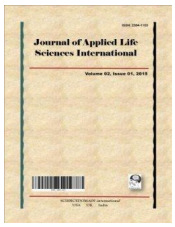

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Maternal Sleep Deprivation Alters Reproductive Capability of Male Offspring in Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OOA and YR designed the study. Author OOA wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Author OTKA edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Existing data suggest a negative correlation between maternal sleep deprivation and male offspring reproductive capability. However, there is dearth of information on the critical period of development during which reproductive organs may be programmed. Thirty pregnant rats were divided into six groups based on the Gestation Days (GD) during which they were sleep deprived as follows: GD 1-7 control, GD 1-7 sleep deprived, GD 8-14 control, GD 8-14 sleep deprived, GD 15-21 control and GD 15-21 sleep deprived. Sleep deprivation was induced using the Modified Multiple Platform Method. Morphometric indices of pups were measured at parturition. Testes descent and preputial separation were monitored. Fertility index was determined on Post Natal Week (PNW) 17. Reproductive organs were harvested at sacrifice on PNW 25. Organs were weighed on electronic scale; histology of the testes and epididymes was done; sperm profile was assessed by microscopy; FSH, LH and testosterone were measured using ELISA kits. Offspring of GD 15-21 sleep deprived dams had significantly reduced birth weight and increased crown-rump length. Crown-rump length in the GD 8-14 sleep deprived group was increased. Testes descent occurred later in the GD15-21 sleep deprived group. Sperm motility, sperm count and serum testosterone

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were reduced in the GD 15-21 sleep deprived group. Fertility index was 0% in the GD 15-21 sleep deprived group. The histology of the testes and epididymes of the GD 15-21 sleep deprived group offspring showed severe aberrations. These suggests that the critical period during which fetal male reproductive organ development is adversely affected by maternal sleep deprivation is GD 15-21.

Keywords: Maternal sleep; critical period; deprivation; testis descent; sperm profile.

1. INTRODUCTION

Sleep is an active state of reversible unconsciousness with relatively reduced responsiveness and alertness [1]. The functions of sleep have not been fully explicated, however, many studies have shown its vital involvement in maintenance of the internal environment [2]. Due to its requisite role in homeostasis, adequate sleep is essential for the wellbeing and survival of an individual [3]. However, sleep has been relegated to a secondary level of importance, because the global population has adopted the popular contemporary lifestyle of the 21st century [4,5]. The impact of this lifestyle on sleep is a state of inadequate quantity and quality of sleep referred to as "sleep deprivation" [6].

Sleep deprivation is gradually becoming a public health issue of global dimensions [7]. Women are prone to experience sleep deprivation probably because they often try to balance family and career demands [8]. Also, it is not uncommon for sleep deprivation to increase during pregnancy because of the pregnancy-related sleep disorders caused by physical, hormonal and behavioural changes [9]. Sleep restriction during pregnancy not only increases the risk of psychiatric disorders in mothers [10], it also causes preterm birth [11].

Epidemiological and experimental studies have shown that adverse early life events may increase the risk of reproductive dysfunction in adult life [12]. In view of this, there have been concerns on the effects of early life on reproductive health, particularly in the light of declining male fertility [13]. Furthermore, altered hormonal levels and sexual behaviours of adult offspring of rats whose mothers were subjected to sleep deprivation throughout the whole length of pregnancy have been reported [14]. However, there is dearth of information on the critical period of development during which maternal sleep deprivation programs the male offspring reproductive functions. The study therefore examined the effects of maternal sleep deprivation at different gestation periods on reproductive capability of adult male offspring of Wistar rats.

2. MATERIALS AND METHODS

2.1 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. They were housed in well aerated plastic cages and had access to rodent's pelletized feed (Ladokun feed mill, Ibadan, Nigeria) and drinking water *ad libitum*. All animals were acclimatized to the environmental condition of the laboratory for two weeks before the commencement of the study. The experimental protocols and procedures used in this study conformed to the guide for care and use of laboratory animals [15] and were approved by the Departmental Committee on the Use and Care of Animal.

2.2 Sleep Deprivation Protocol

The paradigm used for the induction of sleep deprivation was the Modified Multiple Platform Model [16]. It is made up of a glass tank consisting of sixteen circular platforms of 6.5 cm in diameter. The glass tank 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 chamber had a wire mesh cover to ensure proper aeration. Feeders and drinkers were attached to the cover. The control rats were placed in chambers which had similar features as the test chamber, the difference being that it consisted of a glass barrier placed on the platforms. This glass barrier enabled the animals to sleep at will.

