PROTECTIVE EFFECTS OF MELATONIN ON BISPHENOL A-INDUCED REPRODUCTIVE TOXICITY IN MALE WISTAR RATS

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ABSTRACT

Bisphenol A (BPA), a known Endocrine Disrupting Chemical (EDC) used in the production of plastics has been demonstrated to induce marked reproductive toxicities in animals, while melatonin is a known antioxidant capable of ameliorating EDC-induced toxicity. Studies have demonstrated the protective effect of MLT on male reproductive functions following subacute exposure to BPA. However, little is known about the protective mechanisms. This study was designed to evaluate the mechanisms of MLT protection against reproductive toxicity in adult male Wistar rats exposed to BPA.

Forty adult male Wistar rats (180 ± 10 g) randomly assigned into 4 groups (n=10) were used for the study. Group I (control) received 0.2 ml olive oil orally, group II received MLT (10 mg/kg) intra-peritoneally, group III received BPA (10 mg/kg) orally, while group IV was co-treated with BPA (10 mg/kg) and MLT (10 mg/kg). All rats were treated daily for 45 days. On day 46, blood samples were collected for serum hormone assay [testosterone (T), dehydroepiandrosterone (DHEA), Follicle Stimulating Hormone (FSH), estradiol (E2)]. Testes and epididymides were thereafter harvested. Biochemical markers of oxidative stress (MDA, H_2O_2, Catalase, SOD, GSH, GPx) and semen characteristics (sperm motility, liveability sperm count) were studied from epididymal tissue. Light Microscopy (LM) and Transmission Electron Microscopy (TEM) were used to study morphological changes in testes and epididymides. Testicular and epididymal immunoreactivities to alpha smooth muscle actin (αSMA), vimentin (Vm) and S-100 proteins were also estimated using standard methods. Data were analyzed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

Melatonin co-administered with BPA significantly increased the serum levels of T (13.84±1.59 nmol/L), DHEA (0.002±0.0002 μmol/L) compared to BPA-treated rats (6.59±1.23 nmol/L, 0.001±0.00 μmol/L, respectively), but significantly decreased FSH (0.11±0.02 μmol/L) and E2 (13.22±0.59 pmol/L) compared to BPA alone (0.15±0.02 μmol/L, 18.23±0.03 pmol/L, respectively). Similarly, BPA+MLT significantly increased the activities of catalase (6.51±1.07 μmol of H_2O_2 consumed/min/mg), SOD (5.25±0.09 Units/mg protein), GSH (41.49±9.73 μg/mg protein) and GPx (5.35±0.56 nmol of GSH residual/mg protein) compared to BPA alone

(5.87±1.53 μmol of H_2O_2 consumed/min/mg, 4.76±0.31 Units/mg protein, 24.63±4.92 μg/mg protein, 5.19±0.21 nmol of GSH residual/mg protein, respectively). Melatonin co-administered with BPA significantly decreased the levels of H_2O_2 (163.58±24.71 to 110.43±7.74 nmole/mg protein) and MDA (23.97±0.62 to10.06±3.04 μmole MDA formed/mg protein) compared to BPA-treated rats. Also, Melatonin co-administered with BPA significant increased sperm motility (71.67±3.07%), livability (88.83±2.77 %) and sperm count (144.00±2.90 x 10^6 sperm/ml) compared to BPA alone (49.17±2.71 %, 48.33±3.07 %, 137.17±2.57 x 10^6 sperm/ml, respectively). Melatonin co-administered with BPA prevented necrosis and epithelial sloughing in the testes and epididymides and distortion in the 9+2 axoneme arrangement of the flagellar apparatus of spermatozoa observed in BPA-treated rats. BPA+MLT caused increased expression of αSMA, Vm and S-100 proteins in the testes and epididymides which were significantly reduced in the BPA-treated rats.

Melatonin protected against Bisphenol A-induced male reproductive toxicity. The probable mechanisms were prevention of oxidative stress and upregulation of alpha smooth muscle actin, vimentin and S-100 protein expressions in the testes and epididymides.

Keywords: Bisphenol A, Melatonin, Spermatozoa, Oxidative stress, Vimentin expression

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DEDICATION

This project work is dedicated to God who sustained my life and delivered me from untimely death. I am alive today just because He kept me, and unto Him be all the glory.

CERTIFICATION

We certify that this work was carried out by Dr O. S. Ajani in the Department of Theriogenology, University of Ibadan.

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TABLE OF ABBREVIATIONS

BPA	Bisphenol A
MLT	Melatonin
NHWT	Normal Head Without Tail
NTWH	Normal Tail Without Head
RT	Rudimentary Tail
BT	Bent Tail
CT	Curved Tail
BMP	Bent Mid Piece
CMP	Curved Mid Piece
LT	Looped Tail

CHAPTER ONE

INTRODUCTION

Bisphenol A (Bis(p-hydroxyphenyl)propane)is a chemical used in the production of polycarbonate plastic and is a constituent of epoxy and the polystyrene resin (Doerge *et al*., 2011). BPA exhibits hormone-like activities that raise concern about its suitability in consumer products and food containers. It is present in several products, including the interior coatings of food cans, milk containers, and baby formula bottles, as well as in dental sealants (Welshons *et al*., 2006). It can be detected in food, water, dust, and other similar media (Kleywegt *et al*., 2011; Noonan *et al*., 2011) by which animals are also at risk of exposure. Humans are especially and unknowingly exposed to BPA, and it can be detected in the majority of individuals in many nations worldwide (Vandenberg *et al*., 2007).

BPA has attracted scientific attention due to its sizeable presence in numerous consumer products and its deleterious effects on reproduction. Reproductive and endocrine disruptions are the main toxicities caused by BPA (Erler and Novak, 2010).Testicular toxicity prompted by BPA has been suggested as a possible cause of increasing frequency of infertility (Takahashi and Oishi, 2001; Tohei *et al.,* 2001). The reproductive toxicity of BPA is triggered via multiple signaling pathways (D'Cruz *et al.,* 2012) and may contribute to alterationsin the process of spermatogenesis in rats (Qiu *et al.,* 2013). Moreover, BPA reversibly perturbs the integrity of the blood– testis barrier in Sertoli cells in-vitro (Li *et al.,* 2009). The reproductive toxicity of BPA is due to interplay with androgen and estrogen receptors (Matthews *et al.,* 2001). It also induces uterotrophic effects in rats following high oral and/or subcutaneous dosing (Goloubkova *et al.,* 2000). In rodents, developmental exposure to BPA results in increased prostate weight (Nagel *et al.,* 1997), reduced epididymal weight (vom Saal *et al.,* 1998) and reduced daily sperm production (Sakaue *et al.,* 2001).

When BPA is ingested through food, it can be rapidly absorbed from the digestive tract and can be detected in various body fluids, organs, and tissues (Dekant and Volkel, 2008; Fisher *et al*., 2011; Vandenberg *et al*., 2007). The documented concentrations of BPA in blood and urine of

occupationally and environmentally exposed individualshave been extensively reviewed (Dekant and Volkel, 2008; Li *et al*., 2010; Vandenberg *et al*., 2010).
BPA toxicity in mammals manifests through increasing the hydroxyl-radical formation in the rat striatum, depletes the endogenous antioxidants in epididymal sperms and impairs hepatic detoxification mechanisms of rats (Richter *et al*., 2007). Agents with potent anti-oxidant properties have been found useful in ameliorating BPA-induced reproductive toxicity in various studies (Anjum *et al*., 2011; El-Beshbishy *et al*., 2013).

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a neuro-hormone derived from tryptophan and is mainly released from the pineal gland. MLT participates in various homeostatic functions, such as regulation of reproduction and circadian rhythms (Reiter *et al*., 2000), by its action at various portions of the hypothalamic– pituitary–gonadal axis (Pandi-Perumal *et al*., 2006). Also, it possesses antioxidant and prophylatic properties against oxidative stress in many experimental and clinical conditions with a wide margin of safety upon its administration (El-Missiry, 2000; Reiter *et al*., 2004). In addition to its use as an anti-stress, anti-aging, and immune-modulatory agent, MLT has been used for sexual dysfunctions, gallbladder stones, obesity, and even neoplastic conditions (Altun and Ugur-Altun, 2007; El-Missiry and Abd El-Aziz, 2000).
Studies have demonstrated that reactive oxygen species (ROS) play an important role in the defense mechanisms against pathological conditions but excessive generation of free oxygen radicals may damage tissues (Kitas *et al*., 1991).
BPA has been shown to induce ROS generation in cells, resulting in cell apoptosis and tissue injury (Ho *et al*., 1998). There are a plethora of evidence that environmental toxicants like BPA alter antioxidant system in testis (Sujatha *et al*., 2001; Latchoumycandane *et al*., 2002), epididymis and epididymal sperm of adult rats (Chitra *et al*., 2001, 2002; Latchoumycandane *et al*., 2002).

Research reports on the effect of BPA on the fertility of male rats within the last few decades remained contradictory (Al-Hiyasat *et al*., 2002; Richter *et al*., 2007). For example, while Toyama and Yuasa (2004) observed detrimental effects on the spermatogenesis and fertility of neonatal male rats exposed to BPA, Kato *et al*. (2006) observed no adverse effect of BPA on the reproductive indices of adult male rats after neonatal exposure. These observed discrepancies

had been attributed to differences in the doses, mode of administration, duration of treatment as well as the species used (Richter *et al*., 2007; Salian *et al*., 2009).
Oral administration of BPA (20 µg/kg, 2 mg/kg, or 200 mg/kg) to male Sprague-Dawley (SD) rats covering postnatal days 91–97, produced no marked differences in testicular weight as well as daily sperm production at 5 weeks post-treatment in the rats (Ashby *et al*., 2009). There is, probably, no explanation for these conflicting reports on the effects on sperm production in adult male SD rats challenged with low doses of BPA, except there are subtly different genetic make-ups leading to different sensitivities of SD rats to BPA.

However, recent research on the effect of BPA on the fertility of male rats have consistently shown that it impaired male fertility causing a number of testicular dysfunctions, including induction of apoptosis of testicular germ cells, disruption of the junctional proteins of the blood-testis barrier, alterations in the levels of androgen binding protein and steroidogenic enzymes (Peretz *et al*., 2014; Wang *et al*., 2015; Quan *et al*., 2016; Durando *et al*., 2016; Tian *et al*., 2017). In adult male rats, exposure to BPA decreased sperm characteristics, induced apoptosis of germ cells, impaired Sertoli cell structure, altered steroidogenesis, caused oxidative stress and impaired spermatogenesis (Jin *et al*., 2013; Peretz *et al*., 2014; Wang *et al*., 2015; Quan *et al*., 2016).
To date, little is known about the mechanisms by which agents with potent anti-oxidant properties mitigate BPA-induced male reproductive toxicity. Anjum *et al*. (2011), demonstrated that melatonin has a potential role in ameliorating BPA-induced mitochondrial toxicity and that the protection is due to its antioxidant property or by the direct free radical scavenging activity. There is the need to investigate the ameliorative effects of melatonin on BPA-induced male reproductive toxicity especially as it relates to sperm morphology and reserves, epididymal spermatozoa oxidative stress, spermiogenesis and steroidogenesis, as well as testicular and epididymal protein localization. This study was therefore designed to investigate the ameolirative effects of melatonin against BPA-induced male reproductive toxicity in the Wistar rats.

1.2 STATEMENT OF PROBLEM

The last few decades have witnessed a progressive depreciation in male reproductive health due to exposureto endocrine-disrupting chemicals such as Bisphenol-A (BPA)into the environment leading to unfavourable effects observed in animals and man (Dekant and Colnot, 2013; Ahbab *et al*., 2017). Despite the short half-life of BPA, its continuous release into the environment makes it ubiquitous (Oehlmann *et al*., 2009). Thus, BPA, acting via several physiological receptors, including estrogen receptors α and β (ERα and ERβ), membrane-bound ERs, androgen receptor, peroxisome proliferator-activated receptor gamma and thyroid hormone receptor, represents a significant potential risk for man and animals (Richter *et al*., 2007; Crain *et al*., 2007; Flint *et al*., 2012; Peretz *et al*., 2014).

1.3 RESEARCH QUESTION

Does melatonin offer protective effects on BPA-induced male reproductive toxicity especially as it relates to sperm morphology, epididymal spermatozoa oxidative stress, spermiogenesis and steroidogenesis, as well as testicular and epididymal protein localization?

1.4 RESEARCH HYPOTHESIS

Melatonin protects against BPA- induced reproductive toxicity in adult male Wistar rats.

1.5 GENERAL OBJECTIVE

This study aims at investigating the protective effects of melatonin against BPA-induced male reproductive toxicity in the Wistar rats.

1.6 SPECIFIC OBJECTIVES

This study shall investigate the protective effects of melatonin against:

1. BPA-induced epididymal sperm anomalies.
2. BPA-induced epididymal spermatozoa oxidative stress.
3. BPA-induced epididymal injury and altered protein localization
4. BPA-induced alterations in spermatogenesis

1.7 JUSTIFICATION

Several studies have shown the devastating toxic effects of BPA on reproduction with very few reports establishing the potentials of melatonin in the mitigation of testicular oxidative stress and consequent alterations in spermatogenesis. There is therefore the need to investigate the protective mechanisms of melatonin on BPA-induced male reproductive toxicity especially as it relates to sperm morphology, epididymal spermatozoa oxidative stress, spermiogenesis and steroidogenesis, as well as testicular and epididymal protein localization. Data to be made available from the study is expected to go a long way in developing strategies for protecting against male infertility especially those induced by endocrine disrupting agents.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.1 BISPHENOL-A BACKGROUND

Bisphenol-A (BPA) is an organic compound, first synthesized by a Russian scientist named Dianin in 1891 (Rubin, 2011). In the 1930's, its estrogenic properties were discovered when scientists conducted BPA feeding experiments on ovariectomized rats. It was screened as a possible compound to replace synthetic estrogen (Cavalieri and Rogan, 2010). Although BPA expresses estrogenic properties, it is 10,000 to 100,000 times weaker than estradiol in its affinity for traditional estrogen receptors pathways (Vandenberg *et al*., 2007). Due to this, the pharmaceutical industry chose another synthetic estrogen, diethylstilbestrol, or DES, for use with pregnant women to prevent miscarriage and premature birth (Rubin, 2011). DES is now an infamous illustration of the harmful effects of endocrine disruption. Fetal exposure to DES is capable of causing offsprings to develop reproductive disorders and post pubertal female offspring to develop a rare clear-cell carcinoma.

BPA has two phenol functional groups that are well suited for use as a monomer base for polycarbonate plastic (PC). PC is widely used due to its durability, shatter resistance, transparency, thermo-stability, and lightweight nature. BPA is additionally used as a linkage in manufacturing epoxy resins, compounds that provide corrosion resistance, flexibility, and heat resistance. BPA is also employed as a color developer in thermal receipts (Geens *et al*., 2012; Liao and Kannan, 2011), as a component of dental composites (Joskow *et al*. 2006; Olea *et al*., 1996), and in medical devices (Calafat *et al*., 2009).

The constant use of polycarbonate plastics and epoxy resins in many consumer products has led to the annual manufacturing of over six billion pounds of BPA, making it one of the world's highest production volume chemicals (Melzer *et al*., 2010; Willhite *et al*., 2008). Due to its widespread use, BPA has been detected in household dust and air (Wilson *et al*., 2007) and in water contaminated by landfill leachate and wastewater effluent (Tsai, 2006; Vandenberg *et al*., 2007; vom Saal *et al*., 2007). It is also found in detectable levels in a variety of paper products, unintentionally incorporated into goods during the recycling process (Geens *et al*., 2012; Liao and Kannan, 2011; Ozaki *et al*., 2004). Recycled paper products contain ten times more BPA

than virgin paper products. The source of BPA in recycled products can be traced back to thermal receipts with BPA being introduced into the recycling stream (Ozaki *et al*., 2004).

With so many contributing sources, BPA exposure in the human population is ubiquitous. BPA has been measured in the urine of 92.6 % of the American population (Calafat *et al*. 2008). Although BPA exposure contributions include many sources, diet is the main contributor of BPA exposure (Kang *et al*., 2006; von Goetz *et al*., 2010; Wilson *et al*., 2007).

2.1.2 BPA AS ENDOCRINE DISTRUPTOR

Bisphenol A (BPA) is an identified endocrine disruptor and high volume production chemical with more than 5000 of safety-related studies published on the effects of its exposure to human and animals (Alonso-Magdalena *et al*., 2015; Medwid *et al*., 2016; Tian *et al*., 2017). BPA is a plasticizer present in everyday-used items, including refillable drinking containers, plastic utensils, dental sealants, the linings of metal cans, electronics, medical devices, infant feeding bottles and toys, pharmaceuticals, compact disks, waxes, food and beverage packaging, adhesives, building materials, and paper coatings (Ma *et al*., 2017). Despite the short half-life of BPA, its continuous release into the environment makes it ubiquitous (Oehlmann *et al*., 2009). Thus, BPA, acting through several physiological receptors, including estrogen receptors α and β (ERα and ERβ), membrane-bound ERs, androgen receptor, peroxisome proliferator-activated receptor gamma and thyroid hormone receptor, represents a significant potential risk for humans and wildlife (Flint *et al*., 2012; Peretz *et al*., 2014).

Bisphenol A (BPA) as a synthetic was reported to have the efficacy of estrone in stimulating the female reproductive system, although BPA possesses estrogen-like activity and binds to estrogen receptors (ER) such as ER alpha and ER beta (Krishnan *et al*., 1993), but the relative binding affinity of BPA to these receptors is not so strong. It is about 10,000 times less than natural estrogen (Kuiper *et al*., 1998).

2.2.0 SOURCES OF BPA EXPOSURE

Sources of BPA exposure are many, as it easily sips from the inner lining of food cans and microwave containers while heating, from dental sealant into saliva, and into beverages from incessant usage or contact with any acidic/alkaline content in polycarbonate bottles (Brotons *et al*. 1995; Olea *et al*., 1996).

The continuous exposure to BPA could lead to sexual dysfunction (Rahman *et al*., 2015), anomalies of reproduction (Liu *et al*., 2013) and behavior (Roen *et al*.,2015). BPA affects the reproductive system of both sexes. In male, it affects testosterone production by inhibiting the steroidogenic acute regulatory protein (StAR), and cytochrome P450 side chain cleavage (CYP 11A1) (Peretz *et al*., 2013;Xi *et al*.,2011).

Research has shown that sensitivity to BPA is not the same at all developmental stages of life, there are certain critical levels of development that are more sensitive to BPA exposure (Welshons and Nagel, 2006). The neonatal period is such an expedient stage which is largely influenced by steroid hormones and their receptors, and during which the programming of the hypothalamus–pituitary–testicular (HPT) axis occurs. This implies that the neonatal period is one of the most crucial periods that may be a determinant of fertility in adulthood (Goyal*et al*., 2003).

Neonatal exposure to low doses of BPA is known to cause deleterious effects on male reproduction including reduction in epididymal weight, sperm motility and daily sperm production (vom Saal *et al.,* 1998; Aikawa *et al.,* 2004)

Meanwhile, because of the high production level and massive consumption of BPA in most developed and developing countries, there have been many issues raised on the use of this chemical (Chouhan *et al.,* 2014).

2.2.1 HUMAN EXPOSURE TO BISPHENOL A

Bis(p-hydroxyphenyl)propane) being constituent of plastic, epoxy and polystyrene resins which is commercially used for coating the inner lining of food cans, manufacturing plastic bottles and composite dental sealants (Ranjit *et al*., 2010), is ubiquitous. In addition to the above mentioned,

BPA is also used in the production of receipt paper and other commonly used products (Qian *et al*., 2014). It is through this means that BPA has been detected in the human body; in blood, urine, saliva, breast milk, semen, amniotic fluid, and follicular fluid (Vandenberg *et al*., 2007, 2010).Even though BPA is rapidly metabolized, it has been discovered in nearly the whole population at any given time in which human exposure to BPA is significant, continuous, and from multiple sources (vom Saal and Hughes, 2005). An exposure to BPA has contributed to an increased occurrence of heart diseases, diabetes (both type A and type B),excessive increase in body weight thereby leading to obesity, developmental problem of the brain, cancer of the reproductive system and changes in behavior (Melzer *et al*., 2010; Vandenberg *et al*., 2007).

Research has also shown the relationship between exposure to BPA and cardiovascular diseases as well as increased body mass index in humans (Jeng, 2014; Wang *et al*., 2012).

Numerous exposure of BPA to humans is of a great concern to health. Several studies reflect concern over the disrupting properties of BPA on endocrine system, and its adverse effect on reproductive development in both sexes.The early sexual development of children and some cancer of the reproductive tract has been associated with infants having a limited capacity to metabolize BPA on exposure. Just little changes in hormone activity during development can lead to permanent effects (Welshons *et al*. 2003).

Though, reproductive and developmental issues for fetuses have been found to be due to the prenatal maternal exposure to BPA (Soto and Sonnenschein, 2010), it has also been noted that the exposure of adults to BPA affects the male genitalia and that long-lasting, organizational effects in response to developmental exposure to BPA occur in the metabolic processes, the male reproduction as well as in the brain(Rochester, 2013)

Humans are exposed to bisphenol A from plastic beverage bottles and from saliva of individuals receiving dental sealants but the primary source of human exposure to BPA is the leachate from food containers and beverage plastics (Olea *et al*., 1996). It has also been recorded that small quantity of BPA can sipinto water or food from polymers especially when being heated (Le *et al*., 2008; Olea *et al*., 1996).

Research has shown that in developing countries, the usage of plastic wares in most of human activities has tremendously increased such that certain population groups may be at higher risk

than others (Lahera *et al*., 2006) and this is of crucial concern for human health hazards of BPA.The entire population is likely to be exposed to BPA in everyday life by using BPA-containing materials,such as baby bottles, food cans, water containers, and dental sealants (von Goetz *et al*., 2010).

It has been documented that perinatal exposure to Bisphenol Aproduceddeleterious effect on target organs like the mammary gland and brain, which resulted in changes in morphogenesis and disturbances in sexual and mating behavior (Farabollini *et al*., 2002; Negishi *et al*., 2004; Muñoz-de-Toro *et al*., 2005). Thus, the perinatal period being one of the stages for organ differentiation and development,is susceptible to any form of disturbance following exposure to BPA which can influence development and adult function.

2.2.2 EXPOSURE OF WILDLIFE TO BPA

Wildlife are exposed to a combination of environmental pollutants rather than a single chemical. The release of BPA into the environment has been a major source of exposure to wildlife. This can occur during chemical manufacture, transport, and processing of plastic products. Also, post-consumer releases of BPA are mainly through effluent discharge from municipal wastewater treatment plants, leaching from landfills, combustion of domestic waste, as well as from natural breakdown of plastics in the environment (Crain *et al*., 2007; Kang *et al*., 2007). Hence, wildlife are exposed to BPA in their natural habitats, including polluted air, contaminated soil and water bodies.

2.2.3 EFFECTS OF BISPHENOL A ON FISH

Studies of BPA in the aquatic environment are numerous. BPA has been detected in leachates from industrial and municipal waste disposal sites at 8400 g/l and 10,300 g/l, respectively, and these levels are higher than maximum tolerable aquatic toxicity levels. BPA can also be detected in wastewater effluents from paper recycling plants (Fukazawa *et al*., 2001). BPA is considered to be readily biodegradable by bacteria in rivers under aerobic conditions with a half-life in freshwater of 3-5 days, although in seawater, BPA stays much longer, about thirty days. In both aquatic environments, aquatic organisms have been shown to have higher levels of BPA in their systems than is measurable in their aqueous environment. Caution should therefore be taken in consuming seafood and freshwater fish from contaminated waters (Kang *et al*., 2006). Due to the

method of drinking water treatment, through chlorination, ultraviolet light radiation, and ozonation, estrogenic compounds are destroyed. Even if BPA does leach from a PVC pipe, it will be destroyed by the chlorine in the water (Lee *et al*., 2004; Sajiki and Yonekubo, 2002).

Research carried out in Taiwan and Germany has shown that BPA concentrations are higher in the soil than water, showing evidence of its partitioning into the soil (Heemken *et al*., 2001; Lin, 2001; Stachel *et al*., 2005).

2.2.4 EFFECTS OF BISPHENOL A ON REPTILES

Negative effects on reptiles following exposure to BPA have been documented (Stoker *et al.*2003). In reptilian species that have temperature-dependent sex determination, such as the broad-snouted caiman (Caiman latirostris), BPA has been demonstrated to elicit sex changes in embryos. Stoker *et al.* (2003) reported that BPA exposure of 1400 mg/L (90mg/egg) produced abnormal seminiferous tubules in eggshells during the periods for gender determination. Stoker *et al.*(2003) observed complete sex reversal from male to female in eggs incubated at the male-determining temperature. Such malformations and sex reversals have the capacity to lower reproductive potential.Where BPA pollution is suspected, a skewed sex ratio in temperature-dependent sex determination species could be used as an indicator of environmental health (Crain and Guillette, 1998).

2.2.5 EFFECTS OF BISPHENOL A ON BIRDS

A few reportsinvestigating the effects of Bisphenol A on birds have been published to date but only one has shown effects at environmentally relevant levels. Berg *et al*. (2001) found high mortality rate in chicken (Gallus domesticus) embryos and that male embryos experienced feminization of the left testes when eggs were injected with a single dose of 200mg BPA/g egg early in incubation.

Furuya *et al*. (2002)noteda delay in growth of comb, wattle, and testes in male chickens that received oral doses of BPA at 2mg/1000g body weight every two days for 23 weeks. The study reported no difference between controls and juvenile chickens fed high doses of BPA (200,000 mg/1000g body weight) weekly from ages 2 to 16 weeks. Meanwhile, chickens receiving BPA showed reduced weights of combs and testes having smaller seminiferous tubules and exhibiting

abnormal spermatogenesis. The authors opined that an endocrine-disrupting mechanism might trigger these effects and that reproductive function was likely altered (Furuya *et al*., 2002). Estrogenic effects have also been reported in Japanese quail (Coturnix japonica). Unlike chickens, quail eggs administered with 200 mg of BPA/g egg were observed to produce females with oviduct anomalies (Berg *et al*., 2001; Halldin *et al*., 2001),However, it was discoveredthat quail eggs with BPA exposure at 67 and 200mg/g per egg, did not produce individuals with altered testicular weight symmetry, testosterone levels, male sexual behavior, or female fecundity.

2.26 FEECTS OF BISPHENOL IN INVERTEBRATES

The reproductive effects of BPA in variety of invertebrates have been demonstrated (Oehlmann *et al*., 2000). For instance, in the freshwater Ramshorn snail (Marisa cornuarietis), exposure levels >1.0mg/L were found to cause super-feminization (additional female organs, enlarged sex glands, oviduct deformities, and increased fecundity), oviduct rupture, and mortality (Oehlmann *et al*., 2000). Spawning induction, oocyte and ovarian follicle damage were observed following BPA exposure in the mollusc Mytilus edulis, for 3 weeks at 50mg/L (Aarab *et al*., 2006). Marine copepoda. tonsa, showed reduced egg production, reduced hatchability of offspring from exposed adults, and elevated offspring mortality was observed at exposures exceeding environmentally tolerable levels (>100mg/L) (Andersen *et al*., 1999).

Also, in the sea urchin Paracentrotus lividus a 30-min BPA exposure (300mg/L) decreased fertilization outcome approximately 42%, and increased larval deformities in the offspring of BPA exposed sperm (Arslan and Parlak, 2008).

