puncture to aspirate blood samples. The blood was drawn into lithium heparinised bottles for hormonal analysis.

The scalp was then incised and reflected laterally, then the skull was immediately opened using forceps to remove the whole brain. The hypothalamus as well as the pituitary gland (in the floor of the sellae turcica) were identified, harvested and dropped in cryovials, which were subsequently stored in liquid nitrogen tanks. These organs were subsequently used for genetic (RNA) studies. The ovaries were also harvested, using the left ovary for histology (follicular count) which was fixed in 4% paraformaldehyde for 48 hours. The rightovary of each rat was homogenised in 0.25M ice cold phosphate buffer and the homogenates were then centrifuged for enzyme studies.

3.5 Tissue Processing

Histological tissue processing was carried out using the methods of Pearse (1960) and modified by Drury and Wallington in 1980.

Dehydration

The tissues which had been fixed in 4% paraformaldehyde, were dehydrated at room temperature by immersing them in ascending grades of ethanol in order to remove the water molecules as follows:

i. 50% ethanol for 1 hour
ii. 70% ethanol for 1 hour
iii. 90% ethanol for 1 hour
iv. Absolute ethanol I for 1 hour
v. Absolute ethanol II for 1 hour

Tissue Clearing

Dehydrated tissues were cleared at room temperature in two changes of xylene for 1 houreach.

The clearing agent (xylene) is miscible with both the dehydrant (ethanol) and the embedding medium, therefore facilitating the transition between dehydration and infiltration steps.

Impregnation and Embedding

The ovarian tissues were then infiltrated in two changes of molten paraffin wax at a controlled temperature between 55 – 58^{O}C for one hour each. The tissue specimen were taken from their separate labelled specimen bottles and completely immersed in molten wax. The

tissues were embedded in paraffin wax using the embedding moulds and tissue cassettes to allow for sectioning.

Sectioning of tissues

The Rotary Leica microtome was used for sectioning at 4µ per section obtained serially, so that replicate counting or measurements was not an issue. Thereafter, the sections were transferred into a water bath of $40^{O}C$ temperature to allow uniform spreading of folded sections. The sections were then mounted on new clean slides, by dipping the slides into the water bath at an angle to the plane of the water. A hot plate was then used to melt the wax around the tissues by placing the slides on it. The sections were then dried on a slide drier to enhance adherence of the sections to the slides, then were stored on the slide racks for staining.

Histological Staining Principle (Haematoxylin and Eosin)

Haematoxylin is a dark blue to violet stain, that is basic and binds to basophilic substances while eosin is a red to pink stain that is acidic and binds to acidophilic substances. The oxidation product of haematoxylin is haematin whose active ingredient is same.

3.5.1 Haematoxylin and Eosin Procedure

Sections were paraffinised in 2 changes of xylene for 3 minutes each. The sections were rehydrated in 2 changes of descending grades of alcohol (absolute I, absolute II, 90% ethanol, 70% ethanol, then 50% ethanol) for 2 minutes each. They were then rinsed in distilled water for 3 minutes. Staining was done in iron haematoxylin for 10 – 15 minutes. Excess stain was removed by washing under running tap water for 5 minutes.The sections were differentiated in 1% acid alcohol for 1 minute. After on, the sections were counter-stained with eosin for 2 minutes. Sections were then dehydrated through ascending grades of alcohol for 2 minutes

each, cleared in two changes of xylene and mounted in synthetic resin medium (D.P.X.), a mixture of distyrene, a plasticizer and xylene.

3.6 Photomicrography

The images were obtained using Amscope microscope. A 5.0 mega pixel Amscopecamera was used to take the photomicrographs at objective lens 4 and 10.

3.7 Biochemical Analysis

FSH Serum Analysis (Catalogue No: LS-F6305)

Assay Principle

This assay depended on the ELISA standard. Each well of the provided microtiter plate was pre-covered with an objective explicit catch neutraliser. Models or tests were added to the wells just as a fixed amount of biotin-conjugated objective antigen.

The antigens in the guidelines or tests contend with the biotin-conjugated antigen to tie to the catch counter acting agent. Unbound antigen is washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate was then added which ties to the biotin. Unbound HRP-conjugate was washed away. A TMB substrate was then included which responded with the HRP chemical bringing about shading advancement.

A sulfuric corrosive stop arrangement was added to end shading advancement response and afterward the optical thickness (OD) of the well was estimated at a wavelength of 450 nm ± 2 nm. The OD of an obscure example was then contrasted with an OD standard curve produced utilising known antigen focuses so as to decide its antigen fixation.

Assay Procedure

All reagents and tests were brought to room temperature without extra warming then blended completely by delicately twirling before pipetting (abstain from frothing). All reagents, working models and tests were set up.

50µl of standard, blank, or sample was included per well. 50µl of detection reagent,a working answer for each well was quickly included, spread with a plate sealer, delicately shook to guarantee intensive blending, and brooded for 1 hour at 37°C. The fluid from each well was suctioned then washed multiple times. Approximately, 350µl of wash buffer utilising a squirt bottle was used to wash. Each wash was allowed to sit for 1-2 minutes before totally suctioning. After the last wash, suctioning was done to evacuate any residual wash buffer, thenthe plate was modified by tapping against clean retentive paper.100µl of Detection Reagent B working answer for each well was included, delicately enough to guarantee exhaustive blending, then spread with another plate sealer, and hatched for 30 minutes at 37°C. 90µl of TMB Substrate was included for each well, delicately disturbed to guarantee exhaustive blending, then spread with another plate sealer, and hatched for 10-20 minutes at 37°C. It was shielded from light and screened occasionally until ideal shading improvement was accomplished.50µl of stop solution was added to each well in a similar request and timing as the TMB Substrate arrangement,then tenderly unsettled to guarantee exhaustive blending.The optical thickness (OD estimation) of each well was promptly decidedutilising a microplate peruser set to 450 nm.

LH Serum Analysis(Catalogue No: MBS729873)

Assay Principle

LH ELISA unit applied the protein immunoassay system using a polyclonal against LH immuniser and a LH-HRP conjugate. The measure test and support were hatched together with LH-HRP conjugate in pre-covered plate for 60 minutes. After the hatching time frame, the wells were tapped and washed multiple times. The wells were then hatched with a

substrate for HRP compound. The result of the compound substrate response was a blue shaded complex. Then a stop arrangement was added to stop the response, which at that point turned the arrangement to yellow. The force of shading was estimated spectrophotometrically at 450nm in a microplate peruser. The force of the shading was contrarily corresponding to the LH focus since LH from tests and LH-HRP conjugate go after the counter LH immune response restricting site. A standard curvewas plotted relating the force of the shading (O.D.) to the convergence of models. The LH focus in each example was introduced from this standard curve.

Assay Procedure

All Standards and Samples were assayed in duplicate. Preliminary experiment was carried out before measuring all samples.The chosen numbers of coated wells in the holder were secured then 100 µL of standards (the bottle of each standard gently shaken by hand and pipette up and down the solution of standard for 3 times before adding) or samples were added to the appropriate well. 100 µL of PBS (pH 7.0-7.2) in the blank control well was added.50 µL of conjugate to each well was added without adding to the blank control well, which was thenmixed well. The plate was covered and incubated for 1 hour at 37°C. The microtiter plate was washed using the specified manual washing method. The incubation mixture was removed by aspirating contents of the plate into a sink. Each well was completely filled with 1× wash solution, and then contents of the plate were aspirated into a sink.This procedure was repeated five times for a total of five washes. After washing, the plate was inverted, and blotted dry by hitting the plate onto absorbent paper towels until no moisture appeared. The sides of the plate frame were firmly held when washing the plate to assure that all strips remain secured in the frame. Complete removal of liquid at each step was essential for good performance outcome.50 µL substrate A and 50 µL substrate B was added to each well including blank control well, subsequently. It was covered and incubated

for 10-15 minutes at 37°C. (Sunlight was avoided and the longest incubation period was 30minutes to get the desired colour).50 µL of stop solution was added to each well including blank control well, then mixed well. The optical density (O.D.) was determined at 450 nm using a microplate reader immediately.

Testosterone

Principle of the Test

The testosterone EIA depends on the standard between testosterone in the test example and testosterone-HRP conjugate for a steady measure of bunny against testosterone. In the hatching, goat hostile to bunny IgG-covered wells are brooded with 10µl of testosterone principles, controls, understanding examples, 100 µl testosterone-HRP conjugate reagent and 50µl hare against testosterone reagent at 37° C for an hour and a half. During the brooding, a fixed measure of HRP-marked testosterone contends with the endogenous testosterone in the standard, example, or quality control serum for a fixed number of restricting locales of the particular testosterone neutraliser. In this way, the measure of testosterone peroxidase conjugate immunologically bound to the well continuously diminishes as the convergence of Testosterone in the example increments. Unbound testosterone peroxidase conjugate was then expelled and the wells washed. Next, TMB reagent was then included and brooded at room temperature for 20 minutes, bringing about the advancement of blue shading. The shading improvement is halted with the expansion of 1N HCl, and the absorbance was estimated spectrophotometrically at 450nm. The power of the shading framed corresponded to the measure of protein present and was conversely identified with the measure of unlabeled testosterone in the example. A standard curve was gotten by plotting the centralisation of the

standard versus the absorbance. The testosterone convergence of the examples and controls run simultaneously with the principles, and this was determined from the standard curve.

Assay Procedure

The desired number of coated wells were secured in the holder. 10 µl of standards, specimens and controls into appropriate wells was dispensed.100 µl of Testosterone-HRP conjugate reagent into each well was dispensed. 50 µl of specimen anti-testosterone reagent to each well was dispensed,then thoroughly mixed for 30 seconds. Incubation at 37° C for 90 minutes was done. The microwells were rinsed and flicked 5 times with distilled water. 100 µl of TMB reagent into each well was dispensed, then gently mixed for 10seconds. Incubation at room temperature (18-25°C) for 20 minutes was done. The reaction was stopped by adding 100µl of stop solution to each well. Then resulting solution was gently shaked for 30 seconds. It wasensured that all the blue colour changed to yellow colour completely.Absorbance was read at 450 nm with a microtiter well reader within 15 minutes.

Oestradiol

Assay Principle

Oestradiol is one of the principle segments of normally happening oestrogens and itis the significant oestrogen discharged during the menstrual cycle. The serum levels of oestradiol are low during the follicular stage rising slowly until around one day before ovulation when a stamped ascend in the oestradiol level happens (ovulatory peak). The oestradiol level falls quickly at, or directly after ovulation and is again inside the degrees of the follicular stage. There is a second ascent of oestradiol around day 21 of the cycle (luteal peak). The levels at that point decrease progressively to the most reduced level at the beginning of the following menstrual cycle.

Assay Procedure

All reagents were brought to room temperature before use. Calibrators, controls and example tests were examined. When the system had begun, interefrence was avoided to finish the work. Working solutions of the oestradiol-biotin; avidin-HRP conjugate and wash buffer were prepared. The required number of microwell strips were removed. 50 µl of each calibrator, control and specimen samples were pipette into correspondingly labelled wells in duplicate.100 µl of the conjugate working solution into each well was pipette.It was then incubated on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

The wells were washed three times with 300 µl of diluted wash buffer per well thenthe plate was tapped firmly against absorbent paper to ensure that it was dry.150 µl of TMB substrate was pipette into each well at timed interval.Incubation for 10-15 minutes at room temperature, and the desired dark blue colour obtained for OD.50 µl of stop solution was pipette into each well at the same timed intervals.The plate was read on a microwell plate reader at 450nm within 20 minutes after addition of the stop solution.

Superoxide Dismutase (CatalogNumber:KT-60703)

Assay Procedure

All Calibrators and Samples were added in duplicate to the Microtiter Plate.

The chosen number of coated wells were secured in the holder then100 µl of calibrators or samples were added to the appropriate well of the antibody pre-coated microtiter plate.50 µl of conjugate was added to each well and was mixed well. The plate was covered and incubated for 1 hour at 37°C.The microtiter plate was washed manually.50 µl substrate A and 50 µl substrate B was added to each well. It was then covered and incubated for 15minutes at 20-25°C. (sunlight was avoided).50 µl of stop solution was added to each well and mixed well.The optical density (OD) at 450 nm was read using a microtiter plate reader immediately.

Uridyl Diphosphate Glucuronyl Transferase

Assay Procedure

The desired numbers of coated wells were secured in the holder.Then100 µl of standards 100µlofPBS (pH 7.0-7.2) was added in the blank control well.10µl of balance solution was dispensedinto 100µl samples only, and mixed well. 50 µl of conjugate was added to each well (NOT blank control well). It was mixed wel as it is very important in this step. The plate was covered and incubated for 1hour at 37℃.50 µl of stop solution was added to each well and mixed well.The optical density (OD) at 450 nm was read using a microtiter plate reader immediately.

Anti-mullerian Hormone

Assay Procedure

Concentrations of AMH were determined in serum samples using a recently validated, commercially available enzyme immunoassay kit designed for use with serum or plasma samples (AMH Gen II ELISA, Beckman Coulter #A73818, Brea, CA, USA).

Samples were analysed in duplicate using materials and procedures provided with the kit.Samples, calibrators and controls were pipetted into microtiter plate wells coated with anti-AMH capture antibody, incubated for 60 min, and then washed five times with a

microplate washer (BioTek Instruments ELx50, Winooski, VT, USA). Biotinylated anti-AMH detection antibody was then added. The wells were incubated for 60 min and washed, followed by the addition of streptavidin–horseradish peroxidase (HRP). The wells were incubated for 30 min, washed and tetramethylbenzidine chromogen solution was added to promote color change in proportion to the amount of bound AMH.Sulfuric acid was added to each well to stop the reaction. Absorbance was measured at 450 nm with 630 nm reference wavelength in a microplate reader (BioTek Instruments Elx808). Sample concentrations were calculated from the best-fit four-parameter logistic standard curve (KCJunior, BioTek) based on absorbancevalues derived from calibration materials with known concentrations of AMH (Beckman Coulter #A73819).

3.8 Quantitative Real Time Polymerase Chain Reaction Procedure

3.8.1 Extraction of Total RNA from Samples

Total RNA was prepared with EuroGoldTriFast solution (EuroClone). Extraction was performed as per instructions of the manufacturer.Carefully dissected tissues were weighed out on a sensitive weighing scale and placed in a 2 ml microfuge tube, after which EuroGoldTriFast solution was added to the samples in ratio 1:10 respectively. The tissue/RNA extraction solution was then pulverised with a tissue homogeniser at 6,000 rotation per minute (RPM). Integrity of extracted RNA was verified on 1 % agarose gel. In order to eliminate genomic DNA contamination from total RNA preparation, DNase treatment was performed on total RNA samples extracted. 1 unit of RNAse-free DNase (Promega, 1 U/µL) was added per every microgram of total RNA and incubated at 37°C for 30 minutes in DNase buffer. RNA was purified through acid phenolchloroform, precipitated and suspended in distilled water (dH_2O).

3.8.2 Reverse transcription (RT)

For expression analysis by quantitative real time polymerase chain reaction (qRT-PCR), complementary DNA (cDNA) was prepared by reverse transcription of total RNA using M-MLV reverse transcriptase (Invitrogen), per manufacturer's instructions. 1 µg of total RNA were retrotranscribed for quantification of mRNA expression in experiments. Total RNA, 1 µL of 10 mM dNTP mix (10 mM of each dATP, dTTP, dCTP and dGTP at neutral pH) and 1 µL of oligo dT12-18 (500 µg/mL) was mixed and brought to a volume of 12 µL with double distilled water(dH_2O). The mixture was heated to 65°C for 5 min and quickly chilled on ice. Contents were shortly spun in a centrifuge to collect them at the bottom of the tube. Then 4 µL of 5X First-Strand buffer, 2 µL 0.1 M DTT and 1 µL of water (H_2O) were added to the mixture. Samples were well mixed by pipetting and incubated for 2 min at 37°C. 1 µL of MMLV RT was added (200 units), samples were mixed by gentle up and down pipetting and incubated at 37°C for 50 min. M-MLV RT was inactivated for 15 min at 70°C. cDNA was kept at -20°C until further use.

3.8.3 Quantification and quality control of nucleic acid isolates

cDNA and RNA concentrations were measured by NanoDrop™ 1000 spectrophotometer (Thermo Scientific) at 260 nm. Ratios of absorbance at 260 nm/280 nm and 260 nm/230 nm were used as indication of nucleic acids quality. At 260 nm/280 nm cDNA with ratio above 1.7 was considered sufficiently pure, while RNAin the range of 1.9 – 2.2 were considered pure.

3.8.4 Quantitative real-time PCR (Qrt-PCR)

To measure relative amounts of transcript of a specific gene,Qrt-PCR was used. Reactions were performed in CFX96 real-time system (Bio-Rad). IQ™ SYBR® Green Supermix 2x cocktail (Bio-Rad) was used for the reactions. The reactions were performed in duplicates in

the final volume of 15 µL on the 96-well real-time PCR plate. The protocol consisted of two steps, the amplification reaction and subsequent generation of melting curves of the amplicons. The reaction mixture contained 7.5 µL IQ™ SYBR® Green Supermix 2x cocktail, 0.5 µL of each primer (100 ng/µL), 5.5 µL of dH_2O and 1 µL of Cdna. The following conditions were used for all reactions: one denaturation step at 98°C for 30 seconds, then 40 cycles of denaturation step at 95°C for 5 seconds and annealing/extension at 62°C for 25 seconds, following which each cycle fluorescence was measured. After the amplification protocol amplicons were denatured for 10 seconds at 95°C, then melt curve was generated by increasing temperature from 65°C to 95°C at 0.5°C increment every 5 seconds. Prior to the quantification of the transcripts, in order to obtain quantitative results, primer pairs were tested to verify their specificity and efficiency. The specificity was evaluated by melting curve profile from complimentary DNA (cDNA) amplification. The melting curve was expected to show a single peak. If more than one peak was observed, the primer pair was changed. In addition, the PCR product was spread on 2 % agarose gel to verify melting curve data. The efficiency of primers was tested in reactions with six serial 1:10 dilutions of cDNA as a template to perform a calibration curve. After the reactions, dilution vs. threshold cycle (Ct, the cycle at which the signal of the amplicon exceeds the background signal) was plotted. The efficiency of the primers was derived from the slope of the curve and only primers whose efficiency was 100±5 % were used for quantification. The primers used to quantify the expression are listed in Table 3overleaf.

Table 3.3: Primers (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	
	RT-mAr-REV	TTTCCGGAGACGACACGATG	229
3	**Anti-mullerian hormone receptor (Amhr)**		
	mAmh-DIR	CTGGGAGCAAGCCCTGTTAG	180
	mAmh-REV	GGTTGAAGGGTTAGGGCGAG	
4	**KISS1 receptor (Kiss1r)**		
	mKiss1r-DIR	GCTAGTCGGGAACTCACTGG	
	mKiss1r-REV	ACGCAGCACAGAAGGAAAGT	120
5	**Gonadotropin releasing hormone 1 (Gnrh1)**		
	mGnrh1-DIR	TGGTATCCCTTTGGCTTTCACA	
	mGnrh1-REV	GATCCTCCTCCTTGCCCATC	192

6	**Gonadotropin releasing hormone receptor (Gnrhr)**		
	mGnrhr-DIR	GCCTCAGCCTTGTCTCATGT	140
	mGnrhr-REV	TATGTTGGGCTTTCCCGGTC	

3.9 Statistical Analysis

3.9.1 Quantitative Analysis

All data were expressed as the mean ± standard error of mean (mean ± SEM). The differences among the experimental groups were considered statistically significant when p value is less than 0.05, using the one-way analysis of variance (ANOVA).

Statistical analysis was performed using Graph pad prism version 16.

3.9.2 Qualitative Analysis

Histological slides were prepared using haematoxylin and eosin stains, under light microscope. Photomicrographs were taken using Amscope microscope and Amscope eye piece camera, and a qualitative comparison was done between the control groups and the treated groups.

CHAPTER FOUR: RESULTS

4.1 Results of Analysis in Newborn Wistar rats (Group A)

4.1.1 Alterations inHormonal Responses following Bisphenol-A exposure

Bisphenol-A exposure caused increases in follicle stimulating hormone, luteinising hormone, oestradiol and testosterone levels while a decrease in antimullerian hormone level was observed when compared with the control. These changes were however not appreciable reversed by the oral administration of melatonin (Figures 4.1 to 4.6).

