TESTICULAR HISTOLOGY AND OXIDATIVE STRESS IN MODELS OF SLEEP DEPRIVATION: THE ROLE OF ZINC SUPPLEMENTATION.

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ABSTRACT

Background: The current global population works and lives under a 24/7 lifestyle which is characterized by increased physical activities, reduced rest and sleep. About 30 percent of employed adults are Sleep deprived, either by deliberately extending working hours into the night as in Insufficient Sleepers (IS) or works during the night-time as in Night Shift Workers (NSW). Coincidentally, there is remarkable decline in male fertility rates in these modern societies, attributed to testicular failure which is believed to be due to lifestyle modifications.

Aim: The Aims of the study were to create IS and NSW models of SD to study their effects on Testicular morphology and Testicular tissue oxidative stress biomarkers, and to study the role of concomitant Zinc supplementation on those effects.

Methods: Forty (40) adult male Wister rats were randomly divided into five groups: Control, NSW, NSWZ, IS and ISZ models. NSW and NSWZ models were subjected to 12 hours SD, while IS and ISZ models were subjected to 18 hours SD using Modified Multiple Platform (MMPM) method daily for 56 days. ISZ and NSWZ models were supplemented with Zinc sulphates (5mg/animal/day). Testicular tissue MDA, Total Antioxidant Capacity (TAC) and Testicular histology were evaluated and compared.

Results: SD in both NSW and IS models resulted in significant increase (p<0.05) in testicular tissue MDA compared to control. SD in both NSW and IS models, resulted in significant decrease (p<0.05) in testicular tissue TAC compared to control. Plate I (Control): The histology Photomicrograph shows normal testicular histoarchitecture. Plate II (NSW) and Plate IV (IS): shows seminiferous tubules (ST) with relatively scanty spermatids and Sertoli cells with area of cellular degeneration in Plate IV (IS).

Conclusion: The IS model of SD is more detrimental to testicular integrity than the NSW model, with Zinc supplementation ameliorating some of these effects.

Keywords: Oxidative Stress**;** Sleep Deprivation; Total Anti-oxidant Capacity; Sertoli cells; Testosterone;

INTRODUCTION

Sleep is a universal, dynamic brain process that is present in organisms ranging from invertebrates to mammals (Brown and Naidoo, 2010) associated with important restorative functions for every organ in the body (Liu *et al*., 2017). For optimal health, the American Academy of Sleep Medicine and the Sleep Research Society 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 becomes necessary part of the regular work hours, while insufficient sleep occurs when a person chronically fails to obtain the amount of sleep required to maintain normal alertness and wakefulness (AASM, 2014). Sleep deprivation is becoming increasingly common in today's society compared to few decades ago (CDC 2014). It was estimated that about 30% of adults are chronically sleep deprived, hence, SD has been declared to be modern-day 'public health epidemic' (Bixler, 2009: CDC, 2014).

Coincidentally, remarkable decline in fertility rates was 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 (Sharlip *et al*., 2002). A bidirectional relationship between SD and oxidative stress has been documented (Noguti *et al*., 2013; Hill *et al*., 2018). Oxidative stress is postulated as one of the major factors that negatively affects male fertility status (Sharlip *et al*., 2002). Many studies conducted both in vitro and in vivo demonstrated the beneficial effects of antioxidants on fertility and recommend their use for the treatment of male infertility (Gambini *et al*., 2015). It was documented that Zinc supplementation promotes spermatogenesis and sperm motility (Cheah and Yang, 2011) thus, improves fertility.

Most previous studies focused on total sleep deprivation which is hardly obtainable in real human lifestyle (Goel *et al*., 2013). Therefore, we set up rat models of SD to simulates both duration and timing of SD in NSW and IS, to study their effects on testicular histology, testicular tissue oxidative stress and the influence of concomitant Zinc supplementation on those effects.

Materials and Methods

Fifteen plastic cages measuring (55×35×35 cm) with MMPM structure installed in ten of them, digital weighing balance (American weigh triple beam scale, model: TB-2610, with readable load of 610 g and sensitivity of 0.1g), commercially available dispersible Zinc sulfate tablet 20mg (Emzor Pharmaceuticals, Nigeria). The Zinc sulfate was reconstituted in deionized distilled water to form $ZnSO_4$ 5mg/1mL suspension.