Female *Porcellio Scaber*in soils exhibited increased abortions and decreased reproductive function following 10,000 mg/kg soil exposure (Lemos *et al*., 2009). Juvenile*Porcellio Scaber*showed female-biased sex ratios after BPA challenge at the same level (Lemos *et al*., 2009).

Although, developmental and reproductive pertubationsfollowing BPA exposure in invertebrates have been reported, many were observed at levels currently higher than the environmentally tolerable concentrations. There are notable discrepancies in the effects of BPA among related

taxa, and it suggests that some invertebrates may be hypersensitive to BPA exposure (freshwater molluscsinsect larvae, and marine copepods in particular).

2.27 EFFECTS OF BISPHENOL- A IN RODENTS (RATS AND MICE)

2.2.7.1 STUDIES IN THE FEMALE

Numerous toxicological experiments have been conducted to investigate the effects of BPA exposure on rats and mice in an effort to understand possible human health effects. Part of the earliest low-dose studies was on the effect of BPA exposed gravid mice to 2 and 20 microg/kg-BW/day in which fetal exposure to the male mouse resulted in an increased adult prostate weight. This study was the first research to show that fetal exposure to BPA at the pre-parturient level could alter adult reproductive systems in mice (Nagel *et al*., 1997). These early findings were confirmed by research that exposed pregnant mice to 50 microg/kg-BW/day of BPA. This fetal exposure caused the offspring to have increased prostate size, increased anogenital distance, and decreased epididymis weight. The androgen receptor binding activity of the prostate was also permanently increased (Gupta, 2000).

The prenatal effect of BPA exposure has been closely studied in animals. At this stage in development, the fetal and neonatal liver produces high levels of alpha fetoprotein (AFP). In rodents, AFP is the major estrogen binding plasma protein. This binding mechanism is believed to protect perinatal rodent tissues from overexposure to estradiol (Toran-Allerand, 1984). BPA does not rapidly bind to AFP like estradiol, leaving it free to cause harm to sensitive tissues in the developing fetus or neonate. A region particularly susceptible to exogenous estrogens is the developing brain. When pregnant mice were exposed to BPA, sexual differentiation controlled by the brain was affected and the female offspring showed masculine behavior (Rubin *et al*., 2006).

Rodents exposed to BPA in adulthood have shown altered glucose homeostasis. After only a few consecutive days of BPA exposure, adult male mice experienced an elevation in pancreatic insulin levels, hyperinsulinemia, and insulin resistance. When pregnant female mice were exposed, they showed decreased glucose tolerance, and increased plasma insulin, triglycerides and leptin concentrations. Even after giving birth, these females retained their glucose

intolerance, increased plasma insulin, leptin, and triglycerides, as well as experiencing an increase in their body weight (Alonso-Magdalena *et al*., 2006; Alonso-Magdalena *et al*., 2010).

Greater sensitivity to BPA has been shown in animal studies during the perinatal period. In a study where pregnant dams were given low doses of BPA in their water, alterations in their offspring's morphology and reproductive system were seen. Neonatal rats had an increase in body weight that was measurable soon after birth and continued into adulthood. The offspring also had altered estrous cycle and decreased of plasma luteinizing hormone. To see if these health effects could be seen if the BPA doses were applied to adult rats, the research team exposed post pubertal ovariectomized female rats with the same dose and a dose ten times higher. There were no uterotrophic responses in the test population, showing that the perinatal period had greater sensitivity. The study also showed the importance of investigating health effects from low-dose animal toxicology testing (Rubin *et al*., 2001).

Animal studies (Durando *et al*., 2007; Murray *et al*., 2007)have investigated the relationship between BPA exposure and carcinogenesis, particularly breast cancer. In low dose experiments, rats prenatally exposed to BPA had increased incidence of changes in mammary gland structure and developing precancerous lesions during adulthood (Durando *et al*., 2007; Murray *et al*., 2007).

It has been established (vom Saal *et al*., 2007) that low dose BPA exposures to animals during the prenatal and neonatal period "results in organizational changes in the prostate, mammary glands, testis, body size, brain structure and chemistry, and behavior of laboratory animals." In addition, this consensus statement is also confident that adult exposure of laboratory animals to BPA at low doses can cause neurobehavioral effects and reproductive effects in both sexes. The study also emphasizes that life stage can impact the pharmacokinetics of BPA (vom Saal *et al.,*2007).

2.2.7.2 STUDIES IN THE MALE

Bisphenol A has been shown by Chitra*et al.* (2003) to produce deleterious effectson the reproduction of adult male rats and which manifest through generation of free radicals and subsequent induction of oxidative stress on spermatozoa. For instance, Chitra *et al*. (2003)

reported that administration of Bisphenol in male rats at 0.2, 2 and 20 microgrm/Kg body weight per day for 45 days led to a markedreduction in the weight of the testis andepididymis; increased weight of ventral prostate and caused a decrease in epididymal sperm motility and sperm count in a dose-dependent manner. The authors noted that graded doses of bisphenol Acaused depletion of antioxidant defence system and induced oxidative stress in epididymal spermatozoa of adult male rats. Othman *et al*. (2016) also established that administration of BPA significantly increased oxidative stress in the testes and epididymal sperm of rats when administered at 50 mg/kg bw for 3 and 6 weeks. This was associated with decreased serum testosterone level as well as sperm quality, chromatin condensation/de-condensation level, and the percentage of haploid germ cells in the semen

Bisphenol A, when given concurrently at low-dose with diethylstilbestrol (DES) produced significantly high sperm deformity ratios and histological lesions of the testes of rats and decreased sperm motility. Sakaue *et al*. (2001) also described how oral exposure of sexually mature male rats to BPA between postnatal days 91–97 led to decreased daily sperm production at 18 weeks of age. When mice were neonatally exposed at 50 microgram/kg (for the first 5 days of life) decreased sperm motility and an increase in the sperm abnormalities, in the epididymides of mice at 10 weeks of age were observed (Aikawa *et al.,* 2004).

Anjum *et al*. (2011) reported that 10mg/kg administration in Swiss albino mice for 14 days produced a marked reduction in activities of mitochondrial enzymes such as malate dehydrogenase, succinate dehydrogenase, isocitrate dehydrogenase, NADH dehydrogenase and monoamine oxidase. Activities of antioxidant enzymes such as glutathione reductase, superoxide dismutase and glutathione peroxidase were also affected. BPA also caused increased lipid peroxidation (LPO) and decreased glutathione (GSH) content of mitochondria. Pertubations of mitochondria enzymes in the testes has well been established by El-Beshbishy *et al.* (2013) using 10mg/kg oral BPA for 14 days in rats. However, other authors have shown that reproductive failures induced by BPA exposure in male rats could be mediated by apoptosis in the mitochondrial pathway (Wang *et al*., 2014). Some studies also confirmed thataltered spermatogenesis and reproductive damage will be elicited at BPA concentrations of 50, 100, and 200 mg/kg body weight in rats.

Histological assessment revealed testicular injury followingBPA exposure in mice. Microscopic examination under light and transmission electron microscopes showed disorders in the process of spermatogenesis after BPA exposure. These include rough basal lamina of seminiferous tubules and damage of tight junctions between Sertoli cells (Tian *et al.,* 2017). Immunohistochemical assessmentshowed that the expression of androgen-binding protein (ABP) was significantly redued in BPA-treated mice. The study also revealed that distortion of the basal lamina of seminiferous tubules and tight junctions might contribute to BPA-induced cell injury and reduction in the level of Androgen Binding Protein(ABP) could be the possible mechanism for the reproductive toxicity of BPA (Tian *et al.,* 2017). Histopathological observation showing degeneration of seminiferous tubules and loss of elongated spermatids, disorganization, distortion and degeneration of late spermatids were demonstrated after dietary 235mg/kg BPA in male F344 rats for 44 days (Takahashi and Oishi (2001).

Evidence of altered spermatogenesis in rats by a marked reduction in the epithelial height and numbers of round spermatids in seminiferous tubules, sperm count, androgen receptor localization, and the expression of the spermatogenesis-related genes, outer dense fiber protein 1 (ODF1) and transition protein-1 wereobserved in some studies (Qiu*et al.* 2013; Toyama and Yuasa, 2004). Also, a decline was reported in spermatogenesis in mice treated with low dose of BPA by slowing down of meiotic progression of germ cells in the testes (Zhang *et al*., 2013).BPA-induced reproductive toxicity at dosages considered to be safe (5 or 25mg BPA/kg/day) were reported by Wisniewski *et al*. (2015) to compromise the spermatozoa and disrupts the hypothalamic–pituitary–gonadal axis, causing a state of hypogonadotropic hypogonadism.

2.3 MECHANISM OF ACTION OF BISPHENOL A

When BPA is ingested by humans, it is bio-transformed in the liver being the site for the breaking down of food particles into its first pass. The bisphenol A-glucuronide is a highly water soluble metabolite. This metabolite is then rapidly excreted by the kidneys with urine (Volkel *et al*., 2002). By monitoring BPA doses in healthy adults from ingestion to excretion, evidence shows that BPA’s half-life in the body is less than six hours and it is completely cleared from the body in 24 hours (Tsukioka *et al*., 2004; Volkel *et al*., 2002).

This rapid clearance from the body through urine makes total urinary species, comprised of free plus conjugated BPA, the most appropriate BPA exposure assessment marker (Melzer *et al*. 2010).

Dermal absorption and inhalation of BPA are part of the major ways of getting in contact with excessive amount of BPA and these are of great concern because exposures from these pathways are able to avoid the first-pass metabolism of the liver and enter the circulatory system directly (Vandenberg *et al*., 2007).

BPA exposure in infants and children is of great concern because their liver and kidneys are still developing. It is an established fact that the kidneys do not reach full maturation until two years of age. During their first year, the glomerular filtration volume of the kidneys develops while in the second year, the renal tubular function that excretes toxins increases to adult capacity (Yamano *et al*., 2008). As a result of the incomplete liver maturation, infant systems are unable to metabolize BPA through glucuronidation as adults do. It is theorized that infants metabolize BPA through a combination of glucuronidation and sulfation.

This mechanism for metabolizing BPA is plausible because BPA is a substrate for sulfation; and sulfotransferases, responsible for sulfation, develop earlier in neonates than UDP-glucuronosyltransferases, responsible for glucuronidation (Ginsberg and Rice, 2009).Regulations should consider, according to Ginsberg and Rice, (2009), the ability of fetal and neonatal deconjugation of BPA.

Research has shown that the fetus and placenta have glucuronidase, which has the ability to deconjugate BPA. Also in rats, the placenta has highlevels of glucuronidase activity resulting in fetal exposure to deconjugated BPA. Also, although neonates conjugate BPA with sulfate using sulfotransferases, research on endogenous hormones has shown that biological activity does not end with sulfation. There is no reason to believe that BPA is completely de-activated by sulfation either (Ginsberg and Rice 2009), and that this theory does not have enough scientific support (Vandenberg *et al*., 2007).It is an established fact that, mitochondria are the targets of several environmental toxicants (Michelangeli *et al*., 2008), a slight mitochondrial disorder may affect vital testicular functions (Chattopadhyay *et al*., 2010).

It has been observed that BPA exposure induces oxidative stress through generation of reactive oxygen species (Kabuto *et al*., 2004; Richter *et al*., 2007) and mitochondrial dysfunction (Nakagawa and Tayama,2000) in rats. However, the prevention of BPA-induced toxicity has beenshown to be mediated through the use of several antioxidants (Anjum *et al*., 2011; Jain *et al*., 2011). Some of the antioxidants reportedly used in combating the effect of BPA includes Lipoic acid (LA) as a protective agent against BPA-induced disruption of mitochondrial enzyme activities and oxidative stress state elaborated upon oral intake of BPA to rats. After oral intake of BPA in rats, it has been noted that it resulted in a declined serum testosterone, although this was in accordance with another reportshowing decreased testosterone production by BPA that was associated with inhibitionof luteinizing hormone secretion and low steroidogenic enzyme gene (Hardy,2004).

The intake of BPA reducedtesticular weight in rats; this is also in accordance with another study that reported a decrease in the testes weight due to BPA exposure (Yang *et al*., 2010). Although, the testicular total protein levels, was said to be on an increase in BPA-treated rats, the testicular fluid contains several stimulatory and inhibitory factors that selectively alter the protein secretion (Brooks, 1983). Thus an alteration in testicular protein level may be due to a reduction in the secretory activity of testes. Moreso, the report of rats treated with oral dose of BPA indicatinga reduction in testicular enzymes levels such as ACP, ALP and LDH, was affirmed. ACP is an enzyme that enact it effect through hydrolysis of compounds in acidic medium. Testicular ACP gene is very much constant or better still stable and is usually up-regulated by androgens and down-regulated by estrogens (Yousef *et al*., 2001). Testicular ALP enzyme on the other hand is primarily of testicular and epididymal origin (Turner and McDonell, 2003). The decline in ALP activity has been established, which indicated that BPA treatment may show decreased activity of testicular tissues. Although, testicular LDH enzyme is associated with the maturation of germinal epithelial layer of seminiferous tubules and associated with post-meiotic spermatogenic cells (Sinha *et al*., 1997). A reduction in testicular LDH enzymes of rats following BPA administration points to the interference of BPA with the energy metabolism in testicular tissues (Mollenhauer *et al*., 1990).

Research has shown an elevated mitochondrial LPO after BPA intoxication which is a peculiar manifestation of tissues oxidative damage (Esterbauer *et al*., 1991). The administration of BPA

resulted in the decline of the testicular antioxidant enzymes such as CAT, SOD, GR and GPx in rats. The depletion of the enzymatic antioxidative system strengthens the oxidative damage of testicular cellular membranes (Qu *et al.*, 2008). A decline in SOD activity helps the accumulation of superoxide radicals, which will also inhibit CAT enzyme (Kono and Fridovich, 1982). The declined CAT activity led to the augmentation of H_2O_2 generation (Aitken and Roman, 2008). The H_2O_2generated needs to be rapidly eliminated to prevent theoxidative damage to lipids, proteins and DNA (Aitken and Roman, 2008). The removal of H_2O_2 is affected by CAT or GPx enzymes, with the GPx playing a predominating role in testes (Peltola *et al.*, 1992).

The GPx enzyme is involved in catalyzing the reduction of H_2O_2 at the expense of low GSH

(Peltola *et al.*, 1992).The decline in GPx activity in the BPA-treated rats can be attributed to the high H_2O_2 generation or low GSH concentration (Scibior *et al.*, 2008). In addition, it has been affirmed that, LPOs resulted in binding with the nucleophilic amino acids leading to GPx depletion (Kehrer and Biswal, 2000).

GSH is the most essential freely available antioxidant, which acts mainly as an antioxidant and also play a role in catalytic cycles of several antioxidant enzymes such as GPx, GR and GST (Biswas and Rahman, 2009). The low level of GSH in BPA intoxicated rats may be due to the increased utilization of GSH in order to metabolize LPO by GPx or the interaction of GSH with free radicals (Flohe, 1982). This will results in increased superoxide radicals which in turn leads to oxidation and depletion of GSH associated with lipid peroxidation (Reed, 1990).

Marked decline in the activities of the testicular mitochondrial marker enzymes such as IDH, MDH, MAO, NDH and SDH was observed after exposing adult rats to BPA. Shaffi and Dubey, (1989) observed that reduction in activities of these mitochondrial marker enzymes could be due to slower enzyme synthesis which enhanced accumulation of metabolites and binding of the toxicants to the active site of enzymes.Alteration by BPA exposure in testicular mitochondria indicates an imbalance in mitochondrial energetics, especially the ATP production (Nakagawa and Tayama, 2000), because a reduction in ATP production can be a primary event caused by a direct effect on mitochondria, or a secondary event due to altered metabolism through any of several interactions of the compounds within the cell (Nakagawa and Tayama, 2000).

It has been shown that LA increased the TAS levels, reduced LPO and increased GSH and antioxidant enzymes levels, indicating its antiperoxidative and antioxidative effects (Chidlow *et al*., 2002; El-Beshbishy *et al*., 2011; Sivaprasad *et al*., 2004). Oehlmann *et al*., (2009)affirm that some invertebrates appear to be quite sensitive to BPA, and the adverse effects havebeen documented at environmentally tolerable concentrations (Oehlmann *et al*., 2009). However, the question is whether invertebrate estrogen receptors functionthe same manner as vertebrates (Brennan *et al*., 2006). Thus, it is not clear whetherBPA toxicity in these organisms occurs through the endocrine system or other mechanisms (Hutchinson, 2002).

Many reports have shown developmental effects in invertebrates at various exposure levels Both midge (Chironomus riparius) larvae and the marine copepod Tigriopus japonicus exhibited developmental inhibition at very low concentrations of BPA. (Marcial *et al*., 2003;Watts*et al*., 2003). However, it is not clear if these effects have any long-term impacts.

Biggers and Laufer, (2004) reported that higher exposure (11.4 mg/L BPA for one hour) induced premature larval metamorphosis and settlement in the marine polychaete worm Capitella capitata. Likewise, BPA concentrations of 12.5 and 60mg/L stimulated larval development in T *japonicus* (Mariager, 2001;Oehlmann *et al*., 2009). Conversely, the copepod Acartia tonsa exhibiteddevelopmental inhibition at BPA concentrations above environmentallyrelevant levels (100 mg/L) (Andersen *et al*., 1999). Even higher doses of BPA exposure (>300mg/L) caused developmental arrest and mortality in the sea urchin*Paracentrotuslividus* (Arslan and Parlak, 2008). At excessively high exposures (16,000 to 80,000mg/L), abnormal growth and inhibition of gemule germination was found in freshwater sponges *Heteromyeniasp*. and*Eunapius fragilis* (Hill *et al*., 2002). Extremely high exposures (10,000mg/kg to 1,000,000mg/kg soil) also resulted in reduced timeto molt and decreased overall growth in the terrestrial isopod Porcellio scaber(Lemos *et al*., 2009). Protein over-expression in the hepatopancreas, gut, and testes was also found at 10,000mg/kg soil inP. scaber(Lemos *et al*., 2009).

2.4 USES OF BISPHENOL A

Despite individual and industrial awareness about the danger(s) posed by the usage of Bisphenol A, materials and equipment containing Bisphenol A are still being widely used on a daily basis

across the globe.In manufacturing epoxy resins BPA is used additionally as a linkage, also in compounds that provide corrosion resistance, flexibility, and heat resistance. BPA is also employed in thermal receipts as a color developer (Geens *et al*., 2012; Liao and Kannan, 2011), serves as a component of dental composites (Joskow *et al*., 2006; Olea *et al*., 1996) , and also in medical devices (Calafat *et al*., 2009).Bisphenol A (2,2-bis-(4-hydroxyphenyl)propane; BPA) is broadly used as an industrial raw material for polycarbonate and epoxy resins(Brotons *et al*., 1995; Hashimoto *et al*., 2001; Olea *et al*., 1996).

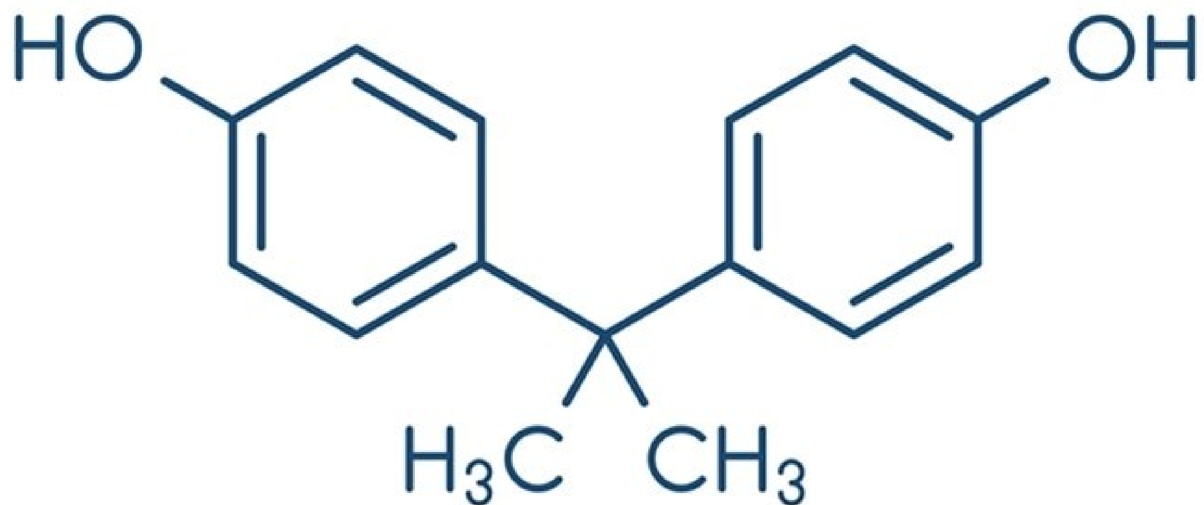
A halogenated derivative of Bisphenol A known as Tetrabromobisphenol A (2,2-bis-(3,5-dibromo-4-hydroxyphenyl)propane; (TBBPA), is also widely used globally as a flame retardant in numerous products. TBBPA was developed as a relatively non-toxic flame retardant (Helleday *et al*., 1999; Sellström and Jansson, 1995; Sjödin *et al*., 2001; Thomsen *et al*., 2001; Watanabe *et al*., 1983). Another derivative of Bisphenol A known as Tetrachlorobisphenol A (2,2-bis-(3,5-dichloro-4-hydroxyphenyl)propane; TCBPA) has been found in the effluent from waste-paper recycling plants (Fukazawa *et al*., 2001)

The purpose of can-coating is to protect the metal can from rust or corrosion which could allow microbes to enter its contents. Can linings are also added in order to protect food from acquiring a metallic taste and to maintain food's colour and texture. Additional performance requirements of can coatings are that they must be able to withstand the stresses of food processing including can distortion and high temperature during sterilization, as well as bending, without degrading or separating off of the metal can walls (Lakind, 2013; Noonan *et al*., 2011).Polycarbonate plastics containing BPA are often used in consumer products that are in contact with foods. These can be commonly found in temporary food storage containers such as Tupperware and reusable water bottles, as well as in plastic serving dishes and plastic serving utensils.

2.5 CHEMICAL STRUCTURES AND PROPERTIES OF BISPHENOL A

BPA is formed through the process of condensation of phenol with acetone; it has a low vapor pressure, high melting point and moderate solubility (Cousins *et al*., 2002; Howard, 1989; Shareef *et al*., 2006).It has been reported that base on the log KOW values that range from 2.20 to 4.16 (Dorn *et al*., 1987;Shao *et al*., 2007; Staples *et al*., 1998; Tsai, 2006; Yoon *et al*., 2003),

BPA is considered to have low (Heinonen *et al*., 2002) or moderate (Cousins *et al*., 2002) hydrophobicity and thus a modest capacity for bioaccumulation.Bisphenol is a colourless liquid at room temperature and has been estimated to have about 3.4*10-5 pa at 20°C. The natural constituents of Bisphenol A in water influence its solubility.it readily forms more or less colloidal dispersion in water.



Source: Howard, 1989

Table 2.1 PHYSICO-CHEMICAL PROPERTIES OF BISPHENOL A

Property	Value
Physical state	Colourless oily liquid
Melting point	-55oc or -50oc
Boiling point	230oc at 5mmHg 385oc at 1013hpa
Density	0.98g/cm3 at 20oc
Vapor pressure	0.000034 Pa at 20oc
Water solubility	3ug/l at 20oc
Partition coefficient	7.5
Conversion factor air	1ppmv= 16.2 mg/m3 at 20oc and 1013 hPa
Flash point	200oc
Auto flammability, ignition temperature	370oc
Explosive properties Explosive limits	0.15-0.18 vol. % 0.3-49vol.%
Viscosity, dynamic	81mPa s at 20oc 58mPa s at 25oc
Henry's constant	4.43Pa m3/mol

Source: Santos *et al.* (2005)

2.6 REGULATIONS ON THE USE OF BPA

Different countries have assessed the risk of environmental exposure to BPA. Developed nations such as the United States of America and those of the European Union has nearly one-third and one-quarter of global BPA production respectively (International Conference on Information System, 2008). But the most amazing thing is despite the adverse effect and the risk level of BPA and also considering the level of development of the above mentioned nations, BPA is still released into the environment and not strongly regulated in either the US or EU (National Institute of Health, 2008).

Since more than 50 endocrine disrupting chemicals are approved for use in US and EU, (Muncke, 2009) it is essential that the regulatory toxicology testing levels reflects what is needed to accurately assess risk of food contact with endocrine disrupting chemicals. Despite the effort of the legislative to ban BPA for years, the United States government was much slower to act in removing BPA from infant feeding systems. Bisphenol A is one of a burgeoning class of chemicals that do not fit well into the current US chemical regulatory structure. Out of almost 87,000 chemicals manufactured or imported into the US, just about 1000 are regulated under any specific US chemical policy (US Government Accounting Office, 1994).

Although the safety of BPA used in food packaging has been the topic of discussion in the scientific community since the 1990's, the ban enacted on BPA by the Canadian government in the year 2008 made BPA to be moved into the mainstream spotlight, and this also resulted in the ban of the usage of BPA in production of baby bottles, since it has been reported that the most common route of exposure to BPA is ingestion or parenteral and the most affected set of people are the prenatal and neonates which then leads to all manner of abnormalities most especially reproductive effect.

After the successful ban of BPA by the Canadian government, many countries quickly followed suit in proposing bans of BPA, with success in Turkey, France, Sweden, and Denmark. Also In 2011, bans of BPA in China and Malaysia were enacted. BPA has been approved for use in food packaging under food additive regulations since the 1960's. There is no specific migration limit for BPA or a restriction in the amount of BPA that can be in a final product.Bisphenol-A, which may leach or migrate into food from its packaging, is regulated by the Food and Drug

Administration (FDA). Also in 2009, FDA commenced regulation of BPA containing epoxy-based enamels and coatings used as inner linings for canned foods.

But it has been shown that current regulations for new packaging materials being added to the marketplace are more stringent, but since BPA has already been approved for use, any manufacturer can start using BPA without approval from the FDA. They also do not need to disclose any information about their formulations. The manufacturer's submittal of a food contact notification for their current uses of BPA is voluntary and not mandatory (FDA, 2013).

Pharmaceuticals and food additives may be strictly assessed with regards to their major intended uses and exposures. However, in the absence of convincing evidence of acute toxicity, the hundreds of new chemicals produced each year are generally put into use with little or no regulation and end-of-life issues are not usually addressed (Breggin and Pendergrass, 2007). In 2010, the US EPA released a report acknowledging the large amounts of BPA released into the environment (US Food and Drug Administration, 2010). The report states that while there is uncertainty in the interpretation of low-dose effects of BPA, environmental concentrations of BPA may pose some threat to aquatic organisms. The US Food and Drug Administration has also changed its rating of BPA from "generally considered safe" to a chemical of "some concern," indicating that US regulatory agencies are concerned about potential effects of BPA on human (US Food and Drug Administration, 2010).

2.7 MELATONIN

Melatonin (N-acetyl-5-methoxytryptamine) is the major secretory product of the pineal gland and has powerful antioxidant activity (Reiter, Manchester, and Tan, 2010). Anjum *et al*. (2011) reported that Melatonin at 10mg/kg administered intra-peritoneally for 14 days mitigated Bisphenol A-induced biochemical pertubations in testicular mitochondria of mouse due to its antioxidant property or by the direct free radical scavenging activity by lowering lipid peroxidation in the mitochondria. It also restored the activity of mitochondrial marker enzymes and ameliorated decreased enzymatic and non-enzymatic antioxidants of mitochondria. Melatonin readily scavenges the most toxic free radical, the hydroxyl radical, and directly the peroxynitrite anion, nitric oxide, singlet oxygen, and the peroxyl radical. Melatonin stimulates the mRNA levels of antioxidant enzymes including superoxide dismutase (Kolter *et al*., 1998).