Outturns of Follicle Stimulating Hormone production following BPA and melatonin exposure

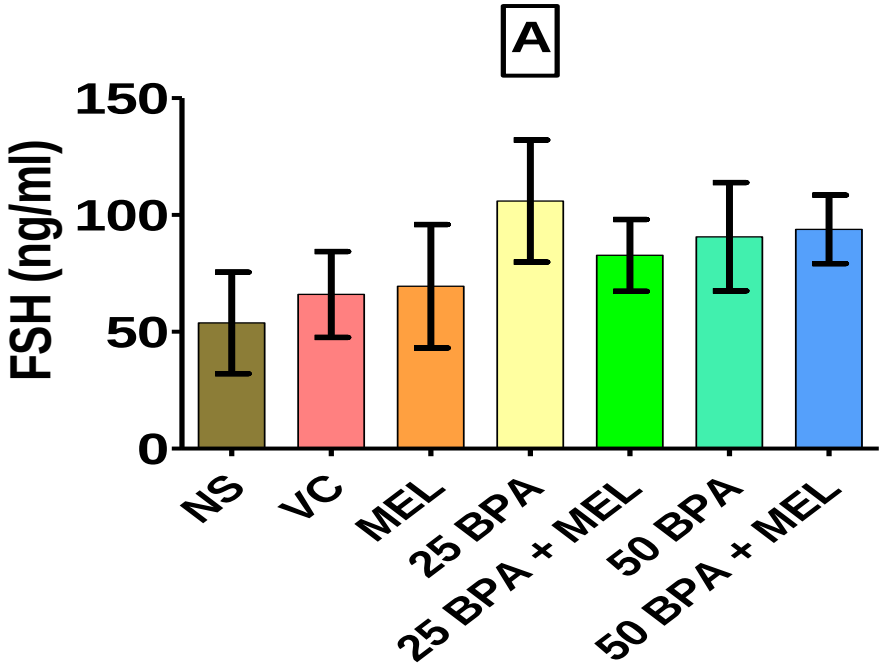


Figure 4.1: Serum FSH levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A),Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin(MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL).Data are presented as Mean ± SEM

Outturns of Luteinising Hormone production following BPA and melatonin exposure

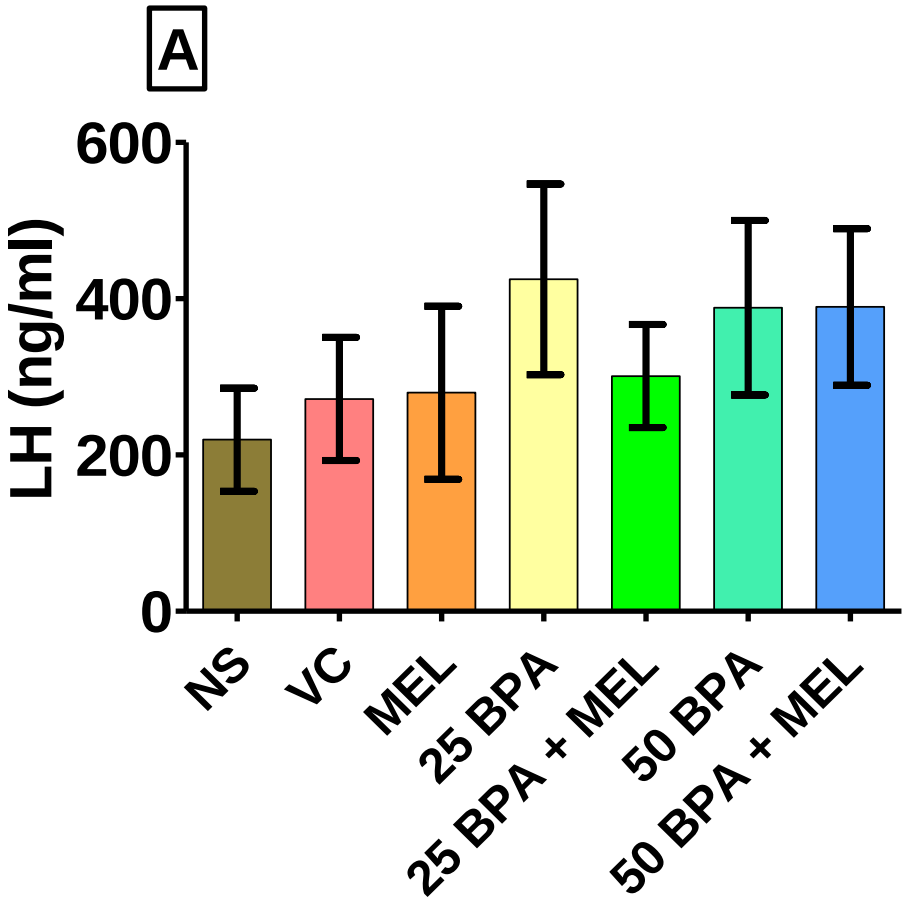


Figure 4.2: Serum LH levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A),Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM

Outturns of Oestradiol production following BPA and melatonin exposure

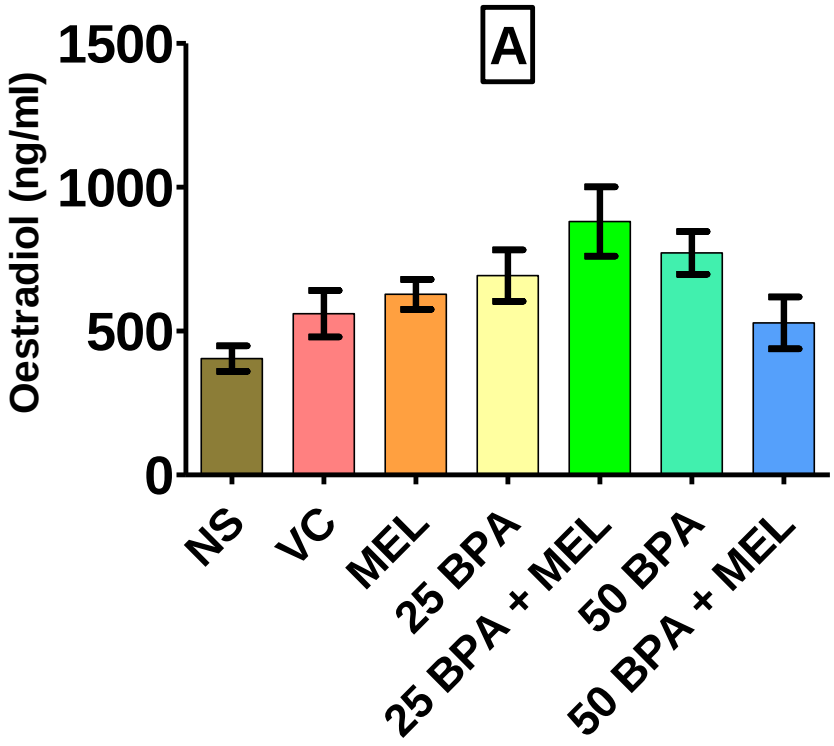


Figure 4.3: Serum Oestradiol levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A),Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM

Outturns of Progesterone production following BPA and melatonin exposure

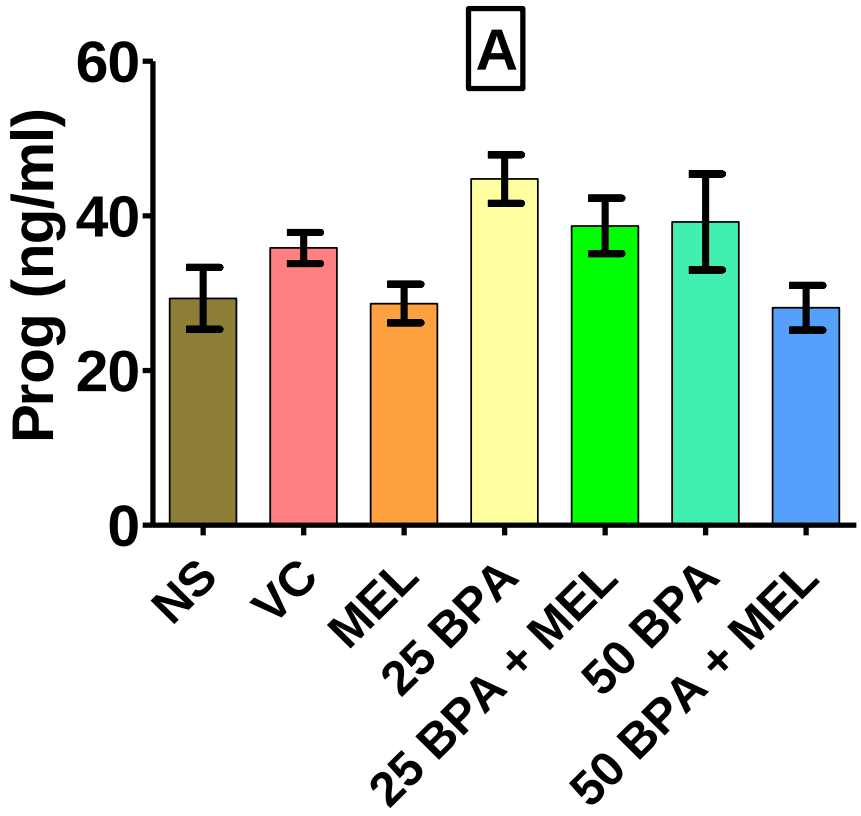


Figure 4.4: Serum Progesterone levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A),Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM

Outturns of Testosterone production following BPA and melatonin exposure

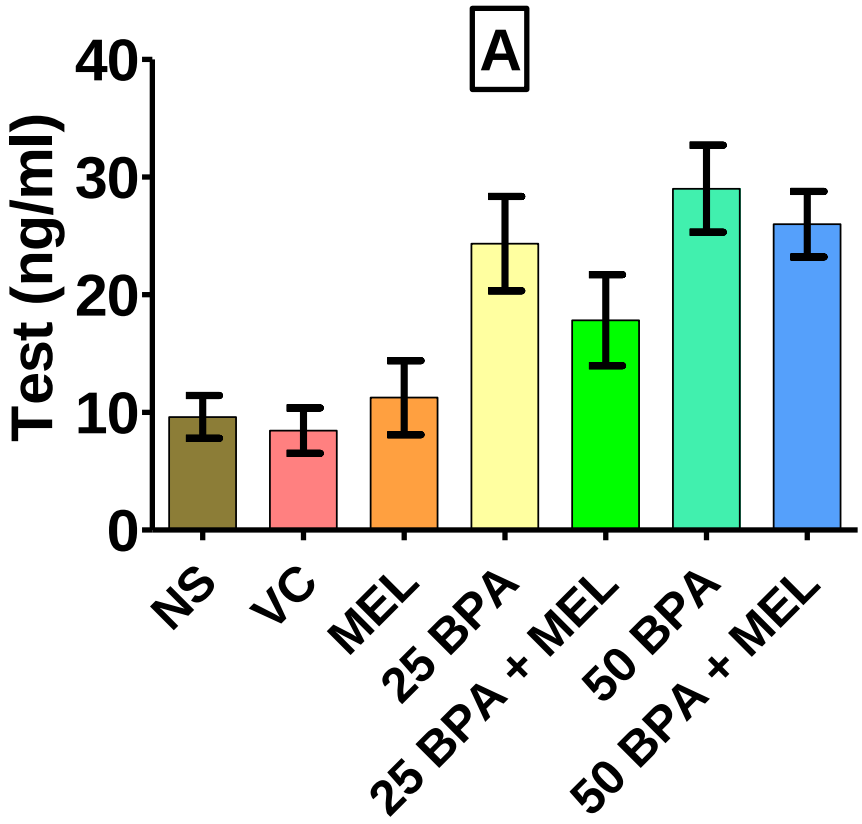


Figure 4.5: Serum Testosterone levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A), Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM

Outturns of Anti-Mullerian Hormone production following BPA and melatonin exposure

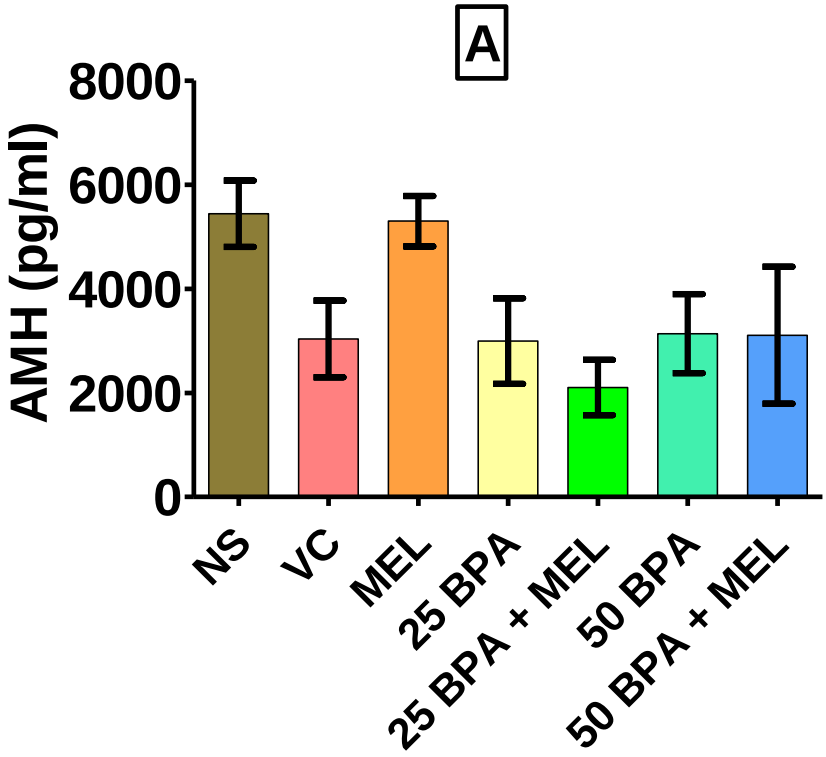


Figure 4.6: Serum AMH levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A), Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM

4.1.2 Changes in ovarian antioxidant and metabolic enzyme levels following BPA exposure

Exposure to bisphenol-A caused marked increases in superoxide dismutase and nitric oxide synthase levels while levels of glutathione peroxidase and uridyl diphosphatase glucoronyl transferase were significantly decreased when compared to the control. Administration of melatonin did not show appreciable changes in the observed effects in these antioxidants and enzyme (Figures 4.7 to 4.10)

Superoxide Dismutase values following BPA and melatonin exposure

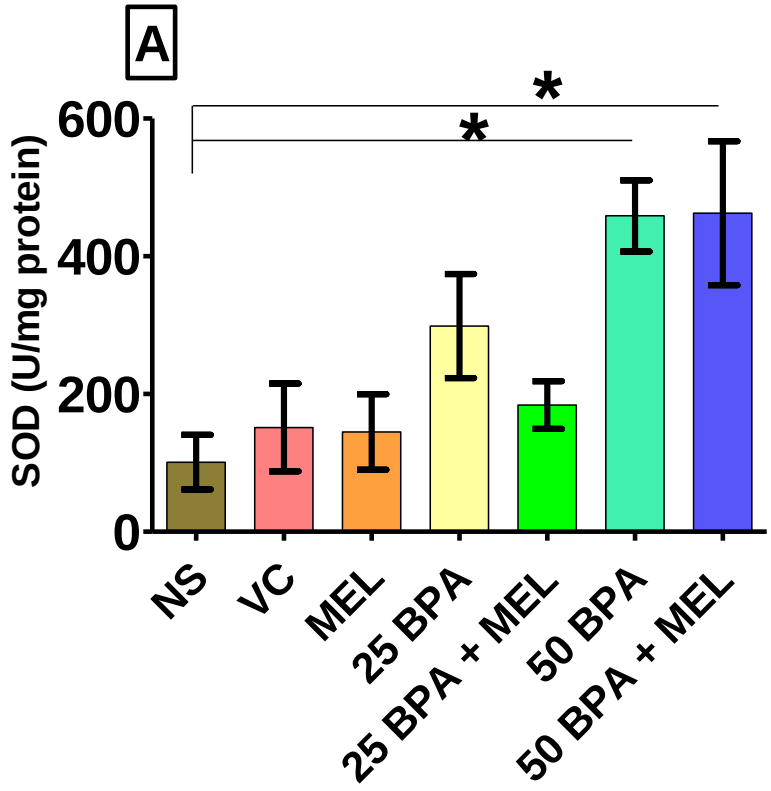


Figure 4.7: Ovarian SOD levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A), Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$

Glutathione Peroxidase values following BPA and melatonin exposure

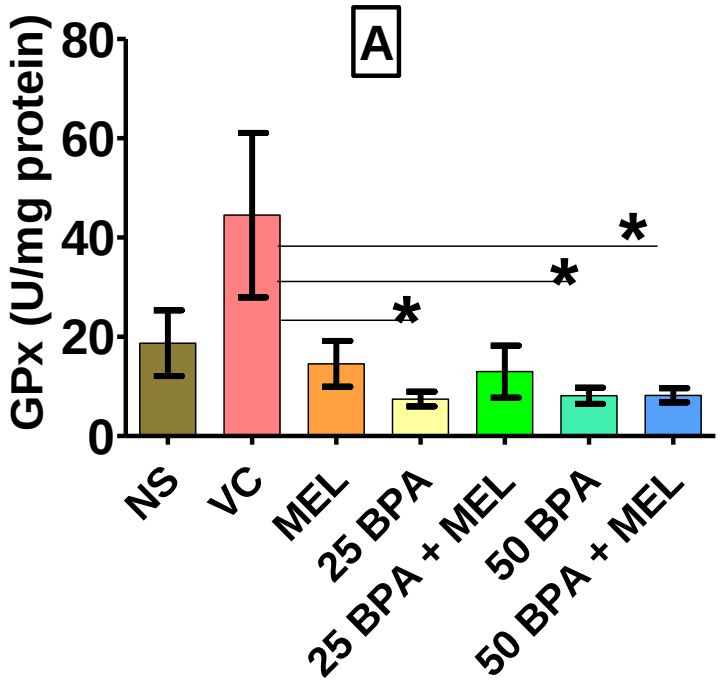


Figure 4.8: Ovarian GPx levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A), Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$

Nitric Oxide Synthase values following BPA and melatonin exposure

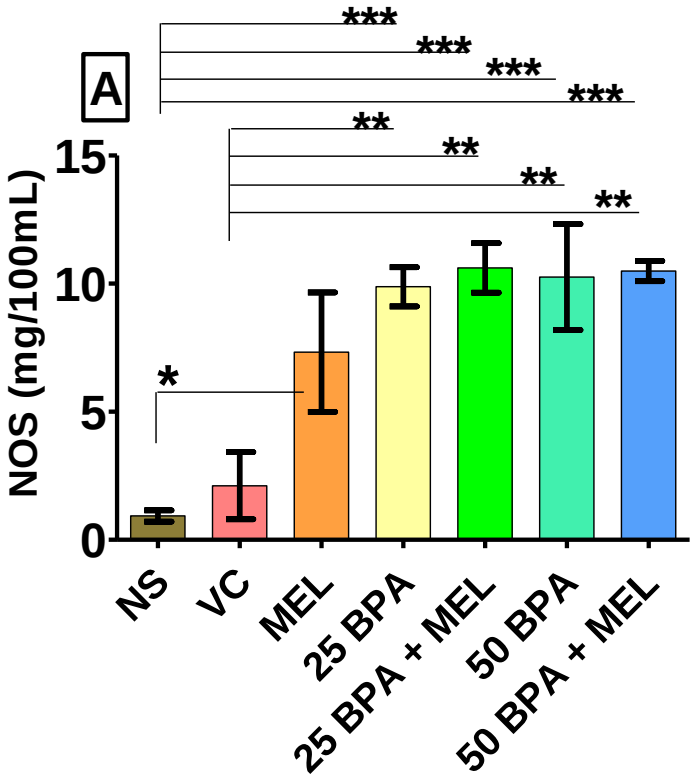


Figure 4.9: Ovarian NOS levels in neonatal female rats exposed to BPA and melatonin.

Newborn group (A), Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$,Asterix (**) $P < 0.01$, Asterix (***) $P < 0.001$

Uridine DiphosphoGlucuronyl Transferase values following BPA and melatonin exposure

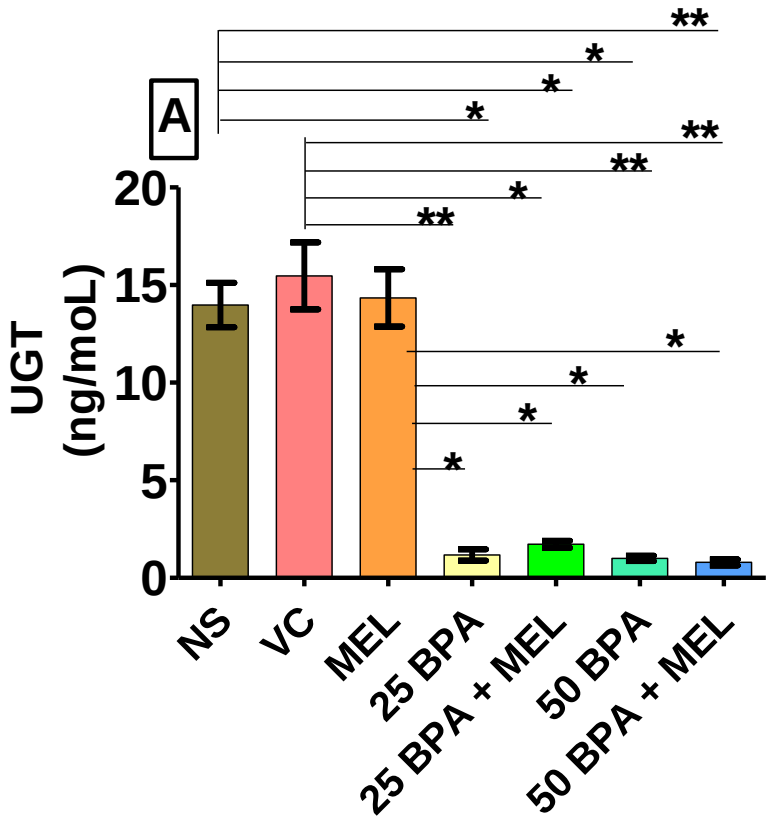


Figure 4.10: Ovarian UDP levels in neonatal female rats exposed to BPA and melatonin.

Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$,Asterix (**) $P < 0.01$.

4.1.3 Overexpression of Reproductive receptor genes following exposures to Bisphenol-A and Melatonin

Administration of Bisphenol-A led to marked increases in reproductive genes of the hypothalamus (AR, ER, AMH mRNA and Kiss 1) and pituitary glands (GnRH mRNA and GnRHr). Similarly, coadministration of melatonin with BPA did not show amelioration in the increased effects observed with BPA administration(Tables 4.1 to 4.2).

Table 4.1: Quantitative PCR of hypothalamic genes following neonatal exposure to bisphenol-A and melatonin. Androgen receptors (AR), oestrgen receptors (ER), Kiss 1 receptors (Kiss 1r) and antimullerian mRNA (AMH mRNA).