Source and Characteristics of Animals

Forty male Wistar rats (aged 10- 12 weeks, weighing 190-210 g) obtained from the animal house of Department of Human Physiology, Bayero University Kano, where the study was carried out. 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, Ahmadu Bello University Zaria, granted ethical clearance for the study (ABUCAUC/2020/65).

Animal Grouping

The forty male Wistar rats were randomly divided into five groups of eight animals each.

Control model: No SD + 1ml/animal/day of distilled water

NSW model: 12 hours of SD + 1ml/animal/day of distilled water

NSWZ model: 12 hours of SD + Zinc sulphates 5mg/animal/day (Dissanayake *et al*., 2009)

IS model: 18 hours SD + 1ml/animal/day of distilled water

ISZ model: 18 hours SD + Zinc sulphates 5mg/animal/day (Dissanayake *et al*., 2009)

Experimental Design

The research is a longitudinal interventional study designed to simulate the two most common modes of SD (NSW and IS models) in global 24/7 society. However, to cope with the modern-day societal demands, some degree of SD is almost inevitable, so we set up Zinc supplemented SD models (NSWZ and ISZ) receiving Zinc sulphates as intervention. Each rat was given either distilled water (Control, NSW and IS models) or $ZnSO_4$ (NSWZ and ISZ models) by gavage between 07:00 - 08:00am daily for the 56 days of the study.

NSW and NSWZ models were subjected to 12 hours SD (07:00am – 07:00pm) and returned to their home cages (07:00pm-07:00am) for 12 hours of sleep/rest window every day. The 12 hours of SD (07:00am – 07:00pm) which is the biological night of the rats, simulates night shift workers.

IS and ISZ were subjected to 18 hours SD (07:00pm – 01:00pm next day), which is the whole of the rats biological day-time (07:00pm – 07:00am) and first half their biological night-time (07:00am – 01:00pm), and returned to their home cages for 6hrs (01:00pm-07:00pm) sleep/rest window every day. The IS model simulates those who works throughout the day time and forced themselves to stay awake for the first half of the night, due to their contemporary lifestyle, work-related pressures and the growth of round-the-clock entertainment televisions and Internet services. Food and water was *ad libitum* during the sleep deprivation periods.

Sleep Deprivation Induction

SD was induced using our customized modified multiple platform method (MMPM). It 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 as a result of muscle atonia 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 MMPM

Animals Sacrifice and Testicular Tissue Handling

Live weight the rats measured before sacrifice. The animals anaesthetized by intraperitoneal injection of a cocktail of diazepam and ketamine hydrochloride at a dose of 2 and 20 mg/kg body weights respectively (Flecknell, 1993). The testes surgically removed and weighed. The left testes stored frozen for testicular oxidative stress biomarkers assessment, while the right testes fixed in 10% formol saline for histological examinations.

Testicular Histology

The right testes were fixed in 10% formol saline for 5–7 days. The specimens were washed with water and then dehydrated in an ascending grade of ethanol solutions. The specimens were then cleared in xylene and embedded in melted paraffin wax.

Tissue blocks were cut into section of 5 µm thickness using microtome. Tissue sections were then stained with haematoxylin and eosin stain for general testicular histology assessments.

Gonadosomatic Index

Gonadosomatic index by weight was calculated as described by Silva *et al.* (2014). The average weight of the right and left testicles was divided by the live weight before sacrifice, multiplied by one hundred (100).

GSI = (average weight of testes ÷ live weight before sacrifice) x 100

Testicular Oxidative Stress Biomarkers

The left testicle of each subject was homogenized in phosphate buffer saline (10 mM pH - 7.4) with potter- Elvenhjem tissue homogenizer. The crude tissue homogenate was centrifuged at 10,000 rpm, for 15 minutes in cold centrifuge (at 4^{o}C), and the resultant clear supernatant was divided into two aliquots for MDA and TAC assays.

