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Effects of Zinc supplementation on male reproductive function in night shift work model of chronic sleep deprivation.

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Abstract

Modernization and industrialization have changed human lifestyle with night shift work (NSW) becoming necessary part of regular working hours. NSW is invariably associated with sleep deprivation (SD). Coincidentally, there is remarkable decline in fertility rates in these modern societies, which is believed to be due to lifestyle modifications. NSW model of SD, was therefore created to study its effects on male reproductive functions and role of concomitant Zinc supplementation on those effects. Twenty four (24) male Wistar rats (aged 12- 14 weeks) were equally divided into three groups; Control, NSW and NSWZ models. NSW and NSWZ models were subjected to SD for 12 hours (07:00am – 07:00pm) using Modified Multiple Platform Method (MMPM) every day for the 56 days of the study. Each rat received either distilled water (1ml/animal/day) for Control and NSW models, or Zinc sulphates (5mg/animal/day) for NSWZ model, by gavage daily respectively. Testicular tissue Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC), male reproductive hormones (FSH, LH, Testosterone and Estradiol) and Sperm parameters (sperm count, morphology and motility) were evaluated and statistically compared. Sleep deprivation in NSW model resulted in significant ($p<0.05$) increase in testicular tissue MDA, serum FSH and significant ($p<0.05$) decrease in TAC, serum Testosterone, serum Estradiol, sperm count and percentage of sperm with active progressive motility (APM) compared to the control. Concomitant Zinc supplementation significantly ($p<0.05$) increases testicular tissue TAC, serum Estradiol and significantly ($p<0.05$) decreases testicular tissue MDA and serum FSH. The NSW model of Sleep deprivation negatively affects male reproductive functions, while concomitant Zinc supplementation ameliorates some of these negative effects.

Keywords: Active Progressive Motility; Night Shift Work**;** Follicle Stimulating Hormone; Testosterone; Total Anti-oxidant Capacity; Sleep Deprivation;

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INTRODUCTION

Sleep is a universal, dynamic brain process that is present in organisms ranging from invertebrates to mammals, which is associated with important restorative function for every organ in the body (Brown and Naidoo, 2010; Liu *et al.,* 2017). For optimal health, the American Academy of Sleep Medicine (AASM) and the Sleep Research Society (SRS) have recommended a regular seven or more hours of night sleep for adults aged 18 to 60 years (Watson *et al*., 2015). But modernization and industrialization resulted in global 24/7 society characterized by increased physical activities, reduced rest and sleep (Rodrigues *et al.,* 2015). To cope with the global 24/7 societal demand, night shift work (NSW) which is invariably associated with sleep deprivation (SD) became a necessary part of the regular work hours. It was estimated that over 20% of adults were chronically sleep deprived, hence, SD was labelled as modern-day epidemic (Bixler, 2009).

Coincidentally, a remarkable decline in fertility rates has been reported in industrialized areas (Pearce *et al.,* 1999). Infertility affects approximately 15% of all couples trying to conceive, and male factor infertility accounts for roughly half of these cases, while no identifiable cause can be found in over 25% of male infertility (Sharlip *et al.,* 2002).

Therefore, our study was designed to simulate duration and timing of SD in NSW to study its effects on male reproductive functions and the role of concomitant Zinc supplementation on those effects.

METHODOLOGY

Animals

Twenty four (24) male Wistar rats (aged 10- 12 weeks) were obtained from the animal house of Department of Human Physiology, Bayero University Kano. The rats were housed in plastic cages, adequate ventilation and natural light/dark cycle maintained, with food and water *ad libitum* in accordance with the National and International Regulations on Use of Animals for Research and Teaching 2017. Animal Research Committee of Ahmadu Bello University Zaria, granted ethical clearance (ABUCAUC/2020/65).

Animal Grouping

The 24 adult male Wistar rats were randomly divided into three groups of eight animals each as follows.
Control model: distilled water (1ml/animal/day)
NSW model: 12 hours of SD + distilled water (1ml/animal/day)
NSWZ model: 12 hours SD + $ZnSO_4$ at dose of 5mg/animal/day (Dissanayake *el al.*, 2009)

Experimental Design

The research was a longitudinal interventional study designed to simulate NSW time schedule which is the most common cause of SD in our global 24/7 society. Each rat was given either distilled water (Control and NSW models) or $ZnSO_4$ (NSWZ model) by gavage between 07:00 - 08:00am daily for the 56 days of the study. NSW and NSWZ models were subjected to 12 hours of SD (07:00am – 07:00pm) during their biological night-time. After each SD episode, the rats were returned to their group home cages (07:00pm-07:00am) for 12hrs of sleep/rest window during their biological day-time.