2.3 Animal Grouping

Pregnant rats were randomly assigned into six groups (n=5) as described below:

Group 1 -	GD 1-7 CONTROL -	Pregnant rats were placed in the control tank from GD 1 - GD 7.
Group 2 -	GD1-7 SLEEP DEPRIVED -	Pregnant rats were placed in the sleep deprivation tank from GD 1 - GD 7.
Group 3 -	GD8-14 CONTROL-	Pregnant rats were placed in control tank from GD 8 - GD 14.
Group 4 -	GD8-14 SLEEP DEPRIVED -	Pregnant rats were placed in the sleep deprivation tank from GD 8 - GD 14.
Group 5 -	GD15-21 CONTROL -	Pregnant rats were placed in control tank from GD 15 – GD 21.
Group 6 -	GD15-21 SLEEP DEPRIVED -	Pregnant rats were placed in the sleep deprivation tank from GD 15 – GD 21.

2.4 Parturition and Postnatal Studies

All pregnant rats were allowed to litter naturally and the day of parturition was designated as Post-Natal Day (PND) 1. Morphometric indices were taken within 24 hours of post-natal life. Only the male pups were retained in the study and allowed to be nursed by their natural mothers. Testes descent and preputial separation were monitored for each offspring. They were weaned on PND 28 (Post-Natal Week (PNW) 4) and were pooled into groups depending on maternal gestational treatment. On PNW 17, the male offspring were paired with proven female breeders for determination of fertility index.

2.5 Pup Morphometry

Pups were weighed individually on an electronic scale (Lisay, China). The anogenital distance (AGD) and crown-rump length were measured using the digital Vernier caliper (Mitutoyo, Japan).

2.6 Determination of Testes Descent and Preputial Separation

Beginning from PND 15, the scrotal sac was gently palpated daily for testicular presence. Monitoring of preputial separation began on PND 35 through daily manual retraction of the prepuce until it separated totally from the shaft of the penis [17].

2.7 Fertility Test

The rats were paired with female rats at ratio 1:2 (male to female) during PNW 17 for two weeks. Fertility index (number of cohabited females becoming pregnant / number of non-pregnant couples * 100) and gestation index (number of females delivering live young / number of females with evidence of pregnancy * 100) were calculated [17].

2.8 Specimen Collection

Animals were anaesthetized with thiopental (i.p., 50 mg kg^{-1}) [18] after which they were bled via cardiac puncture. The testes, epididymes, seminal vesicles and prostate glands were harvested from the adult offspring and freed of adherent tissues before being weighed on the digital electronic scale. The testes were fixed in Bouin's fluid and the epididymes were fixed in 10% formalin in preparation for histological examination.

2.9 Epididymal Sperm Profile Analysis

One of the caudal epididymes was collected for sperm profile analysis [19]. Epididymal Sperm viability was carried out as follows: two drops of eosin / nigrosin stain was added to a drop of the epididymal fluid which was placed on a glass slide. A thick smear was made, dried and studied under a microscope (XS2 107, China) using 40x objective lens. The live sperm cells were unstained while the dead sperm cells were stained. The percentage of live sperm cells was calculated. Sperm motility was done immediately and quickly. 2 drops of warm 2.9% sodium citrate was added to a drop of epididymal fluid on a glass slide. This was then covered with a cover slip and examined under the microscope using 40x objective lens of the light microscope to assess motility. Sperm motility was expressed in percentage. Sperm count was done using the Neubauer counting chamber. The results were

expressed in million/ml. The caudal epididymis was homogenized in a known volume of formal saline. It was further diluted to a total dilution factor of 200. Using the Neubauer counting chamber, spermatic cells in five big squares were counted. Sperm count (Million/mL) = Number of cells counted in 5 squares * 250 * 200 (dilution factor) * 1000.

2.10 Preparation of Histological Slides

Tissue preparation was carried out using the conventional paraffin embedding method. Tissue sections were stained with Haematoxylin and Eosin (H&E) to determine the general morphology.

