J. Afr. Ass. Physiol. Sci. 6 (2): 119-125, December 2018

Journal of African Association of Physiological Sciences

Official Publication of the African Association of Physiological Sciences
http://www.jaaps.aapsnet.org

Research Article

Oestrous cyclicity disruption in Wistar rats subjected to partial sleep deprivation

O.O. Akindele*, O.T. Kunle-Alabi, J.U. Asogwa and Y. Raji

Laboratory for Reproductive Physiology and Developmental Programming Department of Physiology, College of Medicine, University of Ibadan. Ibadan

Keywords:
Sleep deprivation, Oestrous cycle, Adrenal medulla, rats,

ABSTRACT

Background: Chronic partial sleep deprivation continues to be a global problem. Studies have shown that oestrous cyclicity is disrupted by total sleep deprivation. The relationship between partial sleep deprivation – the predominant form of sleep deprivation in the current global society – and the oestrous pattern remains elusive. This study examined oestrous cycle patterns of rats subjected to partial sleep deprivation. **Methods:** Thirty female Wistar rats were divided into control and Sleep-Deprived (SD) groups of fifteen rats each. Oestrous patterns were monitored for two weeks before sleep deprivation and throughout the study. Sleep deprivation was induced using the modified multiple platform method. Five animals were sacrificed under thiopental anaesthesia during proestrus on days 7, 14 and 21. The ovaries, uteri and adrenal glands were examined. Data were compared using t-test at $p<0.05$. **Results:** Proestrus and oestrus frequencies reduced in the SD groups; metestrus frequency increased in the 7 days and 14 days SD and diestrus frequency increased in 21 days SD group. Cycle lengths increased in the 14 days and 21days SD groups. The SD groups had inflammation of ovary and uterus, as well as adrenal medulla hyperplasia. **Conclusion:** Partial sleep deprivation reduces frequencies of phases characterized by sexual activity and ovulation while increasing cycle lengths in Wistar rats. Thus, adequate sleep is important for women who are trying to get pregnant.

INTRODUCTION

Sleep is an age old behaviour that is exhibited by everyone across the globe. Disruption in the normal rhythm and pattern of sleep as well as shortening of sleep duration are detrimental to well-being and body homeostasis (Ezenwanne, 2011). The most common causes of sleep deprivation are those associated with modern-day lifestyle and work-related factors (Orzeł-Gryglewska, 2010). Majority of the global populace has embraced the modern-day lifestyle such that a considerable number of people are sleep deprived, consequently increasing the number of people suffering from adverse effects of sleep deprivation (Luyster et al., 2012). One of such devastating effects of sleep deprivation is its effect on the endocrine system (Tufik et al., 2009).

There have been reports that sleep deprivation alters circulating levels of testosterone consequently affecting fertility in apparently healthy men (Alvarenga et al., 2015). Also, a study proposes that sleep deprivation may be able to modulate levels of ovarian hormones (Tufik et al., 2009), suggesting a possible negative effect of sleep deprivation on reproductive cycle and fertility.

Female infertility is a primitive problem which affects the quality of life of its victim especially in the developing countries where such infertile women are stigmatized (Agarwal et al., 2015). Besides, regular and normal length of ovarian/menstrual cycles are considered an important indication of women's wellness (AAP et al., 2006). As such, factors that modulate ovarian hormones and alter women's reproductive cycles may also affect their well-being.

Evidence suggests that sleep debt is more common in women than in men (Hublin et al., 2001). Despite this, 75% of studies involving sleep deprivation have focused on the male (Chang et al., 2011). For instance, various studies reporting that sleep deprivation disrupts hormone balance and affects reproductive functions have been conducted in the male (Akindele et al., 2014; Alvarenga et al., 2015). However, very few studies

*Address for correspondence:
Email: oo.akindele@mail.ui.edu.ng

have considered the effects of sleep deprivation on female reproductive functions. Even worse, these studies examined the effects of total sleep deprivation (Antunes et al., 2006; Tufik et al., 2009)]. Total sleep deprivation is hardly obtainable in humans while chronic partial sleep deprivation is a prevalent form of sleep deprivation in the general global populace (Goel et al., 2013). Therefore, there is dearth of knowledge on the association between female reproductive cyclicity (which is a determinant of female reproductive functions) and partial sleep deprivation; a pattern of sleep deprivation that is prevalent in the current modern-day society. Thus, this study reports the patterns of oestrous cycle of female Wistar rats subjected to partial sleep deprivation.