PND 0 – 3

Groups	**AR**	**ER**	**Kiss 1r**	**AMH mRNA**
Control	1.01±0.10	1.01±0.09	1.00±0.05	1.01±0.07
Vehicle control	2.06±0.48	0.58±0.13	0.81±0.04	1.19±0.30
10 mg/kg Melatonin	0.57±0.26	0.95±0.27	0.50±0.06	5.42±0.82
25 mg/kg BPA	1.16±0.28	4.40±1.37*#+	1.51±0.59	10.68±0.47
25 mg/kg BPA + 10 ml/kg Melatonin	1.75±0.01	6.64±0.19*#+	1.68±0.75	14.39±0.00*#
50 mg/kg BPA	2.83±0.54*#	1.19±0.22	3.78±0.55*#	19.16±6.19*#+
50 mg/kg BPA + 10 ml/kg Melatonin	4.77±0.17*#	3.16±0.08	2.55±0.14#	42.17±135*#+

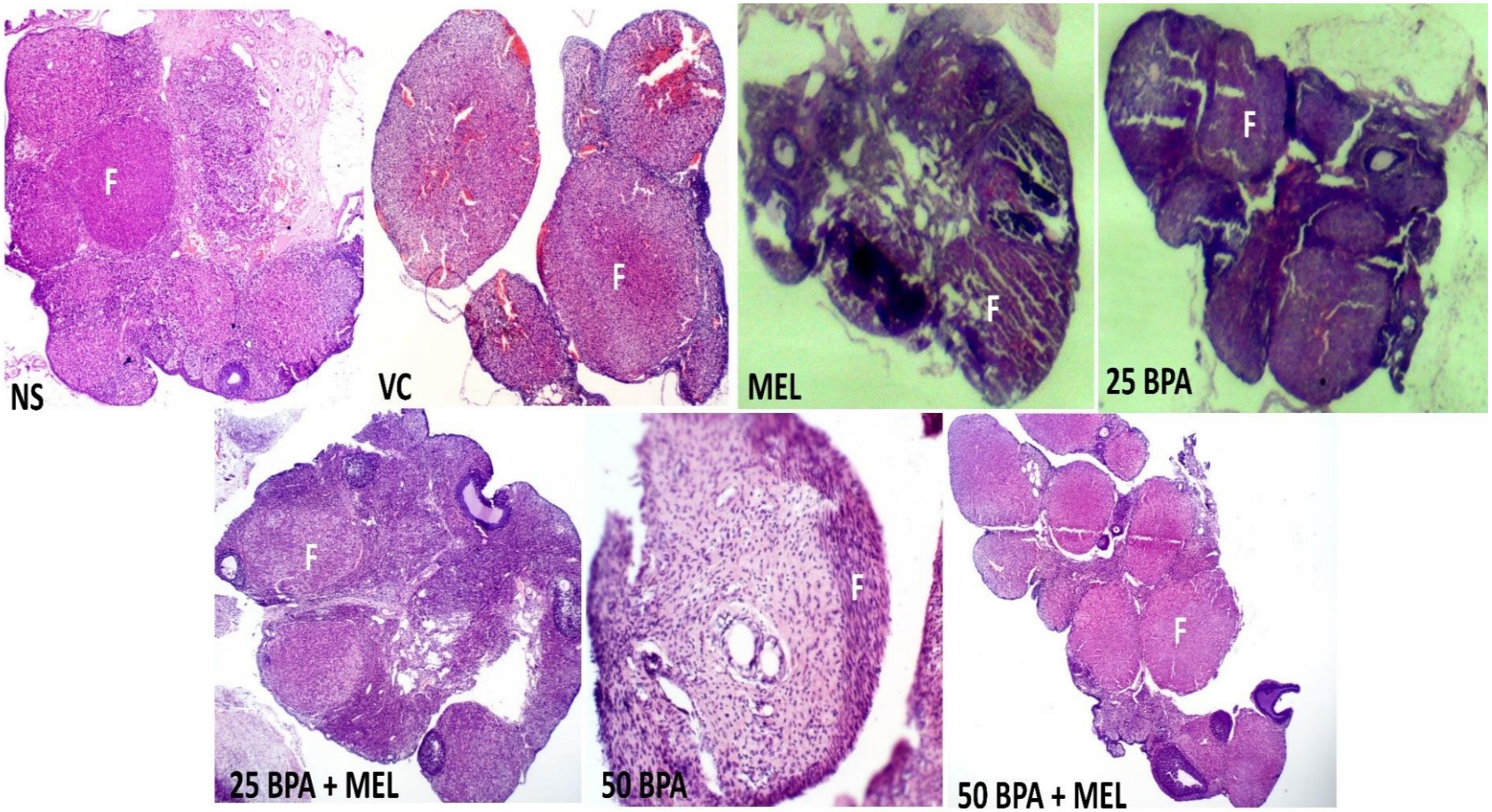
Table 4.2: Quantitative PCR of pituitary genes following neonatal exposure to bisphenol-A and melatonin. Gonadotropin releasing hormone mRNA (GnRH mRNA) and Gonadotropin releasing hormone receptors (GnRHr).

Groups	GnRH mRNA	GnRHr
Control	1.05±0.24	1.12±0.39
Vehicle control	1.29±0.41	1.04±0.35
10 mg/kg Melatonin	1.32±0.16	1.20±0.43
25 mg/kg BP-A	1.18±0.24	1.86±0.75
25 mg/kg BP-A + 10 ml/kg Melatonin	1.61±0.24	1.12±0.35
50 mg/kg BP-A	24.68±22.51	0.75±0.36
50 mg/kg BP-A + 10 ml/kg Melatonin	35.55±2.13	3.26±1.78

4.1.4 Ovarian histology and histomorphometry following BPA and Melatonin exposure at birth.

There was a significant decrease in the corpora count inthe groups administered bisphenol-A and melatonin when compared to the groups administered normal saline and vehicle conyrol groups (see Figures 4.11 a and b). There were decreases in the primary and secondary follicles with associated increase in the defective follicles in the treated groups as well as the melatonin group when compared to the control groups. These defective follicles include degenerated follicles and degenerated oocytes (see figures 4.12 a and b). Melatonin was observed to have marked distortion of the corpora cytoarchitecture, hence not shownto have ameliorative effects on bisphenol-A induced toxicity.

Figure 4.11a: Ovarian histology of Wistar rats exposed to bisphenol-A and melatonin at birth showing the ovarian corpora. Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), F(Follicle) 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). H&E stainX 10.


F
NS
F
VC
F
MEL
F
25 BPA
F
25 BPA + MEL
F
50 BPA
F
50 BPA + MEL

Histomophometry of the ovary following BPA and melatonin exposure

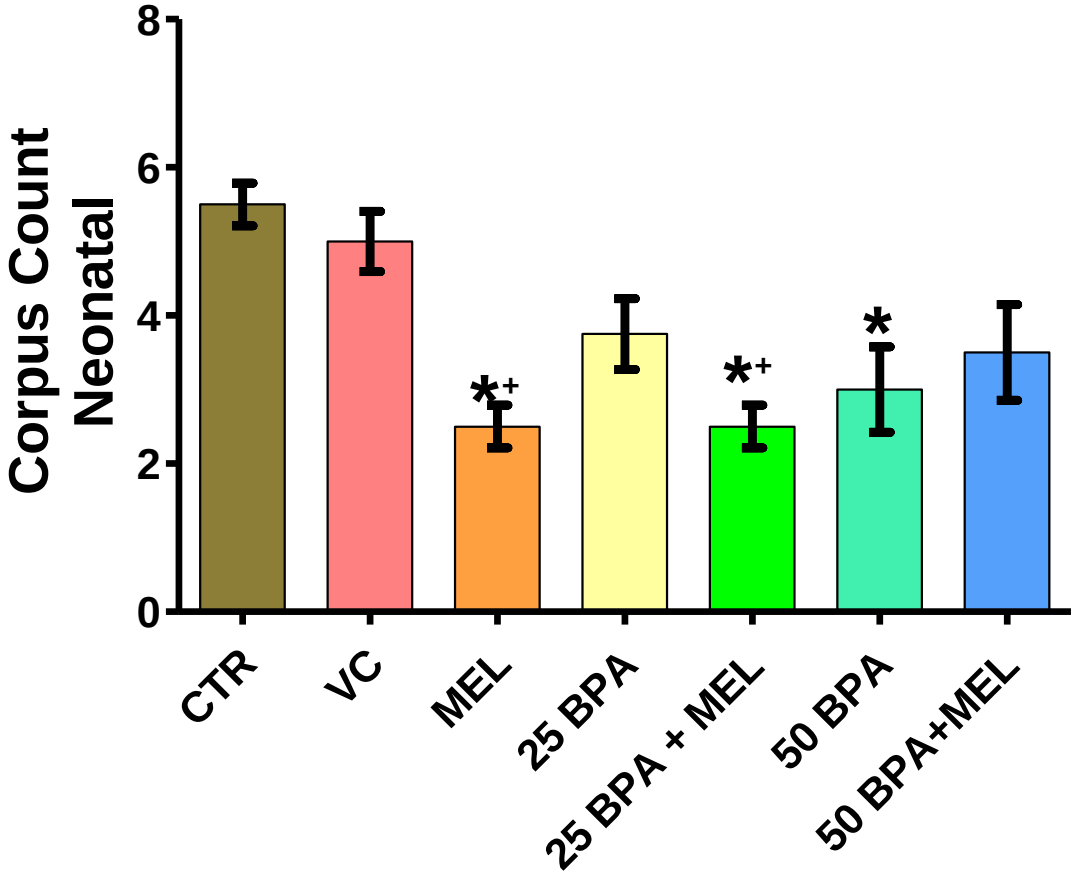
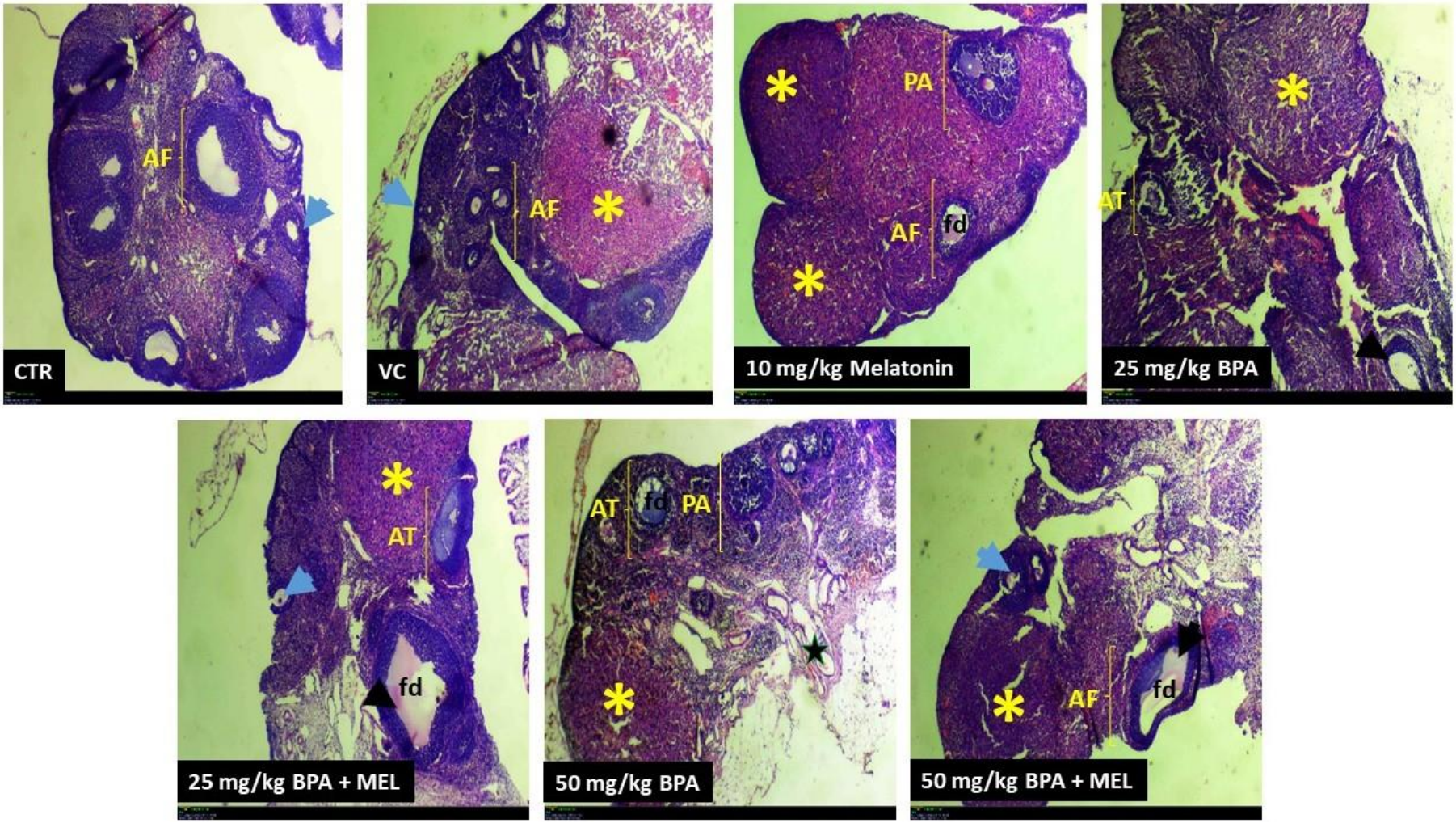


Figure 4.12b: Histomorphometry of the ovarian corpora of Wistar rats exposed to bisphenol-A and melatonin at birth. Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Asterix (*) $P < 0.05$ compared to NS control. + $P < 0.05$ compard to the vehicle control.

Figure 4.13a: Ovarian histology of Wistar rats exposed to bisphenol-A and melatonin at birth showing various types of follicles. Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). H&E stain X 100.


AF
CTR
AF
VC
PA
AF
fd
10 mg/kg Melatonin
AT
25 mg/kg BPA
AT
fd
25 mg/kg BPA + MEL
AT
fd
PA
50 mg/kg BPA
AF
fd
50 mg/kg BPA + MEL

Histomophometry of the ovary following BPA and melatonin exposure

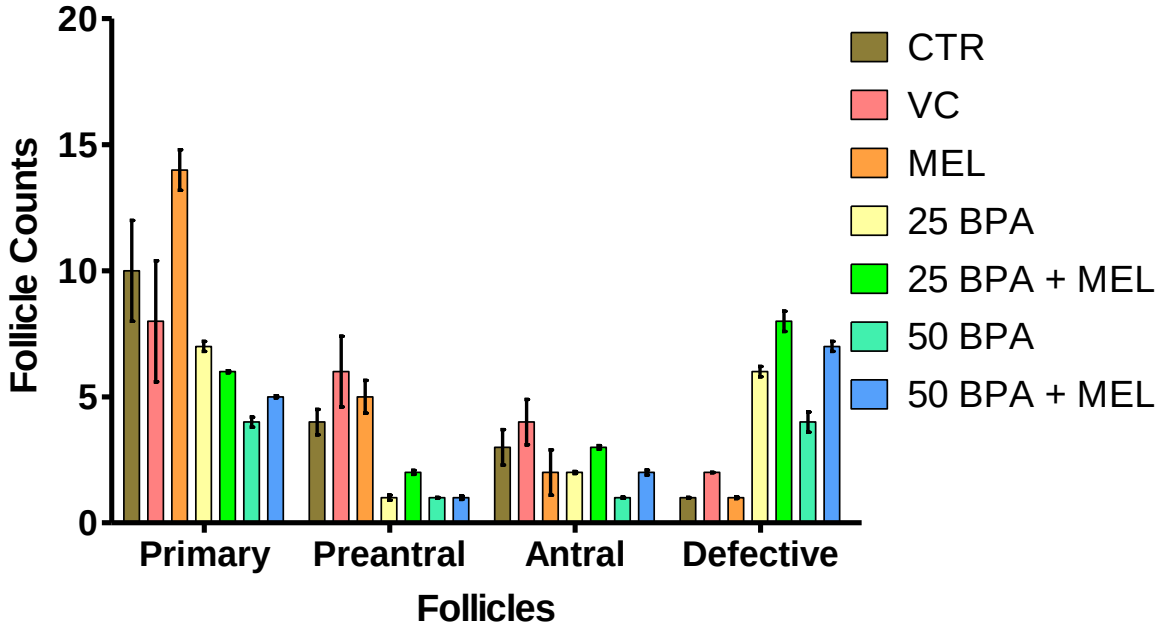


Figure 4.14b: Histomorphometry of the various types of ovarian follicles of Wistar rats exposed to bisphenol-A and melatonin at birth.

Post-natal day 0 (PND 0), Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL).

4.2 Results of analysis in adolescent Wistar rats (Group B)

4.2.1 Alterations in Hormonal Responses following Bisphenol-A exposure

Exposure to bisphenol-A was associated with significant increases in serum follicle stimulating hormone, luteinising hormone, oestrogen and testosterone levels in comparison to the control group, while there was associated decrease in antimullerian hormone level. Similarly, coadministration of BPA and melatonin showed no appreciable ameliorative effects (Figures 4.13 – 4.17).

Outturns of Follicle Stimulating Hormone production following BPA and melatonin exposure

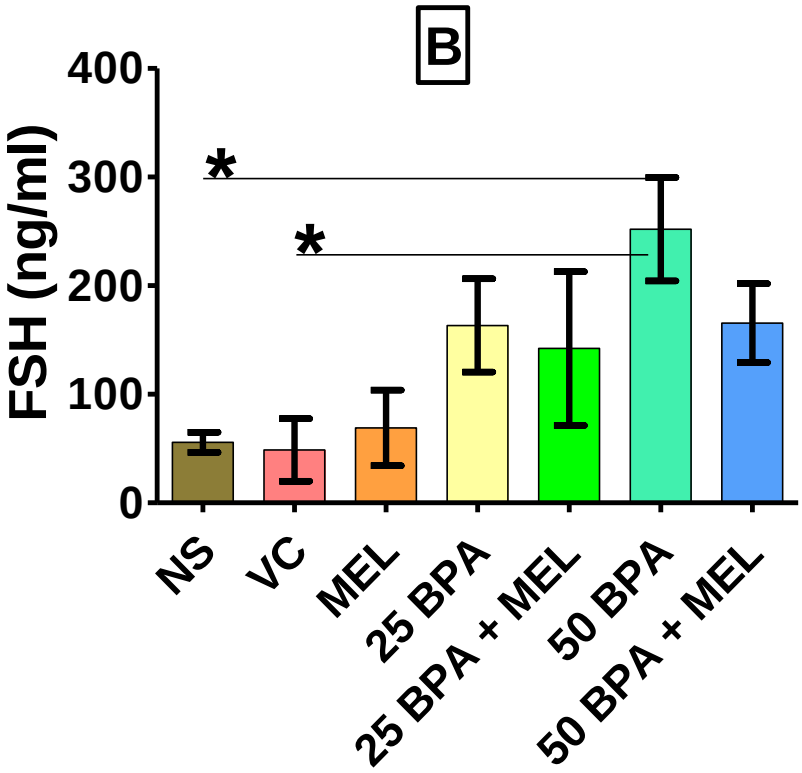


Figure 4.15: Serum FSH levels in adolescent female rats exposed to BPA and melatonin.
Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$ compared to NS control.

Outturns of Luteinising Hormone production following BPA and melatonin exposure

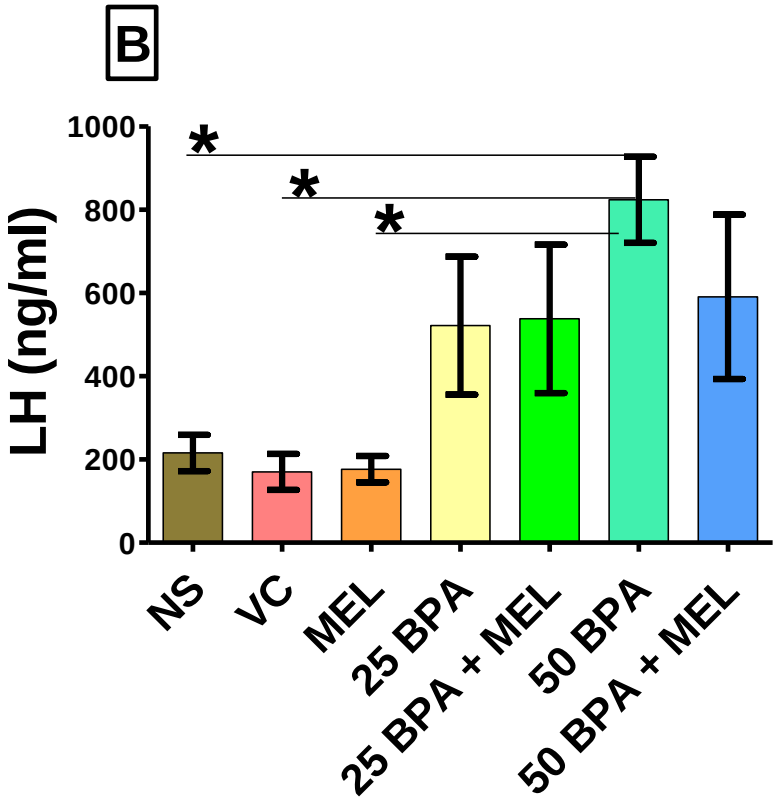


Figure 4.16: Serum LH levels in adolescent female rats exposed to BPA and melatonin.
Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$ compared to NS control.

Outturns of Oestrogen production following BPA and melatonin exposure

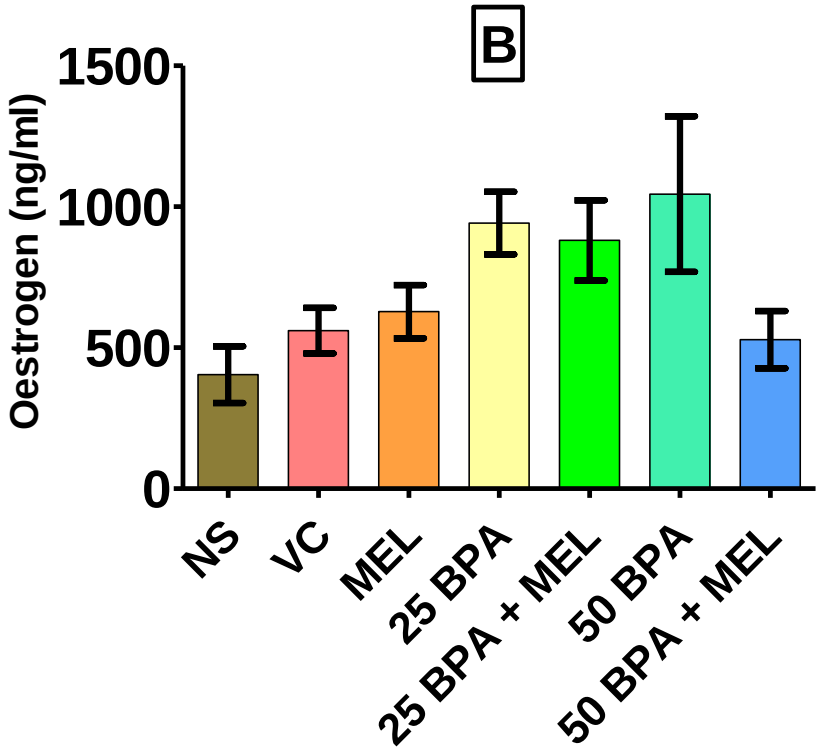


Figure 4.17: Serum Oestradiol levels in adolescent female rats exposed to BPA and melatonin.

Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM.