Sleep deprivation induction
SD was induced using our customized modified multiple platform method (MMPM). The MMPM paradigm consist of a plastic tank (55×35×35 cm) containing 10 round platforms (made from metallic pipe with plastic cap welded to iron base) of 7cm height, 5cm diameter, and placed 7cm apart, improvised from Zager *et al.* (2009) and Choi *et al.* (2016) descriptions (plate 1). The tank was filled with water to about 1 cm below the platform surface. The rats can move around by leaping from one platform to another. Whenever the rat sleeps, it falls into the water and then wakes up. The water in the tank was changed daily throughout the period of the experiment. The Control rats were placed in similar plastic tank, but filled with saw dust instead of water, so they can sleep well on it.

Plate 1: Customized Modified Multiple Platform Method of sleep deprivation induction

Animals Sacrifice, Samples Collection and Tissue handling
Each rat was anaesthetized by intraperitoneal injection of cocktail of diazepam (2 mg/kg) and ketamine (20 mg/kg). Blood was collected via cardiac puncture and then discharged into plain sample bottle. The collected blood sample was allowed to clot at room temperature and then centrifuged at 2000g for 15 minutes. The serum collected was used for hormonal assays. Testes were removed, cleared of adhering connective tissue and weighed as described by Valença *et al.* (2013). The left testicle of each rat was stored frozen for testicular oxidative stress biomarkers assessment. The right cauda epididymis of each rat was used for semen analysis.

Semen Analysis
The right cauda epididymis of each rat was cut into pieces in a petri dish containing 5 ml warm saline solution and incubated for three minutes, with frequent shaking to yield semen suspension. About ten microliter (10μl) of the semen suspension was loaded on to the Neubauer hemocytometer (Deep 1/10 mm, Labart, Germany) for semen analysis at x100 magnification. Spermatozoa were counted in five random squares in triplicate per sample according to WHO laboratory manual for the Examination and processing of human semen (WHO, 2010). Total sperm count was calculated and reported as millions of sperm cells/ml. A small drop of the semen suspension was smeared on a warmed glass slide and covered with a glass slip. A minimum of three different fields were examined to determine the mean percentage of sperm motility and reported as percentage of motile sperm according to WHO laboratory manual for the Examination and processing of human semen (WHO, 2010). The relative proportions of abnormal sperm cells were analysed according to WHO laboratory

manual for the Examination and processing of human semen (WHO, 2010). The relative proportions of normal and abnormal sperm cells were expressed in terms of percentages.

Hormonal assays

The serum was used for testosterone, estradiol, FSH and LH assays. Serum testosterone and estradiol were measured using individual commercial ELISA kits (Sunlong Biotech Co.,Ltd: China) according to manufacturer's instructions. LH and FSH were determination using their Immunoenzymometric Assay kits (Sunlong Biotech Co.,Ltd: tel: 0086-571-56623320: China.) respectively according to manufacturer's instructions.

Testicular Oxidative Stress Biomarkers

The left testicular tissues were individually homogenized in ice cold phosphate buffer saline (10 mM pH - 7.4) using potter- Elvenhjem tissue homogenizer. The crude tissue homogenate was centrifuged at 10,000 rpm, for 15 minutes in cold centrifuge, and the resultant clear supernatant was divided into two aliquots for MDA and TAC assays. Testicular tissue MDA was determined using Colorimetric TBARS as described by Ohkawa *et al.,* (1979). TBARS assay is reported in MDA equivalents. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm. Total Antioxidant Capacity (TAC) determination was done by the use of Rat total antioxidant status Elisa kit obtained from (Sunlong Biotech Co.,Ltd: China. SL1402Ra). This method is based on the decolourization of ABTS (2-2 azinobis (3-methybenzothiazoline-6-sulfonate)) radical cation, Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations, which can be monitored spectrophotometrically, and the bleaching rate is inversely related to the Total anti-oxidant capacity (TAC) of the sample.

