EFFECTS OF MATERNAL SLEEP DEPRIVATION ON REPRODUCTIVE FUNCTIONS OF MALE OFFSPRINGS IN WISTAR RATS

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ABSTRACT

Maternal Sleep Deprivation (MSD) has been reported to alter sexual performance in offsprings of rats. There is paucity of information on the critical period during gestation at which MSD affects reproductive functions. This study was designed to investigate the effects of MSD at different gestation periods on reproductive functions of male offsprings in Wistar rats.

Sixty pregnant rats were assigned into six (Control = C; Sleep Deprived = SD) groups (n=10) at different Gestation Days (GD) as follows: GD1-7C, GD1-7SD, GD8-14C, GD8-14SD, GD15-21C and GD15-21SD. The MSD was induced using the modified multiple platform method. Caesarean section was performed on five animals from each group on GD 8, 15 and 21 for placental morphometric and biochemical analyses. The remaining five dams from each group littered naturally and only their male offsprings were studied. The offsprings were weighed on post-natal day 1 on a digital electronic balance. Reproductive functions of male offsprings were determined by assessing; testes descent, fertility index, sperm count and motility, testosterone, corticosterone, melatonin and reproductive organ histology. The testes descent day was determined by daily palpation of the scrotal sac for the presence of testes. Fertility index was determined on post-natal week 17 after mating, as percentage of female rats (of proven fertility) impregnated by the male offsprings according to the standard technique. Epididymal fluid was analysed by microscopy to determine the sperm motility and sperm count. Serum testosterone, corticosterone and melatonin were quantitated by ELISA. Histological assessment of the testes and epididymes was done using paraffin section processing and microscopy for tissue microscopic examination. Placental malondialdehyde, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were assayed by spectrophotometry to determine the redox status. Immunohistochemistry of placental *Bcl*$_2$ and *p53* was done to determine apoptotic status. Data were subjected to descriptive statistics and analysed using Student's t-test at $\alpha_{0.05}$.

Birth weight reduced in GD15-21SD (5.3±0.1 vs 5.7±0.2 g). Testes descent occurred late in GD15-21SD (25.8±0.4 vs 23.0±0.8 days). Fertility index of GD15-21SD was 0 %. Sperm motility and count decreased in GD15-21SD (72.0±4.9 vs 89.0±2.9 %; 64.4±14.9 vs 114.5±3.0 million/mL). Offspring's testosterone reduced in GD15-21SD (2.9±1.1 vs 7.0±1.3 ng/mL). Offspring's corticosterone increased in GD1-7SD, GD8-14SD and GD15-21SD (106.0±3.0, 115.0±8.2, and 131.6 ± 6.6 nmol/L) compared with their controls (57.2±19.5, 65.8±5.9, 96.6±9.3 nmol/L), respectively. Melatonin increased in GD1-7SD (482.1±33.5 vs 355.3±19.21

nmol/L) and decreased in GD8-14SD (237.3±15.1. vs 353.6±20.5 nmol/L). The testicular and epididymal sections of GD15-21SD showed aberrant seminiferous tubules and atrophic ducts respectively. Dam's testosterone reduced in GD15-21SD (216.0±5.8 vs 370.0±38.2 pg/mL). Placental malondialdehyde increased in GD1-7SD (0.1±0.0 vs 0.0±0.0 nmol/mg) and GD15-21SD (0.2 ± 0.0 vs 0.1 ± 0.0 nmol/mg). Placental SOD decreased and GPx increased in GD15-21SD (62.9±5.6 vs 103.0±11.7; 53.0±5.7 vs 35.3±4.7 U/mg), respectively. *Bcl_2* and *p53* decreased in GD15-21SD decidua (7.8±0.7 vs 16.4±0.2; 0.4±0.1 vs 9.4±0.3 %), respectively.

Reproductive functions were adversely affected during the fifteenth to twenty-first days of gestation.

Keywords: Maternal sleep deprivation, Male rat offsprings, Reproductive functions.
Word count: 478

DEDICATION

To HIM Who knew me before I was formed in my mother's womb;
for HIS thoughts of peace, for the hope HE has given and for the future HE has perfected.

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CERTIFICATION

I certify that this work titled: '**Effects of maternal sleep deprivation on reproductive functions of male offsprings in Wistar rats'** was carried out by Mrs. Opeyemi Oreofe, **AKINDELE** in the Department of Physiology, College of Medicine, University of Ibadan.

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LIST OF ABBREVIATIONS

CVD	Cardiovascular Disease
NCD	Non-Communicable Disease
11βHSD$_2$	11-β-Hydroxy-Steroid Dehydrogenase II
HPA	Hypothalamic-Pituitary-Adrenal
IUGR	Intra-Uterine Growth Restriction
IGF	Insulin-like Growth Factor
PEPCK	Phospho-Enol Piruvate Carboxyl Kinase
EDC	Endocrine Disrupting Compounds
TDS	Testicular Dysgenesis Syndrome
CDC	Centre for Disease Control
NHIS	National Health Interview Survey
SRS	Sleep Research Society
AASM	American Academy of Sleep Medicine
NSF	National Sleep Foundation
NREM	Non-Rapid Eye Movement
REM	Rapid Eye Movement
GD	Gestation Days
C	Control
SD	Sleep Deprived
MMPM	Modified Multiple Platform Method
LGA	Large for Gestational Age
MAP	Mitogen Activated Protein

i.p.	Intraperitoneal
PND	Post-Natal Day
PNW	Post-Natal Week
AGD	Ano-Genital Distance
VPR	Volume Pressure Recording
O Cuff	Occlusion cuff
BCG	Bromocresol Green
TBARS	Thiobarbituric Acid Reactive Substances
TBA	Thiobarbituric Acid
MDA	Malondialdehyde
TCA	Trichloroacetic acid
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
DCF	Dichlorofluorescin
TAC	Total Antioxidant Capacity
TAS	Total Antioxidant Status
ABTS	1, 2'-Azino-di-(3-ethylbenzthiazoline sulphonate
EDTA	Ethylenediaminetetraacetic acid
GPx	Glutathione Peroxidase
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
GSH	Reduced glutathione
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
PBS	Phosphate Buffer Solution

SEM	Standard Error of Mean
SPSS	Statistical Package for Social Sciences
BW	Birth Weight
HC	Head Circumference
ABC	Abdominal Circumference
CRL	Crown-Rump Length
AGD*i*	Anogenital Distance index
LS	Litter Size
FW	Foetal Weight
PW	Placental Weight
PT	Placental Thickness
PV	Placental Volume
PCSA	Placental Chorionic Surface Area
PC	Placental Coefficient
FPR	Foeto-placental Ratio
GFR	Glomerular Filtration Rate
HPG	Hypothalamic-Pituitary-Gonadal

CHAPTER ONE

1.0 INTRODUCTION

The concept of developmental programming of adult health and disease, originally known as the 'Barker hypothesis' is an established biological concept that associates adverse environmental conditions in early life with risk of disease in adult life (Lucas, 1991). The 'Barker hypothesis' was postulated about 25 years ago from large retrospective epidemiological studies that revealed a positive correlation between infant and adult mortality rate and certain classes of adult diseases (Barker and Osmond, 1986). These deaths were associated with low birth weight (Barker *et al.*, 1989), as well as altered birth anthropometric indices (Barker, 1995) caused by maternal undernutrition during gestation rather than post-natal factors (Barker, 1995). Based on these observations, it was concluded that foetal experience during intra-uterine life can predict trajectories of adult health and disease (Barker, 2007). These findings of Barker have since been validated by several epidemiological, clinical and experimental studies which also revealed that, aside from undernutrition, a variety of stressful events during pregnancy may cause low birth weight with disproportionate body size at birth and subsequent postnatal disease (Myatt, 2006; Currrie, 2011).

Developmental programming has contributed greatly to the progressive increase in the prevalence of major chronic diseases over the last 20-40 years in our society (Barouki *et al.*, 2012). For instance, in the developing countries, the prevalence of the non-communicable diseases and reproductive disorders have been on the rise (Boutayeb and Boutayeb, 2005). Globally, Cardiovascular Disease (CVD) contributes mostly to the occurrence of Non-Communicable Disease (NCD)-related mortality (Unwin and Alberti, 2006). CVD is responsible for 30% of total global mortality (Unwin and Alberti, 2006) and the risk of mortality resulting from CVDs is often increased by co-existing chronic kidney disease (Couser *et al.*, 2011). The prevalence of chronic kidney disease has also risen over the past few decades

(Wattanakit and Cushman, 2009) contributing greatly to the poor prognosis of CVDs (Couser *et al.*, 2011). It is therefore understandable if the recent growth of interest in the field of developmental programming of physiological systems has been focused on these NCDs (Zambrano *et al.*, 2014). While it is alarming that NCDs kill more people every year than all causes of death combined (Alwan *et al.*, 2010; Misganaw *et al.*, 2014), it is even more appalling that reproductive dysfunction and infertility may threaten the survival and continuity of the human species (Comizzoli *et al.*, 2010; Silber, 2011). To date, relatively few studies have considered the effects of early life events on reproductive functions, even though, it is clear that the full range of affected phenotypes of offsprings in response to maternal insults includes reproductive diseases (Zambrano *et al.*, 2014).

The abnormal stimuli and stressful events to which mothers are exposed during gestation or lactation are referred to as maternal insults (Fowden *et al.*, 2006a). Developmental programming of physiological systems has been demonstrated experimentally in many species using different forms of stress to produce sup-optimal intra-uterine environment and alter foetal development (McMillen and Robinson, 2005). These controlled animal studies have established that the nature and intensity of the stressful stimuli, the duration of exposure and their timing of occurrence during pregnancy are important determinants of the pattern of foetal growth and the ensuing disease in later life (Bertram and Hanson, 2001). Examples of maternal insults which have been implicated in programming of cardiovascular, renal and reproductive functions include; hypoxia (Giussani and Davidge, 2013), nutritional stress (Armitage *et al.*, 2004; Armitage *et al.*, 2005), endocrine disruption (Rhind *et al.*, 2001; Padmanabhan and Veiga-Lopez, 2014), excessive exercise (Hopkins and Cutfield, 2011) and sleep restriction (Chang *et al.*, 2010; Alvarenga *et al.*, 2013).

Sleep is the state of reversible unconsciousness with relative responsiveness from which an individual can be aroused by either sensory or other forms of stimuli (Rasch and Born, 2013). It engages about a third of human life and it is essential for wellbeing and survival (Punjabi and Polotsky, 2005). Sleep is necessary for the conservation of energy (Penev, 2007), detoxification of the brain (Inoue *et al.*, 1995), control of thermoregulation (Libert, 2003) learning and consolidation of memory (Maquet, 2001), maintenance of metabolic-caloric balance (St-Onge and Shechter,

2014), immune competence (Besedovsky *et al.*, 2012) and maintenance of optimal functional levels in various systems (Aldabal and Bahammam, 2011). Sleep needs may vary from person to person, as such, it is difficult to quantify the amount of sleep needed by individuals (Ferrara and De Gennaro, 2001). However, the National Sleep Foundation (NSF) recommends that adults should have 7-9 hours of sleep per 24 hours to ensure the maintenance of health and wellbeing (Hirshkowitz *et al.*, 2015). Nonetheless, the current global population works and lives under a 24/7 lifestyle, relegating sleep to a secondary level of importance (Shochat, 2012; Aldabal and Bahammam, 2011). The result of this lifestyle is a state of inadequate quantity and quality of sleep called sleep deprivation (Mullington *et al.*, 2009).

Sleep deprivation was originally thought to be a problem of the developed nation alone (Van Cauter and Knutson, 2008). However, a recent epidemiological study of sleep-associated problems in Asian and African countries suggested that the world is in the midst of a "global sleeplessness epidemic" (Stranges *et al.*, 2012). In 1998, WHO carried out a worldwide project on sleep and health and discovered that about 50 % of the people in the world encountered one or more sleep disorders including insomnia, narcolepsy and somnambulism (WHO, 1998). Likewise, the percentage of people who slept for 6 hours or less increased significantly during the early 21st century (CDC, 2005; Chang *et al.*, 2010). The increasing adverse effects of sleep deprivation in the population have also been documented (Luyster *et al.*, 2012). While it is difficult to conduct studies of chronic sleep deprivation in humans, experimental studies in animals have shown the association between chronic sleep restriction and altered gene expression (Cirelli *et al.*, 2006), cardiovascular diseases (Fang *et al.*, 2015), renal changes (Periasamy *et al.*, 2015), reproductive dysfunction (Alvarenga *et al.*, 2015) and even death (Rechtschaffen *et al.*, 1983).

One of the major fallouts of reproductive dysfunction is infertility (Zegers-Hochschild *et al.*, 2009). Men reportedly contribute up to 50 % to the prevalence of infertility (Jarow, 2007). However, the commonness of infertility varies across the different geographical zones. Globally, there are approximately 30 million infertile men and 20 % - 70 % of the infertility cases is attributable to these men (Agarwal *et al.*, 2015). Documented evidence suggests that the declining trends in global semen quality is related to the world-wide declining conception rates (Jensen *et al.*, 2008). Sleep deprivation has a strong positive correlation with reduced testosterone

and low sperm quality in healthy men (Jensen *et al.*, 2013; Jauch-Chara *et al.*, 2013) and in rats (Akindele *et al.*, 2014; Alvarenga *et al.*, 2015). Thus, one may infer that sleep deprivation contributes a great deal to the world-wide declining fertility rate (Wittert, 2014).

Accumulating evidence suggests that sleep debt is more common in women than in men (Hublin *et al.*, 2001; Chang *et al.*, 2010). In the current society, many women occupy prominent positions in the work force, in addition to the numerous responsibilities they have at home (Swanson, 2000; Chang *et al.*, 2010). Consequently, their sleep time is used up for other activities and the remaining time (at the end of their daily activities) does not suffice for adequate quality and quantity of sleep (Chang *et al.*, 2010). Besides their overloaded schedules, women often experience pregnancy-related sleep disorders caused by physical, hormonal and behavioural changes during pregnancy (Pien and Schwab, 2004; Miller *et al.*, 2012). On this account, sleep deprivation is often common during pregnancy (Miller *et al.*, 2012). About two third of pregnant women have been reported to experience sleep deprivation globally (Lopes *et al.*, 2004). There is some information that sleep restriction during pregnancy not only increases the risk of psychiatric disorders in the mothers (Ross *et al.*, 2005), it also causes difficult labour and preterm birth (Chang *et al.*, 2010). A direct effect of maternal sleep deprivation on the health of offspring in human has not been reported, however, some postnatal effects of maternal sleep deprivation in rat's offspring have been documented (Thomal *et al.*, 2010; Alvarenga *et al.*, 2013; Lima *et al.*, 2014; Peng *et al.*, 2016). Also, the ability of sleep deprivation to cause hormonal disruption during pregnancy is suggestive of its plausible adverse effects on growth and development of foetal organs, even in humans (Fowden and Forhead, 2009; Aldabal and Bahammam, 2011).

Foetal hormone availability is regulated by the placenta which serves as the link between the maternal environment and foetal environment (Myatt, 2006). The placenta produces peptides and steroid hormones which influence maternal, placental and foetal metabolism (Jansson and Powell, 2013). There is growing appreciation that maternal insults are transferred to the foetus through alterations in placental functions (Fowden *et al.*, 2009). The placenta adapts to changes in the maternal environment as it develops, in order to perform its functions optimally (Myatt, 2006). Consequently, perturbations in the maternal system caused by

unfavourable changes in the external environment modify the placental structure and functions (Zeltser and Leibel, 2011), thereby impacting the growth and development of the foetal tissues (Myatt, 2006). For instance, maternal stress reduces placental 11-β-Hydroxyl-Steroid Dehydrogenase II (11βHSD$_2$) activity that normally protects the foetus from harmful effects of maternal glucocorticoids (Wyrwoll *et al.*, 2009). Maternal hyperglycaemia triggers upregulation of placental glucose transporter (Jansson and Powell, 2007). Maternal protein restriction alters placental gene methylation status (Serman and Dodig, 2011; Chen *et al.*, 2013) and maternal hypoxia increases placental oxidative/nitrative stress resulting in nitration of placental proteins (Myatt, 2006).

The relationship between maternal sleep deprivation, developmental programming of health and disease and altered placental physiology has been established. Despite the important role the placenta assumes in programming, it has attracted only limited attention. The adaptations made by the foetus in response to maternal perturbations are always permanent (Nathanielsz, 2006) and little or nothing may be done during adult life to restore the normal physiology of offspring. It implies that if programming is preventable at all, strategies for intervention may be directed toward the intra-uterine life. Thus, understanding the changes involved during placental adaptation to maternal sleep deprivation will therefore be crucial in designing strategies for interventions required to prevent such placental changes and ultimately to prevent the ensuing foetal programming.

Statement of problem

The prevalence of male infertility is on the rise and it appears to be positively correlated with declining testosterone level in the male population (Agarwal *et al.*, 2015). Many of these infertile men have coexisting cardio-renal diseases (Holley and Schmidt, 2013) and experience early mortality (Eisenberg *et al.*, 2014).

Justification for the study

Masculinization of foetal reproductive organs is androgen-dependent (Macleod *et al.*, 2010) and sleep deprivation has been reported to reduce circulating testosterone level in humans (Wittert, 2014) and in rats (Akindele *et al.*, 2014). This suggests that maternal sleep deprivation may interfere with the process of foetal masculinization. Maternal sleep deprivation has attracted much attention over the last few decades, however, only few studies have explored its effects on reproductive functions of male offsprings with no report on the placenta which has been established to be the conduit of stress from the mother to the foetus (Barker and Thornburg, 2013). Also, the critical period during which the male reproductive functions may be programmed by maternal sleep deprivation remains elusive.

Aim of the study

The research work was therefore designed to examine the effects of maternal sleep deprivation at different gestation periods on reproductive functions of male offsprings in Wistar rats.

Specific objectives of the study

To examine the effects of maternal sleep deprivation during gestation days 1-7, 8-14 and 15-21 on:

1. Birth morphometric indices of male offsprings in Wistar rats,
2. Cardio-renal functions of male offsprings in Wistar rats,
3. Reproductive functions of male offsprings in Wistar rats,
4. Maternal hormone levels in Wistar rats and
5. Placental morphometric indices, redox status, apoptotic markers and epigenetics in Wistar rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Developmental programming

The concept that adverse events in early life that act during precise, sensitive windows of development can program long-lasting changes in structure and physiology of offspring with consequences for health and disease status in adult life is supported by a wealth of data in many species (Seckl and Holmes, 2007). Developmental programming of adult health and disease, originally known as the 'Barker hypothesis' is an established biological concept that associates adverse environmental conditions in early life with risk of disease during adult life (Lucas, 1991). Before the advent of the Barker hypothesis, the congenital rubella syndrome of the early 1940s (Dunn, 2007) and the thalidomide episode of late 1950s (Mcbride, 1961; Lenz and Knapp, 1962) were ground shaking occurrences in the medical world that suggested that foetal experience during intra-uterine life could impact health trajectories during extra-uterine life. These two events defied the previous beliefs that the foetus was a perfect parasite (Susser and Stein, 1994) and that the placenta was a perfect filter (Almond and Currie, 2011). However, not enough publicity was given to the subject matter at the time. In 1986, the 'Barker hypothesis' was postulated and it proposed a relationship between prenatal undernutrition during the mid and late gestation and early onset of coronary heart disease in postnatal life (Barker and Osmond, 1986). Unlike the previous occurrences which had suggested the association between early life events and postnatal health, the 'Barker hypothesis' was explicit and very well publicized (Almond and Currie, 2011). The hypothesis attracted a lot of attention from both proponents and critics, consequently leading to a quick corroboration of Barker's findings in different parts of the United Kingdom (Hales *et al.*, 1991; Osmond and Barker, 2000). In order to find answers to those questions generated by the study that birthed the hypothesis, Barker carried

out further studies and discovered a relationship between prenatal undernutrition and slow rate of cell division which eventually manifested as low birth weight (Barker *et al.*, 1989) and disproportionate body growth that ultimately altered birth anthropometry (Barker, 1995). Further studies by Barker *et al.*, (1990) also revealed that other than foetal nutrition, neonatal and infantile nutrition may also affect health state later in life (Barker *et al.*, 1990).

The word 'programming' was introduced by Lucas in 1991, to describe the long-term consequences of nutrition (Lucas, 1991). In 1994, Martyn further expounded programming as a phenomenon whereby a long-term and irreversible alteration in structure or metabolism is induced by a relatively brief stimulus (Martyn, 1994). The concept that the foetus undergoes physiological programming to adapt to its environment was then called 'foetal programming' (Hales and Barker, 1992; Desai and Hales, 1997; Barker *et al.*, 2002). Concurrently running studies at the time and even recent ones have shown that aside from undernutrition, a variety of stressful events such as hypoxia (Giussani, 2007; Mehta and Mehta, 2008; Li *et al.*, 2011) nutritional stress (Bertram and Hanson, 2001; Armitage *et al.*, 2004), endocrine disruption (Fowden, 1995; Fowden *et al.*, 2009), excessive exercise (Mottola *et al.*, 2010; Hopkins and Cutfield, 2011) and sleep restriction (Chang *et al.*, 2010; Chen *et al.*, 2013) may cause low birth weight with disproportionate body size at birth and subsequent postnatal disease (Currrie, 2011; Almond and Currie, 2011). In support of the study by Barker *et al.* (1990), several researchers have demonstrated the importance of perinatal events (Kirk *et al.*, 2009; Vickers and Sloboda, 2012) and the heritability of these traits across generations (Mathers and McKay, 2009). Thus, the biological concept of 'developmental programming' evolved. Thence, the concept has been described as a phenomenon that occurs when the normal pattern of foetal/neonatal development is disrupted by an abnormal signal (insult) at a critical period during gestation or infanthood causing a permanent alteration in the structure and function of developing tissues with consequent development of chronic health problems during postnatal life (Myatt, 2006).

2.1.1 Principles of developmental programming

The concept of developmental programming has become accepted and basic principles have been developed based on the convincing outcomes of animal studies that have described the precise postnatal effects of specific exposures. These principles of developmental programming are fundamental, irrespective of the exposure and the physiological system affected in the outcome. The principles are as follows:

i. **During development, there are critical periods of vulnerability to suboptimal conditions**

The critical window periods are periods of foetal vulnerability during which environmental insults can cause programming of specific foetal tissues (Fowden *et al.*, 2006a). Generally, during the periconceptual and preimplantation periods, sub-optimal intra-uterine environment may affect the oocyte and blastocyst environment with consequences for cell distribution between the inner cell mass and trophoblast (Fowden *et al.*, 2006b). Many cell lineages may be affected by insults during this period, however, adaptations such as upregulation of placental nutrient and oxygen later in gestation may compensate for the early disruption and also normalize birth weight (Fowden *et al.*, 2006b). Dietary restriction during this period was shown to cause hypertension and abnormal Hypothalamic-Pituitary-Adrenal (HPA) function in the offsprings of sheep (Kumarasamy *et al.*, 2005). Also, in one typical study, female pups administered a single dose of androgen in the first five days of postnatal life failed to exhibit normal oestrous cycles at puberty and thereafter. However, female pups exposed to male steroid around 20th day of postnatal life did not show any persistent effect on reproductive cycles (Barraclough and Gorski, 1961). Therefore, studies designed to determine systemic effects of exposures during development must consider the timing of exposure in relation to the system being investigated (Nathanielsz, 2006). Additionally, many studies have shown differential results of maternal stress exposure at different phases of development in the rhesus monkey (Schneider and Suomi, 1992; Clarke and Schneider, 1993). The critical periods of foetal growth are marked by hyperplasia and hypertrophy and the different tissues of the body grow during different periods of rapid cell division or cell enlargement (Barker, 1995; Nathanielsz and Thornburg, 2003). During these critical window periods, a single genotype may produce a number of phenotypes

(Gluckman *et al.*, 2005) and the phenotypic outcomes depend on which trait presents the best chance for the survival of the foetus during intra-uterine life (Hocher *et al.*, 2001). Foetal growth and development depend primarily on nutrient and oxygen supply (Harding and Johnston, 1995), a tissue in its critical period being exposed to low oxygen tension and nutrient restriction may slow down its rate of cell division or cell enlargement to match the oxygen supply, leading to a deficit in cell number or cell size (Barker, 1995). However, since nutrient and O_2 availability invariably affect the endocrine environment, the role of hormones as programming signals has also been examined in humans and experimental animals (Fowden and Forhead, 2004). This malleable adaptation is believed to allow the expression of traits that best ensures survival and wellbeing of foetus in the conditions of the intra-uterine environment, which is very different from postnatal environment thereby resulting in postnatal disease (Vo and Hardy, 2012; Gluckman and Hanson, 2004). For instance, the hypersensitivity of the HPA axis programmed by suboptimal conditions *in utero* is essential for survival in poor conditions during intra-uterine life but is inappropriate in adult life when nutrients and O_2 are plentiful and may cause hypertension and metabolic disorders in the adult (Fowden *et al.*, 2006a).

ii. **Programming has permanent effects that alter responses in later life and can modify susceptibility to diseases**

This is subject to the first principle which states that during development, there are critical periods of vulnerability to suboptimal conditions.The scientific explanation is that, beyond the critical window periods during which organs are modifiable by exposures to insults, any alteration in the molecular structure of the organ remains established and permanent (Nathanielsz, 2006). This altered molecular structure is responsible for the differential response to situations presented by the postnatal life. For example, insults that cause impairment in nephrogenesis result in reduced nephron number (Woods *et al.*, 2001; Moritz *et al.*, 2003; Mitchell *et al.*, 2004; Hughson *et al.*, 2006). Beyond the critical window period for nephrogenesis, the nephron number has been established and the offspring remains deficient in nephron number predisposing it to hypertension later in life (Moritz *et al.*, 2003; Bagby, 2007; Jones *et al.*, 2011).

iii. **Foetal development is activity-dependent**

One phase of development is required for subsequent development. Normal activity of developing foetal organs is pre-requisite and vital to normal development (Nathanielsz, 2006). For instance, a decrease in foetal breathing movement leads to foetal lung maturation retardation (Harding *et al.,* 1993). This principle is similar to the concept of "use it or lose it" as is well recognized that persistently immobilized muscles waste. In relation to total growth and development, some studies have shown that one phase of development sets the required conditions for subsequent ones. Thus, when a stage of development is affected by exposures to insults, a cascade of events may be initiated such that may ultimately lead to the mal-development of a whole system and mal-adjustment of this system to postnatal environment.

iv. **Programming involves structural changes to important organs**

Organ development during intra-uterine life may involve hyperplasia or hypertrophy (Mancuso and Palla, 1996). When an organism develops in a suboptimal intra-uterine environment, the growth and development of organs becomes altered. The total cell number in a particular organ may decrease or increase resulting from growth and cell division impairment in response to maternal insult (Vonnahme *et al.,* 2003). Organ growth restriction may also cause restriction of certain components. For instance, smaller number of blood vessels per unit area were observed in pancreas of offsprings of dams fed low protein diet (Snoeck *et al.,* 1990). Thus, pancreatic islet functions may be impaired thereby predisposing the offsprings to diabetes in adult life (Nathanielsz, 2006).

v. **The placenta plays a key role in programming**

The placenta is a highly specialized transient organ of pregnancy that is expelled along with the foetus at parturition. The growth of the placenta is well coordinated and precisely regulated (Gude *et al.,* 2004). The coordinated growth of the placenta ensures the exchange of nutrients, oxygen and waste products between the maternal and foetal circulations works at optimum efficiency (Gude *et al.,* 2004). Aside from this function, the placenta provides immune interface and secretes hormones needed for foetal metabolism and development (Myatt, 2006). There is growing

appreciation that maternal insults are transferred to the foetus by alterations in placental functions (Jansson and Powell, 2007; Fowden *et al.*, 2009). In order to function at its best, the placenta adapts in structure to the cues in maternal environment as it develops (Myatt, 2006). Consequently, perturbations in the maternal system caused by unfavourable changes in the external environment alters placental structure and functions (Zeltser and Leibel, 2011), thereby impacting the growth and development of the foetal tissues (Myatt, 2006). This is because foetal nutrient, substrate and hormonal environment which determine the rate and magnitude of growth and development of foetal tissues are maintained by the placenta and once the placenta itself is altered, foetal organ development becomes compromised (Barker and Thornburg, 2013).

vi. **The developing baby will attempt to compensate for deficiencies in the womb but that compensation carries a price in later life**

The foetus may be able to compensate, however, the mechanisms underlying foetal compensation changes the course of development and may positively or negatively impact postnatal functions depending on the postnatal environment (Nathanielsz, 2006). One suggested mechanism is that, when an insult causes a decrease in nutrient and oxygen supply to the foetus, growth and development of the key organs such as the brain are spared at the expense of other less vital organs like the pancreas, kidney, and bones (Fowden *et al.*, 2006a). Thus, the 'thrifty phenotype' baby has increased head circumference to body length or body weight ratio and decreased ponderal index, being thin for its weight (Giussani, 2016). The disproportionate body growth proposed by Barker (Barker, 1995) in response to Intra-Uterine Growth Restriction (IUGR) is marked by increased foetal brain weight relative to body (Morrison, 2008; Camm *et al.*, 2010). Studies in chronically instrumented foetal sheep revealed that the foetus can redistribute its blood flow when trying to protect its vital organs such as the brain, heart, and adrenals (Giussani, 2016). The protected organs are said to be "spared" as a response to shortage of oxygen. Nonetheless, the so-called protected organs have only experienced a "relative sparing," rather than "absolute sparing." Thus, even the spared organ remains slightly deficient in oxygen because the redistributed blood remains deficient in oxygen and nutrients (Nathanielsz, 2006). Therefore, normal growth and function may still be compromised even in the spared

organs such that the other organs which have not been spared fare even worse. For example, in a study, smaller abdominal circumference resulted when there is impaired growth of liver and this is evident in increased head to abdominal circumference ratio observed in growth-restricted neonates (Bueno *et al.*, 2010). Thus, redistribution of blood in regions of the body is the cause of the asymmetric IUGR observed in babies with placental insufficiency (Nathanielsz, 2006). This is why birth weight may be a poor marker of foetal programming because in the above instance, the birth weight may be normal or slightly deviated from the normal (Nathanielsz, 2006). The extrapolation of the above study to humans has been challenged because foetal primate brain was programmed by moderate reduction in maternal nutrient availability (Antonow-Schlorke *et al.*, 2011). The study appears to be more relevant than rodent's study since it was carried out in non-human primate. Howbeit, certain things are similar in human and in rodent species. For instance; brain development continues after birth in human and rodent's species unlike what is obtainable in the non-human primate (Giussani, 2011).

vii. **Attempts made after birth to reverse the consequences of programming might have their own unwanted consequences**

When the postnatal conditions are different from what the foetus is prepared for, as will always be, there are consequences. Laboratory studies have been carried out in the rat and mouse in which neonates that are growth restricted have been well fed postnatally to allow catch-up growth (Ozanne *et al.*, 2004; Zambrano *et al.*, 2005). The growth restricted mice in the catch-up group exhibited rapid catch-up growth and died earlier than controls. While control mice lived 57% longer than the catch-up group. The authors indicated that the difference between their life span is equivalent to 25 years in humans.

viii. **Foetal cellular mechanisms often differ from adult processes**

The chronically instrumented foetal sheep model has been most useful in this study because of its long gestation period of approximately 150 days and the large birth weight of the offspring which is approximately 5-8 kg. The pregnant sheep allows instrumentation of the foetus under general anaesthesia in the second half of gestation with subsequent recovery. Experimental studies in the sheep have shown that foetal responses are very different from those observed in the adult sheep. The

response of the foetus to hypoxia and the effects of glucocorticoids in increasing activity of the thyroid axis are two examples (Nathanielsz, 2006). Foetal sheep breathing activity are suppressed in response to moderate hypoxemia (Harding *et al.*, 2000). This is in contrast to the stimulation of breathing which follows hypoxemia in adult mammals. Administration of glucocorticoids to the foetal sheep increases thyroid activity while glucocorticoids administration in the adult sheep decreases thyroid activity (Thomas *et al.*, 1978).

ix. **Effects of programming might pass across generations by mechanisms that do not involve changes in the genes**

Genomic heritability alone cannot explain the development of complex and chronic diseases (Manolio *et al.*, 2009). Epigenetics involves heritable changes in gene expression that does not involve changes in underlying DNA sequence (Vickaryous and Whitelaw, 2005). This modification is important for the maintenance of different patterns of gene expression, parental imprinting (i.e. the choice of parental allele expressed) and epimutation erasure (Rakyan *et al.*, 2001). Evidence has revealed that epigenetic modifications such as DNA methylation and histone modifications of regulatory genes and growth related genes, mostly those involved in expressions of Insulin-like Growth Factor (IGF) II are imprinted and contribute to developmental programming (Drake and Walker, 2004). In humans, loss of imprinting of IGF-II gene causes Beckwith-Wiedemann syndrome which is associated with high birth weight (Weksberg *et al.*, 2003). Furthermore, it has been proposed that the IGF-II gene is imprinted in the placenta and may be involved in the control of foetal supply of nutrient (Constancia *et al.*, 2002; Reik *et al.*, 2003). A programmed trait may pass from one generation to another generation through epigenetic modifications (Drake and Walker, 2004). Also, male offsprings belonging to the daughters of dexamethasone-treated mothers had lower birth weights, glucose intolerance, and elevated hepatic PEPCK activity. Remarkably, those male rats that were exposed *in-utero* also had female offsprings that displayed similar changes (Drake *et al.*, 2005).

x. **Programming often has different effects in males and females**

The effect of programming often depends on the offspring's gender (Khan *et al.*, 2003). Laboratory experiments involving controlled animal experimentation have described a number of sex specific effects of foetal programming (Hales *et al.*, 1996;

Smith and Waddell, 2000; Lingas and Matthews, 2001; Owen and Matthews, 2003) with the males showing more sensitivity to certain effects of programming (Mueller and Bale, 2008). The differences to response to sup-optimal intrauterine environment are believed to be dependent on the severity and timing of the insult (Grigore *et al.*, 2008). For instance, moderate maternal protein restriction caused the programming of hypertension in adult offsprings of male rat but failed to cause the same in the female offsprings (Woods *et al.*, 2005). Human studies have also shown some sex differences in disease risk associated with low birth weight (Forsen *et al.*, 2000). Sex hormones are believed to play a key role in mediating offspring sexual dimorphism in response to sup-optimal intrauterine environment (Grigore *et al.*, 2008)

2.2. Infertility

Infertility is a disease of the reproductive system which causes failure to achieve clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild *et al.*, 2009). Infertility is a serious factor of reproductive health (Cui, 2010). The inability to reproduce affects men and women all over the world. It causes depression and distress, as well as ostracism, abandonment and stigmatization in some parts of the world (Chachamovich *et al.*, 2010). There are no absolutely dependable statistics for global prevalence of infertility, however, to a considerable extent, some important inferences may be drawn from the existing data. In 1994, 8–12 % of couples globally were estimated to be affected by infertility (Sciarra, 1994). In 2002, the prevalence increased to 15 % of couples, amounting to approximately 48.5 million couples globally (Sharlip *et al.*, 2002). In 2004, World Health Organization (WHO) reported that 60–80 million couples suffer from infertility (Rutstein and Iqbal, 2004). Another study in 2007 reported that about 72.4 million couples experienced fertility problems (Boivin *et al.*, 2007). These commonly cited statistics reflect one thing; fertility rate is on the decline. However, the limitation in these studies is that they do not reflect the prevalence in different regions of the world and in specific countries. Nevertheless, more recent studies have shown that the majority of infertile couples are populaces of the developing countries (Ombelet, 2011). According to WHO, more than 180 million couples in the developing countries have primary or secondary infertility (Rutstein and Iqbal,

2004). To date, care for the infertile is perhaps the most neglected and underrated health care subject in developing countries (Ombelet, 2011). Also, the undesirable consequences of infertility are much more pronounced in developing countries compared with the Western societies. Nonetheless, focus of the local health care providers and international community is still deficient (Ombelet *et al.*, 2008).

2.2.1. Male infertility

Male infertility refers to the failure of a mature male to impregnate a fertile female (Kumar and Singh, 2015). Male infertility is generally not well reported particularly in countries where cultural variances and patriarchal civilizations may prevent correct statistics from being collected, calculated and compiled (Agarwal *et al.*, 2015). For example, in the Middle East and Northern Africa, the wife is always held responsible for infertility (Agarwal *et al.*, 2015). Due to the patriarchal cultures in these nations, men do not usually accept to undergo fertility evaluation. As a result, many cases of male infertility are not reported. Additionally, certain tradition in some African Countries, allows the brother of an infertile male to impregnate his wife (Matetakufa, 1998). In this system, the man saves his status and reputation as a fertile man in his community. Also, many studies only consider females while others only consider the men visiting infertility clinics, which are not true representative of the bigger population of infertile men (Agarwal *et al.*, 2015). Males reportedly contribute 50 % of the infertility cases worldwide. However, this number does not exactly represent all regions of the world. Agarwal *et al.* (2015) used meta-analyses, systematic reviews and population-based studies and reported that 30 million men worldwide are infertile and that the distribution of infertility resulting from male factor ranged from 20 % - 70 % with the highest rates in Africa and Eastern part of Europe.

Male infertility is generally due to deficits in the semen quality and quantity, which is commonly used as a measure of male fertility. According to WHO, the normal values of semen indices are as follows:

Volume: 1.4 – 1.7 mL;

Sperm concentration: 12 - 16 million spermatozoa/mL

Total sperm number: 33 - 46 million spermatozoa per ejaculate;

Morphology: 3 – 4 % normal forms;

Vitality: 55 - 63 % live;

Progressive motility: 31 – 34 %;

Total motility: 38-42 % (Cooper *et al.*, 2010).

Males whose sperm parameters fall below the normal values given by the WHO are considered to have male factor infertility (Cooper *et al.*, 2010). The more important variables of these factors are sperm concentration, sperm motility, and sperm morphology. Other factors less well associated with infertility include semen volume and seminal markers of accessory organs of reproduction such as markers for epididymal, prostatic, and seminal vesicle function (Harris *et al.*, 2011). The origin of Infertility problems associated with sperm count, motility, and morphology may be related to pre-testicular, testicular, and post-testicular factors (Wamoto *et al.*, 2007). Scientists believe that the rate of fertility in men younger than 30 years of age has decreased globally by approximately 15% (Martin *et al.*, 2006). A number of studies support this finding while some others dismiss it (Carlsen *et al.*, 1992; Fisch *et al.*, 1996; Fisch and Goluboff, 1996; Lackner *et al.*, 2005). Nevertheless, some retrospective studies have indicated that sperm counts may have declined in certain parts of the world, implying the existence of geographical differences in the semen quality (Jørgensen *et al.*, 2001; Swan, 2006). The reason for these variations in semen quality is not clear, but it may be due to nutritional, environmental, socioeconomic, or other unknown causes which may constitute maternal insult during pregnancy and result in programming of foetal reproductive organs (Fowden *et al.*, 2006a). The decline in the semen parameters coincides with an increasing occurrence of anomalies of the male genital tract which includes testicular cancer, hypospadias and cryptorchidism in several countries (Giwercman *et al.*, 1993).

2.3. Embryogenesis of the testis

Foetal sexual differentiation begins at first trimester and continues till mid-gestation in humans and it involves complicated sequence of events at proper critical periods of foetal life. It is controlled by both genetic and hormonal factors. These genetic and hormonal factors usually alternate to cause transformations of the primary gonads, the internal (accessory) sex structures and the external genitalia. Sex chromosomes promote the growth and the differentiation of the testis but the decisive and critical influences are the production of testosterone and antimüllerian hormone. Feminization results from the absence of masculinizing genetic factor and/or testosterone acting during the critical period of differentiation (Welsh *et al.*, 2008).

The undifferentiated primordial gonad located at the anterior surface of the mesonephros or the primitive kidney is already noticeable in the 5 mm human embryo and consists of the coelomic epithelium. The first step is not dependent on the genetic sex and it involves the colonization of the gonadal primordium by the primordial germ cells advancing from the allantoid sac. When the primordial germ cells have reached the primordial gonad, they fuse with the epithelium called gonadal ridge. The gonocytes are present within the epithelium. A layer of mesenchymal cells separates the epithelium from the mesonephros. According to the traditional Witschi's theory, seminiferous tubules are originated from the mesonephros. (Witschi, 1951). Sex differentiation begins at about the 7th week of intra-uterine life (Francavilla *et al.*, 1990).

Testicular tissues, precisely the seminiferous tubules, are visible in the human embryo at 7th week of foetal life (Jirasek, 1977). The germ cells within the primitive seminiferous tubules divide mitotically repeatedly but do not enter meiosis. The Sertoli cells surround the germ cells in preparation for future seminiferous tubules. A basal membrane forms and isolates the tubules from the neighbouring mesenchymal tissue. These processes precede Leydig cell differentiation (Tran *et al.*, 1977). Leydig cells differentiate from the surrounding interstitial tissue at about the 8th to the 9th week. They begin to secrete testosterone from the 8th week (Winter *et al.*, 1977). Maximal foetal serum testosterone concentration is detected from the 14th to the 16th week. Levels of testosterone during this period are similar to those observed in adult males. Maternal serum testosterone also rises during this period

(Klinga *et al.*, 1978). After the 20th week of gestation, Leydig cells involute, and testosterone level decreases. At birth, blood testosterone levels are higher in males than in females (Forest *et al.*, 1974). Descent of foetal testes start at the 12th week of foetal life and the process is androgen-dependent (Welsh *et al.*, 2008).

2.4. Programming of reproductive functions

Steroids play a key role in regulating the course of differentiation of tissues and organs during development, as such, the foetus is very sensitive to steroid hormone exposure during early development (Padmanabhan and Veiga-Lopez, 2014). Endocrine Disrupting Compounds (EDC) that can signal through the steroid-transduction pathways during critical periods of development can cause developmental programming (Padmanabhan and Viega-Lopez, 2014). Reports have it that excess steroidal exposure during the critical period of reproductive organ development adversely affect reproductive capacity in rodents (Drake *et al.*, 2009; Wyrwoll and Holmes, 2012), ruminants and non-human primates (Abbott *et al.*, 2005; Abbott *et al.*, 2008) and humans (Cottrell and Seckl, 2009).

Accumulating evidence suggests that reductions in foetal testosterone production is another important cause of adult male reproductive disorder (Macleod *et al.*, 2010). Recently, Kilcoyne *et al.* (2014) provides evidence suggesting that the mechanism through which the male reproductive system may be programmed *in utero* involves reduction in Leydig stem cell number after birth and associated Leydig cell failure in adult life. This confirms that foetal androgen deficiency causes developmental programming of adult Leydig cell function, which may even have implications for systems beyond the reproductive system (Teerds *et al.*, 2015). The testicular dysgenesis syndrome has been associated with prenatal exposure to EDC and prenatal exposure to deficient-testosterone (Skakkebaek *et al.*, 2001; Macleod *et al.*, 2010).

2.5. Testicular dysgenesis syndrome

The 'Testicular Dysgenesis Syndrome (TDS) hypothesis' proposes that the four conditions; cryptorchidism, testicular cancer, hypospadias and impaired spermatogenesis may all be indicators of disturbed prenatal testicular development (Skakkebaek *et al.,* 2001). Epidemiological and experimental studies suggest that the aetiology of TDS is related to developmental programming of gonadal development during foetal life caused by environmental factors including endocrine disruptors (Skakkebaek *et al.,* 2001; Skakkebaek, 2002). Few human studies have found associations between endocrine disruptors and the different components of TDS (Bay *et al.,* 2006). Howbeit, for ethical reasons, proof of an underlying relationship between prenatal exposure and postnatal TDS is difficult to establish in human studies. As such, animal researches are mostly used to study the pathogenesis of TDS (Bay *et al.,* 2006). Nevertheless, testicular dysgenesis has been established in biopsies of the contralateral testis of men with infertility and/or testicular cancer (Wohlfahrt-Veje *et al.*, 2009). Clinically, the most common indicator of TDS is perhaps a reduced sperm count, while the more severe form may comprise a high risk of testicular cancer (Bay *et al.,* 2006). Recent advances in understanding the pathogenesis of TDS revealed the central role of suboptimal androgen production and action during foetal testis development (Sharpe and Skakkebaek, 2008). In the rat, factors that reduce testosterone during the masculinization window are known to cause TDS and reproductive impairment in adult life (Welsh *et al.,* 2008; Macleod *et al.,* 2010; Van den Driesche *et al.,* 2015).

2.6. Developmental programming of cardiovascular and reproductive functions

Before the 'Barker hypothesis' was postulated, Anders Forsdahl proposed a relationship between childhood and adolescence poor social condition and ischemic heart disease in adulthood (Forsdahl, 1977). Although David Barker and Anders Forsdahl both noted a strong positive association between heart disease and infant mortality, David Barker was the first ever to report the inverse correlation between birth weight and blood pressure (Barker and Osmond, 1988; Barker *et al.,* 1989). Further studies on foetal origins of heart disease have linked adult cardiovascular disease with prenatal excess of glucocorticoid (Langley-Evans, 1997; Edwards *et*

al., 2001) and low birth weight (Palinski *et al.*, 2008). These two factors are also involved in the programming of male reproductive functions.

Programming of adult cardio-metabolic disorders in men are associated with lowered testosterone levels (Kilcoyne *et al.*, 2014). In men, testosterone and systolic blood pressure have inverse relationship (Grigore *et al.*, 2008). Testosterone appears to play an important role in control of blood pressure. This suggests that reduced testosterone may be a connection between the programming of cardiovascular functions and reproductive functions. Similarly, animal experiments have associated the programming of cardiovascular diseases to excessive maternal corticosterone (Singh *et al.*, 2007), a key factor in the programming of reproductive functions (Cottrell and Seckl, 2009). Although, the mechanism leading to the programming of adult cardiovascular disease still remains elusive, nonetheless, reports have shown that the mechanism may include reduction in the level of placental 11βHSD$_2$ (Langley-Evans, 1997; Dodic *et al.*, 2002; Roghair *et al.*, 2005), reduction in foetal nephron number (Woods *et al.*, 2001), impaired vascular function (Gopalakrishnan *et al.*, 2004; Williams *et al.*, 2005), placental insufficiency (Alexander, 2003), alteration in renin-angiotensin system (Zimmermann *et al.*, 2003; Grigore *et al.*, 2007) and oxidative stress (Dusting and Triggle, 2005; Pashkow, 2011).

2.7. Developmental programming of renal and reproductive functions

The adverse effect of low birth weight on the progress of primary kidney disease is clearly acknowledged in a variety of animal studies (Luyckx and Brenner, 2005; Xu and Zuo, 2010). Adult renal diseases have been associated with maternal protein restriction (Woods *et al.*, 2004), maternal malnutrition (Woods, 2007; Wood-Bradley *et al.*, 2015), and maternal glucocorticoid excess (Singh *et al.*, 2007). According to the traditional Witschi's theory, seminiferous tubules are originated from the mesonephros; the primitive kidney. (Witschi, 1951). Also, the reproductive system develops in concert with the renal system during foetal life (Michos, 2009). This suggests that the critical periods of development of these two organs during development may be similar. For instance, during the differentiation of the Wolffian duct (the precursor of male genitals), it swells and an epithelial thickening called the ureteric bud forms (Michos, 2009). The interaction between the ureteric bud and metanephric nephron eventually forms the renal collecting system and later, renal

vesicles. The renal vesicles are the precursors of the nephrons which are basic functional units of the kidney (Dressler, 2006). Reduced nephron endowment which often results from maternal exposure to corticosterone has been proposed to be a major mechanism involved in the programming of acquired renal disease (Moritz *et al.*, 2009; Luyckx *et al.*, 2011). Also, the expression some elements of renal function involving the renin-angiotensin-aldosterone system are testosterone dependent. Apparently, manipulation of endogenous steroids during pregnancy can cause the programming of cardiovascular, renal and reproductive systems of the developing offspring (Woods *et al.*, 2001; Kilcoyne *et al.*, 2014). In adults, renal disease often co-exists with erectile and gonadal dysfunctions (Rathi and Ramachandran, 2012; Holley and Schmidt, 2013). One such stimulus that may alter the levels of steroids in the body is sleep deprivation (Tufik *et al.*, 2009).

2.8 Programming by excessive glucocorticoid

Normal glucocorticoid levels are crucial during the development of foetal organs for maturation of foetal tissue and organ thereby promoting cellular differentiation (Cottrell and Seckl, 2009). However, excessive glucocorticoid exposures has been reported to cause low birth weight (Seckl and Meaney, 2004). Maternal corticosterone levels are much higher than foetal levels (Beitins *et al.*, 1973). This is because 11βHSD$_2$ which is highly expressed in the placenta catalyses the rapid conversion of active cortisol/corticosterone to inert cortisone. However, the enzyme allows only about 10-20% passage of active hormone reflecting a physiological bypass of the hormone (Seckl and Meaney, 2004). Nevertheless, in certain situations, the level of 11βHSD$_2$ may become compromised. This gives room for excess of glucocorticoids to cross the foeto-placental barrier. Studies have reported negative association between placental 11βHSD$_2$ and birth weight (Stewart *et al.*, 1995; Lindsay *et al.*, 1996; Murphy *et al.*, 2002).