Outturns of Progesterone production following BPA and melatonin exposure

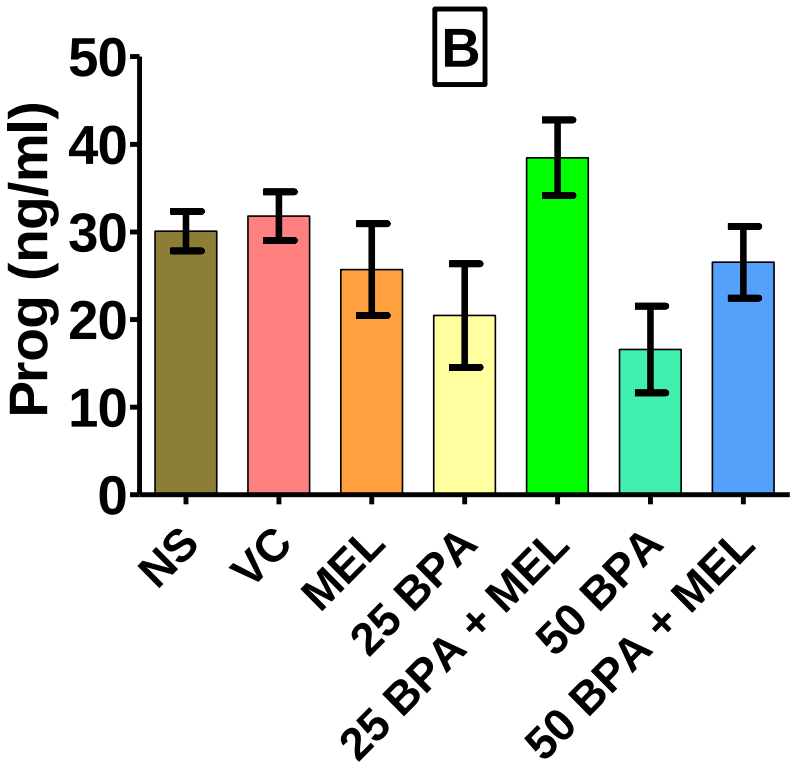


Figure 4.18: Serum Progesterone levels in adolescent female rats exposed to BPA and melatonin.

Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM.

Outturns of Progesterone production following BPA and melatonin exposure

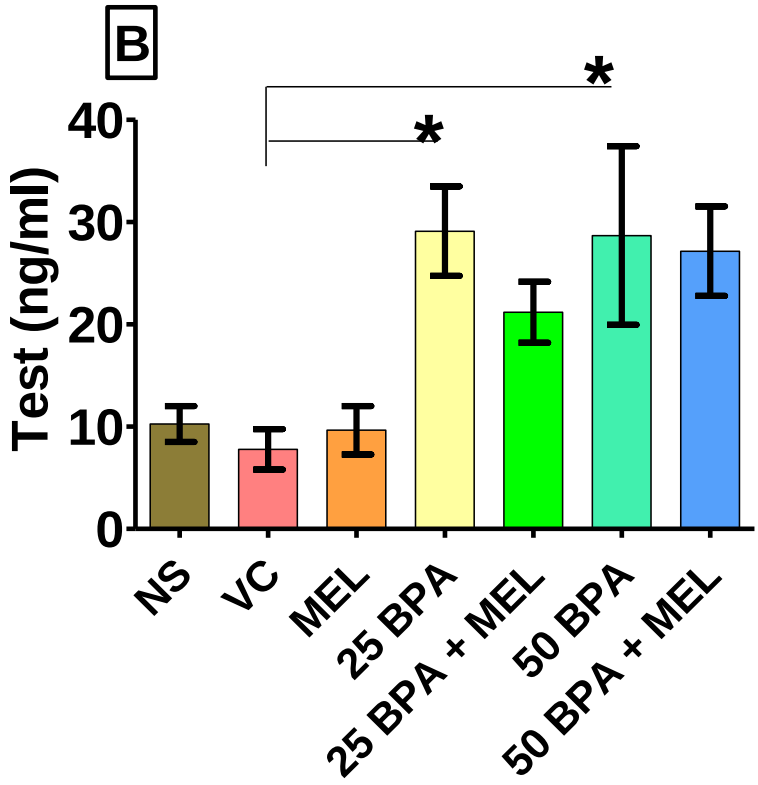


Figure 4.19: Serum Testosterone levels in adolescent female rats exposed to BPA and melatonin.

Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL),50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$ compared to NS control.

Outturns of Antimullerian Hormone production following BPA and melatonin exposure

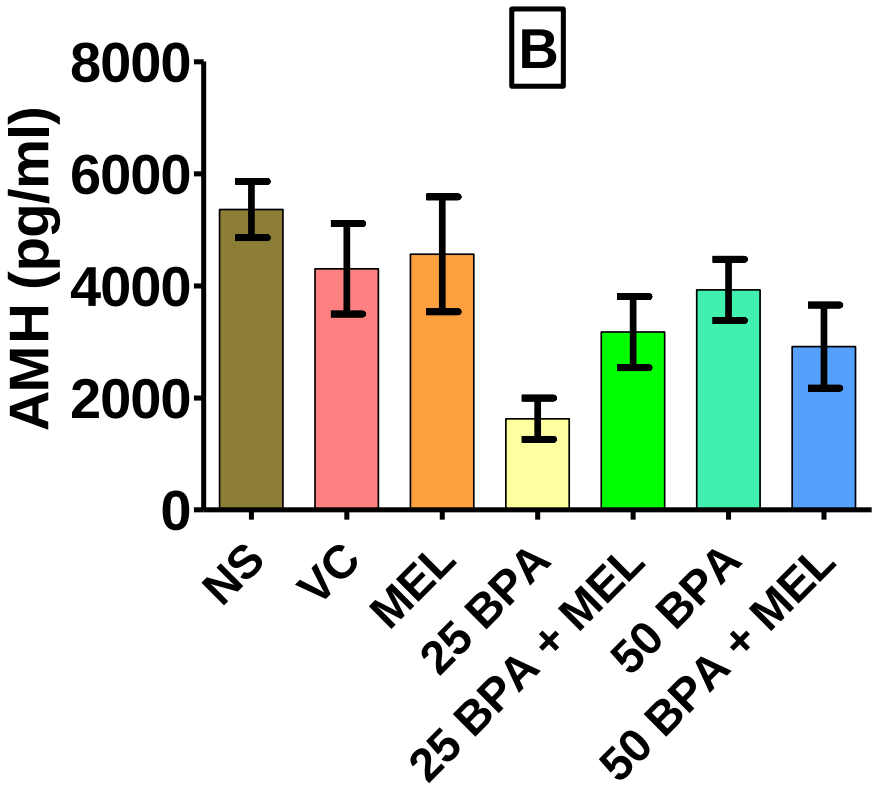


Figure 4.20: Serum AMH levels in adolescent female rats exposed to BPA and melatonin.

Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM.

4.2.2 Changes in ovarian antioxidant and metabolic enzyme levels following BPA exposure

There were marked increases in antioxidant levels of superoxide dismutase and nitric oxide synthase, while a decrease in glutathione peroxidase was observed. The uridyl diphosphate level was similarly markedly elevated. Melatonin had no significant repairative effects in BPA induced toxicity.

Superoxide Dismutase values following BPA and melatonin exposure

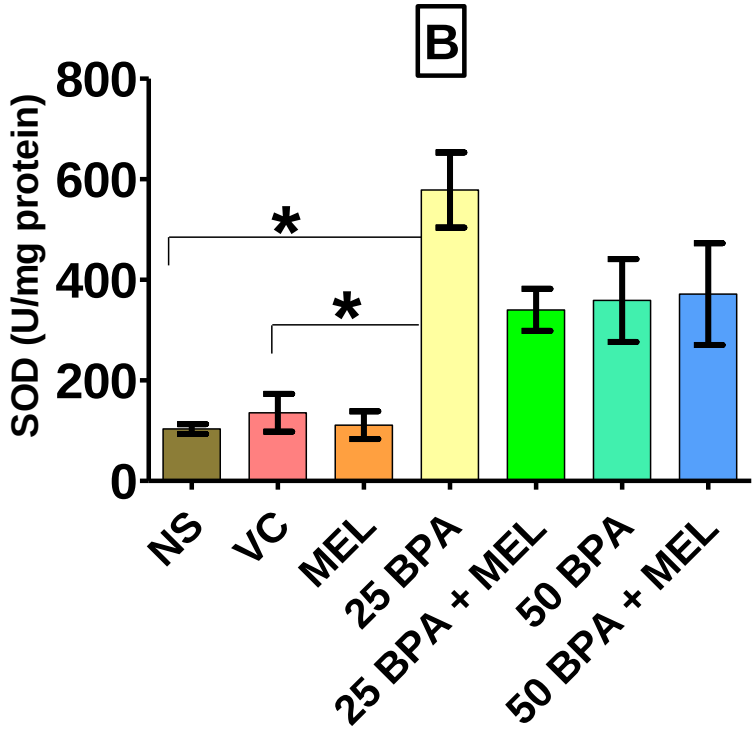


Figure 4.21: Ovarian SOD levels in adolescent female rats exposed to BPA and melatonin.

Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$ compared to NS control.

Glutathione Peroxidase values following BPA and melatonin exposure

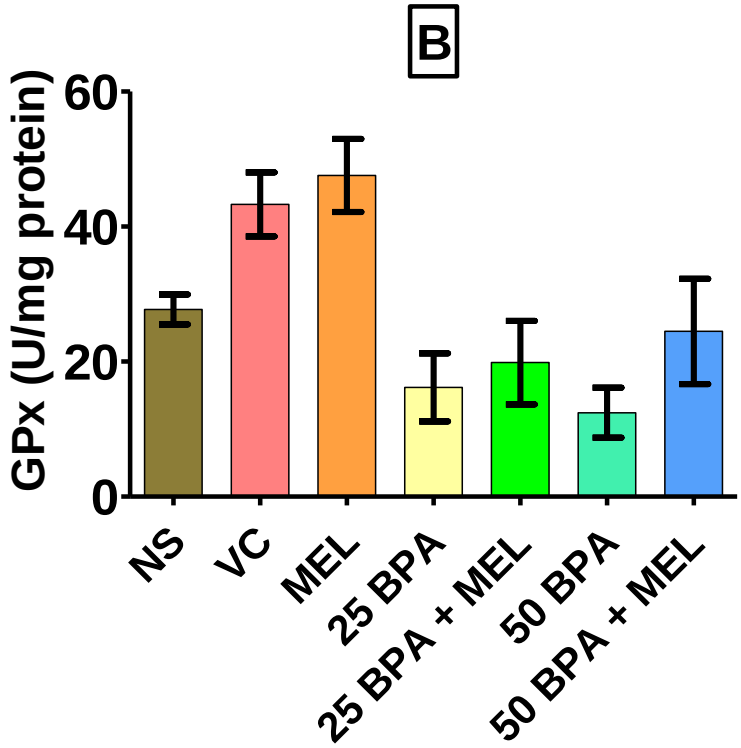


Figure 4.22: Ovarian GPx levels in adolescent female rats exposed to BPA and melatonin.

Adolescent group (B), Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM.

Outturns of Nitric Oxide Synthase production following BPA and melatonin exposure

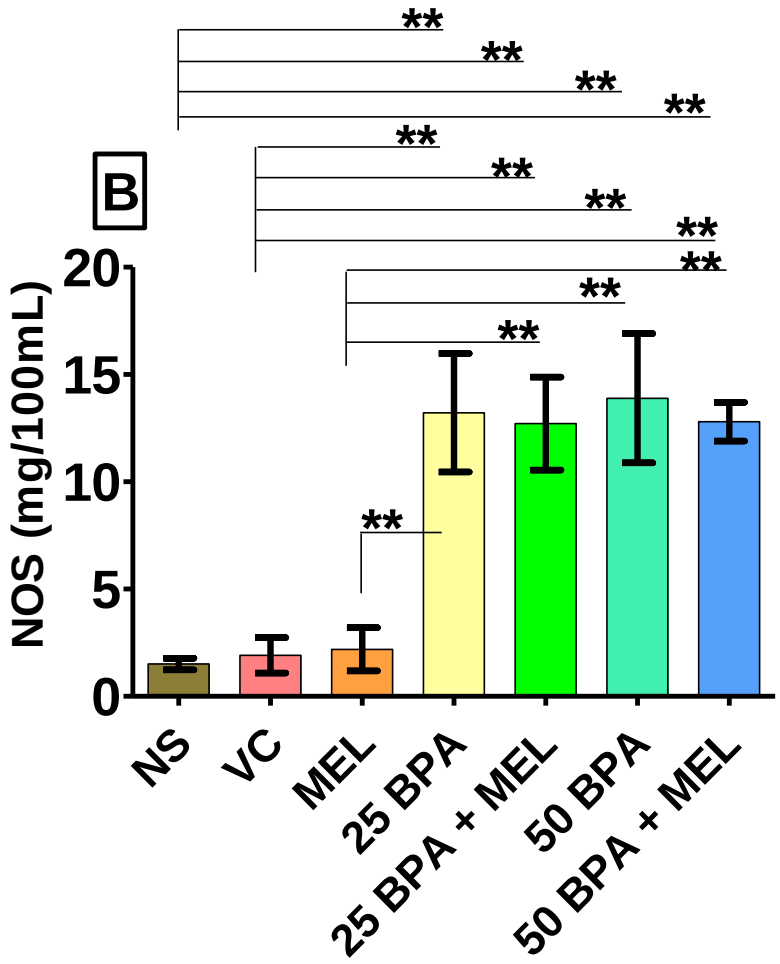


Figure 4.23: Ovarian NOS levels in adolescent female rats exposed to BPA and melatonin.

Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$; Asterix (**) $P < 0.01$ compared to NS control.

Uridine DiphosphoGlucuronyl Transferase values following BPA and melatonin exposure

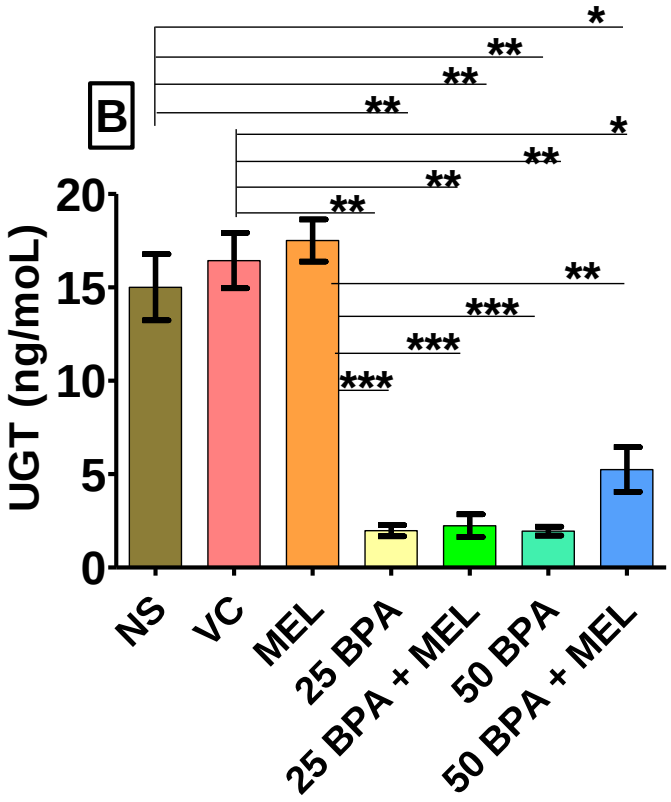


Figure 4.24: Ovarian NOS levels in adolescent female rats exposed to BPA and melatonin.

Normal Saline (NS), Corn oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Data are presented as Mean ± SEM. Asterix (*) $P < 0.05$; Asterix (**) $P < 0.01$; Asterix (***) $P < 0.001$ compared to NS control

4.2.3 Overexpression of Reproductive genes following exposures to bisphenol-Aand melatoninin adolescent period

Bisphenol-A and melatonin were associated with overexpression in hypothalo-pituitary reproductive genes (ER, Kiss 1, GnRHr, GnRH mRNA and AMH mRNA). However, the androgen receptors levels were essentially preserved.

Table 4.3: Quantitative PCR of hypothalamic genes following adolescent exposure to bisphenol-A and melatonin. Androgen receptors (AR), oestrgen receptors (ER), Kiss 1 receptors (Kiss 1r) and antimullerian mRNA (AMH mRNA). *#+ P≤0.05. * - compared to control; # - compared to vehicle control; + compared to melatonin.

Adolescent				
Groups	**AR**	**ER**	**GPR54**	**AMH mRNA**
Control	1.01±0.10	1.01±0.09	1.00±0.05	1.01±0.07
Vehicle control	0.97±0.22	$0.97\pm0.15^{\#+}$	1.37±0.05	$38.13\pm3.14^{+}$
10 mg/kg Melatonin	1.01±0.11	$4.79\pm0.54^{*\#}$	3.74±0.91	$144.4\pm35.2^{*\#}$
25 mg/kg BP-A	1.09±0.14	$0.24\pm0.03^{\#+}$	3.56±0.60	$32.51\pm4.23^{+}$
25 mg/kg BP-A + 10 ml/kg Melatonin	1.90±0.51	$0.30\pm0.06^{\#+}$	4.80±0.87*	$35.35\pm5.67^{+}$
50 mg/kg BP-A	0.82±0.31	3.45±0.30*	4.42±0.56*	71.19±1.00*
50 mg/kg BP-A + 10 ml/kg Melatonin	1.06±0.19	$1.60\pm0.23^{\#+}$	1.99±0.21	$43.25\pm3.26^{+}$

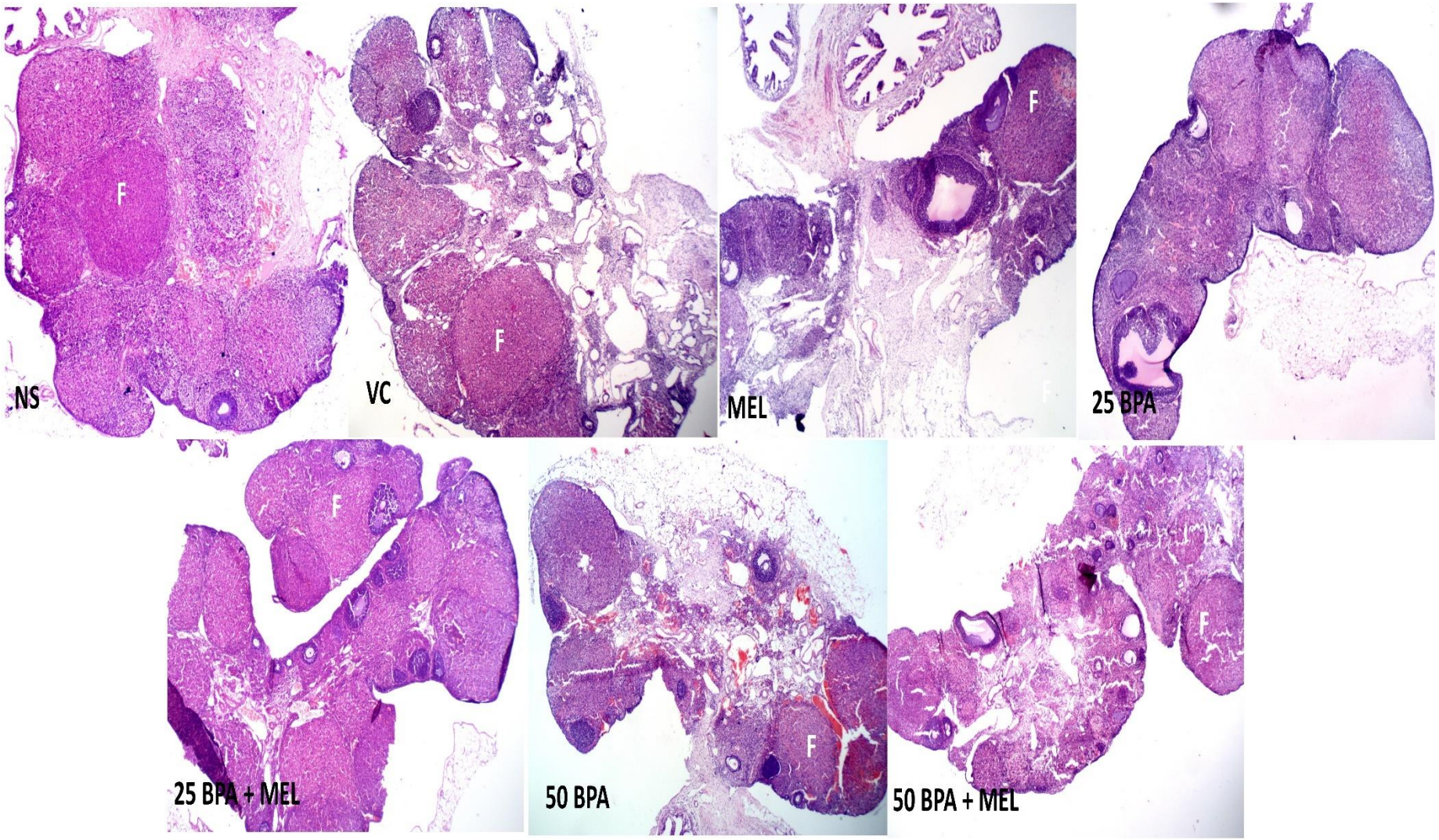
Table 4.4: Quantitative PCR of pituitary genes following adolescent exposure to bisphenol-A and melatonin. Gonadotropin releasing hormone mRNA (GnRH mRNA) and Gonadotropin releasing hormone receptors (GnRHr). *#+ P≤0.05. * - compared to control; # - compared to vehicle control; + compared to melatonin.

Groups	GnRH mRNA	GnRHr
Control	1.05±0.24	1.12±0.39
Vehicle control	48.16±15.86	3.93±0.50
10 mg/kg Melatonin	36.85±15.3	4.32±1.33
25 mg/kg BPA	70.79±7.52*	2.33±0.55
25 mg/kg BPA + 10 mg/kg Melatonin	79.57±13.89*#	4.13±1.00
50 mg/kg BPA	21.65±2.11	1.25±0.33
50 mg/kg BPA + 10 mg/kg Melatonin	128.8±13.56*#+	7.45±2.59*

4.2.4 Ovarian histology and histomorphometry following BPA and Melatonin exposure.

Marked decrease in the corpus count in the treated groups, as well as the melatonin group compared to the control groups (see Figures 4.23 a and b). Similarly, there were increased number of abnormal shaped and degenerated follicles on the histomorphometry as compared to the controls (see Figures 4.24 a and b)

Figure 4.25a: Ovarian histology of Wistar rats exposed to bisphenol-A and melatonin during adolescent period showing the ovarian corpora. Normal Saline (NS), Follicle (F), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). H&E stain X10.