Glutathione peroxidase and glutathione reductase antioxidant enzymes are also stimulated by melatonin (Reiter, 1998).Melatonin hinders the pro-oxidative enzyme, nitric oxide synthase, chelates transition metal ions, inhibits the deterioration of cellular membranes (Catala, 2007) and reduces lipid peroxidation(Reiter, 1998).

Othman *et al*. (2016) found out that Melatonin at a dose of 10 mg/kg body weight protected against BPA-induced apoptosis by controlling Bcl-2 expression and ameliorating oxidative stress in the testes and sperm of rats. Melatonin abrogates nonylphenol-induced testicular dysfunction in Wistar rats (Tabassum *et al*., 2016). Melatonin can clear off the hydroxyl, peroxyl radical, hypochlorous acid and singlet oxygen, all of which cause cellular damage. The direct effects of melatonin on the male reproductive system and testosterone synthesis from Leydig cells have also been studied on animals. Because melatonin binding sites have been detected in the reproductive system of many species, it also seems reasonable to assume that melatonin exerts its actions through direct interaction with the steroidogenic cells of the reproductive organs (Oner-Iyidogan, Gurdol, & Oner, 2001). Such properties have suggested the potential use of MLT as a therapeutic agent.

2.7.1 DIRECT ANTIOXIDANT ACTIONS OF MELATONIN

Melatonin seems to function via a number of means to reduce oxidative stress. Thus, the experimental evidence supports its actions as a direct free radical scavenger (Hardeland et al., 1993; 1995; Allegra et al., 2003), as an indirect antioxidant when stimulating antioxidant enzymes (Reiter et al., 2000c; Rodriquez et al., 2004), its stimulation of the synthesis of glutathione (an essential intracellular antioxidant) (Urata et al., 1999), its ability to augment the activities of other antioxidants (or vice versa) (Gitto et al., 2001a), its protection of antioxidative enzymes from oxidative damage (Mayo et al., 2002; 2003), and its ability to increase the efficiency of mitochondrial electron transport chain (ETC) thereby lowering electron leakage and reducing free radical generation (Acu a-Castroviejo et al., 2002; Okatani et al., 2003a). While melatonin has proven highly effective in lowering molecular damage under conditions of elevated oxidative stress (Reiter, 1998; Reiter & Tan, 2003), the contribution of each of the above-mentioned processes to the ability of this indole to restrain the resulting molecular mutilation that accompanies exaggerated free radical generation remains unknown.

CHAPTER THREE

MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Forty (40) adult male albino rats weighing 160 ± 10g were used in this study. The animals were obtained from the Experimental Animal House of the Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria. Animals were kept in cages (60 × 60 × 50 cm). All animals were kept under controlled conditions of temperature (25 ± 2.0°C), relative humidity (50 ± 15%) and normal photoperiod (12 hour light and 12 hour dark). The animals were fed on a standard rat diet (commercial pellet) and water provided *ad libitum*.

3.2 CHEMICALS

BisphenolA (BPA) and Melatonin (MLT) were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). All other chemicals used in this study were of the highest available grades.

3.3 EXPERIMENTAL PROTOCOL

Anjum *et al*. (2011) demonstrated that BPA at standardized oral dose of 10 mg/kg body weight per day in rats exposed for 45 days produced marked decrease in testes weight, total testicular protein content, testicular enzymes, serum testosterone, total antioxidant status and decline in activities of mitochondrial enzymes as well as reduction in spermatozoa parameters. Hence this study adopteda dose of 10 mg/kg BW/day for reproductive and developmental toxicity (El-Beshbishy *et al*., 2013). Also, melatonin (10 mg/kg body weight/ day) was administered via the intra-peritoneal route as a preventive antioxidant in rats (Anjum *et al*., 2011; Othman *et al*., 2016). The rats were exposed to BPA and concomitant melatonin supplementation for 45 days.

The 40 adult male rats were randomly assigned into four groups of ten animals each as follows:

- **Group A:** Rats were orally administered daily with 0.2 ml of olive oil as the control.
- **Group B.** received 10 mg/kg/day intra-peritoneal melatonin (Sigma–Aldrich P98% pure, dissolved in 0.5% ethanol in normal saline).
- **Group C**. (BPA-intoxicated rats): dose were 10 mg/kg per day body weight suspended in 0.2 ml olive oil, orally
- **Group D**(BPA-Melatonin-treated rats): Rats were orally administered BPA (10 mg/kg per day body weight) and concomitantly with intra-peritoneal Melatonin (10 mg/kg body

weight).

All treatments lasted for45 days in order to evaluate the effect of BPA through a complete spermatogenic cycle which takes approximately 45 days in rodents (Chitra *et al*., 2003). About 24 hours after the last treatment, the rats were weighed and blood samples were collected for serum hormonal analysis before the rats were euthanized according to Chitra *et al*.(2003).

3.4 SAMPLE COLLECTION

3.4.1 Blood sampling and steroid hormone analysis

Blood samples were collected via peri-orbital venous bleeding (venipuncture) into sterile heparinized sample tubes and kept on ice. The blood samples were subsequently transferred into microfuge tubes and centrifuged at 3000 rpm for 15 min at 25 °C. The plasma fraction (supernatant) were collected and stored in 1.5 mL Eppendorf vials at −20 °C.

Following animal sacrifice, a mid-caudoventral abdominal incision was made with sterilized scissors, permitting instant access to the testis once pushed upward from the scrotum. The testes were then separated from the epididymis as described by Oyeyemi and Fayomi, (2011).

Semen samples for spermatozoa analysis were collected from the left caudal epididymis while samples for histology, immunohistochemistry as well as transmission electron microscope were taken from the right testis of each animal in the respective groups.

SECTION ONE

3.5 PROTECTIVE EFFECTS OF MELATONIN AGAINST BPA-INDUCED EPIDIDYMAL SPERM ANOMALIES

Semen sample were collected from the left caudal epididymis through an incision made with a scalpel blade as described by Oyeyemi and Fayomi, (2011). The semen was dropped on warm glass slide and stained using warm Wells and Awa stains for morphological studies. Staining for live/dead ratio were carried out using Eosin-Nigrosin stain. Also, percentage motility were carried out using 2 to 3 drops of 2.9% warm buffered sodium citrate kept at body temperature as described by Zemjanis (1970). The carcasses of the rats were disposed and burnt in an incinerator at the Pathology Department, Faculty of Veterinary Medicine, University of Ibadan

3.5.1 Sperm Motility

This was evaluated using a drop of semen with a drop of 2.9% warm sodium citrate on a clean warm glass slide covered with a glass slip and viewed under a light microscope at a magnification of x40. Only sperm cells moving in a unidirectional progressive motion were included in the motility counts, while sperm cells moving in circles, in backward direction or pendulating movement were excluded as described by Oyeyemi and Fayomi, (2011).

3.5.2 Percentage Sperm Viability

This was carried out as described by Zemjanis, (1970). It involves staining one drop of semen and a drop of warm Eosin-Nigrosin stain on a clean, warm slide. A thin smear was then made of mixture of semen and stain. The smear was air dried and observed under the microscope. The ratio of the *in vitro* dead sperm cells was observed and it were based on the principle of Eosin penetrating and staining the dead autolysing sperm cells whereas viable sperm repelled the stain.

3.5.3 Epididymal sperm morphology assessment

A drop of the sperm suspension was placed on a glass slide, smeared, dried, fixed with absolute ethyl alcohol, and stained with Eosin-Nigrosin for microscopy. One thousand sperm cells per animal were observed and classified into normal and different types of abnormal sperm using an optical microscope. Then, the percentage of abnormal sperm was calculated.

3.5.4 Sperm count

Sperm from the left and right testes and epididymis was kept separate and assessed using an inverted light microscope. The spermatozoa were counted by hemocytometer using the improved Neubauer (Deep 1/10 mm, LABART,Germany) chamber as described by Pant *et al.*(2004).

3.5.4 Statistical Analysis

The data generated was analyzed using the Test of Homogeneity of variance, multiple comparisons and Analysis of variance (One-Way ANOVA). SPSS Version 15 for Windows (SPSS Inc, 2006) and Microsoft Excel Professional Plus (Microsoft Corporation, 2010) were used to carry out all procedures.

SECTION TWO

3.6 PROTECTIVE EFFECTS OF MELATONIN AGAINST BPA-INDUCED EPIDIDYMAL SPERMATOZOA OXIDATIVE STRESS

Epididymal sperm suspension

The epididymal sperm suspension was centrifuged at 225 x g for 10 min at 4 °C and the pellet were re-suspended in normal saline. The sperm was homogenized with the help of a glass-teflon homogenizer for 10 second and centrifuged at 800 x g for 10 min at 4 °C. The supernatant was used as an enzyme source for biochemical studies. Proteins were estimated by the method of Lowry *et al*. (1951) and DNA by the method of Burton (1956).

The antioxidant parameters were estimated by the following methods:

3.6.1 DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

The levels of SOD activity was determined by the method of Misra and Fridovich,(1972).

Principle:

Superoxide anion generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adenochrome and the yield of adenochrome produced per superoxide anion introduced increasing concentration of epinephrine. The ability of superoxide dismutase to inhibit the autoxidation at pH 10.2 makes this reaction a basis for a simple assay of the enzyme. The result led to the proposal that autoxidation of epinephrine proceeds by at least two (2) distinct pathways only one of them is a free radical chain reaction involving superoxide radicals and hence inhibitable by superoxide dismutase.

Preparation of reagents

a) 0.05M Carbonate buffer (pH 10.2)

3.58 g of $Na_2CO_3.10H_2O$ and 1.05 g of $NaHCO_3$ were dissolved in 200ml of distilled water. The pH was adjusted to 10.2 and then made up to 250 ml with distilled water.

b) 0.3mM Adrenaline

0.01 g of adrenaline (epinephrine) was dissolved in 200ml distilled water. It was prepared fresh when needed.

Protocol

1 ml of sample was diluted in 9 ml of distilled water to make a 1 in 10 dilution. An aliquot of0.2 ml of the diluted sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds (Misra and Fridovich, 1972).

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_0}{2.5}$$

Where A_0 = absorbance at zero second

A_3 = absorbance after 150 seconds

$$\% \text{ inhibition} = 100 \times \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance for blank}}$$

The unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

3.6.2 DETERMINATION OF CATALASE ACTIVITY

Catalase activity was determined according to the method of Claiborne (1985).

Principle

The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this

wavelength, its absorbance correlated well enough with concentration to allow its use for quantitative assay. An extinction coefficient of0.0436 $mM^{-1}cm^{-1}$ (Noble and Gibson, 1970) was used.

Reagents

Phosphate buffer (0.05 M, pH 7.4)

Dipotassium hydrogen phosphate trihydrate (0.696g) and potassium dihydrogen phosphate v (0.265g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

Hydrogen peroxide (19 mM)

194 µl of 30% ofH_2O_2 was added to 50 ml of 0.05 M phosphate buffer, pH 7.4and the volume made up to 100 m with the same.

Procedure

Hydrogen peroxide (2.95 ml of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 50 µl of sample was added. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240nm every minute for 5 min.

Calculation

$$\text{Catalase activity} = \frac{\Delta A_{240}/\text{min x reaction volumr x dilution factor}}{\text{0.0436 x sample volume x mg protein/ml}} = \mu\text{mol H2O2/min/mg protein}$$

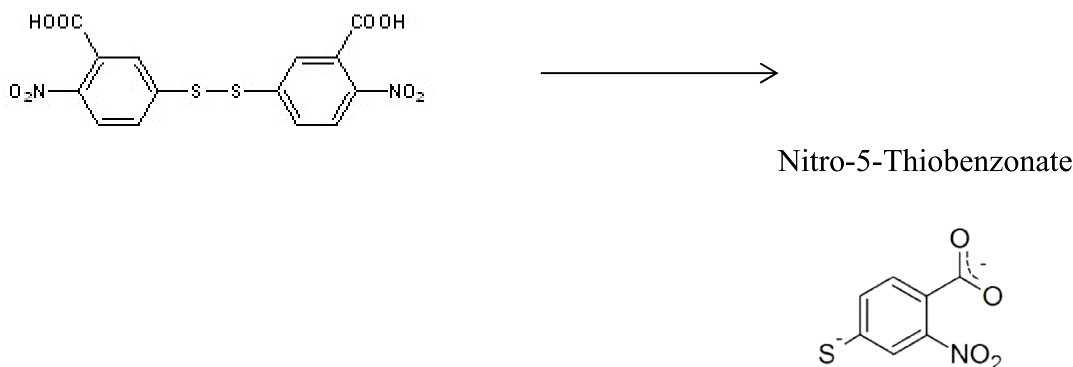
3.6.3 ESTIMATION OF REDUCED GLUTATHIONE (GSH) LEVEL

The method of Beutler, (1963) was followed in estimating the level of reduced gluthathione (GSH).

Principle

This method is based upon the development of a relatively stable yellow coloured product when 5.5-dithiobis-2-nitrobenzoic acid (DTNB; Ellman's reagent) is added to sulfhydryl compounds of which gluthione comprises the bulk in tissues. The resulting chromophoric product possess maximum absorbance at 412 nm.

GSH



Ellman Reagent

Figure 3.6.3.1: Reaction of reduced GSHwith Ellman Reagent

PREPARATION OF REAGENTS

1. **GSH working standard**

   40mg GSH (Sigma, Mol. Weight 307.3g) was dissolved in 100ml of 0.1M phosphate buffer, pH 7.4, and then stored in the refrigerator.

2. **Phosphate buffer (0.1 M, pH 7.4)**

   Dipotassium hydrogen phosphate trihydrate (2.098 g) and potassium dihydrogen phosphate (0.791 g) were dissolved in 120 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 150 ml with distilled water.

3. **Ellman's Reagent**

   60 g Ellman's reagent was dissolved in 0.2 M phosphate buffer, pH 7.4 and made up to150 ml with the same.

4. **Precipitating agent, Sulphosalicylic Acid (4% solution)**

   0.8 g of sulphosalicylicacid was dissolved in 20 mlof distilled water.

   Calibration of GSH standard curve

   Procedure

   Serial dilutions of the GSH stock solution were prepared as shown in the table 3.6.3.1 below. The absorbance of the yellow colour formed upon the addition of Ellman's reagent was read within 30 min at 412 nm against a blank of 1.5 ml of Ellman's reagent and 0.5 ml phosphate buffer. A plot of absorbance against concentration of reduced GSH was then plotted.

Table 3.6.3.1: preparation of GSH Standard curve

GSH Stock (ml)	Phosphate Buffer (ml)	Ellman's Reagent (ml)	GSH Conc. (µg/ml)
0.01	0.49	1.5	1
0.03	0.47	1.5	3
0.05	0.45	1.5	5
0.10	0.40	1.5	10
0.15	0.35	1.5	15
0.20	0.30	1.5	20

Procedure for samples

0.4 ml of sample was added to 0.4 ml of precipitating solution which was vortexed and centrifuged at 4000 rpm for 5 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman's reagent. The absorbance of the reaction mixture was read at 412 nm against reagent blank.

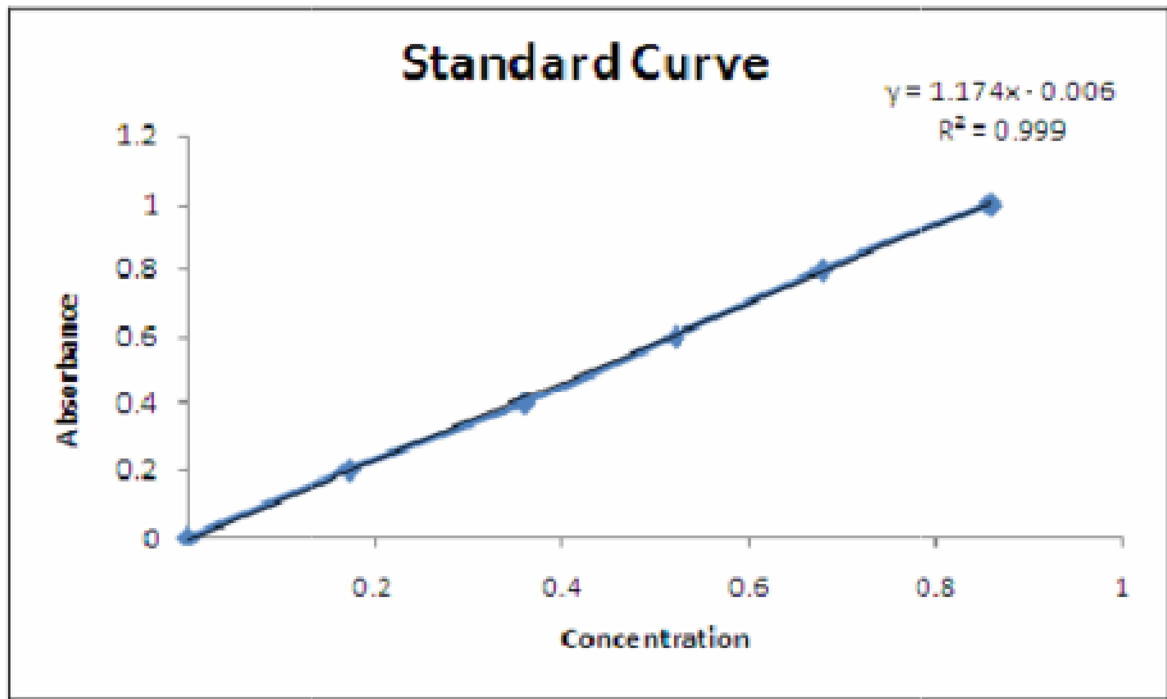


Figure 3.6.3.2: GSH Standard curve

3.6.4 HYDROGEN PEROXIDE GENERATION

Hydrogen peroxide generation was determined according to the method of Wolff (1994)

Principle

This is based on the conversion of ferrous oxide to ferric ion in the presence of xylenol orange as indicator.

PREPARATION OF REAGENT (FOX-1)

1) **100µmol/L Xy;enol orange(MW = 760.6)**

   1.015 of xylenol orange was dissolved in 200mls of distilled water

2) **250mMol/L ammonium ferrous sulfate (MW = 392.14)**

   0.02g of ammonium ferrous sulfate was dissolved in 200mls of distilled water.

3) 100 mM/L sorbitol (MW = 182.2)

3.64g of sorbitol was dissolved in 200mls of distilled water

4) 250mMol/L H_2SO_4

1ml of 1M H_2SO_4 was made up to 40mls with distilled water.

Table 3.6.4.1: Hydrogen peroxide general Protocol

Reagents	Volume
Buffer	2.5mls
AFS	250µL
Sorbitol	100 µL
Xylenol	100ml
H2SO4	25 µl
Sample	50 µL

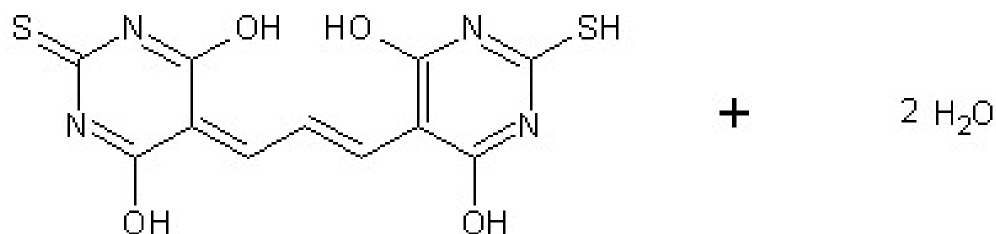
The mixture was vortexed and incubated at room temperature for a mimimun of 30 minutes before reading the absorbance at 560nm.

3.6.5 ASSESSMENT OF LIPID PEROXIDATION

Lipidperoxidation was determined by measuringthe thiobarbituric acidreactive substances (TBARS) produced during lipid peroxidation according to Varshney and Kale (1990).

Principle

This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde; an end product of lipid peroxide during peroxidation. On heating in acidicpH, the product is a pink complex which absorbs maximally at532nm and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often usedto calibrate this test and thus the resuts are expressed as the amount of free MDA produced.



Structure of TBA + MDA -------- MDA-TBA (pink coloured complex)

Figure 3.6.5.1: MDA reaction in lipid peroxidation assay

PREPARATION OF REAGENTS

1. **30% Tricholoroacetic acid (TCA)**
   9g of TCA (CCL3COOH) was dissolved in distilled water and made up to 30 m with same.
2. **0.75% Thiobarbituric acid(TBA)**
   This was prepared by dissolving 0.23g of TBA in 0.1M HCL and made up to 30ml with same.
3. **0.1 5mM Tris-KC1 buffer (pH 7.4)**
   1.12g of KC1 and 2,36g of Tris base were dissolved separately in distilled water and made up to 100ml with same. The pH was then adjusted to 7.40.

   Procedure

   An aliquot of 0.4ml of the sample was mixed with 1.6ml ofTris-KC1 buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% of TBA was added and placed in a water bath for 45 minutes at 80°C.This was then cooled in ice and centrifuged at 3000g. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm. Lipid peroxidation in units/mg protein or gram tissue was computed with molar extinction coefficient of 1.56 x 105M-1Cm-1

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532mm} \text{ x volume of sample x mg protein}}$$

3.6.6 GLUTATHIONE PEROXIDASE ASSAY (GPX)

Glutathione peroxidase assay was determined according to the method of Rotruck *et al.*(1973), with some modifications, whichis based on the reaction between glutathione remaining after the reaction of GPx.

PREPARATION OF REAGENTS

1. **10mM Sodium azide (NaN_3)**
   0.0325g of NaN_3 was dissolved in 50ml of distilled water

2. **4mM Reduced glutathione (GSH)**

   0.0246g of GSH was20ml of phosphate buffer

3. **2.5mM Hydrogen peroxide (H2O2)**

   28 µl H_2O_2 was diluted in 100ml of distilled water

4. **10% Trichloroaccetic acid (TCA)**
   2g of TCA was dissolved in ml of distilled water and made up to 10m

5. **0.3M K_2HPO_4**
   5.23g was dissolved in 70m of distilled water and made up to 100ml

6. **DTNB**

   0.04g DTNB was dissolved in 100ml of phosphate buffer

7. **Phosphate buffer**

   0.992g of K_2HPO_4 and 1.946g of KH_2PO_4 was dissolved in 200ml of distilled water, and pH was adjusted to7.4

Table 3.6.6.1: Procedure for GSH-Px Assay

Reagents	Volume
Phosphate Buffer	0.5ml
NaN3	0.1ml
GSH	0.2ml
H2O2	0.1ml
Sample	0.5ml
Distilled water	0.6ml

The mixture was incubated at 37°C for 3 minutes and 0.5ml of TCA was added. This was Spined at 3,000gfor5 minutes. To 1ml of supernatant 2ml of K_2HPO_4 and 1ml of DTNB was added. Absorbance was at 412nm against a blank. Blank (2ml ofK_2HPO_4 + 1 ml of DTNB).

3.6.7 Statistical analyses

Statistical analyses were performed using Oneway Analysis of Variance (ANOVA) followed by unpaired Student's t -test. Differences were considered to be significant at PB/0.05 against control group. The data were presented as mean± SD

SECTION THREE

3.7 PROTECTIVE EFFECTS OF MELATONIN AGAINST BPA-INDUCED EPIDIDYMAL INJURY AND ALTERED PROTEIN LOCALIZATION

This study involved histology, histomorphometry, immunohistochemistry as well as Transmission Electron Microscopy (TEM) of the epididymal samples (taken from the right testes).

3.7.1 Morphological analysis of epididymis using H & E staining

Epididymal samples (from the right testes) were collected immediately fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for haematoxylin and eosin (H & E) or immunohistochemical staining.

Paraffin-embedded epididymis were sectioned on a microtome into 5 μm-thick sections and stained with hematoxylin and eosin for histological examination of testis morphology. Stained sections were individually examined under a bright field Olympus BX63 light microscope (Olympus Corporation, Tokyo) at ×40 and ×100 (oil immersion) magnification. All testicular evaluations were performed in accordance with the recommended approaches for the evaluation of testicular toxicity (Creasy, 2003).

3.7.2 Immunohistochemistry

Immunohistochemistry for the localization of Smooth Muscle Actin (SMA), S-100 protein and Vimentin (Vm) was carried out as reported by Olukole *et al*. (2018). Epididymal sections were placed on super frost slides, de-waxed in xylene and rehydrated through decreasing concentrations of alcohol. Following initial blocking of endogenous activity using 3% hydrogen peroxide, antigen retrieval from slides were carried out by heating epididymal sections in 0.1M Citrate buffer ($_P$H 6.0) in a microwave at 750 W for three cycles of 7 minutes each. Slides were then allowed to cool for 20 minutes after which they were washed with Phosphate-buffered solution (PBS, $_P$H 7.2) thrice for 5 minutes each. Further permeabilization of slides was carried out in 0.1 M phosphate buffer containing 0.3% (v/v) Triton X-100 (Sigma, USA) for 10 minutes. Normal goat serum supplied with the Immunocruz mouse staining kit was used in blocking the slides for 1 hour prior to incubation with primary antibodies to SMA (1:200), S-100 (1:2000) and Vm (1:200), being incubated overnight at 4 °C in a humidified chamber. For the negative control, adjacent section on same slide was incubated with preimmune sera. This was followed

by washing of slides with PBS after which slides were incubated with biotinylated goat anti-mouse secondary antibody for 90 minutes. The slides were then washed with PBS thrice for 5 minutes each followed by incubation with a streptavidin-horseradish peroxidase complex (Immunocruz kit) for 30 minutes. Again, sections were them washed with PBS thrice for 5 minutes each and immunostaining was visualized following the addition of 0.05% (w/v) 3, 3, 9-diaminobenzidene (DAB) tetra-hydrochloride solution (Sigma, USA). Counterstaining of sections was carried out using Mayer's haematoxylin, mounted and visualized using a bright-field light microscope. Thereafter, images were captured using a light microscope (Olympus BX63 with a DP72 camera). Using 10 non-overlapping fields for each animal, the percentage positive contributions of each antibody in the epididymis was determined with the aid of the image analyser computer system, Lecia Qwin 500 C (Cambridge, UK) as reported by El-Ghamrawy et al. (2014).

3.7.3 Transmission Electron Microscopy

Slide preparation for TEM was carried out as reported by Olukole and Oke (2018). Briefly, testicular tissues were fixed in glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hours at 4 ^{0} C. The samples were then thoroughly washed in the same buffer, post-fixed in 1% osmium tetroxide, and subsequently dehydrated in a graded series of ethanol solutions. Tissues were then cleared with propylene oxide, infiltrated with a 1:1 solution of propylene oxide:epoxy resin, 1:2 solution of propylene oxide:epoxy resin, and then placed in 100% epoxy resin for 36 hour under vacuum. The samples were embedded in fresh epoxy resin and cured at 60 ^{0} C for 48 h. Semi-thin sections were stained with toluidine blue and observed under the light microscope (Olympus BX63 with a DP72 camera). Utra-thin sections (70-80 nm) were cut with a diamond knife on an ultramicrotome (Ultracut- Reichert, Austria). The sections were then double-stained with uranyl acetate and lead acetate. The copper grids were examined under a transmission electron microscope (Philips CM 10 TEM) operating at 80 kv. Representative micrographs of different sections of the testis were taken using a Gatan 785 Erlangshen digital camera (Gatan Inc., Warrendale, PA). Analysis and assembling of composite micrographs were carried out using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

3.7.4 Statistical analyses

Statistical analyses were performed using Oneway Analysis of Variance (ANOVA) followed by unpaired Student's t -test. Differences were considered to be significant at PB/0.05 against control group. The data were presented as mean± SD. for ten animals per group.