Data Analysis

The collected data was analyzed using the Statistical Package for Social Sciences (SPSS for Windows, Version 23, SPSS Inc., Chicago, IL, USA). Values were recorded as mean ± standard error mean (SEM). Mean values were compared using one-way ANOVA analysis, followed by Bonferonni's post hoc test for multiple comparisons. The significance level was set as $p < 0.05$.

RESULTS

Testicular tissue oxidative stress biomarkers

The level of TAC in NSW model significantly decreased by 17% compared to the control model. The level of TAC in NSWZ model significantly increased by 53% compared to NSW model (Table 1). Interestingly, the level of TAC in NSWZ model was significantly higher than that of the control. The level MDA in NSW model doubled ($p<0.05$) that of the control model. On the other hand, the MDA level in NSWZ model significantly dropped by 68% compared to that of NSW model.

Male sex hormones

There is significant increase in both LH (48%) and FSH (208%) concentrations in NSW model compared to those of Control model. While significant reduction in both LH (55%) and FSH (66%) concentrations in NSWZ model were recorded when compared to those of NSW model. The mean Testosterone concentration in NSW model was significantly reduced by 36% compared to that of control model. The mean Estradiol concentration in NSW model also significant decrease by 14% compared to that of control model. While the mean Estradiol

concentration in NSWZ model significantly increased by 13% when compared to that of NSW model.

Semen Analysis
The mean Sperm Counts of NSW ($p<0.05$) model was significantly lower than that of the control model. However, there was significant increase in SC of NSWZ model compared to NSW model. The differences in percentage NM among the models were not statistically significant. There was significant decrease ($p<0.05$) in percentage APM of NSW model by 58% compared to that of control. While a significant ($p<0.05$) increase in %APM of NSWZ was recorded when compared to NSW model.

Table 1. Comparison of serum testicular tissue TAC and MDA concentrations among the models

Parameters	Control	NSW	NSWZ	*f*	*p*
TAC (U/ml)	3.88±0.12	3.22±0.09[a]	4.91±0.17[b]	44.389	0.00
MDA (µmol/l)	5.77±0.88	12.67±0.97[a]	4.03±0.59[b]	33.009	0.00

Mean ±S.E.M, n=8, $p>0.05$, a = significant compared to control, [b] = significant compared to NSW

Table 2: Comparison of LH, FSH, Testosterone and Estradiol hormones concentrations among the models

Parameters	Control	NSW	NSWZ	*f*	*p*
LH(mIU/L)	1.62±0.15	2.39±0.18[a]	1.07±0.16[b]	16.583	0.00
FSH(mIU/L)	1.31±0.03	4.04±0.22[a]	1.36±0.08[b]	134.563	0.00
Testosterone (ng/ml)	3.88±0.04	2.48±0.13[a]	2.81±0.12	49.345	0.00
Estradiol (pg/ml)	25.48±0.30	21.95±0.75[a]	24.84±0.31[b]	14.026	0.00

Mean ±S.E.M, n=8, $p>0.05$, [a] = significant compared to control, [b] = significant compared to NSW

Table 3: Comparison of Sperm Count, percentage of sperm with Normal Morphology and percentage of sperm with Active Progressive Motility among the models

Parameters	**Control**	**NSW**	**NSWZ**	***f***
Sperm Count $\times 10^6$/ml	60.50±1.52	51.13±0.44[a]	53.38±0.42[b]	26.694
Normal Sperm Morphology in %	76.13±2.92	68.50±1.39	75.38±1.88	3.791
Active Progressive Sperm Motility in %	35.00±2.49	14.88±1.79[a]	23.38±1.30[b]	27.624

Mean ±S.E.M, n=8, $p>0.05$, [a] = significant compared to control, [b] = significant compared to NSW

DISCUSSION

Sleep is very important for most of the biological processes in the body, so invariably, sleep deprivation (SD) adversely affects health (Medic *et al.,* 2017). When the intricate balance between ROS and antioxidants is disrupted, oxidative stress occurred (Agarwal *et al.,* 2014). Total antioxidant capacity (TAC) measures the total antioxidant capacity of all antioxidants in a biological sample. In our study, SD resulted in significant reduction in levels of TAC in NSW model compared to that in the control model. This indicated that, there is decrease in both enzymatic and non-enzymatic anti-oxidants activities in testicular tissue as a result of SD. Similarly, Rizk *et al*. (2020) reported significant reduction in TAC levels of SD groups when compared to the control group. De Oliveira *et al.* (2002) also recorded significant decrease of GSH level in testicular tissue of sleep deprived rats. GSH plays multiple roles in cellular antioxidant defence system, hence it decrease implies decrease in TAC (Debnath and Mandal, 2000). In similar development, Pasqualotto *et al.* (2000) reported that Control subjects had seminal TAC values 1.41 fold higher than that found in infertile males. Reimund, (1994) hypothesised that free radicals, which accumulate during wakefulness are removed during sleep. The removal of excess free radicals during sleep is accomplished by decreased rate of formation of free radicals and increased efficiency of antioxidant mechanisms. Interestingly, Zinc supplementation significantly increased the level of TAC more than both NSW and Control models. This finding reaffirmed that Zinc is a potent antioxidant.