NS
F
VC
F
MEL
F
25 BPA
25 BPA + MEL
F
50 BPA
F
50 BPA + MEL
F

Histomophometry of the ovary following BPA and melatonin exposure

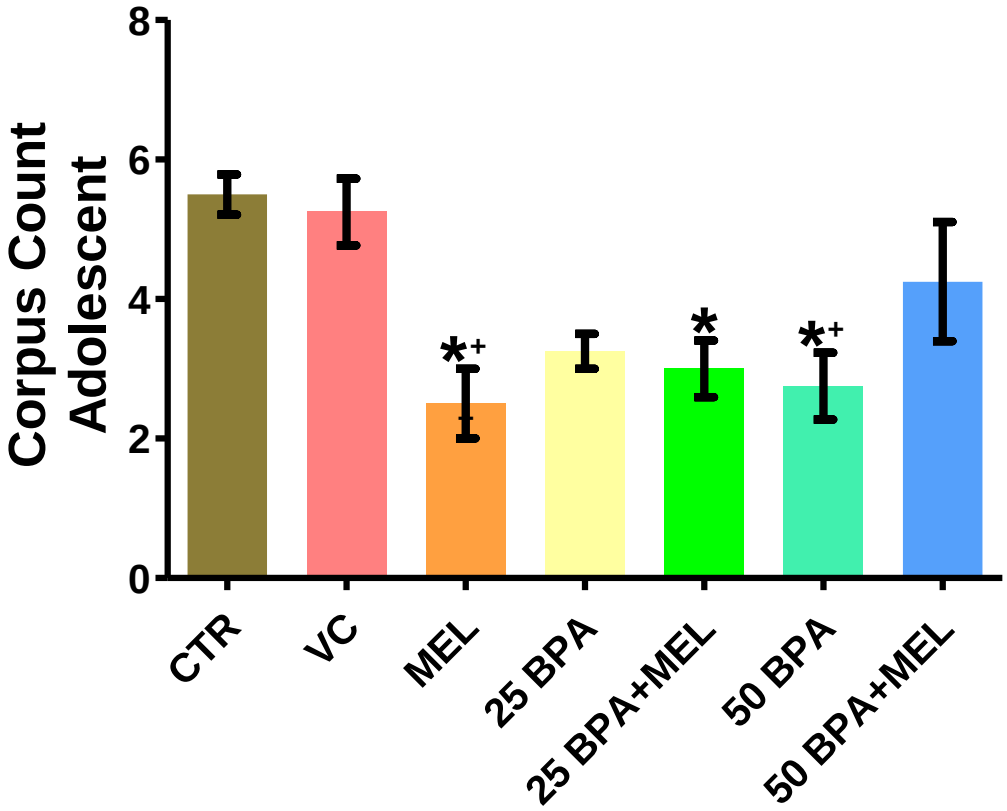
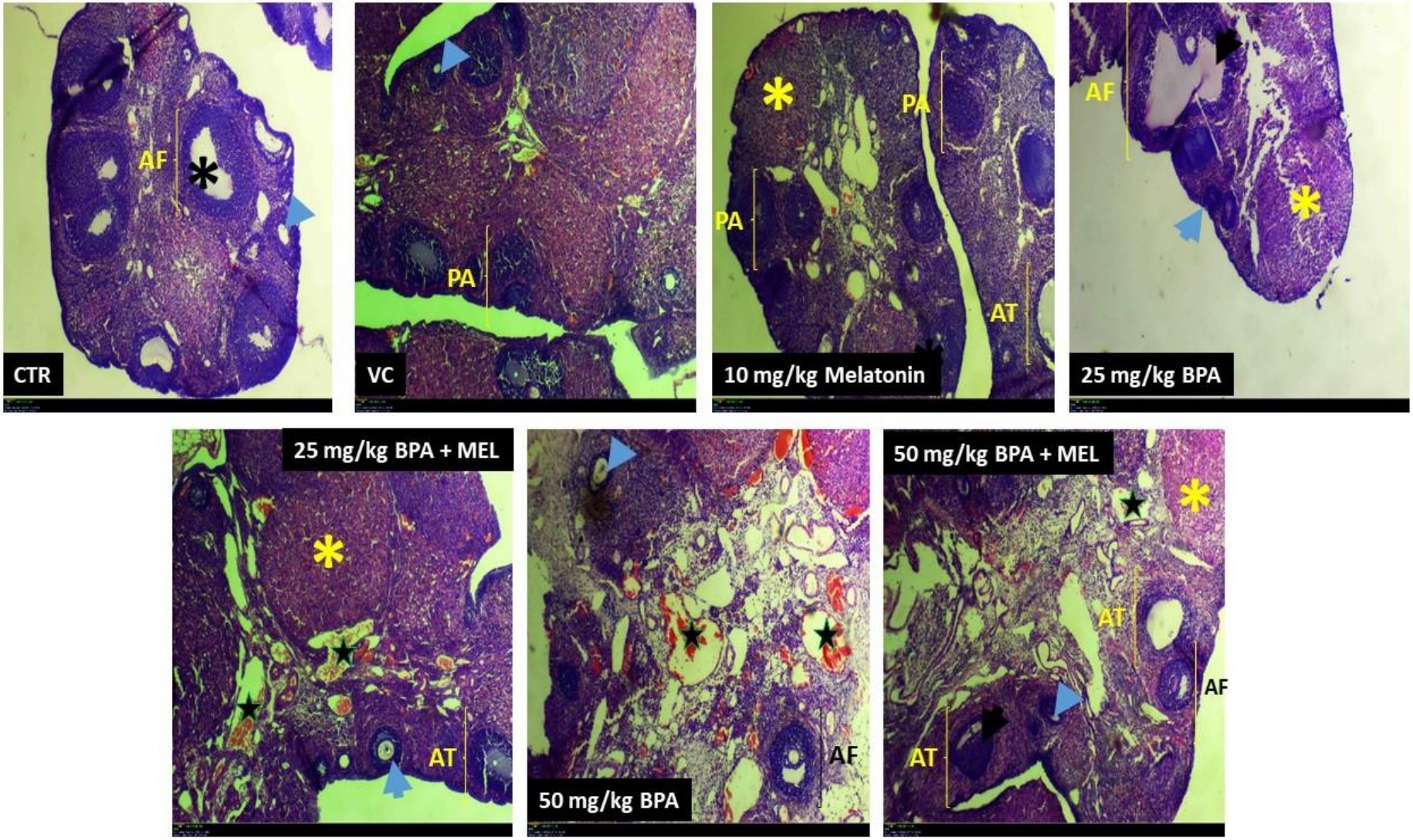


Figure 4.26b: Histomorphometry of the ovarian corpora of Wistar rats exposed to bisphenol-A and melatonin at birth.

Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). Asterix (*) $P < 0.05$ compared to NS control. + $P < 0.05$ compard to the vehicle control.

Figure 4.27a: **Ovarian histology of Wistar rats exposed to bisphenol-A and melatonin during adolescent period showing various types of follicles.Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL). H&E stain X 100.**


CTR
AF
VC
PA
10 mg/kg Melatonin
PA
PA
AT
25 mg/kg BPA
AF
25 mg/kg BPA + MEL
AT
50 mg/kg BPA
AF
50 mg/kg BPA + MEL
AT
AF
AT

d

Histomophometry of the ovary following BPA and melatonin exposure

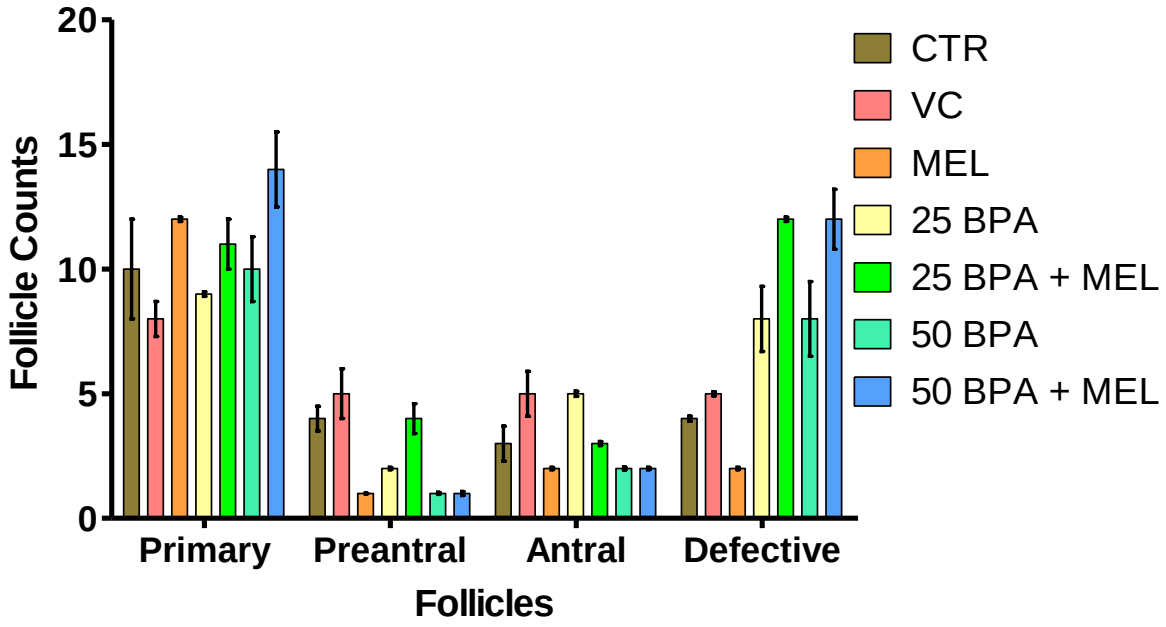


Figure 4.28b: Histomorphometry of the various types of ovarian follicles of Wistar rats exposed to bisphenol-A and melatonin during adolescent period.

Normal Saline (NS), Ethanol hydroxide + sesame oil (Vehicle Control, VC), Melatonin (MEL), 25mg Bisphenol-A (25BPA), 25mg BPA + Melatonin (25BPA +MEL), 50mg Bisphenol-A (50 BPA) and 50mg BPA + Melatonin (50 BPA + MEL).

CHAPTER FIVE :DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Bisphenol-A has been reported to potentially have adverse effects at lower doses and induce effects in a non-monotonic dose-response (Vandenberg *et al.,* 2013). It has also been implicated in numerous endocrine disorders such as early onset puberty, metabolic disorders such as obesity, diabetes, and polycystic ovarian syndrome in human and animal studies (Vandenberg *et al.,* 2013). Studies have suggested that activities of BPA may be dependent on dosage tested, route of administration and/or timing/length of BPA exposure (Peretz *et al.,* 2014). Some studies have however suggested that BPA is unable to exert its debilitating effects on steroidogenesis in experimental animals except at 100 mg/kg and 300 mg/kg doses (Rebuli *et al*., 2014).

5.1.1 BPA effects on Steroidogenesis

Assessment of BPA interference on plasma reproductive hormones in Wistar rats was first undertaken. Bisphenol-A caused hormonal alterations in the adolescent rats used, evidenced in the marked increase in the plasma levels of FSH, LH, estradiol and testosterone in the exposed rats. Further strengthening the reproductive affective activities of BPA, the plasma anti-Mullerian and progesterone hormones were depleted.

The gonadotropins, follicle stimulating hormone (FSH) and luteinising hormone (LH), are glycoproteins secreted and released in a pulsatile manner by the anterior pituitary cells in response to the actions of gonadotropin releasing hormone (GnRH) secreted by the hypothalamus. Follicle stimulating hormone and luteinizing hormone act on the gonads, controlling gametogenesis and gonadal sex hormone production (Casarini and Crépieux, 2019). FSH and LH concentrations according to these findings, increased in the experimental animals exposed to BPA. Substantial increase was observed in adolescent animals exposed to

50 mg/kg BPA compared to control, vehicle and melatonin treated animals. This can be attributed to reduction in functions and efficacy as well as alterations in the negative feedback mechanism of these gonadotropins, whereby the brain is signaled to produce more gonadotropins to boost FSH and LH hormones in the experimental animals (Casarini and Crépieux, 2019). This finding is in line with some other studies who reported that BPA at a low dose stimulated increased production of FSH and LH in exposed male rats (Venugopal and Yerramilli, 2019).

Bisphenol-A is an endocrine disruptor that mimic oestrogen activities and also compete for oestrogen receptors in a biological system. Investigation has shown that BPA interacted with oestrogen receptors and interfered with endocrine metabolism leading to harmful effects (Warner *et al.,* 2020). This is corroborated by this study as unutilisedoestrogen concentrations soared, though insignificantly, in both age groups treated with BPA. Substantial increment in oestradiol concentration has also been implicated in BPA exposure (DiDonato *et al.,* 2017). A possible explanation is that BPA interfers with the normal actions of oestrogen and competes for binding receptors, thereby increasing the oestrogen level. This is contrary to the report of Kuiper and others where he stated that the binding affinity of oestrogen to its receptors is much higher than that of bisphenol-A, which also further adds to the controversy of BPA being an endocrine disruptor (Kuiper *et al.,* 1998).

Progesterone is produced by lutein cell filled corpus luteum, a mature follicle that has shed its ovum during ovulation. Progesterone prepares the uterus for potential implantation, nourish the growing embryo and suppress maternal immune system to tolerate the growing embryo. Reduction in progesterone concentration induced by BPA action may result in abortion in exposed animals if unchecked. Progesterone concentration decreased in the adolescent exposed, though these were statistically insignificant. This however explains that there is a

possible role of decrease progesterone level, a marker of ovulation, when there is prolonged exposure to bisphenol-A. Miao *et al.*, in 2015, also documented similar findings. Exposure to BPA results in antagonism of oestradiol effect on progesterone. Through decrease in progeterone receptor expression, BPA decreases the ability of progesterone to inhibit oestradiol action. Therefore, this affects the balance between oestrogen and progesterone, leading to a heightened oestrogen response and a marked decrease in progesterone level compared to controls (Grasselli, Baratta, Baioni, & Bussolati, 2010)

Testosterone in females is produced by the ovaries and adrenal glands. It plays vital role in growth and maintenance of bone mass and female reproductive tissues (Bienenfeld *et al.,* 2019). Testosterone concentration, according to this study, increased insignificantly in BPA treated animals compared to other experimental groups. Testosterone secreted in females is usually in small amount compared to what is obtainable in males, which is mostly converted to female sex hormones, oestrogen. An excess production of testosterone will ultimately culminate in virilisation since the body cannot keep up with the metabolism (Bienenfeld *et al.,* 2019). A significant and positive relationship was reported between BPA exposure and circulating androgen concentrations in a small study of 26 normal women and 47 women with ovarian dysfunction (Caserta *et al.*, 2014).

Anti-Mullerian hormone (AMH), is an important glycoprotein whose major role is in growth differentiation and folliculogenesis(Marca, Emilia, Modena, Fauser, & Macklon, 2009). AMH is produced by the preantral and antral granulosa cells (Tal *et al.,* 2014). It inhibits recruitment of follicles from the resting pool during follicle maturation as well as serving as a molecular marker for ovarian reserve or ovarian dysfunction (Pelosi *et al.,* 2015). In this study, the level of AMH concentrations reduced in all experimental animals exposed at both neonatal age and adolescent. This reduction is likely due to the toxic actions of BPA on the

ovarian follicular pool or general deterioration in ovarian health. Another possible explanation is that bisphenol-A may cause arrest in the ovarian follicular maturation or degeneration of granulosa cells within the follicles as observed in the ovarian photomicrographs. This finding is in keeping with reports that AMH secretion from the granulosa cells can be altered (Sonigo *et al.,* 2019). This alteration may have resulted in increased apoptotic events i.e. meiotic arrest in germ cells which then results in granulosa cell depletion and follicular atresia. Lower concentrations of AMH can cause primordial follicles to grow at a faster rate, leading to the premature maturation of the follicular pool and a shortened reproductive lifespan.

Bisphenol-A, at different doses, has also been reported to alter negatively hormonal secretion in exposed humans and laboratory animals (Miao *et al.,* 2015). These negative hormonal alterations can be observed at environmentally relevant low dose. This alteration may lead to disruption in the hypothalamo-pituitary-ovarian axis of females as seen in this study, which can culminate into disturbance with fertility. An association between BPA and miscarriage has also been reported, indicating BPA's possible disturbance to hormone homeostasis (Lathi *et al.,* 2014). The alterations noticed in the steroidogenesis of animals exposed to BPA may be as a result of impairment to a rate limiting steroidogenic enzyme responsible for cholesterol transfer to the inner mitochondrial membrane of steroidogenic cells where it is converted to pregnenolone, the steroidogenic acute regulatory protein (StAR).Peretz and Flaws in 2013 reported that BPA down-regulated the expression of StAR in an in-vitro cell culture culminating in alterations in hormone synthesis in exposed experimental subjects.

5.1.2 Interference with oxidative stress markers

Postulations have been that one of the mechanisms through which BPA exert its debilitating properties is via induction of oxidative stress (Hassan *et al.,* 2012; Chouhan *et al.,* 2015;Avci

et al., 2016). Oxidative stress is said to be induced when a number of beneficial anti-oxidants is negatively altered and free radicals are upregulated, through metabolic redox cycling between quinone and hydroquinone forms of BPA, in cells more than the quantity that these cells can metabolise which may result in cellular apoptosis (Amraoui *et al*., 2018). In this study, the oxidative markers assayed for were superoxide dismutase (SOD), glutathione peroxidase (GPx) and nitrogen oxide synthase (NOS). The concentrations of SOD and NOS increased while GPx concentrations tanked in the animals exposed to different low dose BPA. Melatonin, however showed a mild reversal of the observed oxidative stress caused by ROS generated due to actions of BPA, though these were not appreciable significantly.

Superoxide dismutase (SOD) is an important anti-oxidant in a living cell that converts superoxide radicals into ordinary oxygen or hydrogen peroxide. The hydrogen peroxide generated is further degraded by catalase. Superoxide radicals are by-products of oxygen metabolism in nearly all living cells and can be detrimental to cells if superoxide production is not regulated.Superoxide dismutase protects oocytes from oxidative stress. This explains the increased levels of SOD following BPA administration. As BPA may bind to the SOD sites, thereby inhibiting the enzymatic activity of SOD and decreasingits activities, thereby leading to increase concentration of SOD (Taylor*et al*., 2012). The significant rise noticed in SOD concentration can be attributed to the innate cellular response to sporadic superoxide level due to toxic assault of BPA. This finding negates the reports of Jehane*et al.,* (2014) who concluded that low doses, 0.5 and 50 mg/kg, of BPA induced a substantial drop in SOD concentration in exposed animals. Similarly, Hassan *et al*., who studied the effects of varying low doses BPA (0.1, 1, 10 and 50 mg/kg) documented a gradual reduction in SOD concentration with increasing dosage of BPA in exposed animals(Hassan *et al*., 2012).

Gluthione peroxidase is an important biological enzyme that catalyses conversion of lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water, thereby protecting organisms from oxidative damage. The lower concentration found in animals exposed to varying low doses at different stage of development is in concert with another study(Abdel-Wahab and Abdel-Wahab, 2014; Eid *et al*., 2015).

Scientists have implicated increased nitrogen oxide synthase (NOS) in ovarian reserve depletion and reduced steroidogenesis in both male and female BPA treated animals. NOS are a family of enzymes responsible for production of nitric oxide (NO). The more NOS in a biological system, the more NO to be synthesised. The higher NOS concentration in BPA exposed animals in this study can culminate into high rate of NO production which will lead to disruption of normal physiologic functioning in experimental Wistar rats. The findings here of the over production of NOS and depletion of GPx and UGT suggesting induced oxidative stress following BPA exposure, can be strengthened with previous reports of increased ROS, lipid peroxidation by increased MDA, depletion of catalase (CAT), glutathione (GSH) and glutathione-s-transferase (GST), decreased antioxidant enzyme activity and induced DNA damage, leading to organ toxicity, including reproductive toxicity (Khan *et al*., 2015; Leem *et al*., 2016;Zhou *et al*., 2016).

In contrast to the observed oxidative events in BPA toxicity above, melatonin administration and its interventions in BPA exposed improved anti-oxidant capacity by reducing NOS, and increasing GPx and UGT productions in the ovaries. This finding confirms the previously reported melatonin's anti-oxidant efficacy (Anjum *et al*., 2011; Wu *et al.*, 2013; Othman *et al*., 2014), and other antioxidants such as vitamins (Haroun *et al.*, 2016; Balakrishnan and Sendhilvadivu, 2018), quercetin (Elwakeel and El-monem, 2019), Gallic acid (Olukole, Ola-davies, Lanipekun, & Oke, 2020), Ginseng (Saadeldin *et al*., 2018), honey (Sarah, Zaid,

Kassim, & Othman, 2015) and others. Thus, it is tempting to speculate that the antioxidant capacity of melatonin could be used as a potential defence and/or treatment regimen against BPA induced toxicity.

5.1.3 Interfernce with Carbohydrate metabolism

Bisphenol-A at varying low doses tested showed significant reduction in uridine glucuronosyl transferase (UGT) in animals exposed neonatally and during adolescent age. Uridine glucuronosyl transferase is an important enzyme in carbohydrate metabolism in cells of a living organism and aids the addition of glucose molecules to glycogen chains for storage in the liver and muscles i.e. glycogenesis. BPA can be said to have interfered with glucose metabolism in exposed animals; this interference, if unchecked, may culminate in aggregation of glucose molecules in the blood stream more than body cells can take up at a time, which may end up in causing diabetes mellitus. The enzyme UDP-glucose pyrophosphorase catalyzes combination of UDP-glucose with uridine triphosphate forming a UDP-glucose unit. Then, the UDP-glucose unit combines with the glycogen synthase enzyme to form glycogen chain.Elevated androgen concentration leads to down regulation of the Uridyine 5-diphospho-glucuronosyltransferase (UDP-GT) as documented in this study, and this results in a decrease in detoxification and clearance of BPA (Huo et al., 2015).

5.1.4Bisphenol-A interference with protein receptors expression of hypothalamus and pituitary

In female mice during the critical windows of pituitary gland development, BPA has been documented to negatively affect pituitary glandular development by upregulating pituitary cellular differentiation and gonadotrope population (Eckstrum *et al.,* 2017). Neonatally, not much studies have demonstrated the actions of endocrine-disrupting chemicals on pituitary development. One of the potent mechanisms of action of BPA is interference with the action of oestrogen in living organisms; the oestrogen receptors (α & β) and G protein-coupled

oestrogen receptors (GPER) are found localised in the pituitary gland and hypothalamus (Chimento*et al.*,2014). Data gotten byEckstrum *et al.*, in their study revealed distinct sex-specific consequence on gene expression (Pit1 and Pomc mRNA) in neonate animals exposed to BPA during pituitary development (Eckstrum *et al.*, 2017).