2.11 Hormonal Assay

ELISA kits were used to measure serum levels of Follicle Stimulating Hormone (FSH) (Fortress Diagnostics, United Kingdom), Luteinizing Hormone (LH) (Fortress Diagnostics, United Kingdom) and testosterone (Cloud Clone Corp., USA). The intra-assay and inter-assay coefficient of variability for FSH are 3.80% and 4.50%. The intra-assay and inter-assay coefficient of variability for LH are 4.80% and 5.6%. The intra-assay and inter-assay coefficient of variability for testosterone are < 10% and < 12% respectively. The detection limit for the FSH, LH and testosterone kits are; 0.8-43.2 mIU/mL, 0.8-42.7 mIU/mL and 0.1-10 ng/mL respectively.

2.12 Statistical Analysis

Data were summarized and expressed as mean and Standard Error of Mean (mean ± SEM). Differences in means were compared by Student's *t* test. 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.

3. RESULTS

3.1 Effects of Maternal Sleep Deprivation on Morphometric Indices at Birth

Birth weight was significantly reduced in the GD15-21 sleep deprived group. Crown - rump length was increased in the GD 8-14 sleep deprived and GD 15-21 sleep deprived group.

3.2 Effects of Maternal Sleep Deprivation on Testes Descent and Preputial Separation

Testes descent occurred significantly later ($p<0.05$) in offspring of GD15-21 sleep deprived dams compared with the control group. Preputial separation was however, not affected (Fig. 1).

3.3 Effects of Maternal Sleep Deprivation on Relative Organ Weight

Relative organ weights of offspring of control and sleep deprived groups did not show any significant different.

3.4 Effects of Maternal Sleep Deprivation on Histology of the Testis

The testes belonging to the sleep deprived groups showed different levels of abnormalities. The testes of GD1-7 Sleep Deprived group had some seminiferous tubules with partial loss of basal membrane and germinal layer. GD8-14 Sleep Deprived had normal seminiferous tubules, however, the interstitial spaces appear wide. GD15-21 Sleep Deprived group had total aberration of testicular tissue (Fig. 2).

Table 1. Morphometric indices of offspring of control and sleep deprived rats at birth

Group	Birth weight (g)	Crown-rump length (mm)	Anogenital distance index ($mm g^{-3}$)
GD 1-7 control	5.44 ± 0.22	45.63 ± 1.40	2.1 ± 0.40
GD 1-7 sleep deprived	5.60 ± 0.37	45.67 ± 0.78	2.1 ± 0.10
GD 8-14 control	5.91 ± 0.31	46.75 ± 0.07	1.7 ± 0.19
GD 8-14 sleep deprived	6.12 ± 0.25	47.49 ± 0.07 *	1.6 ± 0.28
GD 15-21 control	5.70 ± 0.20	47.14 ± 0.05	1.8 ± 0.13
GD 15-21 sleep Deprived	5.27 ± 0.10*	48.96 ± 0.10 *	1.8 ± 0.38

*Data are presented as mean ± SEM. n=5. * represents significant difference from corresponding control (*p<0.05) based on Student's t test*

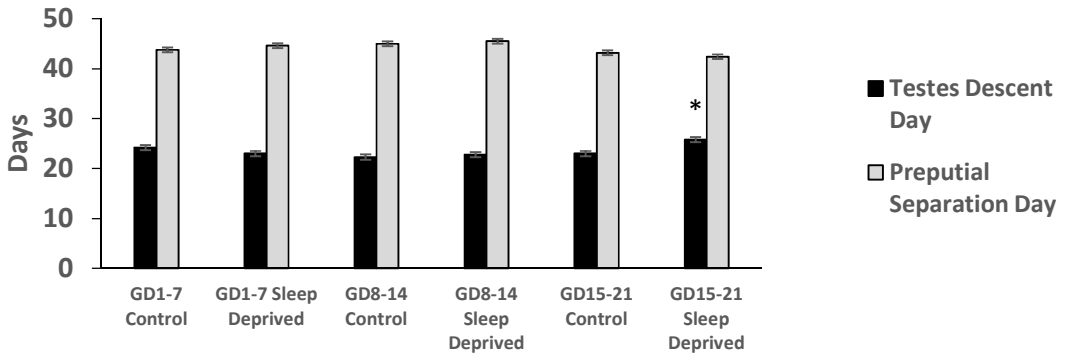

Fig. 1. Testes descent and preputial separation in male offspring of control and sleep deprived dams

*Data are presented as columns and error bars which represent mean ± SEM. n=5. * represents significant difference from corresponding control. (p<0.05) based on Student's t test. GD=Gestation Day*