Determination of Testicular Tissue MDA Concentration

Malondialdehyde (MDA) is a secondary product of lipid peroxidation and its concentration used as an index to monitor lipid peroxidation. The testicular tissue MDA was determined colorimetrically by thiobarbituric acid reactive substances TBARS method of Buege and Aust (1978). The principle of this method is as follows; 2-Thiobarbituric acid reactive substances (TBARS) are naturally present in biological specimens and include lipid hydroperoxides and aldehydes, which increase in concentration as a response to oxidative stress. One molecule of MDA reacts with two molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm.

Determination of Testicular Tissue TAC

Rat total antioxidant status Elisa kit was obtained from (Sunlong Biotech Co.,Ltd: tel: 0086-571-56623320: 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 with the TAC of the sample.

Data Analysis

The collected data was analysed using the Statistical Package for Social Sciences (SPSS for Windows, Version 23, SPSS Inc., Chicago, IL, USA). All values are presented as mean ± standard error mean (SEM). One-way ANOVA analysis and Bonferonni's post hoc test were performed to determine the differences among the models. The significance level was set at $p< 0.05$.

RESULTS

The present study simulated the most common modes of SD in modern day society; NSW model simulate Night Shift Workers (NSW) while IS group simulate Insufficient Sleepers (IS). The final body weight (FBW) before sacrifice, average testicular weight (ATW) and Gonadosomatic index (GSI) of study models were shown in Table 1. The ATW was statistically different across the models ($F = 11.203$, $p<0.05$). The mean ATW of IS model (1.86±0.03) was significantly lower compared that of Control (2.22±0.03) and NSW (2.07±0.04) models respectively. With Zinc supplementation, there was significant increase in mean ATW of ISZ model (2.03±0.05) compared to that that of IS Model (1.86±0.03). The GSI of IS (0.62±0.01) model was significantly lower compared to that of control (0.75±0.05) model ($F = 5.859$, $p<0.05$). While GSI of other models were insignificantly lower than the control model.

Table 1: Comparison of Final Body Weight (FBW), Average Testicular Weights (ATW) and Gonado-Somatic Index (GSI) among the study models of SD.

	Control	NSW	NSWZ	IS	ISZ
FBW(g)	296.00±3.69	305.75±6.34	295.38±5.86	299.13±4.33	290.88±4.91
ATW(g)	2.22±0.03	2.07±0.04	2.11±0.03	1.86±0.03[a,b]	2.03±0.05[a,c]
GSI	0.75±0.05	0.68±0.02	0.72±0.05	0.62±0.01[a]	0.70±0.02

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

Testicular Tissue Oxidative Stress Biomarkers

On comparing the mean Total Antioxidant Capacity (TAC) among the SD models, it was observed that the mean TAC of both IS (2.03±0.09) and NSW (3.23±0.09) models of SD were significantly ($p<0.05$) lower than that of the control (3.63±0.20) model. Similarly, when compared to NSW model, the mean TAC of IS model was significantly ($p<0.05$) lower. Interestingly, the Zinc supplemented models NSWZ (4.91±0.16) and ISZ (3.09±0.18) recorded significantly higher TAC when compared to NSW (3.23±0.09) and IS (2.03±0.09) models respectively. (F = 46.375, $p<0.05$) Fig 1.

Figure 2 shows mean Malondialdehyde (MDA) among the SD models. The mean MDA of both IS (33.39±2.04) and NSW (12.42±0.97) models were significantly ($p<0.05$) higher than that of the control model (5.97±1.00). When compared with NSW model the mean MDA of IS model was also significantly ($p<0.05$) higher. The Zinc supplemented SD models, ISZ (21.57±2.47) and NSWZ (4.03±0.60) recorded significantly lower MDAs compared to SD models IS (33.39±2.04) and NSW (12.42±0.97) respectively. (F = 58.522, $p<0.05$)

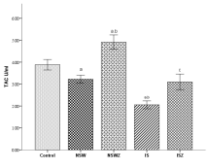

Figure 1: Effects of zinc supplementation on Testicular tissue Total Antioxidant Capacity (TAC) of models of sleep deprivation

Mean ±S.E.M, n=8, $p>0.05$, [a] = significant compared to control, [b] = significant compared to NSW [c] = significant compared to ISS Group (F = 46.375, $p<0.05$)