Although there have been contradictory reports on the genotoxic efficacies of BPA, some scientists reported that BPA is non-genotoxic while other concluded that BPA is genotoxic based on the data gotten from laboratory experiments. After some basic genotoxic tests, BPA failed to induce gene mutation (Ribeiro, 2017) or deviations in chromosomal arrangements. In other conflicting reports, Edmonds and others concluded that in a cellular system, BPA metabolites were found bound to DNA (Wu *et al*., 2016). In cultured Syrian hamster embryo cells, BPA was reported to induce numerical chromosomal aberrations and structural alterations; hence BPA may be genotoxic (Eckstrum *et al.*, 2017). BPA has also been documented to exert both prenatal and postnatal genotoxic and immunohistochemical alterations in experimental animals (Moustafa and Ahmed, 2016).

5.1.5 Anti-Mullerian Hormone mRNA

Anti-Mullerian hormone (AMH) is a time dependent hormone that is very vital in the development and maturation of mammals. Any impairment in AMH expression at different stages of development may be as a result of conditions like persistent Mullerian duct syndrome, polycystic ovarian syndrome, hermaphroditism etc. Anti-Mullerian hormoneis greatly expressed in follicular cells in a female mammal and its expression decreases with apoptosis or non-differentiation of primordial and primary follicles. AMH also play an important role in the growth and development of the ovarian follicular cells. Yuming and others reported that lower AMH expression was seen in atretic follicles which indicated that the differentiation of primordial follicles into graafian follicles was arrested in mice ovary

(Cao*et al.,* 2018). Anti-Mullerian hormone mRNA was substantially higher in BPA treated rats in this study, especially rats exposed during their neonatal age which is indicative of the fact that lower AMH level is being secreted. The rise in AMH mRNA expression may be triggered by negative feedback mechanism due to lower concentration of AMH in circulation.

5.1.6 KiSS 1 receptor

Bisphenol-A may have contributed to GPR54 over-expression. A report suggested that varying low doses of BPS (50 μg/kg & 50 mg/kg) upregulated the expression of KiSS1 gene in the hypothalamus of rat.Johnson*et al.*in 2018 reported an increase in hypothalamic expression of Esr1, Esr2, and KiSS1 genes in BPA-exposed parents' mice. In zebra fish according to Mehwish and others scientists,KiSS mRNA and GPR54 expression were upregulated in the hypothalamus of BPA exposed animal across the doses administered compared to control animals, though the expressions reduced with increasing concentrations of BPA (Faheem*et al.,* 2019). The mechanism of action of BPA in the upregulation of GPR54 is not fully elucidated yet.

The primary events that precedes puberty in living system is GnRH neurons activation (Abreu, 2017).This event has been reported to involve the signaling between kisspeptin and its receptor, KiSS1-derived peptide receptor (GPR54), which directly activates the hypothalamic GnRH neurons. KiSS fibers are majorly localised in the hypothalamus and in close proximity with the GnRH neurons, KiSS is also expressed in the dentate gyrus and it is involved in regulation of endocrine function and the onset of puberty (Skorupskaite *et al.,* 2014). GPR54 is a G protein-coupled receptor expressed in a variety of endocrine and gonadal tissues (Trevisan *et al.,* 2018). Activation of KiSS1 receptor triggers the release of GnRH and release of kisspeptin (Kauff and Editors, 2013). Secretion of KiSS is enhanced by GnRH and inhibited by oestradiol(Skorupskaite et al., 2014). Damage to GPR54 signaling

can result in hypogonadotrophic hypogonadism. However, the results obtained in this study did not show disturbance in the KiSS receptor, signifying its preservation, implying BPA did not lead to its alteration.

5.1.7 Gonadotropin Releasing Hormone (mRNA and receptor)

The hypothalamus produces and release GnRH which stimulates the anterior pituitary gland to synthesis and pulsatile release of gonadotropins i.e. LH and FSH (Kurian *et al.*, 2015). Irregularities in pulsatile patterns of GnRH secretion and in extension gonadotropins secretion have been implicated in reproductive anomalies i.e. polycystic ovarian syndrome (PCOS) and amenorrhea (Kurian *et al.*, 2015). The findings revealed elevated expression of GnRH mRNA in BPA and melatonin treated adolescent animals, which implies more GnRH would be synthesised compared to what is seen in control animals. The GnRH mRNA expression was highest in adolescent animals treated with concurrent 50 mg/kg BPA and melatonin. The GnRH mRNA expression seen in the adolescent with BPA and melatonin negate what was seen in neonate animals exposed to the same substance treatment.

Gonadotropin-releasing hormone receptor (GnRHR) is a G-protein coupled receptor found primarily on the surface of pituitary gonadotrope cells and responsible for evoking the actions of hypothalamic GnRH. The receptor, upon activation, triggers the release of gonadotropins from the anterior pituitary gland (Barnett*et al.*, 2006). In neonatal animals, no noticeable significanceoccured in the GnRHR.In contrast the GnRHR expression was significantly upregulated compared to control in animals exposed to BPA during adolescent stage especially in groups exposed to melatonin and 50 mg/kg BPA and melatonin. Defects in GnRHR may result in hypogonadotropic hypogonadism.

5.1.8 Oestrogen receptor (ER)

Some researchers concentrated on the actions of BPA on nuclear receptors i.e. oestrogen, androgen and thyroid receptors (Acconcia*et al.,* 2015). Bisphenol-A possess antioestrogenicproperties by competing with endogenous oestrogen, antagonising its responses (Vandenberg*et al.,* 2017). An *in vivo* study suggested that BPA does not exert its toxicity through the genomic oestrogenic pathway in mouse ovarian follicles (Huo et al., 2015). In contrast, *in vitro* studies showed that BPA has strong affinity to bind the oestrogen receptors thereby altering oestrogen-dependent gene expression (Lecomte*et al.,* 2017). Another report suggested that BPA has oestrogenic activity and binds to α- and β-oestrogen receptors in female humans (Caserta *et al.,* 2014).

These are cellular receptors activated by the hormone oestrogen.Similar to androgen receptors, ER translocate to the nucleus and bind to DNA to regulate gene activities i.e. a transcription factor. The oestrogen receptors assayed for in this study showed no significant deviation in expression in animals exposed at adolescent stage compared to control animals. Oestrogen receptor is most expressed in melatonin group among animals exposed at adult stage while ERs is most expressed in animals treated with 25 mg/kg BPA only and concurrent 25 mg/kg BPA + melatonin. BPA caused over expression of ERs in animals exposed during neonatal age while ERs expression reduced compared to control in animals exposed at adolescent stage. Unregulated alterations in production and expression of oestrogen and its receptors have been implicated in breast, ovarian, endometrial and ovarian cancers(Camacho *et al.*in 2015).

5.1.9 Androgen receptor (NR3C4)

Androgen receptor is a nuclear receptor that is activated by binding with androgenic hormones i.e. testosterone and dihydrotestosterone.It also functions as a DNA binding

transcription factor that regulates gene expression (Bleachand McIlroy, 2018). In this study, AR expression in animals exposed during neonatal age increased with increasing BPA concentration and melatonin was shown to further enhance AR expressions in groups concurrently treated with BPA and melatonin. Androgen receptor is most expressed in animals treated concurrently with 50 mg/kg and melatonin. No significant alteration seen in AR expression of animals treated with BPA and melatonin at adolescent stage. Reports have revealed that the development and functionality of ovarian follicles and ovulation in females is partly dependent on AR.Hence, AR is vital for normal female cycle and fertility (Su*et al.,* 2017). Thefindings show that androgen receptors were not affected by BPA administration. However, testosterone levels were significantly increased. This is in keeping with previous study(Galloway *et al*., 2010). A blockage of androgen binding sites, alters the feedback control mechanisms. Therefore, this leads to an elevation of circulating testosterone level (Galloway et al., 2010; Lassen, Frederiksen, Jensen, Petersen, & Joensen, 2014). Another explanation for this increase in testosterone level is that BPA increase testosterone concentration by stimulating the ovaries to produce testosterone, and it also inhibits T-hydroxylase activity (Huo et al., 2015). Bisphenol-A at varying lower doses tested is noted to have an age dependent effect on both AR and ER expression in female Wistar rats as depicted in these findings.

5.1.10 Histomorphology of the ovaries

Oogenesis is a specialised process involving the growth and maturation of oocyte within the ovary, the process usually involves the interplay of ovarian steroids, genes and other proteins. Can *et al.*, in 2005 reported severe debilitating actions of BPA on the meiotic cell division machinery and oocyte maturation in laboratory animals. BPA caused delay in meiotic cell division and disruption in chromosome alignment on the meiotic spindle. They further postulated that BPA is a reproductive toxicant due to its potency in sex cells than in somatic

cells. In human studies, scientists found that there was a significant trend towards lower antral follicular count (AFC) with higher urinary concentration of BPA, suggesting that exposure to environmental levels of BPA might have an adverse impact on ovarian function in this group of women (Lowther *et al.,* 2014; Zhou *et al.,* 2017).

In this study, the histological observations in the ovary of animals exposed to varying low doses of BPA showed varying degenerative features ranging from increased number of atretic follicles, distortive shrinkage of ovarian follicles, reduced population of corpus luteum and congestion of blood vessels. Ovarian follicles are said to be atretic due to degeneration of the outer theca cells, inner granulosa cells, follicular degenerations and the oocyte itself as well as accumulation of inspissated substance within its cavity. The theca and granulosa cells play a vital role in hormone secretion and oocyte maturation and survival such that when affected by toxins, oogenesis becomes affected. The histological finding in this study is consistent with the documented literature(Moustafa and Ahmed, 2016). Another denilitating feature observed in 2003 reported formation of blood filled ovarian bursae in 6-month-old CD-1 in the ovary of BPA treated experimental animals was congestion of ovarian blood vessels of mice exposed to BPA prenatally, which was further asserted that BPA induced postnatal changes consistent with reproductive aging.

The histological finding in this study is backed up by the follicular count carried out on the photomicrographs of experimental animals. The number of primary, preantral and antral follicles reduced in animals exposed to BPA at neonatal and adolescent ages. This is likely due to the apoptotic actions of BPA or its direct actions on steroidogenesis and maturation process of affected ovarian follicles. The number of defective or unhealthy follicles were increased, though insignificantly, in experimental animals such that if BPA actions are not checked may impact the animals' fertile life span (Wang *et al.,* 2017).

Melatonin has been reported by numerous scientists to possess protective efficacies in body tissues and organs against toxicity (Eid *et al.,* 2015). In this study, melatonin was unable to revert or mitigate the debilitating actions of BPA on the ovarian histology of exposed animals due to increase in defective follicular count and toxic features observed in the photomicrographs in animals treated concurrently with BPA and melatonin. These can be attributed to melatonin being adminstered at an ineffective dose against BPA actions or melatonin synergistically with BPA exerted toxic effects on the ovarian cyto-architecture and follicle counts. Also, there were traces of follicular atresia and reduced antral follicles compared to control in animals treated with melatonin only which may further indicate that melatonin, at dose tested, lacked ovo-protective properties or demonstrated harmful effects to the ovary and its follicles in this study. Studies have demonstrated that melatonin does not improve maturation rate in oocytes from sheep ovaries (Abecia*et al*., 2008).Cristina *et al*. in 2012 also did not find any significant difference in follicle numbers between controls and group treated with melatonin.(Cristina et al., 2012)(Cristina et al., 2012) This suggest that melatonin does not necessarily affect the follicular counts, as also documented in this study. These findings negate the report of (Aranega and Boulaiz, 2005) which suggested that melatonin may have positive effects on rat ovary and follicle count damaged by neonatal exposure to BPA .

Findings showed that exposure to varying low dose BPA altered the proliferation and maturation of the ovarian follicles and these effects were serious even at low doses as observable in the ovarian cytoarchitecture and histomorphometry of the ovary. Thus, one can infer that actions of BPA may have non-monotonic dose effects i.e. BPA doses below the widely accepted safety reference dose of 50 mg/kg can also cause serious impairment in body tissues of exposed subjects. This therefore shows that early exposure to BPA causes histological alternations in ovary, disrupts folliculogenesis resulting in degenerative changes

of ovarian follicles, however, melatonin administration is not shown to have obvious histological reparative effects on these follicular counts.

5.2 Summary

Bisphenol-A is a type of endocrine disrupting chemical, which is a synthetic chemical that is primarily used as a monomer in the production of polycarbonate plastics and epoxy resins. These are generally used for production of many consumables and equipments of daily use such as drinking bottles, food coolers, dental sealants, etc.

Infertility is the inability to conceive within a year, with hormonal imbalances being a major contributory factor to infertility. It is associated with enormous psychosocial stress, especially in Africa where much emphasy is placed on procreation and stigmatisation of infertile people.

Endocrine disrupting chemicals are exogenous agents that interfere with the actions of natural blood hormones . Bisphenol-A being an endocrine disruptor, is associated with distortion of normal hormonal milieu, and may contribute to unexplained infertility. Therefore, this study proposed an hypothesis that melatonin will not be effective against possible BPAs toxicity on some reproductive markers of the HPO axis in Wistar rats. The aim of the study was to evaluate the effects of BPA at even lower doses compared to the recommended LOAEL in animal studies.

Eighty fourwistar rats were divided into two main groups A and B, then each further subdivided into seven groups. They were administered various doses of BPA and melatonin at birth (Group A) and adolescent period (Group B), then were sacrificed at 120 ± 4days during which blood samples and tissues (ovary, hypothalamus and pituitary gland) were excised for reproductive hormonal, biochemical, histological and genetic studies.

The results show alterations in the hormonal profile of BPA and melatonin exposed Wistar rats. The biochemical analysis also showed marked elevated and reduced antioxidants compared to the controls, while there were distortions in the ovarian cytoarchitecture as well as overexpression of hypothalamic and pituitary genes that regulate reproduction.

It is concluded that exposure to bisphenol-A is associated with reproductive imbalances at both neonatal and adolescent periods in Wistar rats. Melatonin was also associated with imbalances in these assessed parameters and was not shown to possess ameliorative effects in this regard. However, melatonin showed some regenerative roles in regards to oxidative stress. It is recommended that BPA should be used with caution in order to mitigate its contribution to reproductive distortion.

5.3 Conclusion

The endocrine disruptor bisphenol A (BPA) at low doses tested, affected the structural integrity, induced oxidative stress, altered steroidogenic activities and impacted negatively on gene and receptor expression in the brain and the ovary of female rats exposed to it during neonatal and adolescent stages of development. The actions of 25 mg/kg BPA actions on female rats were similar to those of the benchmark low BPA dose (50 mg/kg). It could be said that BPA exposure even at lower dose than the benchmark 50 mg/kg is repro-toxic; it also has a potential long-term effect irrespective of age/period, dose and duration of exposure. Melatonin at dose administered however did not significantly improve the adverse reproductive effects induced by BPA, however its antioxidant effects gives some promising results against BPA induced toxicities.

5.4 Contributions to Knowledge

This study has demonstrated that exposure to bisphenol-A

1. distorts the serum reproductive hormonal profile;
2. induces marked oxidative stress in the ovaries;
3. causes overexpression of genes that regulate reproduction in the hypothalamus and pituitary glands; and
4. distorts the ovarian histoarchitecture; and
5. is associated with some reversal effects by melatonin on the BPA induced oxidative stress.

All these ultimately will affect reproductive ability. This can be one of the causes of unexplained infertility in females.

5.5 Limitations

Unavailability of needed laboratory equipment and lackof research grant from Nigerian government and other grant organisations are the major limitations I struggled with during the course of this research work. Tissue samples for polymerase chain reaction (PCR) to quantify genetic expression in the experimental animals had to be processed at a laboratory in Italy. There was no such facility and equipment available in Nigeria where the study could be conducted. A major limitation was to have carried out electron microscopic study on the ovary and pituitary tissues of BPA and melatonin treated animals to check if there were ultrastructural alterations in the tissues, which I was unable to actualise due to limited laboratory equipment and funding.

5.6 Recommendations

- Policies banning or regulating the usage of BPA in Nigeria should be publicized, to protect the Nigerian populace from the scourge of Bisphenol-A.There is need for policies that would regulate the production and marketing of BPA products to protect unsuspecting victims from becoming subfertileas evidences including findings from this study has implicated BPA to be reprotoxic.
- There is need for researches to come up with alternatives to be utilised instead of BPA in domestic products. Bisphenol-S (BPS), a substitute for BPA that has been adopted in most countries today is also being thought to be as harmful, if not potentially more dangerous than BPA. There is need to explore other materials than can be used instead of BPA and BPS.

5.7 Suggestions for Further Studies

1. Further studies regarding the possible ameliorative effects of BPA induced toxicity should be done

2. Alternatives to bisphenol-A use should also be researched.

3. In addition, further studies are encouraged to elucidate the BPA induced ultrastructural alterations of the HPG-axis in both sexes using electron microscopy.

REFERENCES

Abdalla, H., & Thum, M. Y. (2006). Repeated testing of basal FSH levels has no predictive value for IVF outcome in women with elevated basal FSH. *Human Reproduction*, *21*(1), 171–174. https://doi.org/10.1093/humrep/dei288

Abdel-Wahab, W. M., & Abdel-Wahab, W. M. (2014). Thymoquinone attenuates toxicity and oxidative stress induced by bisphenol a in liver of male rats. *Pakistan Journal of Biological Sciences*, 17(11) 1152-1160. https://doi.org/10.3923/pjbs.2014.1152.1160

Abecia, J. A., Forcada, F., & Casao, A. (2008). Effect of exogenous melatonin on the ovary , the embryo and the establishment of pregnancy in sAbecia, J. A., Forcada, F., & Casao, A. (2008). Effect of exogenous melatonin on the ovary, the embryo and the establishment of pregnancy in sheep.*Animal*, 399–404. https://doi.org/10.1017/S1751731107001383

Aboul Ezz, H. S., Khadrawy, Y. A., & Mourad, I. M. (2015). The effect of bisphenol A on some oxidative stress parameters and acetylcholinesterase activity in the heart of male albino rats. *Cytotechnology*, *67*(1), 145–155. https://doi.org/10.1007/s10616-013-9672-1

Abreu, A. P. & K. U. B. (2017). Pubertal development and regulation. HHS Public Access. *Lancet Diabetes Endocrinology*, *4*(3), 254–264. https://doi.org/10.1016/S2213-8587(15)00418-0.Pubertal

Acaroz, U., Ince, S., Arslan-Acaroz, D., Gurler, Z., Demirel, H. H., Kucukkurt, I., … Zhu, K. (2019). Bisphenol-A induced oxidative stress, inflammatory gene expression, and metabolic and histopathological changes in male Wistar albino rats: Protective role of boron. *Toxicology Research*, *8*(2), 262–269. https://doi.org/10.1039/c8tx00312b

Acconcia, F., Pallottini, V., & Marino, M. (2015). Molecular mechanisms of action of BPA, *Dose Response*,1–9. https://doi.org/10.1177/1559325815610582

Adewale, H. B., Jefferson, W. N., Newbold, R. R., & Patisaul, H. B. (2009). Neonatal Bisphenol-A Exposure Alters Rat Reproductive Development and Ovarian Morphology Without Impairing Activation of Gonadotropin-Releasing Hormone Neurons1. *Biology of Reproduction*, *81*(4), 690–699. https://doi.org/10.1095/biolreprod.109.078261

Agarwal, A., Virk, G., Ong, C., & du Plessis, S. S. (2014). Effect of Oxidative Stress on Male Reproduction. *The World Journal of Men's Health*, *32*(1), 1.

https://doi.org/10.5534/wjmh.2014.32.1.1

Ajayi, A. F., & Akhigbe, R. E. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertility Research and Practice*, *6*(1), 1–15. https://doi.org/10.1186/s40738-020-00074-3

Al-Asmakh, M. (2007). Reproductive functions of progesterone. *Middle East Fertility Society Journal*, *12*(3), 147–152.

Allocati, N., Masulli, M., Di Ilio, C., & Federici, L. (2018). Glutathione transferases: Substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. *Oncogenesis*, *7*(1). https://doi.org/10.1038/s41389-017-0025-3

Amraoui, W., Adjabi, N., Bououza, F., Boumendjel, M., Taibi, F., Boumendjel, A., … Messarah, M. (2018). Modulatory role of selenium and vitamin E, natural antioxidants, against bisphenol A-induced oxidative stress in wistar albinos rats. *Toxicological Research*, *34*(3), 231–239. https://doi.org/10.5487/TR.2018.34.3.231

Andersen, C. Y. (2017). Inhibin-B secretion and FSH isoform distribution may play an integral part of follicular selection in the natural menstrual cycle. *Molecular Human Reproduction*,*23*(1), 16–24. https://doi.org/10.1093/molehr/gaw070

Andrabi, S. S., Parvez, S., & Tabassum, H. (2015). Melatonin and ischemic stroke: Mechanistic roles and action. *Advances in Pharmacological Sciences*, *2015*. https://doi.org/10.1155/2015/384750

Andrews, C. D., Foster, R. G., Alexander, I., Vasudevan, S., Downes, S. M., Heneghan, C., & Plüddemann, A. (2019). Sleep-wake disturbance related to ocular disease: A systematic review of phase-shifting pharmaceutical therapies. *Translational Vision Science and Technology*, *8*(3). https://doi.org/10.1167/tvst.8.3.49

Anet, A., Olakkaran, S., Kizhakke Purayil, A., & Hunasanahally Puttaswamygowda, G. (2019). Bisphenol A induced oxidative stress mediated genotoxicity in Drosophila melanogaster. *Journal of Hazardous Materials*, (March), 42–53. https://doi.org/10.1016/j.jhazmat.2018.07.050

Anjum, S., Rahman, S., Kaur, M., Ahmad, F., Rashid, H., Ahmad, R., & Raisuddin, S. (2011). Melatonin ameliorates bisphenol A-induced biochemical toxicity in testicular mitochondria of mouse. *Food and Chemical Toxicology*, *49*(11), 2849–2854.

https://doi.org/10.1016/j.fct.2011.07.062

Aranega, A., & Boulaiz, H. (2005). The effect of melatonin on oxidative stress and prevention in primordial follicles loss via activation of mTOR pathway in the rat ovary. *Cellular and Molecular Biology*, *51*(1), 1.

Aritonang, T. R., Rahayu, S., Sirait, L. I., Karo, M. B., Simanjuntak, T. P., Natzir, R., … Kamelia, E. (2017). The role of FSH, LH, estradiol and progesterone hormone on estrus cycle of female rats. *International Journal of Sciences: Basic and Applied Research (IJSBAR)*, *35*(1), 92–100.