2.9. Birth morphometric indices

One of the numerous observations stated from the earlier studies of Barker was that morphometry at birth is prognostic of adult disease (Barker *et al.*, 1989; Barker *et al.*, 1990; Barker *et al.*, 1993). Birth morphometry is easily taken, non-invasive, not expensive and reliable surrogate marker of foetal programming. Retrospective and observational studies by David Barker first showed the association between low birth weight and disease state in England and Scandinavia (Barker *et al.*, 1989). Many studies in different nations have since confirmed the relationship between lower birth weight and disease in later life (Law *et al.*, 1991; Phillips *et al.*, 1994; Fall *et al.*, 1995; Kaijser, 2015). Recent animal studies however, showed that when a foetus is programmed, its birth weight may not be influenced (Thone-Reineke *et al.*, 2006) or may even be high (Skilton *et al.*, 2014). A closer look at the popular Dutch Hunger Winter Study by Schultz (2010) also revealed that foetal exposures that impacted adult health did not automatically result in altered birth weight (Schulz, 2010). Although birth weight is the most widely studied morphometric measure of foetal future health, it may not be a particularly sensitive measure in many situations (Almond and Currie, 2011). That being noted, it is difficult to find a single measure of concealed foetal health impacts at birth. Nevertheless, in Gluckman's words, "We have to be extremely careful not to view body size and shape at birth as measures of developmental outcome" (Gluckman *et al.*, 2005). Birth weight should not to be treated in isolation but rather, as a component of other morphometric indices of intra-uterine programming. For instance: weight: height ratio determines thinness (Eide *et al.*, 2005; Nascimento *et al.*, 2011); crown-rump length is a marker of shortness (Mongelli *et al.*, 2012); abdominal circumference indicates liver size (Haller *et al.*, 1995; Shi *et al.*, 2005; Li *et al.*, 2011); head circumference is a pointer of brain size (Barker *et al.*, 1993); head: abdominal circumference is an indicator of brain sparing (Risnes *et al.*, 2009) and anogenital distance is a marker of reproductive status (Eisenberg *et al.*, 2011; Welsh *et al.*, 2008). All these together have been considered to generate a holistic measure that gives a comprehensive status of latent foetal health and adult disease. Thus, indices of body asymmetry and organ growth serve as a more sensitive tool in detecting programmed infants as compared with birth weight alone.

2.10. Sleep deprivation

Sleep is the state of reversible unconsciousness with relative inactivity and reduced responsiveness from which an individual can be aroused by either sensory or other stimuli (Rasch and Born, 2013). It is important and essential for proper maintenance of homeostatic functions, wellbeing and survival. However, sleep has been relegated to a secondary level of importance, because the current global population works and lives under a 24/7 lifestyle which is characterized by increased physical activities, reduced rest and less sleep (Shochat, 2012). This inadequate sleep syndrome termed sleep deprivation, as well as its adverse effect on daytime performance are experienced globally and have been labelled a modern epidemic (Durmer and Dinges, 2005). Chronic sleep deprivation may result from medical conditions, academic and work demands, social responsibilities, domestic responsibilities, constant exposure to artificial light, unrestricted access to the internet, television and sleep disorders (Durmer and Dinges, 2005).

Sleep disorders include; periodic leg movement disturbance, sleep apnea, restless leg syndrome, insomnia and jet lag (Aldabal and Bahammam, 2011). It was estimated that about 20% of adults experienced sleep deprivation globally (CDC, 2005; Chang *et al.,* 2010). Moreover, the increasing adverse effects of sleep deprivation has been documented (Tufik *et al.*, 2009; Luyster *et al.*, 2012). In 2010, National Health Interview Survey (NHIS) reported that over 40 million employed U.S. adults regularly experienced sleep deprivation (Elliott *et al.,* 2014). Sleep deprivation experienced over a short period called 'acute sleep deprivation' may not have serious adverse effects on health, but, the prolonged state of shortened or mistimed sleep well-defined as '*chronic sleep deficiency'* by the Sleep Research Society (SRS) and the American Academy of Sleep Medicine (AASM) may result into serious negative health effects (Luyster *et al.,* 2012). Even though sleep is a vital process required for homeostasis, it is not fully clear when a person is sleep deprived or how many hours are needed to achieve restorative sleep (Aldabal and Bahammam, 2011). On this account, the National Sleep Foundation (NSF) recommends that all adults should have 7-9 hours of sleep per 24 hours to ensure the optimal maintenance of health (Hirshkowitz *et al.*, 2015). While it is impossible and even unethical to conduct researches of chronic sleep deprivation in human, growing evidence over the past decades have suggested that having shorter duration of sleep

regularly is associated with hallucinations (Devillieres *et al.*, 1996; Mistraletti *et al.*, 2008), visual perception disruption (Kendall *et al.*, 2006), attention deficit (Caldwell *et al.*, 2003) diabetes and obesity (Gottlieb *et al.*, 2005; Aldabal and Bahammam, 2011), hypertension (Gottlieb *et al.*, 2006), cardiovascular diseases (Leeuwen *et al.*, 2009; Sauvet *et al.*, 2010), reduced testosterone (Wittert, 2014), oxidative stress (Villafuerte *et al.*, 2015), altered melatonin (Davis *et al.*, 2014) and increased mortality (Luyster *et al.*, 2012). Furthermore, animal experimentation revealed the grave consequences of sleep deprivation to include: aggressive behaviour (Kamphuis *et al.*, 2012), alteration in gene expression (Cirelli *et al.*, 2006) impaired cognition (Alhaider *et al.*, 2010), cardiovascular and renal diseases (Kamperis *et al.*, 2010; Sauvet *et al.*, 2014), inflammation (Vetrivelan *et al.*, 2012), reduced testosterone and reproductive capability (Akindele *et al.*, 2014) and even death (Rechtschaffen *et al.*, 1983). There is hardly any system in the body that is not affected by both partial and total sleep deprivation (Tufik *et al.*, 2009).

2.11. Sleep deprivation in pregnancy

In the 21st century society, women of reproductive age are involved in numerous activities. They occupy prominent positions in the labour force and also have several responsibilities at home. As such, they are likely to experience more sleep deprivation (Chang *et al.*, 2010). Many women habitually engage in various activities relating to their careers, household responsibilities, children's school events, play group obligations and other commitments in order to balance their career, home and social life (Swanson, 2000). Because of the time expended on these activities, they do not have enough time for adequate quantity and quality of sleep which results in sleep deprivation (Mullington *et al.*, 2009; Williams and Aderanti, 2014). Sleep deprivation is particularly more common during pregnancy (Miller *et al.*, 2012) because of pregnancy-related sleep disorders caused by physical, hormonal and behavioural changes in addition to the daily busy schedule of a typical 21st century woman (Miller *et al.*, 2012). There is some information that sleep deprivation in pregnant women has more deleterious health effect than in adults of similar age (Miller *et al.*, 2012) and that sleep deprivation is a form of maternal insult since it is associated with adverse pregnancy outcome in human (Chang *et al.*, 2010). Similarly in animals, partial sleep restriction throughout the

whole duration of gestation has led to adult offspring hypertension and renal abnormality (Thomal *et al.*, 2010; Lima *et al.*, 2014), reproductive dysfunction (Alvarenga *et al.*, 2013) and impairment of cognitive functions (Peng *et al.*, 2016).

2.12. Experimentally induced sleep deprivation

Sleep deprivation was first studied by Pieron, in 1913 (Morrison, 2014) and since then, the effects of sleep deprivation has been extensively studied, both in humans and animals. Laboratory methods (associated with the multiple platform technique) employed for the induction of sleep deprivation in animal researches are as follows:

i. The Flower Pot Techniques

This laboratory sleep deprivation technique involves the awakening of experimental animals by external stimulus at the onset of each paradoxical sleep period. This method was first developed by Jouvet *et al.*, (1964) for cats and it was known as the flower pot technique (Jouvet *et al.*, 1964). The method was later modified and used to deprive rats of sleep (Cohen and Dement, 1965). The rat to be deprived is placed on an inverted flowerpot with a narrow platform (6.5 cm in diameter) surrounded by water. Once the rat is accustomed to the condition in its new environment, it can achieve a Non-Rapid Eye Movement (NREM) sleep. However, at the onset of Rapid Eye Movement (REM) sleep, there is loss of muscle tone and the animal makes contact with the water or falls into the water and awakens. The animal climbs back on the platform and enters NREM sleep again. The degree of sleep deprivation in each animal may vary considerably because of the diameter of the platform relative to the size of the animal (Steiner and Ellman, 1972; Mendelson *et al.*, 1974; Hicks *et al.*, 1977). Furthermore, this model has been criticized because the method subjects the animal to restriction of movement which in itself, is an additional stress that could cause many of the observed effect (Tufik *et al.*, 2009).

ii. The Multiple Platform Method (with single rat)

In order to correct the deficiency of the single flower pot technique, the multiple platform method was developed. In this model, a rat is placed inside a large water tank containing multiple platforms and this eliminates the restriction of movement and also allows the animal to jump from one platform to another (van Hulzen and

Coenen, 1981). The method was criticized because of the social isolation stress that may be induced by placing one rat alone in the large tank. Additionally, the control animal was placed in a normal cage which means the control and test animals were placed in dissimilar environments.

iii. The Multiple Platform Method (with many rats)

The paradigm was further modified in an attempt to eliminate the social isolation experienced by the animal in the flower pot technique and the multiple platform technique (Nunes and Tufik, 1994). In this model, 10 animals were placed in a water tank containing 18 narrow platforms (6.5 cm). This method therefore eliminates both movement restriction and social isolation. Additionally, the control animals were placed in a tank containing wider (14 cm in diameter) platforms, thus eliminating the disparity in environment. This technique is simple and allows for many animals to be sleep deprived at the same time. This paradigm did not consider the social instability caused by picking animals from different cages.

iv. Modified Multiple Platform Method (MMPM)

In an attempt to eliminate the social instability, Suchecki and Tufik in 2000 proposed the Modified Multiple Platform Method (MMPM) using socially stable groups (10 animals from one cage). The stress caused by social instability was attenuated by this method. The control animals were placed in a tank containing wider (14 cm in diameter) platforms as earlier done by Nunes and Tufik (Nunes and Tufik, 1994). Although the animal could curl up and experience both NREM and REM sleep without contacting the water thereby addressing confounding factors, it remains controversial if indeed it is an appropriate control (Landis, 1996; Machado *et al.*, 2004). Control animals placed on the wide platforms showed adrenal hypertrophy similar to that seen in the test animals (Nunes and Tufik, 1994). Furthermore, some degree of sleep deprivation is experienced by the supposed control animals, as implied by amplified rebound seen in the recovery period (Machado *et al.*, 2004). Another recommended control group for the sleep deprivation consisted of animals placed on a grid in place of the wider platform (Suchecki and Tufik, 2000). This was a good control as the adrenal hyperplasia was abrogated. However, sleep rebound was also observed (Tufik *et al.*, 2009).

v. Chronic Sleep Deprivation Paradigm

Chronic sleep restriction paradigm consists of rats subjected to 18 hours of sleep deprivation with a 6 hour sleep window for 21 days (Machado *et al.*, 2005) or 20 hours of sleep deprivation and 4 hour sleep window for 21 days (Thomal *et al.*, 2010). This restriction caused complete suppression of paradoxical sleep and loss of slow wave sleep. A major consolidation of sleep was observed during the 4 to 6 hour sleep window as reflected by an intense reduction of arousals (Tufik *et al.*, 2009).

2.13. Placenta

The placenta is the circular organ that anchors the foetus to the uterus and serves as a link between the maternal environment and the foetal environment. The function of the placenta is to maintain foetal homeostasis since most of the foetal organs have not acquired functional capability *in-utero*. It performs the functions of the kidneys, gastrointestinal tract, lungs and endocrine glands of the foetus before birth. These functions include supply of nutrient and oxygen, excretion of waste product, production of peptides and steroids influencing both maternal, placental and foetal metabolism. It also provides immune interface allowing a successful coexistence between the mother, itself and the foetal allograft which expresses both maternal and paternal genes (Kanellopoulos-Langevin *et al.*, 2003).There is growing appreciation that maternal insults are transferred to the foetus by alterations in placental function (Jansson and Powell, 2007; Fowden *et al.*, 2009). This is because the placenta adapts to the predominating maternal environment as it develops (Myatt, 2006). Consequently, agitations in the maternal system caused by unfavourable changes in the external environment alters the structure of the placenta (Zeltser and Leibel, 2011). Functions are based on structures; therefore, the functions of the placenta are also affected by perturbations in the maternal environment (Zeltser and Leibel, 2011). This means that the amount of oxygen, nutrients, hormones and substrates available to the foetus as well as rate of elimination of waste products are affected when the placental development is altered (Barker and Thornburg, 2013). These elements are the determining factors of foetal growth and development, thus, the placenta plays a major role in transferring insults from the maternal environment to the foetus (Myatt, 2006). Maintenance of the structure of the placenta which is necessary for optimal function is therefore central and crucial to the regulation of

foetal nutrient/substrate and hormonal bioavailability, hence, growth and development of the foetus. The mechanisms linking placental response to intra-uterine programming are currently under study.

2.14. Adaptations of the placenta in developmental programming

In human, the placental growth and differentiation is a cautiously coordinated process leading to a 40 fold increase in foetal/placental weight ratio at term (Benirschke and Kaufmann, 1990). Disruption of this developmental cascade may cause abnormal development of placental structure (Myatt and Cui, 2004). As there are critical window periods of foetal tissue growth, so are there critical periods of placental tissue growth and development during which unfavourable environmental conditions may impact placental development (Myatt, 2006). These are periods of implantation, invasion of trophoblast, vasculogenesis, angiogenesis, differentiation of trophoblast and formation of syncytium (Fowden *et al.*, 2006b). Human studies have shown that growth restricted placentas are not merely smaller than normal placenta, they exhibit alterations in; placental expression of transporters (Jansson *et al.*, 2002), vasculogenesis and angiogenesis (Krebs *et al.*, 1996) and hormone production and enzyme activity (McMullen *et al.*, 2004). The functions of placenta may be altered in response to changes in trophoblast thickness, surface area and expression of transporters and more importantly, placental blood flow causing the programming effect in the foetus (Myatt, 2006). Angiogenesis and vasculogenesis are particularly important because transport of flow limited substances to and from the placenta depends on placental blood flow. In addition, the trophoblast also plays a vital role in production of hormone and metabolism of substrate. The role of both the trophoblast and vasculature and in transport activities of the placenta has been emphasized in many animal experimental studies. (Wallace *et al.*, 2002; Vonnahme and Ford, 2004).

2.14.1. Placental morphometric indices

Appropriate foetal growth relies upon adequate nutrient transfer by placenta which has been said to be an active participant in foetal programming (Myatt, 2006; Fowden *et al.*, 2008). As the placenta grows, it adapts to changes in the maternal environment (Zeltser and Leibel, 2011). It is therefore likely that changes in placental growth which may be reflected in its weight, shape, size and surface area could provide information on programmed health of the infant (Myatt, 2006; Jansson and Powell, 2007). Placental insufficiency caused by abnormal maternal placental blood flow is the most essential cause of IUGR in clinical studies (Longtine and Nelson, 2011) and in animal studies likewise, in which the placental growth was affected before foetal growth (Jansson and Powell, 2000).

In a retrospective study carried out by Barker *et al.*, (2010), a strong association was observed between adult hypertension, placental weight and placental surface area. The use of placental morphometric indices as a marker of foetal programming was thence advocated (Barker *et al.*, 2010). In a placental efficiency experiment, increased birth weight: placental weight ratio indicated an increase in nutrient transfer per gram placenta and vice versa (Hayward *et al.*, 2016). Mid-pregnancy placental volume was shown to be directly associated with maternal height, as well as to a high-calorie diet (Robinson *et al.*, 1999). Adolescent mothers also have offspring with low placental ratio and lower range of birth weights (Lurie *et al.*, 1999). When a placenta is disproportionately small, the capacity of the placenta to supply nutrients may be impaired. Conversely, if the placenta is disproportionately large, the foetus may experience foetal catabolism in trying to supply amino acid to the placenta (Jones *et al.*, 2011). Generally, placental ratios are associated with foetal growth pattern (Ruangvutilert *et al.*, 2002). However, using placental size as an indicator of foetal programming may be complex and should be used with caution. For instance, perturbations in early pregnancy may yield an increased placental size especially if placental efficiency is greatly improved during the latter part of pregnancy (Godfrey *et al.*, 1996) as exemplified by the Dutch winter hunger in which there was increased placental growth following severe calorie reduction in the first trimester and high calorie intake during the remaining part of pregnancy (Lumey, 1998).

2.14.2. Role of imprinted genes

The role of imprinted genes in placental adaptive response cannot be overemphasized. More than 60 imprinted genes have been discovered in humans. Imprinted genes that are of paternal origin enhance foetal growth while those imprinted genes which are of maternal origin suppress foetal growth (Reik *et al.,* 2003). When paternally expressed *Igf2* was knocked out, there was reduction in placental growth while placental hyperplasia resulted when there was knockout of maternally expressed *p57kip2* (Myatt, 2006). Imprinted genes may control placental supply of nutrients and foetal demand for nutrients thereby regulating both foetal and placental growth. In addition, many imprinted genes also encode for specific trophoblast transporters. The *Ata3* gene which is paternally expressed encodes an element of system A amino acid transporter (Mizuno *et al.,* 2002), while *Impt/Slc22a11 which is* maternally imprinted gene encodes a transporter of organic cation (Dao *et al.,* 1998).

2.14.3. Role of placental nutrient transporters

Studies have reported the modifications in the expression of amino acid and glucose transporters in the placentas of pregnancies complicated by intra-uterine growth restriction, diabetes and pre-eclampsia. For instance, in individuals with type 1 diabetes with Large for Gestational Age (LGA) babies, the trophoblast basement membrane showed increase in *GLUT 1* expression coupled with increase in system A amino acid transporter (Jansson *et al.,* 1999). Contrarily, only system A was increased in the basement membrane of individuals with gestational diabetes who also had LGA babies (Jansson *et al.,* 1999). This implies that glucose and amino acid transporters can be programmed by hyperglycaemia at different gestational periods (Jansson *et al.,* 2003). Hypoxia which is associated with IUGR has also been reported to decrease expression of system A in the trophoblast (Nelson *et al.,* 2003). Inhibition of system A causes IUGR (Cramer *et al.,* 2002), however, upregulation of amino acid transport I seen in the small placenta of growth-restricted placental-specific *Igf2* knockout mouse. All these reflect an adaptive response of the placental transporters to varying intra-uterine conditions.

2.14.4. Role of glucocorticoids

Glucocorticoids are central regulators of organ growth and maturation. Rat's offsprings that were exposed to excessive maternal glucocorticoids had growth restriction, hyperglycaemia, hypertension and increased hypothalamic pituitary adrenal axis activity (Lindsay *et al.,* 1996). Glucocorticoids, which prevent the expression and function of GLUT transporters are produced by the trophoblast and regulated by 11βHSD$_2$ (Hahn *et al.,* 1999). 11βHSD$_2$ converts active cortisol to inactive cortisone protecting the foetus against high levels of maternal cortisol (Krozowski *et al.,* 1995). In humans, mutations in the *11βHSD$_2$* gene is associated with low birth weight probably caused by intra-uterine growth restriction (Seckl *et al.*, 2000). *11βHSD$_2$* expression increases with gestational age (Murphy and Clifton, 2003) and changes in oxygen tension alter *11βHSD$_2$* expression and activity (Alfaidy *et al.,* 2002). Thus, the placenta plays a central role in foetal programming.

2.14.5. Oxidative and nitrative stress

Many placental pathologies that are associated with intra-uterine programming are also associated with placental hypoxia, oxidative and nitrative stress. During early trophoblast differentiation in human, low oxygen tension is physiological for organogenesis and it is a vital regulator of cellular events (Genbacev *et al.,* 1997). However, the establishment of intervillous (oxygen rich) blood flow at 10–12 weeks of gestation causes oxidative stress in the trophoblast (Jauniaux *et al.,* 2000). Oxidative stress is a feature of normal pregnancy because of the increased metabolic activity of placental mitochondria. Oxidative stress is however aggravated in complicated pregnancies (Wang *et al.,* 1992; Giugliano *et al.,* 1996) and it is evidenced by the increased level of reactive oxygen species and reactive nitrogen species and/or decreased level of antioxidant enzymes. The more damaging reactive oxygen species that are produced by the trophoblast and placental vascular endothelium are superoxide anions and nitric oxide. The interaction between nitric oxide and superoxide anion yields a powerful pro-oxidant known as peroxynitrite. Peroxynitrite causes nitration of placental protein leading to the loss or gain of their functions. There are reports that peroxynitrite is increased in pregnancy complicated by diabetes and pre-eclampsia (Myatt *et al.,* 1996) and *in vitro* treatment with peroxynitrite reportedly altered placental vascular function (Kossenjans *et al.,*

2000). Nitration may affect enzymes like p38 MAP kinase, poly ADP-ribose polymerase, acetyl coA transferase (Myatt, 2006) and these enzymes are involved in regulation of placental development (Myatt, 2006).

2.14.6 Epigenetic modifications

Epigenetic modification refers to heritable changes in gene expression that are not mediated by alterations in DNA sequence (Jaenisch and Bird, 2003). Epigenetic mechanisms include DNA methylation, acetylation and histone phosphorylation etc. A particularly important epigenetic mechanism is methylation of cytosine residue of the DNA at the CpG islands which is associated with gene promoter regions. Changes in placental DNA methylation during placental development may lead to alteration of specific gene expression and placental function which ultimately affects foetal growth and development. Precisely, hypermethylation of promoter regions normally results in suppression of transcription, whereas hypomethylation commonly increases transcription (He *et al.*, 2003). Laboratory research on the placentas of cat has revealed that placental hypermethylation may be associated with decrease in cloning success rates (Cho *et al.*, 2007) and that certain cytochrome p450 enzyme gene is partially controlled by varying methylation status in sheep and cattle placentas (Vanselow *et al.*, 2008). There has also been focus on the relationship between abnormal methylation patterns of placental gene promoters and disease progression. For instance, it was suggested that peculiar methylation pattern may be a distinctive mechanism leading to preeclampsia (Maccani and Marsit, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental animals

Adult male (230 - 250 g) and female (170 - 200 g) Wistar rats obtained from the Central Animal House, College of Medicine, University of Ibadan were used for the study. The male rats were proven breeders certified fertile by isolated mating techniques while the female rats were virgin rats with regular oestrous cycle. The rats were conveyed to the Laboratory for Reproductive Physiology and Developmental Programming, Department of Physiology, College of Medicine, University of Ibadan where the research was carried out. The rats were housed in well aerated plastic cages and had access to rodent's pelletized feed (Ladokun feed mill, Ibadan) and water *ad libitum*. All animals were acclimatized to the environmental condition of the laboratory for two weeks before the commencement of the study. Rats were chosen for this study because they are have short gestation periods, have litter size that can easily be standardized and have short life span.

3.2 Sleep deprivation model

Test chamber

The paradigm used for sleep deprivation was the Modified Multiple Platform Model (MMPM) of Suchecki and Tufik, (2000) (Figure 1). It is made up of a glass chamber (70 x 70 x 35 cm) consisting of sixteen circular platforms of 6.5 cm in diameter. The glass chamber was filled with water up to 1 cm mark below the top of the platform. The rats to be sleep deprived were placed on the narrow platforms where they freely ambulated from one platform to another. The loss of muscle tone associated with the onset of sleep resulted into arousal when the rats fell into the water. The cover of the chamber is made of wire mesh to ensure proper aeration of the chamber. Attached

to the wire mesh are feeders and drinkers. The feeders are perforated panels made of acrylic materials while the drinkers are plastic bottles with metal pipes.

Control chamber

The control rats were placed in chambers with similar features as the test chamber. The difference being that it additionally consists of a glass barrier placed on the platforms closest to the wall of the chamber (Figure 2). The centre of the glass barrier is cut open so that the control rats are subjected to the same environment as the sleep deprived rats. The glass barrier served as bed where the pregnant rats were able to sleep at will. The barrier was such that rats would not fall into the water even if there were movements during sleep. This was so designed because the control tank for the pre-existing sleep deprivation model was made of larger platforms which did not prevent the animals from having some degree of sleep deprivation as reflected by adrenal hypertrophy similar to that produced in the sleep deprived animals (Nunes and Tufik, 1994).

Figure 3.1. The test chamber of the Modified Multiple Platform Method (Suchecki and Tufik, 2000). It consists of glass chamber (black arrow), multiple platforms (blue arrow), feeding panel (red arrow) and drinkers (green arrow)

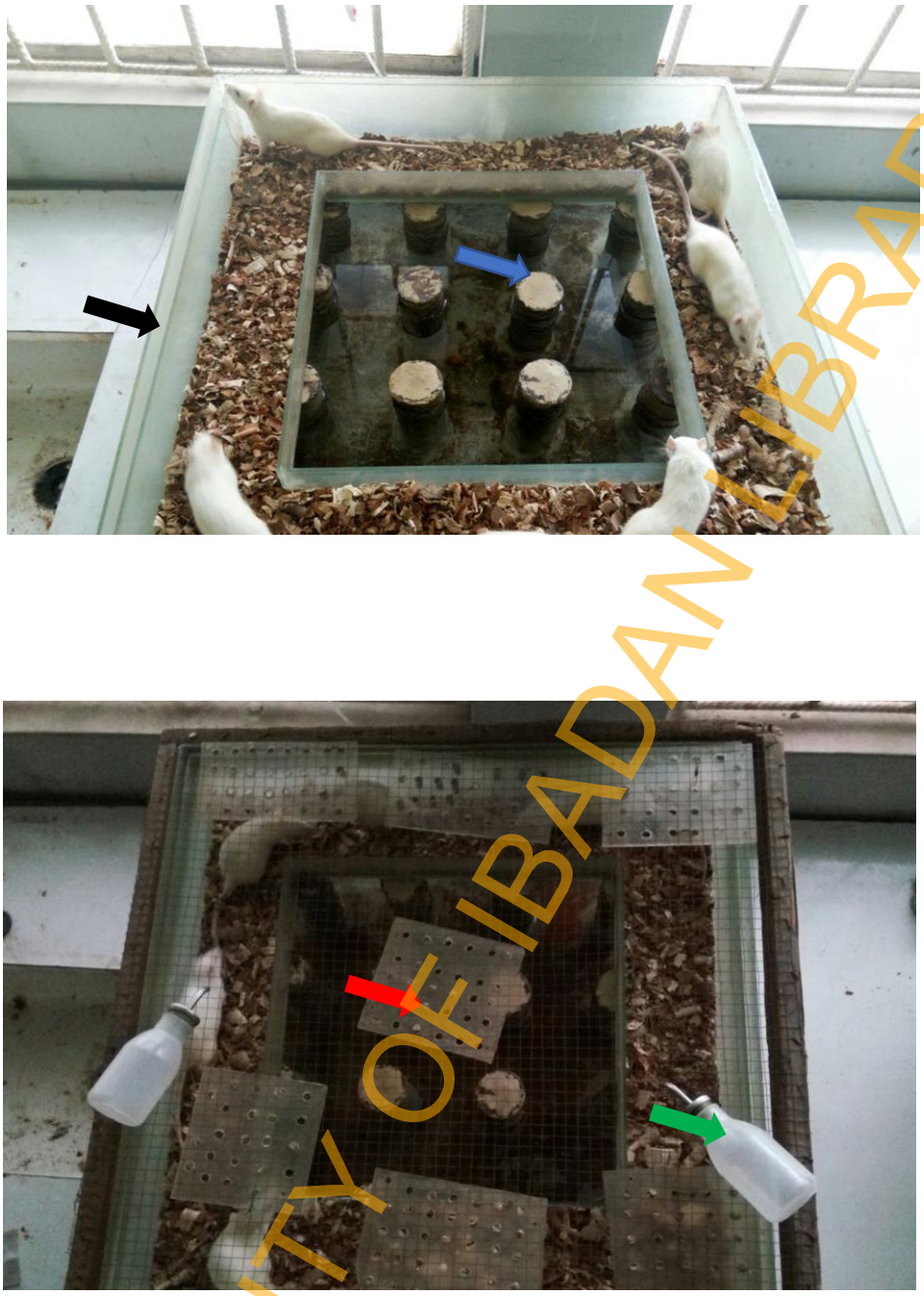


Figure 3.2. The control chamber of the Modified Multiple Platform Method (Suchecki and Tufik, 2000). It consists of glass chamber (black arrow), multiple platforms (blue arrow), feeding panel (red arrow), drinkers (green arrow) and a glass barrier with wood shavings (white arrow)

3.3 Experimental protocol

The study was carried out in three phases: preliminary study protocol (study one) examined the effects of sleep deprivation on oestrous cycle and reproductive organs of female Wistar rats; study two examined the cardio-renal and reproductive functions of male offsprings of dams subjected to sleep deprivation during different gestation periods; study two explored the effects of sleep deprivation during different gestation periods on maternal hormones and placental physiology.

3.4 Preliminary study

Thirty mature nulliparous female Wistar rats (170 - 200 g) with normal oestrous cycle pattern were used for the study. They were divided into two groups; Control (C) and Sleep Deprived (SD). Control group consisted of fifteen (15) rats that were not sleep deprived while the sleep deprived group consisted of fifteen (15) rats. Five animals were sacrificed in each group on days 7, 14 and 21 of sleep deprivation.

3.5. Sleep deprivation protocol

Rats were sleep deprived using the MMPM (Suchecki and Tufik, 2000). The control and test rats were placed in their respective chambers daily at 2:00 pm. and removed from the chamber at 10:00 am. in the morning of the next day into their home cages. This affords the test rats four hours of sleep during the day. The time interval (between 10:00 am - 2:00 pm) was especially important because it is when paradoxical sleep is at its greatest incidence, thus creating partial compensation for sleep (Kumar and Singh, 2009).

3.6. Determination of oestrous cycle pattern

The determination of oestrous cycle was done by Marcondes' technique (Marcondes *et al.*, 2002). Oestrous pattern was studied by determining the oestrous phase of each animal every morning between 7:00 am and 8:00 am throughout the study. Vaginal content was collected with a Pasteur pipette containing about 0.1 mL of normal saline (0.9 % NaCl) by gently inserting the tip of the pipette into the rat's vagina.

The pipette was pressed to release the fluid content 2 or 3 times in order to make a vaginal lavage which contained some of the vaginal cells of the rat. The pipette was thereafter withdrawn and its content was placed on a glass slide. A new and clean glass slide was used for each animal. The slide was thereafter viewed using the x40 magnification objective lens of the microscope (Olympus, Japan). The cell types and the proportion among them was used to define the oestrous cycle phase of the rat.

A proestrus smear consists of a predominance of nucleated epithelial cells; an oestrous smear primarily consists of a nucleated cornified cells; a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells, and a diestrus smear primarily consists of a predominance of leukocytes (Long and Evans, 1922; Mandl, 1951). Both the control and sleep deprived groups were placed in their respective cages as described above. Five animals in each group were sacrificed during proestrus on days 7, 14 and 21. The study lasted for 21 days.

3.7. Sacrifice

Rats were sacrificed under thiopental anaesthesia (50 mg/kg, i.p.) (Pereda *et al.*, 2006). They were surgically opened along the linea alba of the anterior abdominal wall to the thoracic cavity to expose the heart and the organs. The ovaries, uteri and adrenal glands were harvested and freed of adherent tissues. All harvested organs were fixed in 10% formalin for histological examination.

3.8. Experimental protocol for the effects of maternal sleep deprivation on dams, placentas and male offsprings

Sixty mature nulliparous female Wistar rats (170 - 200 g) with normal oestrous cycle pattern and thirty proven male breeders (230 – 250 g) were used for the study.

3.9. Co-habitation and confirmation of mating

Female rats were paired with male rats at ratio 2:1 (female: male) during the proestrus phase of the female rats. On the next morning after pairing, vaginal lavage was collected with Pasteur pipette filled with about 0.1 mL of normal saline (0.9 % NaCl) by gently inserting the tip of the pipette into the rat's vagina. The withdrawn vaginal content was placed on a glass slide and the smear was spread out evenly. The glass slide was examined using the x10 and x40 objective lens of the light microscope (Olympus, Japan) to determine the presence of spermatozoa. Mating was confirmed by the presence of spermatozoa in vaginal smear. The day on which spermatozoa were found in the vaginal lavage was designated as gestation day (GD) 1.

3.10. Animal grouping

Table 3.1. Animal Grouping

Group	Name of Group	Number of animals	Description of group
Group 1	GD1-7C	10 (5 animals for offspring's study and 5 animals for placental study)	Pregnant rats were placed in the control chamber from GD 1 to GD 7
Group 2	GD1-7SD	10 (5 animals for offspring's study and 5 animals for placental study)	Pregnant rats were placed in the sleep deprivation chamber from GD 1 to GD 7
Group 3	GD8-14C	10 (5 animals for offspring's study and 5 animals for placental study)	Pregnant rats were placed in control chamber from GD 8 to GD 14
Group 4	GD8-14SD	10 (5 animals for offspring's study and 5 animals for placental study)	Pregnant rats were placed in the sleep deprivation chamber from GD 8 to GD 14
Group 5	GD15-21C	10 (5 animals for offspring's study and 5 animals for placental study)	Pregnant rats were placed in control chamber from GD 15 to GD 21
Group 6	GD15-21SD	10 (5 animals for offspring's study and 5 animals for placental study)	Pregnant rats were placed in the sleep deprivation chamber from GD 15 to GD 21

3.11. Sleep deprivation protocol for pregnant dams

Pregnant rats were sleep deprived using the MMPM (Suchecki and Tufik, 2000) on the gestation days designated to their individual groups. The control and test pregnant dams were placed in their respective chambers daily at 2:00 pm and removed from the chamber at 10:00 am in the morning of the next day into their home cages. This affords the test pregnant rats four hours of sleep during the day. At the end of the seventh day of sleep deprivation, five pregnant dams were sacrificed from each group. The remaining five rats in each group were removed into their home cages and left there until parturition.

3.12. Caesarean section

Five pregnant dams were randomly selected from each group at the end of seven days of sleep deprivation and Caesarean section was performed under thiopental anaesthesia (50 mg/kg, i.p.) (Pereda *et al.*, 2006). They were surgically opened along the linea alba of the abdominal cavity to the thoracic cavity to expose the beating heart. The incision was extended to the pelvic region to fully expose the gravid uterus. Using a 5mL syringe and needle, they were bled out into plain serum bottles through cardiac puncture. Each gravid uterus was carefully removed from the root. The pups and their placentas were dissected from the uterus (figure 3). Pup and placental morphometry were estimated. The placentas of the GD 1-7 groups have just begun to develop, therefore the site of implantation along with its contents were extracted. Two placentas per dam were fixed in 10 % formalin for immunohistochemical assessment. Another one was fixed in RNA later for analysis of global DNA methylation status. Two other placentas were homogenized in ice cold phosphate buffer (pH 7.4) for redox status analysis.

3.13. Parturition and postnatal studies

Animals were allowed to litter naturally and the day of parturition was designated as Post-Natal Day (PND) one. Birth weight, anogenital distance, head circumference, abdominal circumference and crown-rump length were measured within 24 hours of postnatal life. Only the male pups were retained in the study and allowed to be nursed by their natural mothers during which testicular descent and

preputial separation were monitored. They were weaned on PND 28 (Post-Natal Week (PNW) 4) (Thiels *et al.*, 1990) and were pooled into groups depending on maternal gestational treatment. On PNW 17, the male offsprings were paired with proven breeders for determination of fertility and gestational index. During the PNW 25, cardiovascular variables were measured, renal function was evaluated and the male offsprings were sacrificed.

3.14. Placental morphometry

The Placenta of each pup was weighed on digital electronic weighing scale (Lisay, China). Placental thickness was measured with the use of Vernier caliper by measuring from the widest surface to the concave surface (figure 4a). Placental circumference was measured by placing a piece thread round the perimeter of the widest surface of the placenta (figure 4b). This length of the thread that represents the perimeter of the placenta was then measured using the Vernier caliper. This was done because the rat's placental surface has at least two different diameters and the circumference cannot be calculated from the diameter since it is not a perfect circle. Placental chorionic surface area was calculated from this value. Placental volume was measured using Archimedes' principle (figure 4c) by dropping each placenta into a measuring cylinder containing a known volume of phosphate buffer (pH 7.4). The displaced volume of phosphate buffer was recorded as the volume of placenta.

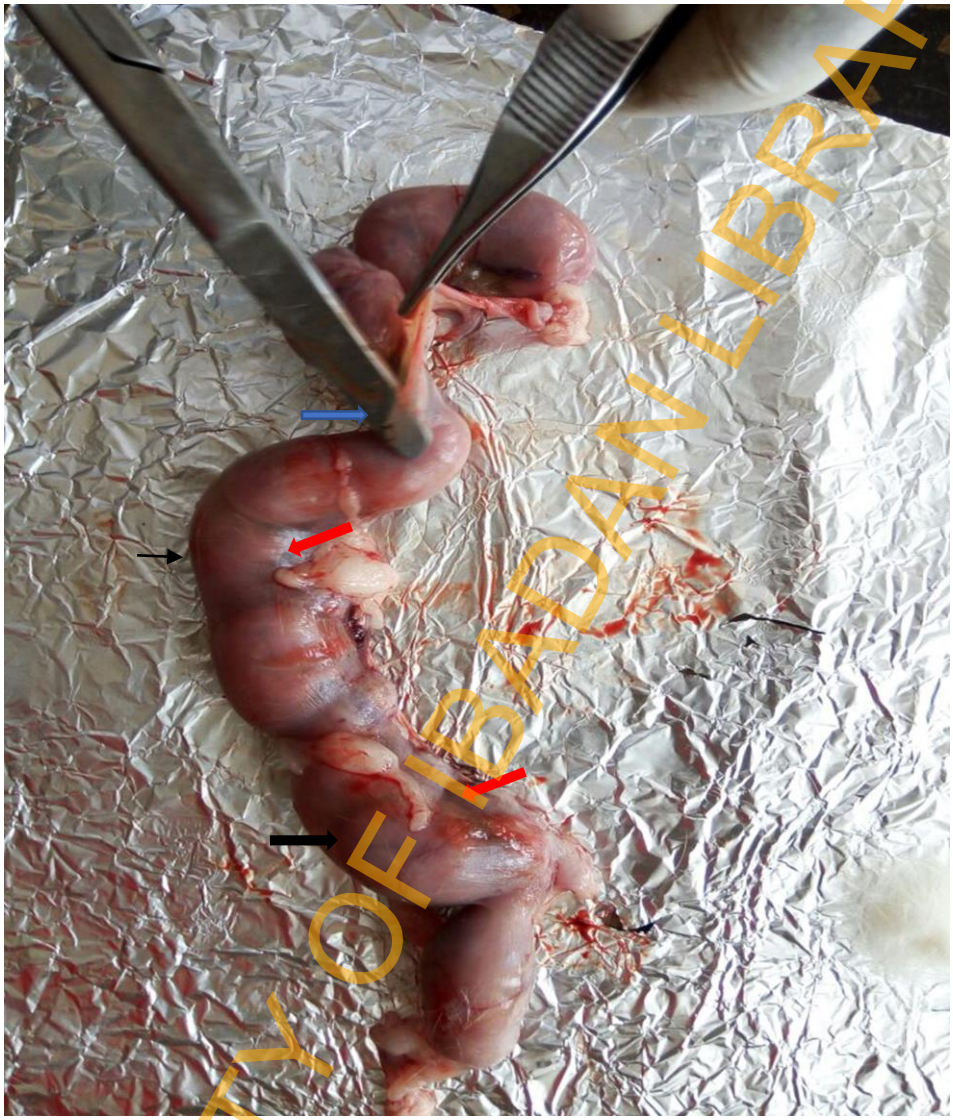
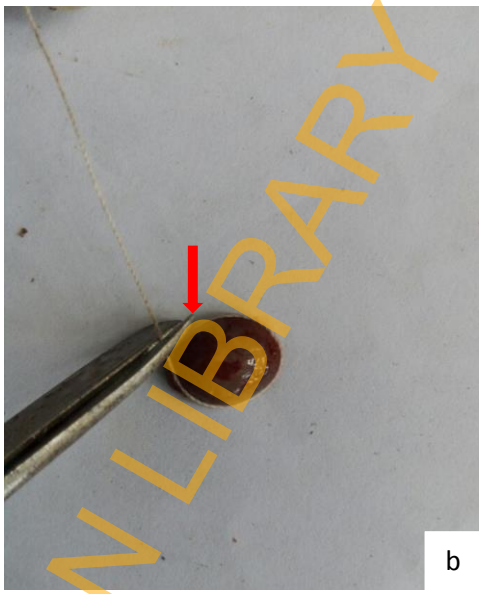
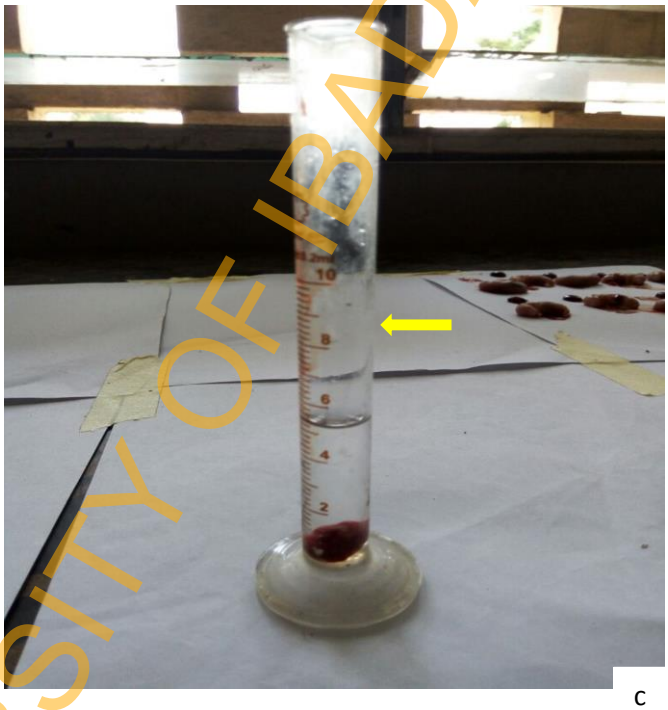


Figure 3.3. Dissection (blue arrow) of pups (black arrow) and placentas (red arrow) from the uterus.

a



b



c

Figure 3.4. Placental morphometry a) Measurement of placental thickness from the widest surface (blue arrow) to the concave surface (black arrow) with a Vernier caliper (green arrow) b) Placental circumference determination using a piece of thread round the perimeter of the placenta (red arrow) c) Placental volume determination using Archimedes' principle (yellow arrow)

3.15. Pup morphometry

Pup morphometry was carried out within 24 hours of post-natal life for the pups that were naturally littered and immediately after surgery for the pups obtained by Caesarean section. They were weighed on electronic scale (Lisay, China). The head diameter was measured from one ear to the other ear, abdominal diameter was measured at the centre of the waist, Ano-Genital Distance (AGD) was measured from the genital ridge to the anus and crown-rump length was measured from the tip of the nose to the root of the tail. All measurements were done using the digital Vernier caliper (Mitutotyo, Japan) while the pups were gently held firmly (Gray *et al.*, 2004). Head circumference (2Πr), abdominal circumference (2Πr) and anogenital distance index (AGD/body weight$^{1/3}$) (Gallavan *et al.*, 1999) were calculated from the morphometric data obtained.

3.16. Determination of testes descent and preputial separation and weaning

Starting from PND 15, testicular descent was monitored by daily palpation of the scrotal sac for testicular presence. Daily manual retraction of the prepuce was done starting from PND 35 until the prepuce separated totally from the shaft of the penis (Korenbrot *et al.*, 1977). Pups were weaned on PND 28 (Thiels *et al.*, 1990).

3.17. Fertility test

The male offsprings were paired with virgin rats at ratio 1:2 (male: female) during PNW 17 (PND 120) for two weeks in the first instance. At the end of the two-week session, the process was repeated with a set of proven female breeders. The virgin and proven breeders were monitored for pregnancy for at least 21 days after their pairing session. Fertility and gestational indices were calculated as follows:

$$\text{Fertility Index} = \frac{\text{Number of cohabited females becoming pregnant}}{\text{Number of cohabited}} \times 100$$

$$\text{Gestational Index} = \frac{\text{Number of females delivering live young}}{\text{Number of females with evidence of pregnancy}} \times 100$$

Desesso *et al.*, 2014

3.18. Measurement of cardiovascular variables

Cardiovascular parameters were measured using a computerized non-invasive tail cuff system (figure 5) (Kent Scientifik, USA). After setting up the system, the animals were placed in a warming holder for 10 minutes prior to obtaining measurements. The Occlusion (O) cuff was then slid up near the base of the animal's tail followed by the Volume Pressure Recording (VPR) tail cuff and the system was run. The system was used to measure systolic pressure, diastolic pressure, heart rate, blood volume and blood flow.

3.19. Evaluation of renal functions

After the measurement of cardiovascular variables, male offsprings were placed individually in the metabolic cages (figure 6) in the morning for 24- hour collection of urine. After 24 hours, animals were anesthetized and blood was collected from the heart. Volume of urine in 24 hours was measured, Serum levels of creatinine, urea and albumin were estimated and urinary levels of creatinine and albumin were assayed using colorimetric assay kits (Fortress diagnostics, UK).

Figure 3.5. The computerized non-invasive tail cuff device. Processor (white arrow), O cuff (blue arrow), VPR cuff (yellow arrow), Holder with rat (red arrow), System output (black arrow).

Figure 3.6. The Metabolic cage (black arrow), feeder (blue arrow), calibrated drinkers (red arrow) and calibrated urine collector (white arrow).

3.20. Blood collection and serum preparation

Animals were anaesthetized with thiopental (50 mg/kg., i.p). They were cut open from the linea alba of the abdominal cavity to the thoracic cavity. Using a 5mL syringe and needle, they were bled out into plain serum bottles through cardiac puncture. The blood was allowed to clot for at least 45 minutes after which it was centrifuged at 3000 rpm for 15 minutes. The serum portion which was the supernatant was decanted from the centrifuged blood and stored at -20 ^{o}C for ELISA of hormones. Serum levels of progesterone, prolactin, testosterone, corticosterone and melatonin were assayed in dams while serum levels of FSH, LH, testosterone, corticosterone and melatonin were assayed in the male offsprings.

3.21. Organ harvest

The heart, kidneys, adrenal glands, the testes, epididymes, seminal vesicles and prostate glands were harvested from the male offsprings and freed of adherent tissues before being weighed on the digital electronic scale (Lisay, China). The testes were fixed in Bouin's fluid while the other organs were fixed in 10 % formalin in preparation for histological assessment.

3.22. Epididymal Sperm profile analysis

The left caudal epididymis was collected for sperm analysis. Epididymal fluid was prepared for analysis as described by Raji *et al.*, (2003). Sperm analysis was carried out as described by Zemjanis (1970).

3.22.1. Epididymal Sperm viability

Sperm viability was done using the eosin/nigrosin stain which contained 1.00 g of Eosin blue and 4.00 g of nigrosin in 100 mL phosphate buffer. A drop of the epididymal fluid was placed on the slide and 2 drops of the stain were added, after which a thick smear was made and dried. The resulting stain was then studied using the x40 objective lens of the light microscope (Olympus, Japan). The live spermatic cells were unstained while the dead spermatic cells were stained. A minimum of 100

spermatic cells, stained and unstained sperm cells were counted and an average was taken for the percentage of live sperm.

3.22.2. Epididymal Sperm motility

Sperm motility was done immediately and quickly after sacrifice. The caudal epididymis was cut open, and a drop of the content was placed on a slide. Two drops of warm 2.9 % sodium citrate was added. This was then covered with a cover slip and examined using x40 objective lens of the light microscope (Olympus, Japan) to assess motility. Sperm motility was expressed in percentage.

3.22.3 Epididymal Sperm count

Sperm count was done using a Neubauer counting chamber. The caudal epididymis was homogenized in a known volume of formal saline. It was further diluted to a total dilution factor of 200. A cover slip was fixed firmly on the Neubauer counting chamber and a drop of the homogenized epididymis was released on the counting chamber, below the cover slip. Spermatic cells in five big squares were counted. The volume of 5 squares is 1/50 µL. The number of sperm in 1 mL was calculated and the results were expressed in million/mL as stated below;

Sperm count (million/mL) = Number of cells counted x 50 x 200 (dilution factor) x 1000/mL

3.23 Histological assessment of the organs

Fixation

The testes were fixed in Bouin's fluid for about 20 hours while the other samples were fixed in 10 % formalin for at least 5 hours, immediately after they were collected from the animal.

Dehydration

The tissues were dehydrated by passing them through graded alcohol (70 %, 80 %, 90 % and 2 changes of absolute (100 %) alcohol for one hour each). This procedure was undertaken so as to remove the inherent water content in a gradual way considering osmotic dynamics.

Clearing

The tissues were then cleared of the ethanol that they have been bathed so as to initiate and complete the process that would make cells transparent at microscopic level. This is done by placing them in two changes of xylene for one hour each. Upon removal from xylene, it was placed in the wax bath, at least 2 changes for one hour each.

Embedding

The tissues were infiltrated by placing them in molten paraffin wax which served as support to the tissues for the stage of sectioning. The infiltrated tissues were then embedded in molten paraffin wax within an enclosure called the mould. The embedded tissues were left until the wax solidified. The tissues were then cut into blocks and they were held in position by paraffin wax. Afterward, the blocks were clamped and positioned for sectioning.

Microtomy

It was trimmed, nicked and sectioned at 3-5 micron with a microtome. The section was floated with 20 % alcohol on water at a temperature of 50 ^{0}C below paraffin wax melting point. The water was drained and placed on microscope slide for at least one hour. The satisfactory sections were picked with a clean, grease-free microscope slide that has been coated on one side with glycerine egg albumin. The slides carrying the sections were labelled by diamond pencil, arranged in a slide carrier and put in an oven to dry.

Staining

Staining was done with Haematoxylin and Eosin (H&E) for the purpose of determining the general morphology. The tissues were dewaxed in 2 changes of xylene for 3 minutes each and sections were taken into water. They were then rinsed in absolute alcohol for 2 minutes and rinsed in 95 %, 90 % 80 %, 70 % alcohol each for two minutes after which they were rinsed in water and stained with Harris-haematoxylin for 5 minutes. They were rinsed in water again and differentiated in 1 % acid alcohol in 3 dips. They were again rinsed briefly in water and were blued in running tap water for 3 minutes. They were counterstained with aqueous eosin for 3 minutes and dehydrated using ascending grades of alcohol, i.e.70 %, 8 0%, 90 %, 95

% and 100 % for 1 minute each. They were then cleared in xylene and mounted on the microscope for histological examination. Photomicrograph of the sections were taken at different magnifications in order to observe morphological changes.

3.24 Determination of serum and urinary creatinine levels

Kit: Fortress Diagnostics Limited, United Kingdom

Test principle: Creatinine reacts with picric acid in an alkaline medium to form a deep yellow complex. The amount of complex formed is directly proportional to the level of creatinine in the sample.