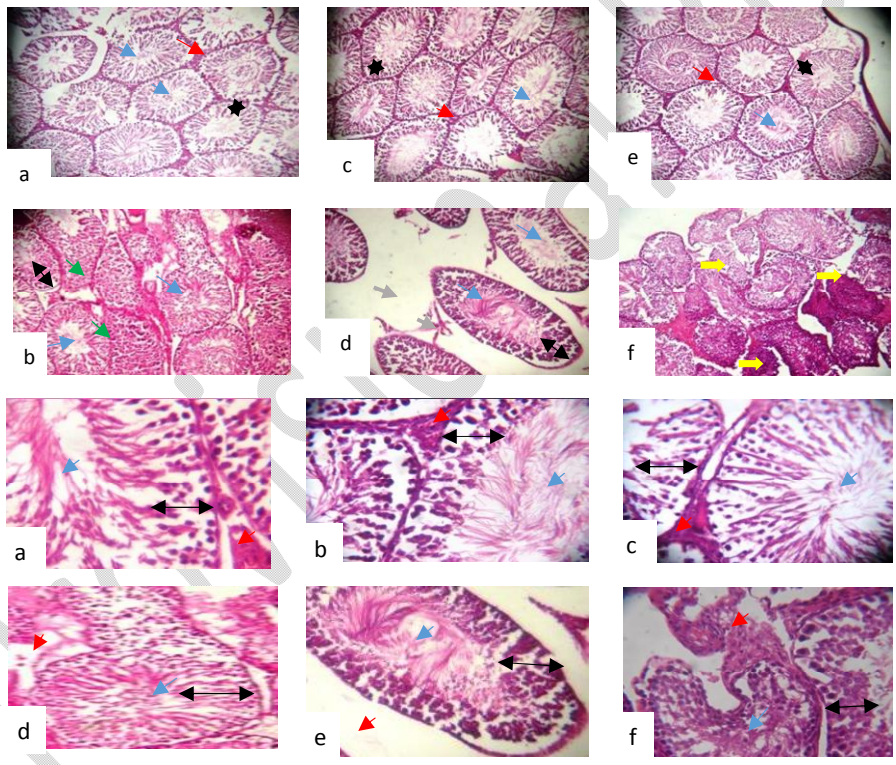

Fig. 2. Photomicrographs of testicular sections of adult male offspring of control and sleep deprived Wistar rat dams. a=GD1-7 Control, b=GD1-7 Sleep Deprived, c=GD8-14 Control, d=GD8-14 Sleep Deprived, e=GD15-21 Control, f=GD15-21 Sleep Deprived. It shows regular seminiferous tubules with lumen containing mature spermatozoa (blue arrow), maturing germinal cell layer (black arrow) in a, b, c, d &e. Normal interstitial spaces (red arrow) are observed in a, b, c &e. The interstitial spaces appear wide with reduced interstitial cells (gray arrow) in d. The irregularly shaped seminiferous tubules have collapsed lumen, no distinct geminal cell layer and signs of necrosis (yellow arrow) in f. Tissues were stained by H&E and presented at 100x and 400x magnifications

Table 2. Relative organ weight of male offspring of control and sleep deprived dams

	Testis	Epididymis	Seminal vesicle	prostate gland	Adrenal gland
GD1-7 control	0.49 ± 0.02	0.19 ± 0.02	0.41 ± 0.08	0.12 ± 0.02	0.01 ± 0.001
GD1-7 sleep deprived	0.47 ± 0.02	0.25 ± 0.03	0.29 ± 0.05	0.11 ± 0.02	0.02 ±0.001
GD8-14 control	0.41 ± 0.02	0.22 ± 0.01	0.38 ± 0.07	0.12 ± 0.02	0.01 ± 0.002
GD8-14 sleep Deprived	0.50 ± 0.05	0.22 ± 0.03	0.34 ± 0.04	0.15 ± 0.02	0.01 ± 0.001
GD15-21 Control	0.46 ± 0.02	0.23 ± 0.02	0.34 ± 0.08	0.11 ± 0.03	0.02 ± 0.002
GD15-21 sleep deprived	0.50 ± 0.01	0.19 ± 0.04	0.46 ± 0.03	0.17 ± 0.01	0.01 ± 0.001

Data are presented as mean ± SEM. n=5. GD=Gestation Day

Table 3. Serum FSH, LH and testosterone of offspring of control and sleep deprived dams