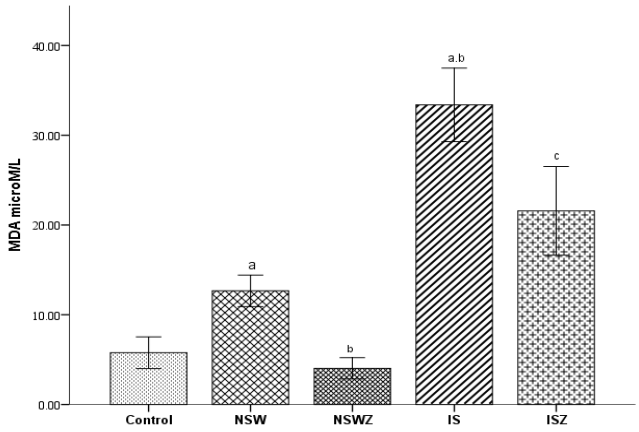

Figure 2: Effects of zinc supplementation on Testicular tissue Malondialdehyde (MDA) among models of sleep deprivation.
Mean ±S.E.M, n=8, $p>0.05$, a = significant compared to control, ^{b} = significant compared to NSW ^{c} = significant compared to IS Group($F = 58.522$, $p<0.05$)

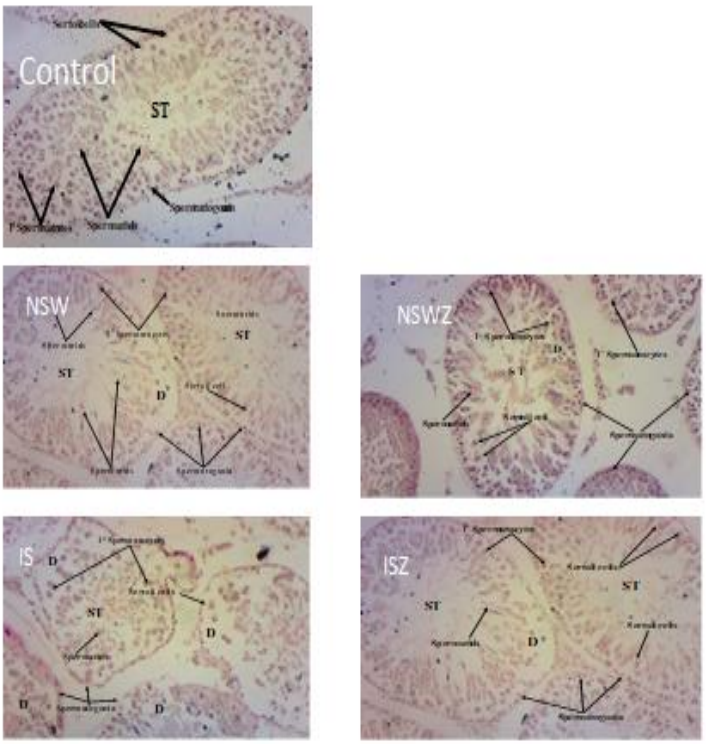

Plate 1: Photomicrograph of testicular section (H and E stained and presented at x40 magnification) **Control model** - The histology shows normal testicular histoarchitecture presenting seminiferous tubule (ST) studded with spermatids. **NSW**-The testicular histoarchitecture shows seminiferous tubules (ST) with relatively scanty spermatids and Sertoli cells. Although there were few patches cellular degeneration (D) in their seminiferous tubules. **NSWZ model** - The testicular histology shows seminiferous tubules (ST) studded with good numbers of spermatids and Sertoli cells. **IS model** - Although a few primary spermatocytes, spermatids and Sertoli cells were present, the luminal structure of the seminiferous tubules collapsed with fewer spermatogonia. Numerous patches of cellular degeneration (D) were observed in their seminiferous tubules. **ISZ model** – the testicular histology was fairly reserved with primary spermatocytes, spermatogonia, and intact seminiferous tubule (ST) . The Sertoli cells were clearly present. However, few patches of cellular degeneration (D) were still present in the seminiferous tubules.