SECTION FOUR

3.8 PROTECTIVE EFFECTS OF MELATONIN AGAINST BPA-INDUCED ALTERATIONS IN SPERMATOGENESIS

3.8.1 ELISA for Steroidogenic Hormones

Commercial kits were used to quantify serum hormones in triplicatesto avoid errors due to inter-assay. Testosterone (T) and Dehydroepiandrosterone (DHEAS) concentrations in serum were determined using ELISA kits (MP Biomedicals, Ohio, USA) according to instructions by manufacturer (Wei *et al..,* 2011). The Beckman Coulter ACCESS 2 immunoassay system (Beckman Coulter, Fullerton, CA USA) kit was used to quantify estradiol (E). FSH levels in serum were assayed using FSH ELISA kit according to the manufacturer's instructions (Rapid Labs. Ltd, Colchester, Essex, UK) (Wei *et a*l.., 2011). Data for T and DHEAS were expressed in nmol/L and µmol/l respectively while those for E and FSH were expressed in Pmol/L and Iµ/L respectively.

3.8.2 Histopathology of the testis

At necropsy, the location of the testis was observed in all the groups and was rapidly excised and visually inspected for any evidence of gross morphology. Each testis was then weighed using an electronic weighing scale. The gonadosomatic index (GSI) was estimated: testis weight / body weight] × 100.

Testicular samples (from the right testis) were collected immediately fixed in 4% buffered neutral formalin, embedded in paraffin, and sectioned for haematoxylin and eosin (H & E).

Paraffin-embedded testes were sectioned on a microtome into 5 µm-thick sections and stained with hematoxylin and eosin for histological examination of testis morphology. Stained sections were individually examined under a bright field Olympus BX63 light microscope (Olympus Corporation, Tokyo) at ×40 and ×100 (oil immersion) magnification for histo-pathological changes such as inflammation, testicular atrophy, lymphocytic infiltration, degeneration and necrosis of Sertoli, Leydig and spermatogenic cells. All testicular evaluations were performed in accordance with the recommended approaches for the evaluation of testicular toxicity (Creasy, 2003).

3.8.3 Transmission Electron Microscopy

Additional testicular tissues were fixed in glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hours at 4 ^{0}C. The samples were then thoroughly washed in the same buffer, post-fixed in 1% osmium tetroxide, and subsequently dehydrated in a graded series of ethanol solutions. Tissues were then cleared with propylene oxide, infiltrated with a 1:1 solution of propylene oxide:epoxy resin, 1:2 solution of propylene oxide:epoxy resin, and then placed in 100% epoxy resin for 36 hour under vacuum. The samples were embedded in fresh epoxy resin and cured at 60 0 C for 48 h. Semi-thin sections were stained with toluidine blue and observed under the light microscope (Olympus BX63 with a DP72 camera). Ultra-thin sections (70-80 nm) were cut with a diamond knife on an ultramicrotome (Ultracut- Reichert, Austria). The sections were then double-stained with uranyl acetate and lead acetate. The copper grids were examined under a transmission electron microscope (Philips CM 10 TEM) operating at 80 kv. Representative micrographs of different sections of the testis were taken using a Gatan 785 Erlangshen digital camera (Gatan Inc., Warrendale, PA). Analysis and assembling of composite micrographs were carried out using Adobe Photoshop CS5, Adobe Systems, San Jose, CA (Olukole and Oke, 2018).

3.8.4 Immunohistochemistry

Immunohistochemistry for the localization of αSmooth Muscle Actin (SMA), S-100 protein and Vimentin (Vm) was carried out as reported by Olukole *et al.* (2018). Testicular sections were placed on super frost slides, de-waxed in xylene and rehydrated through decreasing concentrations of alcohol. Following initial blocking of endogenous activity using 3% hydrogen peroxide, antigen retrieval from slides were carried out by heating sections in 0.1M Citrate buffer (pH 6.0) in a microwave at 750W for three cycles of 7 min each. Slides were then allowed to cool for 20 min after which they were washed with Phosphate-buffered solution (PBS, pH 7.2) thrice for 5 min each. Further permeabilization of slides was carried out in 0.1M phosphate buffer containing 0.3% (v/v) Triton X-100 (Sigma, USA) for 10 min. Normal goat serum supplied with the Immunocruz mouse staining kit was used in blocking the slides for 1 h prior to incubation with primary antibodies to αSMA (1:200), S-100 (1:2000) and Vm (1:200), being incubated overnight at 4 °C in a humidified chamber. For the negative control, adjacent section on same slide was incubated with preimmune sera. This was followed by washing of slides with

PBS after which slides were incubated with biotinylated goat anti-mouse secondary antibody for 90 min. The slides were then washed with PBS thrice for 5 min each followed by incubation with a streptavidinhorseradish peroxidase complex (Immunocruz kit) for 30 min.
Again, sections were then washed with PBS thrice for 5 min each and immunostaining was visualized following the addition of 0.05% (w/v) 3, 3, 9-diaminobenzidene (DAB) tetra-hydrochloride solution (Sigma, USA). Counterstaining of sections was carried out using Mayer's Haematoxylin, mounted and visualized using a bright-field light microscope.
Thereafter, images were captured using a light microscope (Olympus BX63 with a DP72 camera). Using 10 non-overlapping fields per rat, the percentage positive contributions of each antibody in the testis gland was determined with the aid of the image analyser computer system, Lecia Qwin 500 C (Cambridge, UK) as reported by El- Ghamrawy *et al.* (2016).

3.8.5 Statistical Analysis

The data generated was analyzed using the Test of Homogeneity of variance, multiple comparisons and Analysis of variance (One-Way ANOVA). SPSS Version 15 for Windows (SPSS Inc, 2006) and Microsoft Excel Professional Plus (Microsoft Corporation, 2010) were used to carry out all procedures. Significance was set at ($P < 0.05$)

CHAPTER FOUR
RESULTS

4.1 SECTION ONE

4.1.1 Body and organ weights of rats in different treatment groups

BPA did not induce any significant difference (P>0.05) on the body weight of the rats (Table 1). However, there was general decrease in the body weight of BPA-treated rats compared to the control and MLT-treated rats. Also, there was no significant difference (P>0.05) in the body weight of the control and BPA+MLT-treated rats. There was no significant difference (P>0.05) between theepididymal weight of the control and BPA-treated rats. BPA induced a significant decrease(P<0.05) in testicular weight compared to the control rats. However, no significant differences were observed between the control, MLT-treated and BPA+MLT-treated rats in terms of testicular weight. Concomitant treated with MLT protected against the BPA-induced decrease in testicular weight.

4.1.2 Spermatozoa motility, livability and counts of rats in different treatment groups

The role of MLT on BPA-induced alteration in spermatozoa motility, livability and counts are given in table 2. BPA significantly reduced sperm motility, livability and count in the rats. However, concomitant treated with MLT protected against the BPA-induced reduction in sperm motility, livability and count in the rats (Figures 1-3). There were significant differences in the values for sperm motility and livability between the control and MLT-treated rats (Figures 1 and 2). However, there was no significant difference (P>0.05) between the control and MLT-treated rats in terms of sperm count (Figure 3).

4.1.3 Percentage sperm morphology abnormalities of rats in different treatment groups

The percentage sperm morphological abnormalities of rats are given in Table 3. BPA significantly increased (P<0.05) normal head without tail as well as normal tail without head spermatozoa compared to the control while BPA+MLT protected against the increase (Table 3). BPA also significantly increased spermatozoa abnormalities with rudimentary tail, bent tail, curved tail, bent mid piece, curved mid piece as well as looped tail compared to the control. Concomitant treatment with MLT significantly reduced these increases in sperm abnormalities.

Overall, BPA caused significant increase in total abnormal spermatozoa compared to the control while the concomitant treatment with MLT significantly reduced it (Figure 4).

Table 1: Body weight and reproductive organ weights of rats in different treatment groups

Parameters	Control	MLT	BPA	BPA+MLT
Body weight of animals (g)	157.17±5.87	156.67±3.71	147.33±3.19	154.00±2.25
Epididymal weight (g)	0.59±0.04[a]	0.55±0.03[a]	0.38 ±0.03[b]	0.41±0.06[a]
Testicular weight (g)	1.11±0.06[a]	0.97±0.07[a]	0.76±0.05[b]	0.84±0.13[b]

Values expressed as Means ± Standard Deviation (SD)

Means with different superscriptswithin row are significantly different (P<0.05).

Table 2: Sperm motility, livability and sperm counts of rats in different treatment groups

PARAMETERS	CONTROL	MLT	BPA	BPA+MLT
Sperm Motility (%)	93.33±1.05[a]	94.33±2.11[a]	49.17±2.71[b]	71.67±3.07[c]
Sperm Livability (%)	97.50±0.50[a]	93.50±2.08[a]	48.33±3.07[b]	88.83±2.77[c]
Sperm Count ($X10^6$sperm/ml)	151.50±6.06 [a]	149.17±4.76 [a]	130.17±2.57[b]	144.00±2.90[a]

Values expressed as Means ±Standard Deviation (SD)

Means with different superscripts within row are significantly different (P<0.05)

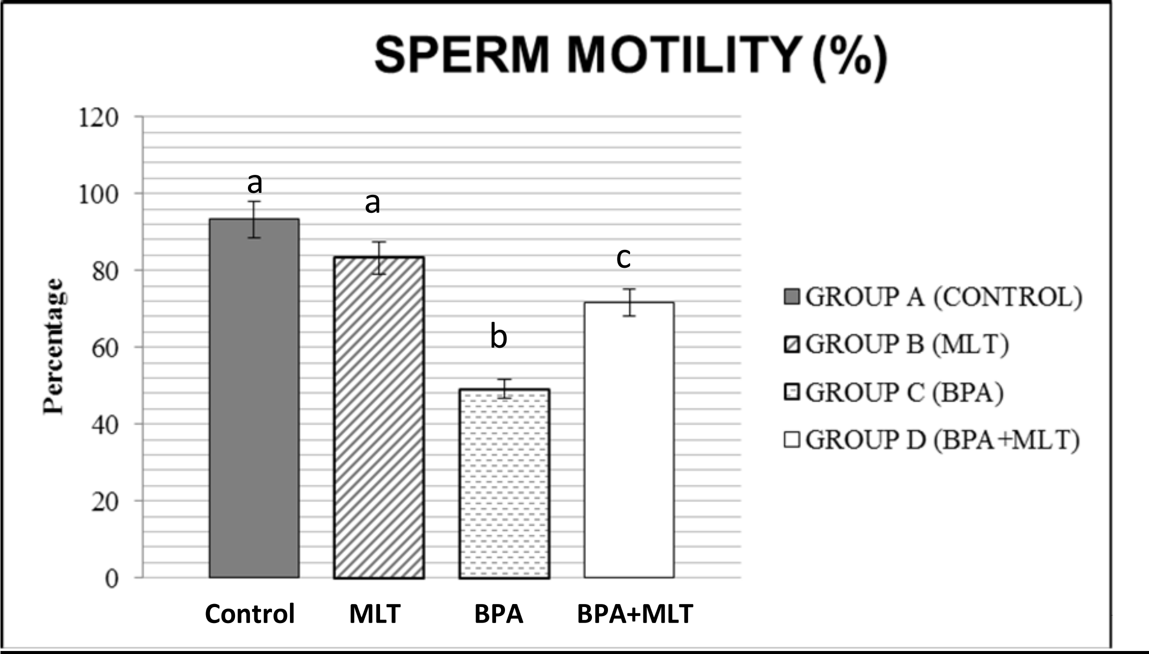


Figure 1: Sperm motility of rats in different treatment groups

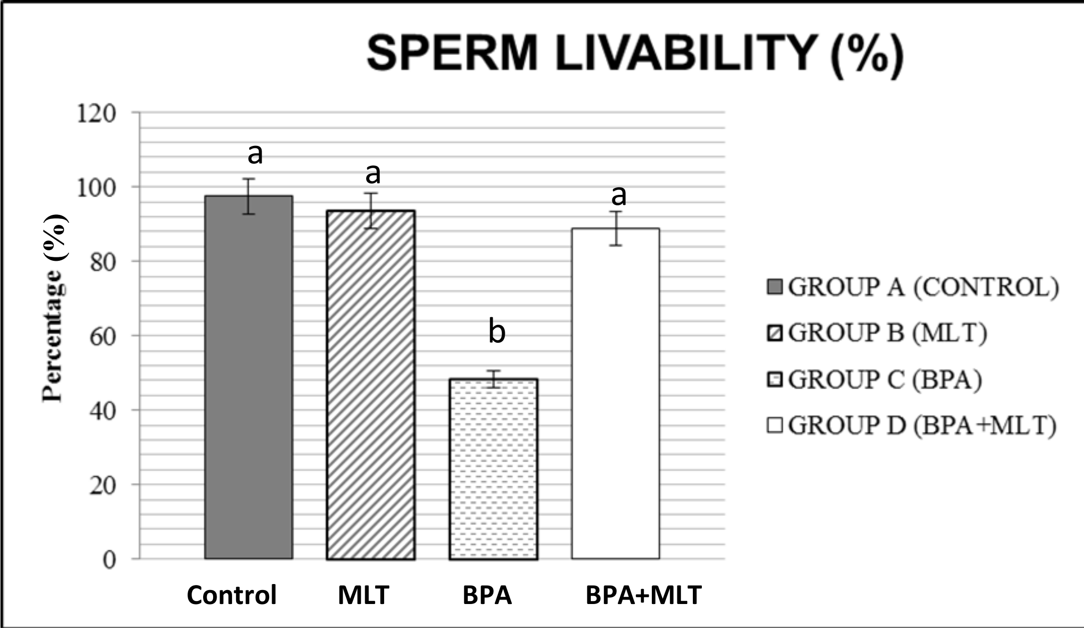


Figure 2. Sperm livabilityof ratsin different treatment groups

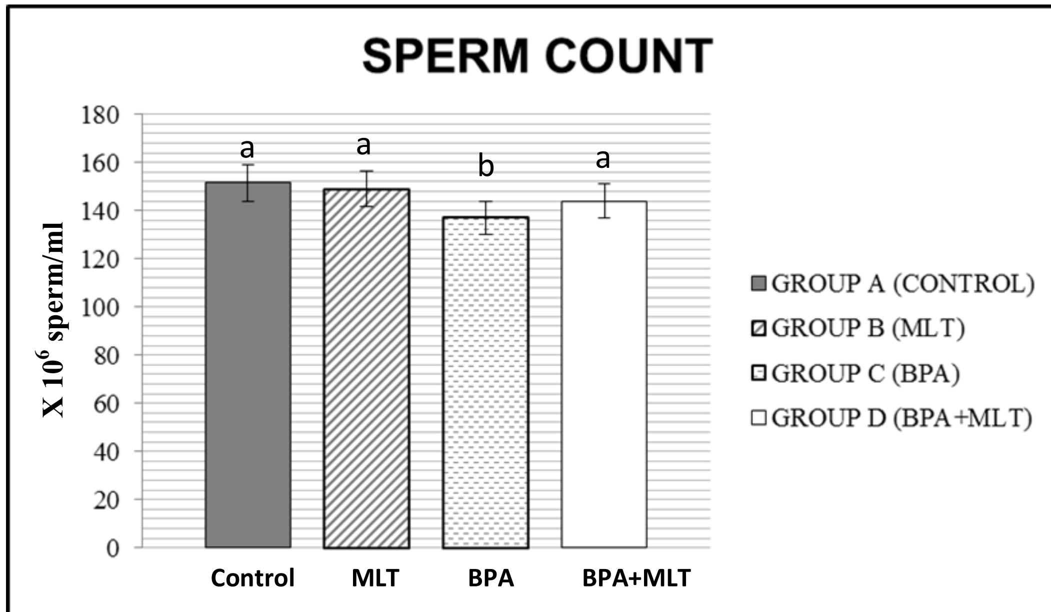


Figure 3: Sperm count of ratsin different treatment groups

Table 3: **Percentagesperm morphological abnormalities of ratsin different treatment groups**

PARAMETERS	Control	MLT	BPA	BPA + MLT
NHWT	0.86±0.13[a]	1.06±0.14[b]	1.56±0.15[c]	0.71±0.09[a]
NTWH	0.78±0.08[a]	0.86±0.15[a]	1.27±0.22[b]	0.71±0.07[a]
RT	0.44±0.11[a]	0.49±0.16[a]	0.82±0.10[b]	0.33±0.06[a]
BT	1.77±0.09[a]	1.91±0.12[a]	2.56±1.13[b]	2.50±0.09[b]
CT	1.72±0.09[a]	2.16±0.12[b]	2.28±0.05[b]	2.27±0.18[b]
BMP	1.72±0.08[a]	2.47±0.19[b]	2.28±0.16[b]	2.35±0.20[b]
CMP	1.81±0.18[a]	1.98±0.23[a]	2.47±0.06[b]	2.36±0.08[b]
LT	0.33±0.05[a]	0.50±0.21[b]	0.95±0.26[c]	0.33±0.06[a]
Total abnormal cells	9.41±0.20[a]	11.41±0.64[b]	14.21±0.08[c]	11.66±0.42[b]
Total cells	410.50±5.65[a]	411.33±9.39[a]	412.40±7.89[a]	424.40±10.55[a]

Values expressed as Means ± Standard Deviation (SD)

Means with different superscripts within row are significantly different (P<0.05)

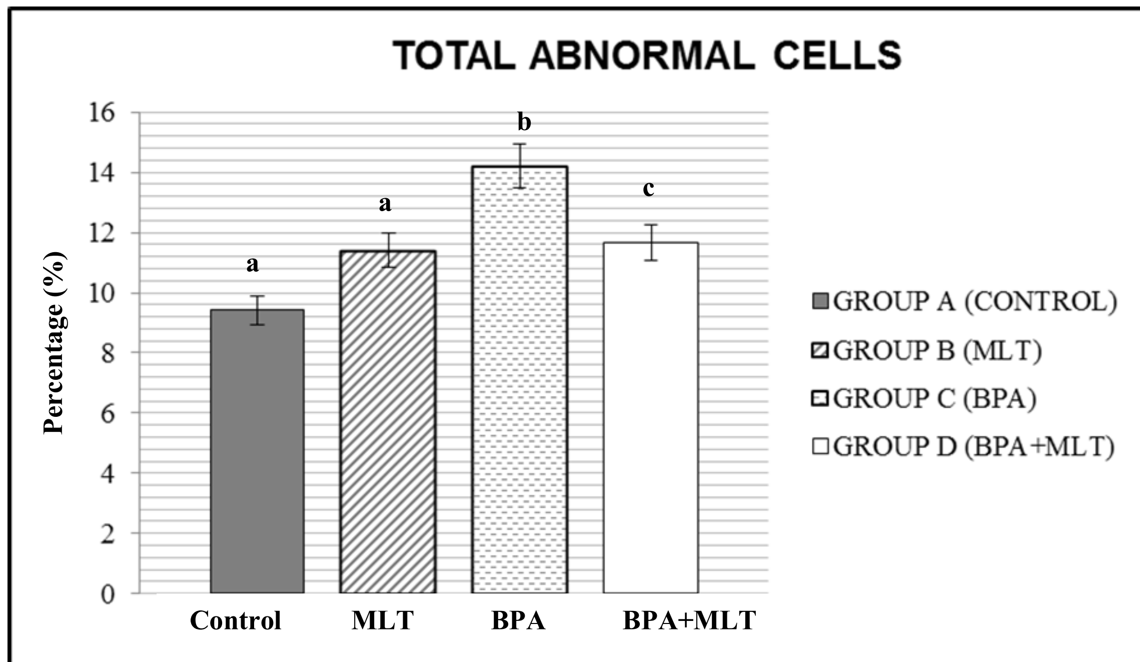


Figure 4: Total Percentage of abnormal sperm cells of rats in different treatment groups

SECTION TWO

4.2. Concentrations of oxidative stress markers in rats undervarious treatment regimes

The results of the effect of MLT on BPA-induced oxidative stress markers are given in figures 5-10. In BPA-treated rats, there were significant decreases ($P < 0.05$) in the activities of catalase, SOD, GSH and GPX compared to the control rats (Figures 5-10). However, BPA-MLT intake resulted in significant increases ($P < 0.05$) in the activities of catalase, SOD, GSH and GPX compared to the BPA-treated rats. Conversely, BPA-treated rats exhibited significant increases ($P < 0.05$) in LPO and H_2O_2activities (figures 8 and 9). In rats treated with MLT, LPO and H_2O_2activities were significantly ($P < 0.05$) reduced. In comparison with the control group, MLT caused significant increases ($P < 0.05$) in the activities of GSH and H_2O_2 (Figures 7 and 9). In all the oxidative stress markers estimated, MLT demonstrated significant protective effect to BPA-induced epididymal sperm oxidative stress.

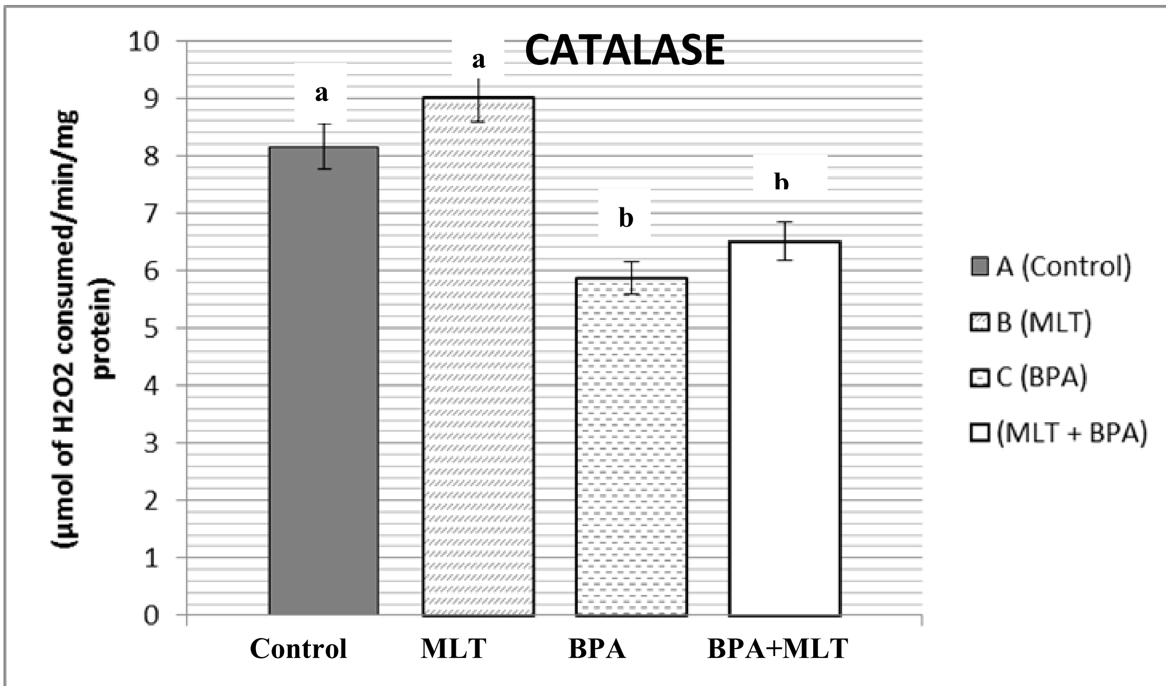


Figure 5. Concentrations of Catalase markers in rats under various treatment regimes