Avci, B., Bahadir, A., Tuncel, O. K., & Bilgici, B. (2016). Influence of α-tocopherol and α-lipoic acid on bisphenol-A-induced oxidative damage in liver and ovarian tissue of rats. *Toxicology and Industrial Health*, *32*(8), 1381–1390. https://doi.org/10.1177/0748233714563433

Balakrishnan, B., Henare, K., Thorstensen, E. B., Ponnampalam, A. P., & Mitchell, M. D. (2010). Basic Science: Obstetricstransfer of bisphenol A across the human placenta.*International Journal of Science and Research*,*202*(4), 393.e1-393.e7. https://doi.org/10.1016/j.ajog.2010.01.025

Balakrishnan, N., & Sendhilvadivu, M. (2018). Vitamin E modulates the oxidant-antioxidant imbalance of BPA induced oxidative stress in albino rats. *American Journal of Obstetrics and Gynaecology*,*7*(1), 900–906. https://doi.org/10.21275/10011803

Barbotin, A.-L., Peigné, M., Malone, S. A., & Giacobini, P. (2019). Emerging Roles of Anti-Müllerian Hormone in Hypothalamic-Pituitary Function. *Neuroendocrinology*, *109*(3), 218–229. https://doi.org/10.1159/000500689

Barnett, D. K., Bunnell, T. M., Millar, R. P., & Abbott, D. H. (2006). Gonadotropin-releasing hormone II stimulates female sexual behavior in marmoset monkeys. *Endocrinology*, 147(1), 615–623. https://doi.org/10.1210/en.2005-0662

Becedas, L., Lundgren, B., & Pierre, J. W. D. E. (1998). Characterization of the UDP-glucuronosyltransferase isoenzyme expressed in rat ovary and its regulation by gonadotropins. *Biochemical and Toxicology Journal*, *55*, 51–55.

Bednarek-Tupikowska, G., Tworowska-Bardzińska, U., & Tupikowski, K. (2008). Effects of estrogen and estrogen-progesteron on serum nitric oxide metabolite concentrations in

post-menopausal women. *Journal of Endocrinological Investigation*, *31*(10), 877–881. https://doi.org/10.1007/BF03346435

Bienenfeld, A., Azarchi, S., Lo Sicco, K., Marchbein, S., Shapiro, J., & Nagler, A. R. (2019). Androgens in women: Androgen-mediated skin disease and patient evaluation. *Journal of the American Academy of Dermatology*, *80*(6), 1497–1506. https://doi.org/10.1016/j.jaad.2018.08.062

Bleach, R., & McIlroy, M. (2018). The divergent function of androgen receptor in breast cancer; analysis of steroid mediators and tumor intracrinology. *Frontiers in Endocrinology*, *9*(OCT), 1–19. https://doi.org/10.3389/fendo.2018.00594

Boafo, A., Greenham, S., Alenezi, S., Robillard, R., Pajer, K., Tavakoli, P., & De Koninck, J. (2019). Could long-term administration of melatonin to prepubertal children affect timing of puberty? A clinician’s perspective. *Nature and Science of Sleep*, *Volume 11*, 1–10. https://doi.org/10.2147/nss.s181365

Book, G. E. (2011). *The national academies press*. https://doi.org/10.17226/12910

Branavan, U., Muneeswaran, K., Wijesundera, W. S. S., Senanayake, A., Chandrasekharan, N. V., & Wijeyaratne, C. N. (2019). Association of Kiss1 and GPR54 gene polymorphisms with polycystic ovary syndrome among Sri Lankan women. *BioMedical Research International*, 2019(1996). https://doi.org/10.1155/2019/6235680

Butts, S., Riethman, H., Ratcliffe, S., Shaunik, A., Coutifaris, C., & Barnhart, K. (2009). Correlation of telomere length and telomerase activity with occult ovarian insufficiency. *Journal of Clinical Endocrinology and Metabolism*, *94*(12), 4835–4843. https://doi.org/10.1210/jc.2008-2269

Camacho, L., Basavarajappa, M. S., Chang, C. W., Han, T., Kobets, T., Koturbash, I., … Delclos, K. B. (2015). Effects of oral exposure to bisphenol A on gene expression and global genomic DNA methylation in the prostate, female mammary gland, and uterus of NCTR Sprague-Dawley rats. *Food and Chemical Toxicology*, *81*, 92–103. https://doi.org/10.1016/j.fct.2015.04.009

Can, A., Semiz, O., & Cinar, O. (2005). Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. *Molecular Human Reproduction*, *11*(6), 389–396. https://doi.org/10.1093/molehr/gah179

Cao, Y., Qu, X., Ming, Z., Yao, Y., & Zhang, Y. (2018). The correlation between exposure to BPA and the decrease of the ovarian reserve. *International Journal of Clinical and Experimental Pathology*, *11*(7), 3375–3382.

Casarini, L., & Crépieux, P. (2019). Molecular mechanisms of action of FSH. *Frontiers in Endocrinology*, *10*(MAY), 1–10. https://doi.org/10.3389/fendo.2019.00305

Caserta, D., Di Segni, N., Mallozzi, M., Giovanale, V., Mantovani, A., Marci, R., & Moscarini, M. (2014). Bisphenol a and the female reproductive tract: An overview of recent laboratory evidence and epidemiological studies. *Reproductive Biology and Endocrinology*, *12*(1), 1–10. https://doi.org/10.1186/1477-7827-12-37

Chimento, A., Sirianni, R., Casaburi, I., & Pezzi, V. (2014). Role of estrogen receptors and G protein-coupled estrogen receptor in regulation of hypothalamus – pituitary – testis axis and spermatogenesis. *Frontiers in Endocrinology*, 5(January), 1–9. https://doi.org/10.3389/fendo.2014.00001

Chouhan, S., Yadav, S. K., Prakash, J., Westfall, S., Ghosh, A., Agarwal, N. K., & Singh, S. P. (2015). Increase in the expression of inducible nitric oxide synthase on exposure to bisphenol A: A possible cause for decline in steroidogenesis in male mice. *Environmental Toxicology and Pharmacology*, *39*(1), 405–416. https://doi.org/10.1016/j.etap.2014.09.014

Combelles, C. M. H., Holick, E. A., Paolella, L. J., Walker, D. C., & Wu, Q. (2010). Profiling of superoxide dismutase isoenzymes in compartments of the developing bovine antral follicles. *Reproduction*, *139*(5), 871–881. https://doi.org/10.1530/REP-09-0390

Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal Cytology of the Laboratory Rat and Mouse:Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicologic Pathology*, *43*(6), 776–793. https://doi.org/10.1177/0192623315570339

Cortès, M., Turco, M., Llasat-Botija, M., & Carmen Llasat, M. (2018). The relationship between precipitation and insurance data for floods in a Mediterranean region (northeast Spain). *Natural Hazards and Earth System Sciences*, *18*(3), 857–868. https://doi.org/10.5194/nhess-18-857-2018

Cristiana, F., Elena, A., & Nina, Z. (2014). Superoxide Dismutase: Therapeutic Targets in

SOD Related Pathology. *Health*, 06(10), 975–988. https://doi.org/10.4236/health.2014.610123

Cristina, C., Fernando, L., Simo, R. S., Chada, E., Oliveira-filho, R. M., Simo, M. D. J., & Jr, M. S. (2012). Effects of melatonin on ovarian follicles. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, https://doi.org/10.1016/j.ejogrb.2012.10.006

Di Donato, M., Cernera, G., Giovannelli, P., Galasso, G., Bilancio, A., Migliaccio, A., & Castoria, G. (2017). Recent advances on bisphenol-A and endocrine disruptor effects on human prostate cancer. *Molecular and Cellular Endocrinology*, *457*, 35–42. https://doi.org/10.1016/j.mce.2017.02.045

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Gore, A. C. (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293–342. https://doi.org/10.1210/er.2009-0002

Dose, H. E., & Daily, T. (2015). How much BPA does a typical person take in.*European Food Safety Authority*, https://doi.org/10.2903/j.efsa.2015.3978/epdf

Duntas, L. H., & Brenta, G. (2018). A renewed focus on the association between thyroid hormones and lipid metabolism. *Frontiers in Endocrinology*, *9*(SEP). https://doi.org/10.3389/fendo.2018.00511

Eckstrum, K. S., Edwards, W., Banerjee, A., Wang, W., Flaws, J. A., Katzenellenbogen, J. A., Raetzman, L. T. (2017). Effects of exposure to the endocrine disrupting chemical bisphenol A during critical windows of murine pituitary development. *Endocrinology*, https://doi.org/10.1210/en.2017-00565

Eid, J. I., Eissa, S. M., & El-Ghor, A. A. (2015). Bisphenol A induces oxidative stress and DNA damage in hepatic tissue of female rat offspring. *The Journal of Basic & Applied Zoology*, *71*(April), 10–19. https://doi.org/10.1016/j.jobaz.2015.01.006

Elhussein, O. G., Ahmed, M. A., Suliman, S. O., Yahya, I., & Adam, I. (2019). Epidemiology of infertility and characteristics of infertile couples requesting assisted reproduction in a low- resource setting in Africa , Sudan, *1*, 7–11.

Eller, L. S., Rivero-Mendez, M., Voss, J., Chen, W. T., Chaiphibalsarisdi, P., Iipinge, S., … Brion, J. M. (2014). Depressive symptoms, self-esteem, HIV symptom management

self-efficacy and self-compassion in people living with HIV. *AIDS Care - Psychological and Socio-Medical Aspects of AIDS/HIV*, *26*(7), 795–803. https://doi.org/10.1080/09540121.2013.841842

Elwakeel, S. H. B., & El-monem, D. D. A. (2019). Ameliorative effect of melatonin and quercetin against bisphenol A induced reproductive toxicity in male albino mice. *Ciencia and Vitivinicultural Technique*, 33(n. 11, 2018).

Environmental Protection Agency. (2015). *Environmental Protection Agency Annual Report and Accounts | 2015*.

Erland, L. A. E., & Saxena, P. K. (2017). Melatonin natural health products and supplements: Presence of serotonin and significant variability of melatonin content. *Journal of Clinical Sleep Medicine*, *13*(2), 275–281. https://doi.org/10.5664/jcsm.6462

Faheem, M., Jahan, N., Khaliq, S., & Lone, K. P. (2019). Modulation of brain kisspeptin expression after bisphenol-A exposure in a teleost fish, Catla catla. *Fish Physiology and Biochemistry*, *45*(1), 33–42. https://doi.org/10.1007/s10695-018-0532-y

FAO/WHO. (2010). *Toxicological and Health Aspects of Bisphenol A Report of Joint FAO / WHO Expert Meeting*. Geneva, Switzerland.

Fernando, S., & Rombauts, L. (2014). Melatonin : shedding light on infertility ? - a review of the recent literature. *Journal of Ovarian Research*,1–14. https://doi.org/10.1186/s13048-014-0098-y

Ferracioli-Oda, E., Qawasmi, A., & Bloch, M. H. (2013). Meta-Analysis: Melatonin for the Treatment of Primary Sleep Disorders. *PLoS ONE*, *8*(5), 6–11. https://doi.org/10.1371/journal.pone.0063773

Flanagan, C. A., Manilall, A., & Flanagan, C. A. (2017). Gonadotropin releasing hormone (GnRH) receptor structure and GnRH binding. *Frontiers in Endocrinology*. 8(October), 1–14. https://doi.org/10.3389/fendo.2017.00274

Franks, S., & Hardy, K. (2018). Androgen action in the ovary. *Frontiers in Endocrinology*, *9*(AUG), 1–7. https://doi.org/10.3389/fendo.2018.00452

Galloway, T., Cipelli, R., Guralnik, J., Ferrucci, L., Bandinelli, S., & Corsi, A. M. (2010). Daily bisphenol A excretion and associations with sex hormone concentrations : Results from the InCHIANTI adult population study. *Environmental Health*

Perspectives,118(11), 1603–1609. https://doi.org/10.1289/ehp.1002367

Gao, H., Yang, B. J., Li, N., Feng, L. M., Shi, X. Y., Zhao, W. H., & Liu, S. J. (2015). Bisphenol A and hormone-associated cancers: Current progress and perspectives. *Medicine (United States)*, *94*(1), e211. https://doi.org/10.1097/MD.0000000000000211

Gillies, G. E., & Mcarthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women : A Case for sex-specific medicines. *Pharmacological Reviews*,62(2), 155–198. https://doi.org/10.1124/pr.109.002071.155

Glanowska, K. M., Burger, L. L., & Moenter, S. M. (2014). Development of gonadotropin-releasing hormone secretion and pituitary response. *Journal of Neuroscience*, *34*(45), 15060–15069. https://doi.org/10.1523/JNEUROSCI.2200-14.2014

Gonzales, G., & Kretser, D. M. De. (1989). Effect of testosterone on serum immunoactive inhibin concentrations in intact and hypophysectomized male rats.*Journal of Reproduction and Fertility*, 87, 795-801.

Gonzalez, J. S., Batchelder, A. W., Psaros, C., & Safren, S. A. (2011). Depression and HIV/AIDS treatment nonadherence: A review and meta-analysis. *Journal of Acquired Immune Deficiency Syndromes*, *58*(2), 181–187. https://doi.org/10.1097/QAI.0B013E31822D490A

Gore, A. C., Crews, D., Doan, L. L., & Merrill, M. La. (2014). Introduction to endocrine disrupting chemicals (EDCs) A guide for public interest organizations and policy makers (December).

Grasselli, F., Baratta, L., Baioni, L., & Bussolati, S. (2010). Bisphenol A disrupts granulosa cell function. *Domstic Animal Endocrinology*, *39*(1), 34–39. https://doi.org/10.1016/j.domaniend.2010.01.004

Grinspon, R. P., Gottlieb, S., Bedecarrás, P., & Rey, R. A. (2018). Anti-Müllerian hormone and testicular function in prepubertal boys with cryptorchidism. *Frontiers in Endocrinology*, *9*(APR). https://doi.org/10.3389/fendo.2018.00182

Gustavo, L., Chuffa, D. A., Lupi-júnior, L. A., Balandis, A., Paulo, J., Amorim, D. A., … Seiva, F. (2017). The role of sex hormones and steroid receptors on female reproductive cancers. *Steroids*, 118, 93–108. https://doi.org/10.1016/j.steroids.2016.12.011

Halder, A., Jain, M., & Kumar, P. (2014). Primary testicular failure: Genotype phenotype

correlation of 140 cases. *Andrology*, 2(Suppl 1)1

Haroun, M. R., Zamzam, I. S., Metwally, E. S., & El-shafey, R. S. (2016). Effect of Vitamin C on bisphenol A induced hepato and nephrotoxicity in albino rats. *The Egyptian Journal of Forensic Sciences and Apllied Toxicology*,*16*(2).

Hassan, Z. K., Elobeid, M. A., Virk, P., Omer, S. A., Elamin, M., Daghestani, M. H., & Alolayan, E. M. (2012). Bisphenol a induces hepatotoxicity through oxidative stress in rat model. *Oxidative Medicine and Cellular Longevity*, *2012*. https://doi.org/10.1155/2012/194829

Health, W., & Metz, C. M. (2016). Bisphenol A: Understanding the Controversy. *Workplace Health and Safety*. https://doi.org/10.1177/2165079915623790

Hengstler, J. G., Foth, H., Gebel, T., Kramer, P., Lilienblum, W., Schweinfurth, H., & Völkel, W. (2011). Critical evaluation of key evidence on the human health hazards of exposure to bisphenol A. *Critical Reviews for Toxicology*,41(January), 263–291. https://doi.org/10.3109/10408444.2011.558487

Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E., & Svendsen, C. (2017). Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. *Science of the Total Environment*, *586*, 127–141. https://doi.org/10.1016/j.scitotenv.2017.01.190

Hoyer, P. B., & Keating, A. F. (2014). Xenobiotic effects in the ovary : temporary versus permanent infertility. *Expert Opin. Drug Metab. Toxicol*, 511–523.

Huo, X., Chen, D., He, Y., Zhu, W., Zhou, W., & Zhang, J. (2015). Bisphenol-a and female infertility: A possible role of gene-environment interactions. *International Journal of Environmental Research and Public Health*, *12*(9), 11101–11116. https://doi.org/10.3390/ijerph120911101

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287–293. https://doi.org/10.1016/j.ajme.2017.09.001

Ishido, M., & Suzuki, J. (2010). Inhibition by rotenone of mesencephalic neural stem-cell migration in a neurosphere assay in vitro. *Toxicology in Vitro*, *24*(2), 552–557.

https://doi.org/10.1016/j.tiv.2009.11.005

Jiwakanon, J., Persson, E., Kaeoket, K., & Dalin, A. M. (2005). The sow endosalpinx at different stages of the oestrous cycle and at anoestrus: Studies on morphological changes and infiltration by cells of the immune system. *Reproduction in Domestic Animals*, 40(1), 28–39. https://doi.org/10.1111/j.1439-0531.2004.00550.x

Johnson, S. A., Ellersieck, M. R., & Rosenfeld, C. S. (2018). Hypothalamic gene expression changes in F1 California mice (Peromyscus californicus) parents developmentally exposed to bisphenol A or ethinyl estradiol. *Heliyon*, 4(6), e00672. https://doi.org/10.1016/j.heliyon.2018.e00672

Karasek, M., Winczyk, K., & Diseases, M. (2006). Mela tonin in humans 1. *Pharmacology*, 19–39.

Kauff, A. S., & Editors, J. T. S. (2013). Kisspeptin Signaling in Reproductive Biology, *784*, 113–131. https://doi.org/10.1007/978-1-4614-6199-9

Kereilwe, O., & Kadokawa, H. (2020). Anti-Müllerian hormone and its receptor are detected in most gonadotropin-releasing-hormone cell bodies and fibers in heifer brains. *Domestic Animal Endocrinology*, *72*, 106432. https://doi.org/10.1016/j.domaniend.2019.106432

Khan, S., Beigh, S., Chaudhari, B. P., Sharma, S., Aliul, S., Abdi, H., … Raisuddin, S. (2015). Mitochondrial dysfunction induced by bisphenol A is a factor of its hepatotoxicity in rats. *Toxicology*, 1–13. https://doi.org/10.1002/tox

Kim, D. J. (2008). Institutional Knowledge at Singapore Management University A trust-based consumer decision-making model in electronic commerce : The role of trust , perceived risk , and their antecedents A trust-based consumer decision-making model in electronic commerce. *Journal of Decision Support Systems.* 544–564. https://doi.org/10.1016/j.dss.2007.07.001

Ko, E. Y., Jr, S. S., Agarwal, A., & Ph, D. (2014). Male infertility testing : reactive oxygen species and antioxidant capacity. *Fertility and Sterility*, *102*(6), 1518–1527. https://doi.org/10.1016/j.fertnstert.2014.10.020

Kuhl, H. (2005). Pharmacology of estrogens and progestogens : influence of different routes of administration. *Climacteric,*8(Suppl 1), 3–63.

https://doi.org/10.1080/13697130500148875

Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., Van Der Saag, P. T., … Gustafsson, J. Å. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology*, *139*(10), 4252–4263. https://doi.org/10.1210/endo.139.10.6216

Kurian, J. R., Keen, K. L., Kenealy, B. P., Garcia, J. P., Hedman, C. J., & Terasawa, E. (2015). Acute influences of bisphenol A exposure on hypothalamic release of gonadotropin-releasing hormone and kisspeptin in female rhesus monkeys. *Endocrinology*, *156*(7), 2563–2570. https://doi.org/10.1210/en.2014-1634

Kurutas, E. B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. *Nutrition Journal*, *15*(1), 1–22. https://doi.org/10.1186/s12937-016-0186-5

Laird, M., Thomson, K., Fenwick, M., Mora, J., Franks, S., & Hardy, K. (2017). Androgen Stimulates Growth of Mouse Preantral. *Endocrinology*,158(April), 920–935. https://doi.org/10.1210/en.2016-1538

Lassen, T. H., Frederiksen, H., Jensen, T. K., Petersen, J. H., & Joensen, U. N. (2014). Urinary Bisphenol A Levels in Young Men : Association with Reproductive. *Environmental Health Perspective*,478(5), 478–484.

Lathi, R. B., Liebert, C. A., Brookfield, K. F., Taylor, J. A., Vom Saal, F. S., Fujimoto, V. Y., & Baker, V. L. (2014). Conjugated bisphenol A in maternal serum in relation to miscarriage risk. *Fertility and Sterility*, 102(1), 123–128. https://doi.org/10.1016/j.fertnstert.2014.03.024

Lecomte, S., Demay, F., Ferri, F., & Pakdel, F. (2017). Phytochemicals Targeting Estrogen Receptors : Beneficial Rather Than Adverse Effects ? *International Journal of Molecular Sciences*, 1–19. https://doi.org/10.3390/ijms18071381

Lee, B. E., Park, H., Hong, Y. C., Ha, M., Kim, Y., Chang, N., Ha, E. H. (2014). Prenatal bisphenol A and birth outcomes: MOCEH (Mothers and Children's Environmental Health) study. *International Journal of Hygiene and Environmental Health*, 217(2–3), 328–334. https://doi.org/10.1016/j.ijheh.2013.07.005

Lee, D. (2018). Review article evidence of the possible harm of endocrine disrupting

chemicals in humans : Ongoing debates and key issues. *Endocrinology Metabolism*, 44–52.

Leem, Y., Oh, S., Kang, H., Kim, J., & Yoon, J. (2016). BPA-Toxicity via superoxide anion overload and a deficit in b -Catenin signaling in human bone mesenchymal stem cells.*Environmental Toxicology*, 1–9. https://doi.org/10.1002/tox

Li, L., Wang, Q., Zhang, Y., Niu, Y., Yao, X., & Liu, H. (2015). The molecular mechanism of bisphenol A (BPA) as an endocrine disruptor by interacting with nuclear receptors: Insights from molecular dynamics (MD) simulations. *PLoS ONE*, *10*(3), 1–18. https://doi.org/10.1371/journal.pone.0120330

Liu, Y. Q., Yuan, L. M., Gao, Z. Z., Xiao, Y. S., Sun, H. Y., Yu, L. S., & Zeng, S. (2016). Dimerization of human uridine diphosphate glucuronosyltransferase allozymes 1A1 and 1A9 alters their quercetin glucuronidation activities. *Scientific Reports*, *6*(February), 1–13. https://doi.org/10.1038/srep23763

Lowther, K., Selman, L., Harding, R., & Higginson, I. J. (2014). Experience of persistent psychological symptoms and perceived stigma among people with HIV on antiretroviral therapy (ART): A systematic review. *International Journal of Nursing Studies*, *51*(8), 1171–1189. https://doi.org/10.1016/j.ijnurstu.2014.01.015

Maggi, R., Cariboni, A. M., Marelli, M. M., Marzagalli, M., Moretti, R. M., Andre, V., & Limonta, P. (2016). GnRH and GnRH receptors in the pathophysiology of the human female reproductive system. *Human Reproduction Update*, 22(3), 358–381. https://doi.org/10.1093/humupd/dmv059

Manibusan, M. K., & Touart, L. W. (2017). Critical Reviews in Toxicology A comprehensive review of regulatory test methods for endocrine adverse health effects. *Critical Reviews in Toxicology*, *0*(0), 440–488. https://doi.org/10.1080/10408444.2016.1272095

Marca, A. La, Emilia, R., Modena, C. E., Fauser, B. C. J. M., & Macklon, N. (2009). ¨ llerian hormone (AMH): what Anti-Mu do we still need to know ?*Human Reproduction*, (July 2014). https://doi.org/10.1093/humrep/dep210

Marcondes, F. K., & Bianchi, F. (2002). Determination of the estrous cycle phases of rats: Some helpful considerations.*Brazillian Journal of Biology*, (June 2014). https://doi.org/10.1590/S1519-69842002000400008

Maria, E., Costa, F., Spritzer, P. M., Hohl, A., & Bachega, T. A. S. S. (2014). Effects of endocrine disruptors in the development of the female reproductive tract. *Endocrinology Metabolism*, 153–161.