Reagents:

- 0.32 mol/L of NaOH
- 35 mmol/L of picric acid
- 177 µmol/L(2mg/dL) of standard

Procedure:

100 µL of Standard/Sample was added to respective test tubes. One mL of working reagent was then added. The absorbance of the standard and samples were read at 492 nm after 30 minutes and after 150 seconds.

Calculation:

Change in absorbance of the sample or standard (Δabs) = abs. at 150 secs – abs. at 30 secs

$$\text{Creatinine concentration in serum (mg/dL)} = \frac{\Delta\text{abs Sample} \times 2}{\Delta\text{abs Standard}}$$

$$\text{Creatinine concentration in urine (mg/dL)} = \frac{\Delta\text{abs Sample} \times 100}{\Delta\text{abs Standard}}$$

$$\text{Creatinine Clearance (mL/min)} = \frac{\text{mg creatinine/dL urine x mL urine 24 hours}}{\text{mg creatinine/dL serum x 1440}}$$

3.25 Determination of serum urea level

Kit: Fortress Diagnostics Limited, United Kingdom

Test principle:

Ammonium ions produced when urea present in the sample reacts with salicylate and hypochlorite to form an indophenol complex which is green in colour.

Reagents:

- 62 mmol/L of sodium salicylate buffer
- 5 mmol/L of sodium nitroprusside buffer
- 18 mmol/L of sodium hypochlorite
- 750 mmol/L of NaOH
- >5000 U/L of urease
- 50 mg/dL of standard.

Procedure:

10 µL of standard/sample was added to respective glass wares free from ammonium ions. 1000 µL of buffer was added to all tubes including blank. A drop of urease was added to all tubes. Content was mixed and incubated for 3 minutes at 30 ^{0}C. 200 µL of hypochlorite was added to all tubes. The mixture was incubated at 37 ^{0}C for 5 minutes and absorbance of standards and samples were read at 578 nm.

$$\text{Urea concentration (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ x standard concentration}$$

3.26 Determination of serum albumin level

Kit: Fortress Diagnostics Limited, United Kingdom

Test principle: Serum albumin binds with the Bromocresol green indicator in an acid medium to form a green BCG complex, the amount of which is directly proportional to the albumin concentration present in the sample.

Reagents:

- 0.24 mmol/L of bromocresol Green (BD concentrate)
- 75 mmol/L of succinate buffer
- 45 g/L (4.5 g/dL) of standard.

Reagent handling and preparation:

Concentrate was diluted by adding 52 mL of DDH2O.

Procedure:

10 µL of DDH_2O was added to blank while 10 µL of sample/standard was added to tubes. 3 mL reagent was added to all tubes. The content of the test tube was mixed and left for five minutes at 20-25 ^{0}C. Absorbances of standards and samples were read against reagent blank at 600-650 nm.

3.27. Determination of serum hormonal levels, placental nitrotyrosine and methylation status

Serum levels of progesterone, prolactin, testosterone, corticosterone, melatonin, follicle stimulating hormone, luteinizing hormone and placental levels of nitrotyrosine and global methylation status were assayed by ELISA. The protocols used for the assay were specified by the kit manufacturers. The kits and their manufacturers are listed below.

Table 3.2. List of ELISA kits used and their manufacturers

S/N	Kit	Manufacturer
1	Progesterone	Calbiotech Inc., 10461 Austin Dr, Spring Valley, CA, 91978, United States of America.
2	Prolactin	
3	Testosterone	Cloud-Clone Corp., 1304 Langham Creek Dr., Suite 226, Houston, TX 77084, United States of America
4	Nitrotyrosine	
5	Melatonin	
6	Corticosterone	Assaypro 3400, Harry S Truman Blvd, St. Charles, MO 63301-4046, United States of America
7	Follicle stimulating hormone	Fortress Diagnotics Limited, Unit 2C Antrim Technology Pack, Antrim, BT41 IQS, United Kingdom
8	Luteinizing hormone	
9	Global DNA methylation Status	Cell Biolabs, Inc. 7758 Arjons Drive San Diego, CA 92126, United States of America

3.27.1 General principle of ELISA used in this study

The direct, competitive and sandwich ELISA were used in this study for the quantitative analysis of the analyte. Generally, the essential reagent required for immunoenzyme methods include the microtitre plate, high affinity and specific antibody, the attached enzyme, the enzyme substrate, wash buffer, stop solution and spectrophotometer. After incubating the test serum in an antigen-coated or antibody-coated microplate (depending on the method of ELISA employed), enzyme labelled anti-immunoglobulin is added. The content is washed after a period of incubation and enzyme substrate is thereafter added. The enzyme remaining in the tube or plate after washing reacts with the enzyme substrate and generates a colour which provides a measure of the amount of specific antibody in the serum. The test relies on the insolubilisation of antigens by passive adsorption to a solid phase, e.g. the polystyrene surface of the microplate.

3.28 Determination of placental redox status

The harvested placentas were homogenized in phosphate buffer (pH 7.4) and the homogenate was centrifuged at 10,000 g x 15 minutes at 4 °C. The supernatant was collected for the assay of malondialdehyde (for lipid peroxidation), total reactive oxygen species, total antioxidant capacity, superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione concentration in the placenta.

3.29 Assessment of placental lipid peroxidation

Placental lipid peroxidation was determined by measuring the Thiobarbituric Acid Reactive Substances (TBARS) produced during lipid peroxidation (Ohkawa *et al.*, 1979).

Principle:

This method is based on the reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA), an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 535 nm and is extractable into organic solvents such as butanol. The MDA level was

calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein was computed with a molar extinction coefficient (E) of 1.56 x 10^5 $M^{-1}cm^{-1}$.

Reagents:

- Stock TCA-TBA-HCl composed of 15 g of trichloroacetic acid (TCA)
- 0.375 g of thiobarbituric acid and 0.25 N hydrochloric acid

Procedure:

1 mL of sample was added to the test tube after which 2 mL of the TCA-TBA-HCl acid reagent was added. Three mL of the reagent was added to the blank test tube. The test tubes were place in boiling water for 15 minutes. After cooling, the flocculent precipitate was removed by centrifugation at 1000 g for 10 minutes. The absorbance of sample supernatant was read at 535 nm against the blank.

$$\text{Calculation: TBARs activity} = \frac{\text{Absorbance x volume of mixture x 1000}}{\text{E x volume of sample x mg protein}}$$

3.30 Assessment of total Reactive Oxygen Species (ROS) and total Reactive Nitrogen Species (RNS)

Total ROS was measured by oxiselect™ in vitro ROS/RNS Assay Kit (Cell Biolabs Inc., USA) which uses the fluorescence technique.

Principle:

In Vitro ROS assay Kit is an *in vitro* assay for measuring total ROS and RNS free radical activity. Unknown ROS or RNS samples or standards are added to the wells with a catalyst that helps accelerate the oxidative reaction. After a brief incubation, the prepared Dichlorodihydrofluorescin (DCF) probe is added to all wells and the oxidation reaction was allowed to proceed. Samples are measured fluorometrically against a hydrogen peroxide or DCF standard. The assay is performed in a 96-well fluorescence plate format that can be read on a standard fluorescence plate reader. The free radical content in unknown samples is determined by comparison with the

predetermined DCF or hydrogen peroxide standard curve. The DCF standard curve is used when measuring free radicals other than hydrogen peroxide.

3.31 Assessment of Total Antioxidant Capacity (TAC)

Total antioxidant capacity was measured by Total Antioxidant Status (TAS) assay kit (Fortress Diagnostics, United Kingdom)

Principle:

The kit uses the principle of colorimetry. ABTS (1, 2'-Azino-di-(3-ethylbenzthiazoline sulphonate) is incubated with a peroxidase and H_2O_2 to produce the radical cation $ABTS^{+}$. This has a stable blue green colour which is measured at 600-660 nm. Antioxidants in the sample, suppress the formation of this colour, to a degree which is proportional to their concentration.

Reagents:

- TAS buffer (80 mmol)
- TAS chromogen 610 Umol/L
- TAS standard.

Procedure:
800 µL of TAS buffer was added to the blank, standard and sample test tubes. 50 µL of standard and sample were added to their respective tubes. The initial absorbance (I abs) was then read at 660 nm. 125 µL of TAS chromogen was added to the blank, sample or standard bottles. The content of each test tube was mixed thoroughly before being incubated for five minutes at 37 ^{0}C. The final absorbance (II abs) was read at 660 nm.

Calculation:
ΔAbsorbance sample = (II abs of sample – I abs of sample) - reagent blank absorbance.
ΔAbsorbance Standard 1= (II abs of Std 1 – I abs of Std 1) - reagent blank absorbance.

ΔAbsorbance Standard 2= (II abs of Std 2 – I abs of Std 2) - reagent blank absorbance.

$$\text{Results (mmol/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Standard concentration}$$

3.32 Assessment of Superoxide Dismutase (SOD) activity

The level of SOD activity was determined by the method of Misra and Fridovich (Misra and Fridovich, 1972).The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per superoxide introduced increased with increasing pH and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide radical and hence inhabitable by SOD.

Reagents:

- 0.05 M carbonate buffer (pH 10.2). This was prepared by dissolving Na_2CO_3 (0.2014 g) and Ethylenediaminetetraacetic acid (EDTA) (0.0372 g) in about 80 mL of distilled water. After adjusting the pH to 10.2 with 0.1 M NaOH, the volume was made up to 100 mL with distilled water.
- 0.03 mM adrenaline solution. This was freshly prepared by dissolving adrenaline (0.01098 g) in 200 mL of 0.005 M HCl solution.
- 0.005 M HCl solution. This was prepared by diluting 5 mL of 0.1 M HCl solution to 100 mL with distilled water.
- 0.10 M HCl solution. This was prepared by diluting 1 mL of commercial concentrated HCl to 120 mL with distilled water.

Procedure:

Placental homogenate (0.2 mL) was added to the reference tube, while 0.2 mL of the appropriate enzyme extracts were added to appropriately labelled test tube.

To each of these was added 2.5 mL of the carbonate buffer followed by equilibration at room temperature. Adrenaline (0.3 mL) solution was added to the reference and each of the test solutions. Content was mixed and absorbance was read at 420 nm.

Calculation:

Change in absorbance per minute = (A_3-A_0)

Where A_0 = absorbance after 30seconds

A_3 = absorbance after 150 seconds

$$\text{Percentage inhibition} = 100 - \frac{100 \times \text{Change in substrate absorbance}}{\text{Change in blank absorbance}}$$

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

3.33 Assessment of catalase activity

Catalase activity was determined according to the method of Sinha, (1972).

Principle:

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570-610 nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Reagents:

- 5 % $K_2Cr_2O_7$ (Dichromate solution)
  5 g of $K_2Cr_2O_7$ (Hopkins and Williams, England) was dissolved in 80 mL of distilled water and made up to 100 mL with saline.
- 0.2 M H_2O_2
  11.50 mL of 30 % (w/w) H_2O_2 was diluted with distilled water in a volumetric flask and the solution made up to 500 mL
- Dichromate/acetic acids
  This reagent was prepared by mixing 5 % solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and could then be used indefinitely.
- Phosphate buffer (0.01 M, PH 7.0)
  3.58 g of Na_2HPO_4 $12H_2O$ and 1.19 g NaH_2PO_4 $2H_2O$ dissolved in 900 mL of distilled water was added to make it up to one litre.

3.34 Assessment of glutathione peroxidase activity

Glutathione Peroxidase (GPx) catalyzes the redox reaction between reduced glutathione (GSH) and hydrogen peroxide (H_2O_2). The amount of GSH utilized is estimated by measuring it in the assay mixture before and after the enzyme activity (Rotruck *et al.,* 1973). GSH reacts with Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid or DTNB) to give a yellow colour which was then measured at 412 nm.

Reagents:

- Sodium phosphate buffer (pH 7.0; 0.4 M, w/v)
- Sodium azide (10 mM, w/v)
- Reduced glutathione (4 mM: w/v)
- Hydrogen peroxide (2.5 mM, v/v)
- 10 % trichloroacetic acid (TCA) (w/v)
- Disodium hydrogen phosphate solution (0.3 M, w/v)
- 5,5'-dithiobis-2-nitrobenzoic acid reagent (40 mg of DTNB in 100 mL of 1 % sodium citrate solution)
- Reduced glutathione standard

Procedure:

Sodium phosphate buffer (0.5 mL), sodium azide (0.1 mL), reduced glutathione (0.2 mL), hydrogen peroxide (0.1 mL) and 1:10 diluted aliquot of the enzyme (0.5 mL) were mixed and the total volume was made up to 2.0 mL with distilled water. The tubes were incubated at 37 ^{0}C for 3 minutes and the reaction was terminated by adding 0.5 mL of 10 % TCA. To determine the residual glutathione content, the supernatant was removed after centrifugation (1500 rpm for 8 minutes) and to this 4.0 mL of disodium hydrogen phosphate (0.3 M) solution and 1 mL of the DTNB reagent were added. The colour developed was read at 412 nm against a reagent blank containing only phosphate solution and DTNB reagent. Suitable aliquots of the standard were also treated similarly.

The enzyme activity was calculated from the formula:

$$\text{Enzyme activity} = \frac{\text{AS2 X CS X DF}}{\text{AS1 X VS X P}}$$

AS2 = Absorbance of sample
CS = Concentration of standard
DF = Dilution factor
AS1 = Absorbance of standard
VS = Volume of homogenate
P = protein content in homogenate
The enzyme activity was expressed as units/mg protein

3.35 Assessment of reduced glutathione concentration

Reduce glutathione level was measured by spectrophotometric assay kit (Oxford Biomedical Research, USA).

Principle:
This kit employs a kinetic enzymatic recycling assay based on the oxidation of GSH by 5, 5'-dithiobis (2- nitrobenzoic acid) [DTNB] to measure the total glutathione (tGSH) content of biological samples. The included glutathione standards or treated

samples are added to the microtiter plate wells, followed by DTNB and glutathione reductase. Addition of NADPH to the wells initiates the progressive reduction of DTNB by GSH, causing a colour increase that is monitored at 405 nm. The rate of colour change, followed over a 5-minute time period, is proportional to the GSH concentration. Consequently, the concentration of GSH in unknown samples may be determined by reference to the standard curve. GSH reacts with DTNB to produce a coloured ion, which absorbs light at 405 nm, and a mixed disulphide. This disulphide reacts with further quantities of GSH present to liberate another ion and GSSG. GSSG is reduced enzymatically to GSH which then re-enters the cycle. Since GSSG represents only a small percentage of total acid-solution free glutathione, the resulting values for GSH (which encompasses both GSH and GSSG) are expressed in units of GSH equivalents.

3.36 Assessment of total protein

Total protein level was measured by spectrophotometric assay kit (Fortress Diagnostics, USA).

Test Principle:

Copper ions react in alkaline solution, with protein peptide bonds to give a purple coloured biuret complex. The amount of complex formed is directly proportional to the amount of protein in the specimen.

Reagents:

- Biuret reagent consisting of NaOH (200 mmol/L)
- Potassium Iodide (30 mmol/L)
- Copper sulphate (18 mmol/L)
- Sodium potassium tartate (32 mmol/L)
- Standard made up of protein solution of 60 mg/mL

Procedure:

Distilled water (20 µL), standard and sample was added to the blank, standard and sample test tubes respectively. Biuret reagent (1000 µL) was added to all test tubes.

It was incubated for 10 minutes at 37 ^{0}C. The absorbance of standard and samples were measured against the blank at 546 nm.

$$\text{Total protein (mg/mL)} = \frac{\Delta \text{ in absorbance of sample}}{\Delta \text{ in absorbance of standard}} \times \text{standard concentration}$$

3.37 Extraction and quantitation of placental DNA

Extraction of DNA was done using the quick-DNA universal kit (Zymo Research, USA) following the manual instruction. DNA was quantified with Nanodrop 2000 (Thermo Fisher Scientific, USA).

Procedure

Placental tissue (25 mg) was homogenized in 500 µL of genomic lysis buffer. The placental lysate was centrifuged at top speed (10,000 g) for 5 minutes. The supernatant was used. The supernatant was transferred to a Zymo-Spin column in a collection tube. Thereafter, it was centrifuged at 10,000 g for one minute. The Zymo-Spin column was transferred to a new collection tube. 200 µL of DNA pre-wash buffer was added to the spin column. Thereafter, it was centrifuged at 10,000 g for one minute. Five hundred microlitre of g-DNA wash buffer was added to the spin column. It was thereafter centrifuged at 10,000 g for one minute. The spin column was transferred to a clean micro centrifuge tube. Fifty microlitre of DNA Elution Buffer was added to the spin column. It was incubated for 2-5 minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was used immediately.

3.38 Procedure for immunohistochemistry

Tissue sections were prepared as described previously (3.23). Sections were transferred onto Superfrost Plus glass slides. The slides were allowed to dry overnight. The next day, Slides were deparaffinized in 2 changes of xylene, 5 minutes each.

Slides were transferred to 100 % alcohol, for 2 changes, 3 minutes each, and then transferred once through 95 %, 70 % and 50 % alcohols respectively for 3 minutes each.

Endogenous peroxidase activity was blocked by incubating sections in 3 % H_2O_2 solution in methanol at room temperature for 10 minutes. They were then rinsed in 300 mL of Phosphate Buffer Solution (PBS) for 2 changes, 5 minutes each.

Antigen retrieval was performed to unmask the antigenic epitope using the citrate buffer method. Slides were arranged in a staining container. 300 mL of citrate buffer (pH 6.0, 10 mM) was added in the staining container and incubated at 95-100 °C for 10 minutes. The staining container was removed to room temperature and slides were allowed to cool for 20 minutes. Slides were rinsed in 300 mL PBS for 2 changes, 5 minutes each.

100 µL of appropriately diluted serum bovine primary antibody were applied to the sections on the slides and incubated in a humidified chamber at room temperature for 1 hour. Slides were washed in 300 mL PBS for 2 changes, 5 minute in each case.

A hundred microlitre appropriately diluted (using the antibody dilution buffer) biotinylated secondary antibody was applied to the sections on the slides and incubated in a humidified chamber at room temperature for 30 minutes. Slides were washed in 300 mL PBS for 2 changes, 5 minute each.

A hundred microlitre of appropriately diluted Sav-HRP conjugates (using the antibody dilution buffer) was applied to the sections on the slides and incubated in a humidified chamber at room temperature for 30 minutes to protect from light. Slides were washed in 300 mL PBS for 2 changes, 5 minutes each.

A hundred microlitre 3,3'-diaminobenzidine substrate solution (freshly made just before use: 0.05 % DAB - 0.015 % H_2O_2 in PBS) was added to the sections on the slides to reveal the colour of antibody staining. The colour was allowed to develop for about 5 minutes until the desired colour intensity is reached.

Slides were washed in 300 mL PBS for 3 changes 2 minutes each. Slides were counterstained by immersing them in hematoxylin for 1-2 min. Slides were rinsed in running tap water for greater than 15 minutes.

The tissue slides were dehydrated through 4 changes of alcohol (95%, 95%, 100% and 100%) for 5 minutes in each case. Tissue slides were cleared in 3 changes of xylene and coverslip using mounting solution.

The colour of the antibody staining in the tissue sections was observed under x10 and x40 objective lens of the microscope. Photomicrographs of sections were made.

3.39 Statistical analysis

Data were expressed as mean ± Standard Error of Mean (SEM) and differences in means were compared by analysis of variance (ANOVA) and Student's t-test where applicable. $P<0.05$ was considered statistically significant. The Statistical Package for Social Sciences (SPSS) software (version 22.0; SPSS Inc., USA) was used for data analysis.

CHAPTER FOUR

4.0 RESULTS

4.1. Effects of sleep deprivation on reproductive functions of female Wistar rats

4.1.1. Effects of sleep deprivation on frequency of proestrus phase of female Wistar rats

Proestrus frequency was significantly reduced in the sleep-deprived group during the first, second and third week ($p<0.05$) of sleep deprivation compared with the frequency of proestrus during the pre-sleep deprivation period in the same group. During the first week of sleep deprivation, proestrus frequency was 10% lower in the sleep-deprived group, but this difference was not statistically significant compared with the control (Figure 4.1). However, there was a statistically significant reduction in proestrus frequency during the second and third week of sleep deprivation when compared with the control group (Figure 4.1).

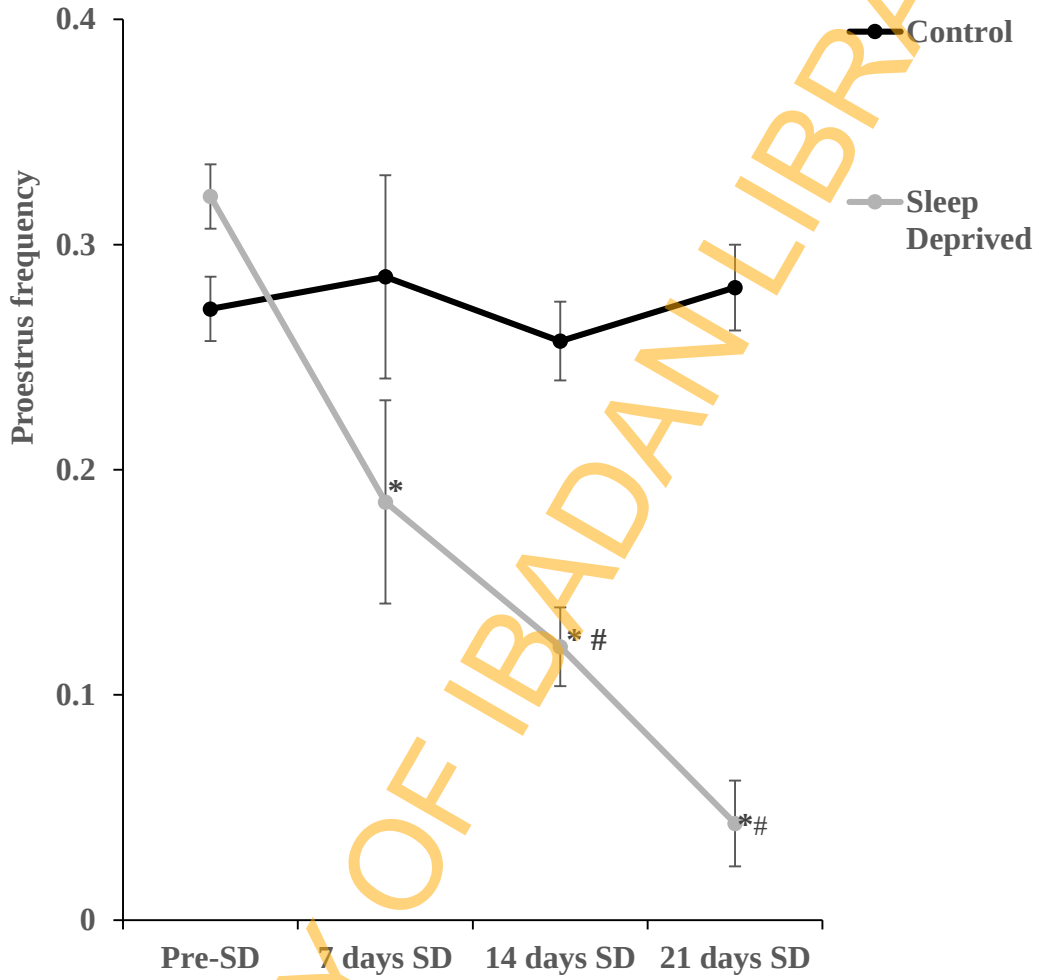


Figure 4.1. Proestrus phase frequency of Control (C) and Sleep Deprived (SD) female Wistar rats. Point estimates represent mean± SEM. n = 5. *p<0.05 when compared with the pre-SD group. #p<0.05 when compared with the control group. Analysis was based on Student's t-test and analysis of variance.

4.1.2. Effects of sleep deprivation on frequency of oestrus phase of female Wistar rats

Frequency of oestrus phase in the sleep deprived group significantly decreased ($p<0.05$) during the first, second and third week post sleep deprivation compared with the oestrus phase frequency during the pre-SD period (Figure 4.2). Oestrus frequency was decreased ($p<0.05$) during the first and third week of sleep deprivation when compared with the control group but not during the second week of sleep deprivation (Figure 4.2).

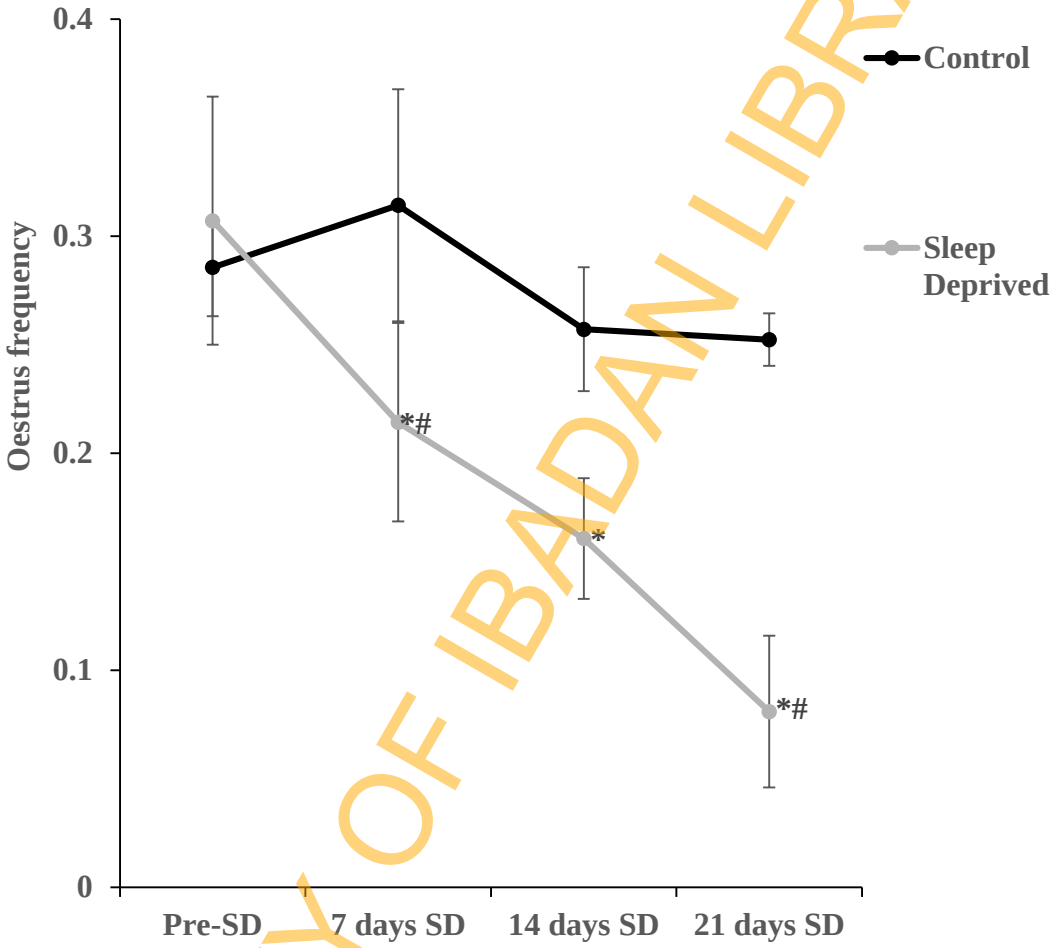


Figure 4.2. Oestrus phase frequency of Control (C) and Sleep Deprived (SD) female Wistar rats. Point estimates represent mean ± SEM. n = 5. *p<0.05 when compared with the pre-SD group. #p<0.05 when compared with the control group. Analysis was based on Student's t-test and analysis of variance.

4.1.3. Effects of sleep deprivation on frequency of metestrus phase of female Wistar rats

Metestrus frequency significantly increased ($p<0.05$) during the second and third week of sleep deprivation in the sleep deprived group when compared with metestrus frequency of the same group before sleep deprivation (Figure 4.3). The increase in frequency of metestrus in the sleep deprived group at the end of the second week (only) was also statistically significant ($p<0.05$) when compared with that of the control group (Figure 4.3).

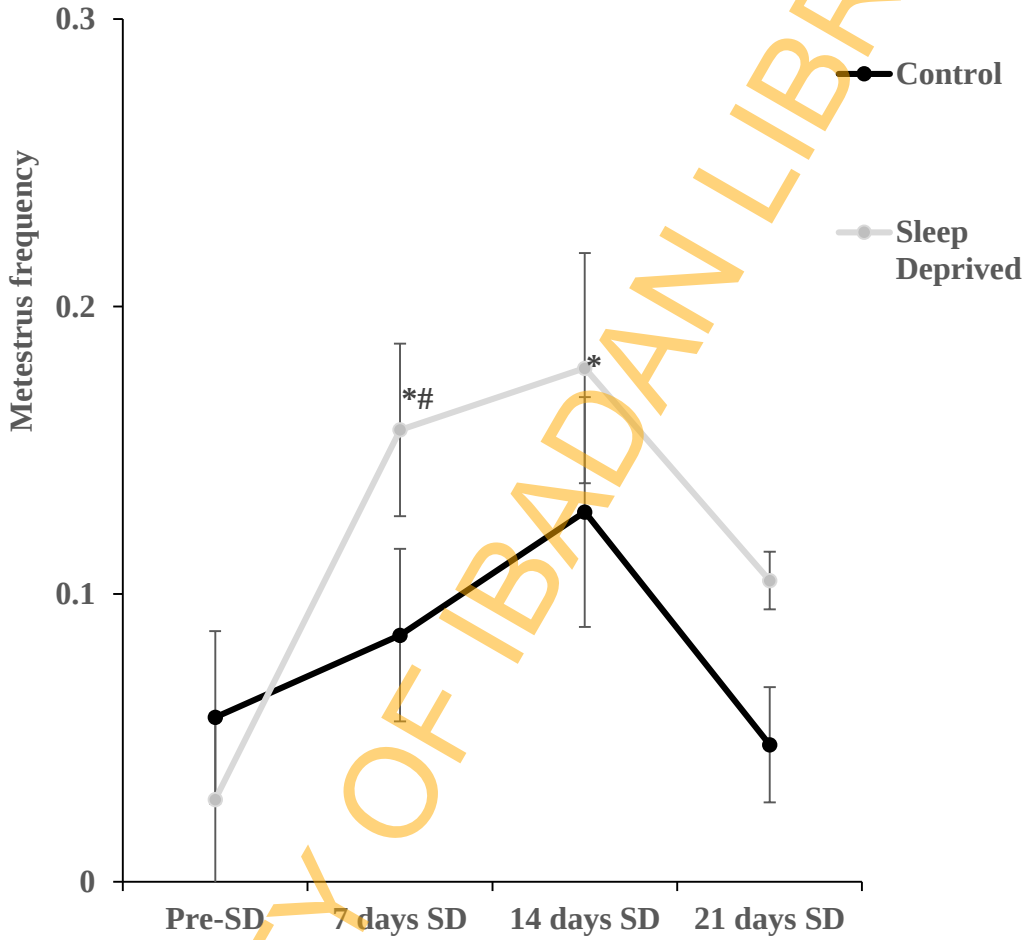


Figure 4.3. Metestrus phase frequency of Control (C) and Sleep Deprived (SD) female Wistar rats. Point estimates represent mean ± SEM. n = 5. *p<0.05 when compared with the pre-SD group. #p<0.05 when compared with the control group. Analysis was based on Student's t-test and analysis of variance.

4.1.4. Effects of sleep deprivation on frequency of diestrus phase of female Wistar rats

The frequency of diestrus was significantly increased ($p<0.05$) at the end of the third week of sleep deprivation in the sleep deprived group compared with the frequency in the pre-sleep deprivation group and with the control group frequency (Figure 4.4).

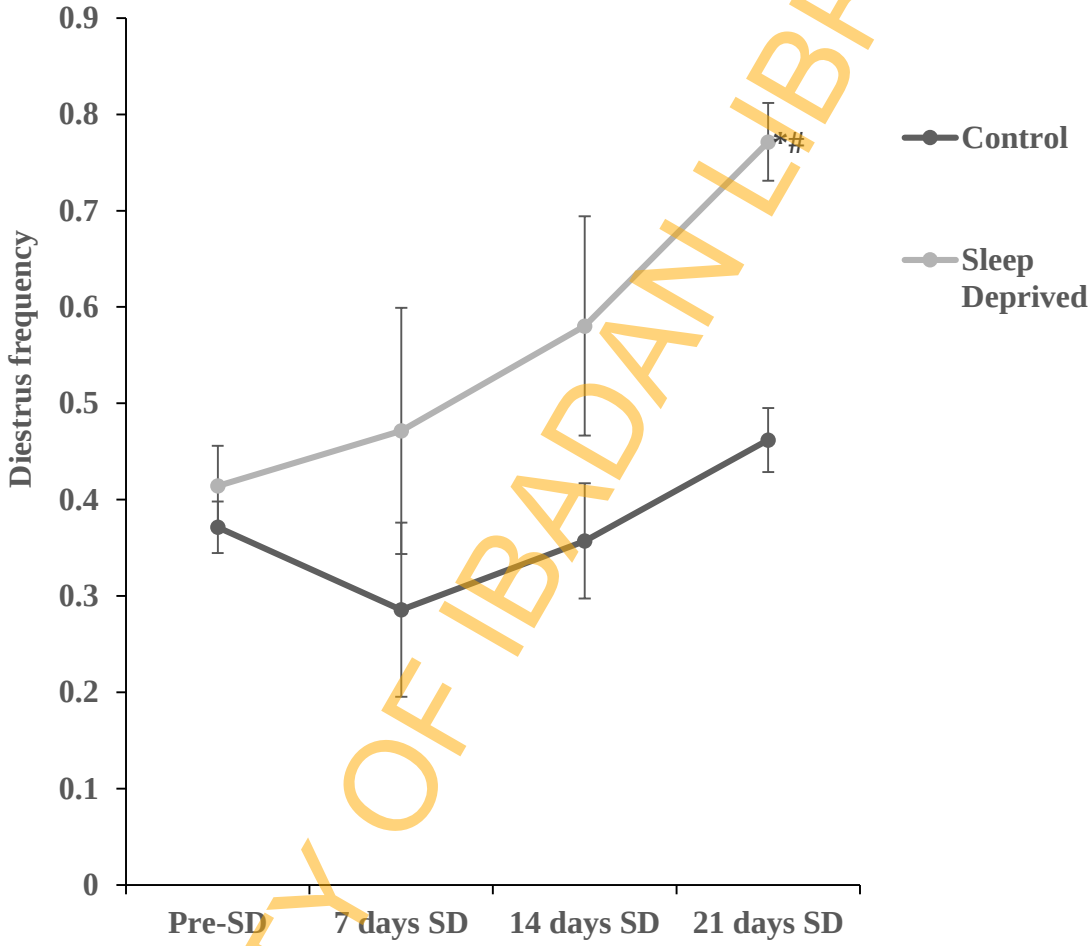


Figure 4.4. Diestrus phase frequency of Control (C) and Sleep Deprived (SD) female Wistar rats. Point estimates represent mean ± SEM. n = 5. *p<0.05 when compared with the pre-SD group. #p<0.05 when compared with the control group. Analysis was based on Student's t-test and analysis of variance.

4.1.5. Effects of sleep deprivation on oestrous cycle length of female Wistar rats

There was no significant difference in oestrous cycle length before sleep deprivation and at the end of first week of sleep deprivation (Figure 4.5). However, length of oestrous cycle increased significantly in the sleep deprived group at the end of the second and third week of sleep deprivation compared with the length of oestrous cycle of the pre-sleep deprivation period and the control group (Figure 4.5).

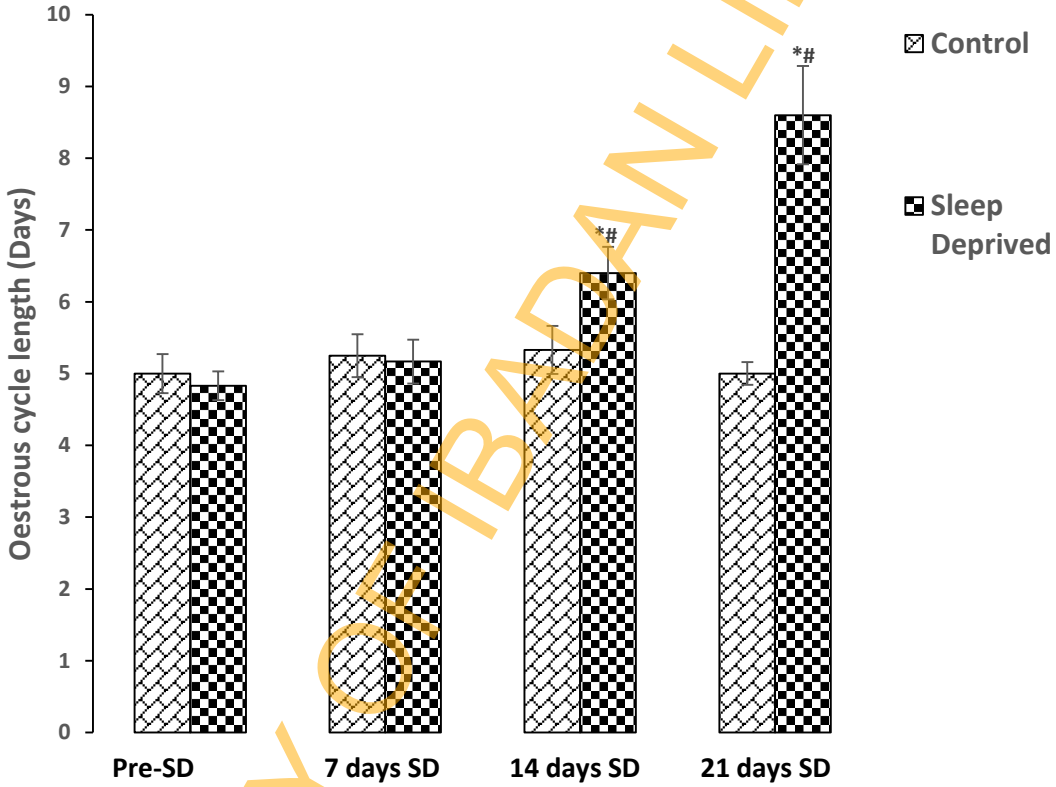


Figure 4.5. Length of oestrous cycle of Control (C) and Sleep Deprived (SD) female Wistar rats. Columns represent mean ± SEM. *p<0.05 when compared with the pre-SD group. #p<0.05 when compared with the control group. Analysis was based on Student's t-test and analysis of variance.

4.1.6 Effects of sleep deprivation on body weight of non-pregnant female Wistar rats

The control rats gained between 2-4% of their body weights, while the sleep deprived rats lost over 6% of their body weight during the first two weeks of sleep deprivation (Figure 4.6). However, at the end of third week of sleep deprivation, the sleep deprived group had about 0.4% increase in body weight but this gain was significantly lower than the weight gained by the control group (Figure 4.6).

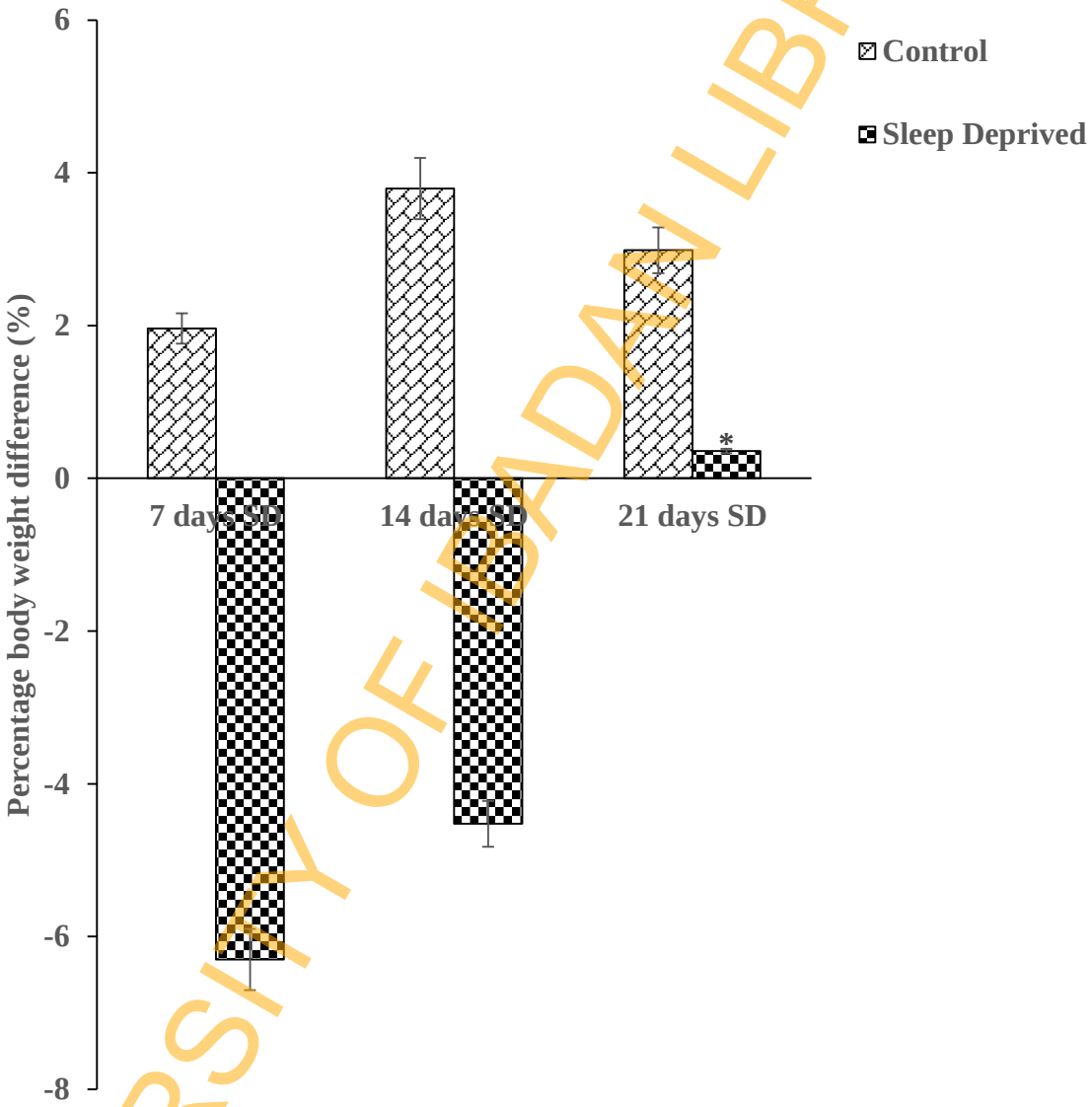


Figure 4.6. Percentage body weight difference of Control (C) and Sleep-Deprived (SD) Wistar rats. Columns represent mean ± SEM. *p<0.05 when compared with the control group. Analysis was based on Student's t-test.

4.1.7. Effects of sleep deprivation on histology of the ovary of non-pregnant female Wistar rats

Control rat had normal ovarian stroma with follicles at different stages of maturation. The ovarian section from the rats that were sleep deprived for 7 days shows no developing follicle (Plate 1). The vessels were congested and the stroma was infiltrated with inflammatory cells. Ovarian sections from rats that experienced 14 and 21 days of sleep deprivation show infiltration of inflammatory cells and different stages of follicular maturation arrest (Plate 1).

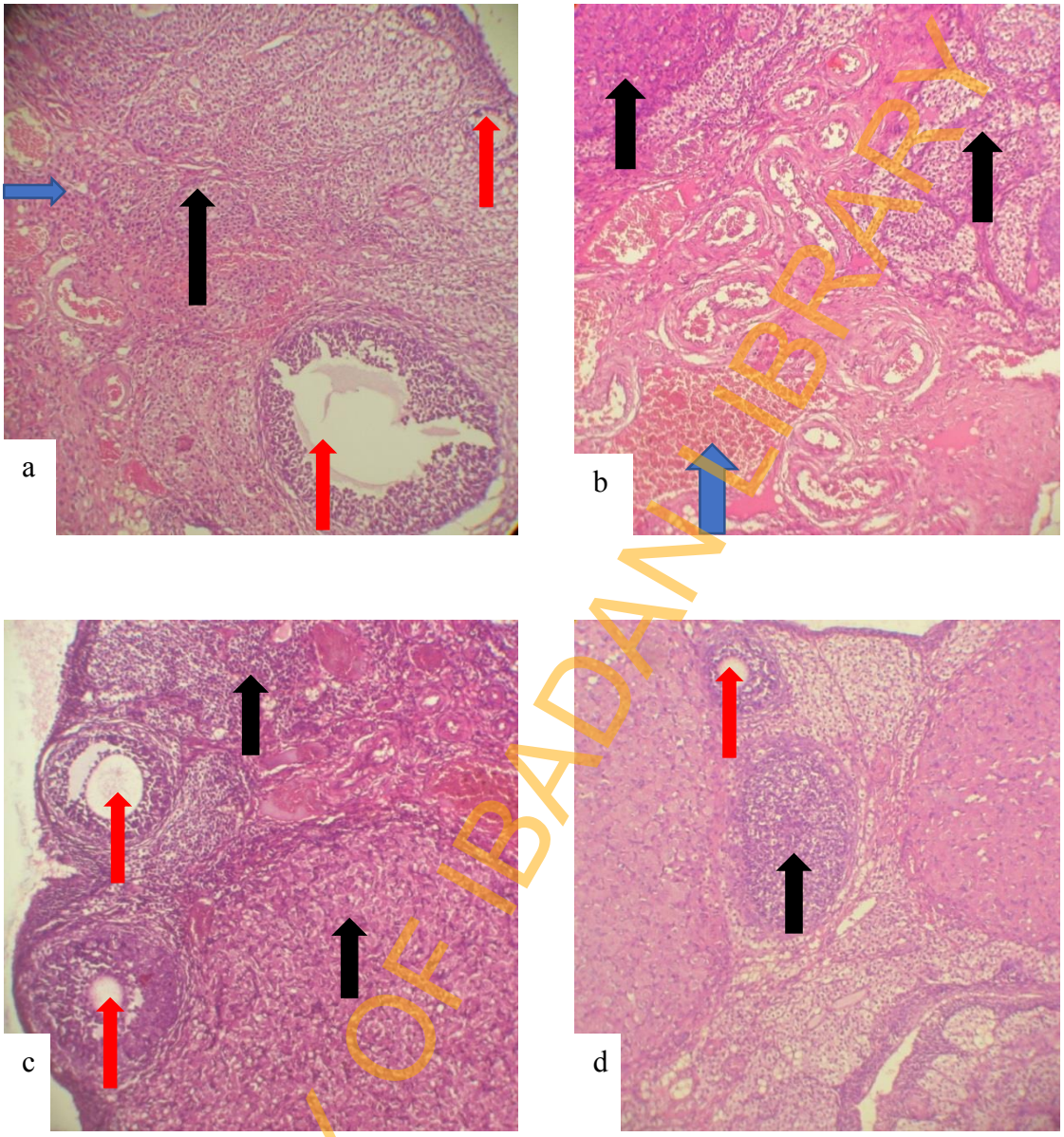


Plate 4.1. Photomicrographs of ovarian sections from control and sleep deprived rats. Tissue sections were stained with H&E and presented at x100 magnification. a) Control: Photomicrograph shows normal ovarian stroma with no infiltration of the stroma (black arrow). There are follicles at different stages of maturation (red arrow) at the cortical region of the ovary. The vessels appear normal (blue arrow). b) 7 days SD rats: Photomicrograph shows no developing follicles. There are several vessels developing in the ovarian stroma with vascular congestion (blue arrow). The stroma is infiltrated with inflammatory cells (black arrow). c) 14 days SD rats: Photomicrograph shows stages of follicular maturation arrest but mostly consisting of secondary follicles with reduced antral cavity (red arrow) and inflammatory cell infiltration (black arrow). d) 21 days SD rats: Photomicrograph shows follicular maturation arrest (red arrow) and infiltrated stroma (black arrow).

4.1.8. Effects of sleep deprivation on histology of the uterus of non-pregnant female Wistar rats

Rats in the control group had normal endometrium and uterine gland with no sign of infiltration of inflammatory cells. The surface epithelial cells appear normal and proliferating. The group sleep deprived for 7 days also showed normal endometrium and uterine gland, however, proliferation of the surface epithelial cells appears to be reduced and the stroma had mild infiltration by inflammatory cells (Plate 4.2). The group sleep deprived 14 days showed endometrial stroma with moderate infiltration of inflammatory cells. The surface epithelial layers appear inflamed (Plate 4.2). The group that was sleep deprived for 21 days showed normal endometrial epithelial lining comprising of simple columnar epithelium. The uterine glands appear normal. There are moderate to severe vascular congestion and inflammatory cells (Plate 4.2).