DISCUSSION

Sleep is very important for most of the biological processes in the body, so invariably, sleep deprivation (SD) can adversely affects health (Medic *et al.,* 2017). Our study revealed the varying extents to which the night shift work (NSW) and insufficient sleep (IS) models of SD deranged the testicular histoarchitecture, through induction of oxidative stress in the testicles, and the ameliorative role of concomitant Zinc supplementation on the derangement.

The weight of male reproductive organs usually provides a useful reproductive function assessment tools in experimental studies, and testicular size is the best primary assessment of efficient spermatogenesis (Raji *et al.,* 2005). The tubules and germinal elements account for approximately 98% of the testicular mass thus, testicular size correlated with the capacity to produce sperm (Lunstra *et al.,* 2002). In our study, SD induced significant reduction in average testicular weight (ATW) when compared to the control model. In accordance to our findings Victor *et al.,* (2018) reported significant reduction in Testicular weight following SD.

The reduction in ATW in IS model was significantly more pronounced than in NSW model, indicating that, the longer the duration of SD per day the more the reduction in testicular weight. But contrary to our finding, Rizk *et al.,* (2020) reported significant increase rather than decrease in the testicular index with increase in duration SD.

Zinc supplementation significantly prevented the SD induced decrease in testicular weight. The gonads are among the rapidly growing tissues in the body, and some of the essential enzymes involved in nucleic acid and protein synthesis are zinc metallo-enzymes (Tapiero and Tew, 2003), hence the conservation of testicular weight with zinc supplementation.

Gonadosomatic index predicts the rates of sperm production as well as sperm function in a given specie (Gomendio *et al.,* 2006; Adebayo *et al.,* 2009). In our study, chronic SD induced significant reduction of GSI in IS model compared to that in control model. The reduction in GSI was not unexpected, owing to the fact that, the model recorded significant testicular weight loss and significant body weight gain. Conversely, Rizk *et al.* (2020) reported significant increase in the gonadosomatic index in the SD group, which they explain to be due to, the marked weight loss observed in test group when compared to the control group.

A small amount of ROS is essential for sperm to obtain fertilizing capabilities, but High ROS levels can affect sperm function by oxidation of lipids, proteins, and even DNA (Agarwal and Prabakaran, 2005; Ebisch, *et al.,* 2007). When the intricate balance between ROS and antioxidants is disrupted, oxidative stress occurred (Agarwal *et al.,* 2014). In our study, SD resulted in significant reduction in levels of TAC in both NSW and IS models compared to that in the control model. This indicates that, there is decrease in both enzymatic and non-enzymatic anti-oxidants activities in testicular tissue due to 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. Everson *et al* (2005) reported decrease in GSH activity in liver after SD. GSH plays multiple roles in cellular antioxidant defense 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. In contrast to our finding, Singh *et al* (2008) reported that SD does not affect the activity of glutathione peroxidase in any of the studied brain regions.

The NSW model has significantly higher level of TAC compared to that of IS model. It is clear that the IS model is more prone to oxidative stress challenge than NSW model of SD. This is due to, partly the 18 hours SD duration in IS model as against only 12 hours SD duration in NSW model, and partly the possibility of rebound or recovery sleep effect in NSW model, which may, partially compensate for the sleep lost. 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 endogenous antioxidant mechanisms. Thus sleep has an anti-oxidative role. Interestingly, Zinc supplementation significantly increased the level of TAC in Zinc supplanted SD models compared SD models.

Lipid peroxidation, is highly detrimental to germ cell membrane structure, process of spermatogenesis, and sperm fertilization capacitation (Leong *et al.,* 2013; Talevi *et al.,* 2013). Spermatozoa in contrast with other cells are particularly susceptible to free radical attack due to the high percentage of polyunsaturated fatty acids (PUFA) in their membrane (Talevi *et al.,* 2013; Agarwal *et al.,* 2014). In addition, the cytoplasmic space of sperm is limited, restricting availability of intracellular antioxidant enzymes significantly, which further compromised its ability to resist oxidative stress challenges (Weir and Robaire 2007). MDA is a stable end product of lipid peroxidation widely used in biomedical research as an index of lipid peroxidation and could serve as a diagnostic tool for male infertility assessment (Sanocka and Kurpisz, 2004; Collodel *et al.,* 2014). In our study, the level MDA in both IS and NSW models were significantly higher than that of the control model, indicating increased lipid peroxidation in their testicular tissues. Our finding is in keeping with those of Rizk *et al.* (2020) where they reported significant increase in testicular MDA level after sleep deprivation. Similarly, Ramanathan *et al.,* (2002) found that SD increase lipid peroxidation in the hippocampal region of the brain of Wistar rats.