MATERIALS AND METHODS

All procedures used in this study conformed to the Guide for the Care and Use of Laboratory Animals, NIH Publication revised 1996: No. 85-23 (NIH, 1996).

Sleep deprivation model

Rats were sleep deprived using the Modified Multiple Platform Method of Nunes and Tufik in which rats were placed in a water tank consisting of multiple circular platforms of 6.5 cm in diameter (Nunes & Tufik, 1994). The rats were placed on the narrow platforms and the loss of muscle tone associated with onset of Rapid Eye Movement (REM) sleep resulted into arousal when the rats fell into the water. The cover of the chamber was made of wire mesh to which feeders and drinkers were attached. The control rats were placed in tanks with similar features as the test chamber; the difference being that it additionally consisted of a glass barrier on which the animals could sleep at will.

Animal grouping

Thirty mature nulliparous female Wistar rats (170 - 200 g) with normal oestrous cycle patterns were used for the study. They were divided into control and Sleep-Deprived (SD) groups; each group consisting of 3 sub-groups; A, B & C (n=5). Sub-groups A, B & C were placed in their respective chambers for 7 days, 14 days and 21 days respectively. i.e. The control animals were placed in a similar environment as the test group in order to validate effects (if any) on oestrous cycle which may be caused by being in an artificial environment for a long duration of time.

Determination of oestrous pattern

Basal oestrous cycle patterns were studied and established for two weeks before sleep deprivation was commenced. This served as the pre-SD data. The determination of oestrous cycle pattern was carried out using Marcondes' technique (Marcondes et al., 2002). Oestrous cycle 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 physiological saline (0.9 % NaCl) by gently inserting the tip of the pipette into the rat's vagina. The pipette was pressed to release and siphon 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 content was viewed using the x40 magnification objective lens of a light microscope (Olympus, Japan). The cell types and the proportion among them were used to identify the oestrous cycle phase of the rat.

Sleep deprivation protocol

The control and test rats were placed in their respective tanks daily from 2:00 pm of one day to 10:00 am of the next day when they were returned to their home cages. Five animals in each group were sacrificed during proestrus on days 7, 14 and 21. Animals whose proestrus did not fall within 7-10 days, 14-17 days or 21-24 days for 7 days, 14 days and 21 days groups respectively, were removed from the study. Oestrous cycles of all the animals were monitored throughout the study.

Animal sacrifice

Rats were sacrificed under thiopental anaesthesia (50 mg/kg; i.p.) (Pereda et al., 2006). The ovaries, uteri and adrenal glands were harvested and freed of adherent tissues. All harvested organs were fixed in 10% formalin for histological examination.

Statistical analysis

Data were expressed as mean ± Standard Error of Mean (SEM) and differences in means were compared with paired and Student's t-test where applicable. $P<0.05$ was considered statistically significant.

RESULTS

Frequency of oestrous phases

During the first week of sleep deprivation, proestrus frequency was 10% lower ($p<0.05$) in the sleep-deprived group relative to the pre-SD, but this reduction was not statistically significant when compared with the control group. However, there was a statistically significant reduction in proestrus frequency during the second and third week of sleep deprivation compared with the pre-SD and the control group (Figure 1a). Oestrus frequency in the sleep deprived group significantly decreased at the end of first,

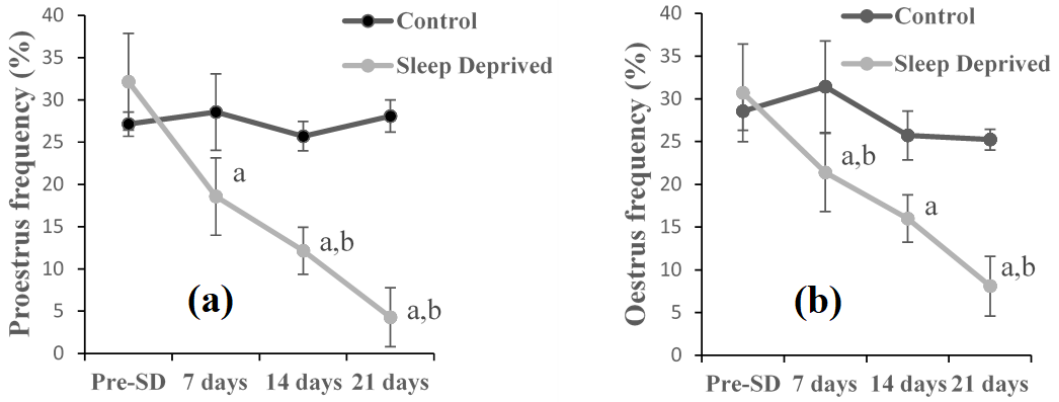

Fig. 1. Proestrus phase frequency (**a**) and Oestrus phase frequency (**b**) of Control and Sleep Deprived (SD) female Wistar rats. Points represent mean ± SEM. n = 5. [a]p<0.05 compared with the pre-SD group, [b]p<0.05 compared with the respective control group.