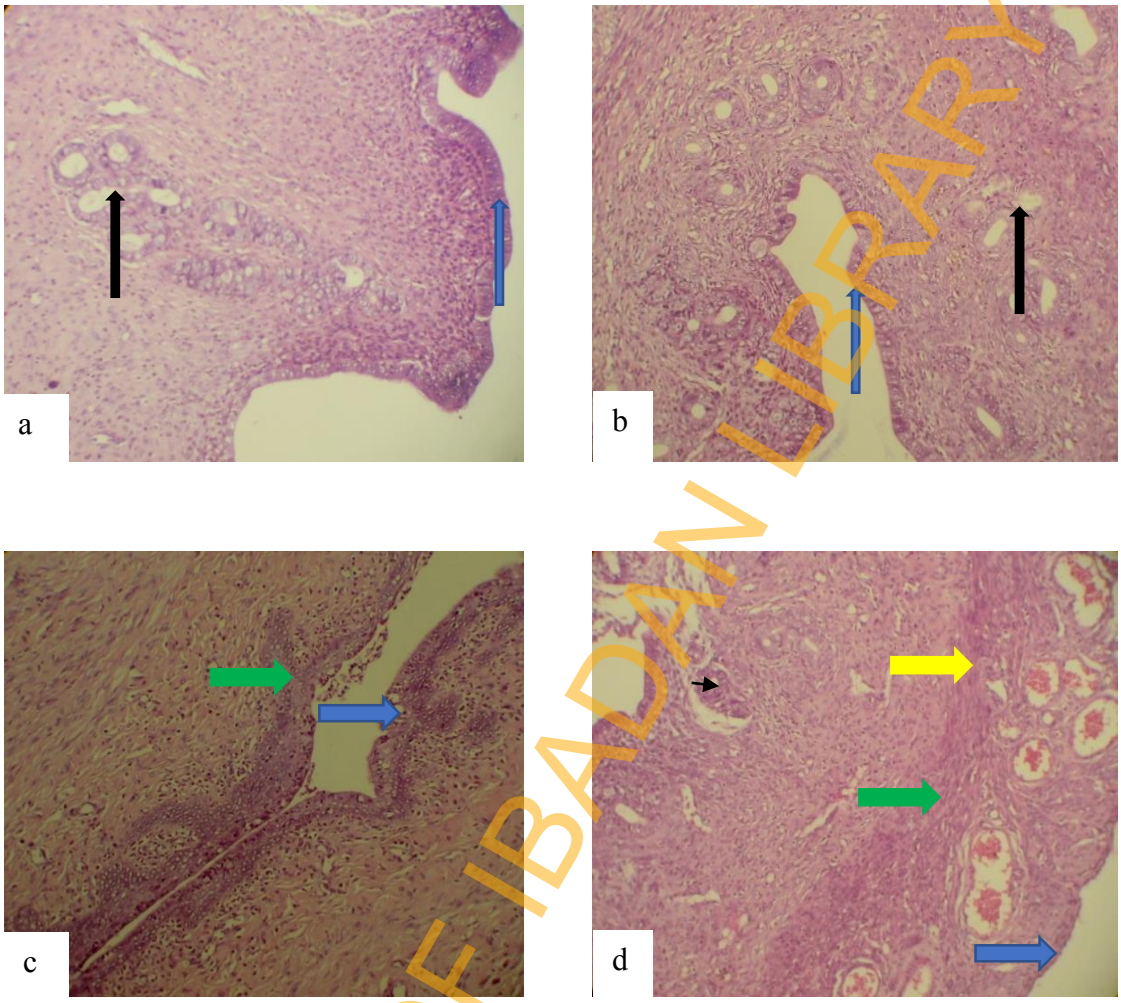


Plate 4.2. Photomicrographs of uterine sections from control and sleep deprived rats. Tissue sections were stained with H&E and presented at x100 magnification. a) Control: Photomicrograph shows endometrium consisting of normal uterine gland (black arrow). The surface epithelial cells are normal and appear to be proliferating (blue arrow). b) 7 days SD: Photomicrograph shows endometrium consisting of normal uterine gland (black arrow). The surface epithelial cells appear normal but with reduced proliferation (blue arrow). The stroma appears to have mild infiltration by inflammatory cells (green arrow). c) 14 days SD: The endometrial stroma shows moderate infiltration of inflammatory cells (green arrow). The surface epithelial layers appear inflamed (blue arrow). d) 21 days SD: Photomicrograph shows normal endometrial epithelial lining comprising of simple columnar epithelium (blue arrow). The uterine glands appear normal (black arrow). There are moderate to severe vascular congestion (yellow arrow). Moderate infiltration of the uterine stroma is seen (green arrow).

4.1.9. Effects of sleep deprivation on histology of adrenal gland of non-pregnant female Wistar rats

The zona fasciculata, zona reticularis and normal adrenal medulla of the control group appear normal. The zona glomerulosa and zona fasciculata layers of the 7 days SD shows normal presentation (Plate 4.3). However, the adrenal medulla appears hyperplastic and compose of clustered cells with basophilic granulated cytoplasm. The vessels also appear mildly dilated in the supporting stroma. In the 14 days SD, the adrenal cortex appears normal while the adrenal medulla is hyperplastic. In the 21 days SD both the cortical and medulla layers are hyperplastic (Plate 4.3).

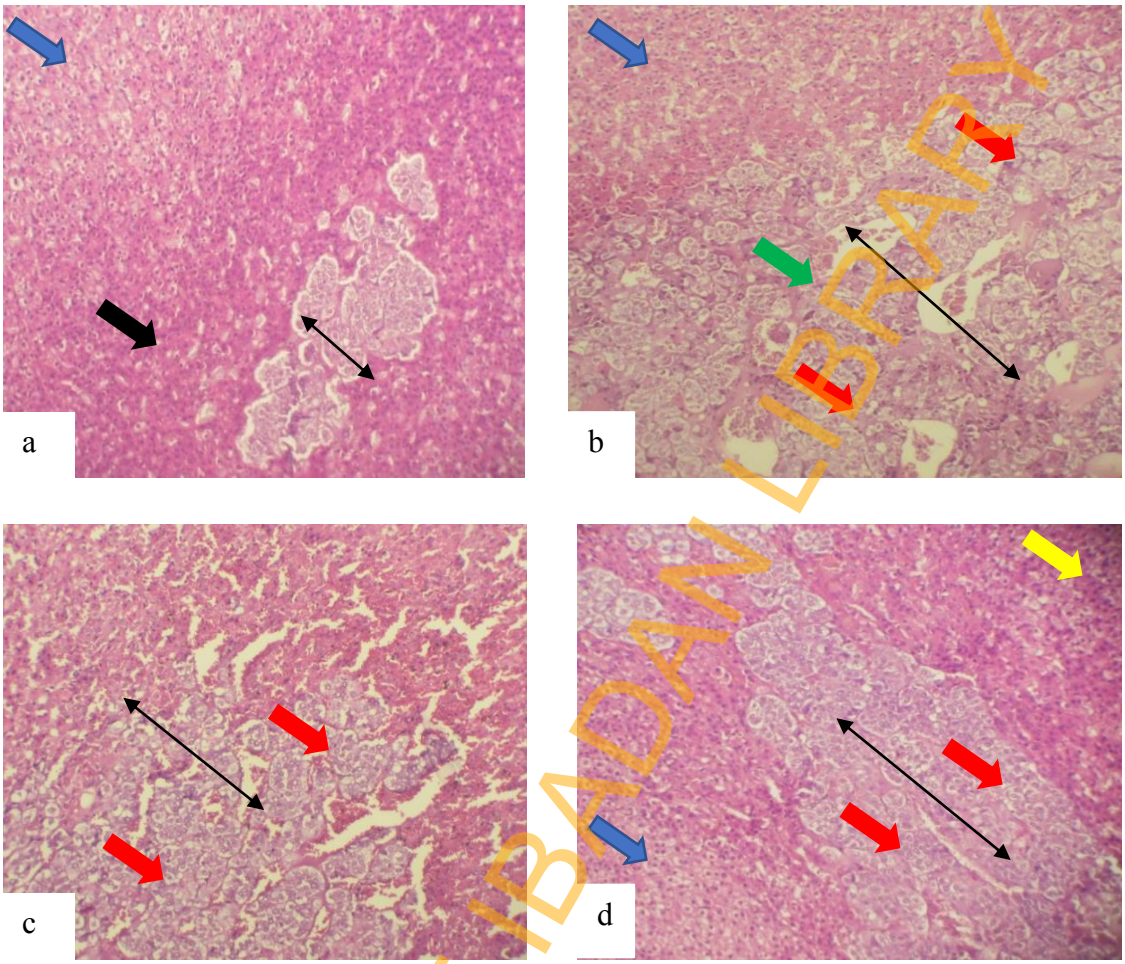


Plate 4.3. Photomicrographs of adrenal gland sections from control and sleep deprived rats. Tissue sections were stained with H&E and presented at x100 magnification. a) Control: Photomicrograph shows normal zona fasciculata (blue arrow) and zona reticularis (black arrow) and normal adrenal medulla (spanned arrow) composing of clustered cells with basophilic granulated cytoplasm. b) 7 days SD: There is normal presentation of zona glomerulosa and zona fasciculata layers (blue arrow). The adrenal medulla appears hyperplastic (spanned arrow) and composed of clustered cells with basophilic granulated cytoplasm (red arrow). There are mildly dilated vessels in the supporting stroma (green arrow). c) 14 days SD: Photomicrograph shows normal zona fasciculata (blue arrow), zona reticularis (black arrow) and hyperplastic adrenal medulla (spanned arrow) composed of clustered cells with basophilic granulated cytoplasm (red arrow). d) 21 days SD: Photomicrograph shows normal adrenal cortex. The cortical layer shows hyperplasia (blue arrow). The hyperplastic medulla (spanned) is composed of clustered cells with basophilic granulated cytoplasm (red arrow).

4.2 Effects of maternal sleep deprivation during gestation on birth morphometric indices of male offsprings of Wistar rats

The birth weight and crown-rump length of adult male offsprings of GD15-21 sleep deprived dams were significantly lower ($p<0.05$) compared with that of GD15-21C group (Table 4.1). While offsprings of GD8-14 sleep deprived dams had significantly lower crown-rump length ($p<0.05$), their birth weight was not significantly different from that of their corresponding control group (Table 4.1).

Table 4.1. Birth morphometric indices of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams

GROUP	GD1-7 C	GD1-7SD	GD8-14 C	GD8-14SD	GD15-21 C	GD15-21 S D
BW (g)	5.44 ±0.22	5.60 ± 0.37	5.91 ± 0.31	6.12 ± 0.25	5.70 ± 0.20	5.27 ± .10*
HC (mm)	37.00 ± 4.95	35.50 ± 0.86	34.40 ±0 .45	35.40 ±0 .84	36.29 ± .24	35.40 ± 1.01
ABC (mm)	7.50 ± 7.10	38.90 ± 4.80	40. 50 ± 6.10	36.80 ± 6.10	40.82 ± .50	38.80 ± 2.20
HC/ABC	1.0 ± 0.11	0.92 ±0.10	0.94 ± 0.10	1.00 ± 0.11	0.89 ± 0.08	0.91 ± 0.08
CRL (mm)	45.63 ± 1.40	45.67 ± 0.78	46.75 ± 0.07	47.49 ± 0.07 *	47.14 ± .05	48.96 ± 0.10 *
AGD*i* **(mmg^3)**	2.1 ± 0.40	2.1 ± 0.10	1.7 ± 0.19	1.6 ± 0.28	1.8 ± 0.13	1.8 ± 0.38

Data are presented as mean ± SEM. n = 5 *p<0.05 when compared with corresponding control group based on Student's t-test. BW=Birth Weight; HC=Head Circumference; ABC=Abdominal Circumference; CRL=Crown-Rump Length; AGD*i*=Anogenital Distance index; GD = Gestation Day.

4.3. Effects of maternal sleep deprivation during gestation on cardiovascular functions of male offsprings of Wistar rats

4.3.1. Effects of maternal sleep deprivation on blood pressure of male offsprings

The male offsprings of GD1-7SD and GD8-14SD groups had significantly higher ($p<0.05$) systolic blood pressure compared with GD1-7C and GD8-14C respectively (Figure 4.7). Diastolic pressure was also increased ($p<0.05$) in the offsprings of GD1-7SD compared with its control. Systolic pressure was lower ($p<0.05$) in the GD15-21SD when compared with GD15-21C group. There were no differences in pulse pressure across the groups (Figure 4.7).

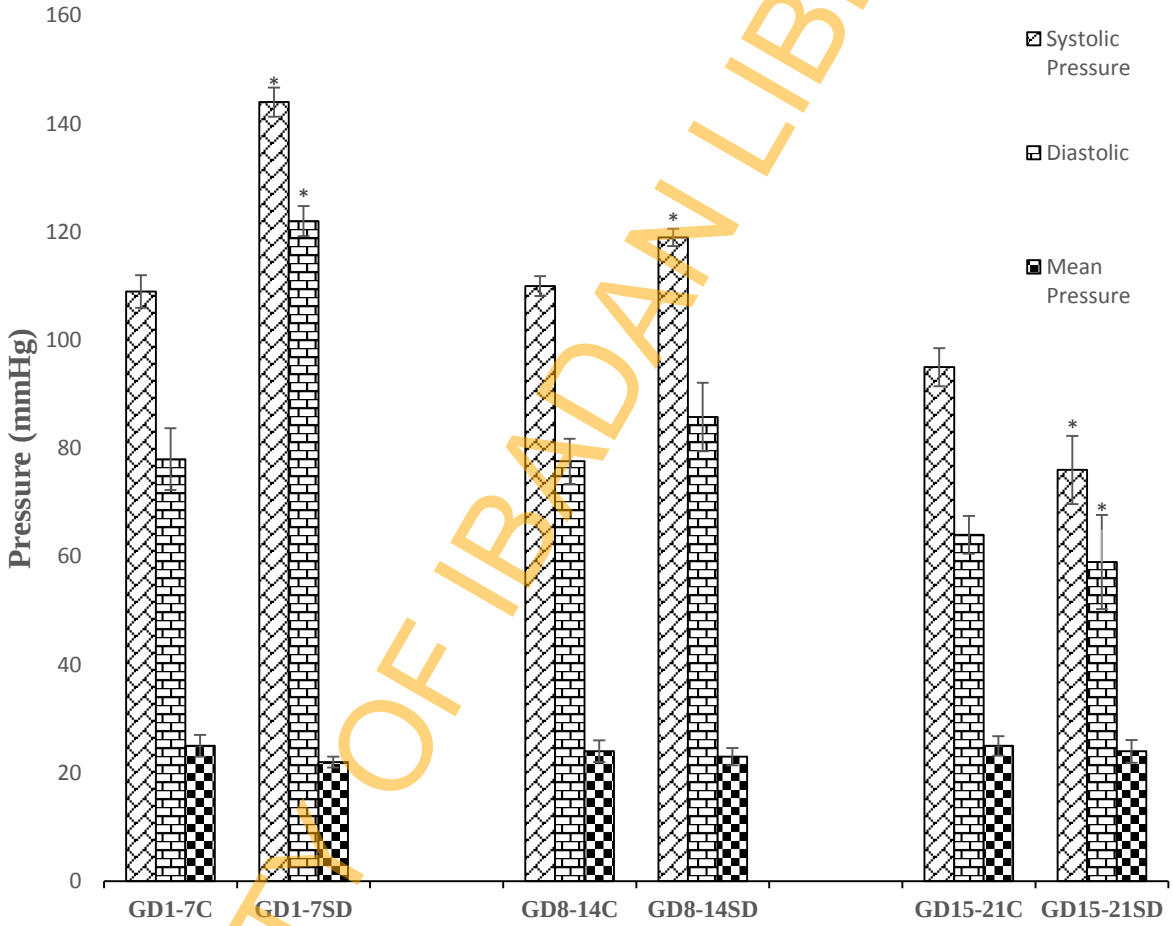


Figure 4.7. Systolic pressure, diastolic pressure and pulse pressure of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 compared with corresponding control group based on Student's t-test. GD = Gestation Day.

4.3.2. Effects of maternal sleep deprivation on heart rate, blood flow and blood volume of male offsprings

Blood flow was significantly higher ($p<0.05$) in the male offsprings of GD8-14SD dams when compared with GD8-14C group (Table 4.2). There was no significant difference in the heart rate and blood volume of offsprings across the groups.

Table 4.2. Heart rate, blood flow and blood volume of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams.

GROUP	HEART RATE (beat/min)	BLOOD FLOW (mL/min)	BLOOD VOLUME (mL)
GD1-7C	454.20 ± 25.00	20.00 ± 6.00	68.00 ± 23.50
GD1-7SD	633.20 ± 93.90	20.80 ± 2.30	52.40 ± 20.80
GD8-14C	584.60 ± 34.30	14.60 ± 2.01	74.60 ± 19.40
GD8-14SD	497.40 ± 66.86	34.60 ± 6.85*	64.20± 13.42
GD15-21C	498.40 ± 54.80	21.80 ± 2.60	76.00± 9.68
GD15-21SD	664.40± 91.60	39.20 ± 2.50	81.20 ± 22.34

Data are presented as mean ± SEM. n = 5. *p<0.05 compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.3.3. Effects of maternal sleep deprivation on relative weight of heart of male offsprings

There were no significant differences in the relative weight of heart of the male offsprings of sleep deprived dams when compared with control dams (Figure 4.8).

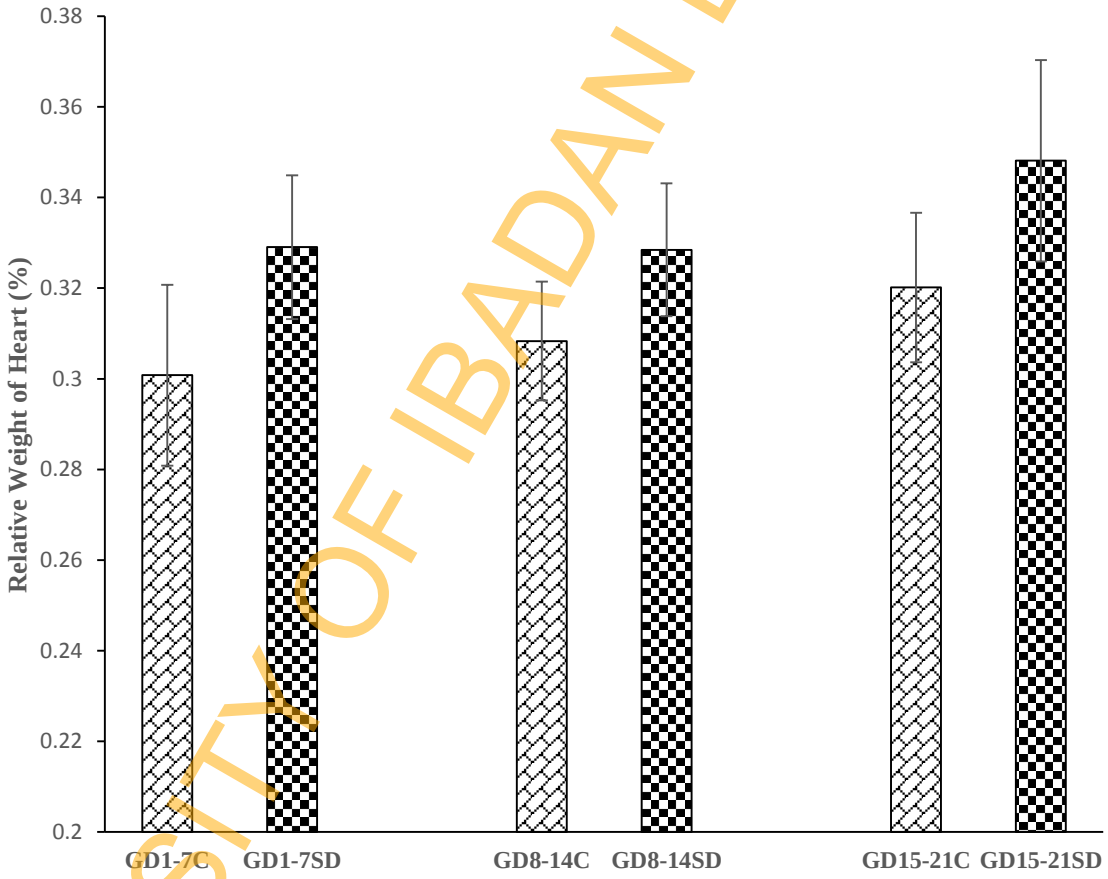
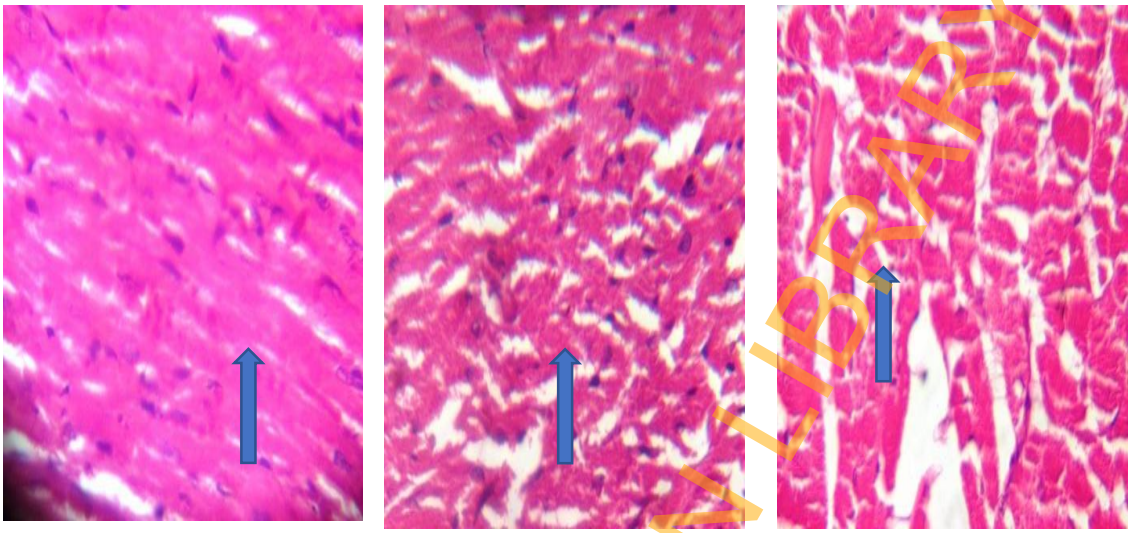


Figure 4.8. Relative heart weight of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. GD = Gestation Day.

4.3.4. Effects of maternal sleep deprivation on histology of the heart of male offsprings

The sections from GD1-7C, GD1-7SD, GD8-14C and GD15-21C groups show normal myocardial structure. There were mild focal points of degeneration in some parts of the myocardium of GD8-14SD. GD15-21SD heart section shows epicardial vascular congestion and myocardial haemorrhage (Plate 4.4) .



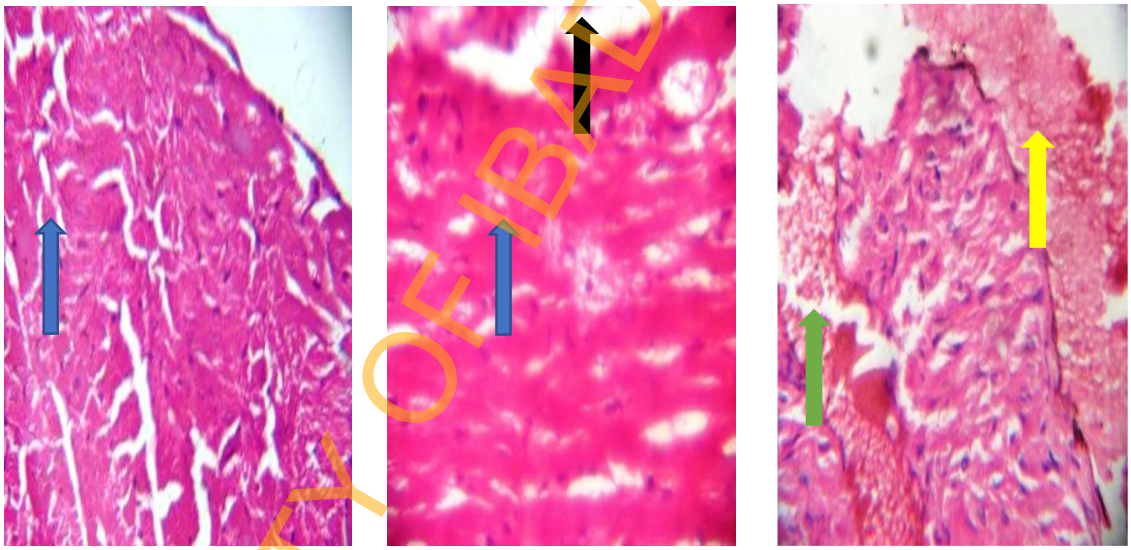


Plate 4.4 Photomicrographs of heart sections from male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Tissue sections were stained with H&E and presented at x100 magnification. GD = Gestation Days. Photomicrograph shows myocardium (blue arrow) with no infiltration in GD1-7C, GD1-7SD, GD8-14C & GD15-21C.There are mild focal points of degeneration in the myocardium (black arrow) of GD8-14SD. The epicardium shows vascular congestion (yellow arrow) and myocardium shows haemorrhage (green arrow) in GD15-21SD.

4.4. Effects of maternal sleep deprivation during gestation on renal functions of adult male offsprings of Wistar rats

4.4.1. Effects of maternal sleep deprivation on serum albumin level of male offsprings

Maternal sleep deprivation did not affect the serum albumin levels of adult male offsprings in all the groups (Figure 4.9).

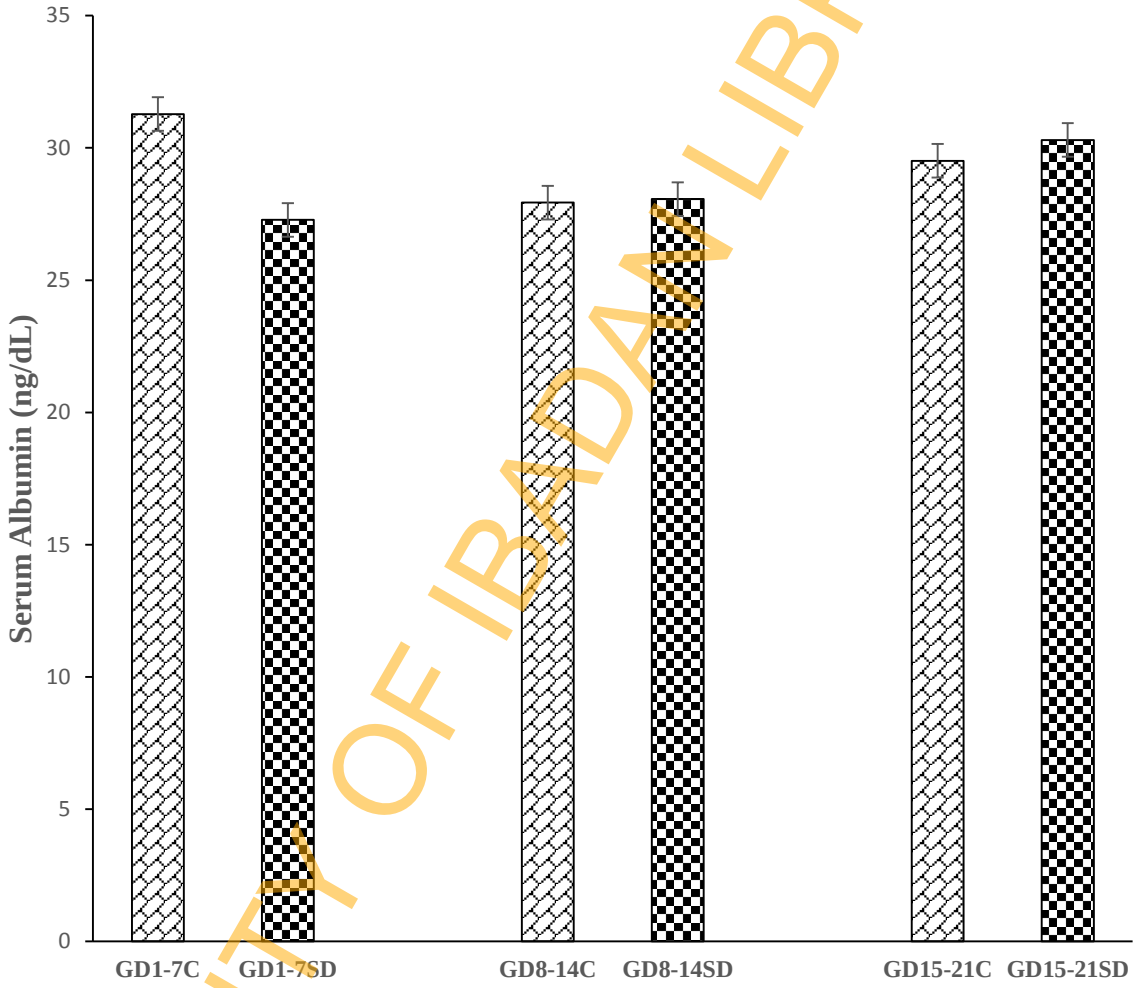


Figure 4.9. Serum albumin of adult male offsprings of control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.4.2. Effects of maternal sleep deprivation on serum creatinine level of male offsprings

Maternal sleep deprivation during GD 15-21 caused a significant increase in serum creatinine level ($p<0.05$) of adult male offsprings when compared with the GD15-21C group male offsprings (Figure 4.10).

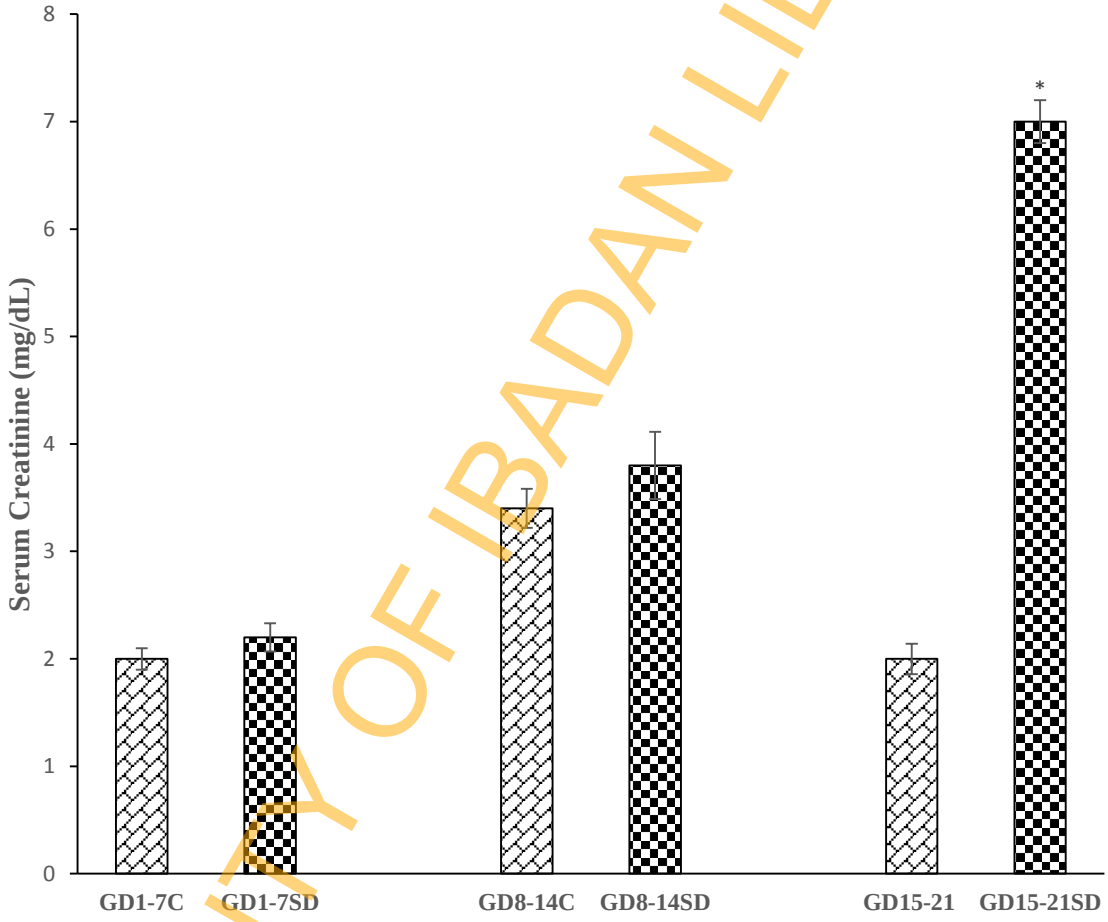


Figure 4.10. Serum creatinine level of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.4.3. Effects of maternal sleep deprivation on serum urea level of male offsprings

Maternal sleep deprivation during the different gestation periods did not cause any significant change in serum urea levels of adult male offsprings when compared with the respective control groups (Figure 4.11).

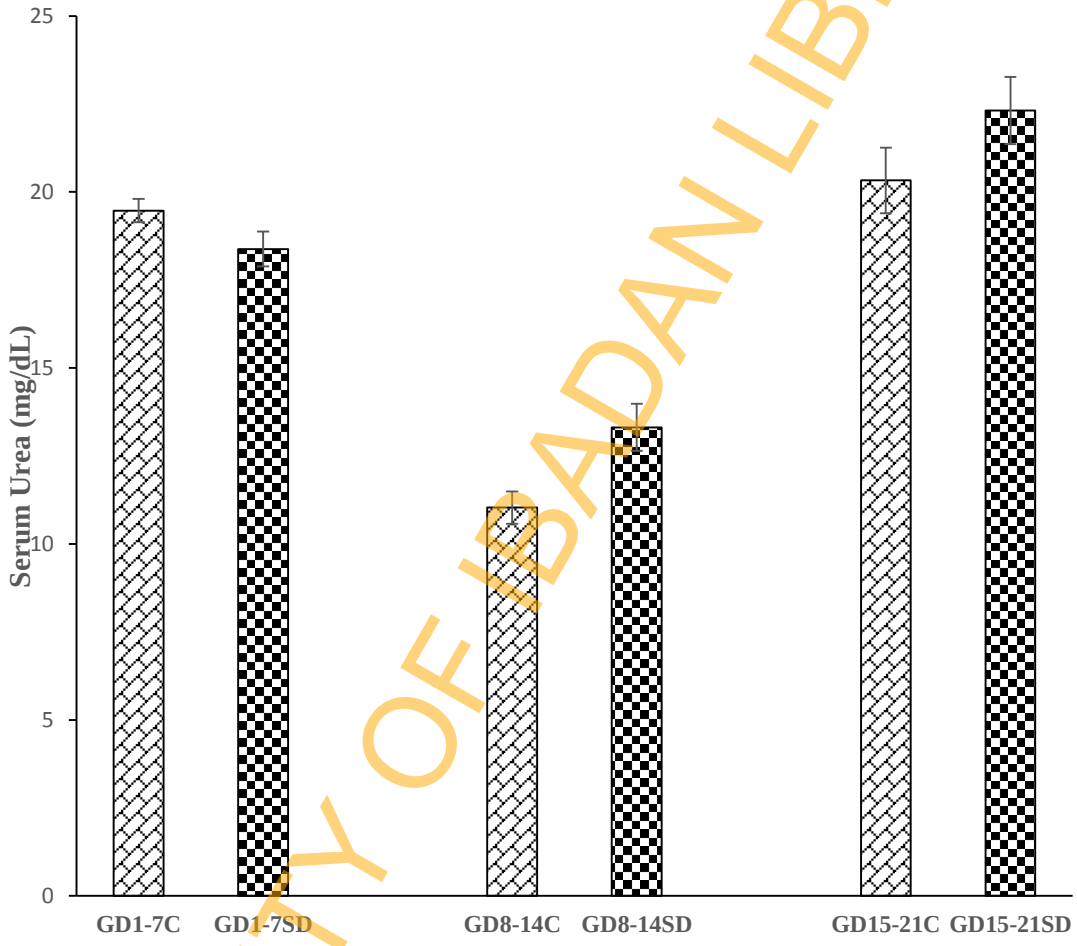


Figure 4.11. Serum urea level of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.4.4. Effects of maternal sleep deprivation on urinary albumin level of male offsprings

Urinary albumin increased significantly ($p<0.05$) in offsprings of GD15-21SD dams compared with urinary albumin level of the offsprings of GD15-21C dams (Figure 4.12).

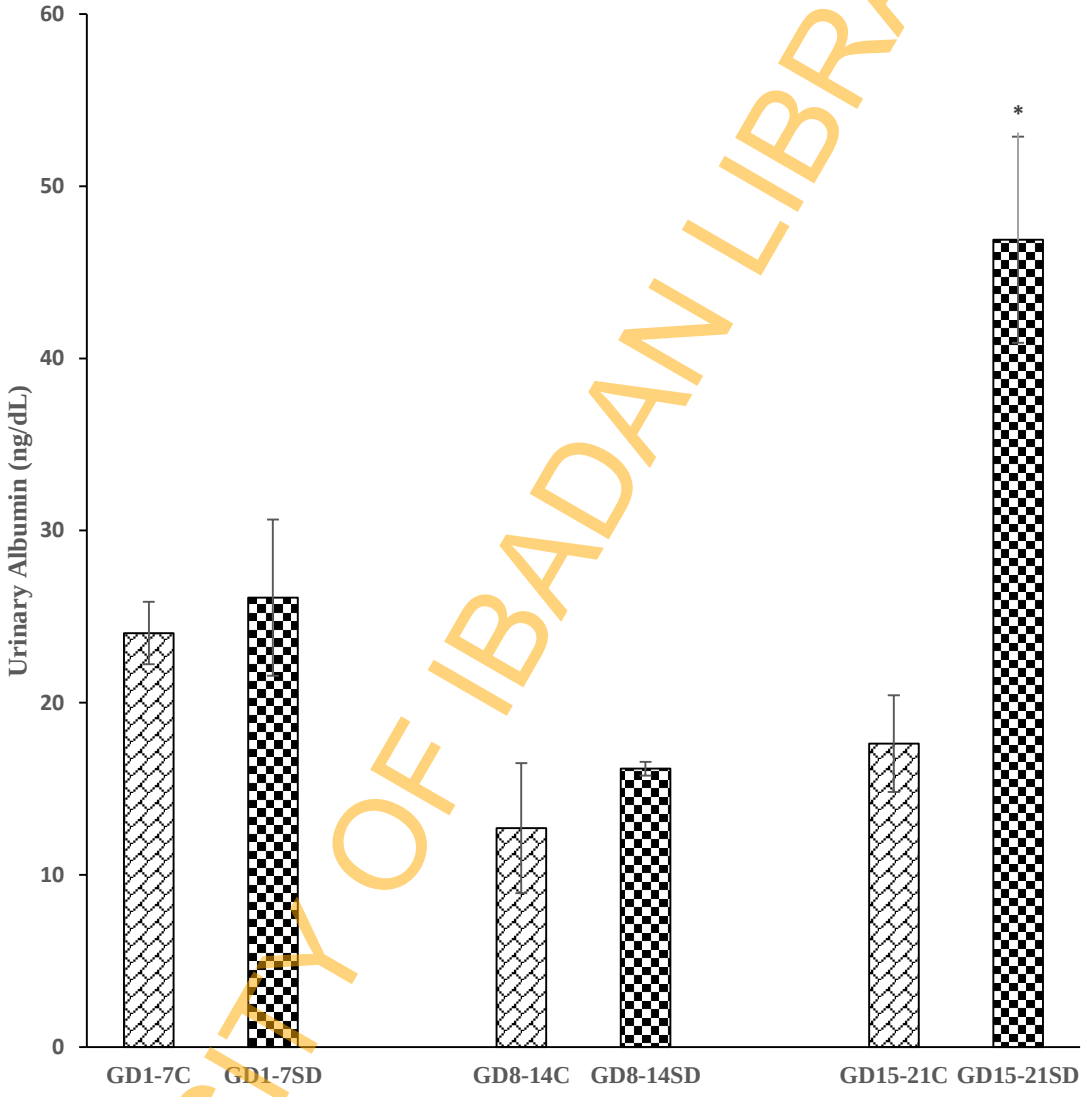


Figure 4.12. Urinary albumin level of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.4.5. Effects of maternal sleep deprivation on renal creatinine clearance of male offsprings

Renal creatinine clearance decreased significantly ($p<0.05$) in the male offsprings of GD15-21SD dams when compared with the offsprings of their corresponding control group (Figure 4.13).

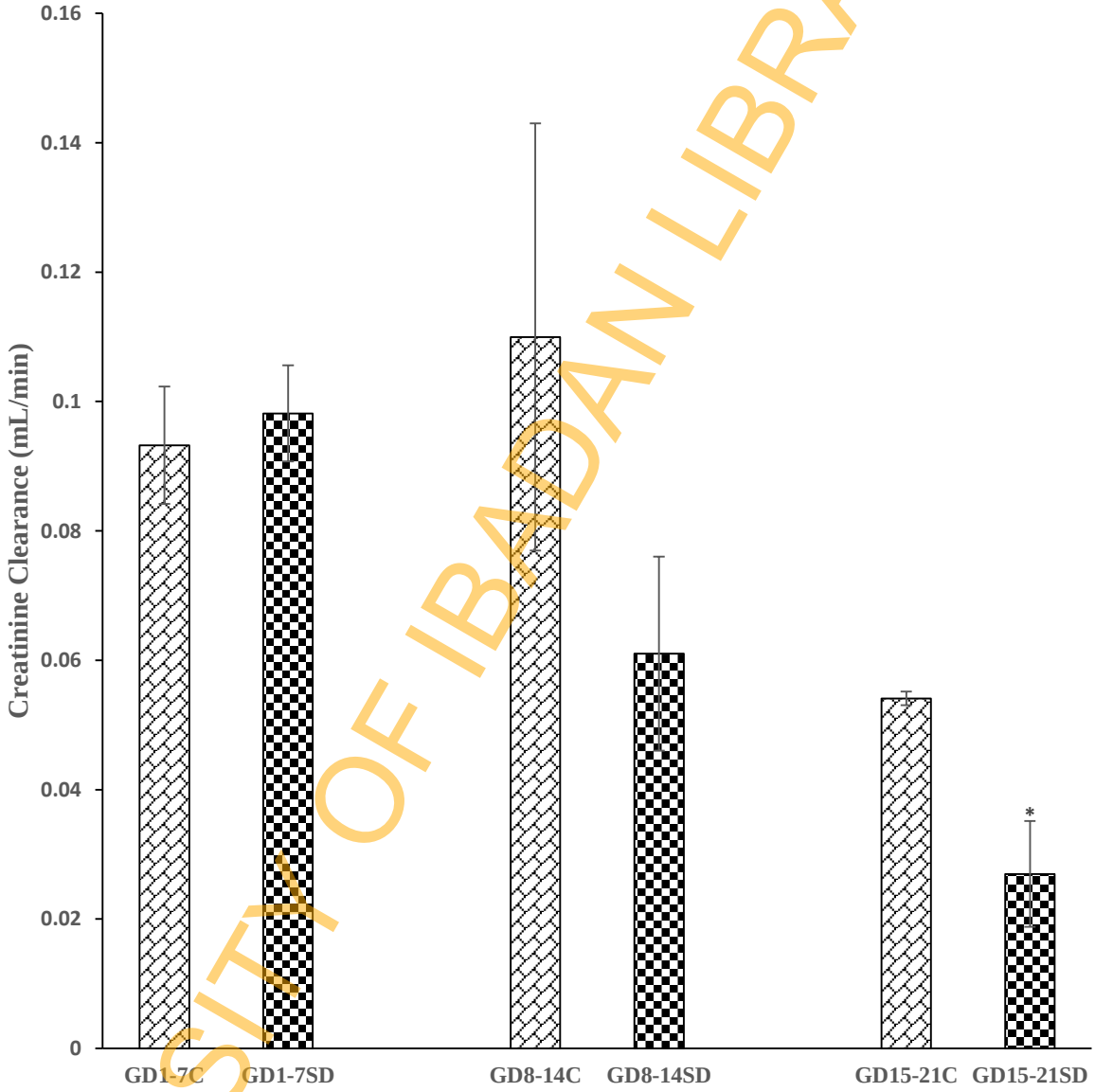


Figure 4.13. Renal creatinine clearance of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.4.6. Effects of maternal sleep deprivation on urea/creatinine ratio of male offsprings

Urea/creatinine ratio was reduced significantly ($p<0.05$) in the offsprings of GD15-21SD dams when compared with GD15-21C (Figure 4.14).

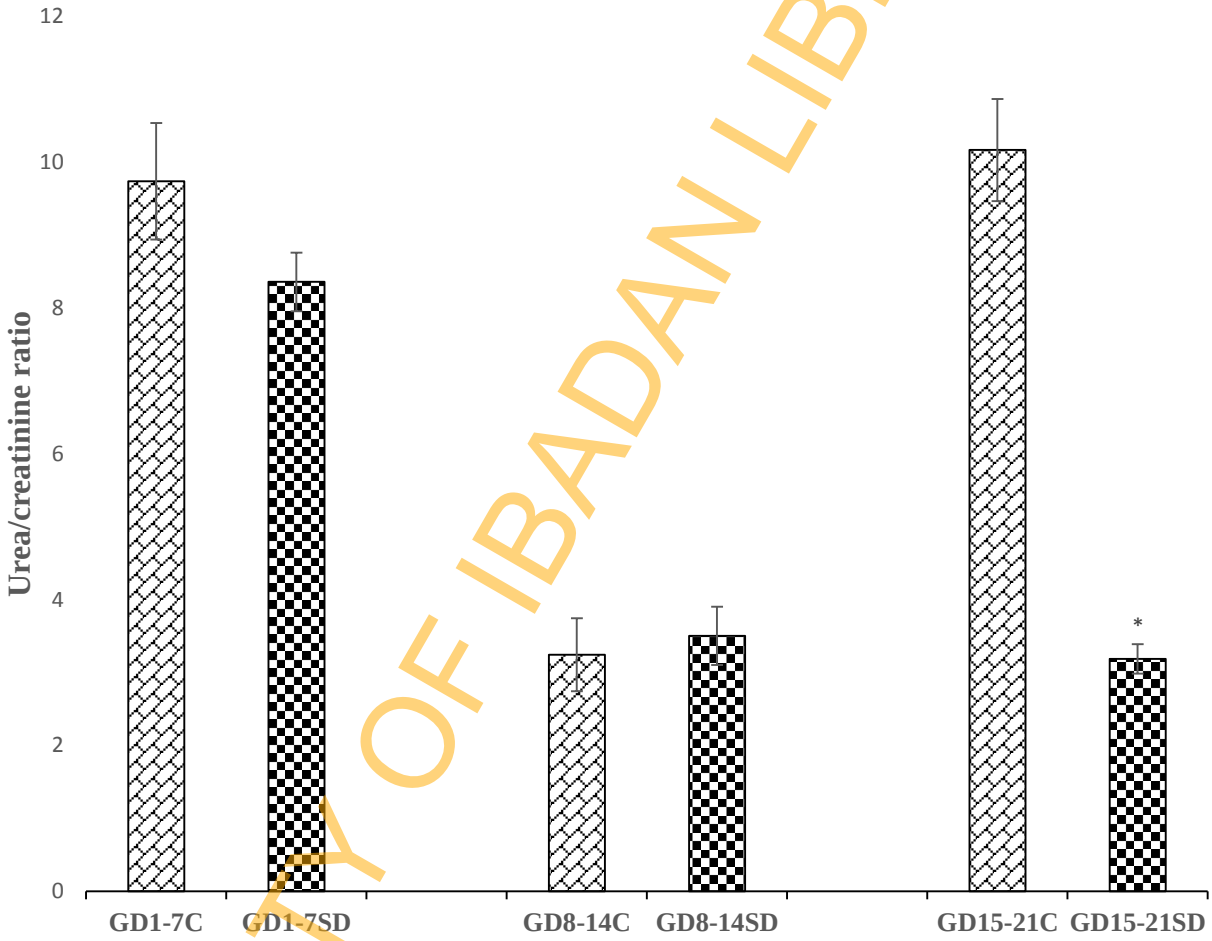


Figure 4.14. Urea/Creatinine ratio of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with corresponding control group based on Student's t-test. GD = Gestation Day.

4.4.7. Effects of maternal sleep deprivation on relative weight of the kidney of male offsprings

There were no significant differences in the relative weights of the kidney of adult male offsprings of sleep deprived dams and control dams (Figure 4.15).

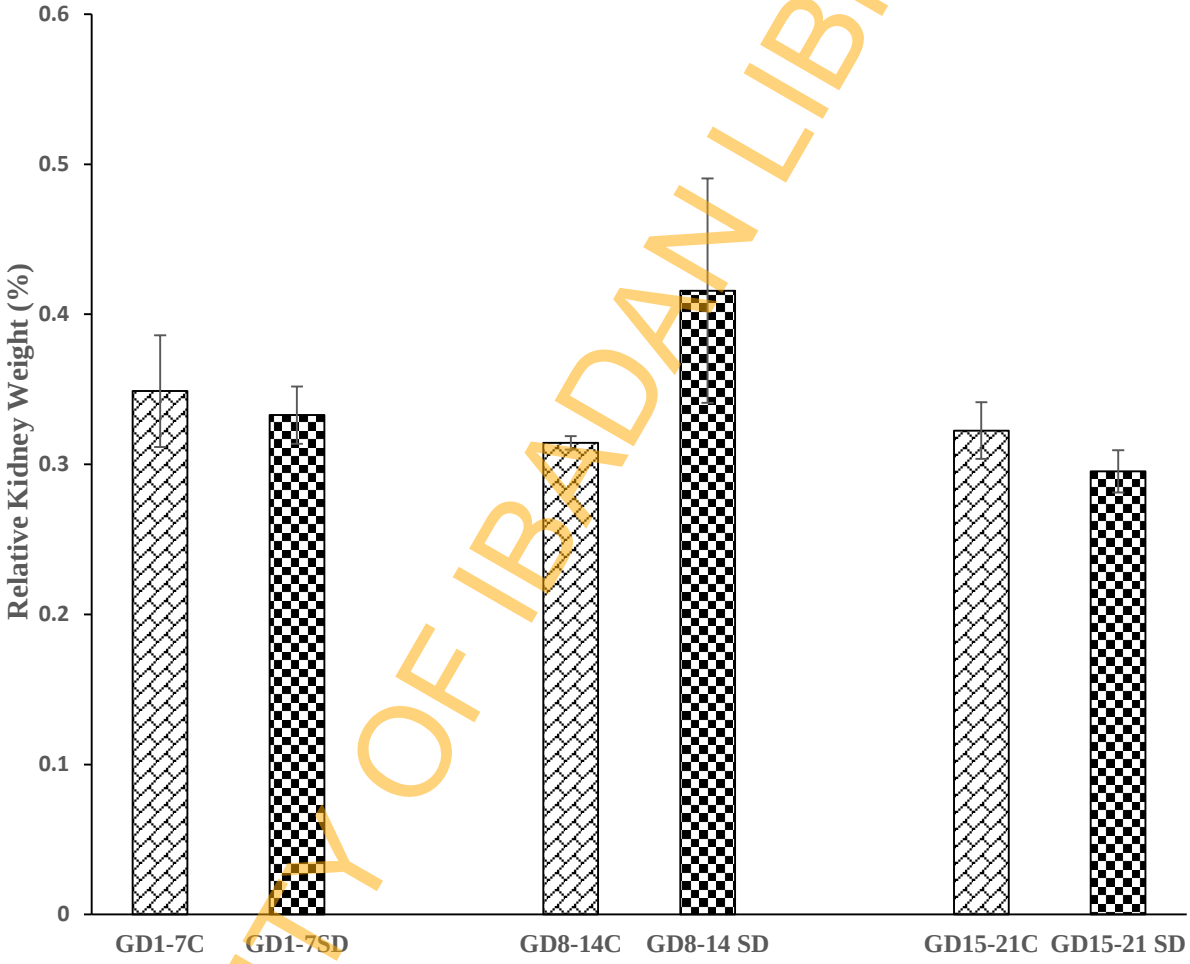


Figure 4.15. Relative weight of kidney of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.4.8. Effects of maternal sleep deprivation on histology of kidney of male offsprings

The photomicrographs belonging to GD1-7C, GD 8-14C and GD 15-21C show normal architecture. The renal cortex of the kidney of GD1-7SD, GD8-14SD and GD15-21SD show varying degrees of pathologies (Plate 4.5).