We also noted that the level MDA in IS model was significantly higher than that of NSW model, denoting the effect of SD duration on MDA production. The difference may be due to partly, the longer hours of SD in IS model, which may favor more generation of ROS, and partly due to rebound sleep present in NSW model, which may enhanced the activity of endogenous anti-oxidants.

In our study, Zinc supplementation at 5mg/animal/day significantly reduce the MDA levels in both Zinc supplemented SD models, signifying reduction in the rate of lipid peroxidation in testicular tissues. Our finding goes well with some previous studies (Nagalakshmi *et al*., 2013; Juneet *et al.,* 2018). However, Singh (2012) reported increased oxidative stress with use of excessive zinc in diet. Although the exert mechanism may not be known, but Zinc is postulated to influence anti-oxidants activity via its role as a cofactor and enhancer of Cu, Zn-SOD, (Powell, 2000; Colagar *et al.,* 2009). It serves through several 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 (Chia *et al.,* 2000; Ebisch *et al.,* 2007). It was also reported that Zinc deficiency can impair antioxidant defenses and makes the spermatozoa more susceptible to OS and inflammatory reactions (Colagar *et al.,* 2009). Omu *et al.* (2015) reported that zinc deficient rats, Cu-Zn SOD activity was significantly decreased as compared to zinc supplemented groups. Zinc supplementation also increased catalase activity in seminal plasma within tolerable limits, and can improve fertility (Egwurugwu *et al.,* 2013).

The detrimental effect of SD on male reproductive system was confirmed by testicular histology findings. From each subject's slide, we examined three most transverse seminiferous tubules sections. The testicular tissue photomicrograph of the control model shows nomral testicular histoarchitecture with intact seminiferous tubule (ST) studded with spermatids, spermatogonia and primary spermatocytes supported by adequate numbers of Sertoli cells. SD resulted in significant alterations in testicular histoarchitecture, with reduced number of spermatogonia, spermatids, and Sertoli cells, and patches of cellular degeneration (D) in irregular seminiferous tubules compared to that of control. Our finding is similar to that of Rizk *et al.,* (2020) where they revealed abnormal morphology of seminiferous tubules, with cellular degeneration and thickening of the basement membrane in the SD group compared to the control group. These alterations in testicular histoarchitecture are more pronounced in IS model when compared to NSW model. The sleep deprivation induced alteration of testicular histoarchitecture is believed to be increased oxidative stress evidenced by high levels of MDA and low levels of TAC in testicular tissue, in our SD models.

Interestingly, with Zinc supplementation the normal testicular histoarchitecture was appreciably preserved. In Zinc-supplemented SD models, a near normal testicular histology was evident showing the primary spermatocytes, spermatogonia, and Sertoli cells in seminiferous tubules.

CONCLUSIONS

Sleep is believed to have important restorative function on body's homeostasis; hence SD is associated with adverse effects on various body systems including reproduction. Despite the multiple challenges in SD studies, this study provides novel insights into the reciprocal interactions between sleep deprivation, oxidative stress, testicular integrity and the anti-oxidant role of Zinc supplementation. SD induced significant alterations in testicular morphology that may negatively affects it`s function in both the SD models. The SD induced alterations were attributed to the induction of oxidative stress evidenced by increased MDA and decreased TAC in testicular tissue. These alterations may results in male reproductive hypo-function and inferentially male infertility. However, concomitant Zinc sulfates supplementation at dose of 5mg/animal/day) significantly ameliorates most of the alterations induced by SD. It is note worthy that, these detrimental changes were more severe in IS model than in NSW model, indicating SD duration dependent effect.

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