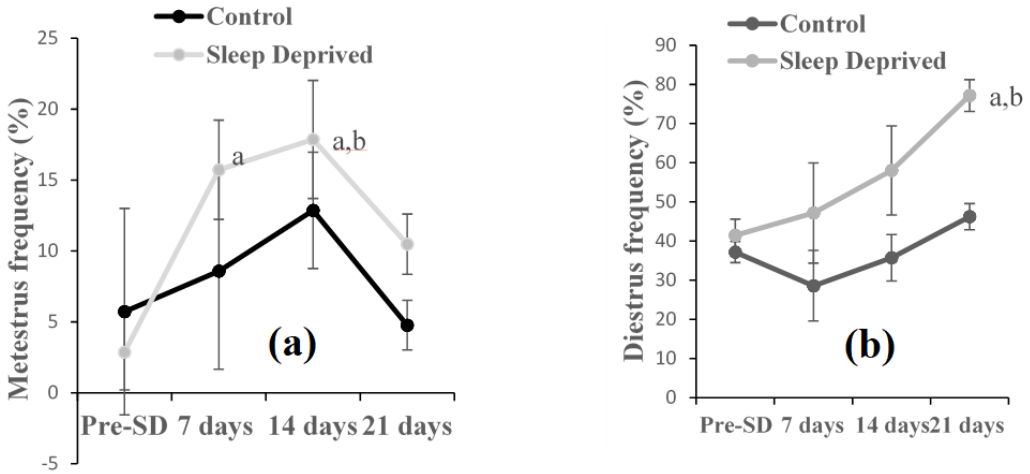

Fig. 2: Metestrus phase frequency (**a**) and diestrus phase frequency (**b**) of Control and Sleep Deprived (SD) female Wistar rats. Points represent mean ± SEM. n = 5. [a]p<0.05 compared with the pre-SD group, [b]p<0.05 compared with the respective control group.

second and third week of sleep deprivation compared with the basal frequency (Figure 1b). Metestrus frequency significantly increased in the SD at the end of first and second week of sleep deprivation compared with the basal metestrus frequency in the same group. The increase in the frequency of metestrus in the SD at the end of the first week (only) was also statistically significant compared with the control group (Figure 2a). The frequency of diestrus was significantly increased at the end of the third week in the SD compared with the basal diestrus frequency in the same group and the control group frequency (Figure 2b).

Oestrous cycle length

There was no significant difference in the oestrous cycle length before sleep deprivation and at the end of first week of sleep deprivation. However, the length of oestrous cycle increased significantly ($p<0.05$) at the end of the second and third week of sleep deprivation compared with the basal oestrous length and the control group (Figure 3a).

Body weight

The control rats gained approximately 3% of their basal body weight, while the sleep-deprived rats lost over 6% of their body weight during the first two weeks of sleep deprivation. However, at the end of the third week of sleep deprivation, the SD group had about 0.4% increase in body weight but this gain was significantly lower ($p<0.05$) than the weight gained by the control group (Figure 3b).

Histology of the ovary

Rats that were sleep-deprived for 7 days (7 days SD) had ovarian vascular congestion. All sleep deprived groups show infiltration by inflammatory cells within the ovarian stroma (Figure 4).