Spermatozoa in contrast to other cells are particularly susceptible to lipid peroxidation due to the high percentage of polyunsaturated fatty acids (PUFA) in their membrane (Agarwal *et al*., 2014). MDA is a stable end product of lipid peroxidation widely used in biomedical research as an index of lipid peroxidation (Collodel *et al.,* 2014). In our study, the level MDA in NSW model is significantly higher than that of the control model, indicating increased lipid peroxidation in testicular tissues. Our finding is in keeping with the study of Rizk *et al.* (2020) where they reported significant increase in testicular MDA level after sleep deprivation. In our study, Zinc supplementation significantly reduces the MDA level, signifying its role as antioxidant in reducing the rate of lipid peroxidation in testicular tissues. Our finding goes well with that of Nagalakshmi *et al*., (2013). However, Juneet *et al.,* (2018) reported increased oxidative stress with excessive use of zinc in diet. Although the exert mechanism of action of Zinc is fully known, but it was postulated that Zinc influence anti-oxidants activity via its role as a cofactor and enhancer of Cu, Zn-SOD, (Colagar *et al.,* 2009). Zinc was also believed to serves through several other mechanisms; reduction of hydroxyl radical (OH•) production, due to its ability to displace Cu and Fe from membrane binding sites (Michalska-Mosiej *et al*., 2016) and stabilization of sperm membrane against oxidative stress challenge (Ebisch *et al.,* 2007). It was also reported that Zinc deficiency can impair antioxidant defence and makes the spermatozoa more susceptible to OS and inflammatory reactions (Colagar *et al.,* 2009). Zinc supplementation also increased catalase activity in seminal plasma within tolerable limits, and can improve fertility (Egwurugwu *et al*., 2013).

Spermatogenesis depends on pituitary gonadotropins, follicle stimulant hormone (FSH) and luteinizing hormone (LH).. In the testes LH binds to receptors on Leyding cells stimulating synthesis and secretion of Testosterone hormone. FSH regulates the mitotic proliferation growth and function of Sertoli cells. Sertoli cells in turn, support many aspects of sperm cell maturation (Simoni *et al.,* 1999). Gonadotropins production is under the feedback control of sex hormones (Ganong, 2003).

In our study SD induced significant increase in serum FSH of NSW model compared to Control. The increase in serum FSH may be secondary to decrease in serum testosterone level

as evident in our study since, Serum LH and FSH are well known to be under the negative feedback control of serum testosterone (Ganong, 2005). Our results are in accordance with other researches, that reported elevated blood levels of FSH and/or LH due to lack of steroid negative feedback from the testes, which are indicators of testicular dysfunction, sub-fertility or infertility (Babu *et al.,* 2004; Martin-du, 2009). Our findings goes contrary to Breen and Karsch, (2006) that reported inhibition of the hypothalamus-pituitary-gonadal (HPG) axis leading to decreased LH and FSH secretion in response to stress.

Our study also revealed significant reduction in serum FSH concentration with Zinc supplementation. The reduction in serum FSH is an indicator about the improvement of testicular function, as decreased levels of gonadotropins usually reflect increase in sex hormones negative feedback regulation. This is in accordance to the findings of Egwurugwu *et al*., (2013) and Mohamad and Hassan, (2014) that reported significant decrease in serum levels of FSH and LH with Zinc supplementation. This is not surprising because previous researches have demonstrated that Zn is needed for the normal functioning and regulation of the hypothalamus-pituitary gonadal axis (Fallah *et al.,* 2018), all though the exact mechanism was not yet clear.