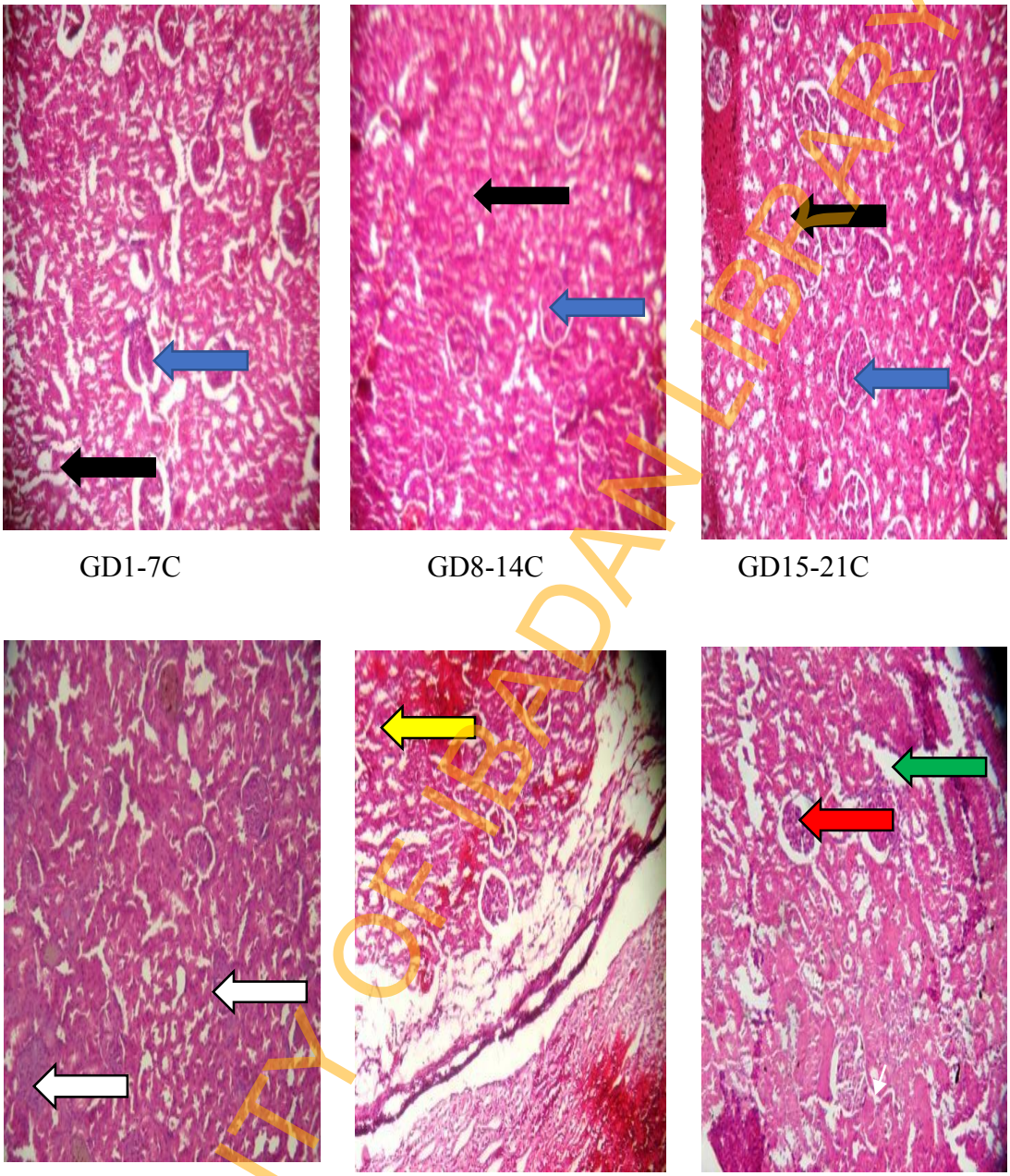


Plate 4.5. Photomicrographs of kidney sections from male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Tissue sections were stained with H&E and presented at x100 magnification. a) Photomicrograph shows normal glomeruli (blue arrow) and renal tubules (black arrow) in GD1-7C, GD8-14C and GD15-21C. GD1-7SD shows several glomeruli with mesangial cell hyperplasia and lack of capsular spaces (white arrow). GD8-14SD and GD15-21SD show cast within their lumen (yellow arrow). GD15-21SD shows renal cortex with few glomeruli (red arrow) and tubules devoid of brush borders (green arrow)

4.5. Effects of maternal sleep deprivation during gestation on reproductive functions of adult male offsprings of Wistar rats

4.5.1. Effects of maternal sleep deprivation on testes descent and preputial separation

Testes descent was significantly delayed ($p<0.05$) in offsprings of GD15-21SD dams compared with the corresponding control group. However, maternal sleep deprivation did not affect preputial separation in all the test groups (Figure 4.16).

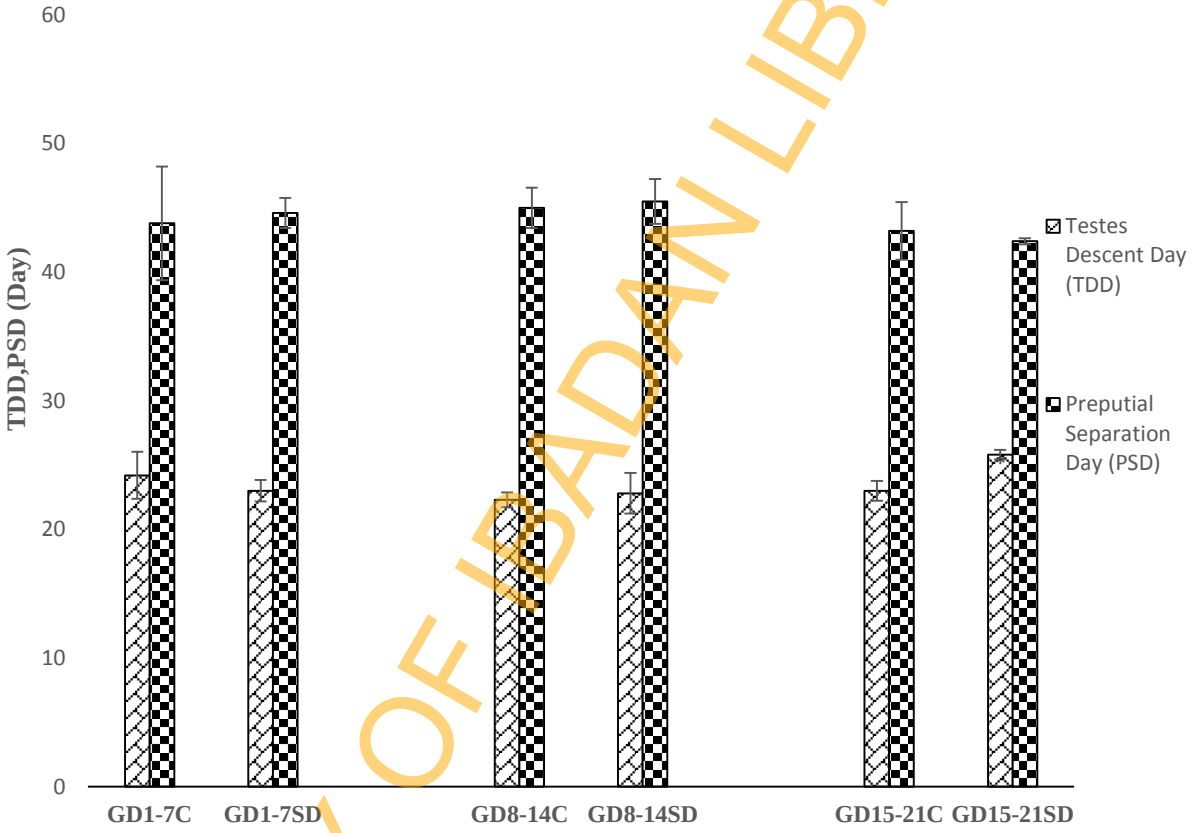


Figure 4.16. Testes descent and preputial separation in male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.5.2. Effects of maternal sleep deprivation on sperm indices of male offsprings

Epididymal sperm viability was not significantly affected across the groups (Figure 4.17). Maternal sleep deprivation significantly reduced ($p<0.05$) epididymal sperm motility (Figure 4.18) and epididymal sperm count (Figure 4.19) in offsprings of GD15-21SD dams. All comparisons were done against their respective control groups.

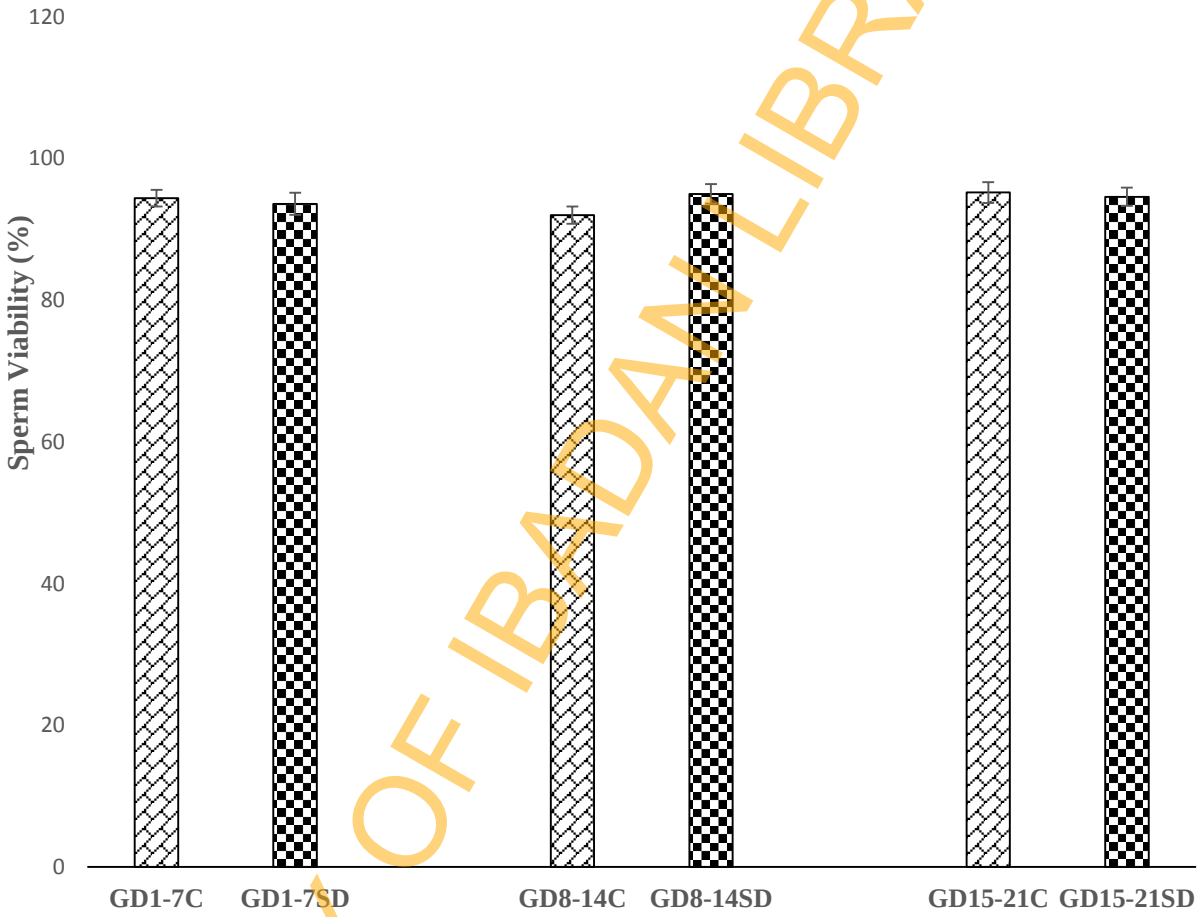


Figure 4.17. Epididymal sperm viability of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

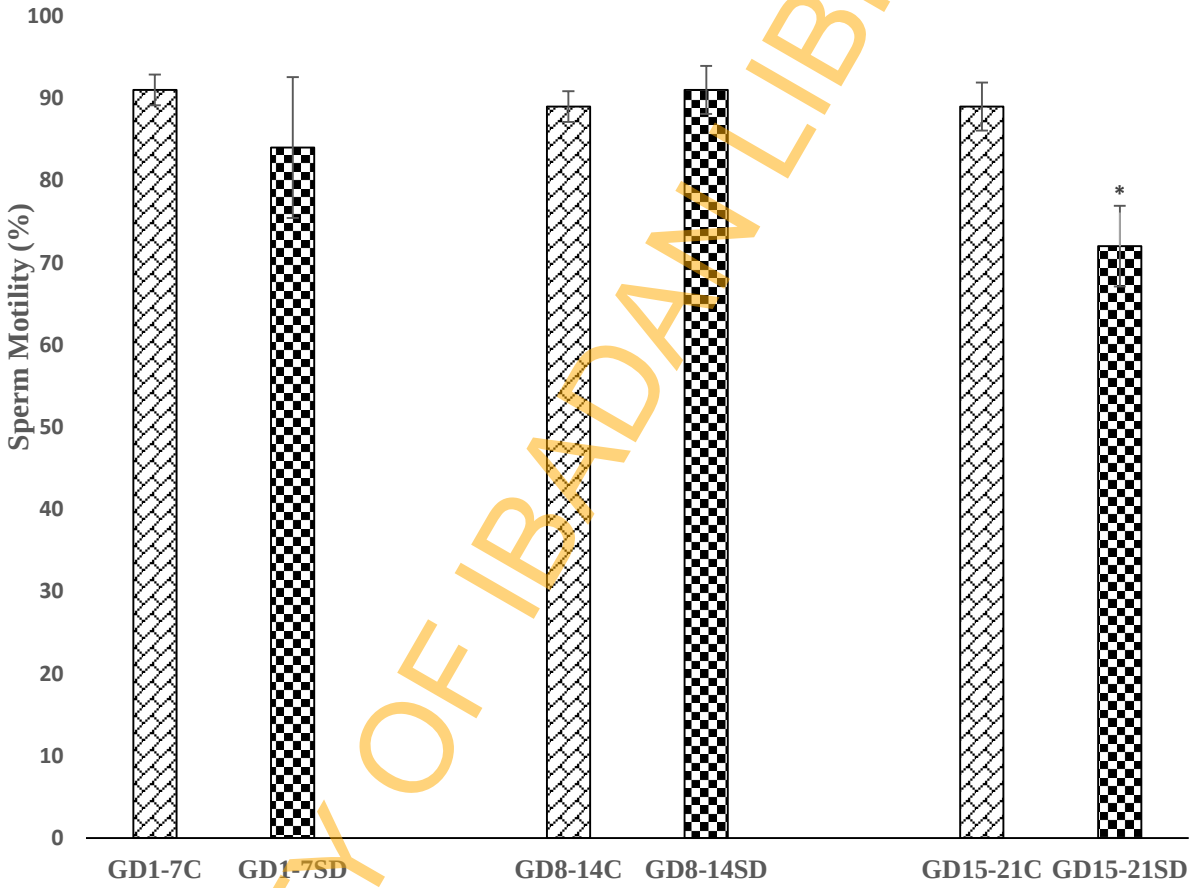


Figure 4.18. Epididymal sperm motility of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

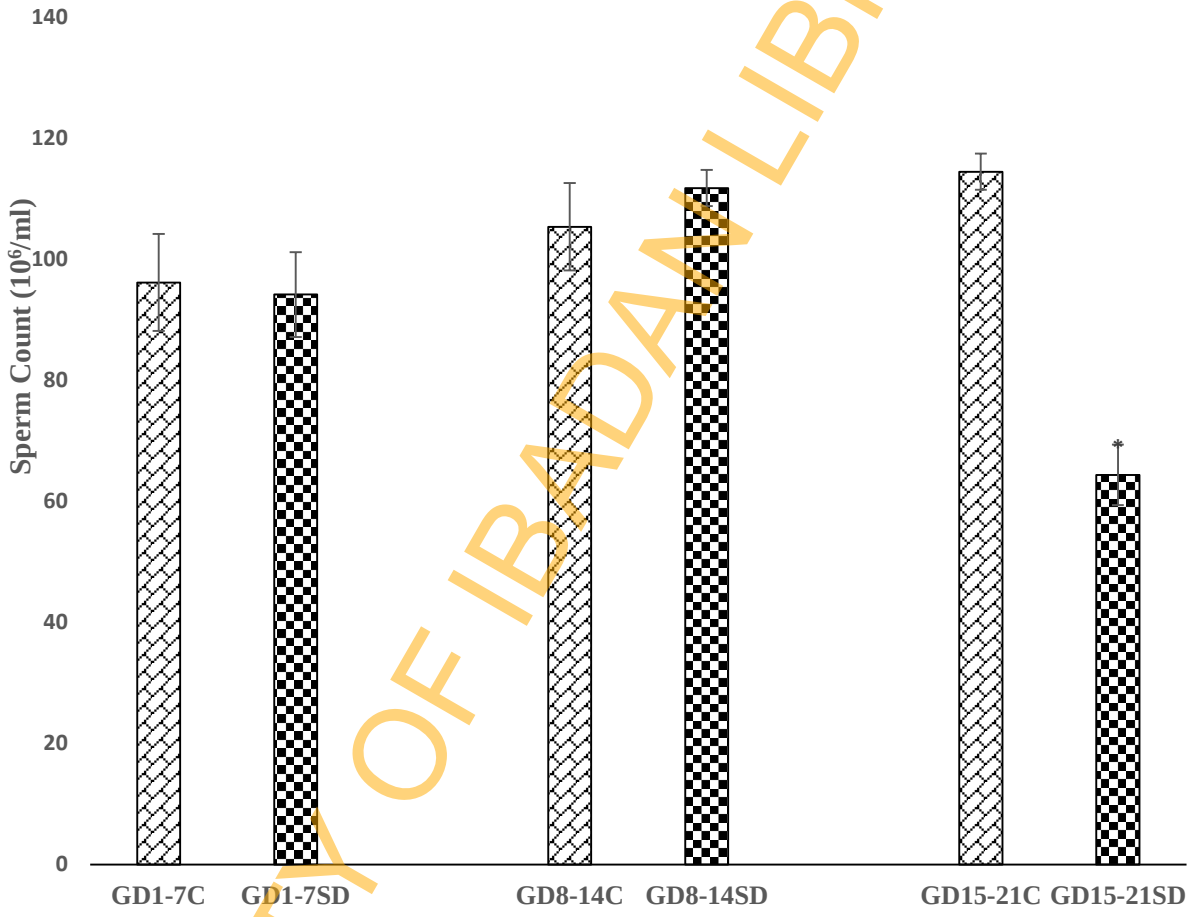


Figure 4.19. Epididymal sperm count of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.5.3. Effects of maternal sleep deprivation on relative weights of reproductive organs in male offsprings

Maternal sleep deprivation did not affect the relative weight of reproductive organs from male offsprings of the sleep deprived dams (Table 4.3).

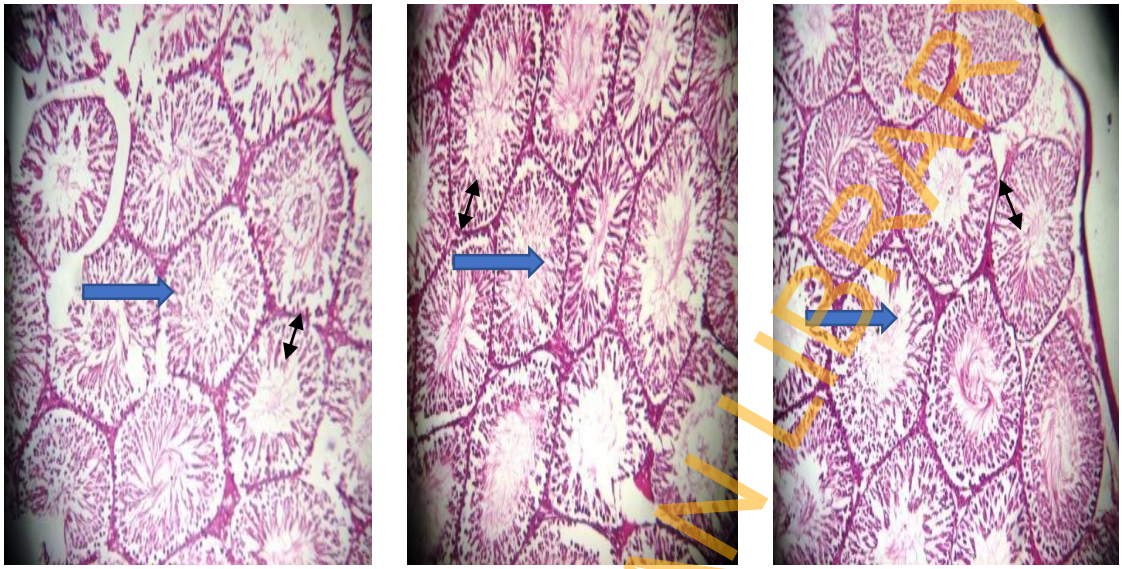
Table 4.3. Relative organ weight of male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams.

GROUP	TESTIS (%)	EPIDIDYMIS (%)	SEMINAL VESICLE (%)	PROSTATE GLAND (%)	ADRENAL GLAND (%)
GD1-7C	0.49 ± 0.02	0.19 ± 0.02	0.41 ± 0.08	0.12 ± 0.02	0.01 ± 0.00
GD1-7 SD	0.47 ± 0.02	0.25 ± 0.03	0.29 ± 0.05	0.11 ± 0.02	0.02 ± 0.00
GD8-14C	0.41 ± 0.02	0.22 ± 0.01	0.38 ± 0.07	0.12 ± 0.02	0.01 ± 0.00
GD8-14SD	0.50 ± 0.05	0.22 ± 0.03	0.34 ± 0.04	0.15 ± 0.02	0.01 ± 0.00
GD15-21C	0.46 ± 0.02	0.23 ± 0.02	0.34 ± 0.08	0.11 ± 0.03	0.02 ± 0.00
GD15-21SD	0.50 ± 0.01	0.19 ± 0.04	0.46 ± 0.03	0.17 ± 0.01	0.01 ± 0.00

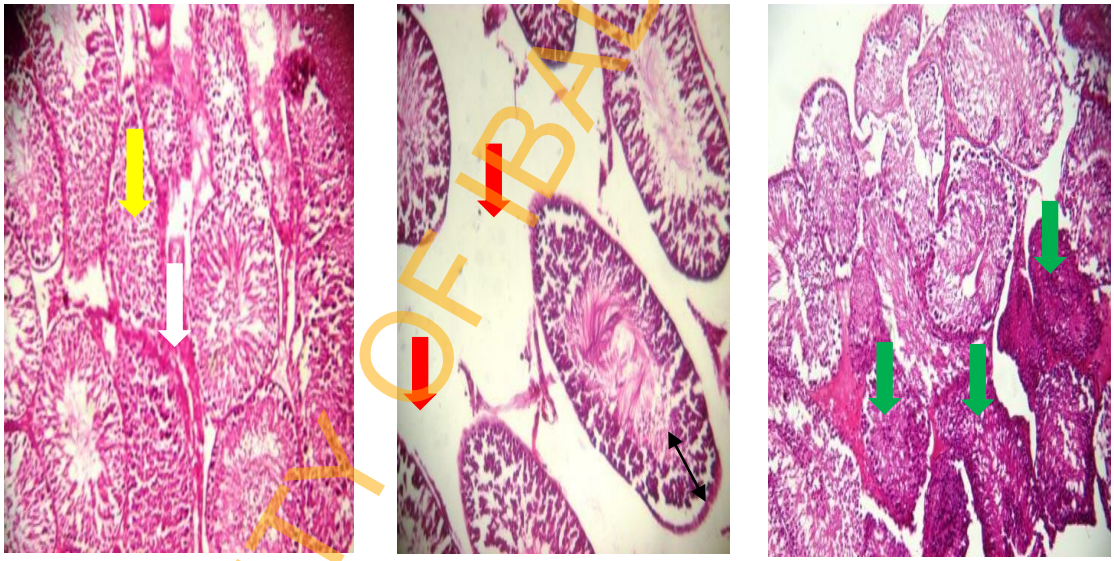
Data are presented as mean ± SEM. n = 5. Analysis was based on Student's t-test.
GD = Gestation Day.

4.5.4. Effects of maternal sleep deprivation on histology of the testis of male offsprings

The male offsprings from all control dams (GD1-7C, GD8-14C and GD15-21C) showed testicular tissue architecture with normal seminiferous tubules containing normal maturing germinal cells layer. GD1-7SD testis shows few abnormal seminiferous tubules with loss of basal membrane and germ cell layer. GD8-14SD testes had wide interstitial spaces. GD15-21SD testis appeared abnormal with very poor testicular architecture and anomalous seminiferous tubules lacking in germ cell layers and lumen (Plate 4.6).



GD1-7C GD8-14C GD15 -21C

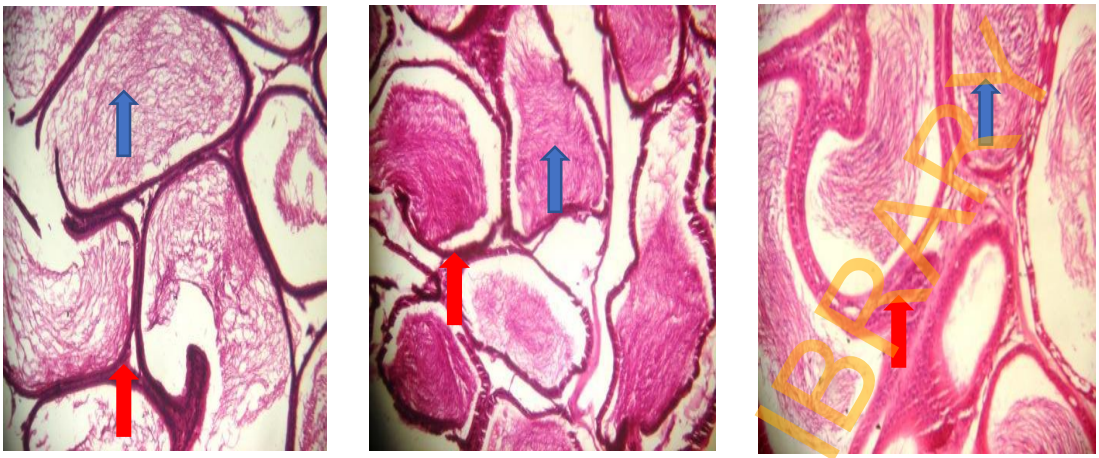


GD1-7SD GD8-14SD GD15-21SD

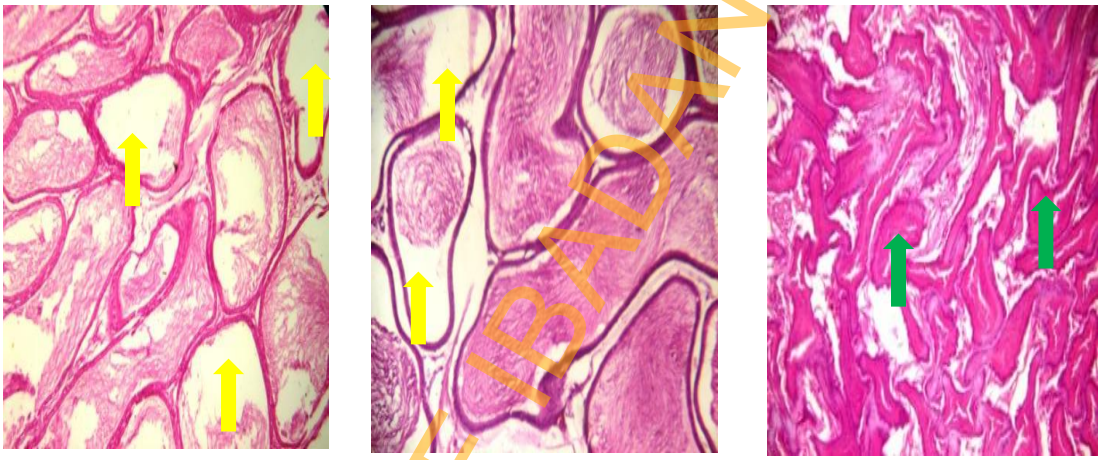
Plate 4.6. Photomicrographs of testicular sections from male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Tissues were stained with H&E and presented at x100 magnification. a) GD1-7C, GD8-14C and GD15-21C show normal seminiferous tubules (blue arrow) containing maturing germ cell layer (black arrow). GD1-7SD testicular section shows seminiferous tubules with loss of basal membrane, germ cell layers and lumen (yellow arrow) and crowded interstitial spaces (white arrow). GD8-14SD shows wide interstitial spaces (red arrow). GD15-21SD testicular section shows poor testicular architecture with abnormal seminiferous tubules lacking in germ cell layers and lumen (green arrow).

4.5.5. Effects of maternal sleep deprivation on histology of the epididymis of male offsprings

The male offsprings of control dams from GD1-7, GD8-14 and GD15-21 had normal epididymal tissue architecture. GD1-7SD group epididymis had some ducts lacking spermatozoa storage. The epididymis of GD8-14SD group shows thickened smooth muscle layer and epithelial layers. The epididymis of GD15-21SD group had atrophic ducts with severe thickening of the smooth muscle layer of the ductus epididymis that are collapsed. The ductus epididymis lacked sperm and appeared empty (Plate 4.7).



GD1-7C GD8-14C GD15 -21C



GD1-7SD GD8-14SD GD15-21SD

Plate 4.7. Photomicrographs of epididymal sections from male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Tissues were stained with H&E and presented at x100 magnification. a) GD1-7C, GD8-14C, GD15-21C and GD8-14SD show normal smooth muscle and epithelial layers (red arrow) and lumen containing spermatozoa (blue arrow). Some ducts lack spermatozoa (yellow arrow) in GD1-7SD and GD8-14SD. GD15-21SD shows empty and atrophic ducts with severe thickening of the smooth muscle layer that are collapsed (green arrow).

4.5.6. Effects of maternal sleep deprivation on histology of the prostate gland of male offsprings

Maternal sleep deprivation did not have any adverse effect on prostate gland architecture of offspring. Sections from GD1-7C, GD1-7SD, GD8-14C, GD8-14SD, GD15-21C and GD15-21SD show normal prostate glands containing secretions and corpora amylacea. The glands are lined by normal tall columnar secretory cells and normal stromal smooth muscle fibres are seen (Plate 4.8).

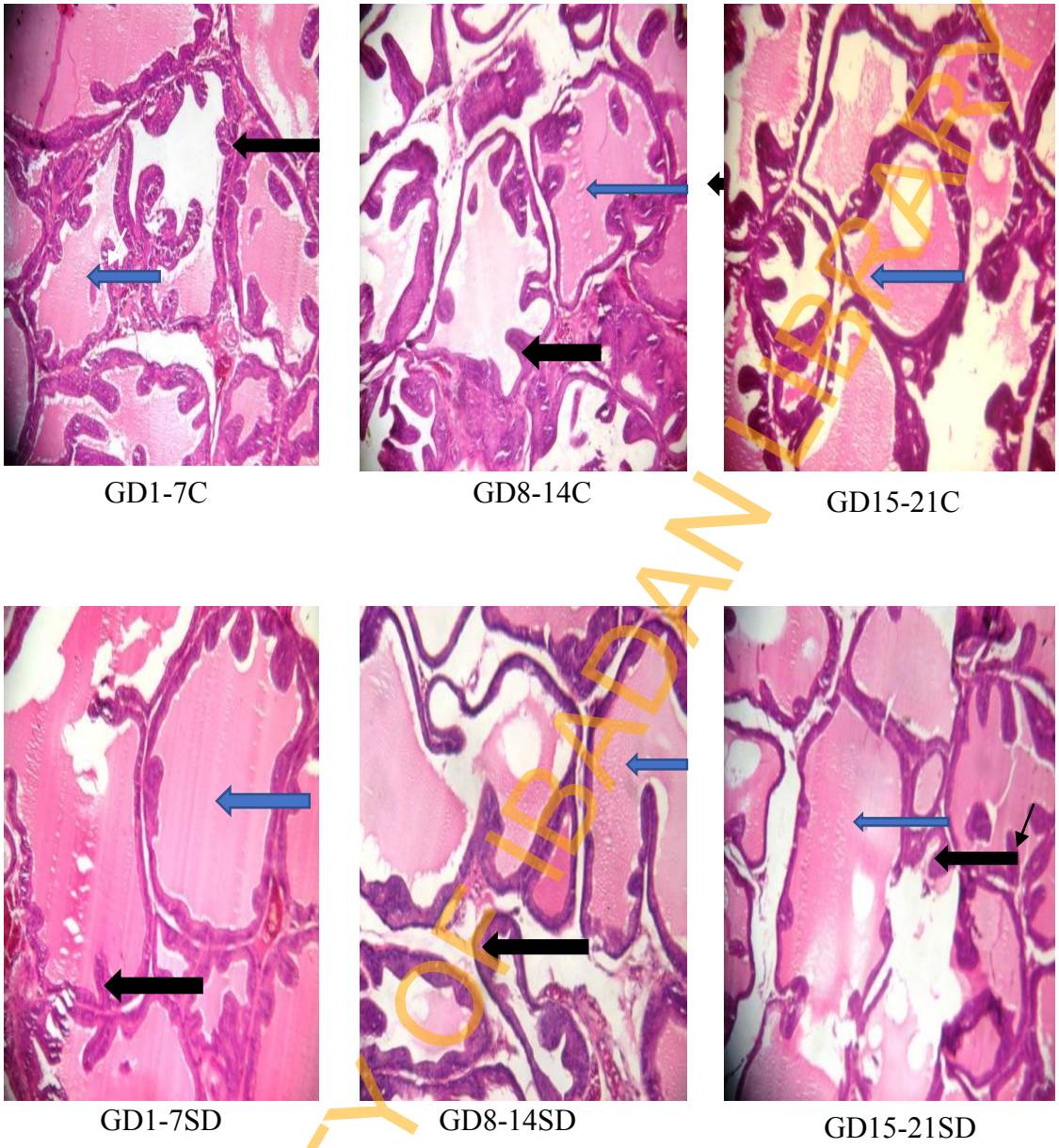
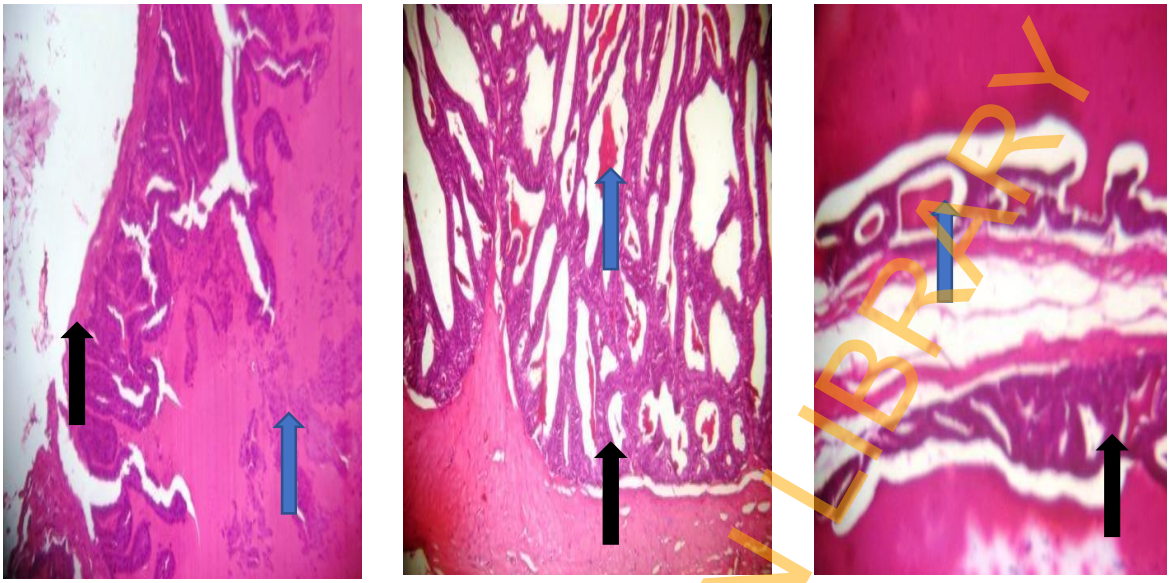


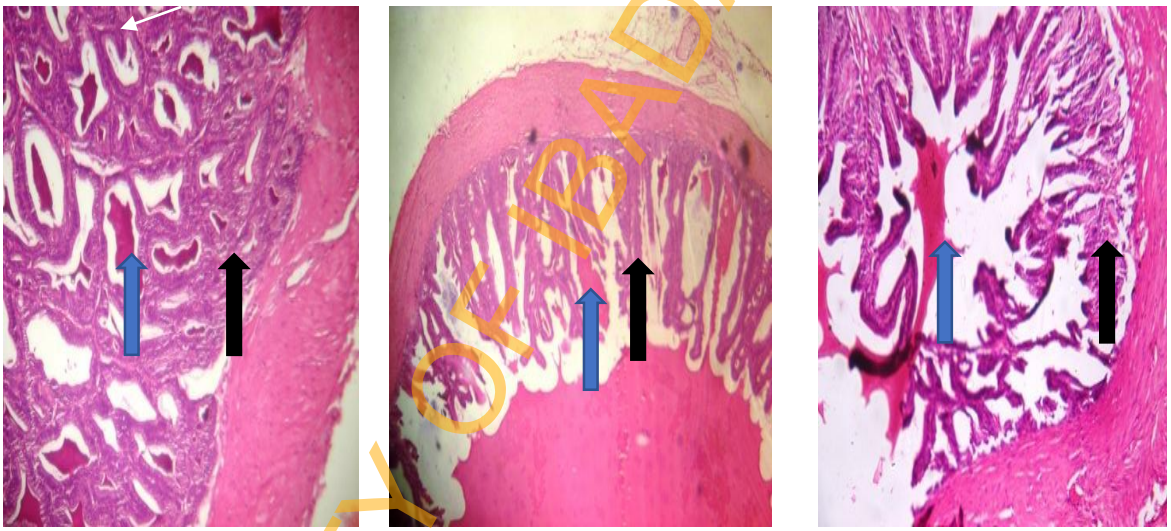
Plate 4.8. Photomicrographs of prostate sections from male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Tissues were stained with H&E and presented at x100 magnification. a) GD1-7C, GD1-7SD, GD8-14C, GD8-14SD, GD15-21C and GD15-21SD show normal prostate glands containing secretions and corpora amylacea (blue arrow). The glands are lined by normal tall columnar secretory cells (black arrow).

4.5.7. Effects of maternal sleep deprivation on histology of seminal vesicle in male offsprings

Sections from GD1-7C, GD1-7SD, GD8-14C, GD8-14SD, and GD15-21C groups show normal architecture of the seminal vesicle. However, maternal sleep deprivation during GD15-21 adversely affected the structure of the seminal vesicles of male offsprings. Photomicrograph shows severely thickened internal and external muscle layers in this group (Plate 4.9).



GD1-7C GD8-14C GD15-21C



GD1-7SD GD8-14SD GD15-1SD

Plate 4.9. Photomicrographs of seminal vesicle sections from male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Tissues were stained with H&E and presented at x100 magnification. a) GD1-7C, GD1-7SD, GD8-14C, GD8-14SD, GD15-21C and GD15-21SD show normal smooth muscle layer (black arrow) and eosinophilic secretion (blue arrow).

4.5.8. Effects of maternal sleep deprivation on fertility of male offsprings of Wistar rats.

Four out of five male offsprings of GD1-7C, GD1-7SD, GD8-14SD and GD15-21C groups mated successfully with the female rats. GD8-14C had 100% fertility and gestational indices while none of the male offsprings of GD15-21SD dams mated successfully with the female rats (Table 4.4).

Table 4.4. Fertility indices of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams

GROUPS	FERTILITY INDEX (%)	GESTATIONAL INDEX (%)
GD1-7C	80	80
GD1-7SD	80	80
GD8-14C	100	100
GD8-14SD	80	80
GD15-21C	80	80
GD15-21SD	0	0

Data are presented as percentage. GD = Gestation Day

4.6. Effects of maternal sleep deprivation during gestation on serum hormone concentration of male offsprings

4.6.1. Effects of maternal sleep deprivation on serum corticosterone concentration of male offsprings

Serum corticosterone level was significantly increased in all male offsprings of sleep deprived dams when compared with their respective control groups (Figure 4.20).

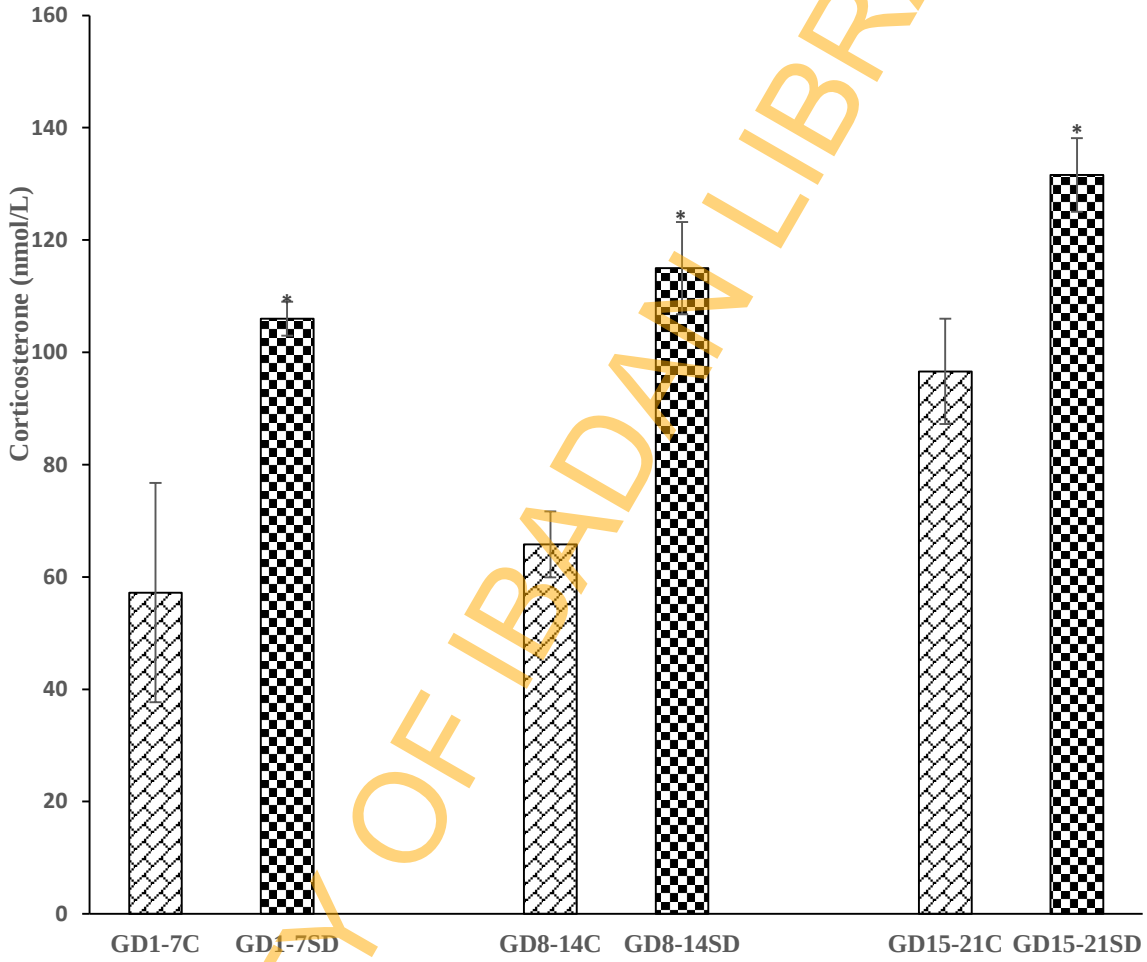


Figure 4.20. Serum corticosterone concentration of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *$p<0.05$ when compared with the corresponding control groups based on Student's t-test. GD = Gestation Day.

4.6.2. Effects of maternal sleep deprivation on serum Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) concentrations of male offsprings

There were no significant differences in levels of follicle stimulating hormone and luteinizing hormone across the groups (Figure 4.21).

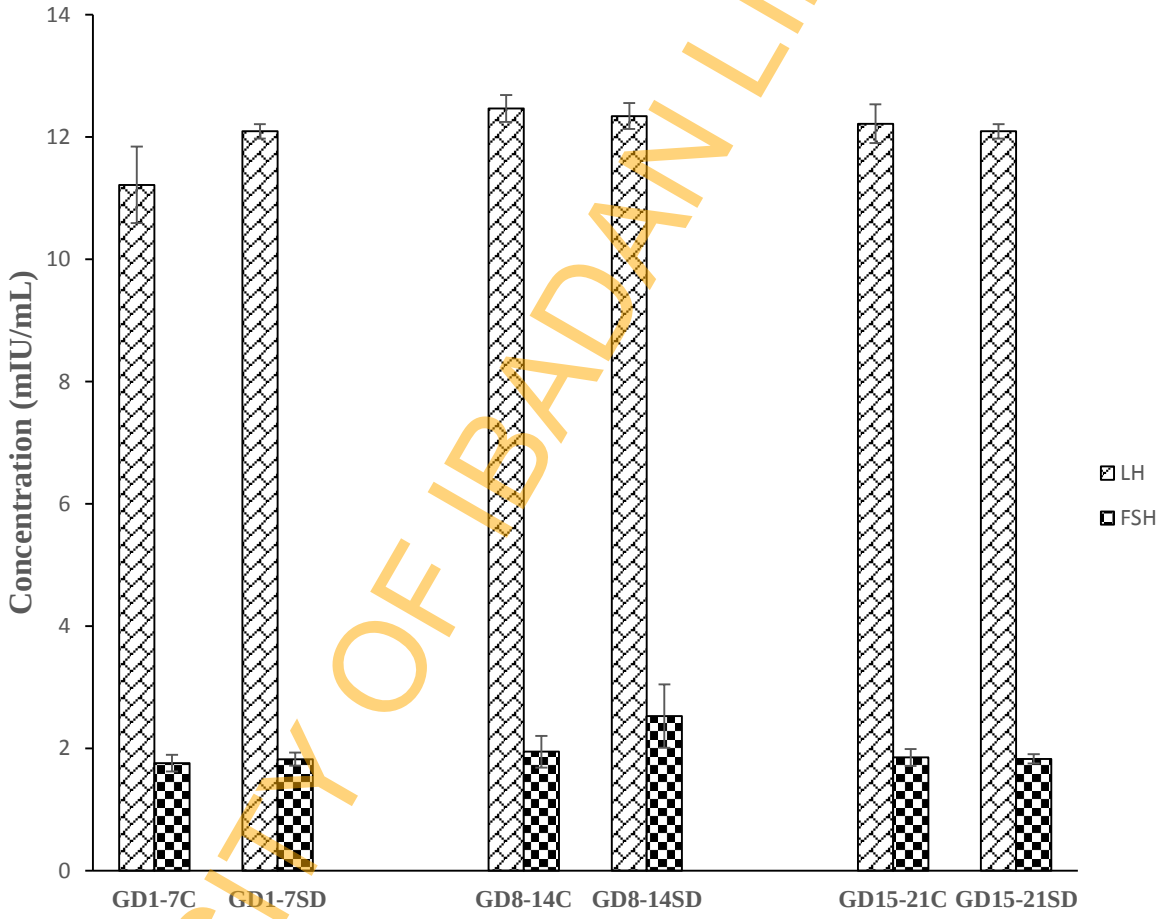


Figure 4.21. Serum Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) concentrations of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.6.3. Effects of maternal sleep deprivation on serum testosterone concentration of male offsprings

Maternal sleep deprivation caused significant reduction ($p<0.05$) in serum testosterone concentration in male offsprings of GD15-21SD dams when compared with the male offsprings of their corresponding control groups (Figure 4.22).