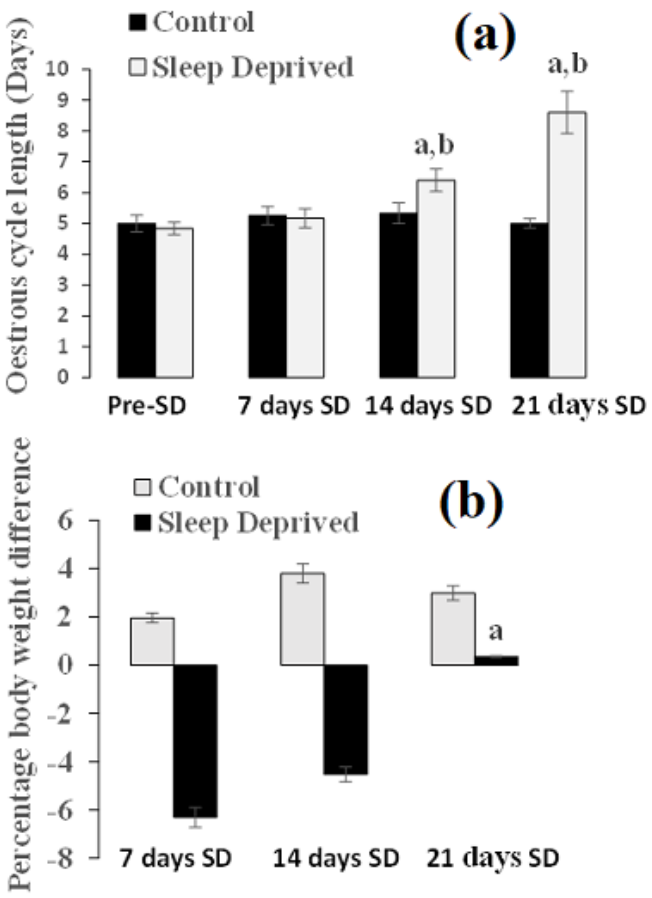

Fig. 3: Length of oestrous cycle (**a**) and Percentage body weight difference (**b**) of Control and Sleep Deprived (SD) Wistar rats. Columns represent mean ± SEM. n = 5. [a]p<0.05 compared with the pre-SD group, [b]p<0.05 compared with the respective control group.

Histology of the uterus

The uterus of rats deprived of sleep for 7 days (7 days SD) had reduced surface epithelial cell proliferation. Twenty-one days of sleep deprivation caused moderate to severe vascular congestion and inflammation in the uterus (Figure 5).

Histology of adrenal gland of Wistar rats

There was adrenal medulla hyperplasia in all groups that were sleep deprived. Adrenal cortices of the sleep deprived groups did not appear different from that of the control groups (Figure 6).

DISCUSSION

The rat's reproductive cycle known as the oestrous cycle is characterized by proestrus, estrus, metestrus and diestrus phases. Proestrus and oestrus are phases of the reproductive cycle in the rat during which ovulation occurs (Marcondes et al., 2002) and female animals are receptive to male animals (Hirshfield, 1985; Kim et al., 2016). The events typical of these phases occur under

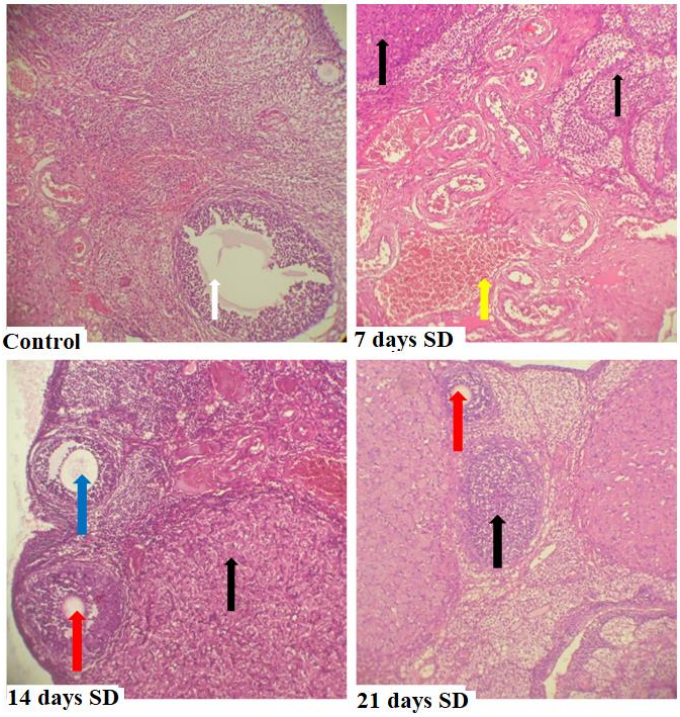

Fig. 4: Photomicrographs of ovarian sections from Control and Sleep Deprived (SD) rats showing: a follicle which just expelled its ovum (white arrow) in control; vascular congestion (yellow arrow) and inflammatory cell infiltration (black arrow) in 7 days SD rats; tertiary follicle with reduced antral cavity (blue arrow) and inflammatory cell-infiltrated stroma (black arrow) in 14 days SD rats; primary follicle (red arrow) and inflammatory cell-infiltrated stroma (black arrow) in 21 days SD rats. Tissue sections were stained with H&E and presented at x100 magnification.

the influence of FSH, LH and oestrogen (Plas-Roser et al., 1977). Thus the reduction in proestrus and oestrus frequencies of the sleep deprived animals may indicate a sup-optimal production of FSH, LH and oestrogen as estrogen and LH surge are requisites for ovulation (Micevych et al., 2003). The predominating primary follicles and antral follicle apparent in the ovaries of the sleep-deprived rats also support the possible involvement of sub-optimal production of these hormones. This implies that chronic partial sleep deprivation may cause anovulatory cycles and ultimately, infertility.