It was reported that SD may disrupts hormone balance and affects male reproductive functions (Alvarenga *et al.,* 2015). In our study, the serum testosterone of NSW model was significantly lower than that of control, indicating that SD resulted in reduction in testosterone production. Our finding is in keeping with many previous SD studies in both humans and animals (Choi *et al.,* 2016; Victor *et al.,* 2018; Rizk *et al.,* 2020). Longstanding decrease in circulating testosterone can consequently affect fertility in apparently healthy men (Alvarenga *et al.,* 2015). The exact mechanism by which SD decrease testosterone level is still unknown. Invariably, it was presumed that SD as a potent stressor, increase circulating level of glucocorticoids in the body. It was reported that Glucocorticoid induce gonadal resistance to gonadotrophins and inhibit testosterone synthesis by inducing apoptosis of Leydig cells and decreases its sensitivity to LH (Chen *et al*., 2012) leading to decrease testosterone production. Never the less, Alvarenga *et al.,* (2015) revealed no significant difference in testosterone concentration in 21 days Sleep-Restricted group compared to the control group. In our study zinc supplementation resulted in slight increase in testosterone level, however, the increase was not statistically significant. This is contrary to the findings Egwurugwu *et al*. (2013) that reported dose dependant increase in testosterone level with Zinc supplementation.

Traditionally, testosterone and estrogen have been considered to be male and female sex hormones, respectively. However, the wide expression of the aromatase enzyme andestrogen’s receptors throughout the male reproductive system and within human sperm underlines the role of estrogens in human male reproductive function (Carreau *et al.,* 2012). In our study, SD resulted in significant decrease in serum Estradiol in NSW model compared to the control model. Our finding is in accordance with that of Andersen *et al.,* (2004) that reported significant reduction in serum Estradiol of SD group compared to the control group. Testosterone is the main substrate for the synthesis of these estrogens and therefore any alteration of its concentration may alter the synthesis of estrogens. In our study, the serum estradiol value in NSW model was insignificantly lower than that of control.

Sperm concentration, motility, and morphology are the most important predictors of an individual’s potential to produce viable sperm and male fecundity reduces with decrease in sperm concentration (Maya, 2010). Our study revealed significant reduction of sperm count in NSW model compared to control model. This is in keeping with the findings of previous

studies (Victor *et al*., 2018; Wise *et al*., 2018; Rizk *et al*., 2020), and contrary to the findings of Alvarenga *et al.,* (2015) and Choi *et al.,* (2016). It was documented that sperm count negatively associate with level reactive oxygen species (ROS) (Pasqualotto *et al.,* 2000). High levels of ROS disrupt the inner and outer mitochondrial membranes, inducing the release of cytochrome C and activating apoptosis of sperm cells, resulting in reduced sperm count (Agarwal *et al.,* 2014).

Sperm motility and morphology are indicators of qualitative spermatogenesis. In our study, SD significantly decreases the percentages of sperm with normal morphology and sperm with active progressive motility in NSW model compared to that of the control model. Our findings are in keeping many previous studies that linked SD to impaired sperm motility, concentration, and morphology (Choi *et al.,* 2016; Victor *et al.,* 2018; Wise *et al*., 2018; Rizk *et al.,* 2020). Although the precise mechanism was not fully elucidated, but oxidative stress it was believed to negatively affects sperm quality and quantity (Wagner *et al.,* 2017). As evident in our study, the NSW model recorded high level of MDA and low level of TAC, which connotes high level of oxidative stress. Collodel *et al.* (2014) reported that MDA level in seminal plasma negatively correlate with sperm motility, morphology and concentration, while TAC level, positively correlates with sperm concentration, motility, and morphology (Subramanian *et al*., 2018).

Interestingly, with zinc supplementation the percentages of sperm with normal morphology and sperm with active progressive motility were significantly improved. Zhao *et al*. (2016) reported that Zn is essential for maintaining the stability of sperm chromatin and membrane, and also for inhibiting apoptosis of sperm cells with normal morphology. However, Egwurugwu *et al.,* (2013) reported that beyond 50mg/kg of zinc there was significant dose dependent decrease in sperm motility and normal morphology.

CONCLUSION

The SD in NSW model induced significant alterations in male reproductive hormones and sperm parameters. The induced alterations were attributed to the induction of oxidative stress evidenced by increased MDA and decreased TAC in testicular tissue. These alterations may result in male reproductive hypo-function and inferentially male infertility. However, concomitant Zinc sulphates supplementation at dose of 5mg daily significantly ameliorates most of the alterations induced by chronic sleep deprivation.

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