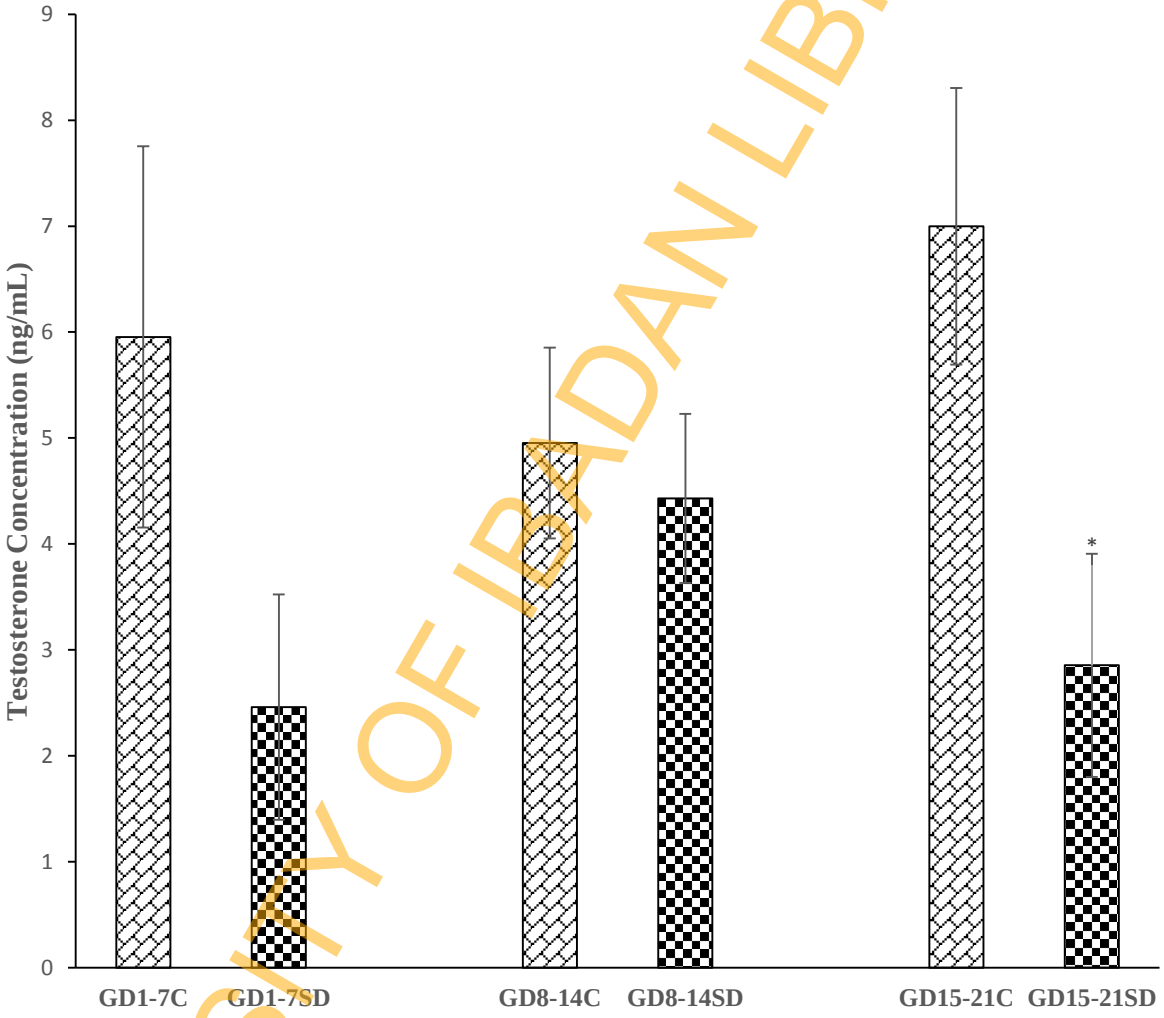


Figure 4.22. Serum testosterone concentration of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.6.4. Effects of maternal sleep deprivation on serum melatonin concentration of male offsprings

Maternal sleep deprivation caused significant increase in serum melatonin level of adult male offsprings of GD1-7SD dams and significant decrease in serum melatonin level of offsprings of GD8-14SD dams when compared with their respective control groups (Figure 4.23).

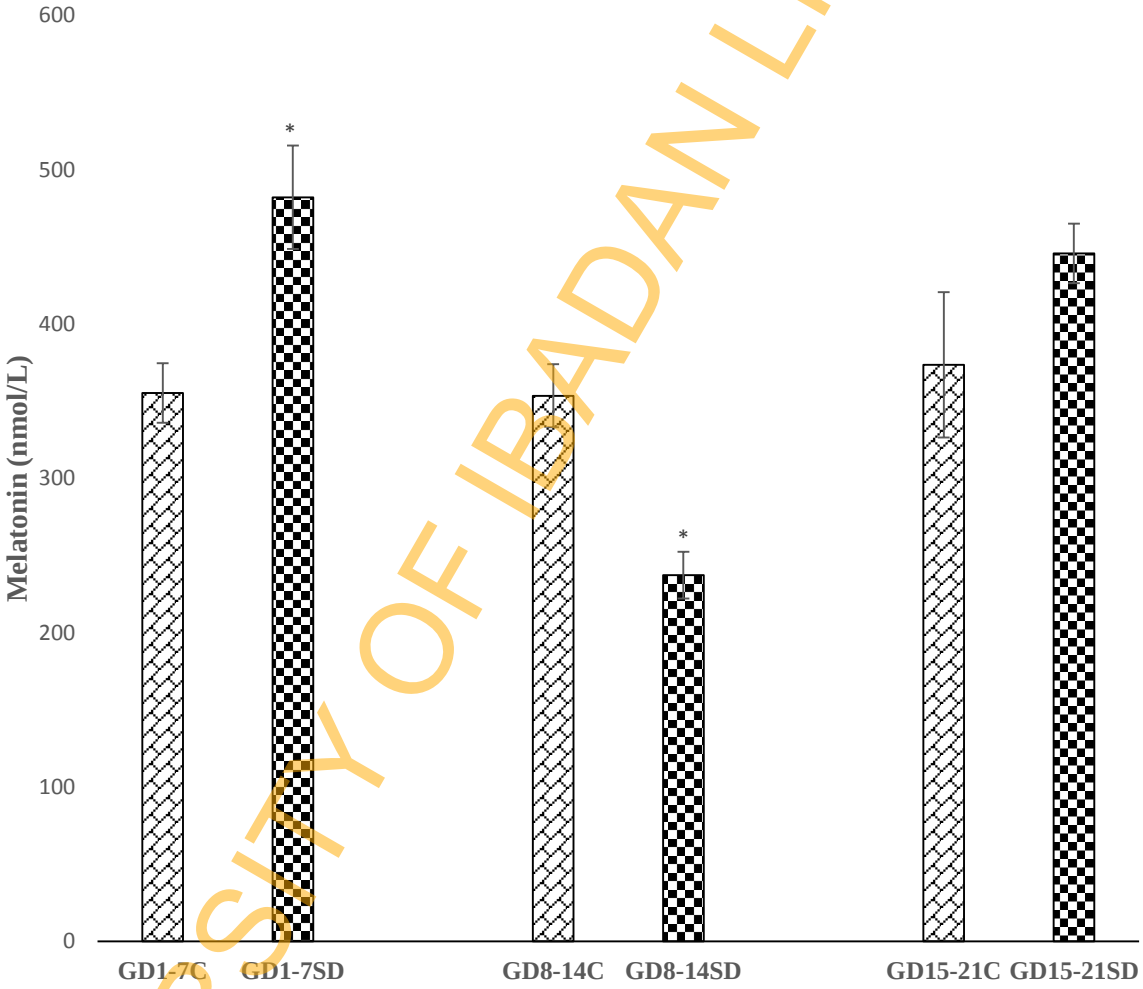


Figure 4.23. Serum melatonin concentration of adult male offsprings of Control (C) and Sleep Deprived (SD) Pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.7. Effects of sleep deprivation on hormone concentration in pregnant Wistar rat dams

4.7.1. Effects of sleep deprivation on serum progesterone concentration of pregnant Wistar rat dams

Maternal sleep deprivation did not have any significant effect on the serum progesterone level of sleep deprived pregnant rats (Figure 4.24).

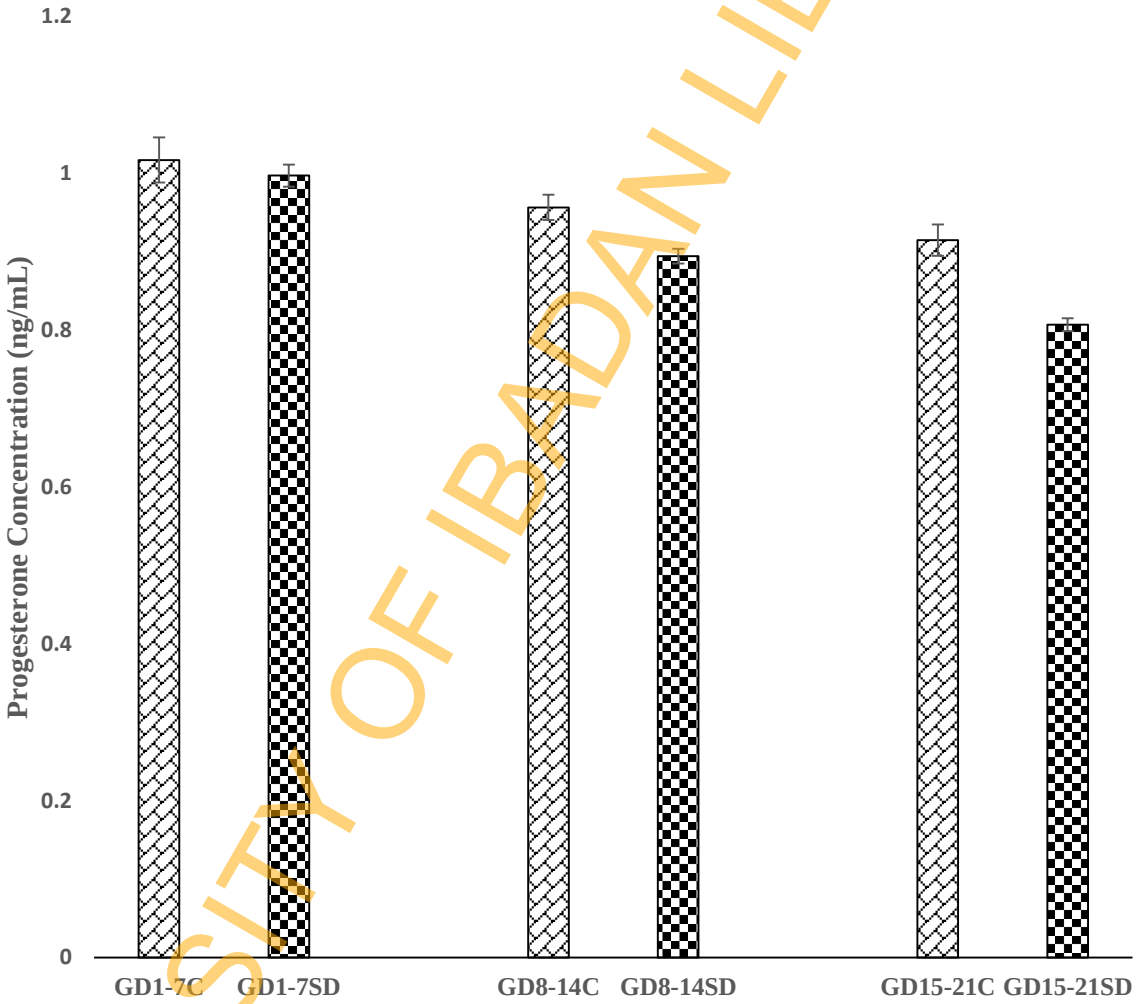


Figure 4.24. Serum progesterone level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.7.2. Effects of sleep deprivation on serum prolactin concentration of pregnant Wistar rat dams

Sleep deprivation during gestation did not significantly affect the serum concentration of prolactin in the pregnant rats (Figure 4.25).

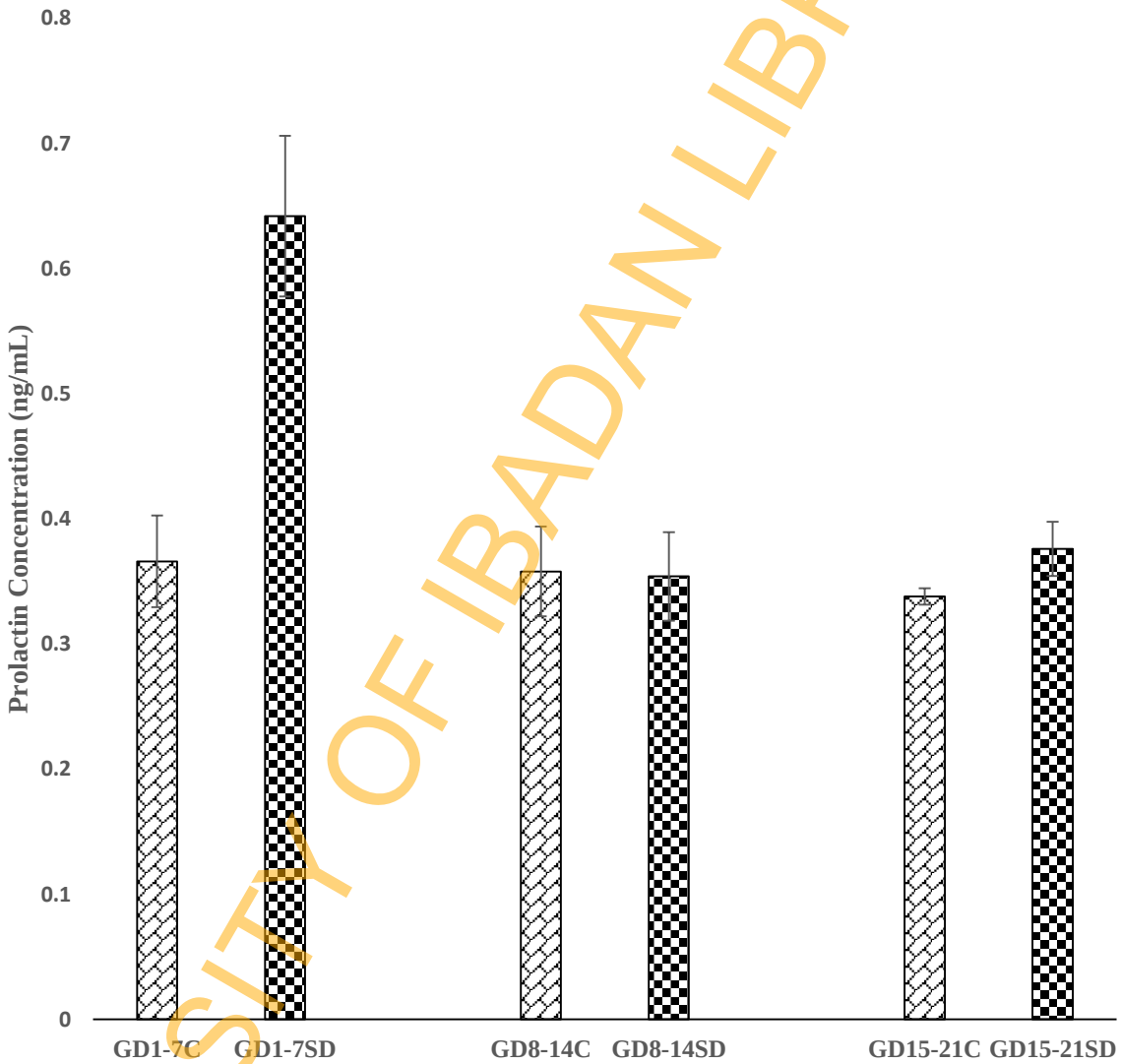


Figure 4.25. Serum prolactin level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.7.3. Effects of sleep deprivation on serum testosterone concentration of pregnant Wistar rat dams

Maternal sleep deprivation caused a decrease ($p<0.05$) in serum testosterone level of pregnant dams subjected to sleep deprivation on gestation days 15-21 when compared with their respective group (Figure 4.26).

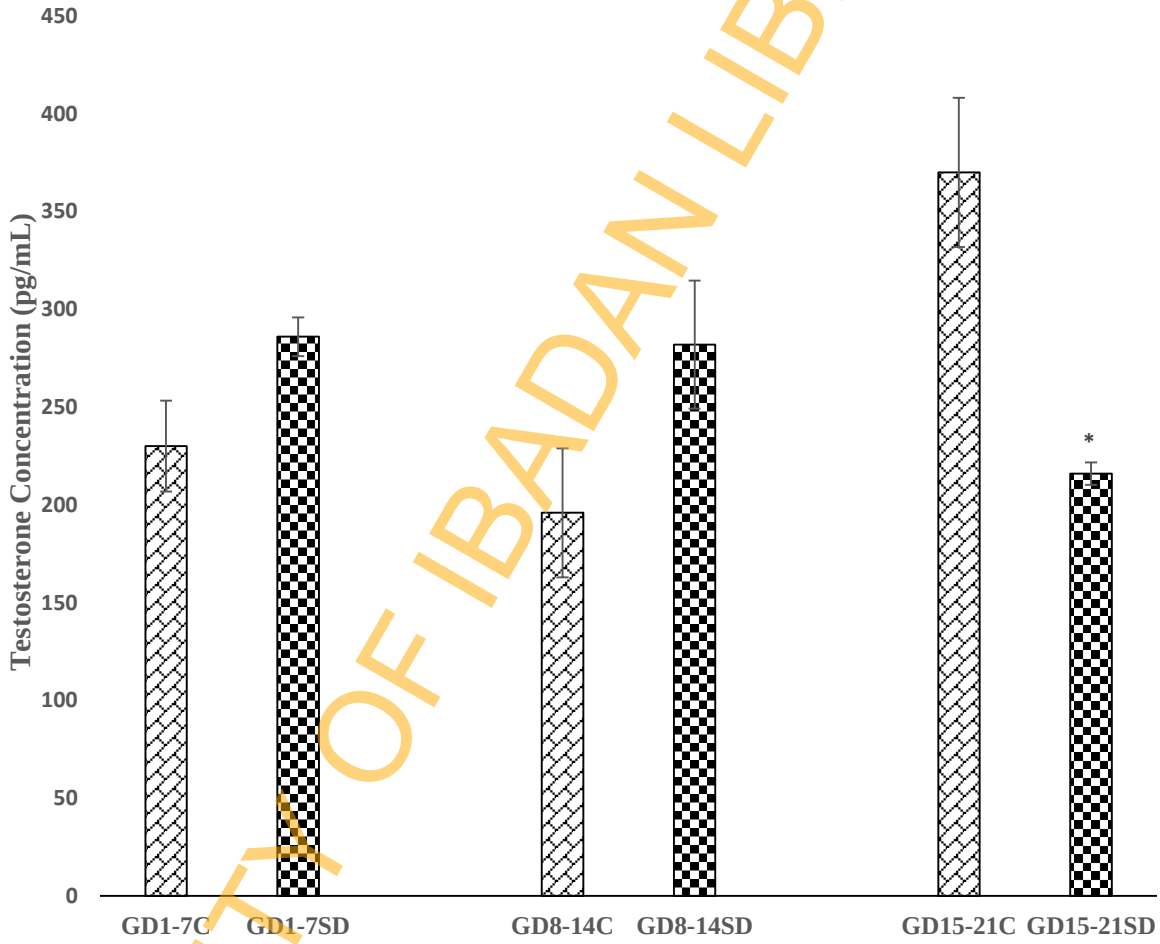


Figure 4.26. Serum testosterone level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.7.4. Effects of sleep deprivation on corticosterone concentration of pregnant Wistar rat dams

Maternal sleep deprivation did not affect serum corticosterone level of all the pregnant dams in all groups (Figure 4.27).

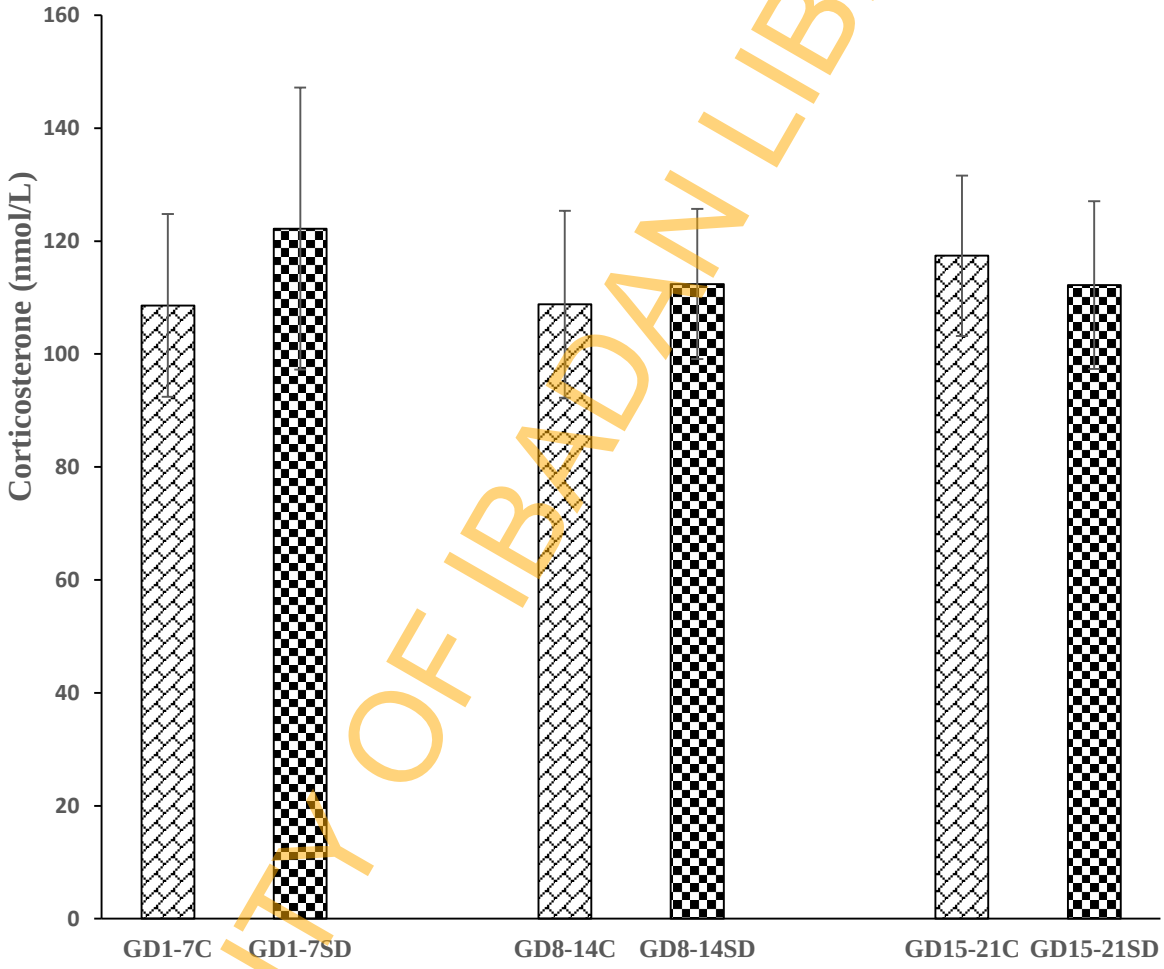


Figure 4.27. Serum corticosterone level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.7.5 Effects of sleep deprivation on serum melatonin concentration of pregnant Wistar rat dams

Maternal sleep deprivation during pregnancy significantly caused decrease ($p<0.05$) of serum melatonin level in the GD1-7SD dams while serum melatonin level of GD8-14SD group increased significantly (Figure 4.28).

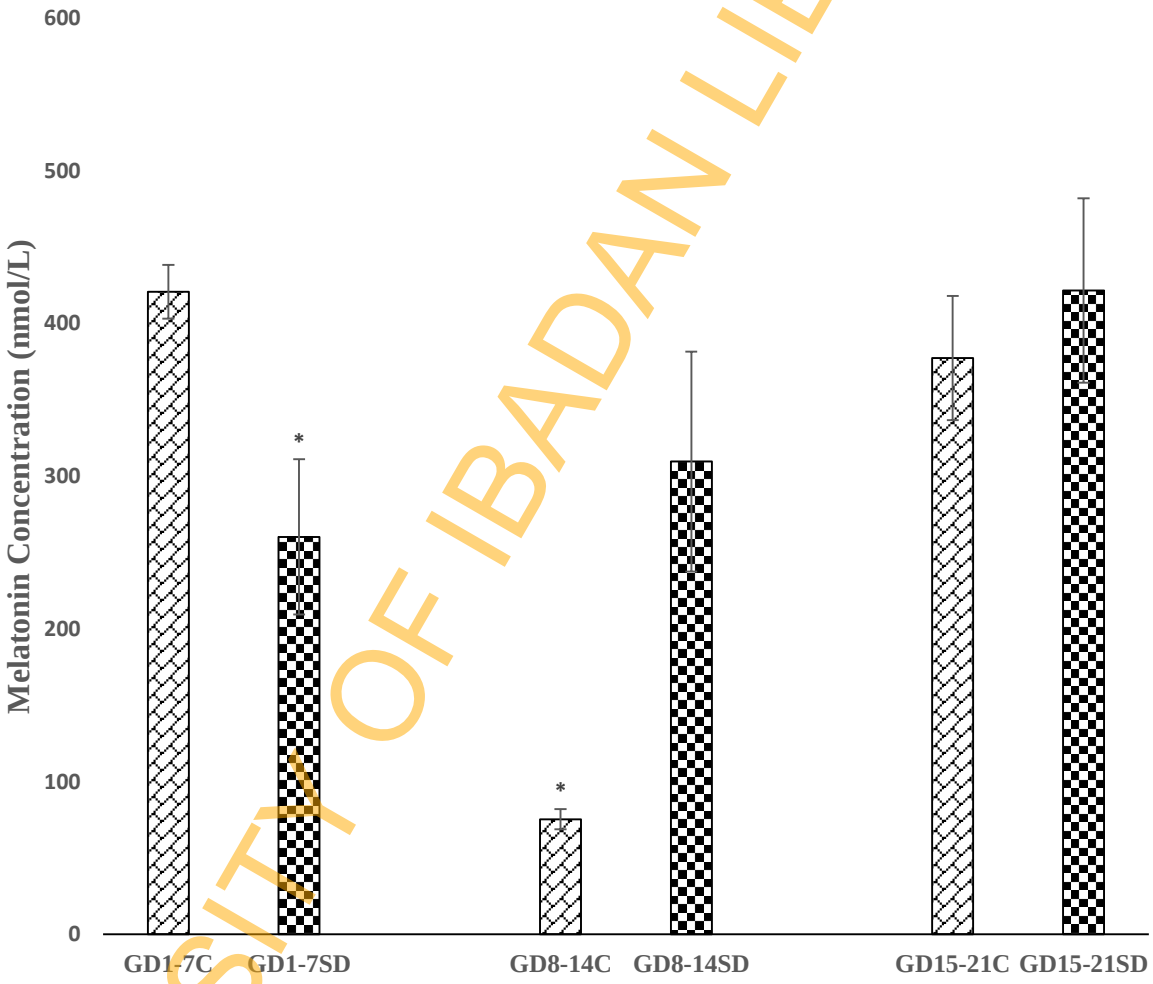


Figure 4.28. Serum melatonin level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.8. Effects of maternal sleep deprivation on foetal and placental morphometric indices in pregnant Wistar rat dams

GD8-14SD group placenta had reduced thickness and increased volume ($p<0.05$) while the foeto-placental ratio was comparable with that of the control group. GD15-2SD pup weight at Caesarean section was significantly lower ($p<0.05$) compared with the respective control group. Similarly, the foeto-placental ratio was reduced ($P<0.05$) in the same group when compared with the GD15-21C group (Table 4.5).

Table 4.5. Foetal and placental morphometric indices in Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams

GROUPS	L S	FW (g)	PW (g)	PT (mm)	PV (mm^3)	PCSA (mm^2)	PC	FPR
GD1-7C	8±1	-	-	-	-	-	-	-
GD1-7 SD	8±1	-	-	-	-	-	-	-
GD8-14C	7±1	0.23 ± 0.02	0.26 ± 0.03	2.91 ± 1.89	0.23 ± 0.00	0.01± 0.00	1.20 ± 0.23	0.95 ± 0.08
GD8-14SD	8±1	0.23 ± 0.02	0.23 ± 0.02	1.89 ± 0.21*	0.26 ± 0.01*	0.01 ± 0.00	1.03 ± 0.07	1.00 ± 0.22
GD15-21C	8±1	4.56 ± 0.22	0.52 ± 0.03	2.99 ± 0.18	0.56 ± 0.04	1.36 ± 0.28	0.12 ± 0.01	8.87 ± 0.86
GD15-21SD	8±1	3.44 ± 0.13**	0.53 ± 0.01	3.20 ± 0.16	0.53 ± 0.04	1.57 ± 0.06	0.15 ± 0.01*	6.55 ± 0.20*

Data are presented as mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day. C=Control. SD=Sleep-Deprived. LS=Litter Size. FW= Foetal Weight. PW= Placental Weight. PT= Placental Thickness. PV= Placental Volume. PCSA= Placental Chorionic Surface Area. PC= Placental Coefficient. FPR= Foeto-placental Ratio

4.9. Effects of maternal sleep deprivation on placental oxidative stress in pregnant Wistar rat dams

4.9.1. Effects of maternal sleep deprivation on placental malondialdehyde level

Placental malondialdehyde level was increased ($p<0.05$) in GD1-7SD and GD15-21SD groups when compared with GD1-7C and GD15-21C respectively (Figure 4.29).

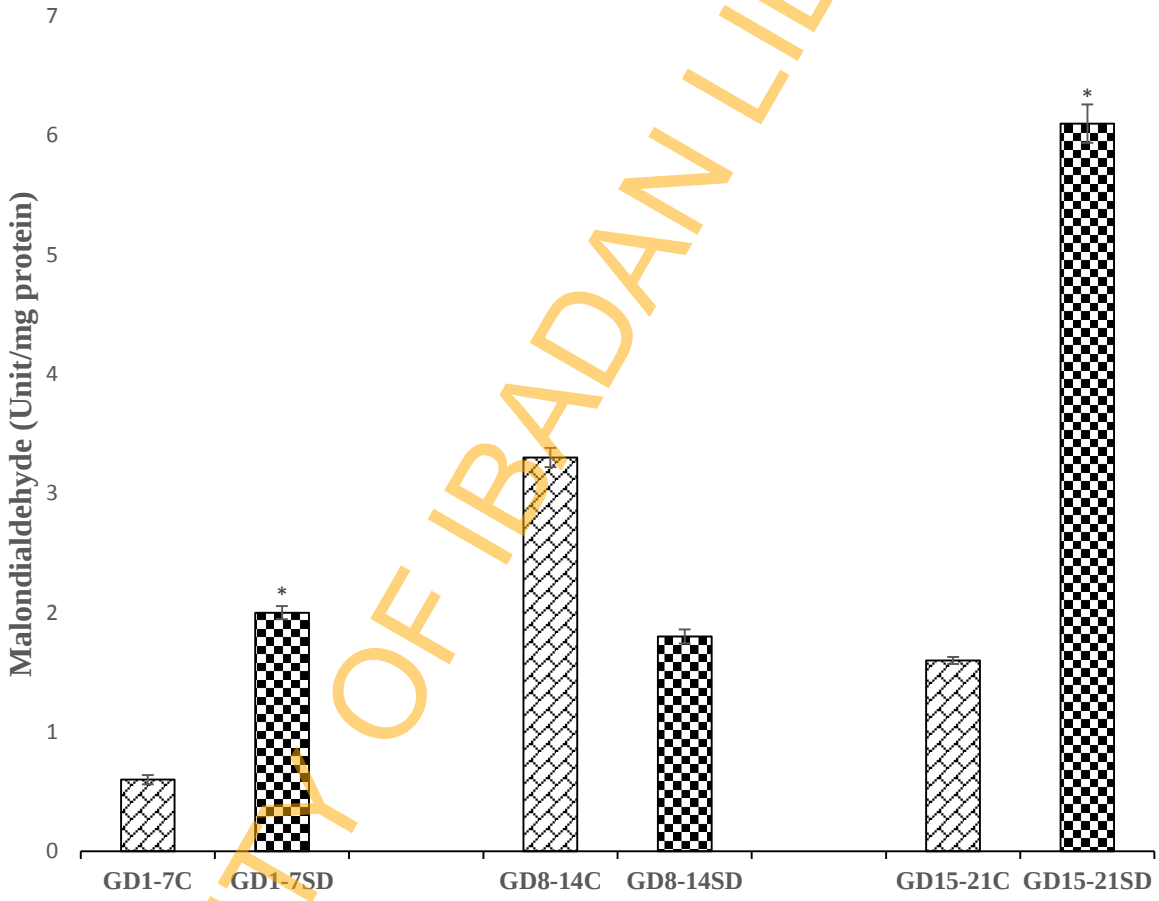


Figure 4.29. Placental malondialdehyde level of Control (C) and Sleep-Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.9.2. Effects of maternal sleep deprivation on placental hydrogen peroxide level

Hydrogen peroxide was significantly increased ($p<0.05$) in the placenta of pregnant dams that were sleep deprived during gestation days 15-21 when compared with the control group (Figure 4.30).

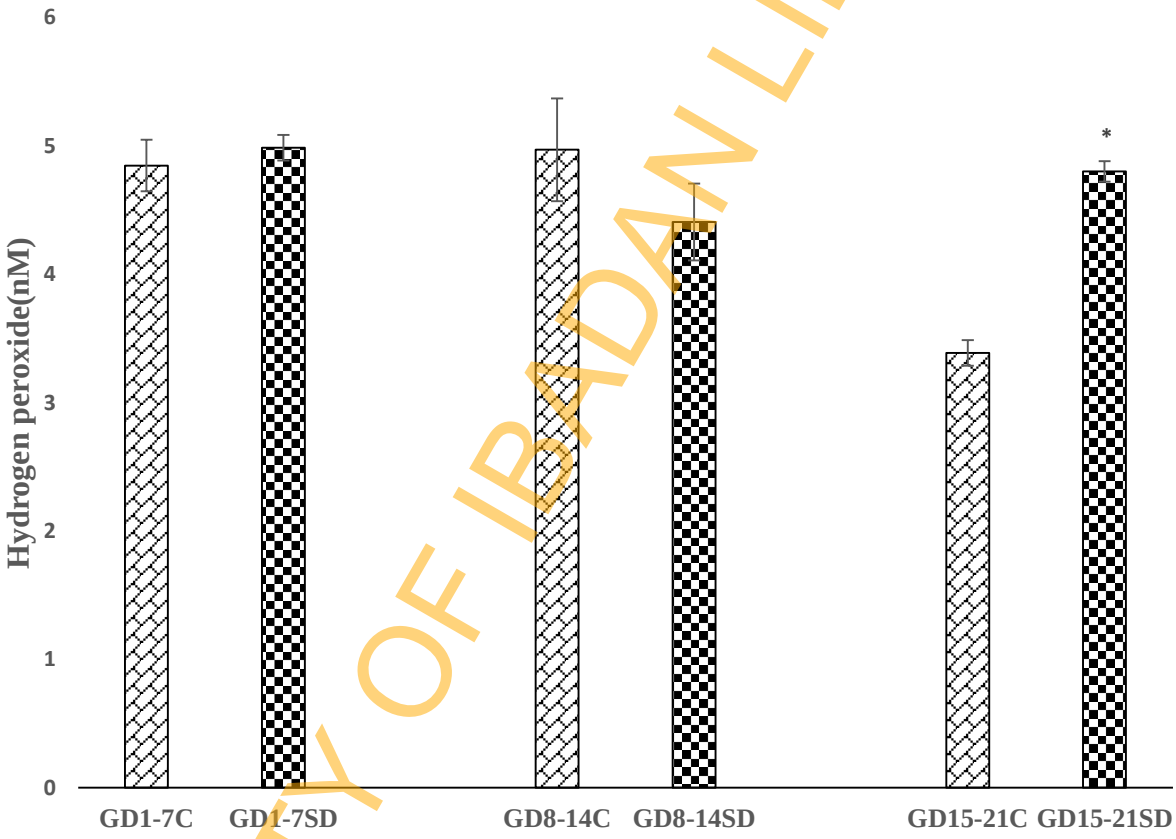


Figure 4.30. Placental hydrogen peroxide (H_2O_2) level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.9.3. Effects of sleep deprivation on placental dichlorofluorescein (DCF) level

Dichlorofluorescein was significantly increased ($p<0.05$) in the placenta of pregnant dams that were sleep deprived during gestation days 15-21 when compared with the control group (Figure 4.31).

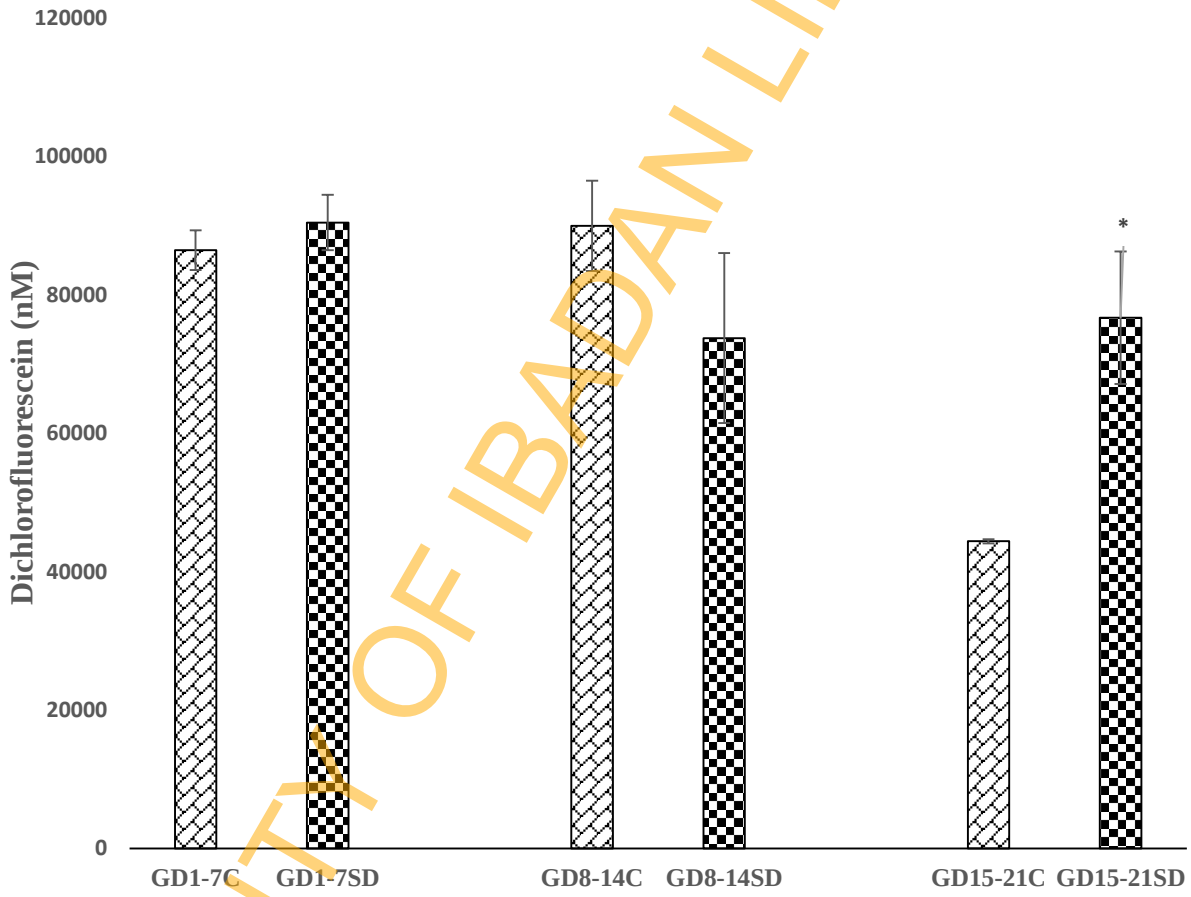


Figure 4.31. Placental Dichlorofluorescein (DCF) level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.9.4. Effects of sleep deprivation on placental total antioxidant capacity

Placental total antioxidant capacity was reduced in the GD15-21SD group compared with GD15-21C group (Figure 4.32). The total antioxidant capacity in the placentas of GD1-7SD and GD8-14SD groups were higher but the difference in means when compared with their control groups was not significant (Figure 4.32).

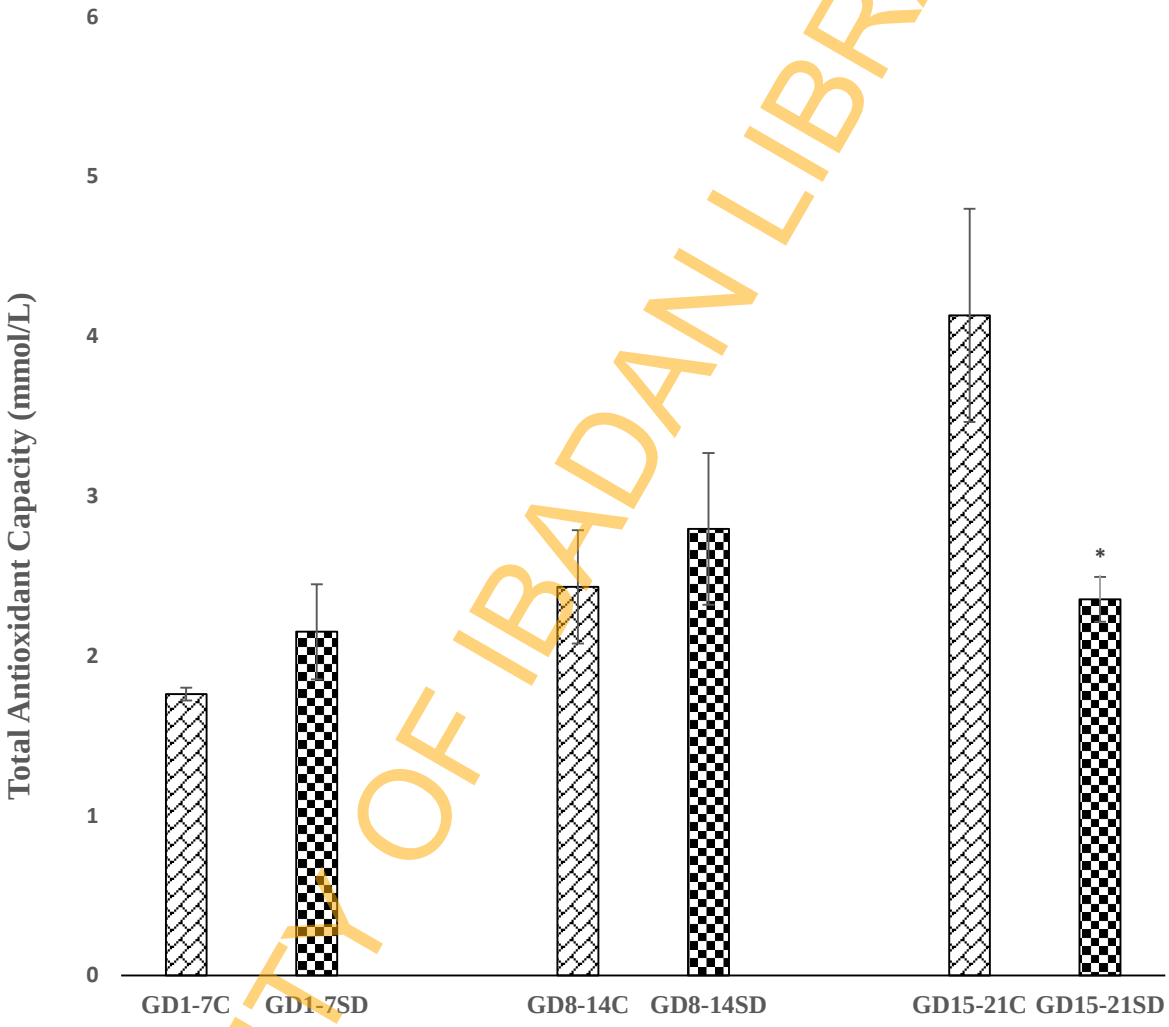


Figure 4.32. Placental total antioxidant capacity of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.9.5. Effects of sleep deprivation on placental glutathione level

Reduced glutathione level was higher in the placentas all of the sleep deprived dams (Figure 4.33). However, the increase was only significant ($p<0.05$) in the placentas of GD8-14SD and GD15-21SD groups compared with their respective control groups (Figure 4.33).

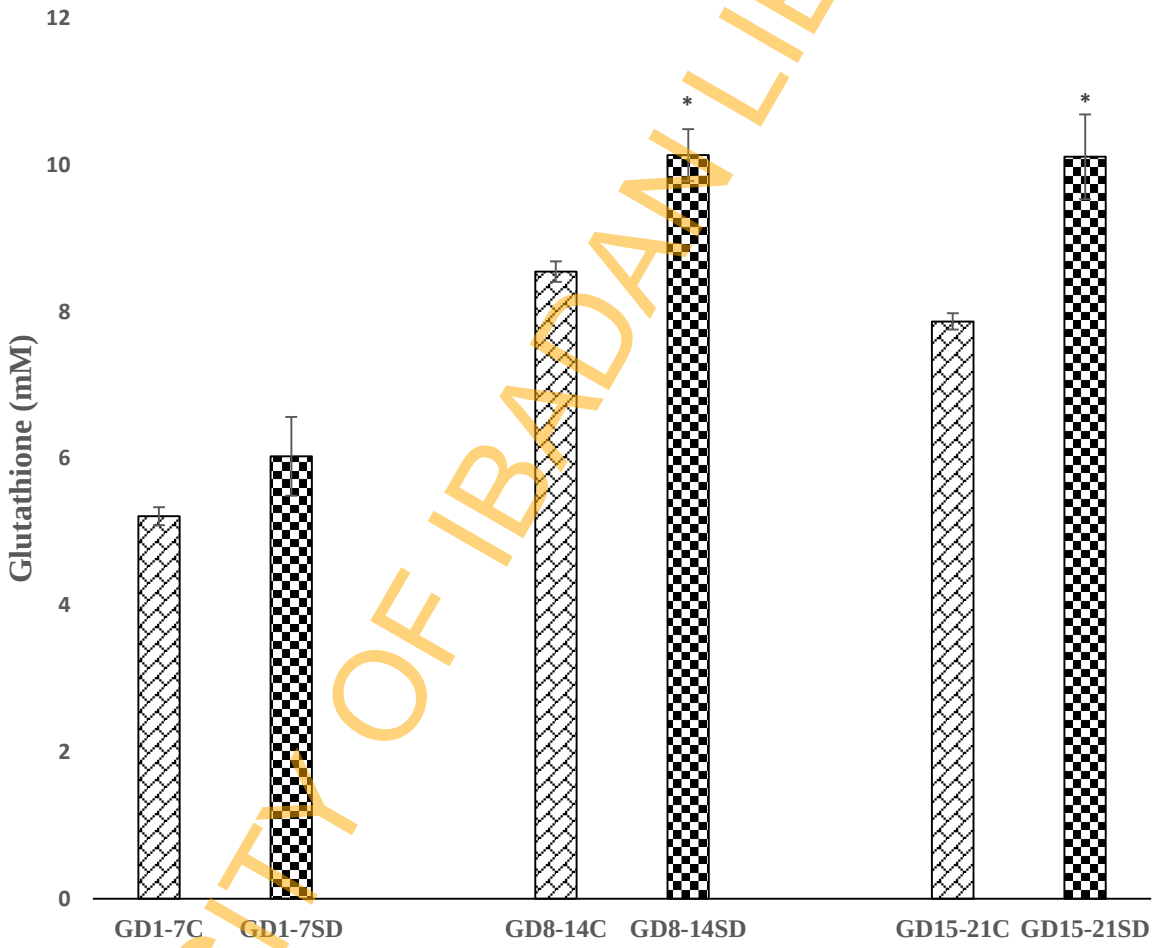


Figure 4.33. Placental glutathione of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.9.6. Effects of sleep deprivation on placental superoxide dismutase activity

Superoxide dismutase level was reduced in all placentas of sleep deprived groups. However, the reduction was only significant ($p<0.05$) in GD15-21SD group when compared with its control group (Figure 4.34).

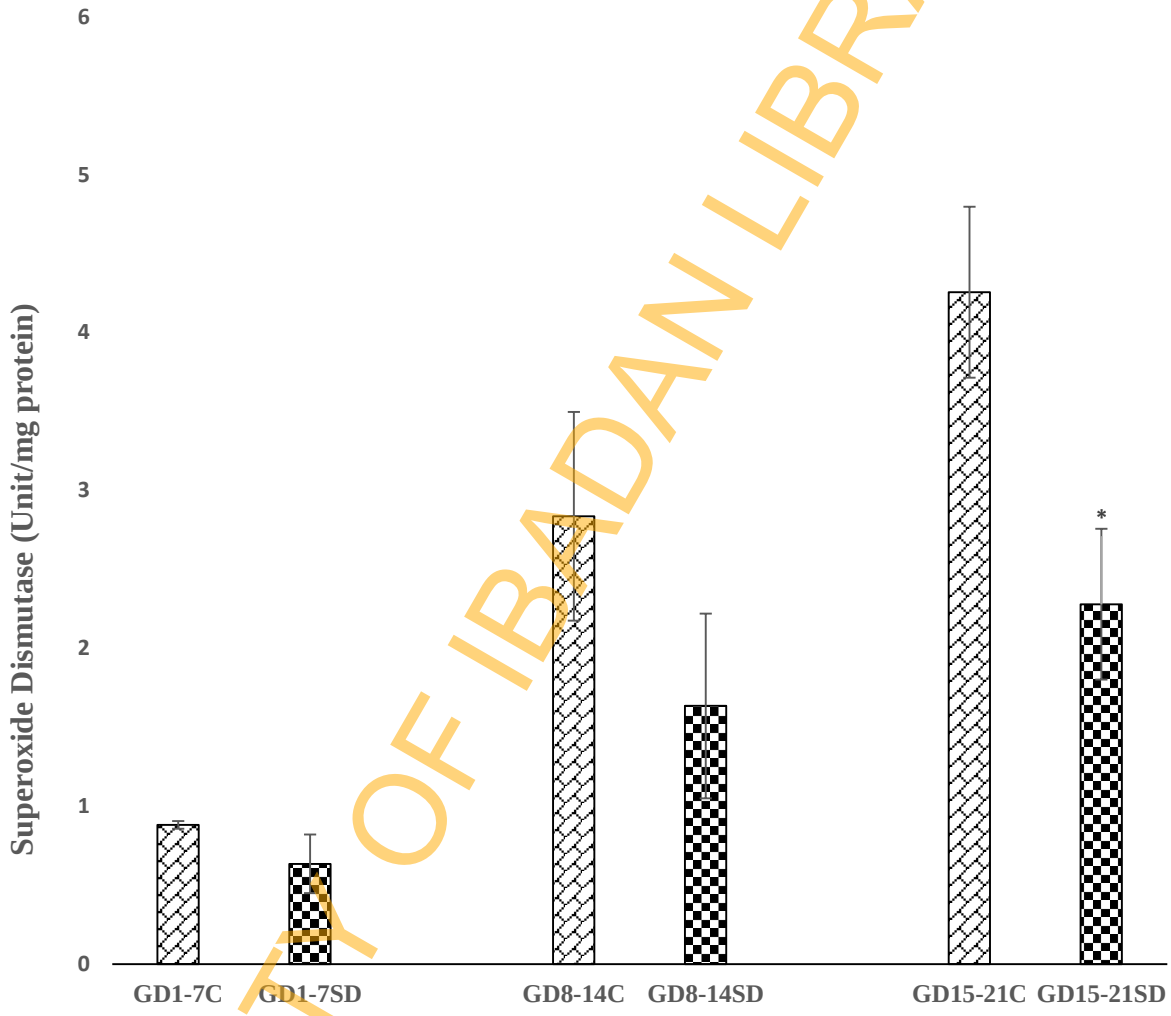


Figure 4.34. Placental superoxide dismutase activity of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.9.7. Effects of sleep deprivation on placental catalase activity

Maternal sleep deprivation did not affect placental catalase activity in all groups (Figure 4.35).