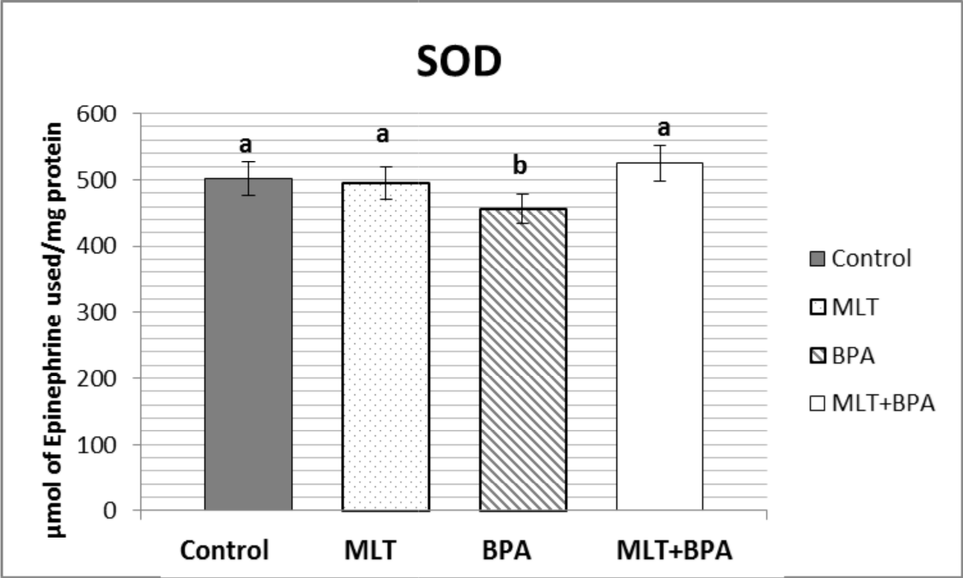


Figure 6.Concentrations of SOD markers in rats under various treatment regimes

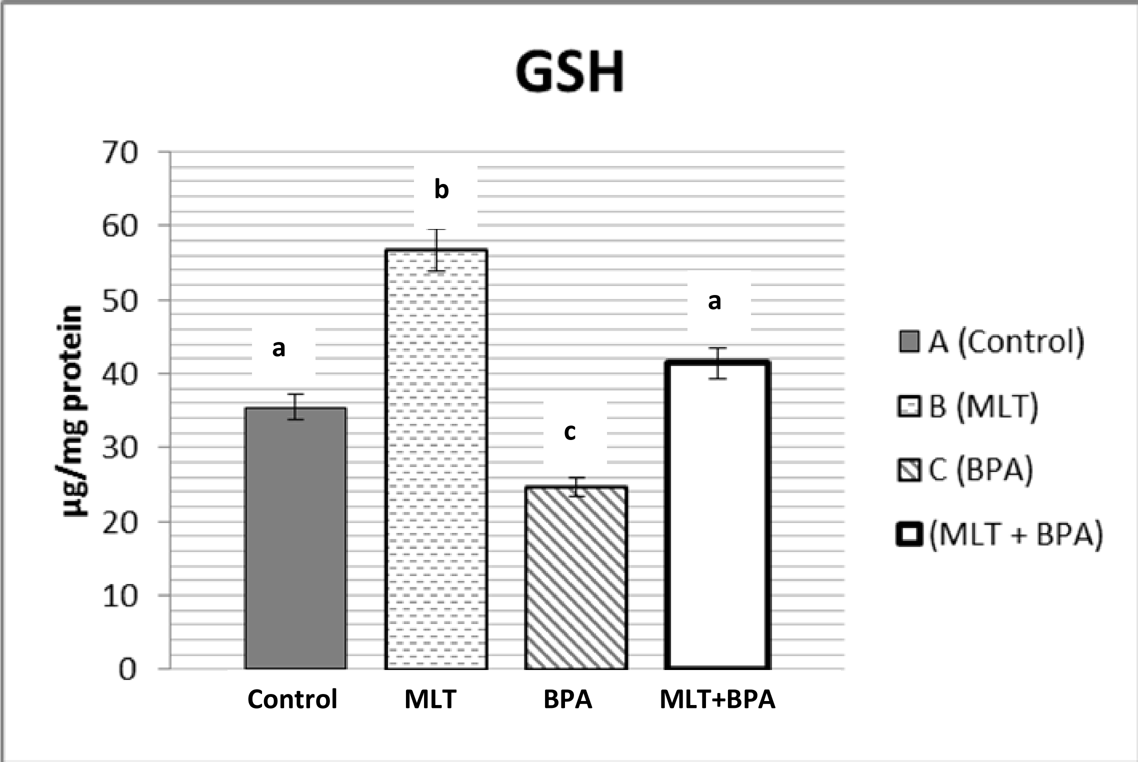


Figure 7. Concentrations of GSH markers in rats under various treatment regimes

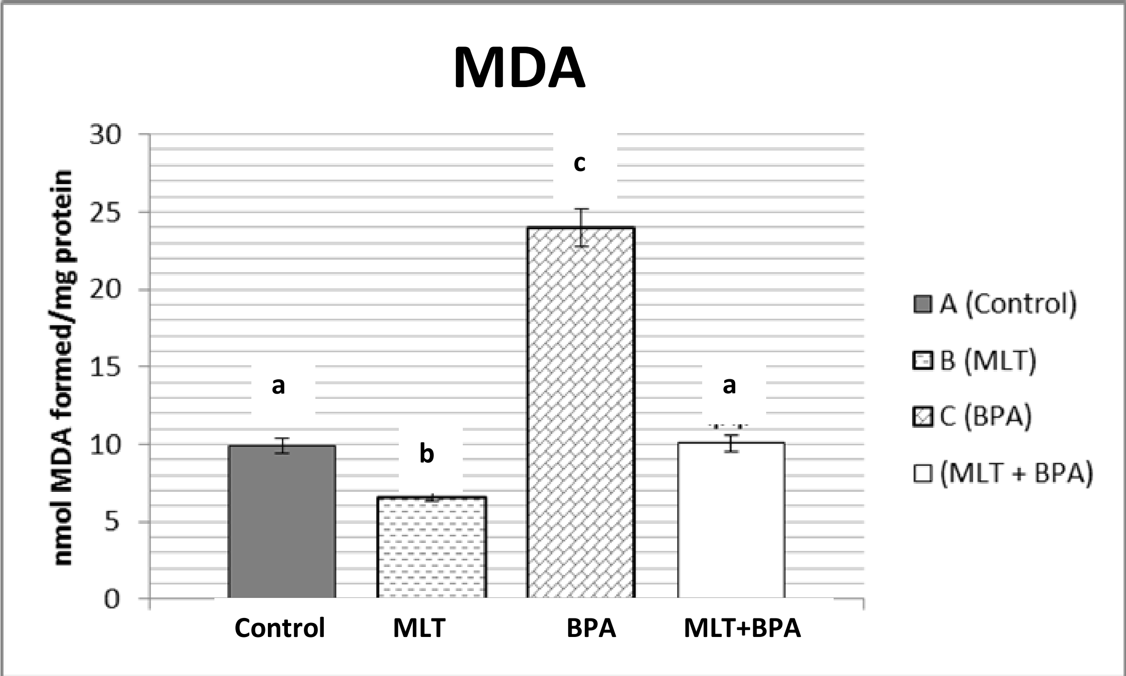


Figure 8. Concentration of MDA in rats under various treatment regimes

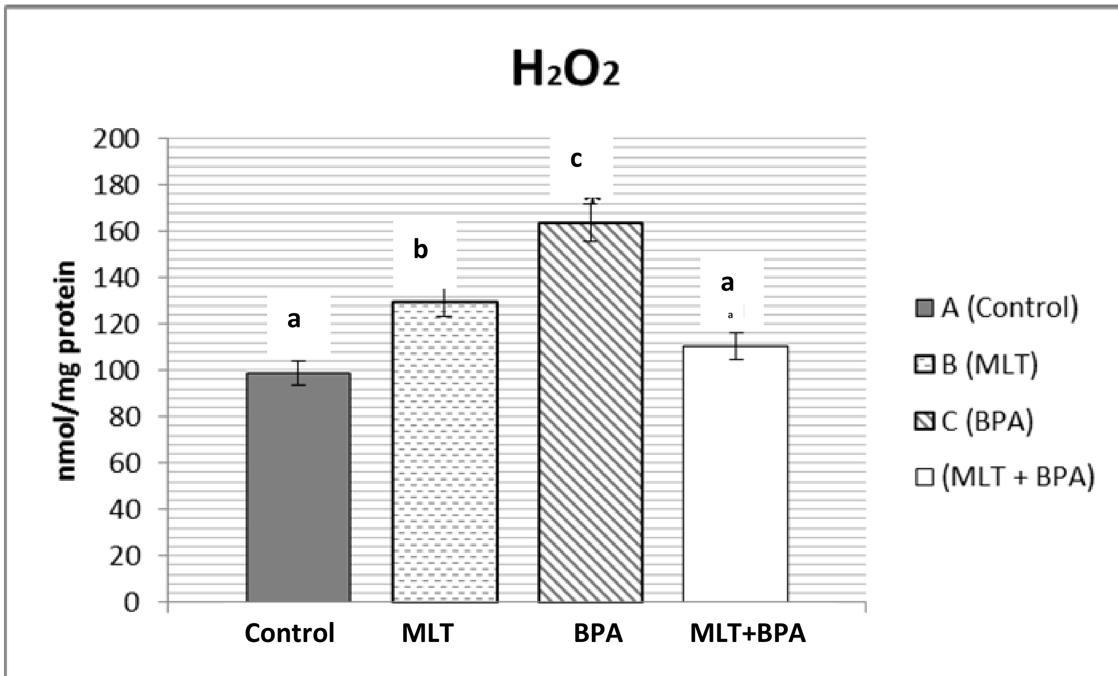


Figure 9.Concentrations of H_2O_2 markers in rats under various treatment regimes

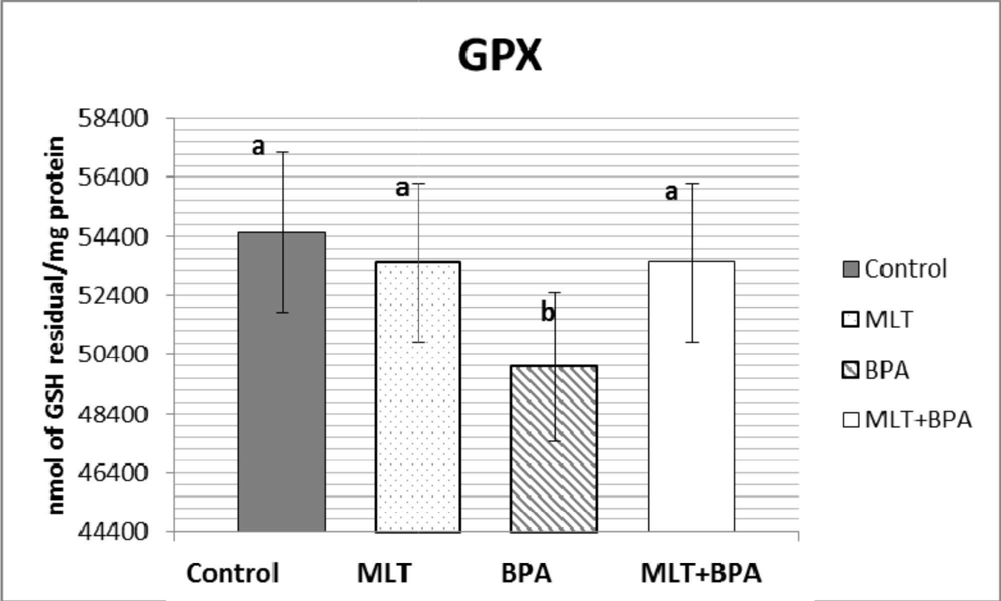


Figure 10.Concentrations of GPX markers in rats under various treatment regimes

SECTION THREE

4.3 Effect of Melatonin on BPA-induced epididymal injury and altered protein localization

4.3.1 Effect on histopathology and histomorphometry

The epididymis of the control and MLT-treated rats showed intact epithelial cells, normal interstitium, normal epididymal membrane and intact lumen filled with spermatozoa (Figure 11A & B). However, the BPA-treated rats revealed a number of histopathological lesions including the disintegration of the duct membrane, sloughing off of epithelial cells as well as spermatozoa within the lumen and moderate erosion of the epididymal interstitium (Figure 11C). In terms of histomorphometrical parameters of the epididymis, rats treated with BPA showed significantly reduced luminal diameter, ductal diameter and epithelial height when compared with the control rats (Table 4).There was no significant difference (P>0.05) between the control and MLT-treated rats in terms of luminal diameter, ductal diameter and epithelial height of the epididymis. However, the BPA+MLT-treated rats showed significant difference amongst these parameters (Table 4).

4.3.2 Immunohistochemical localization of proteins

The percentage contributions of positive reactions toSMA, S-100 and Vm in the epididymis of rats in the present study are given in the Table 5. Rats treated with BPA showeddecreased localization of SMA, S-100 and Vm in the cauda epididymidis (Figures 12-14). Concomitant treatment with MLT improved on the percentage contributions of positive reactions to SMA, S-100 and Vm in the cauda epididymis of the rats. ForSMA and Vm, there were significant decreases in localization at theblood vessels as well as epididymal ductal membrane between the control and BPA-treated rats. S-100 protein localization also significantly decreased at the epididymal sheath as well as blood vessels between the control and BPA treated rats. However, the BPA+MLT-treated rats showed significant increase in the localization of these proteins.

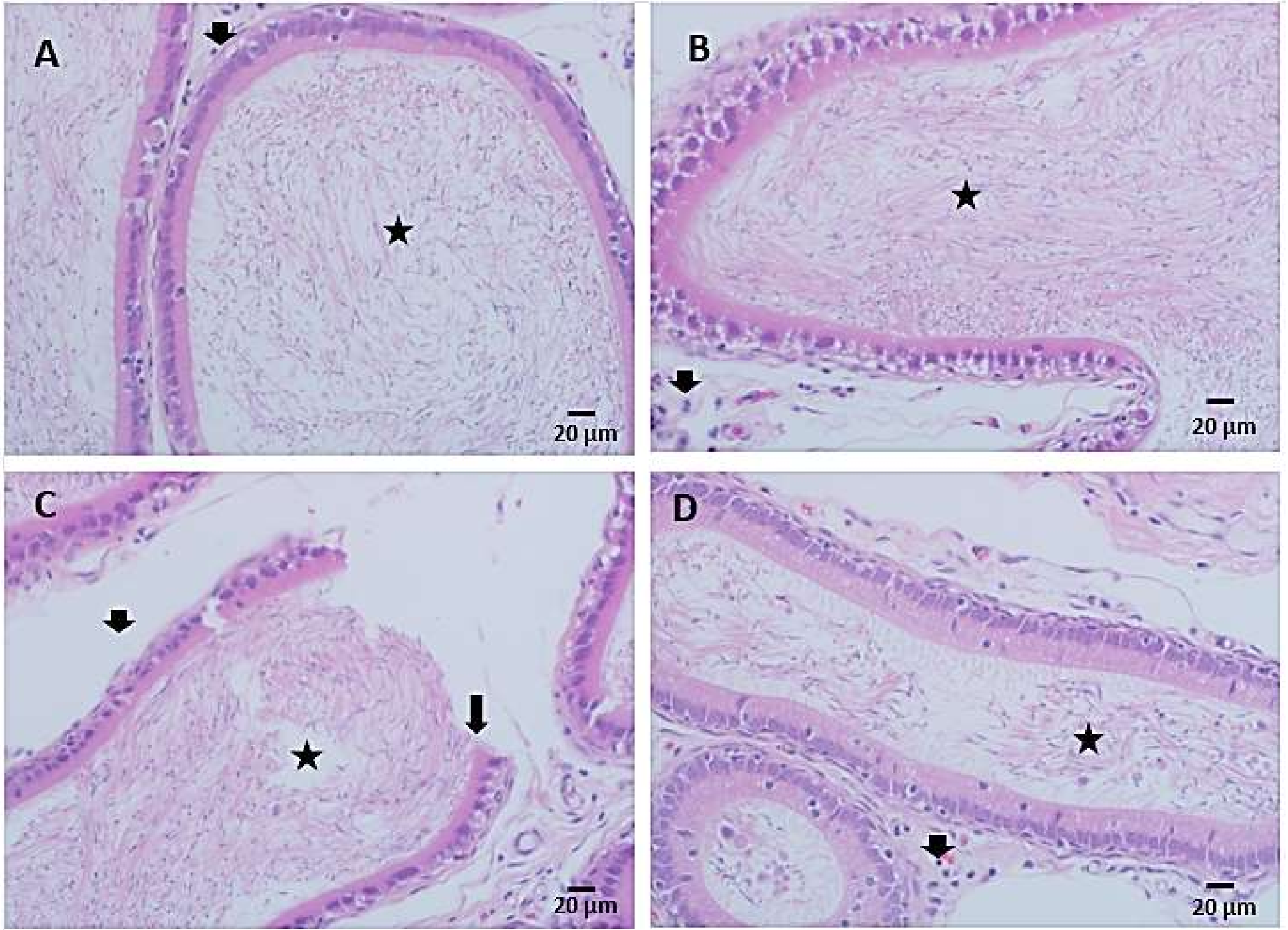


Figure 11: Crosssection of the cauda epididymis (H&E). A. Control group showing epididymal lumen filled with spermatozoa (star) with normal epididymal sheath and intact ductal membrane (arrow head).

B. MLT treated group showing the lumen of epididymis with more compactly packed spermatozoa (star) and normal epididymal sheath and intact ductal membrane (arrow head).

C. BPA treated group showing eroded epididymal sheath (arrow head), disintegration of the ductal membrane of epididymis (arrow) as well as sloughing off of spermatozoa (star) within the lumen of the epididymis.

D. BPA + MLT group showing normal interstitium (arrow head) and spermatozoa (star) within the lumen of the epididymis.

Table 4. Epididymal morphometry in rats under various treatment regimes

Parameters	Luminal diameter	Ductal diameter	Epithelial height
Control	295.34±11.62[a]	382.31±27.37[a]	28.48±2.67[a]
MLT	285.14±17.53[a]	394.74±17.56[a]	24.15±3.17[a]
BPA	255.72 ±14.61[b]	365.72±22.35[b]	21.43±3.21[b]
BPA + MLT	260.13 ±11.73[a]	370.19±17.23[a]	25.93±4.34[a]

Values expressed as Means ± Standard Deviation (SD)

Means with different superscripts within columns are significantly (P<0.05) different.

Table 5:Quantification of proteins expression in the epididymis of rats

Proteins	CONTROL	MLT	BPA	BPA+MLT
SMA (%)	9.14±0.65[a]	8.32±0.61[a]	3.74±0.37[b]	5.72±2.25[a]
S-100 (%)	5.30±0.52[a]	5.83±0.61[a]	1.93±0.02[b]	4.36±0.25[a]
Vm (%)	8.87±1.47[a]	11.26±1.15[a]	2.28±1.12[b]	6.81±0.25[a]

Values expressed as Means ± Standard Deviation (SD)

Means with different superscripts across rows are significantly (P<0.05) different.

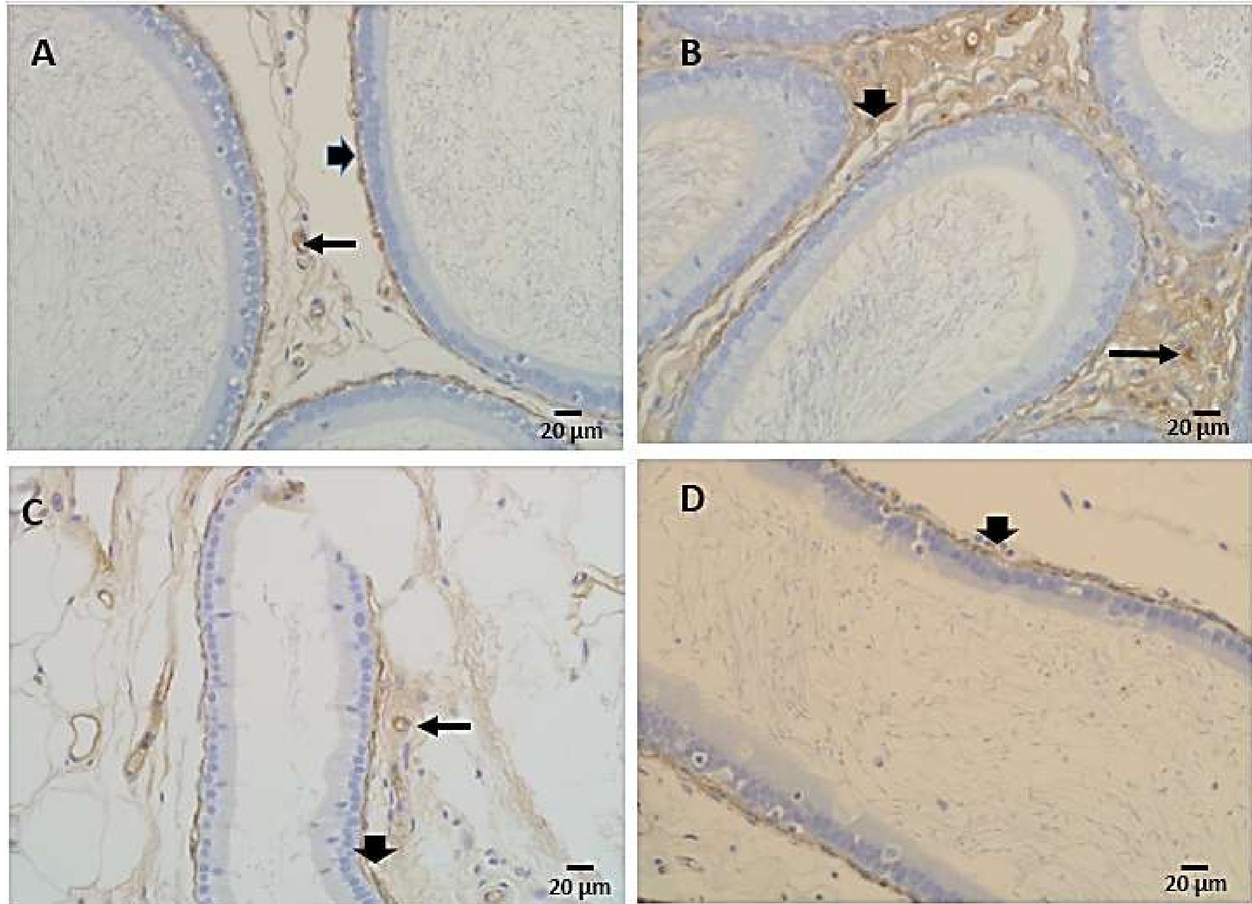


Figure 12.Immunohistochemical localization of SMA in the cauda epididymidis of rats.

A. Control group showing SMA-positive epididymal ductal membrane (arrow head) and endothelium (arrow).

B. MLT treated group showing SMA-positive epididymal sheath (arrow head) and endothelium (arrow)

C. BPA treated group showing weakly SMA-positive epididymal duct (arrow head) and endothelium (arrow)

D. BPA + MLT group showing SMA-positive epididymal duct membrane (arrow head).

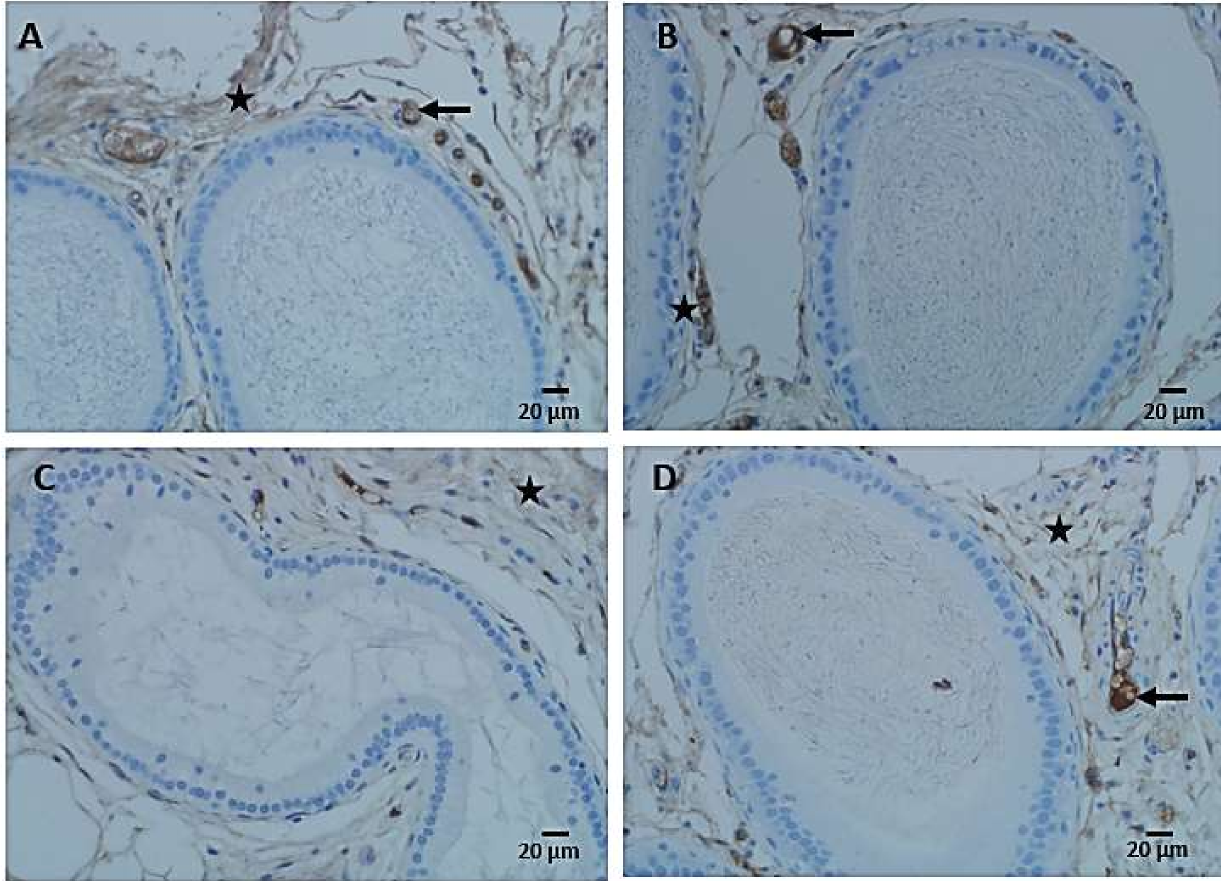


Figure 13:.Immunohistochemical localization of S-100 protein in the cauda epididymidis of rats. A. Control group showing S-100-positive epididymal sheath (star) and endothelium (arrow).

B. MLT treated group showing S-100-positive epididymal sheath(star) and endothelium (arrow)
C. BPA treated group showing weakly S-100-positive epididymal sheath(star).

D. BPA + MLT group showing S-100-positive epididymal sheath (star) and endothelium.

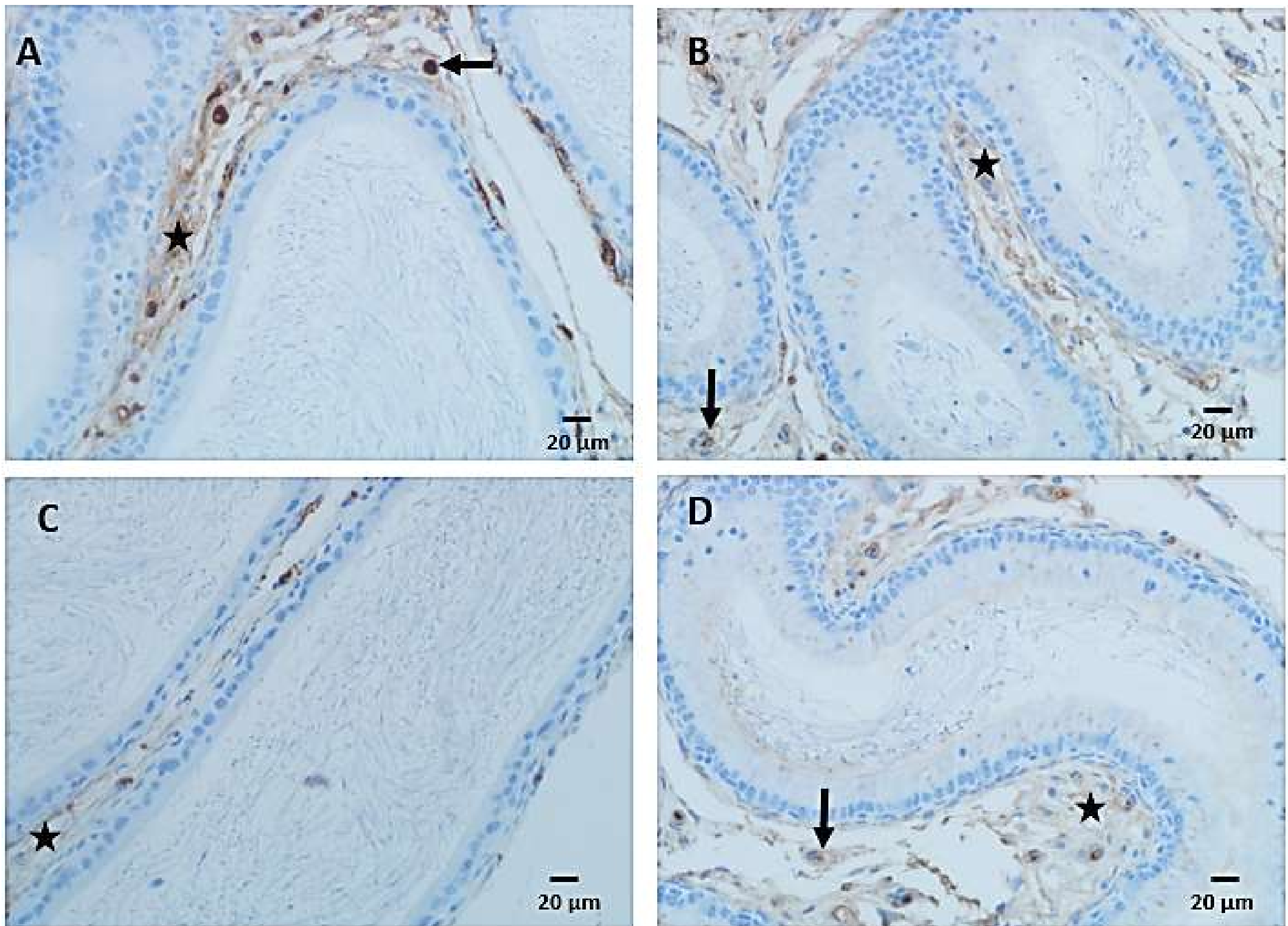


Figure 14: Immunohistochemical localization of Vm in the cauda epididymidis.

A. Control group showing Vm-positive epididymal sheath (star) and endothelium (arrow).

B. MLT treated group showing Vm-positive epididymal sheath (star) and endothelium (arrow).

C. BPA treated group showing weakly Vm-positive epididymal sheath (star).

D. BPA + MLT group showing Vm-positive epididymal sheath (star) and endothelium.

SECTION FOUR

4.4 EFFECT OF MELATONIN ON BPA-INDUCED ALTERATIONS IN SPERMATOGENESIS

4.4.1 Serum concentrations of Testosterone, DHEAS, Estradioland FSH for the rats on the different treatment regimes

Serum concentrations of Testosterone, DHEAS, Estradioland FSH for the rats on the different treatment groups areshown in Figures 15-18. Serum. There was significant decrease ($P<0.05$) in the mean testosterone and DHEAS serum levelbetween the BPA and the control groups (Figures 15 and 16).However, there were no significant differences between the control and MLT-treated rats with respect to testoterone and DHEAS (Figures 15 and 16). Serum FSH and Estradiol levels in the MLT-treated rats were also as low as for the control rats.Serum FSH and Estradiol levels were significantly higher ($P<0.05$) in the BPA-treated rats than BPA+MLT treated rats (Figures 17 and 18).