Mazerbourg, S., Monget, P., Mazerbourg, S., & Monget, P. (2018). Insulin-like growth factor binding proteins and IGFBP proteases: A dynamic system regulating the ovarian folliculogenesis.*Frontiers in Endocrinology*, 9(March), 1–10. https://doi.org/10.3389/fendo.2018.00134

McCallum, J. E., Mackenzie, A. E., Divorty, N., Clarke, C., Delles, C., Milligan, G., & Nicklin, S. A. (2016). G-Protein-Coupled Receptor 35 Mediates Human Saphenous Vein Vascular Smooth Muscle Cell Migration and Endothelial Cell Proliferation. *Journal of Vascular Research*, *52*(6), 383–395. https://doi.org/10.1159/000444754

Merzenich, H., Zeeb, H., & Blettner, M. (2010). Decreasing sperm quality : a global problem ? *BMC Public Health*. doi: 10.1186/1471-2458-10-24

Miao, M., Yuan, W., Yang, F., Liang, H., Zhou, Z., Li, R., … Li, D. K. (2015). Associations between bisphenol a exposure and reproductive hormones among female workers. *International Journal of Environmental Research and Public Health*, *12*(10), 13240–13250. https://doi.org/10.3390/ijerph121013240

Moini, A., Pirjani, R., Rabiei, M., Nurzadeh, M., Sepidarkish, M., Hosseini, R., & Hosseini, L. (2019). Can delivery mode influence future ovarian reserve? Anti-Mullerian hormone levels and antral follicle count following cesarean section: A prospective cohort study. *Journal of Ovarian Research*, *12*(1), 1–7. https://doi.org/10.1186/s13048-019-0551-z

Monti, M. (2013). Bisphenol A, (August), 1–6.http://dx.doi.org/10.6084/m9.figshare.5414593

Moustafa, G. G., & Ahmed, A. A. M. (2016). Impact of prenatal and postnatal exposure to bisphenol A on female rats in a two generational study: Genotoxic and immunohistochemical implications. *Toxicology Reports*, *3*, 685–695. https://doi.org/10.1016/j.toxrep.2016.08.008

Moyosore Salihu Ajao, O. O. A. O. I. et al. (2011). Melatonin potentiates cells proliferation in the dental gyrus following ischemic brain injury in adult rats.*Journal of Animal and Veterinary Advances*, 11(9) 1633-1638.

Mulgund, A., Doshi, S., & Agarwal, A. (2015). The Role of Oxidative Stress in Endometriosis. *Handbook of Fertility: Nutrition, Diet, Lifestyle and Reproductive Health*, 273–281. https://doi.org/10.1016/B978-0-12-800872-0.00025-1

Mulla, A. Al, Fazari, A. B. E., Elkhouly, M., & Moghaddam, N. (2018). Role of Antioxidants in Female Fertility. *Open Journal of Obstetrics and Gynecology*, *08*(02), 85–91. https://doi.org/10.4236/ojog.2018.82011

N'Tumba-Byn, T., Moison, D., Lacroix, M., Lecureuil, C., Lesage, L., Prud'homme, S. M., … Habert, R. (2012). Differential Effects of Bisphenol A and Diethylstilbestrol on Human, Rat and Mouse Fetal Leydig Cell Function. *PLoS ONE*, *7*(12), 4–13. https://doi.org/10.1371/journal.pone.0051579

Naseem, M., & Parvez, S. (2014). Role of melatonin in traumatic brain injury and spinal cord injury. *Scientific World Journal*, *2014*. https://doi.org/10.1155/2014/586270

Naufel, M. F., Ribeiro, E. B., Tufik, S., & Hachul, H. (2019). Influence of Dietary Sources of Melatonin on Sleep Quality : A Review. *Journal of Food Science*, 1–9. https://doi.org/10.1111/1750-3841.14952

Noblanc, A., Kocer, A., Chabory, E., Vernet, P., Saez, F., Cadet, R.,Drevet, J. R. (2011). Glutathione peroxidases at work on epididymal spermatozoa: An example of the dual effect of reactive oxygen species on mammalian male fertilizing ability. *Journal of Andrology*, *32*(6), 641–650. https://doi.org/10.2164/jandrol.110.012823

Olukole, S. G., Ola-davies, E. O., Lanipekun, D. O., & Oke, B. O. (2020). Chronic exposure of adult male Wistar rats to bisphenol A causes testicular oxidative stress : Role of gallic acid. *Endocrinology Regulations*,54(1), 14–21. https://doi.org/10.2478/enr-2020-0003

Othman, A. I., Edrees, G. M., El-missiry, M. A., & Ali, D. A. (2014). Melatonin controlled apoptosis and protected the testes and sperm quality against bisphenol A-induced oxidative toxicity, *Toxicology and Industrial Health*,32(9):1537. 49.https://doi.org/10.1177/0748233714561286

Ouzzine, M., Gulberti, S., Ramalanjaona, N., Magdalou, J., & Fournel-Gigleux, S. (2014). The UDP-glucuronosyltransferases of the blood-brain barrier: Their role in drug metabolism and detoxication. *Frontiers in Cellular Neuroscience*, *8*(October), 1–12. https://doi.org/10.3389/fncel.2014.00349

Parrill, A. L., & Bautista, D. L. (2011). GPCR Conformations : Implications for rational drug design. *Pharmaceuticals*, 7–43. https://doi.org/10.3390/ph4010007

Pelosi, E., Forabosco, A., & Schlessinger, D. (2015). Genetics of the ovarian reserve. *Frontiers in Genetics*, *6*(OCT), 1–20. https://doi.org/10.3389/fgene.2015.00308

Peretz, J., & Flaws, J. A. (2013). Bisphenol A down-regulates rate-limiting Cyp11a1 to acutely inhibit steroidogenesis in cultured mouse antral follicles. *Toxicology and Applied Pharmacology*, *271*(2), 249–256. https://doi.org/10.1016/j.taap.2013.04.028

Peretz, J., Vrooman, L., Ricke, W. A., Hunt, P. A., Ehrlich, S., Hauser, R., Flaws, J. A. (2014). Bisphenol A and reproductive health: Update of experimental and human evidence, 2007-2013. *Environmental Health Perspectives*, *122*(8), 775–786. https://doi.org/10.1289/ehp.1307728

Peretz, J., Vrooman, L., Ricke, W. A., Hunt, P. A., Ehrlich, S., Hauser, R., Vandevoort, C. A. (2014). Review bisphenol A and reproductive health : Update of experimental and human. *Environmental Health Perspective*,122(8), 2007–2013.

Poulsen, L. C., Englund, A. L. M., Andersen, A. S., Bøtkjær, J. A., Mamsen, L. S., Damdimopoulou, P.,Yding Andersen, C. (2020). Follicular hormone dynamics during the midcycle surge of gonadotropins in women undergoing fertility treatment. *Molecular Human Reproduction*, *26*(4), 256–268. https://doi.org/10.1093/molehr/gaaa013

Preethi, S., Sandhya, K., Lebonah, D. E., Prasad, C. V., Sreedevi, B., Chandrasekhar, K., & Kumari, J. P. (2014). Toxicity of bisphenol a on humans : a review. *Inernational Letters of Natural Sciences*,27, 32–46. https://doi.org/10.18052/www.scipress.com/ILNS.27.32

Priyanka Sanjay Deshpande, A. S. G. (2019). Causes and prevalence of factors causing infertility in a public health facility. *Journal of Human Reproductive Sciences*, 287–293. https://doi.org/10.4103/jhrs.JHRS

Raju, G. A. R., Chavan, R., Deenadayal, M., Gunasheela, D., Gutgutia, R., Haripriya, G., Patki, A. S. (2013). Luteinizing hormone and follicle stimulating hormone synergy: A review of role in controlled ovarian hyper-stimulation. *Journal of Human Reproductive Sciences*, *6*(4), 227–234. https://doi.org/10.4103/0974-1208.126285

Rebuli, M. E., Cao, J., Sluzas, E., Barry Delclos, K., Camacho, L., Lewis, S. M., … Patisaul, H. B. (2014). Investigation of the effects of subchronic low dose oral exposure to

bisphenol a (BPA) and ethinyl estradiol (EE) on estrogen receptor expression in the juvenile and adult female rat hypothalamus. *Toxicological Sciences*, *140*(1), 190–203. https://doi.org/10.1093/toxsci/kfu074

Reiter, R. J., Mayo, J. C., Tan, D. X., Sainz, R. M., Alatorre-Jimenez, M., & Qin, L. (2016). Melatonin as an antioxidant: under promises but over delivers. *Journal of Pineal Research*, 253–278. https://doi.org/10.1111/jpi.12360

Requena, A., Cruz, M., Ruiz, F. J., & García-Velasco, J. A. (2014). Endocrine profile following stimulation with recombinant follicle stimulating hormone and luteinizing hormone versus highly purified human menopausal gonadotropin. *Reproductive Biology and Endocrinology*, *12*(1), 1–7. https://doi.org/10.1186/1477-7827-12-10

Ribeiro, E. (1990). Occupational Exposure to Bisphenol A (BPA). *Toxics*,1–16. https://doi.org/10.3390/toxics5030022

Richter, C. A., Birnbaum, L. S., Farabollini, F., Newbold, R. R., Rubin, B. S., Talsness, C. E., Agency, U. S. E. P. (2008). In vivo effect of bisphenol A in laboratory rodent studies. NIH Public Access. *NIH Public Access*, *24*(2), 199–224.

Rina, T., Rahayu, S., Irmawaty, L., & Br, M. (2017). The Role of FSH , LH , estradiol and progesterone hormone on estrus cycle of female rats. International Journal of Sciences,4531, 92–100.

Robertson, T. J., & Farrelly, T. A. (2015). Bisphenol A (BPA) exposure in New Zealand : a basis for discussion. *Journal of Royal Society of New Zealand*,6758. https://doi.org/10.1080/03036758.2015.1071271

Robker, R. L., Hennebold, J. D., & Russell, D. L. (2018). Coordination of ovulation and oocyte maturation. *Endocrinology*,159(September), 3209–3218. https://doi.org/10.1210/en.2018-00485

Rochester, J. R. (2013). Author ' s personal copy Bisphenol A and human health : A review of the literature. *Reproductive Toxicology*, 42, 132– 155.

Saadeldin, I. M., Hussein, M. A., Suleiman, A. H., Abohassan, M. G., Ahmed, M. M., Moustafa, A. A., Swelum, A. A. (2018). Ameliorative effect of ginseng extract on phthalate and bisphenol A reprotoxicity during pregnancy in rats.*Environmental Science and Pollution Research*, 25(21) 21205-21215. doi: 10.1007/s11356-018-2299-1

Sadanandan, N., Cozene, B., Cho, J., Park, Y. J., Saft, M., Gonzales-Portillo, B., & Borlongan, C. V. (2020). Melatonin—A potent therapeutic for stroke and stroke-related dementia. *Antioxidants*, *9*(8), 1–15. https://doi.org/10.3390/antiox9080672

Salmon, D. G. (2013). French Law Banning Bisphenol A in Food Containers Enacted.

Sandoo, A., Zanten, J. J. C. S. V. Van, Metsios, G. S., Carroll, D., & Kitas, G. D. (2010). The endothelium and its role in regulating vascular tone. *The Open Cardiovascular Medicine Journal*, 302–312.

Sarah, S., Zaid, M., Kassim, N. M., & Othman, S. (2015). Tualang honey protects against BPA-induced morphological abnormalities and disruption of ER ?, and C3 mRNA and protein expressions in the uterus of rats. *Evidence-based Complementary and Alternative Medicine,*2015;2015:202874. doi: 10.1155/2015/202874

Sarıkaya, E., & Doğan, S. (n.d.). Glutathione Peroxidase in Health and Diseases.DOI: 10.5772/intechopen.91009

Schierow, L., & Lister, S. (2010). Bisphenol A (BPA) in Plastics and Possible Human Health Effects. *Congressional Research Serive*, 1–10. https://doi.org/10.1007/978-981-10-1673-8

Sever, R., & Glass, C. K. (2013). Signaling by nuclear receptors. *Cold Spring Harbor Perspectives in Biology*, *5*(3), 1–4. https://doi.org/10.1101/cshperspect.a016709

Skorupskaite, K., George, J. T., & Anderson, R. A. (2014). The kisspeptin-GnRH pathway in human reproductive health and disease. *Human Reproduction Update*, *20*(4), 485–500. https://doi.org/10.1093/humupd/dmu009

Song, C., Peng, W., Yin, S., Zhao, J., Fu, B., & Zhang, J. (2016). Melatonin improves age-induced fertility decline and attenuates ovarian mitochondrial oxidative stress in mice. *Nature Publishing Group*, (September), 1–15. https://doi.org/10.1038/srep35165

Sonigo, C., Beau, I., Binart, N., & Grynberg, M. (2019). Anti-Müllerian Hormone in Fertility Preservation: Clinical and Therapeutic Applications. *Clinical Medicine Insights: Reproductive Health*, *13*, 117955811985475. https://doi.org/10.1177/1179558119854755

Spiering, M. J. (2019). On the trail of steroid aromatase: The work of Kenneth J. Ryan. *Journal of Biological Chemistry*, *294*(28), 10743–10745.

https://doi.org/10.1074/jbc.CL119.009620

States, U., Akingbemi, B. T., Belcher, S. M., Agency, U. S. E. P., States, U., Crain, D. A., … Marcus, M. (2010). NIH Public Access, *24*(2), 131–138. https://doi.org/10.1016/j.reprotox.2007.07.005.Chapel

Steinmetz, R., Mitchner, N. A., Grant, A., Allen, D. L., Bigsby, R. M., & Ben-jonathan, N. (2015). The xenoestrogen bisphenol A induces growth, differentiation , and c- fos gene expression in the female reproductive tract. *Endocrinology*,*139*(6), 2741–2747.

Street, M. E., Id, S. A., Bernasconi, S., Burgio, E., Cassio, A., Id, C. C., Id, L. I. (2018). Current knowledge on endocrine disrupting chemicals (EDCs) from animal biology to humans , from pregnancy to adulthood : Highlights from a National Italian meeting. *International Journal of Molecular Science*. 19(6) 1647. https://doi.org/10.3390/ijms19061647

Su, H.-W., Yi, Y.-C., Wei, T.-Y., Chang, T.-C., & Cheng, C.-M. (2017). Detection of ovulation, a review of currently available methods. *Bioengineering & Translational Medicine*, *2*(3), 238–246. https://doi.org/10.1002/btm2.10058

Tal, R., Seifer, D. B., Khanimov, M., Malter, H. E., Grazi, R. V., & Leader, B. (2014). Characterization of women with elevated antimüllerian hormone levels (AMH): Correlation of AMH with polycystic ovarian syndrome phenotypes and assisted reproductive technology outcomes. *American Journal of Obstetrics and Gynecology*, *211*(1), 59.e1-59.e8. https://doi.org/10.1016/j.ajog.2014.02.026

Taraborrelli, S. (2015). Physiology , production and action of progesterone. *Scandinavica*,94, 8–16. https://doi.org/10.1111/aogs.12771

Taylor, P., Prasanth, G. K., M, D. L., & Sadasivan, C. (2012). Human and ecological risk assessment : Bisphenol-A can inhibit the enzymatic activity of human superoxide dismutase. *Human and Economic Risk Assessment*, (January 2015), 37–41. https://doi.org/10.1080/10807039.2012.683720

Thornton, M. J. (2013). Estrogens and aging skin. *Dermato-endocrinology*, 5(2), 264–270.

Trevisan, C. M., Montagna, E., De Oliveira, R., Christofolini, D. M., Barbosa, C. P., Crandall, K. A., & Bianco, B. (2018). Kisspeptin/GPR54 System: What Do We Know about Its Role in Human Reproduction? *Cellular Physiology and Biochemistry*, *49*(4),

1259–1276. https://doi.org/10.1159/000493406

Urrego, R., Herrera-Puerta, E., Chavarria, N. A., Camargo, O., Wrenzycki, C., & Rodriguez-Osorio, N. (2015). Follicular progesterone concentrations and messenger RNA expression of MATER and OCT-4 in immature bovine oocytes as predictors of developmental competence. *Theriogenology*, *83*(7), 1179–1187. https://doi.org/10.1016/j.theriogenology.2014.12.024

Urrestarazu, E., & Iriarte, J. (2016). Clinical management of sleep disturbances in Alzheimer's disease: Current and emerging strategies. *Nature and Science of Sleep*, *8*, 21–33. https://doi.org/10.2147/NSS.S76706

Vandenberg, L. N., Ehrlich, S., Belcher, S. M., Ben-jonathan, N., Dolinoy, D. C., Hugo, E. R., Low, S. (2013). Low dose effects of bisphenol A An integrated review of in vitro , laboratory animal , and epidemiology studies low dose effects of bisphenol A. *Endocrine Disruptors*, 3747. https://doi.org/10.4161/endo.26490

Vandenberg, L. N., Prins, G. S., & Sciences, H. (2017). HHS Public Access. *Andrology*, 4(4), 561–564. https://doi.org/10.1111/andr.12219.Clarity

Varshney, M., & Nalvarte, I. (2017). Genes, gender, environment, and novel functions of estrogen receptor beta in the susceptibility to neurodevelopmental disorders. *Brain Sciences*, *7*(3). https://doi.org/10.3390/brainsci7030024

Venugopal, B., & Yerramilli, V. (2019). Outside Directors at Early-Stage Startups. *SSRN Electronic Journal*, *1*(April). https://doi.org/10.2139/ssrn.3320010

Wagner, H., Cheng, J. W., & Ko, E. Y. (2018). Role of reactive oxygen species in male infertility: An updated review of literature. *Arab Journal of Urology*, *16*(1), 35–43. https://doi.org/10.1016/j.aju.2017.11.001

Wang, W., Zhang, X., Deng, F., Yuan, R., & Shen, F. (2017). Genome-wide characterization and expression analyses of superoxide dismutase (SOD) genes in Gossypium hirsutum. *BMC Genomics*, *18*(1), 1–25. https://doi.org/10.1186/s12864-017-3768-5

Wang, Z., Liu, H., & Liu, S. (2017). Low-Dose Bisphenol A Exposure: A Seemingly Instigating Carcinogenic Effect on Breast Cancer. *Advanced Science*, *4*(2). https://doi.org/10.1002/advs.201600248

Warner, G. R., Mourikes, V. E., Neff, A. M., Brehm, E., & Flaws, J. A. (2020). Mechanisms

of action of agrochemicals acting as endocrine disrupting chemicals. *Molecular and Cellular Endocrinology*, *502*, 110680. https://doi.org/10.1016/j.mce.2019.110680

West, A., Vojta, P. J., Welch, D. R., & Weissman, B. E. (1998). Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1). *Genomics*, *54*(1), 145–148. https://doi.org/10.1006/geno.1998.5566

WHO. (2015). International Chemicals Management : implementation and priorities Strategic Approach to International Chemicals Management : implementation and priorities, (June).

Wisniewski, P., Romano, R. M., Kizys, M. M. L., Oliveira, K. C., Kasamatsu, T., Giannocco, G., … Romano, M. A. (2015). Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. *Toxicology*, *329*, 1–9. https://doi.org/10.1016/j.tox.2015.01.002

Wo, O., Dv, O., Lo, O., & Cl, O. (2019). Pattern of infertility among infertile couple in a secondary health facility in Delta State , South South Nigeria. *Alexandria Journal of Medicine*, 2–6. https://doi.org/10.4103/TJOG.TJOG

Woalder (2017). HHS Public Access. *Physiology & Behavior*, 176(1), 139–148. https://doi.org/10.1016/j.physbeh.2017.03.040

Wu, H., Liu, C., Duan, W., Xu, S., He, M., Chen, C., … Chen, Y. (2013). Mutation Research / Genetic Toxicology and Environmental Mutagenesis Melatonin ameliorates bisphenol A-induced DNA damage in the germ cells of adult male rats. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *752*(1–2), 57–67. https://doi.org/10.1016/j.mrgentox.2013.01.005

Wu, Q., Fang, J., Li, S., Wei, J., Yang, Z., Zhao, H., … Cai, Z. (2016). Interaction of bisphenol A 3,4-quinone metabolite with glutathione and ribonucleosides/deoxyribonucleosides in vitro. *Journal of Hazardous Materials*. https://doi.org/10.1016/j.jhazmat.2016.03.015

Yoshihara, S., Mizutare, T., Makishima, M., Suzuki, N., Fujimoto, N., Igarashi, K., & Ohta, S. (2004). Potent estrogenic metabolites of bisphenol A and bisphenol B formed by rat liver S9 fraction: Their structures and estrogenic potency. *Toxicological Sciences*, *78*(1), 50–59. https://doi.org/10.1093/toxsci/kfh047

Zbucka-Kretowska, M., Zbucki, R., Parfieniuk, E., Maslyk, M., Lazarek, U., Miltyk, W., … Ciborowski, M. (2018). Evaluation of Bisphenol A influence on endocannabinoid system in pregnant women. *Chemosphere*, *203*, 387–392. https://doi.org/10.1016/j.chemosphere.2018.03.195

Zhou, C., Wang, W., Peretz, J., & Flaws, J. (2016). HHS Public Access, 87–99. https://doi.org/10.1016/j.reprotox.2015.05.012.Bisphenol