Group	FSH (mIU/mL)	LH (mIU/mL)	Testosterone (ng/mL)
GD1-7 control	1.76 ± 0.14	11.22 ± 0.63	6.00 ± 1.79
GD1-7 sleep deprived	1.83 ± 0.11	12.09 ± 0.12	2.46 ± 1.06
GD8-14 control	1.95 ± 0.26	12.47 ± 0.22	4.95 ± 0.90
GD8-14 sleep deprived	2.53 ± 0.52	12.34 ± 0.21	4.43 ± 0.79
GD15-21 control	1.85 ± 0.14	12.22 ± 0.32	7.00 ± 1.31
GD15-21 sleep deprived	1.83 ± 0.08	12.09 ± 0.12	2.86 ± 1.05*

*Data are presented as mean ± SEM. n=5. * represents significant difference from corresponding control. (p<0.05) based on Student's t test. GD=Gestation Day*

3.5 Effects of Maternal Sleep Deprivation on Histology of the Epididymis

The epididymes of GD1-7 Sleep Deprived and GD8-14 Sleep Deprived groups had normal epididymal ducts, however, some of the ducts appear empty. GD15-21 Sleep Deprived group had collapsed ducts (Fig. 3).

3.6 Effects of Maternal Sleep Deprivation on Sperm Profile

Epididymal sperm viability was not significantly affected across the groups. Maternal sleep deprivation significantly reduced epididymal sperm motility ($p<0.05$) (Fig. 4) and epididymal sperm count ($p<0.001$) in offspring of GD15-21 sleep deprived dams (Fig. 5). All comparisons were done against their respective control groups.

3.7 Effects of Maternal Sleep Deprivation on Hormone Concentration

There were no significant differences in levels of follicle stimulating hormone and luteinizing hormone across the groups. Testosterone concentration was reduced in all groups. However, this reduction was only significant in the GD 15-21 sleep deprived group.

3.8 Effect of Maternal Sleep Deprivation on Fertility of Male Offspring of Sleep Deprived Female Wistar Rats

Four out of five male offspring of GD1-7 control, GD1-7 sleep deprived, GD8-14 sleep deprived and GD15-21 Control dams mated successfully with the control female rats they were paired with. GD8-14 control had 100% fertility index while none of the male offspring of GD15-21 sleep deprived dams were able to have offspring of their own.

4. DISCUSSION

The increasing growth of interest in the field of developmental programming of physiological systems has been more focused on the non-communicable diseases (NCDs) [20]. While these NCDs may lead to early mortality [21], infertility, resulting from the inability of sperm cells to fertilize an ovum may threaten the continuity of a whole generation. In animals, sleep deprivation resulted into testosterone decrease [22,23] and testosterone decrease during the period of fetal organ differentiation