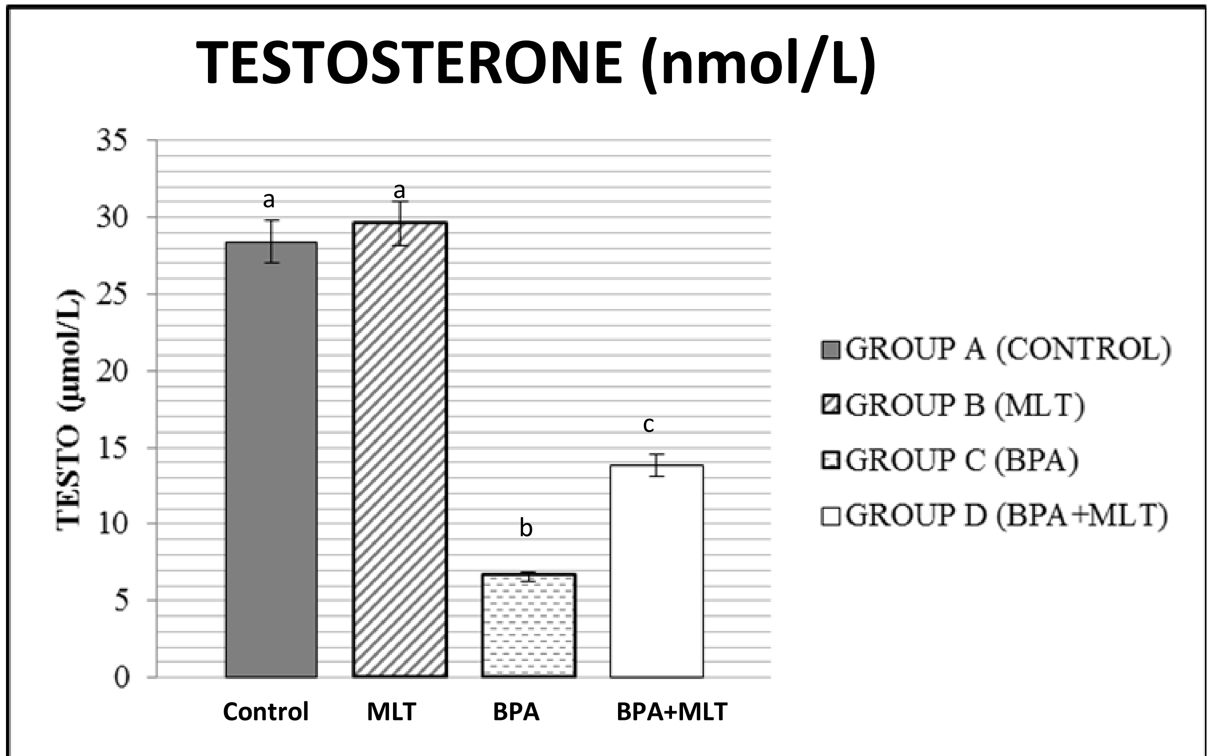


Figure 15: Serum testosteroneconcentrationof rats under different treatment regimes

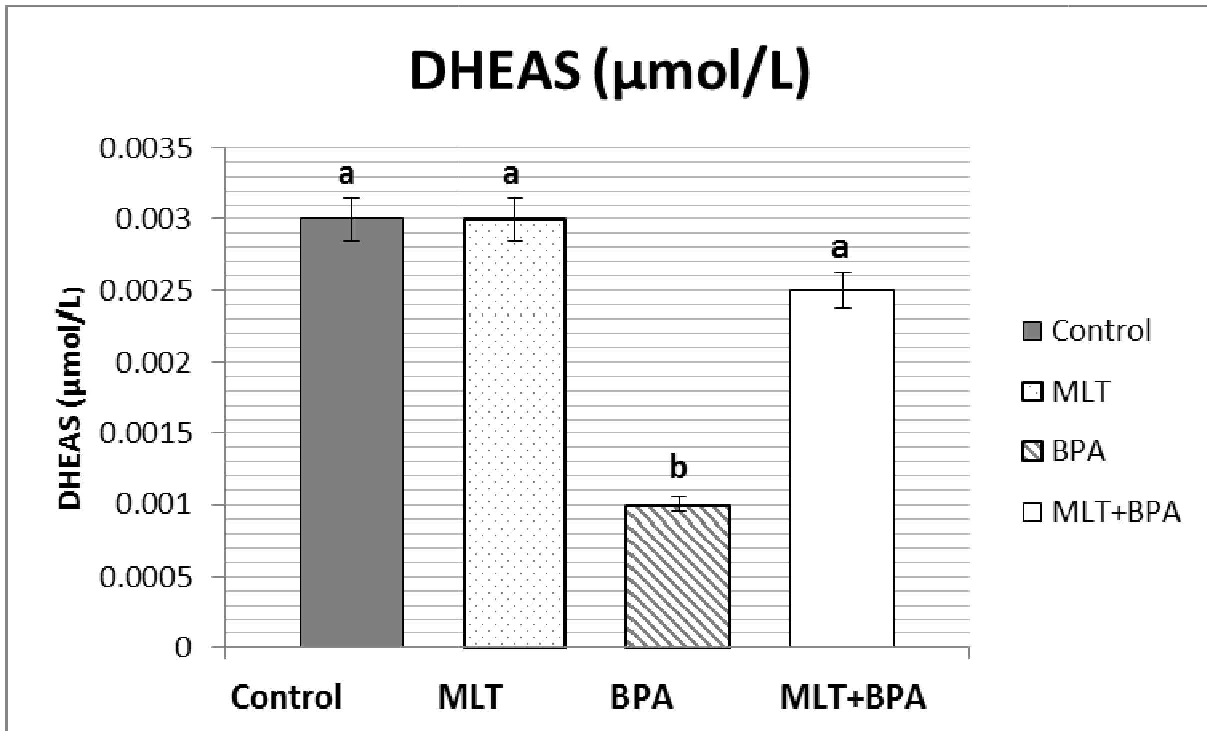


Figure 16: Serum DHEAS concentration ofrats under different treatment regimes

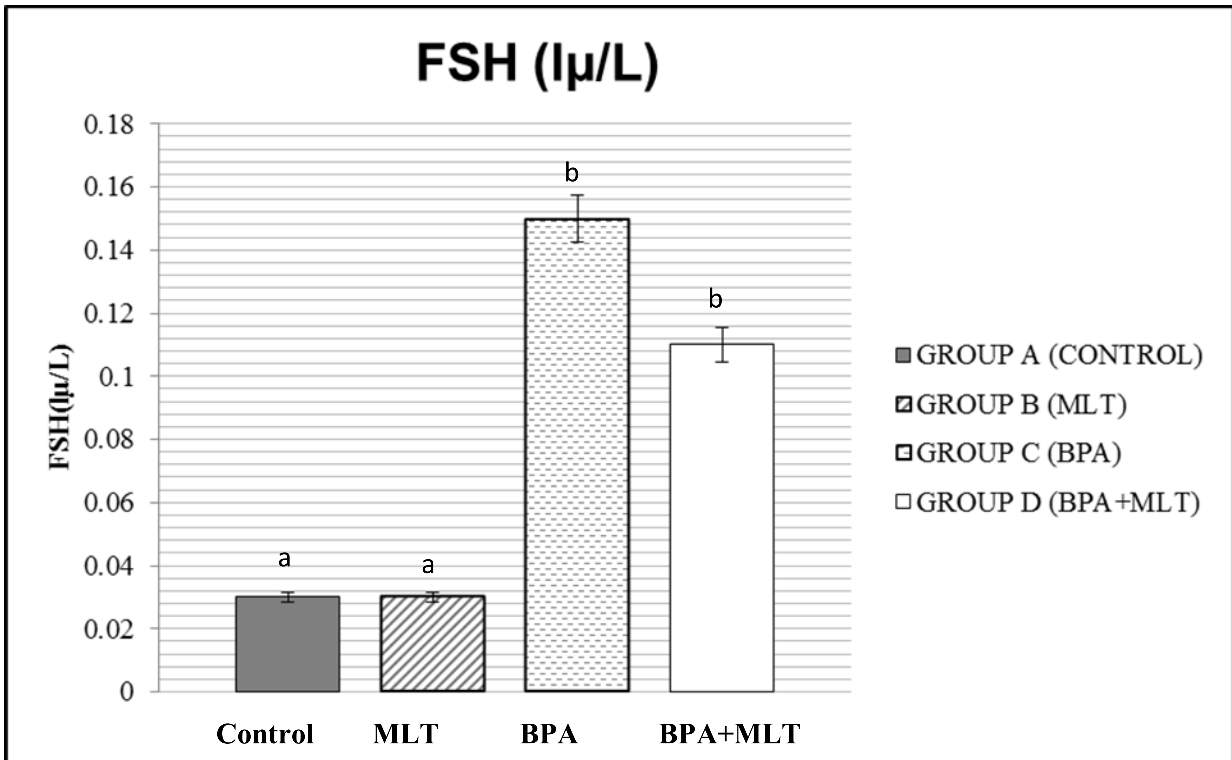


Figure 17: Serum FSHconcentrationsof rats under different treatment regimes

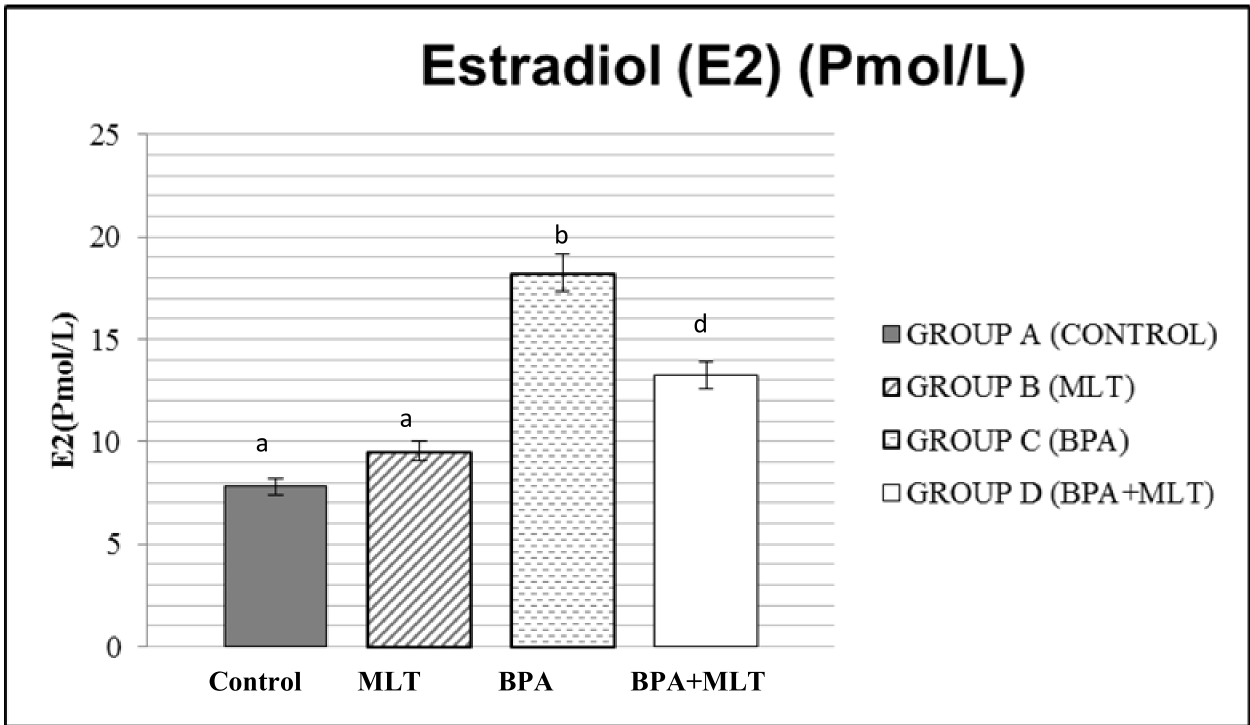


Figure 18: Serum Estradiol (E_2)concentrationof rats under different treatment regimes

4.4.2Testicular lesions in rats under different treatment regimes

The light micrscopic (LM) sections of the testis of both the control and MLT-treated rats revealed normal histology of the testis including intact seminiferous tubules enclosing Sertoli cells and spermatogenic cells in their normal succession (Figures 19A and B). Also, the testicular intersitum in the control and MLT-treated rats were intact possessing Leydig cells as well as blood vessels. The BPA-treated rats showed hyperemia of the interstitium with erosion of interstitial elements. Testicular lesions of the BPA-treated rats include testicular vacuolations within the seminiferous tubules alongside the reduction in the number of elongated spermatids as well as the disintegration of the basement membrane of seminiferous tubules (Figures 19 and 20). The seminiferous tubules presented with eroded spermatogenic cells due to discontinuation of the basement membrane. Also, fewer spermatozoa were found in the lumen of the seminferous tubules of the BPA-treated rats. In the BPA+MLT-treated rats, these lesions became reduced with improved testicular interstitum as well as more Leydig cells and blood vessels. The seminiferous tubules of the BPA+MLT treated rats showed spermatogenic cells in their succession as well as Sertoli cells.

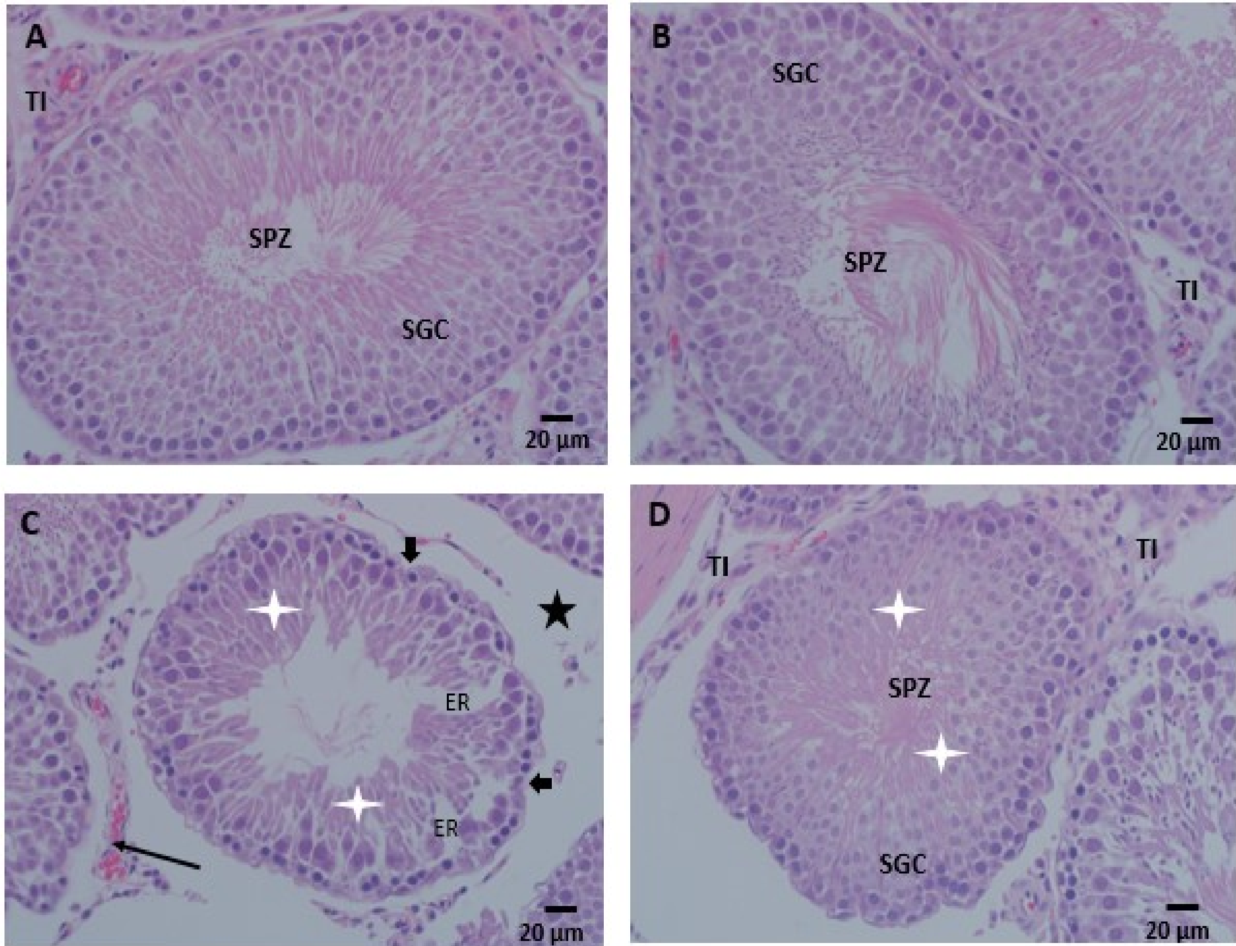


Figure 19: H & E Section of the testes showing the seminiferous tubules in Control (A), MLT treated (B), BPA treated (C) and BPA + MLT treated (D)

A and B show normal spermatogenesis with seminiferous tubules filled with germ cells (SGC) and normal spermatozoa (SPZ), with normal insersttium (IT).

C. BPA-treated ratsshows hyperemia of the interstitium (arrow), erosion of interstitial elements (black star). There is reduction of elongated spermatid (white star) and degeneration (ER) of the germinal cells.

D. BPA + MLT group showing normal Interstitium (IT), spermatogenic cells (SGC) and spermatozoa (SPZ). Bar = 20µm (H & E).

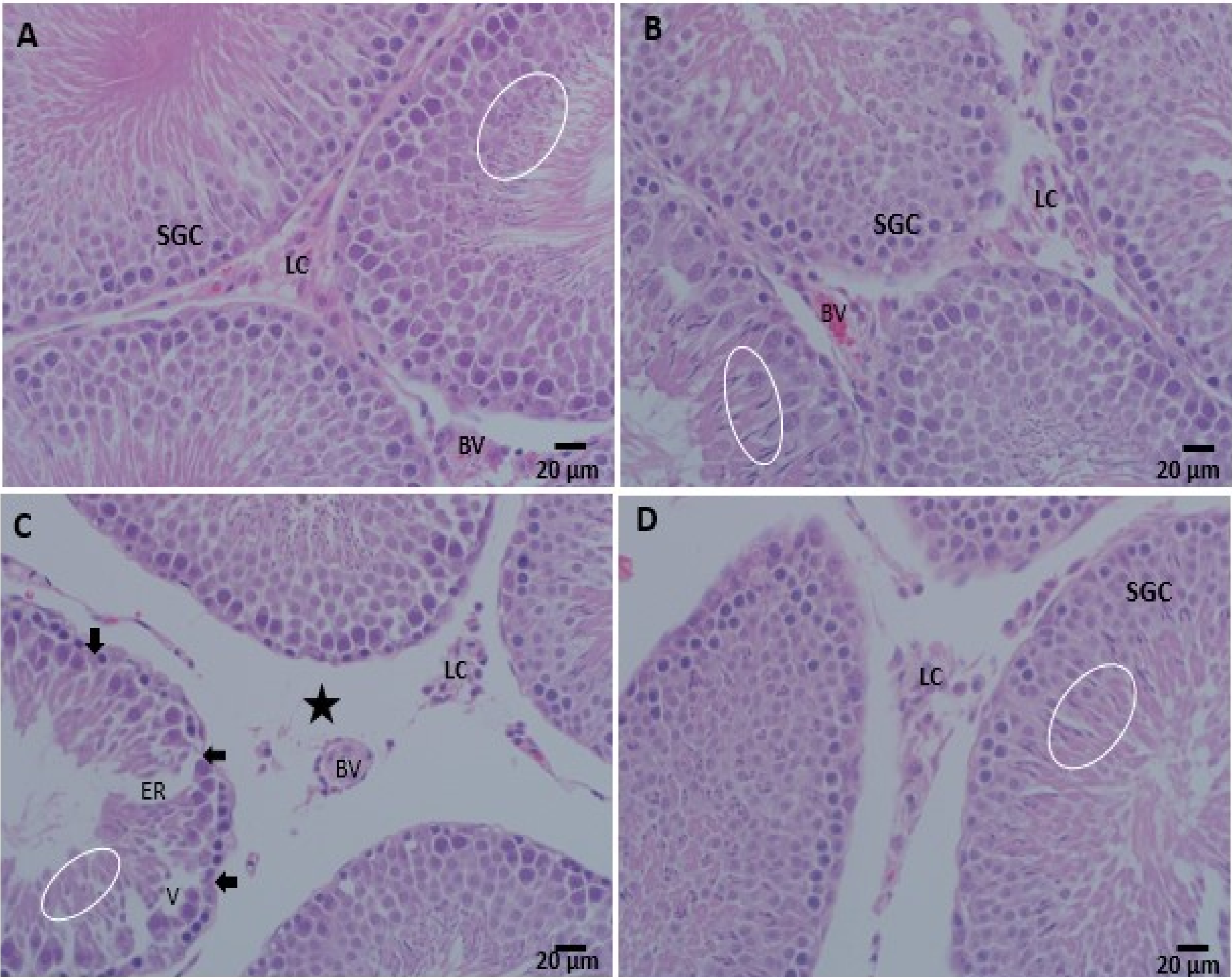


Figure 20: Section of the testes showing the seminiferous tubules in Control (A), MLT treated (B), BPA treated (C) and BPA + MLT-treated group (D).

A and B show the presence of abundant Leydig cells (LC), spermatozoa (SPZ), blood vessels (BV) and normal spermatogenic cells (SGC).

C. BPA treated group showing erosion of testicular interstitium (star) with few Leydig cells (LC), erosion of germ cells (ER) and disintegration of basement membrane of seminiferous tubules (arrow head). There is reduction of elongated spermatid and testicular vacuolations (V)

D. BPA + MLT treated group showing the normal elongated spermatids, spermatogenic cells (SGC) and spermatozoa (SPZ). Bar = 20µm (H & E)

4.4.3 Transmission Electron Microscope (TEM) of the testis of rats exposed to BPA

Results from TEM revealed normal seminiferous tubules containing spermatogenic cells as wellas Sertoli cells in the contro and MLT-treated rats. Spermatogonia were found at the basement membrane of the seminiferous tubules in close opposition with Sertoli cells which extended their cytoplasmic processes into the adluminal surface Figure 21A and B). BPA induced lesions of the Sertoli cell including pyknotic nuclei and the dissolution of Sertoli cell cytoplasmic processes. Also, as evident on LM, there were testicular vacuolations, erosion of germinal cells as well as distortion of the basement membrane of the seminiferous tubules. The control and MLT-treated groups revealed numerous mitochondria as well as lipid droplets in the cytoplasm of Sertoli cells (Figure 22A and B) while these organelles were totally absent in the cytoplasm of the Sertoli cells of the BPA- treated group (Figure 22C). Concomitant treatment with MLT revealed normal Sertoli cells resting in the basement membrane of seminiferous tubules with their cytoplasmic processes extending to the adluminal surface and possessing numerous mitochondria as well as lipid droplets within the cytoplasm (Figure 22D). At the testicular interstitum, the control and MLT-treated groups revealed Leydig cells with normal nucleus and cytoplasm, blood vessels as well as lipid droplets (Figure 23A and B). The BPA-treated group revealed severe erosion of the testicular interstitum with a few Leydig cells possessing nuclei with no visible cytoplasm (Figure 23C). The BPA+MLT treated group revealed a restoration of the cytoplasm of Leydig cells as well as intact testicular interstitium (Figure 23D).

Observing the process of spermiogenesis, the control and MLT-treated groups presented with normal round spermatids possessing normal acrosomal vescicles and granules as well as cytoplasm containing numerous mitochondria (Figures 24A and B) while the BPA-treated group revealed round spermatids with distorted acrosomal vessicles and absence of granules as well as cytoplasm devoid of mitochondria (Figure 24C). The round spermatids of the BPA+MLT group revealed normal acrosomal vesicle and granule as well as cytoplasm possessing mitochondria (Figure 24D). The elongated spermatids of the control and MLT-treatedgroups revealed nuclear condensation and elongation with Sertoli cell cytoplasmic processes surrounding the spermatid, (Figure 25A and B). The BPA-treated group revealed elongated spermatids with eroded nucleus as well as dissolved Sertoli cell cytoplasmic processes (Figure 25C).

However, the BPA+MLT group revealed normal elongated spermatid with intact nucleus and Sertoli cell cytoplasmic processes surrounding it (Figure 25D). The control and MLT-treated groups revealed the normal 9+2 axoneme arrangement of flagellar apparatus of spermatozoa (Figure 26A and B) while the BPA-treated group revealed a distortion of the9+2 axoneme arrangement (Figure 26C). However, the BPA+MLT treated group revealed the restoration of the 9+2 axoneme arrangement of flagellar apparatus of spermatozoa. (Figure 26D).

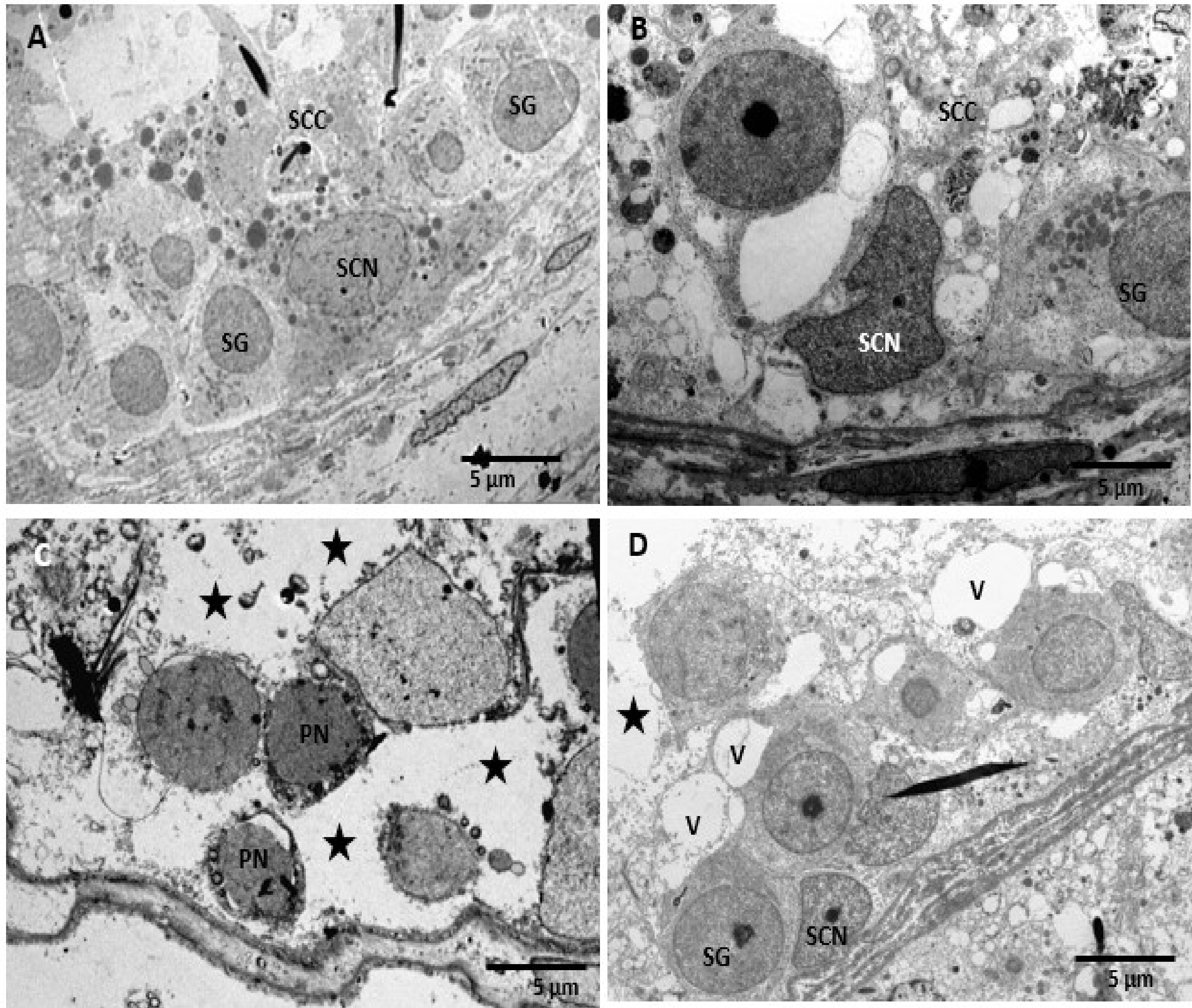


Figure 21. Transmission Electron Microscopy (TEM) Section of the testis of rats exposed to BPA.