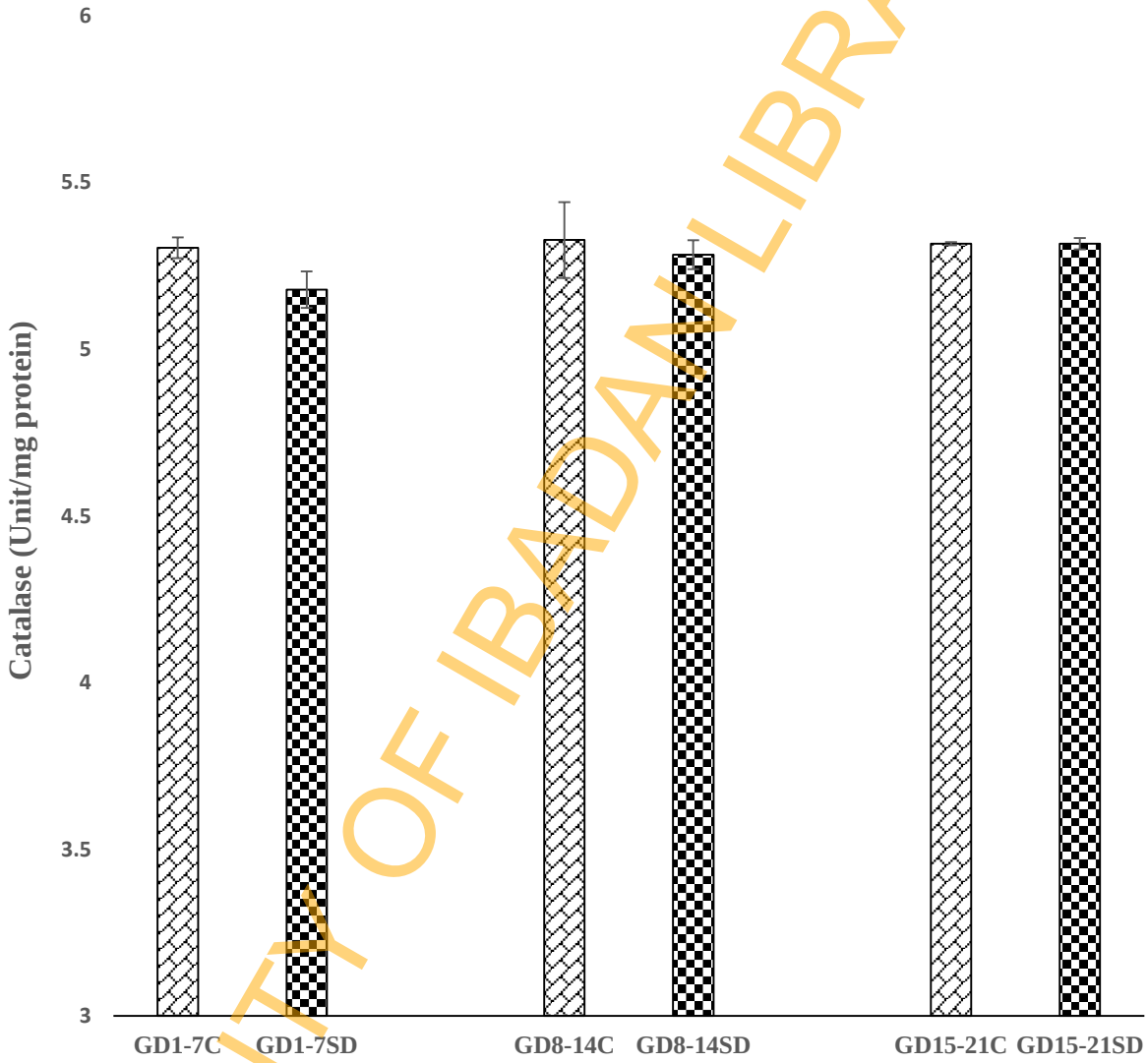


Figure 4.35. Placental catalase activity of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.9.8. Effects of sleep deprivation on placental glutathione peroxidase activity

Glutathione peroxidase activity was significantly increased only in the placenta of GD15-21SD group and reduced in the placenta of GD1-7SD, when compared with their respective control groups (Figure 4.36).

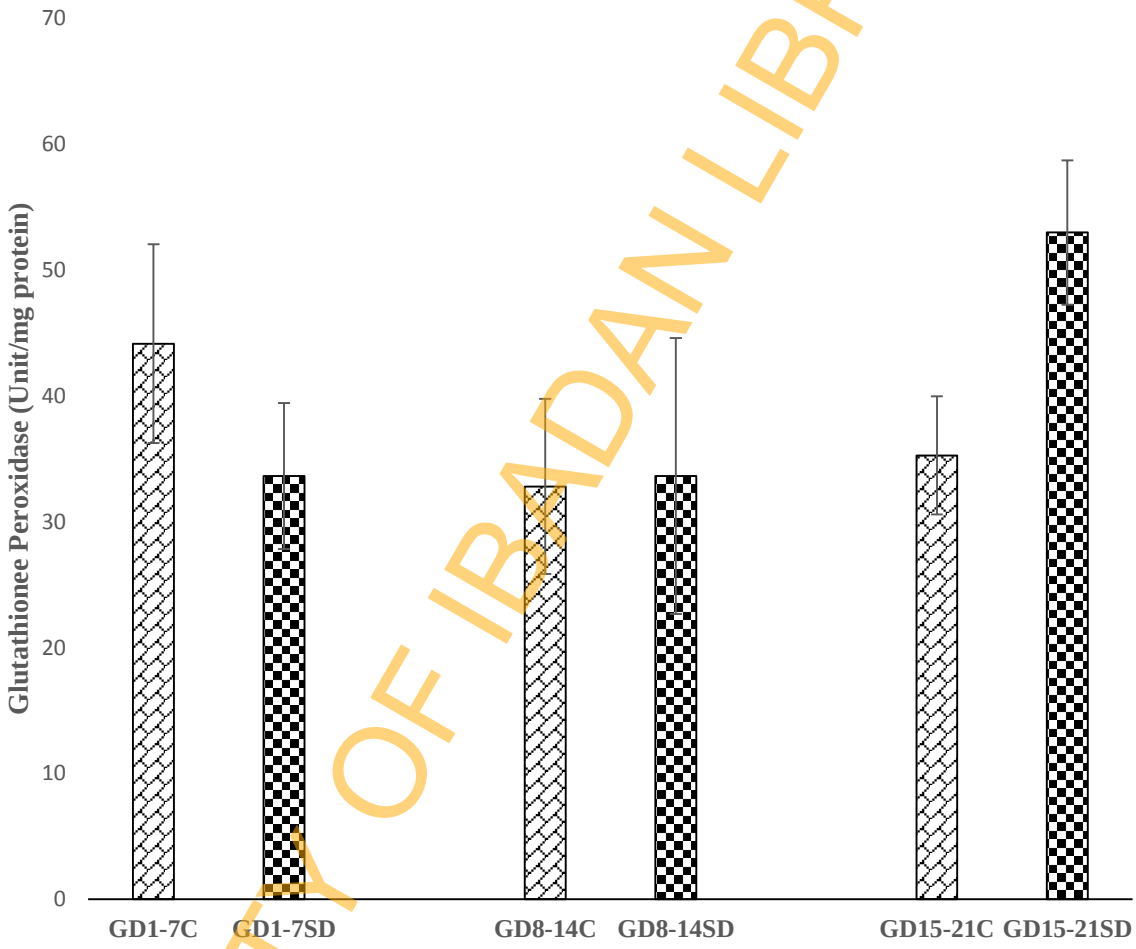


Figure 4.36. Placental glutathione peroxidase activity of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.10. Effects of maternal sleep deprivation on placental nitrotyrosine level in pregnant Wistar rat dams

Maternal sleep deprivation significantly increased ($p<0.05$) placental level of nitrotyrosine in the GD15-21SD group compared with the control group (Figure 4.37).

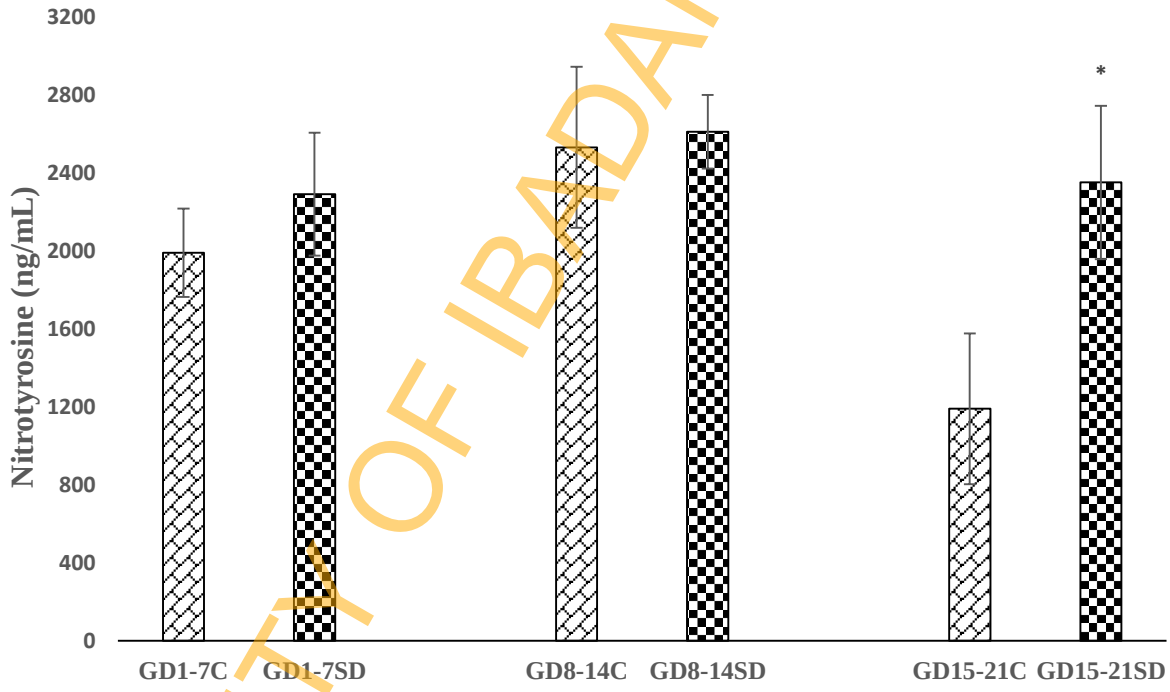


Figure 4.37. Placental nitrotyrosine level of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.11. Effects of maternal sleep deprivation on placental total protein in pregnant Wistar rat dams

The total protein content of each placenta did not show any significant difference across all the groups (Figure 4.38).

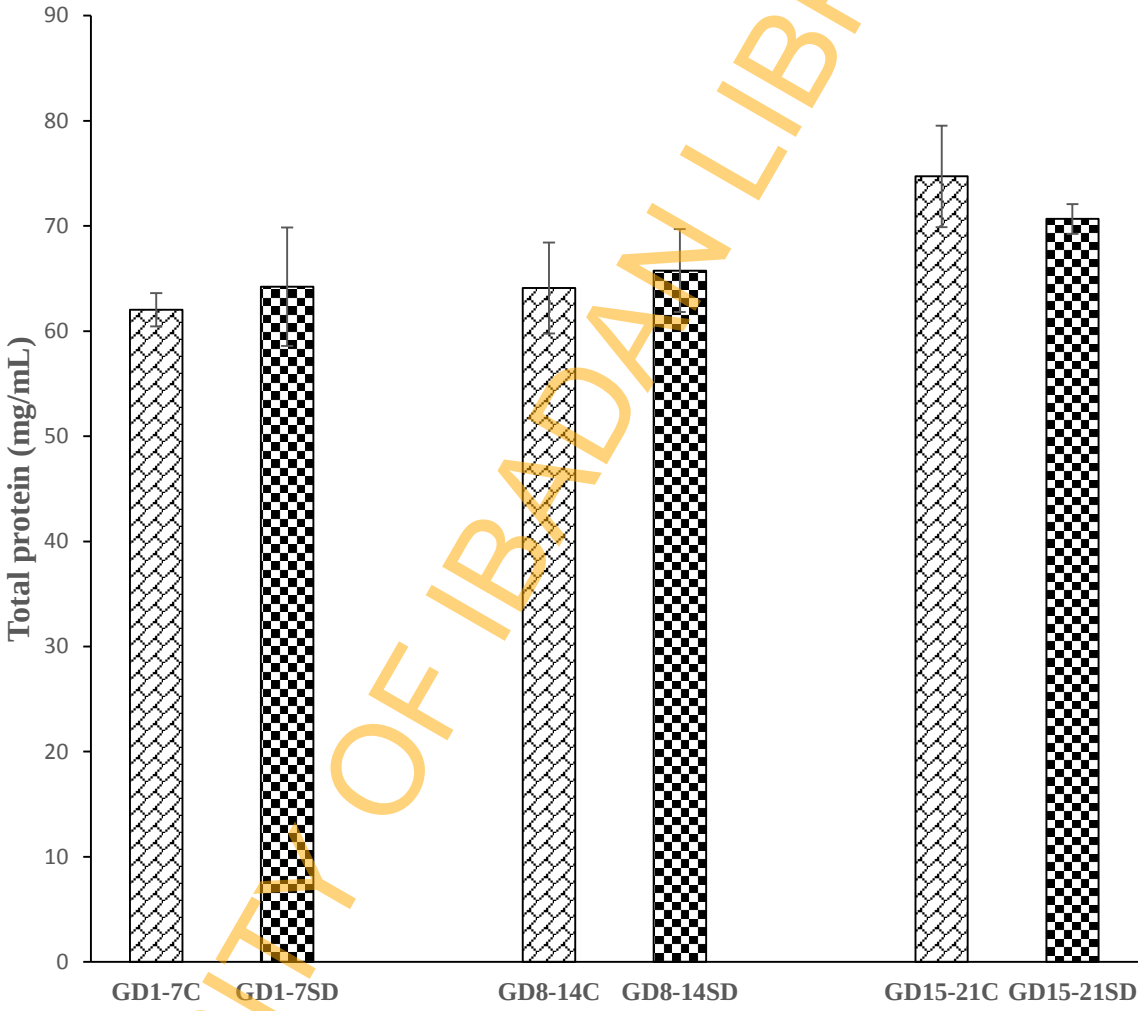


Figure 4.38. Placental total protein of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams**.** Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.12. Effects of maternal sleep deprivation on placental nucleic acid concentration in pregnant Wistar rat dams

There was a significant increase in the placental nucleic acid concentration of the GD15-21SD dams when compared with the control group (Figure 4.39).

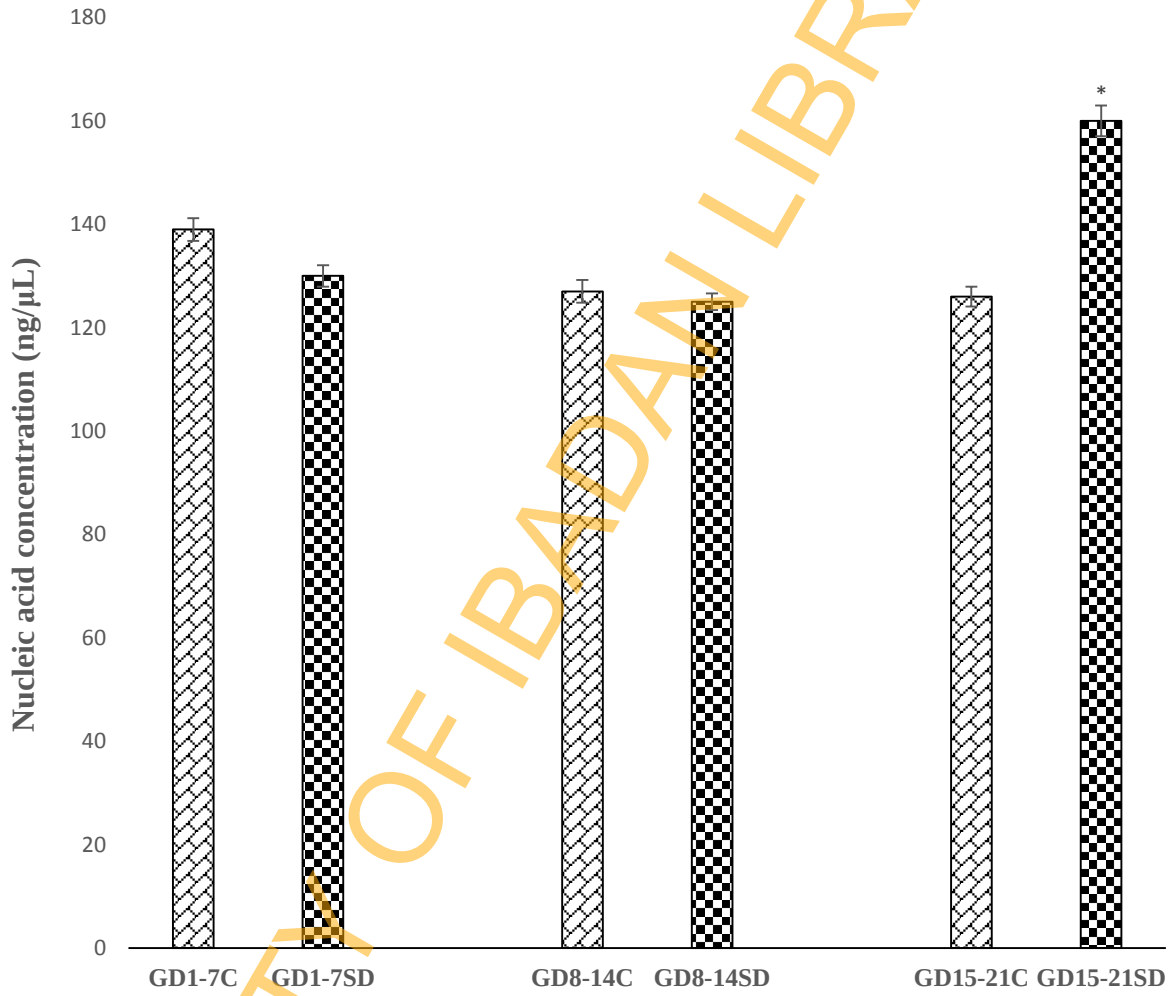


Figure 4.39. Placental nucleic acid of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. *p<0.05 when compared with the corresponding control group based on Student's t-test. GD = Gestation Day.

4.13. Effects of maternal sleep deprivation on placental global DNA methylation status in pregnant Wistar rat dams

Maternal sleep deprivation did not affect Placental global DNA methylation status across all groups (Figure 4.40).

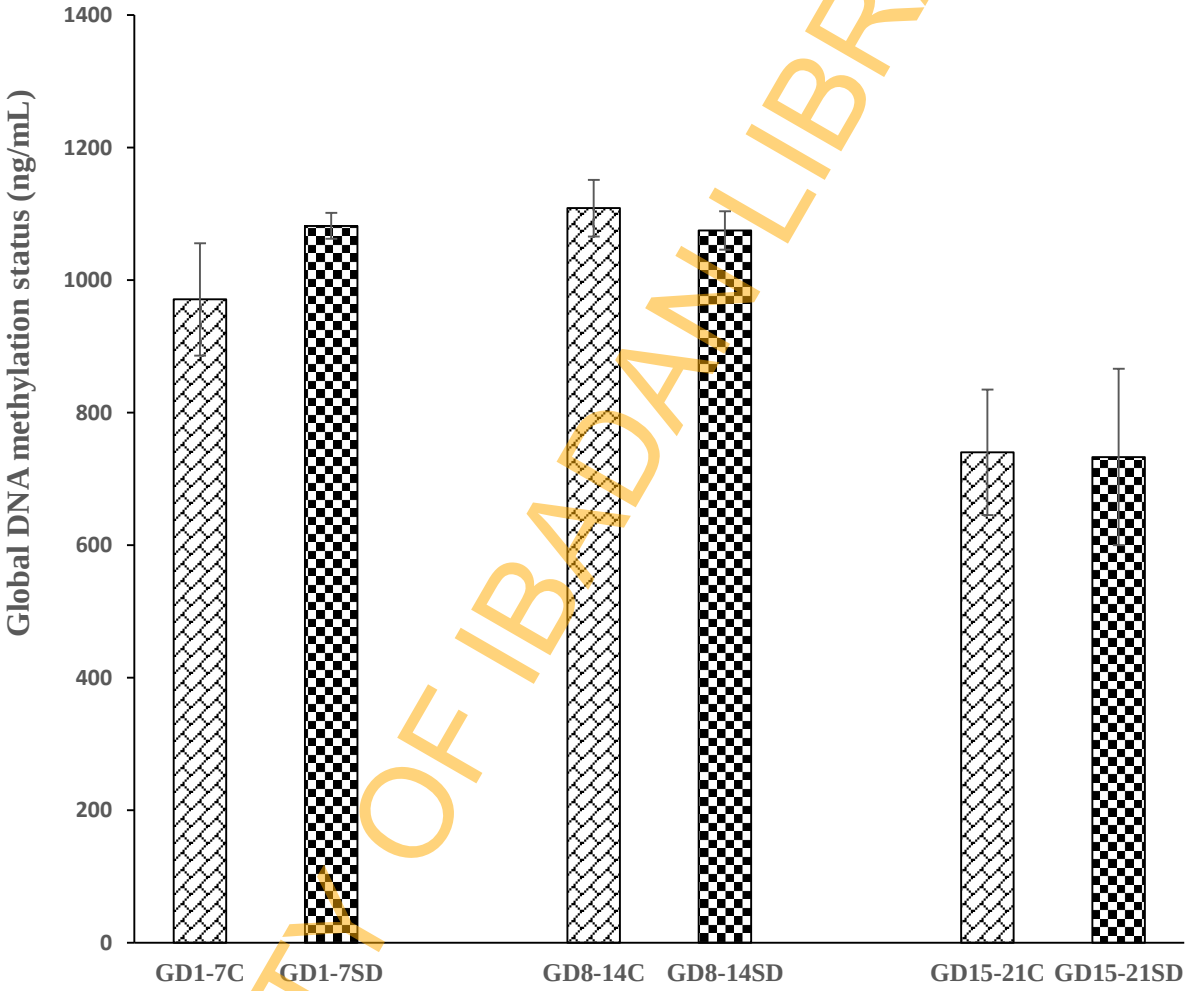


Figure 4.40. Placental global DNA methylation status of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams. Columns represent mean ± SEM. n = 5. Analysis was based on Student's t-test. GD = Gestation Day.

4.14. Effects of maternal sleep deprivation on placental apoptotic markers in pregnant Wistar rat dams

4.14.1. Effects of maternal sleep deprivation on placental expression of *Bcl$_2$*

Qualitatively, GD1-7SD placenta expressed more anti-apoptotic factor; *Bcl$_2$* than GD1-7C group placenta. Similar levels of expression of *Bcl$_2$* were observed in GD8-14C and GD8-14SD on the endometrial glands. GD15-21SD expressed no anti-apoptotic factor on the chorionic villi while GD15-21C did (Plate 4.10). Quantitatively, *Bcl$_2$* was significantly reduced ($p<0.05$) in the placenta of GD15-21SD compared with GD15-21C. *Bcl$_2$* expression was more in the placenta of GD1-7SD than in the placenta of GD1-7C, however, this difference was not significant (Figure 4.41).

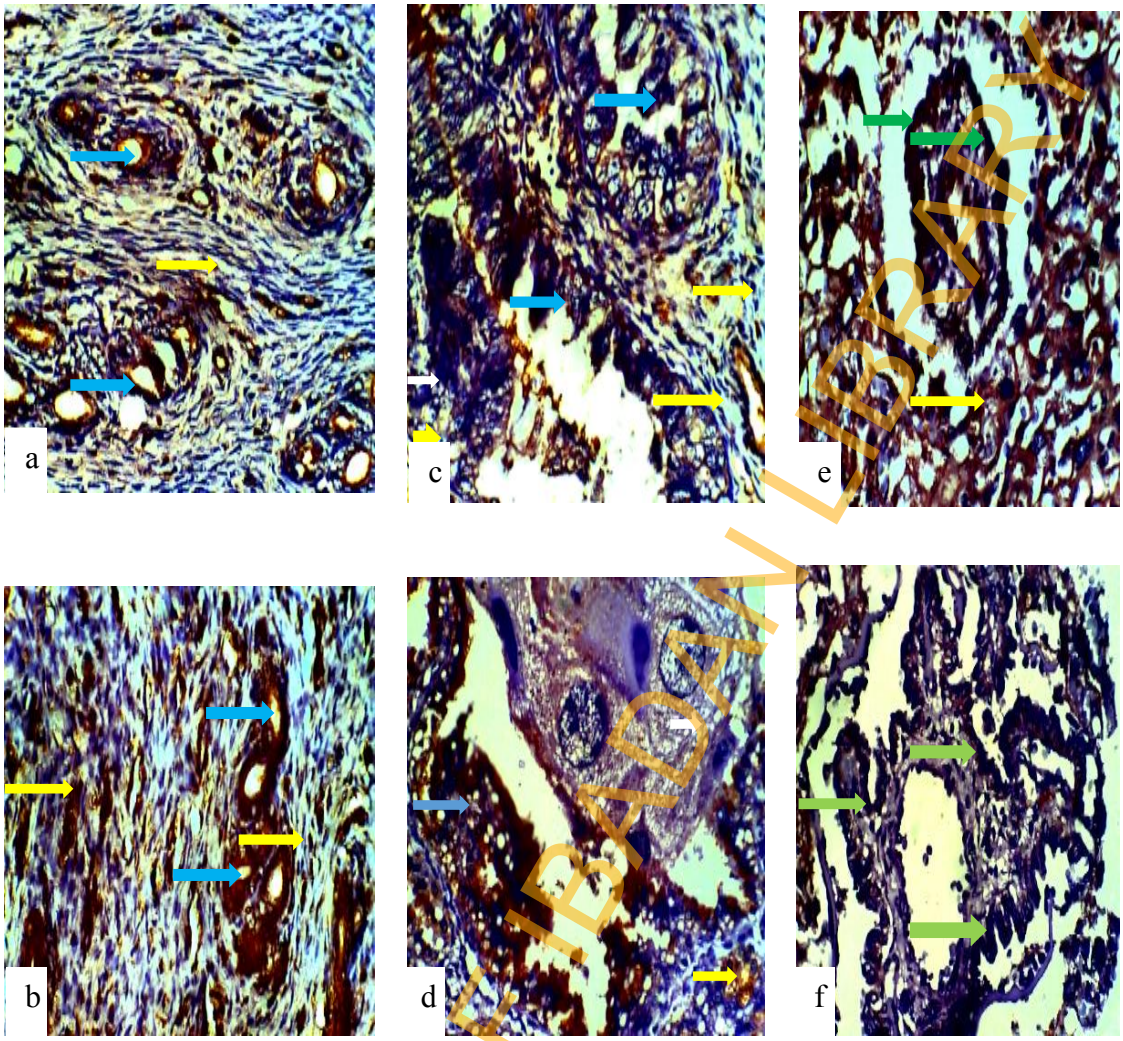


Plate 4.10. Qualitative Immunohistochemistry of *Bcl₂* expression in placental sections from Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams a = GD1-7C, b = GD1-7SD, c = GD8-14C d = GD 8-14SD, e = GD15-21C and f = GD15-21SD. Tissues were stained by 3,3'-diaminobenzidine and presented at x100 magnification. GD1-7C developing placenta shows mild expression of *Bcl₂* on stromal cells (yellow arrow) and endometrial glands (blue arrow). GD1-7SD developing placenta shows moderate expression of *Bcl₂* on stromal cells (yellow arrow) and endometrial glands (blue arrow). GD8-14C and GD8-14SD show negative expression of *Bcl₂* on stromal cells (yellow arrow) and mild expression on endometrial glands (blue arrow). GD15-21C rat placenta shows moderate expression of *Bcl₂* on decidua (red arrow) and mild expression on the chorionic villi (green arrow). GD15-21SD rat placenta shows negative expression of *Bcl₂* on the chorionic villi (green arrow).

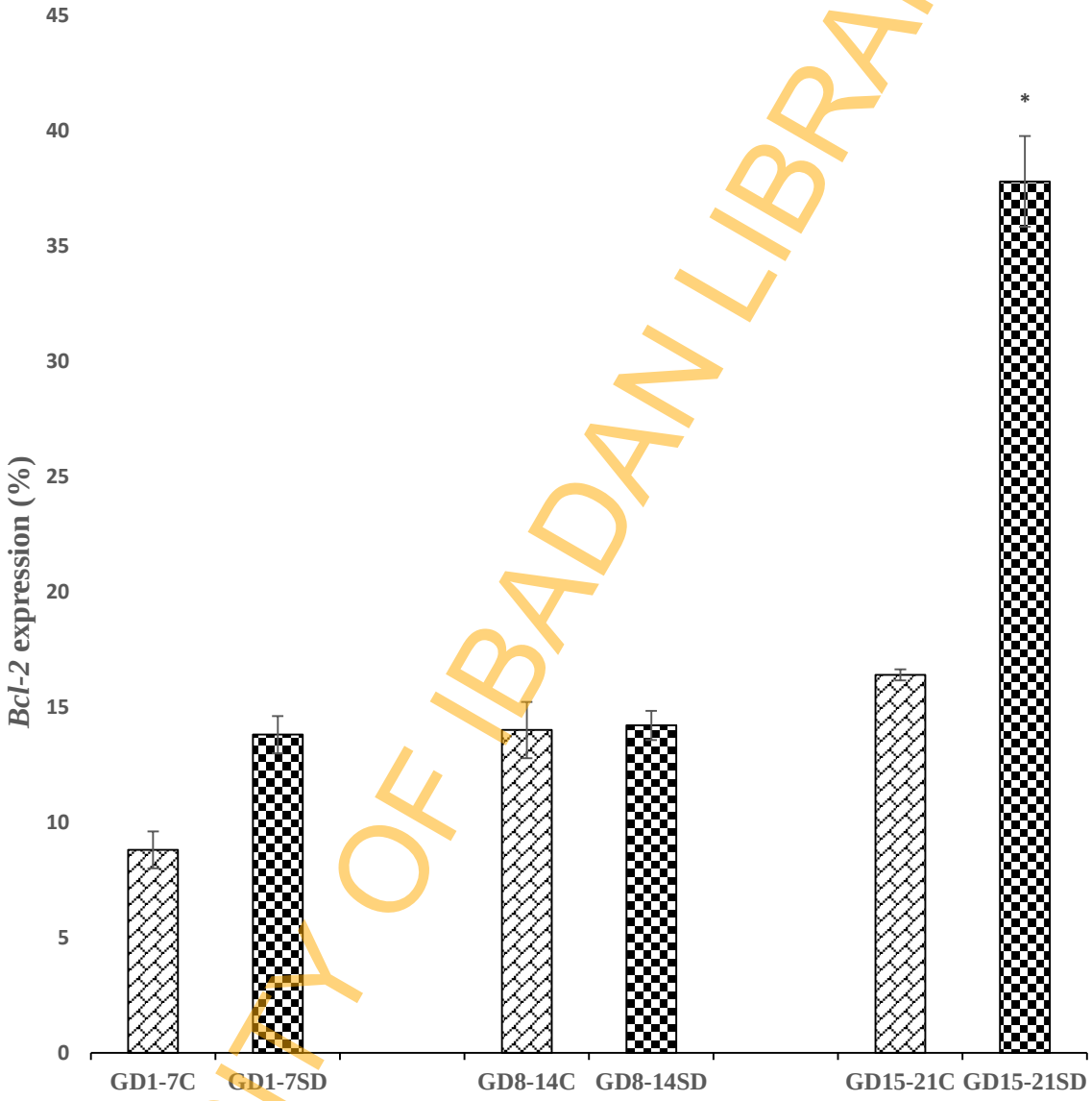


Figure 4.41. Quantitative immunohistochemistry of *Bcl*$_2$ expression in the placenta of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams. *p<0.05 compared with corresponding control group based on data generated from imagej 1.46 software and Student's t-test. GD = Gestation Day.

4.14.2. Effects of maternal sleep deprivation on placental expression of *p53*

Qualitatively, GD1-7C and GD1-7SD showed comparable levels of *p53* on stromal cells and endometrial glands of the placenta. GD8-14SD had less expression of *p53* compared with the control group. While GD15-21C placenta showed expression of *p53* on decidua and stromal cells, GD15-21SD rat placenta did not show any expression of *p53* in the placental tissue (Plate 4.11). Quantitatively, *p53* expression was reduced in the placentas of GD8-14SD and GD15-21SD compared with their respective control groups (Figure 4.42)

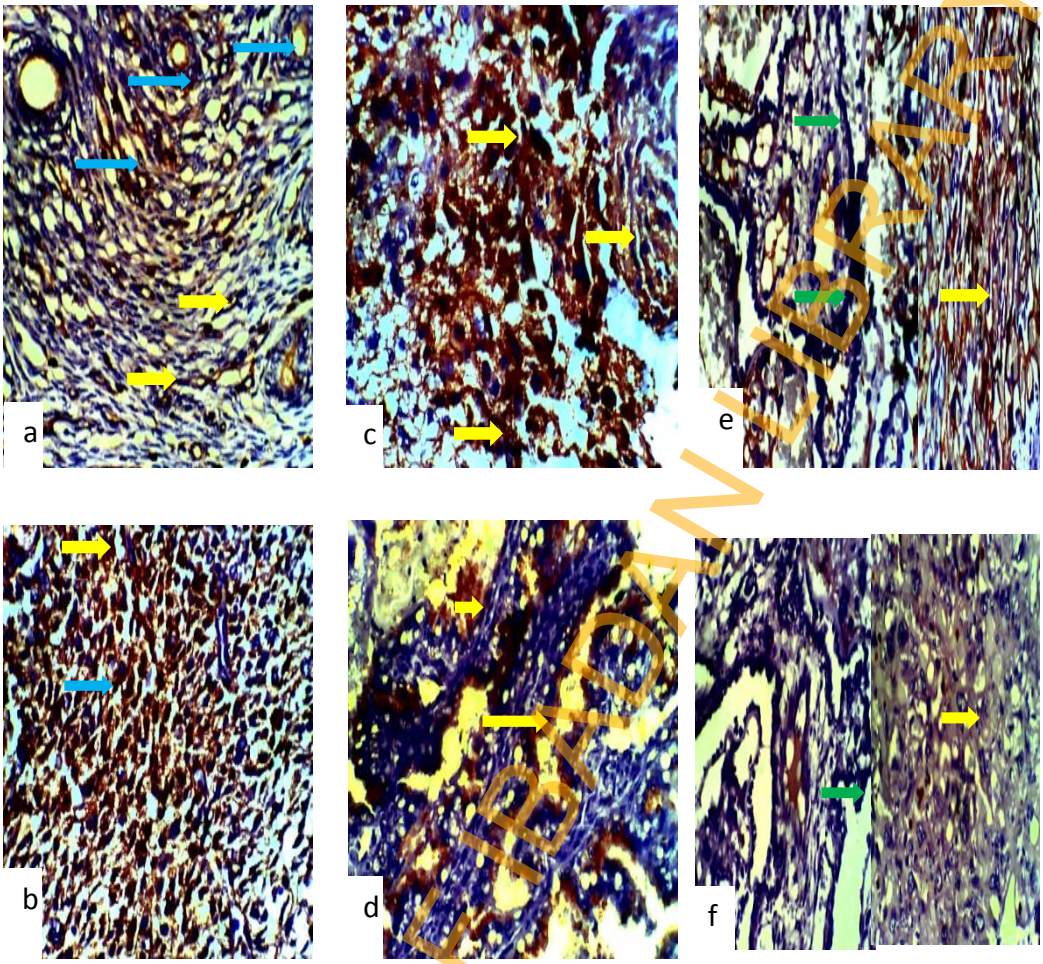


Plate 4.11. Qualitative Immunohistochemistry of *p53* expression in placental sections from Control (C) and Sleep-Deprived (SD) pregnant Wistar rat dams a = GD1-7C, b = GD1-7SD, c = GD8-14C d = GD 8-14SD, e = GD15-21C and f = GD15-21SD. Tissues were stained by 3,3'-diaminobenzidine and presented at x100 magnification. GD1-7C developing placenta shows mild expression of *p53* on stromal cells (yellow arrow) and endometrial glands (blue arrow). GD1-7SD developing placenta shows mild expression of *p53* on stromal cells (yellow arrow) and endometrial glands (blue arrow). GD8-14C placenta shows moderate expression of *p53* on stromal cells (yellow arrow) and glands (blue arrow). GD8-14SD placenta shows mild expression of *p53* on glands (yellow arrow). GD15-21C placenta shows moderate expression of *p53* on decidua (red arrow), mild expression on stromal cells (yellow arrow) and negative expression on the chorionic villi (green arrow). GD15-21SD placenta shows negative expression of *p53* on decidua (red arrow), on stromal cells (yellow arrow) and on chorionic villi (green arrow).

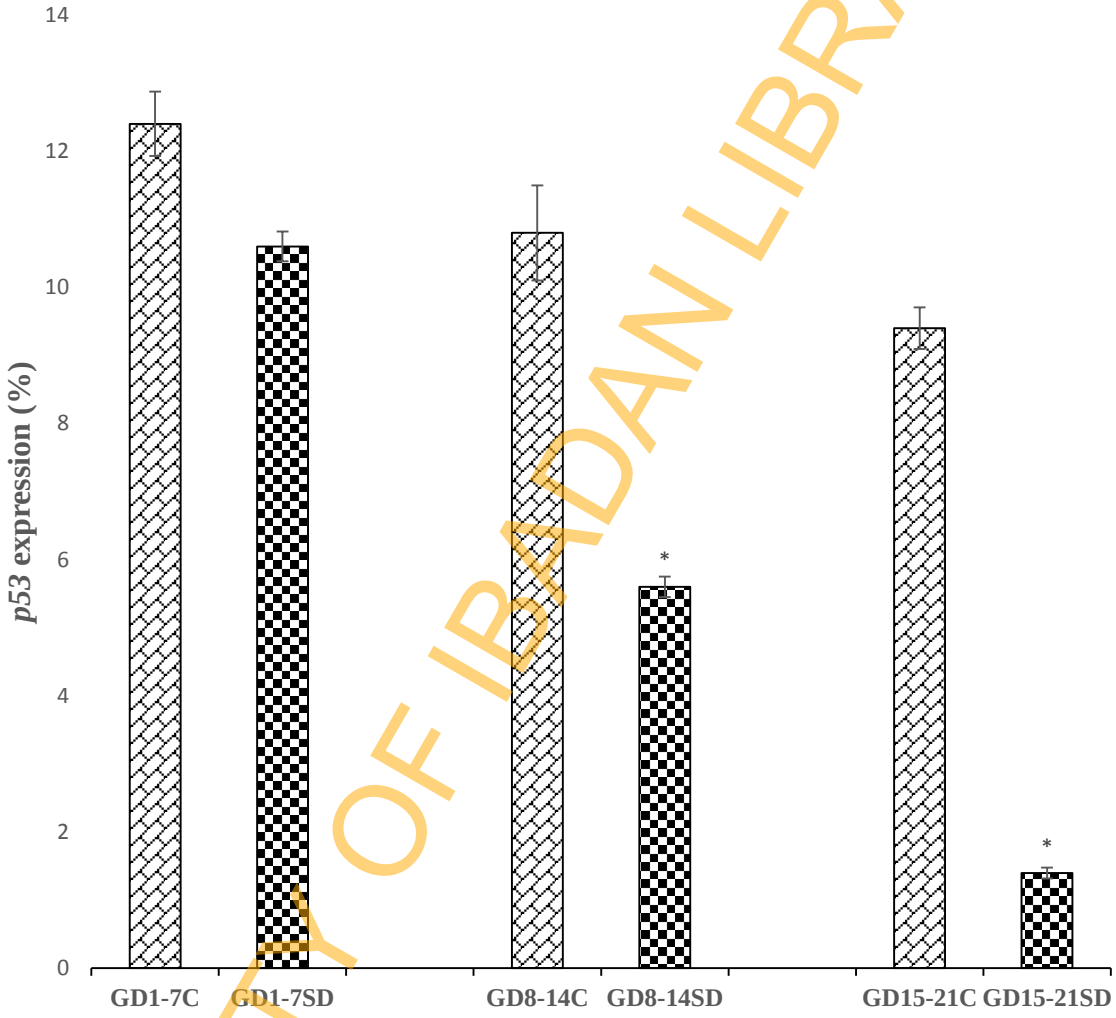


Figure 4.42. Quantitative immunohistochemistry of *p53* expression in the placenta of Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams. *p<0.05 compared with corresponding control group, based on data generated from imagej 1.46 software and Student's t-test. GD = Gestation Day.

4.14.3. Effects of maternal sleep deprivation on placental expression of *Bcl$_{10}$*

Qualitatively, GD1-7 groups and GD15-21 groups show similar levels of expression of *Bcl$_{10}$* on the decidual cells (Plate 4.12). GD8-14C placenta showed strong expression of *Bcl$_{10}$* on glands while GD8-14SD placenta showed moderate expression of *Bcl$_{10}$* on glands (Plate 4.12). Quantitatively, there was no significant difference in the expression of *Bcl$_{10}$* across all groups (Figure 4.43).

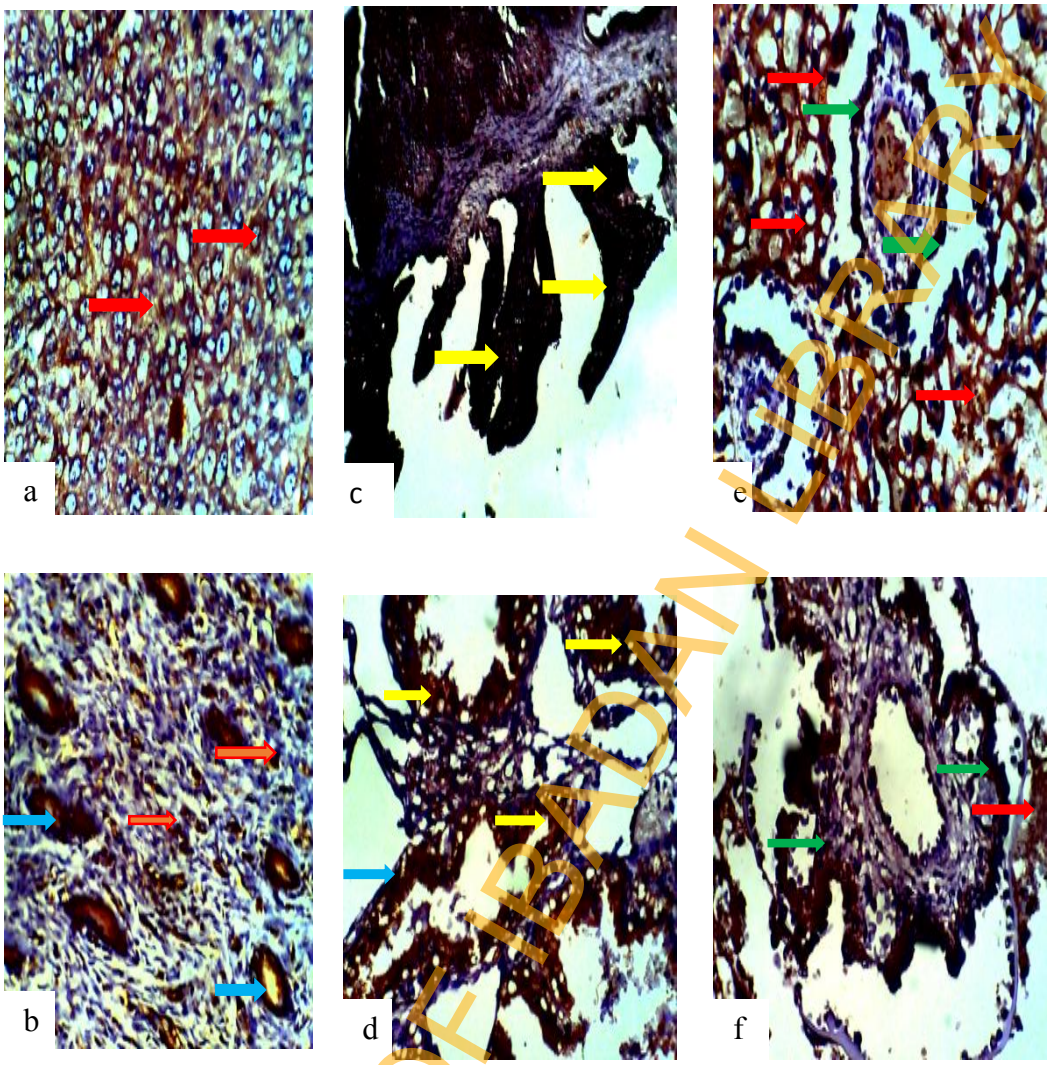


Plate 4.12. Qualitative Immunohistochemistry of *Bcl*$_{10}$ expression in placental sections from Control (C) and Sleep Deprived (SD) pregnant Wistar rat dams. a = GD1-7C, b = GD1-7SD, c = GD8-14C d = GD 8-14SD, e = GD15-21C and f = GD15-21SD. Tissues were stained by 3,3'-diaminobenzidine and presented at x100 magnification. GD1-7C rat developing placenta shows moderate expression of *Bcl-10* on decidual cells (red arrow). GD1-7SD rat developing placenta shows moderate expression of *Bcl-10* on decidual cells (red arrow) and endometrial glands (blue arrow). GD8-14C rat developing placenta shows strong expression of *Bcl-10* on glands (yellow arrow). GD8-14SD rat developing placenta showing moderate expression of *Bcl-10* on glands (yellow arrow). GD15-21C rat placenta showing mild expression of *Bcl-10* on the chorionic villi (green arrow). GD15-21SD rat placenta showing mild expression of *Bcl-10* on the chorionic villi (green arrow).

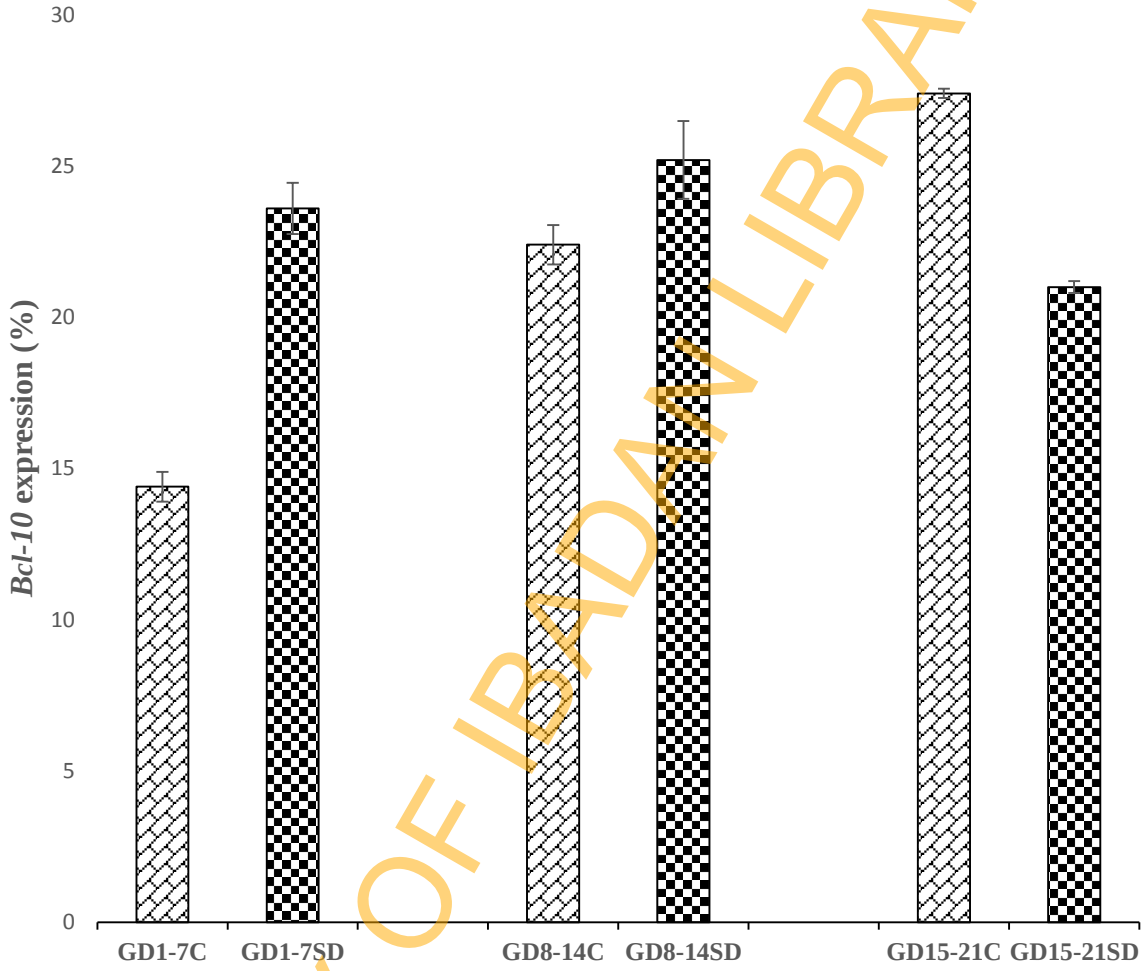


Figure 4.43. Quantitative immunohistochemistry of *Bcl*$_{10}$ expression in the placenta of control (C) and Sleep Deprived (SD) pregnant Wistar rat dams. Analysis was based on data generated from imagej 1.46 software and Student's t-test. GD = Gestation Day.