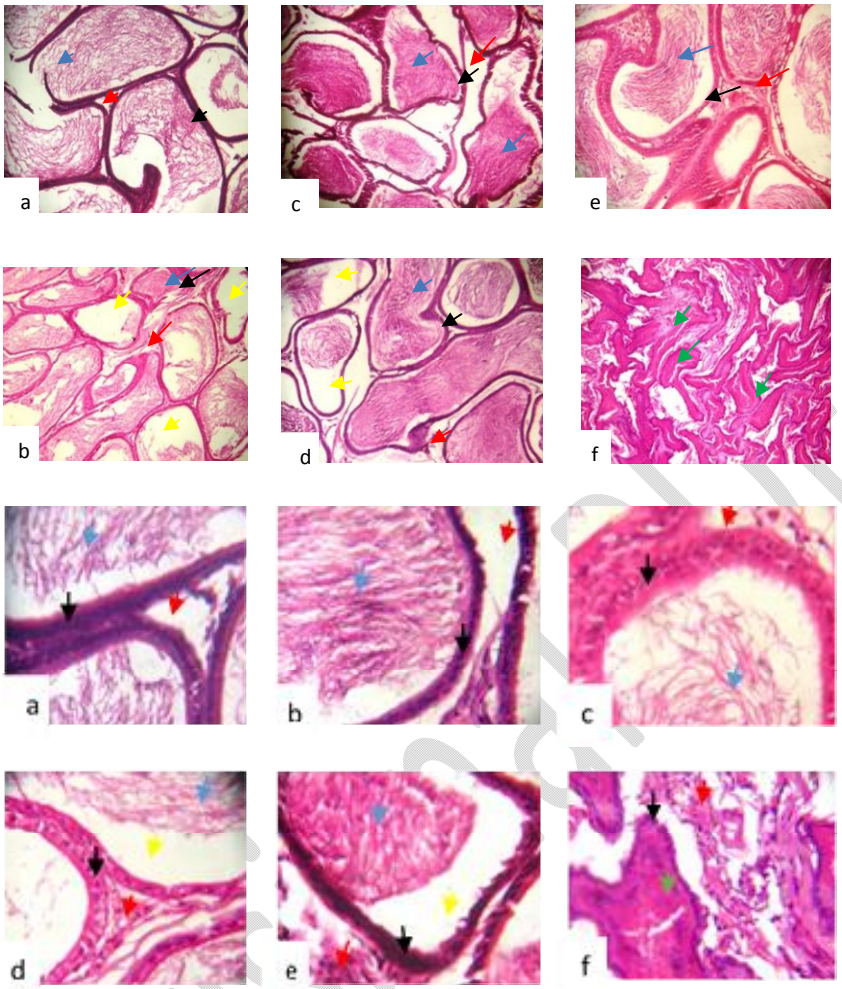

Fig. 3. Photomicrographs of epididymal sections of adult male offspring of control and sleep deprived Wistar rat dams. a=GD1-7 Control, b=GD1-7 Sleep Deprived, c=GD8-14 Control, d=GD8-14 Sleep Deprived, e=GD15-21 Control, f=GD15-21 Sleep Deprived showing regular epididymal duct (black arrow) with lumen containing mature spermatozoa (blue arrow) and normal interstitium (red arrow) in a, b, c, d &e. Some ducts show partially empty lumen (yellow arrow) in b & d. The irregularly shaped epididymal ducts have collapsed lumen (green arrow) and general signs of severe epididymal damage in f. Tissues were stained by H&E and presented at 100x and 400x magnifications

has been reported in association with male offspring reproductive dysfunction [24]. Few studies have examined the impacts of maternal sleep deprivation on the reproductive functions of male offspring, but none of these studies considered the critical period during which the reproductive system of the male offspring may be adversely programmed. The present study evaluated the reproductive functions of male offspring of Wistar dams subjected to sleep deprivation during the three 'thirds' of rat gestation; GD 1-7, 8-14 and 15-21.

The reduced birth weight observed in the GD 15-21 offspring of sleep deprived dams support reports of a recent epidemiological survey which revealed that infants born to mothers who experienced aberrant sleep patterns during pregnancy had intrauterine growth restriction and low birth weight [25]. The reduced birth weight also occurred in concert with increased crown-rump length which means that the pups were thinner, thus, confirming the occurrence of asymmetric intrauterine growth restriction and a high risk of future poor health and early mortality in this group [26]. While the birth weight was not

affected in the GD 8-14 sleep deprived, crown-rump length was increased. Recent animal studies showed that when a fetus is programmed, its birth weight may not be influenced [27]. A more detailed perusal of the popular Dutch Hunger Winter Study also revealed that fetal exposures that affected adult health did not inevitably result in altered birth weight [28]. Therefore, GD 8-14 sleep deprived group offspring may have experienced programming.

Table 4. Fertility index of adult male offspring of control and sleep deprived dams

Groups	Fertility index (%)	Gestational index (%)
GD1-7 Control	80	80
GD1-7 sleep deprived	80	80
GD8-14 Control	100	100
GD8-14 sleep Deprived	80	80
GD15-21 Control	80	80
GD15-21 sleep Deprived	0	0

Data are presented in percentage. GD=Gestation Day

The delayed onset of puberty signified by late testes descent that was observed in the GD 15-21 offspring 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 [29]. The serum testosterone was not measured during the pubertal period, however, the delayed testicular descent and the evidently reduced level of testosterone at maturity suggest that the status of testosterone may have originated long before adulthood.

Aside from the reduced testosterone in the GD 15-21 sleep deprived group, the sperm motility and count were grossly reduced, while severe structural aberrations were seen in the histology of their testes and epididymes. The reproductive dysfunction observed in this group is seemingly limited to the peripheral tissue as the reduction in testosterone concentration was accompanied by normal levels of gonadotropins. Clinical and experimental studies have reported the negative correlation between sleep deprivation and serum testosterone concentration in adult men [30-32] and adult male rat [22,23]. However, there are contradictory reports on the relationship between sleep restriction and activity of the Hypothalamic-Pituitary-Gonadal HPG axis [33,34]. The present results support the study carried out in 2013 which reported a decrease in testosterone level of first filial male offspring of dams subjected to 21 days of sleep deprivation [14]. Low testosterone level is known to reduce sperm quality [35], as such, the reduced sperm quality observed in the male offspring of the GD 15-21 sleep deprived group in the present study may be a consequence of the low testosterone level and/or of the structural deficit acquired during intrauterine life.