A. Control group showing normal spermatogonium (SG), Sertoli cell nucleus (SCN) as well as Sertoli cell cytoplasm (SCC) within the seminiferous tubule.
B. MLT-treated group showing Sertoli cell nucleus (SCN) with its cytoplasm (SCC) in contact with a spermatogonium (SG).
C. BPA-treated group showing distorted basement membrane of seminiferous tubules, Sertoli cell, pyknotic nucleus (PN) with severe erosion of germinal cells as well as absence of sertoli cell cytoplasmic processes (star),
D. BPA+MLTgroup showing normal basement membrane of seminiferous tubule, normal spermatogenium (SG) and Sertoli cell nucleus (SCN) with its cytoplasmic processes intact as well as testicular vacuolations (V).

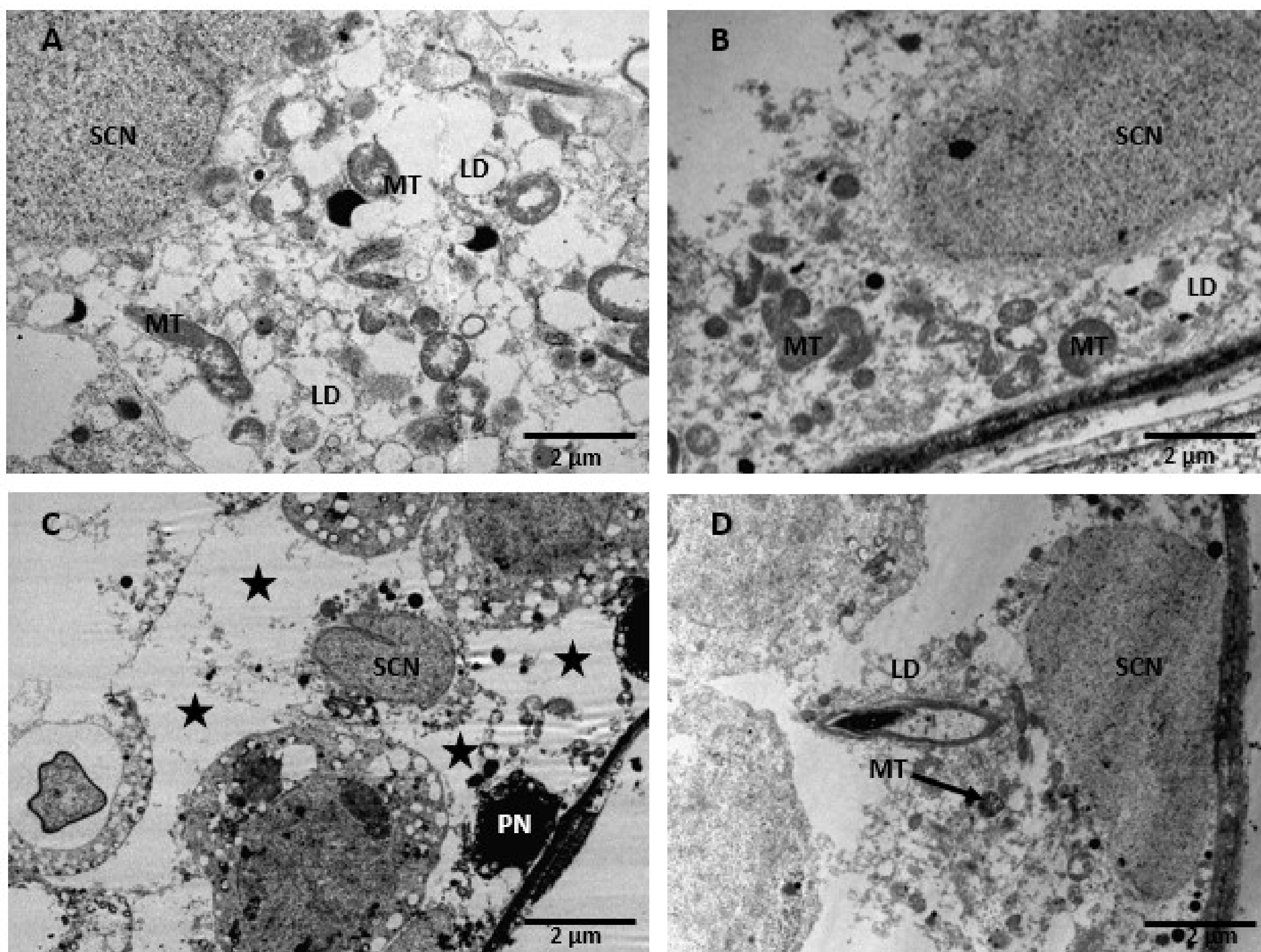


Figure 22. Transmission Electron Microscopy (TEM) Section of the testis at higher magnification.

Control (A), MLT treated (B), BPA-treated (C) and BPA + MLT treated rat.

A and B showing the normal Sertoli cell nucleus (SCN) and Lipid droplets (LD) with presence of numerous Mitochondrion (MT).

C. BPA-treated group showing eroded cytoplasm and pyknotic nucleus of Sertoli cells.

D. BPA + MLT-treated group showing normal mitochondrial activities (MT), lipid droplets (LD) and Sertoli cell nucleus (SCN). Bar = 2µm

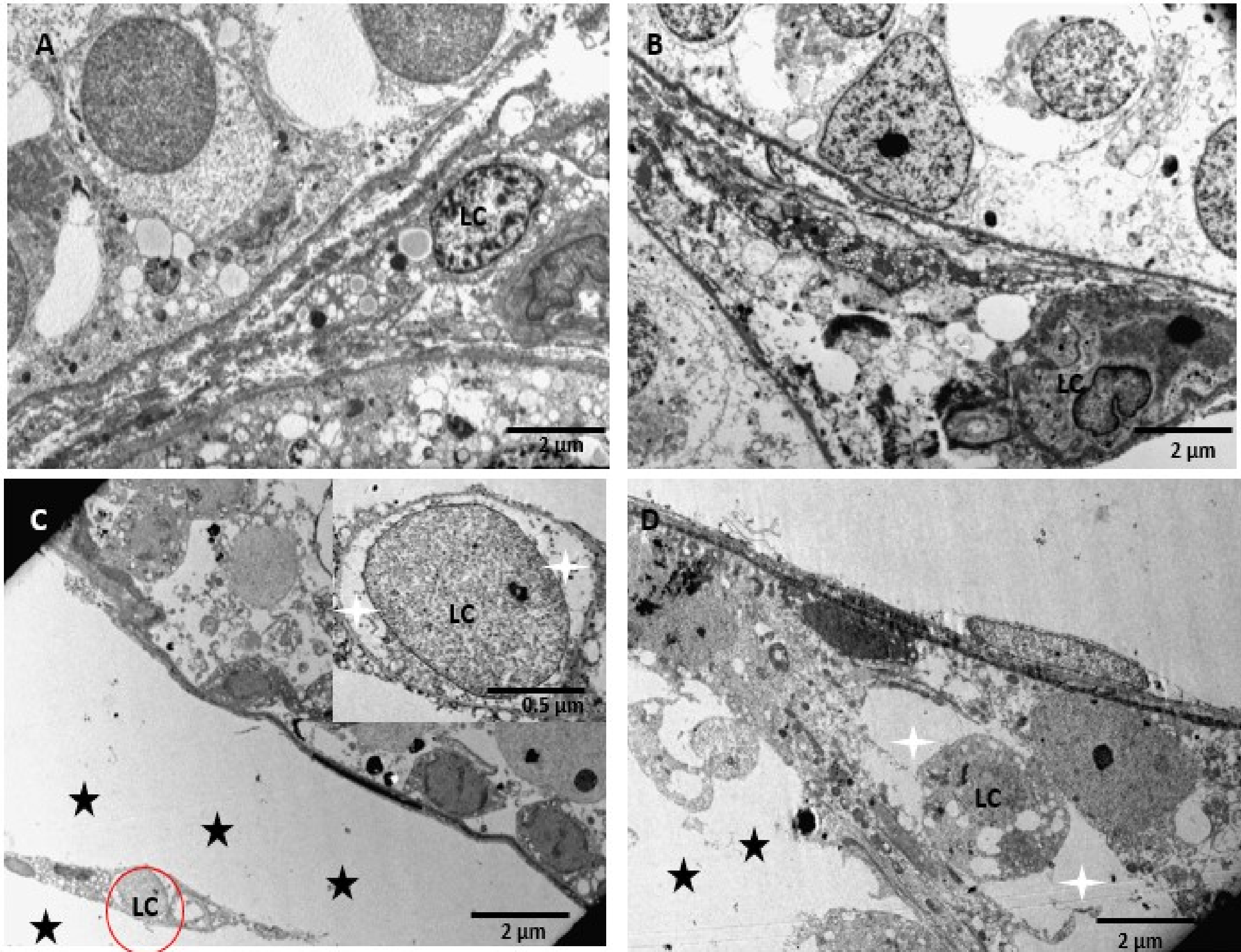


Figure 23. Transmission Electron Microscopy (TEM) section of the testis showing testicular interstitum in Control (A), MLT-treated (B), BPA-treated (C) and BPA + MLT-treated (D).

A and B show the normal Leydig cells (LC).

C. BPA-treated group showing desolution of the cytoplasm of Leydig cells (LC) and severe erosion (star) of the interstitium (see Inset Bar = 5µm).

D. BPA + MLT-treated group showsnormal (arrow) interstitium and Leydig cell cytoplasm (LC). Bar = 2µm

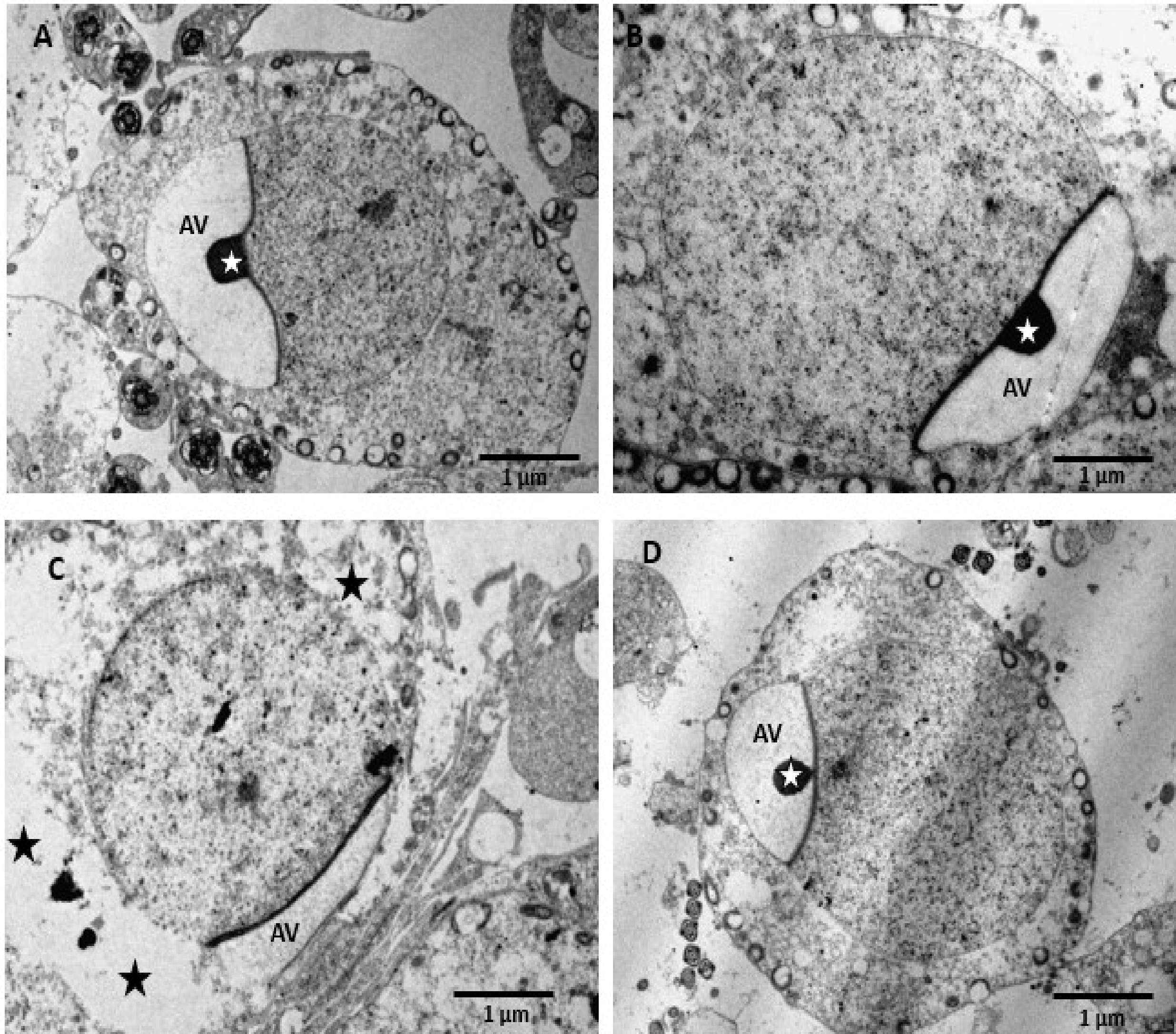


Figure 24. Transmission Electron Microscopy (TEM) Section of round spermatid in Control (A), MLT treated (B), BPA treated (C) and BPA + MLT treated (D).

A and B show normal acrosome vesicle (AV) with acrosomal granule (star) and numerous mitochondria.

C. BPA-treated group showing the distorted acrosome vesicle (AV) and eroded cytoplasm of round spermatid (star).

D. BPA + MLT-treated group showing normal acrosome vesicle (AV) and acrosomal granule (star) with cytoplasm having mitochondria. **Bar** = 1µm

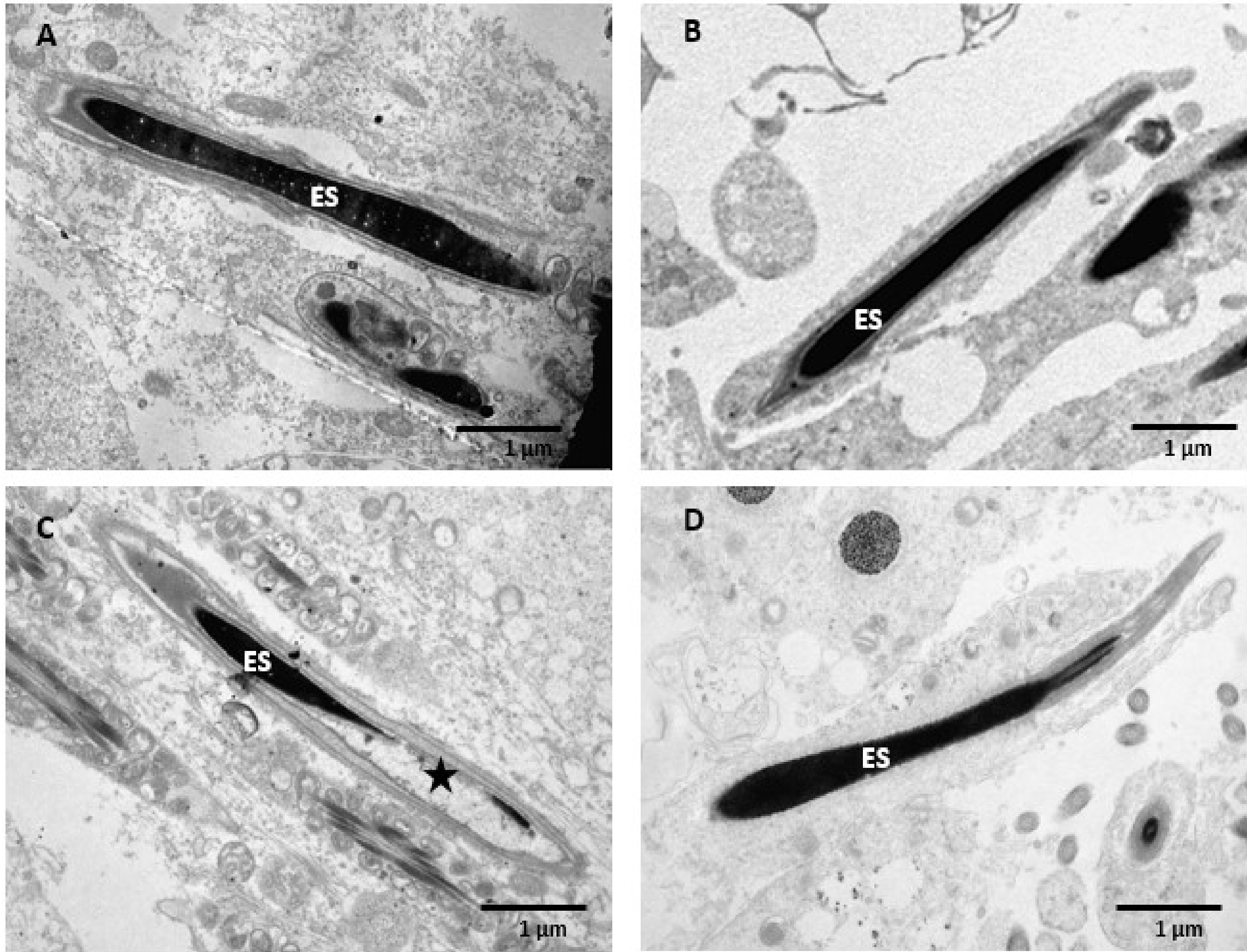


Figure 25. Transmission Electron Microscopy (TEM) section of elongated spermatid (ES) in Control (A), MLT-treated (B), BPA-treated (C) and BPA + MLT-treated (D).

A and B showing normal elongated spermatid (ES)

C. BPA treated group showing karyorhexis (star) of elongated spermatid (ES).

D. BPA + MLT-treated group showing elongated spermatid having normal nucleus. **Bar** = 1µm

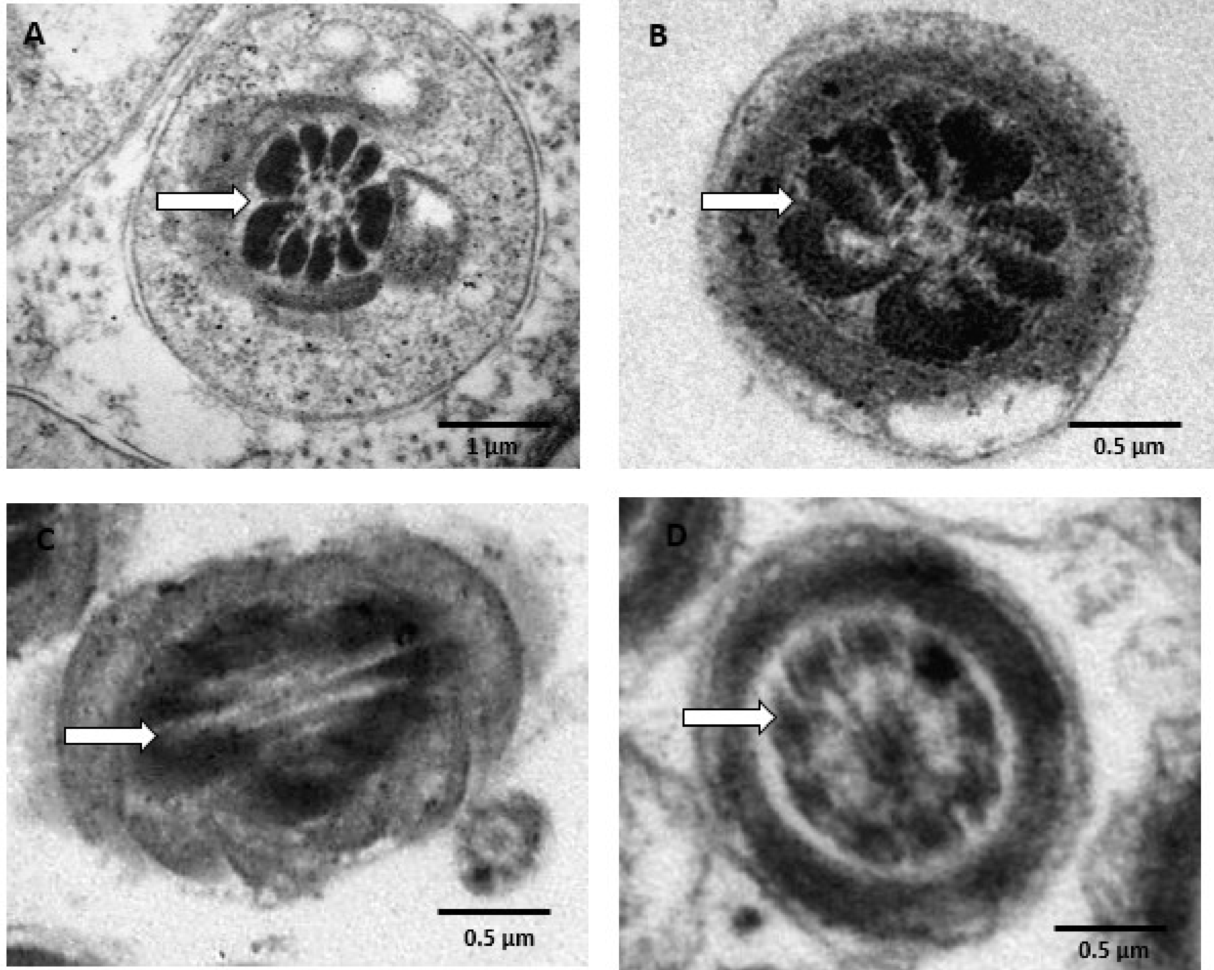


Figure 26: Transmission Electron Microscopy (TEM) transverse section of spermatozum showing the 9+2 Axoneme structure of spermatozum in Control (A), MLT treated (B), BPA treated (C) and BPA + MLT treated (D).

A and B groups show normal 9+2 Axoneme structure (white arrow).

C. BPA-treated group showing a distortion in the axoneme structure.

D. treated BPA + MLT shows the normal 9+2 Axoneme structure of the spermatozum. **Bar** = 0.5µm

4.4.4 Immunohistochemical localization of protein in the testes of rats

The percentage contributions of positive reactions to alpha Smooth Muscle Actin (αSMA), S-100 and Vimectin (Vm) in the testes of rats in the present study are given in Table 8. BPA caused lowexpression of αSMA, S-100 and Vm in testis (figures 27-30). There were significant differences ($P<0.05$) in staining intensities for SMA, Vm and S-100 proteins between the control and BPA-treated groups (Table 8). There were no significant differences ($P>0.05$) in the staining intensities for the 3proteins between the control and MLT-treated groups (Table 8). Concomitant treatment with MLT improved on the percentage contributions of positive reactions to SMA, S-100 and Vm in the testes of the rats especially at the Leydig cells, blood vessels and peritubular membrane level.

Table 6.Quantificantion ofpercentage proteins expression in the testes of rats

Proteins	CONTROL	MLT	BPA	BPA+MLT
SMA (%)	8.85±0.55[a]	9.98±0.51[a]	4.48±0.37[b]	5.80±2.35[a]
S-100 (%)	4.72±0.51[a]	4.88±0.61[a]	3.40±0.01[b]	5.24±0.26[a]
Vm (%)	7.87±1.57[a]	7.35±1.16[a]	4.39±1.13[b]	4.68±0.25[a]

Values expressed as Means ± Standard Deviation (SD)

Means with different superscripts within rows are significantly (P<0.05) different.

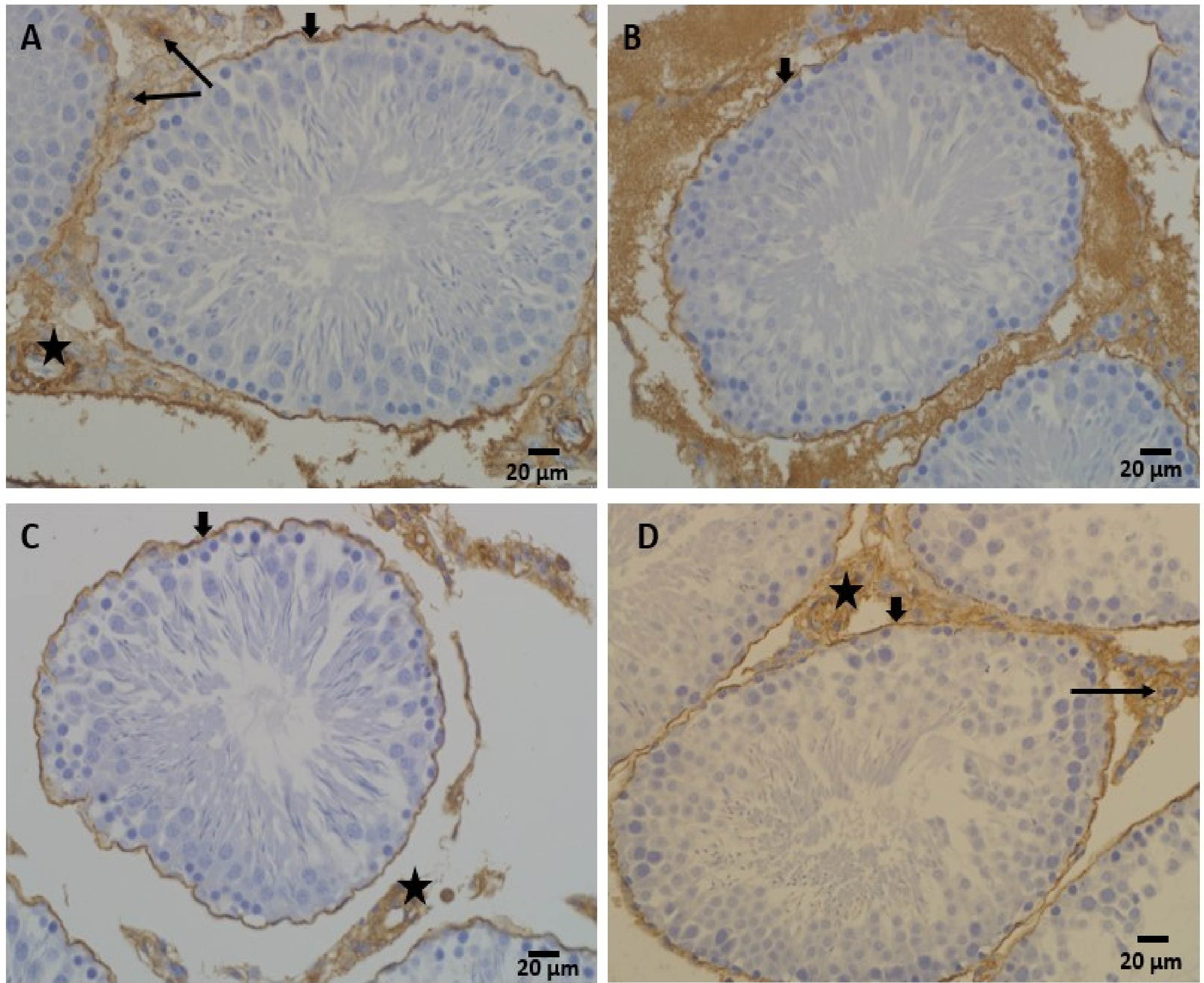


Figure 27. Immunohistochemical localization of SMA in the testis: Control group (A), MLT treated group (B), BPA treated group (C) and BPA + MLT treated (D).