CHAPTER FIVE

5.0. DISCUSSION

5.1. Effects of sleep deprivation on reproductive functions of male offsprings of Wistar Rats

The preliminary study simulated the pattern of sleep deprivation that is common in humans as it affects oestrous cycle in the rat. Due to the high death rate that is associated with total sleep deprivation and long duration of partial sleep deprivation in the rat (Everson *et al.,* 1989; Rechtschaffen and Bergmann 2002), the preliminary study was designed to determine the shortest possible duration of sleep deprivation that would affect the female reproductive cycle without causing unnecessary deaths. Results showed that paradoxical sleep deprivation caused oestrus cycle disruption, ovarian vascular congestion, ovarian haemorrhage, endometrial inflammation, endometrial vascular congestion and loss of body weights of the female Wistar rats. The observed disruption of oestrous cycle corroborates the findings from earlier studies (Antunes *et al.,* 2006; Tufik *et al.,* 2009). These previous studies examined the relationship between reproductive functions and total sleep deprivation which is hardly obtainable in humans (Orzel-Gryglewska, 2010).

The loss of body weight was attenuated during the third week as sleep deprivation progressed. This suggests that certain physiological processes may be able adapt to sleep deprivation. However, the adaptation may be related to the quantity of feed they consumed as sleep deprivation progressed (Koban *et al.,* 2008). Contrarily, the disruption in oestrous cycle was further intensified as the duration of sleep deprivation lengthened, suggesting a possible lack of adaptation to sleep deprivation by the physiological processes associated with the control of reproductive functions. The oestrous cycle in animals and menstrual cycle in humans are regulated by hormones of the Hypothalamic Pituitary Gonadal Axis (Reed and Carr, 2015).

Proestrus is the phase of the reproductive cycle in the rat during which ovarian follicles mature and uterine lining proliferate while oestrus is the phase of the cycle

during which ovulation occurs and female animals are receptive to male animals (Hirshfield, 1985; Kim *et al.*, 2016). The events typical of these phases occur under the influence of FSH, LH and oestrogen. Thus, the reduction in proestrus and oestrus frequency may indicate sup-optimal production of FSH, LH and oestrogen during sleep deprivation (Plas-Roser *et al.*, 1977). Oestrogen and LH surges are requisites for ovulation (Micevych *et al.*, 2003) hence, the follicular maturation arrest and lack of ovulation apparent in the ovaries of the sleep deprived rats also support the possible involvement of sub-optimal production of these hormones. Metestrus and diestrus phases are characterized by the activities of the corpus luteum which produces the predominating hormone; progesterone (Hirshfield, 1985). Sustained metestrus or diestrus in animals is associated with lack of receptive behaviour (Kim *et al.*, 2016). The implication of these observed results in reproduction is that animals that exhibit this type of oestrous cycle pattern are not likely to be fertile. Infertility in the animals may be caused by reduced number of maturing follicles, reduced frequency of ovulation and reduced receptive behaviour. Even though, Andersen *et al.*, (2009) used another approach to investigate the relationship between paradoxical sleep deprivation and reproductive functions in the rat, they also concluded in their study that paradoxical sleep deprivation led to reproductive abnormality in the female rats.

Additionally, longer duration of sleep deprivation caused inflammatory cell infiltration of the ovary and uterus and vascular congestion in the ovary. Inflammation of the reproductive organs has been implicated in the aetiology of ovarian and cervical cancer (Maccio and Madeddu, 2012; Deivendran *et al.*, 2014). This suggests that chronic sleep deprivation may be an indirect cause of these cancers.

The shortest duration of paradoxical sleep deprivation that caused the disruption of oestrous cycle in the study was 7 days. Therefore, the combination of loss of body weight, ovarian inflammation, disrupted oestrous cycle and plausible hormonal disruption caused by sleep deprivation in this study led to the hypothesis that seven days of sleep deprivation during rat's pregnancy is a potential insult which may trigger suboptimal intra-uterine environment, thereby causing foetal programming.

5.2. Effects of maternal sleep deprivation on morphometric indices in male offsprings of Wistar rats

Male offsprings of dams that were sleep deprived during gestation days 15-21 had reduced birth weight and increased crown-rump length. GD8-14SD offsprings had increased crown-rump length while the birth weight was not affected. One of the numerous observations stated from the earlier studies of Barker was that morphometry at birth is prognostic of adult disease (Barker *et al.*, 1989; Barker *et al.*, 1990; Barker *et al.*, 1993). Birth morphometry is easily taken, non-invasive, not expensive and reliable surrogate marker of foetal programming.

The reduced birth weight in the GD15-21SD offsprings suggests the occurrence of foetal programming and probable development of disease in later life (Law *et al.*, 1991; Phillips *et al.*, 1994; Fall *et al.*, 1995; Kaijser, 2015). The reduction in birth weight in these offsprings occurred in conjunction with increased crown-rump length, implying that the pups were thinner, thus, confirming the occurrence of asymmetric intra-uterine growth restriction and a high risk of future poor health in the male offsprings whose mothers were sleep deprived during gestation days 15 to 21 (Haggarty *et al.*, 2004). This result supports the report from a recent epidemiological survey which revealed that infants born to mothers who experienced aberrant sleep patterns during late gestation had intra-uterine growth restriction and low birth weight (Micheli *et al.*, 2011).

The increased crown-rump length without increased birth weight in the GD8-14SD offsprings also indicates that the animals were thinner than their control counterparts (Nascimento *et al.*, 2011). Studies report that thin babies with normal birth weight have distinct metabolic functions (Taylor *et al.*, 1995). Recent animal studies have shown that when a foetus is programmed, its birth weight may not be influenced (Thone-Reineke *et al.*, 2006). For instance, a closer look at the popular Dutch Hunger Winter Study by Schultz (2010) revealed that foetal exposures that impacted adult health did not automatically result in altered birth weight (Schulz, 2010). Although birth weight is the most widely studied measure of foetal future health, it may not be a particularly sensitive measure in many situations (Almond and Currie, 2011). Thus, the available results on birth weight only confirms the presence of foetal programming in the GD15-21SD offsprings but does not establish the absence of the phenomenon in the other groups. It is worth noting also that early defect in

organ development may be compensated for and normalized by adaptations later in gestation especially when there is abundance of placental nutrient and O_2 (Fowden *et al.*, 2006b). This may explain why GD1-7SD offsprings' birth weight was not affected.

5.3. Effects of maternal sleep deprivation on cardiovascular functions of male offsprings in Wistar rats

Male offsprings of GD1-7SD and GD8-14SD groups had high systolic pressure, normal heart rate and normal blood volume at adulthood. This observation supports the reports of previous studies (Thomal *et al.*, 2010; Lima *et al.*, 2014) in which dams that were subjected to 21 days of paradoxical sleep deprivation had offsprings with high systolic blood pressure. The development of cardiovascular system which begins around GD 8-9 (Brand, 2003; Marcela *et al.*, 2012) and the occurrence of rapid hyperplasia and differentiation following the initiation (Marcela *et al.*, 2012) may explain in part, the vulnerability of the embryonic heart at GD 8-14 to the programming effect of maternal sleep deprivation. This critical period excludes the GD 1-7. However, the activities of the 50 founder cells which are the earliest precursors of the heart have begun at GD 6.5 (Tam *et al.*, 1997). This critical period of organogenesis is marked by founder cell hyperplasia (Vogler and Bodmer, 2015) which may be subject to modifications by the physiological changes imposed upon the body in the presence of maternal insults such as sleep deprivation (Fowden *et al.*, 2006a). In any case, many cell lineages may be affected during the periconceptual period and the GD 1-7 in the rat falls within this period (Phillips and Poyser, 1981). However, as cited earlier, early defect in organ development may be compensated for and normalized by adaptations later in gestation, especially when there is abundance of placental nutrient and O_2 (Fowden *et al.*, 2006b). This may be the reason why the heart of GD1-7SD group showed no pathology as opposed to that of the GD8-14SD group which showed mild myocardial degeneration. It is not clear if the myocardial degeneration preceded the high systolic pressure in this group, nonetheless, myocardial degeneration may be a cause of hypertension (O'Rourke *et al.*, 2010) as well as a consequence of hypertension (Nadruz, 2015).

The cause of the lower blood pressure in the offsprings of GD15-21SD dams is not clear, but, there is a possibility that it is just a component of a bigger problem judging

by the epicardial congestion and myocardial haemorrhage in the group. The aetiology of pericardial disease remains idiopathic or is usually difficult to determine (Khandaker *et al.*, 2010).

While the serum level of melatonin in the GD1-7SD male offsprings was increased, it was decreased in the GD8-14SD male offsprings. Melatonin receptors have been identified in the heart of rat (Sallinen *et al.*, 2005; Peliciari-Garcia *et al.*, 2011) and man (Ekmekcioglu *et al.*, 2003; Dominguez-Rodriguez, 2012) and even though the functions remain to be fully elucidated, reports have it that, melatonin may act in the capacity of both anti-oxidant (Reiter *et al.*, 2003; Zhang and Zhang, 2014) and pro-oxidant (Osseni *et al.*, 2000; Albertini *et al.*, 2006; Zhang and Zhang, 2014; Munik and Ekmekçioğlu, 2015). On this account, failure of a biological system to maintain optimal serum level of melatonin may cause oxidative stress (Zhang and Zhang, 2014) which has been said to be a principal actor in the etiopathogenesis of cardiovascular disease (Campos, 2009; Forstermann, 2010).

Another factor that may have contributed to the development of hypertension in these animals is the increased level of serum corticosterone in the offsprings (Whitworth *et al.*, 1989; Mangos *et al.*, 2000; Whitworth *et al.*, 2005; Walker, 2007). Clinical and experimental studies have shown the association between sleep restriction and altered physiology such as hypertension and increased activity of the HPA axis (Spiegel *et al.*, 2004). Interestingly, the present observation showed a similar relationship, albeit, between maternal sleep restriction and offspring's hypertension associated with increased HPA axis activity. In humans, cortisol-induced hypertension is associated with volume expansion (Whitworth *et al.*, 2005) as observed in the GD8-14SD offsprings which exhibited higher serum corticosterone, high systolic blood pressure with increased blood flow. Conversely, GD15-21SD offsprings had reduced blood pressure in spite of increased corticosterone, showing that corticosterone alone may not be enough to trigger hypertension in the offsprings of sleep deprived dams. Nevertheless, considering the myocardial bleeding and low blood pressure, there is a possibility that the offsprings of GD15-21SD group were experiencing heart failure due to a prior hypertension and that the low blood pressure was just a consequence of the supposed heart failure (Drazner, 2011).

5.4. Effects of maternal sleep deprivation on renal functions of male offsprings in Wistar rats

It was observed that maternal sleep deprivation caused a modification in renal structure and functions in all offsprings, however, the effects were mostly pronounced in those belonging to the GD 15-21 group. The results suggest that the intra-uterine process of nephrogenesis was affected during the gestation days 15 to 21 resulting into reduced number of nephrons as demonstrated by fewer number of glomeruli in the GD15-21SD kidney histology. The interstitial cells of reno-medullary tissue are first seen during Embryonic Day (ED) 14 (Bertram *et al.*, 2000; Maric *et al.*, 1997) which is equivalent to GD 14 (Hill, 2007). The presence of interstitial cells of reno-medullary tissue initiates metanephrogenesis and this continues till the end of gestation (Moritz and Wintour, 1999). This means that the critical period of nephrogenesis in the rat lies within GD 14 and GD 21 i.e. the end of gestation. This may explain why, but not how the kidneys of offsprings belonging to the GD15-21SD group were mostly impacted by maternal sleep deprivation. The existing hypotheses regarding how a stressful event during pregnancy may result into fewer number of nephron include: changes in DNA methylation, alteration in apoptosis, alteration in activity of renin-angiotensin system and over-exposure to glucocorticoid (Woods *et al.*, 2001; Welham *et al.*, 2002; Dickinson *et al.*, 2007).

In addition to the fewer number of glomeruli in the GD15-21SD, serum level of creatinine increased significantly. Creatinine is the end product of muscle creatine and almost all creatinine produced in the body is filtered by the kidneys and excreted in urine. This makes the serum level of creatinine a good indicator of how well the kidneys are functioning. Increased serum creatinine level in the GD 15-21 suggests impairment of renal function (Roth, 2012). In agreement with this, creatinine clearance was reduced in this group, implying a reduction in the glomerular filtration rate (GFR).

The reduced GFR is usually one of the sequelae of low blood pressure and albuminuria. Urinary protein is the earliest sign of damage to the vessels in both kidney and heart (Koroshi, 2007). It is a symbol of general dysfunction of the glomerular filtration barrier and leakage of other macromolecules into vascular wall leading to inflammation and resultant arteriosclerotic process (Deckert *et al.*, 1989). Albuminuria as well as low blood pressure causes reduction in the glomerular

filtration pressure resulting in decreased glomerular filtration rate (Currie and Delles, 2013). It may occur secondary to either kidney disease or (Abbate *et al.*, 2006) or heart disease (Currie and Delles, 2013) and both of these are present in the GD 15-21SD group offsprings. Kidney disease and heart disease are related on many grounds and the presence of albumin in urine points to a bad prognosis in both cases (Koroshi, 2007). Thus, the GD15-21SD offsprings may have shorter life span than the others.

5.5. Effects of maternal sleep deprivation on reproductive functions of male offsprings in Wistar rats

The reproductive functions of offsprings belonging to GD15-21SD dams appeared to have been mostly affected by maternal sleep deprivation. They exhibited late testes descent, reduced serum testosterone, sperm motility and sperm count. Their reproductive organ histology also revealed severe abnormality and they had 0% fertility and gestational indices.

The delayed onset of puberty signified by late testes descent that was observed in the GD 15-21 offsprings of the sleep deprived dams may be one of the resultant effects of reduced secretion of testosterone; which is a major determinant of testes descent (Macleod *et al.*, 2010). The serum testosterone was not measured during puberty, however, the delayed testicular descent and the evidently reduced level of testosterone at maturity suggests that the status of testosterone may have originated long before adulthood in these animals.

The reproductive dysfunction in these offsprings was seemingly limited to the peripheral tissue as reduction in testosterone concentration was accompanied by normal levels of gonadotropins. Clinical and experimental studies have reported a negative correlation between sleep deprivation and serum testosterone concentration in men (Leeuwen *et al.*, 2009; Schmid *et al.*, 2012; Jauch-Chara *et al.*, 2013) and in adult male rats (Akindele *et al.*, 2014). However, there are contradictory reports on the relationship between sleep restriction and activity of the Hypothalamic-Pituitary-Gonadal (HPG) axis (Hairston *et al.*, 2001; Suer *et al.*, 2011). The present observations were also consistent with the report of Alvarenga *et al.*, (2013) which showed a decrease in testosterone level and lack of proceptive behaviour of first

filial male offspringss of dams subjected to 21 days of sleep deprivation (Alvarenga *et al.*, 2013).

Low testosterone level is known to reduce sperm quality (Esteves *et al.*, 2011), as such, the reduced sperm quality demonstrated by the male offsprings of the GD15-21SD may be a consequence of low testosterone or of structural deficit acquired during intra-uterine life. The infertility exhibited by these offsprings was not unexpected since low sperm motility and count are well-established causes of infertility (Kumar and Singh, 2009). Additionally, low testosterone level decreases proceptive behaviour in the rats (Stanworth and Jones, 2008) and this may also lead to infertility.

The male offsprings of sleep deprived dams belonging to GD 1-7 and GD 8-14 groups also showed minor reproductive organ aberrations but the effects of sleep deprivation were ostensibly more remarkable in the GD 15-21 group probably because this period encapsulates the masculinization and male organ differentiation window (Macleod *et al.*, 2010; Welsh *et al.*, 2008). Masculinization is androgen dependent and existing data have shown that sleep deprivation results in testosterone reduction (Leproult and Van Cauter, 2011; Schmid *et al.*, 2012; Jauch-Chara *et al.*, 2013).

5.6. Effects of sleep deprivation during gestation on maternal hormones

The impact of sleep deprivation on biochemical variables at different gestation periods are not similar, possibly because of the normal changes in hormonal levels accompanying the different gestational periods (Kumar and Magon, 2012). For instance, in the control dams, melatonin level dropped during GD 8-14 and rose again during GD 15-21 producing a V-shaped curve. Although the functions of melatonin during pregnancy are not fully elucidated, the present observation suggests that less of those functions are needed during mid-pregnancy in the rat. Results showed that sleep deprivation caused a sustenance in melatonin level from GD 1-14. The level at which it was sustained was not high enough during GD 1-7 and not low enough during GD 8-14. During gestation, melatonin exerts pleiotropic effects on a number of tissues (Fildes *et al.*, 2009) controlling a cascade of reactions. This plays crucial role in the regulation of foetal organ development that are

important for the proper adaptation of the neonate during extra-uterine life (Torres-Farfan *et al.*, 2008). As such, the disruption of the pattern of melatonin secretion seen in the GD 1-7 and GD 8-14 groups may contribute to the foetal programming effects observed in these offsprings at adulthood. It is not clear what physiological mechanism(s) is/are responsible, nevertheless, it may be related to oxidative status within the foeto-placental unit.

Fowden *et al.*, (2006a) explained that the pattern of post-natal disease resulting from foetal programming is determined by the nature, severity, timing and duration of maternal insult. The dams and offsprings belonging to GD 15-21 sleep deprived group which exhibited normal levels of melatonin did not have high blood pressure. This suggests that the programming of foetal organs in the GD15-21SD offsprings had no association with maternal melatonin. Also, the offsprings of dams whose melatonin levels were altered during sleep deprivation (GD1-7 and GD8-14 offspring) also had their melatonin secretion pattern affected at adulthood. Furthermore, the levels of melatonin exhibited by the 2 sets of offsprings were opposite to that observed in their mothers.

Corticosterone levels of all the sleep deprived dams were not affected. Similar results have been reported by van der Borght *et al.* (2006) and Hagewoud *et al.* (2010). Conversely, their offsprings exhibited increased serum corticosterone level suggesting that maternal sleep deprivation caused the programming of the HPA axis. Some studies have reported a positive association between sleep deprivation and glucocorticoid production (Aldabal and Bahammam, 2011) while others report a nonexistent association between these two (Hagewoud *et al.*, 2010). Corticosterone is normally converted by 11βHSD$_2$ into cortisone during pregnancy (Seckl and Meaney, 2004). The role of 11βHSD$_2$ in this study was not explored.

Reduced testosterone during the masculinization window in the GD15-21SD group may be partly responsible for the abnormalities seen in the adult offsprings. This is because masculinization is dependent, though not exclusively on testosterone (Welsh et al, 2008). Optimal secretion of foetal testosterone is required for the normal growth and development of the male reproductive organs. In the mammals, before the period of masculinization in the foetus, reproductive system is indistinguishable and even the gonads are called indifferent gonads. The

differentiation of the indifferent gonad into a male gonad involves modification by a chain of events initiated by the *SRY* gene leading to formation of the testis (Osmond and Barker, 2000; Gluckman and Hanson, 2004). Following this is the process of masculinization which is primarily driven by testosterone secreted by the foetal testis (Welsh *et al.*, 2008). Disruption in the process of masculinization has been reported to cause mild to serious male reproductive disorders which may be obvious at birth (Barker *et al.*, 1989; Armitage *et al.*, 2004) or manifesting as low sperm quality and other testicular disease during early adulthood (Langley and Jackson, 1994). In the rat, masculinization of testis, penis, prostate and seminal vesicle occurs during GD 15-21, the same period during which testosterone concentration was reduced in the sleep deprived dams whose offsprings were eventually infertile (Welsh *et al.*, 2008).

The secretion of foetal testosterone is triggered by human chorionic gonadotropin and chorionic gonadotropin has been identified in the serum and placenta of pregnant rat (Blank and Dufau, 1983). Although there are contrary opinions on the effect sleep deprivation may exert on pituitary gonadotropins, many studies have reported inverse relationship between oxidative stress and pituitary gonadotropins (Abou-Seif and Youssef, 2001). Thus, a possible mechanism that led to the reduction of testosterone may be a reduction in placental gonadotropin triggered by placental oxidative/nitrative stress.

5.7. Effects of sleep deprivation during gestation on placental oxidative stress

Many adverse pregnancy outcomes can trace their origin to the placenta (Ilekis *et al.*, 2016). The dams that were sleep deprived during GD 1-7 experienced exaggerated oxidative stress as evident by reduced serum melatonin level, increased placental malondialdehyde and reduced glutathione peroxidase. GD8-14SD offsprings exhibited increased serum melatonin, increased placental glutathione (GSH) and altered placental apoptosis. GD15-21 group showed reduced serum testosterone, decreased placental total antioxidant capacity, decreased placental superoxide dismutase, increased placental malondialdehyde, hydrogen peroxide, dichlorofluorescein, nitrotyrosine, glutathione, glutathione peroxidase, placental nuclei acid concentration and reduced evidence of placental apoptosis.

An aspect of gestational melatonin function that is worthy of exploration is its effects on redox status in pregnancy. Melatonin is known to prevent oxidative stress by direct and indirect mechanisms (Maharaj *et al.*, 2007; Reiter *et al.*, 2010) and this, it does in order to protect the developing embryo from the damaging effects of reactive oxygen species by increasing the level of glutathione peroxidase activity (Okatani *et al.*, 2001). The results suggest that the compromised melatonin level in the GD 1-7 offsprings of sleep deprived dams was responsible for the reduced level of glutathione peroxidase and increased level of malondialdehyde leading to the placental oxidative stress (Sies, 1997). Interestingly, GD 15-21 placentas of sleep deprived dams exhibited increased oxidative stress even in the presence of normal serum melatonin level with increased level of placental glutathione peroxidase thereby suggesting that melatonin may have more profound influence on the earlier gestational events pertaining to redox balance than the latter events.

Pregnancy is associated with physiological oxidative stress in the mother (Gitto *et al.*, 2002; Reiter *et al.*, 2009) and in the placenta (Burton and Jauniaux, 2011). Nevertheless, exaggerated levels of oxidative stress have been reported in pregnancies complicated with diabetes, pre-eclampsia and IUGR (Thompson and Al-Hasan, 2012). The exaggerated oxidative stress in these groups emphasizes the previous reports on the antioxidative functions of sleep (Reimund, 1994) and oxidative consequences of sleep deprivation (Reiter *et al.*, 2010; Periasamy *et al.*, 2015).

5.8. Effects of sleep deprivation during gestation on placental nitrative stress

Oxidative stress occurs when there is imbalance between reactive oxygen species (ROS)/ reactive nitrogen species (RNS) and antioxidants in biological system. The reduced total placental antioxidant capacity in the placentas of GD15-21SD dams possibly resulted from superoxide dismutase decrease since the other antioxidant level remained unaffected, pointing to the possible role of increased superoxide anions and nitric oxide (NO) (Beckman and Koppenol, 1996) during this period. Superoxide anion and nitric oxide radicals normally react when they are produced simultaneously in the same compartment to produce a short-lived but powerful reactive specie with far-reaching damaging effects on the tissues called peroxynitrite

(Szabo *et al.*, 2007). The evidently increased placental nitrotyrosine and hydrogen peroxide in the placenta of GD15-21SD also supports this view.

Nitrotyrosine is a detectable footprint of tyrosine nitration caused by peroxynitrite anion ($OONO^-$) (Szabo *et al.*, 2007). It is a marker of cell damage and inflammation (Murata *et al.*, 2004; Rose *et al.*, 2012). The nitration of placental protein causes placental nitrative stress resulting in alteration of the activity in signal transduction pathways (Greenacre and Ischiropoulos, 2001). Increased placental nitration of phosphorylated mitogen-activated protein kinase (p38 MAPK) has been reported in association with pre-eclampsia (Lindsay *et al.*, 1996; Webster *et al.*, 2006). The physiological consequence of this nitration is signal transduction pathway inhibition, although seldom activation has been observed (Greenacre and Ischiropoulos, 2001). Thus, placental tyrosine nitration in the GD15-21SD group suggests an interference with certain signal transduction pathway in their placentas. Although, the particular pathway(s) affected is/are not clear but this interference is possibly related to the programming of the reproductive organs in this group offsprings because placental tyrosine nitration has been implicated in the molecular pathway leading to foetal programming (Myatt, 2006; Jansson and Powell, 2007). Endothelial NO synthase (eNOS) which has a direct relationship with peroxynitrite is upregulated in placental villous tissue in pregnancy complications associated with foetal programming (Myatt *et al.*, 1996). In fact, alteration in the function of key metabolic and signaling pathways by oxidative and nitrative stress has been proposed as a possible general mechanism underlying foetal programming (Myatt, 2006). However, the link between nitration of definite placental proteins and foetal programming still remains elusive.

5.9. Effects of sleep deprivation during gestation on placental morphometric indices, nucleic acid concentration and apoptosis

GD1-7SD placenta expressed more anti-apoptotic factor than the control. Expression of *p53* was reduced in the GD8-14SD placenta and the expression of both pro- and anti-apoptotic factors were suppressed in the placenta of the GD15-21SD group. Associations between abnormal level of apoptosis and a number of gestational pathologies such as was seen in placentas of ectopic pregnancy,

abortions, pre-eclampsia, post-term babies, IUGR and maternal hypertension have been reported (Halperin *et al.*, 2000; Leung *et al.*, 2001). All these pathologies are associated with increased placental apoptosis, however, decreased placental apoptotic activity has been reported in association with gestational diabetes (Magee *et al.*, 2014). This relationship between placental apoptosis and pregnancy complication indicates that factors controlling proliferative activities and factors controlling catabolic activities in the placenta must be optimally regulated (Sharp *et al.*, 2010). This is key to the regulation of placental functions and growth of the foetus (Hayward *et al.*, 2016).

The results suggest that maternal sleep deprivation caused a decreased placental apoptotic activity in all groups. In normal pregnancy, placental apoptosis increases as pregnancy advances (Smith *et al.*, 1997; Sharp *et al.*, 2010). Maintenance of structural and functional integrity of the placental tissue is dependent on balance of cell proliferation, differentiation and apoptosis (Sharp *et al.*, 2010). Thus, a reduced apoptotic activity will compromise placental structure and function. Compromise in placental structure has been reported in association with altered placental morphometric indices (Barker *et al.*, 2010; Hayward *et al.*, 2016) and foetal growth pattern (Ruangvutilert *et al.*, 2002). One such morphometric indicator affected in the GD15-21SD was the foeto-placental ratio. The reduction in this indices suggests that there was reduced transfer of nutrient from the placental circulation to the foetal circulation (Hayward *et al.*, 2016). The foeto-placental ratio refers to the gram of foetus per gram of placenta (Hayward *et al.*, 2016). It therefore means that maternal sleep deprivation during the late gestation caused the placenta to grow bigger than the foetus. The mechanism is not totally clear, however, results from this study showed that apoptosis was suppressed in the placenta of this group. This implies that programmed cellular death were prevented and the placentas had tissues that should have degenerated. This was corroborated by the increased nucleic acid concentration in the placentas of this group. Large placentas have been reported in association with reduced nutrient transfer because the placenta itself uses up much of the nutrients and oxygen leaving the foetus deficient (Jansson and Powell, 2007).

The suggested mechanism that led to a near zero expression of pro-apoptotic factor *p53* in the GD15-21SD group may be related to the aforementioned placental nitrative stress in this group. The physiological consequence of placental protein

nitration is often an indication of inhibition of signal transduction pathway (Greenacre and Ischiropoulos, 2001). This is because after nitration, the protein is changed in structure, as a result, its functions may be changed or absent (Greenacre and Ischiropoulos, 2001). In an *in vitro* study conducted by Myatt (2016), placental *p53* was nitrated in the presence of placental peroxynitrite. Thus, in this study, nitration of placental *p53* is suggested to be the reason why this protein was almost not expressed in the GD15-21SD placenta. Reduced expression of *p53* led to reduced apoptosis in this group. The activation of the mitochondrial apoptotic pathway normally expands the exchange zone between foetal and maternal circulations (Magee *et al.*, 2014). Consequently, the exchange zone between foetal and maternal circulations became compromised when plausibly, *p53* was nitrated and apoptosis was reduced in the GD15-21SD placenta. Reduction in exchange zone would lead to reduction in blood flow, transporters and nutrient transfer and the resultant intra-uterine growth restriction. This seems to be one mechanism responsible for the low birth weight in the offsprings of GD15-21SD group.

5.10. Conclusion

In conclusion, seven days of maternal sleep deprivation during the different gestation periods adversely altered the cardiovascular, renal and reproductive functions of adult male offsprings in Wistar rat dams. The most severely impacted offsprings were those belonging to the dams that were sleep deprived during gestation days 15-21.

Reproductive functions were programmed by maternal sleep deprivation during gestation days 15-21. The cardio-renal functions of the offsprings of this group were also programmed alongside, although the programming of the cardiovascular functions was not exclusive to the GD 15-21.

Generally, the programming in the GD15-21SD group may be associated with exaggerated placental oxidative stress, increased placental protein nitration, reduced placental apoptosis and intra-uterine growth restriction. However, reduced testosterone during the masculinization window suggests that specifically, reproductive dysfunction was programmed as a result of altered masculinization. These are components of the possible placental mechanisms involved.

5.11. Contributions to knowledge

- Maternal sleep deprivation during gestation days 1-7 resulted in increased systolic and diastolic pressures in adult male offsprings of Wistar rats.
- Maternal sleep deprivation during gestation days 15-21 increased serum creatinine level, urinary albumin level, reduced creatinine clearance and reduced blood pressure in adult male offsprings of Wistar rats.
- Sperm profile, serum testosterone level and reproductive organ histology were severely altered in adult male offsprings of dams, sleep deprived during gestation days 15-21. These offsprings exhibited 0% fertility.
- Maternal sleep deprivation during gestation days 15-21 caused placental nitrative stress.
- Maternal sleep deprivation at the three different gestation periods altered placental apoptotic markers in a pattern that suggests decreased placental apoptosis.

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APPENDICES

Effects of maternal sleep deprivation on cardiovascular functions of male offsprings

GROUP	SYSTOLIC PRESSURE (mmHg)	DIASTOLIC PRESSURE (mmHg)	PULSE PRESSURE (mmHg)
GD1-7C	109.60 ± 3.00	78.90 ± 5.70	30.80 ± 7.43
GD1-7SD	144.60 ± 2.70*	122.00 ± 2.80*	22.60 ± 5.00
GD8-14C	110 ± 1.80	77.60 ± 4.20	32.60 ± 5.50
GD8-14SD	119.40 ± 1.60*	85.80 ± 6.30	33.60 ± 5.90
GD15-21C	113.2 ± 3.70	95.20 ± 3.50	18.00 ± 3.42
GD15-21SD	76.60 ±6.30*	59.80 ± 8.70	16.80 ± 4.80

Data are presented as mean ± SEM. n=5. *=Significant difference from corresponding control ($p<0.05$) based on Student's t-test. GD=Gestation Day. C = Control. SD =Sleep deprived

Effects of maternal sleep deprivation on relative heart weight of male offsprings

GROUP	RELATIVE WEIGHT OF HEART
GD1-7C	0.30 ± 0.02
GD1-7SD	0.33 ± 0.02
GD8-14C	0.31 ± 0.01
GD8-14SD	0.33 ± 0.01
GD15-21C	0.32 ± 0.02
GD15-21SD	0.35 ± 0.02

Data are presented as mean ± SEM. n=5. Analysis was based on Student's t-test. GD=Gestation Day. C = Control. SD =Sleep deprived

Effects of maternal sleep deprivation on serum albumin level of male offsprings

GROUP	SERUM ALBUMIN (ng/dL)
GD1-7C	31.28 ± 3.32
GD1-7SD	27.28 ± 2.25
GD8-14C	27.93 ± 1.12
GD8-14SD	28.07 ± 1.67
GD15 21C	29.51 ± 0.72
GD15-21SD	30.30 ± 2.23

Data are presented as mean ± SEM. n=5. Analysis was based on Student's t-test. GD=Gestation Day. C = Control. SD =Sleep deprived

Effects of maternal sleep deprivation on serum creatinine level of male offsprings

GROUP	SERUM CREATININE (mg/dL)
GD1-7C	2.00 ± 0.10
GD1-7SD	2.20 ± 0.13
GD8-14C	3.40 ± 0.18
GD8-14SD	3.80 ± 0.31
GD15 21C	2.00 ±0.14
GD15-21SD	7.00 ± 0.20*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on serum urea level of male offsprings

GROUP	SERUM UREA (mg/dL)
GD1-7C	19.46 ±0.33
GD1-7SD	18.38 ±0.50
GD8-14C	11.03 ± 0.46
GD8-14SD	13.31 ± 0.67
GD15 21C	20.33 ± 0.93
GD15-21SD	22.31 ± 0.96

Data are presented as mean ± SEM. n=5. ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on urinary albumin level of male offsprings

GROUP	URINARY ALBUMIN (ng/dL)
GD1-7C	24.04 ± 1.81
GD1-7SD	26.10 ± 4.54
GD8-14C	12.71 ± 3.77
GD8-14SD	16.17 ± 0.39
GD15 21C	17.62 ± 2.81
GD15-21SD	46.89 ± 5.99*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on renal creatinine clearance of male offsprings

GROUP	CREATININE CLEARANCE (mL/min)
GD1-7C	0.09 ± 0.01
GD1-7SD	0.10 ± 0.01
GD8-14C	0.11 ± 0.03
GD8-14SD	0.06 ± 0.01
GD15 21C	0.05 ± 0.00
GD15-21SD	0.03 ± 0.00*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep on relative weight of the kidney of male offsprings

GROUP	RELATIVE WEIGHT OF KIDNEY
GD1-7C	0.35 ± 0.04
GD1-7SD	0.33 ± 0.02
GD8-14C	0.31 ± 0.01
GD8-14SD	0.42 ± 0.07
GD15-21C	0.32 ± 0.02
GD15-21SD	0.29 ± 0.01

Data are presented as mean ± SEM. n=5. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on testes descent and preputial separation of male offsprings

GROUP	TESTES DESCENT (DAYS)	PREPUTIAL SEPARATION (DAYS)
GD1-7C	24.20 ± 1.82	43.80 ± 4.42
GD1-7SD	23.00 ± 0.83	44.60 ± 1.16
GD8-14C	22.30 ± 0.58	45.00 ± 1.58
GD8-14SD	22.80 ± 1.58	45.50 ± 1.74
GD15-21C	23.00 ± 0.77	43.2 ± 2.24
GD15-21SD	25.80 ± 0.37*	42.4 ± 0.24

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on serum corticosterone concentration of male offsprings

GROUP	CORTICOSTERONE (nmol/L)
GD1-7C	57.20 ± 19.52
GD1-7SD	106.00 ± 3.03*
GD8-14C	65.80 ± 5.85
GD8-14SD	115.00 ± 8.22*
GD15-21C	96.60 ± 9.38
GD15-21SD	131.60 ± 6.55*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on serum Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) of male offsprings

GROUP	FSH (mIU/mL)	LH (mIU/mL)
GD1-7C	1.76 ± 0.14	11.22 ± 0.63
GD1-7SD	1.83 ± 0.11	12.09 ± 0.12
GD8-14C	1.95 ± 0.26	12.47 ± 0.22
GD8-14SD	2.53 ± 0.52	12.34 ± 0.21
GD15-21C	1.85 ± 0.14	12.22 ± 0.32
GD15-21SD	1.83 ± 0.08	12.0 ± 0.12

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on serum testosterone concentration of male offsprings

GROUP	TESTOSTERONE (ng/mL)
GD1-7C	5.95 ± 1.79
GD1-7SD	2.46 ± 1.06
GD8-14C	4.95 ± 0.90
GD8-14SD	4.43 ± 0.79
GD15-21C	7.00 ± 1.31
GD15-21SD	2.86 ± 1.05*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on serum melatonin concentration of male offsprings

GROUP	MELATONIN (nmol/L)
GD1-7C	355.33 ± 19.21
GD1-7SD	482.14 ± 33.49*
GD8-14C	353.57 ± 20.53
GD8-14SD	237.29 ±15.07*
GD15-21 C	373.57 ±47.09
GD15-21SD	445.78 ±19.34

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on sperm profile of male offsprings

GROUP	SPERM VIABILITY (%)	SPERM MOTILITY (%)	SPERM COUNT (Million/mL)
GD1-7C	94.40 ± 1.16	91.00 ± 1.87	96.20 ± 14.77
GD1-7SD	93.60 ± 1.57	84.00 ± 8.57	94.20 ± 16.99
GD8-14C	92. 00 ± 1.22	89.00 ± 1.87	105.40 ± 7.23
GD8-14SD	95.00 ± 1.38	91.00 ± 2.92	111.80 ± 15.61
GD15-21 C	95.20 ± 1.46	89.00 ± 2.92	114.5 ± 3.00
GD15-21SD	94.60 ± 1.29	72.00 ± 4.90*	64.4 ± 14.88*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on serum progesterone concentration of pregnant dams

GROUP	PROGESTERONE (ng/mL)
GD1-7C	1.02 ± 0.02
GD1-7SD	1.00 ± 0.01
GD8-14C	0.96 ± 0.02
GD8-14SD	0.89 ± 0.01
GD15-21C	0.91 ± 0.02
GD15-21SD	0.81 ± 0.01

Data are presented as mean ± SEM. n=5. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on serum prolactin concentration of pregnant dams

GROUP	PROLACTIN (ng/mL)
GD1-7C	0.37 ± 0.01
GD1-7SD	0.64 ± 0.03
GD8-14C	0.36 ± 0.01
GD8-14SD	0.35 ± 0.01
GD15-21C	0.34 ± 0.01
GD15-21SD	0.38 ± 0.02

Data are presented as mean ± SEM. n=5. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on serum testosterone concentration of pregnant dams

GROUP	TESTOSTERONE (pg/mL)
GD1-7C	230.00 ± 23.23
GD1-7SD	286.00 ± 9.92
GD8-14C	196.00 ± 32.96
GD8-14SD	282.00 ± 32.96
GD15-21C	370.00 ± 38.19
GD15-21SD	216.00 ± 5.77*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on corticosterone level of pregnant dams

GROUP	CORTICOSTERONE (nmol/L)
GD1-7C	108.61 ± 16.18
GD1-7SD	122.20 ± 24.98
GD8-14C	108.80 ± 16.54
GD8-14SD	112.40 ± 13.30
GD15-21C	117.40 ± 14.19
GD15-21SD	118.20 ± 12.44

Data are presented as mean ± SEM. n=5. Analysis was based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on melatonin level of pregnant Wistar rat dams

GROUP	CORTICOSTERONE (nmol/L)	MELATONIN (nmol/L)
GD1-7C	108.61 ± 16.18	420.75 ± 17.59
GD1-7SD	122.25 ± 4.98	260.29 ± 50.7*
GD8-14C	108.80 ± 16.54	75.34 ± 10.63
GD8-14SD	112.40 ± 13.30	309.59 ± 71.92*
GD15-21 C	117.40 ± 14.19	377.41 ± 40.66
GD15-21SD	118.20 ± 12.44	421.65 ± 60.29

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental malondialdehyde level

GROUP	MALONDIALDEHYDE (nmol/mg protein)
GD1-7C	0.03 ± 0.00
GD1-7SD	0.08 ± 0.01*
GD8-14C	0.11 ± 0.01
GD8-14SD	0.06 ± 0.00
GD15-21 C	0.06 ± 0.00
GD15-21SD	0.16 ± 0.01*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental hydrogen peroxide

GROUP	HYDROGEN PEROXIDE (µM)
GD1-7C	4.85 ± 0.09
GD1-7SD	4.99 ± 0.12
GD8-14C	4.97 ± 0.20
GD8-14SD	4.41 ± 0.38
GD15-21C	3.39 ± 0.01
GD15-21SD	4.80 ± 0.09*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental Dichlorofluorescein (DCF) level

GROUP	DCF(nM)
GD1-7C	86445.16 ± 2867.13
GD1-7SD	90446.86 ± 4002.30
GD8-14C	89984.32 ± 6529.36
GD8-14SD	73763.91 ± 12273.86
GD15-21C	44398.07 ± 303.91
GD15-21SD	76717.44 ± 9569.81*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental nitrotyrosine level

GROUP	NITROTYROSINE (ng/mL)
GD1-7C	1990.00 ± 226.71
GD1-7SD	2290.00 ± 315.28
GD8-14C	2530.00 ± 413.04
GD8-14SD	2610.00 ± 188.68
GD15-21 C	1190.00 ± 386.52
GD15-21SD	2350.00 ± 392.94*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on total antioxidant capacity of pregnant Wistar rat dams

GROUP	TOTAL ANTIOXIDANT CAPACITY (mmol/L)
GD1-7C	1.76 ± 0.04
GD1-7SD	2.15 ± 0.30
GD8-14C	2.43 ± 0.36
GD8-14SD	2.79 ± 0.47
GD15-21 C	4.13 ± 0.67
GD15-21SD	2.35 ± 0.14*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental glutathione level

GROUP	GLUTATHIONE(mM)
GD1-7C	5.21 ± 0.12
GD1-7SD	6.03 ± 0.54
GD8-14C	8.55 ± 0.14
GD8-14SD	10.14 ± 0.35*
GD15-21 C	7.88 ± 0.11
GD15-21SD	10.11 ± 0.58*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental superoxide dismutase level

GROUP	SUPEROXIDE DISMUTASE (Unit/mg protein)
GD1-7C	22.90 ± 6.99
GD1-7SD	12.10 ± 1.12
GD8-14C	59.78 ± 7.13
GD8-14SD	27.09 ± 7.59*
GD15-21 C	102.99 ± 11.72
GD15-21SD	62.92 ± 5.57*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental catalase activity

GROUP	CATALASE (Unit/mg protein)
GD1-7C	5.30 ± 0.03
GD1-7SD	5.18± 0.05
GD8-14C	5.33 ± 0.11
GD8-14SD	5.28 ± 0.04
GD15-21C	5.32 ± 0.01
GD15-21SD	5.32 ± 0.02

Data are presented as mean ± SEM. n=5. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental glutathione peroxidase activity

GROUP	GLUTATHIONE PEROXIDASE (Unit/mg protein)
GD1-7C	44.16 ± 7.90
GD1-7SD	33.65 ± 5.80*
GD8-14C	32.80 ± 6.96
GD8-14SD	33.65 ± 10.97
GD15-21C	35.27 ± 4.69
GD15-21SD	52.99 ± 5.74*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on placental total protein content

GROUP	TOTAL PROTEIN (mg/mL)
GD1-7C	62.02 ± 1.58
GD1-7SD	64.23 ± 5.63
GD8-14C	64.09 ± 4.32
GD8-14SD	65.75 ±3.94
GD15-21 C	73.61 ± 4.82
GD15-21SD	70.67 ± 1.41

Data are presented as mean ± SEM. n=5 GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of maternal sleep deprivation on placental nucleic acid concentration

GROUP	NUCLEIC ACID CONCENTRATION (ng/µL)
GD1-7C	136.10 ± 3.09
GD1-7SD	126.54 ± 2.85
GD8-14C	113.44 ± 2.15
GD8-14SD	110.22 ± 3.49
GD15-21 C	117.40 ± 1.38
GD15-21SD	155.68 ± 2.80*

Data are presented as mean ± SEM. n=5. *=significant difference from corresponding control group ($p<0.05$) based on Student's t-test. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of sleep deprivation on placental global DNA methylation status

GROUP	GLOBAL DNA METHYLATION STATUS (mg/mL)
GD1-7C	971.00 ± 84.64
GD1-7SD	1082.00 ± 19.66
GD8-14C	1108.00 ± 42.72
GD8-14SD	1075.00 ± 28.81
GD15-21 C	740.00 ± 94.68
GD15-21SD	733.00 ± 133.14

Data are presented as mean ± SEM. n=5. GD=Gestation Day. C=Control. SD=Sleep Deprived

Effects of Vitamin E and Melatonin on Testosterone, Corticosterone and Melatonin levels in Sleep deprived Wistar Rats.

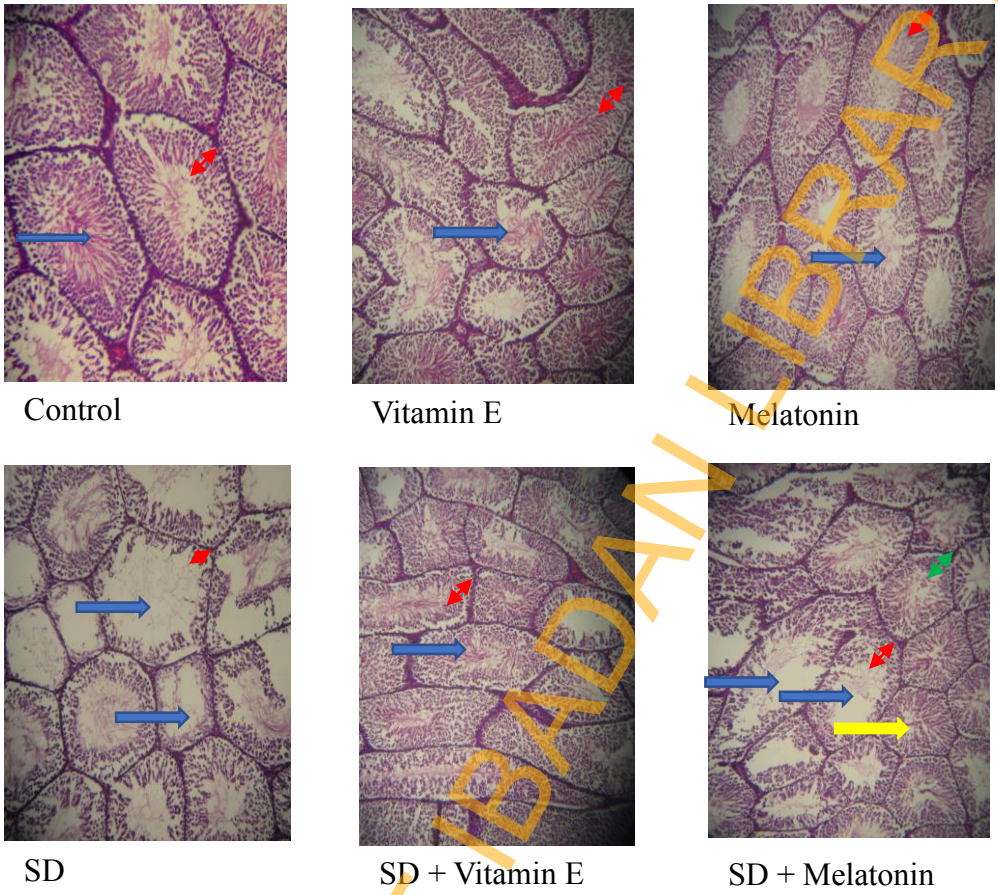
Group	Control	SD	Vitamin E	SD+ Vitamin E	M	SD+ M
T (nmol/ml)	3.32 ± 0.40	0.64 ± 0.30*	3.90 ± 0.60^{+}	2.80 ± 0.50$^{\#}$	3.60 ± 0.60^{+}	2.00 ± 0.30*$^{\#}$
M (nmol/l)	19.30 ± 5.60	15.50 ± 7.0	29.00 ± 3.90	14.70 ± 7.00	8.60 ± 1.90	28.30 ± 12.40
C (nmol/l)	6.30 ± 0.60	24.10 ± 13.20	16.50 ± 7.20	51.60 ± 0.50*	36.60 ± 29.20	10.30 ± 6.60

Data are expressed in mean ± SEM. n=5. *P<0.05 when compared with control, $^{\#}$p<0.05 compared with the SD group based on ANOVA. SD=Sleep deprived. T = Testosterone. M = Melatonin. C = Corticosterone

Effects vitamin E and melatonin on testicular redox status in sleep deprived Wistar rats

Group	MALONDIALDEHYDE (Unit/mg protein)	SUPEROXIDE DISMUTASE (Unit/mg protein)	CATALASE (Unit/mg protein)
Control	0.90 ± 0.00	1.90 ± 0.10	44.30 ± 1.10
SD	1.60 ± 0.10*	3.20 ± 0.20*	49.10 ± 1.10*
Vitamin E	1.10 ± 0.10$^{\#}$	2.20 ± 0.20$^{\#}$	44.10 ± 1.70$^{\#}$
SD + Vitamin E	1.10 ± 0.20$^{\#}$	2.40 ± 0.30$^{\#}$	39.40 ± 1.00 *$^{\#}$
Melatonin	1.70 ± 0.20*	3.50± 0.40*	46.20±1.60
SD + Melatonin	1.30 ± 0.10	2.70 ± 0.20*	45.50 ± 1.80

Data are expressed in mean ± SEM. n=5. *P<0.05 when compared with Control, $^{\#}$p<0.05 compared with the SD group based on ANOVA. SD=Sleep Deprived.



Photomicrograph of testicular sections from control and sleep deprived male Wistar rats

Sections were stained with H&E and presented at x100 magnification. SD = Sleep deprived. Photomicrograph of testicular section from control, Vitamin E, melatonin and SD + Vitamin E rats show seminiferous tubules with germinal layer (red arrow) and normal lumen containing spermatozoa (blue arrow). Photomicrograph of testicular section from SD rat shows seminiferous tubules with wide lumen and few spermatozoa (blue arrow). The height of germinal epithelium is reduced (red arrow). Photomicrograph of testicular section from melatonin treated SD rat shows seminiferous tubules with some reduced germinal epithelium (red arrow). Some lumen appear with few strands of spermatozoa (blue arrow). Some seminiferous tubules appear normal (yellow arrow).