The offspring of GD 15-21 sleep deprived group also had 0% fertility index and 0% gestational index. The infertility exhibited by this group is not unexpected since low sperm motility and count are well-established causes of infertility [36]. However, it remains to be determined whether the male rats mated in the least with the female rats. This is because a decreased testosterone level reduces proceptive behavior [37], thereby preventing the animals from mating and ultimately leading to infertility. Furthermore, in 2013, Alvarenga reported decreased proceptive behaviour in male offspring of dams that were paradoxically sleep deprived all through the entire gestation period. This may be an additional cause of infertility in the GD 15-21 sleep deprived group offspring [14].

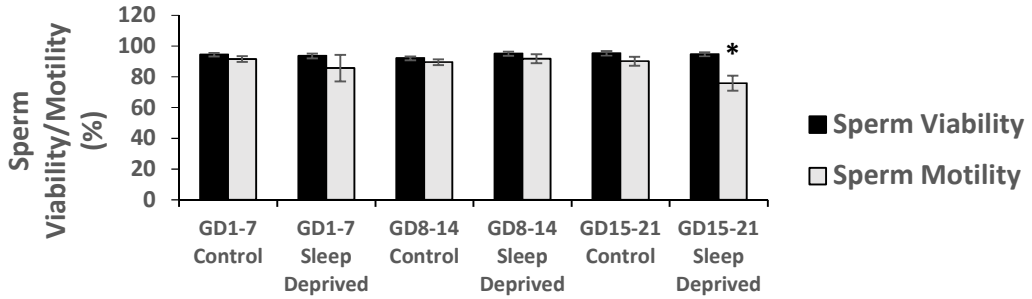

Fig. 4. Epididymal sperm viability of control and sleep deprived dams

*Columns and error bars represent mean ± SEM. n=5. * represents significant difference from corresponding control. (p<0.05) based on Student's t test. GD=Gestation Day*

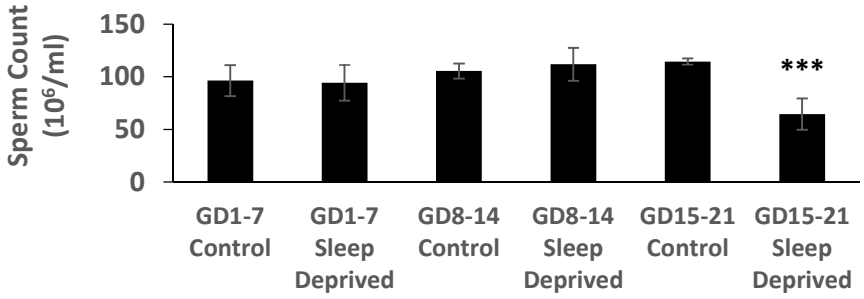

Fig. 5. Epididymal sperm count of offspring of control and sleep deprived dams

*Columns and error bars represent mean ± SEM. n=5. *** represents significant difference from corresponding control. (p<0.001) based on Student's t test. GD=Gestation Day*

The male offspring 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 are ostensibly more remarkable in the GD 15-21 group. The reason for this is probably because this period encapsulates the masculinization and male organ differentiation window in the rat [38]. In mammals, before the period of fetal organ masculinization, the gonads are indifferent gonads which are morphologically indistinguishable. 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 [39,40]. Following this is the process of masculinization which is primarily driven by testosterone secreted by the fetal testis [38]. Existing data have shown that sleep deprivation causes testosterone reduction [22]. Hypothetically, maternal sleep deprivation during GD 15-21 caused reduced testosterone which affected the masculinization process. Disruption in the process of masculinization has been reported to cause mild to serious male reproductive disorders which may be obvious at birth [41] or manifest as low sperm quality and other testicular diseases during adult life [42].

5. CONCLUSION

Maternal sleep deprivation during GD 15-21 caused infertility in the male offspring of Wistar rats. This suggests that the critical period during which fetal male reproductive organ development is adversely affected by maternal sleep deprivation is GD 15-21 which contains the masculinization programming window.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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