A and Bshow SMA-positive section with higher intensity of staining at the basement membraneand blood vessels.

C. BPA-treated group showing reduced intensity of staining at the basement membrane (arrow head) as well as blood vessel (star).

D. BPA+MLT-treated group showing better intensity of staining (compared to the BPA group) at the basement membrane (arrow head) and blood vessel (star).

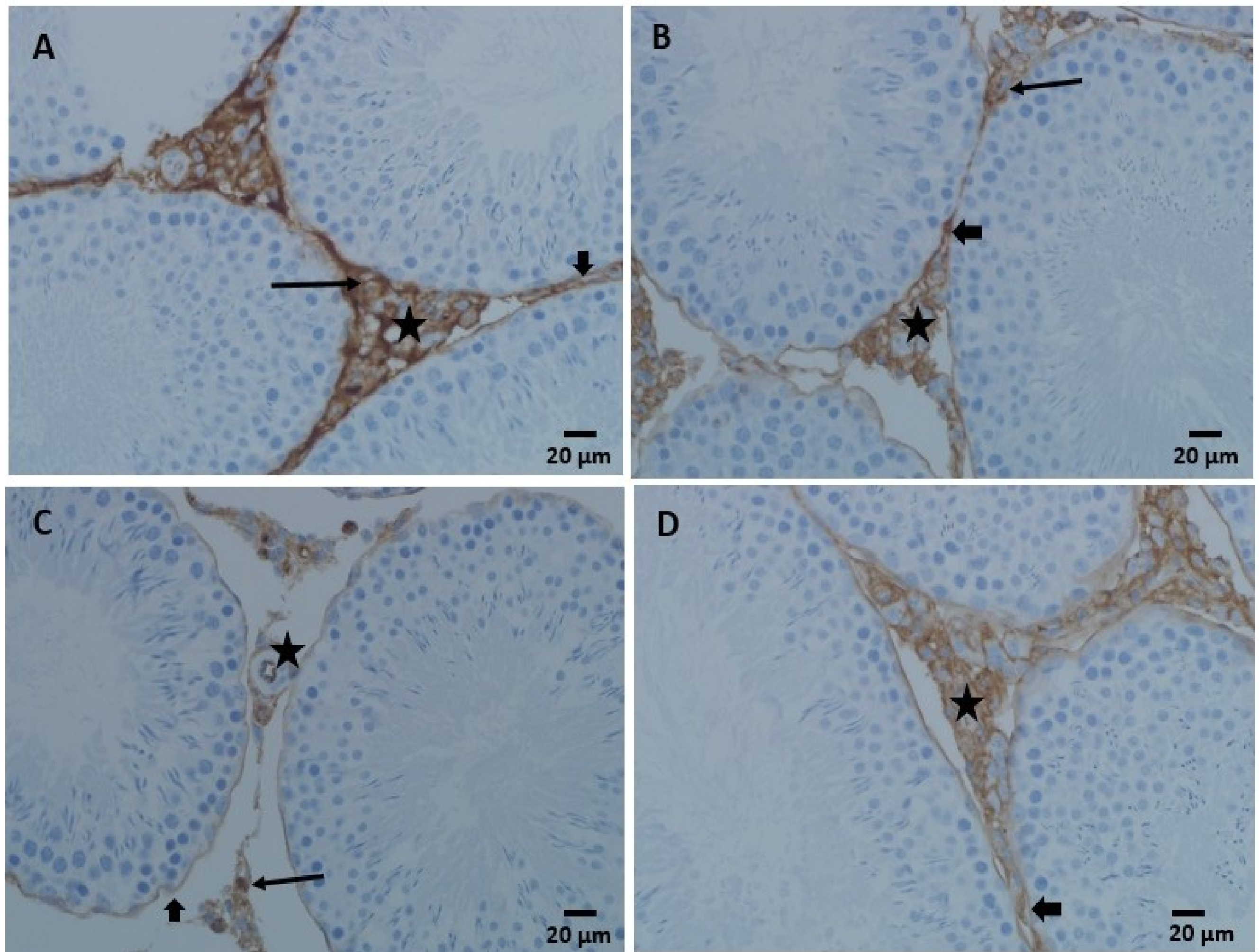


Figure 28. Immunohistochemical localization of Vimectin in the testes: Control (A), MLT treated (B), BPA treated (C) and BPA + MLT treated (D).

A and B showing Vimectin-positive section with higher stain intensity for the blood vessels and Leydig cells.

C. BPA -treated group showing a reduced staining intensity indicating Vimectin collapse.

D. BPA + MLT- treated group showinghigher staining intensity for blood vessels and peritubular membrane.**Bar** = 20µm

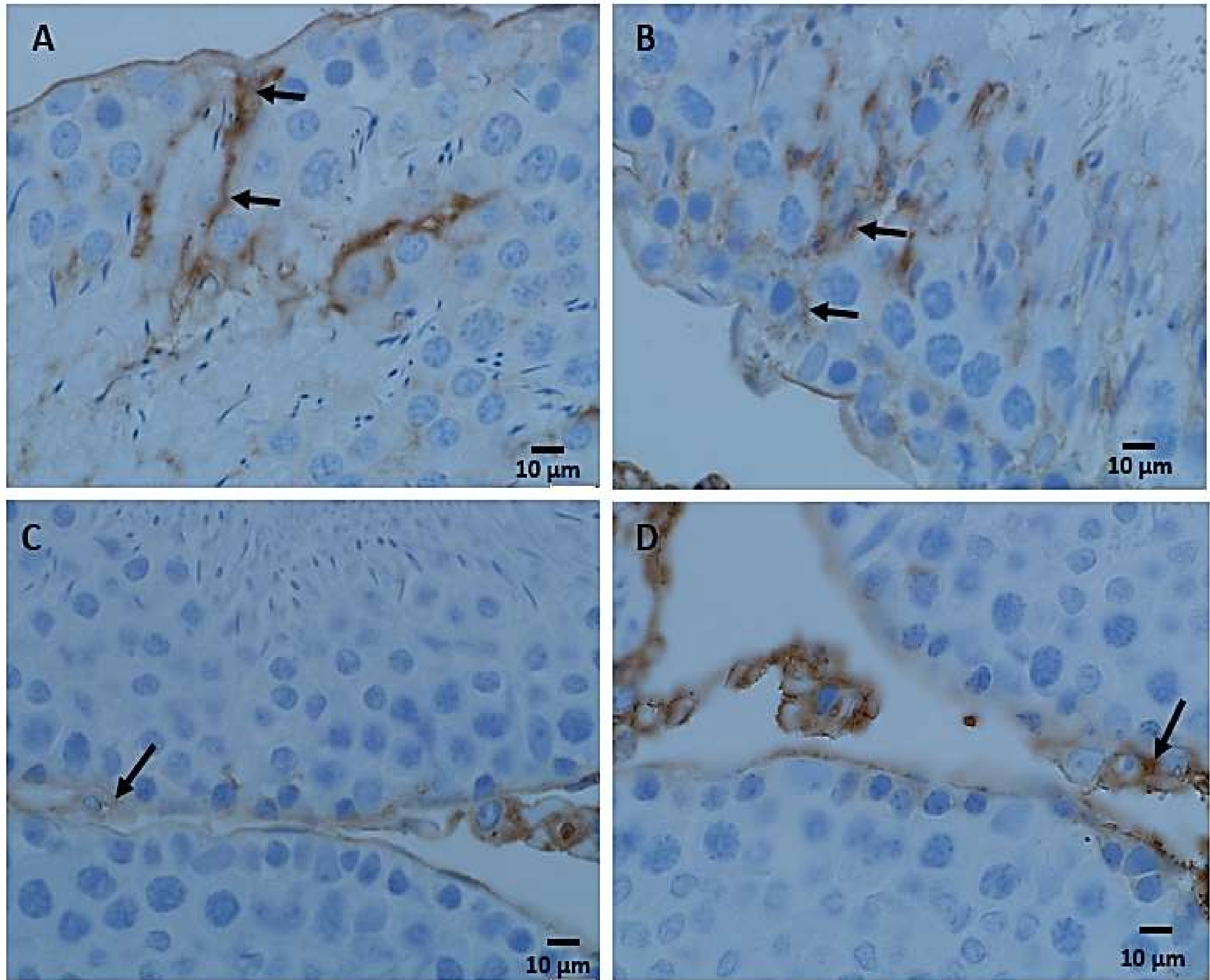


Figure 29. Immunohistochemical localization of Vimectin in the testes at higher magnification showing: Control (A), MLT treated (B), BPA treated (C) and BPA + MLT treated (D).

A and B shows Vimectin-positive-Sertoli cell processes

C. BPA-treated group showing a reduced staining intensity at Sertoli cell level with no Sertoli processes.

D. BPA + MLT-treated group showinghigher staining intensity.**Bar** = 10µm

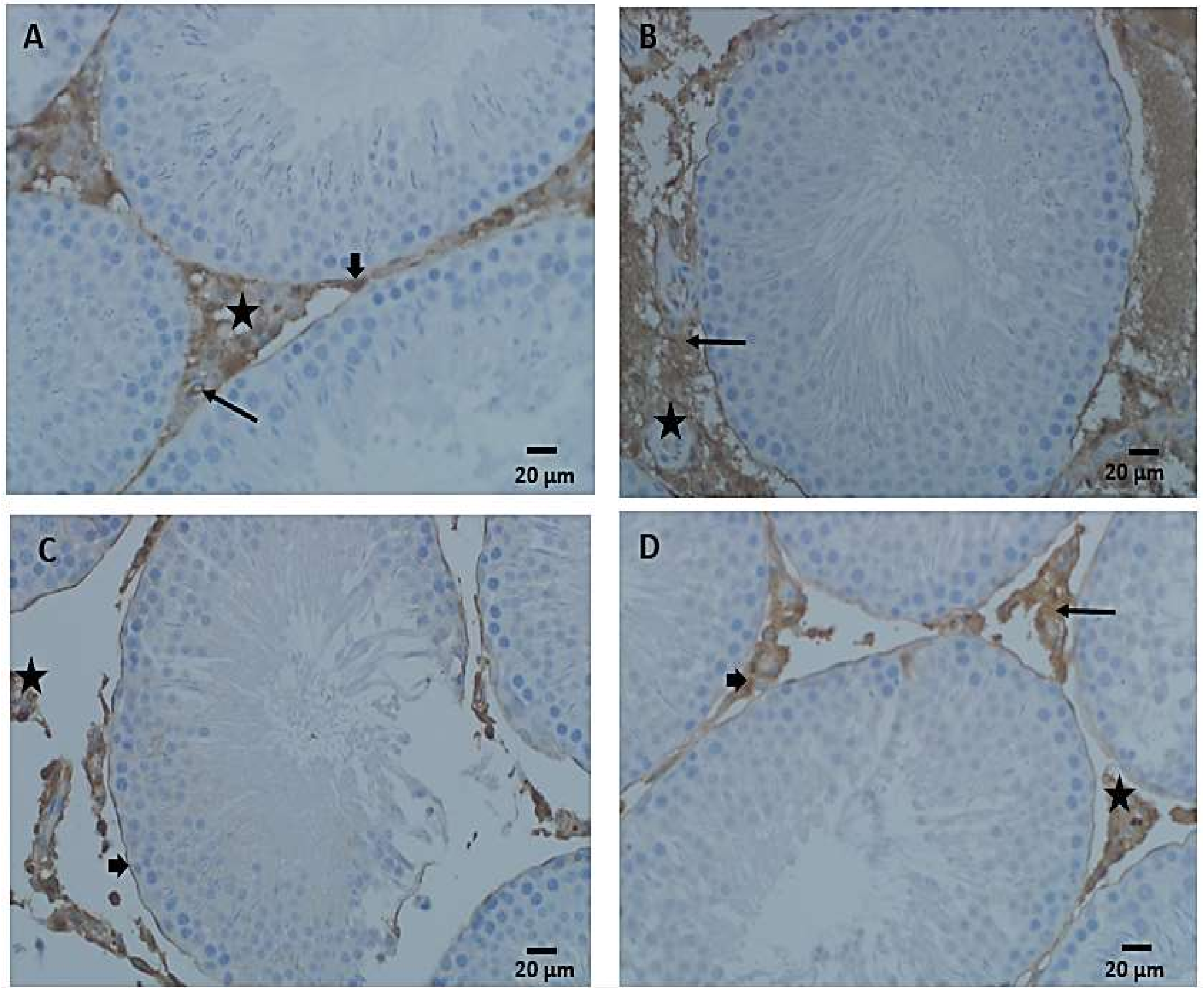


Figure 30. Immunohistochemical localization of S100 in the testes at higher magnification showing: Control (A), MLT treated (B), BPA treated (C) and BPA + MLT treated (D).

A and B shows S100-positive-Leydig cells (arrow), blood vessels (star) and peritubular membrane (arrow head).

C. BPA-treated group showing reduced staining intensity of S-100 collapse for peritubular membrane (arrow head) and blood vessel (star) treated.

D. BPA + MLT-treated group showing higher staining intensity at the peritubular membrane (arrow head) and Leydig cells (star).**Bar** = 20µm

CHAPTER FIVE

5.1 DISCUSSION

Findings from this study showed that a chronic exposure of adult male rats to BPA (10 mg/kg) for 45 days is capable of inducing alterations in their reproductive functionswhich can precipitate infertility. Although a dose of 50 µg/kg/day BPA has been demonstrated as the standard daily tolerable dose for humans while a dose of 50 mg/kg has been demonstrated as not having or showing Observable Adverse Effect Level (NOAEL) for BPA in rodents (Doerge*et al*., 2011), a number of studies in rodents have shown that BPA dose as low as 3 mg/kg/day for 2 weeks resulted in significant alterations in testicular and epididymal structure and weights, as well as in hormonal levels (Chitra *et al*., 2003; Yang *et al*., 2010; Doerge*et al*., 2011; Othman *et al*., 2016). Conversely, Ashby *et al*. (2009) reported that oral exposure of BPA (20 µg/kg, 2 mg/kg) to male Sprague Dawley rats over postnatal days 91–97, produced no significant differences in testicular weight after 5 weeks in the rats.

In the early 2000, there were controversial reports on the effect of the exposure of low doses of BPA on reproductive parameters in adult male rats. While some studies reported that exposure to oral doses of BPA (0.2 µg - 100 mg/kg) to adult male rats had no significant effect on male reproductive parameters (Ema *et al*., 2001; Tyl *et al*., 2002; Tinwell *et al*., 2002), a few other studies maintained that it induced marked changes in male reproductive parameters (Talsness *et al*., 2000; Sakaue *et al*., 2001; Takahashi and Oishi, 2001). With more investigations on BPA, some authors later consistently reported marked adverse effect of low doses of BPA on the male reproductive system (Herath *et al*., 2004; Wu *et al*., 2011; Brandt *et al*., 2014; Bernardo *et al*., 2015; Ahbab *et al*., 2017; Tian et al., 2017). More recently, Olukole *et al*. (2018) demonstrated that the exposure of adult male rats to oral BPA (10 mg/kg) for 14 days produced marked estrogenic effect including increase in prostatic index among other alterations in the structure of the prostate gland of rats.

The BPA-induced significant decreases in testicular and epididymal weights observed in the present study shows that the chemical has adverse effect on reproductive performance of rats. This correlates positively with the previous reports on the effect of BPA on the male

reproductive organs (El-Beshbishy *et al*., 2013; Kazemi *et al*., 2016; Othman *et al*., 2016; Olukole *et al*., 2018).

The absence of significant differences in body weights between the control and BPA-treated groups in the study confirms the report of Chitra *et al*. (2003) that exposure of adult male rats to BPA for 45 days resulted in a significant decrease in gonado-somatic index (GSI) with no significant difference in body weight. BPA-induced decrease in the weight of male reproductive organs has been linked to the ability of BPA to inhibit spermatogenesis, decrease elongated spermatids and alter steroidogenic enzyme activities (Takahashi and Oishi, 2001). However, the observed ability of MLT to protect against BPA-induced decreases in testicular and epididymal weights shows that it is protective against BPA-induced toxicity. This is in accord with previous reports on the effect of MLT in BPA-induced toxicity of the male reproductive system (Anjum*et al*., 2011; Olukole *et al*., 2018).

The BPA-induced significant decrease in epididymal sperm motility, livability and count observed in the present study shows that oral administration of BPA (10 mg/kg per day body weight) in adult rats for a 45day period can result in male reproductive perturbations. Sperm motility, livability and count are key indices of potential fertility in the male animals (Garner and Hafez, 1993).Reduction in the number of actively motile sperm cells in semencan impart negatively on mating outcome. The abundance of sperm cells having tail abnormalities in the BPA-treated group is also a pointer to the reduction in the mean percentage sperm motility observed in this study. Such abnormalities are capable of reducing the fertilization potential of sperm cells. A number of authors have also reported significant decrease in sperm motility, livability and count on the exposure of BPA to adult male rats (Kazemi et al., 2016; Ahbab *et al*., 2017; Tian et al., 2017). Interestingly, MLT protected against all the observed alterations in sperm quality and quantity showing its ability to protect the male reproductive function. This claim is supported by previous authors that the prevention of BPA-induced toxicity has been mediated through the use of several antioxidants (Anjum *et al*., 2011; Jain *et al*., 2011; Othman *et al*., 2016).

The significant decreases in the activities of catalase, SOD, GSH and GPX as well as the increase in LPO and H_2O_2 content in the epididymis due to BPA intoxication observed in the present study are in consonant with the findings of Chitra *et al*. (2003) as well as those of Anjum *et al*. (2011). The ability of MLT to protect against these observed changes in the oxidative stress markers of epididymal sperm of adult rats is a clear indication of the antioxidant properties of MLT. In the present study, BPA induced increase in LPO and H_2O_2 content in epddidymal sperm. Authors have reported that increased levels of H_2O_2 mediate toxic effect through the formation of hydroxyl radical a potent activator of lipid peroxidation (Saradha and Mathur, 2006; Oyagbemi *et al*., 2010). It has been demonstrated that marked reduction in the activity of catalase reflects the inability of epididymal sperm to eliminate H_2O_2 generated due to BPA intoxication as glutathione peroxidase has been shown to directly act as antioxidant enzymes resulting in the inhibition of sperm lipid peroxidation (Lenzi *et al*., 1994; Sikka, 2001; Chitra *et al*., 2003). Thiele *et al*. (1995) has suggested that BPA-induced decrease in epididymal sperm motility, livability and count are as a result of increased lipid peroxidation. Findings from this study confirm increased lipid peroxidation after BPA intoxication in rats. The roles of melatonin in the maintenance of the male reproductive function and the amelioration of a number of pathologic situations associated with male reproductive system dysfunction have been traced to its antioxidant properties (Rocha et al., 2015).

The results of the histopathogical assessment of the epididymis show that BPA perturbs the epididymis significantly and this corroborates observations on biochemical assays. The erosion of epididymal sheath, disintegration of epididymal duct membrane as well as sloughing off of spermatozoa within the lumen of the epididymis observed in the BPA-treated group confirms the findings of previous authors on the effect of BPA with respect to male reproductive organs (Molina-Molina *et al*., 2013; Ullah *et al*., 2016; Feng *et al*., 2016). The sloughing off of epithelial cells of the cauda epididymidis observed in the study is capable of affecting the secretory role of the epididymal epithelium. The mammalian epididymis has been demonstrated to induce biochemical changes in gametes by its catalytic activity of acid hydrolases secreted by the epididymal epithelium (Tulsiani *et al*., 1998; Dacheux *et al*., 2005; Tulsiani & Abou-Haila, 2011; Ullah *et al*., 2016). Some authors have opined that the secretory activity of cauda epididymidis is vital to sperm maturation (Dacheux *et al*., 2005; Tulsiani & Abou-Haila, 2011;

Carvelli *et al*., 2014). Epididymal epithelial cells monitor the secretory and endocytotic activities of the epididymis and thus ensure that adequate amount of proteins as well as water and ions required for sperm maturation are made available (Turner, 2002). The observed BPA-induced injuries to the epididymis have great potential of affecting further maturation and storage of spermatozoa in the cauda epididymidis of the rats. The cauda epididymidis not only stores the sperm but also makes available the enabling environment for holding sperm in a quiescent state of metabolism.

The BPA-induced decreased localization of SMA and Vm in the cauda epididymidis observed in the study implies that the sperm maturation function of the cauda epididymidis will be affected. For example, the BPA-induced collapse of SMA and Vm observed in the rats is capable of reducing the contractile activity of the epididymis and in turn negatively affect sperm transport, a major function of the epididymis. In the present study, BPA induced a decrease in the expression of S-100 protein. The S-100 protein is a multifunctional subfamily of Ca^{2+}-binding proteins that have been demonstrated in a number of metabolic functions including motility, chemotaxis, and secretion (Heizmann *et al*., 2002). Put together, the decreased expression of SMA, S-100 and Vm by BPA in the cauda epididymidis of Wistar rats is capable of affecting the regulation of cell morphology, the dynamics of certain cytoskeleton constituents, and the reciprocal relationships of cytoskeleton element via direct or indirect interactions with microtubules, intermediate filaments, microfilaments, myosin, and tropomyosin (Donato, 2001; Abd-Elmaksoud *et al*., 2014).

Also, the BPA-induced decreases in localization of SMA, Vm and S-100 proteins in the testes observed in the present study positively correlate with findings from the epididymis. The decrease in the localization of SMA and S-100 proteins at the testicular interstitium as well as the basement membrane of the seminiferous tubules implies that the contractile activities of the blood vessels as well as those of the peritubular myoid cells would be impaired. Peritubular myoid cells has been shown to be involved in the regulation of Sertoli cell functions in mammals and are known to stimulate total protein production by Sertoli cells thereby increasing the production of androgen-binding protein and transferrin (Hadley et al., 1985; Anthony et al., 1991). The BPA-induced decrease in the localization of Vm by the Sertoli cells implies that the structural and nutritional support functions of Sertoli cells to the germ cells will be altered in the

rats. This corroborates the BPA-induced degeneration of germ cells observed with histopathology as well as TEM. Similar observations with BPA have been reported by Olukole *et al*. (2018). Hence, a decrease in the localization of Vm is expected to result in the alteration of the process of spermatogenesis. However, the up-regulation of these proteins by a concomitant treatment with MLT shows its protective role against BPA-induced epididymal and testicular protein down-regulation.

The histopathological assessment of the testes in the current study showed that administration of BPA elicited severe pathological damages in the testes. The marked reduction in the number of germ cells as well as their degeneration observed in the BPA-treated group shows its capability of altering the process of spermatogenesis in rats with grave consequences for reproduction. This corroborates the findings of Tian *et al.* (2017) in rats challenged with oral graded doses of BPA. Recent research on the effect of BPA on the fertility of male rats have consistently shown that it impaired male fertility causing a number of testicular dysfunction, including induction of apoptosis of testicular germ cells, disruption of the junctional proteins of the blood-testis barrier, alterations in the levels of androgen binding protein and steroidogenic enzymes (Peretz *et al*., 2014; Wang *et al*., 2015; Quan *et al*., 2016; Durando *et al*., 2016; Tian *et al*., 2017).
The erosion of the testicular interstitium leading to a reduction in the number of Leydig cells is capable of inducing a decrease in circulating testosterone produced by the Leydig cells. Results of morphological injuries on the testes induced by BPA- exposure of rats by TEM in this study are similar to earlier report by Tian, (2017). Severe erosion of the germinal cells, dissolution of the Leydig cell cytoplasm, pyknotic nucleus of the Sertoli cells and karyorhexis of elongated spermatid observed after exposing the rats to BPA further corroborates the histological observation with the testis. The spermatogenic cells are maintained by Sertoli cells to ensure continuity of spermatogenesis (O'Shaughnessy, 2014).

Also, the primary morphological abnormalities of sperm spermatozoa observed in the current study might be attributed to the BPA-induced spermatogenic arrest in the rats. This is further explained by the distortion in the 9+2 axoneme structure of the spermatozoa in the BPA-exposed rats as revealed by TEM. The axoneme arrangement of microtubule doublets of spermatozoa are vital metabolic apparatus for sperm viability. This implies that the distortion of such structural metabolic apparatus will alter spermatogenesis and may result in infertility in the

rats. However, a restoration of these anomalies by a simultaneous treatment with MLT shows its protective role against BPA- induced reproductive toxicity in adult male rats.

The BPA-induced decrease in testosterone and DHEAS as well as the corresponding increases in estradiol and FSH correlate positively with histopathological and semen analysis. The reduction in Leydig cells activity must have been responsible for the decreased production of testosterone in the BPA-treated rats. BPA exposure has been reported to induce the reduction of testosterone in adult male rats (Chitra ea al., 2003; Nakammura, *et al* 2010; Olukole et al., 2018). Testosterone is carried by androgen binding protein (ABP) in the seminiferous tubules of the testis towards the epididymis. Testosterone is converted by the action of 5-alpha-reductase enzyme to dihydro testosterone (DH T) (Robaire *et al*., 2006; Robaire and Hamzeh, 2011). Testosterone is responsible for the initiation of spermatogenesis and the maintenance of certain functions of Sertoli cells after it is being converted to estradiol by Aromatase (O'Donnel *et al*., 2008). The BPA-induced increases in estradiol and FSH show that BPA is capable of eliciting estrogenic changes in rats. This is in conformity with previous reports (Pinto *et al*., 2008; Kazemi et al., 2016; Ahbab *et al*., 2017). However, simultaneous treatment with MLT protected against the BPA-induced alterations in testosterone, DHEAS, estradiol and FSH.

5.2 Conclusions

Findings from this study have shown that chronic exposure to low dose BPA causes marked estrogenic effects in adult male rats including epididymal sperm oxidative stress, sperm anomalies, histopathological perturbations of the testes and epididymis, steroidogenic anomalies as well as proteins downward regulation. The study has also demonstrated the ability of MLT, a potent antioxidant, to protect against BPA-induced toxicities of the male reproductive function. Hence, MLT is recommended in the prevention of BPA-induced toxicities of the male reproductive function in adult male rats especially in chronically exposed population.

5. 3 Contributions to Knowledge

This study has been able to contribute to the body of scientific knowledge in the following areas:

1. That BPA induces epididymal spermatozoa toxicity by altering the antioxidant defense mechanism while MLT prevented it.
2. That BPA-induced spermatozoa morphological abnormality is as a result of the distortion of the 9 + 2 axoneme arrangement of microtubule doublets of the flagellar apparatus of the spermatozoa.
3. The down-regulation of alpha smooth muscle action actin (αSMA), vimentin (Vm) and S-100 proteins in BPA-exposed rats and the ability of melatonin (MLT) to prevent these alterations in protein localization in the testis and epididymis of rats.

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