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

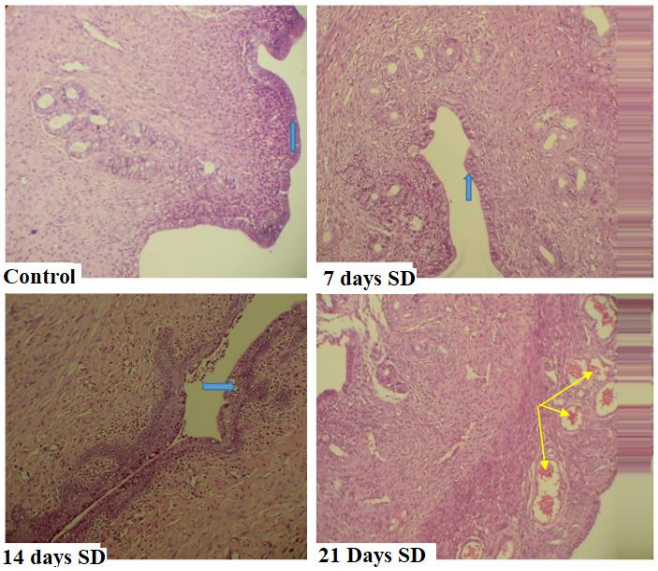

Fig. 5: Photomicrographs of uterus sections from Control and Sleep-deprived (SD) rats showing: proliferating surface epithelial cells (blue arrow) in control; reduced surface epithelial cell proliferation (blue arrow) in 7 days SD rats; inflamed surface epithelial cell layers (blue arrow) in 14 days SD rats; vascular congestion (yellow arrow) in 21 days SD rats. Tissue sections were stained with H&E and presented at x100 magnification.

associated with lack of receptive behaviour (Plas-Roser et al., 1977). In this study, diestrus alone occupied more than 75% of the cycles in the sleep-deprived groups. The implication of this as far as reproduction is concerned is that animals that exhibit this type of oestrous cycle pattern are not likely to be fertile as they tend to remain unreceptive to male animals for most part of their cycles (Hamid & Zakaria, 2013). Although, humans do not typically exhibit this type of behaviour, it has been reported that women are less interested in sexual relations during the luteal phase of their ovarian cycles which is synonymous to the diestrus phase in rats (Bullivant et al., 2004).

The sleep deprived animals also had lengthened cycles in addition to the persisting diestrus phase. It has been reported that when female rodents spend 50% or more of their cycle on a phase or have an average cycle length of 7 days or longer, they are considered to have irregular oestrous patterns (Li et al., 2016). Irregular reproductive cycles in humans and animals are commonly caused by hormonal imbalance which is one of the consequences of sleep deprivation (Tufik et al., 2009). Additionally, longer duration of sleep deprivation caused inflammation and vascular congestion in the ovary. Aside from being a possible cause of the oestrous cycle derangement, inflammation of the reproductive organs has been implicated in the aetiology of ovarian cancer (Deivendran et al., 2011). This suggests that chronic sleep deprivation may be an indirect cause of this cancers.

There was loss of body weight in the sleep deprived group which was attenuated during the third week of sleep deprivation. This type of weight loss may be likened to 'involuntary weight loss' in humans which is a non-specific sign that is common in disorders with chronic or sub-acute courses (Hernández et al., 2003). More frequently, involuntary weight loss is associated with organ specific manifestation that serves as a key pointer to the system affected (Hernández et al., 2003).The fact that the rats began to gain weight during the third week of sleep deprivation suggests a form of internal or external adaptation which may or may not be related to the quantity of feed they consumed as sleep deprivation progressed (Koban et al., 2008). While some studies showed a positive association between sleep deprivation and weight gain, others reported a negative correlation between them. In this study, weight loss; a non-specific sign and disruption of female reproductive cycles; an organ-specific manifestation was present in the sleep deprived rats.

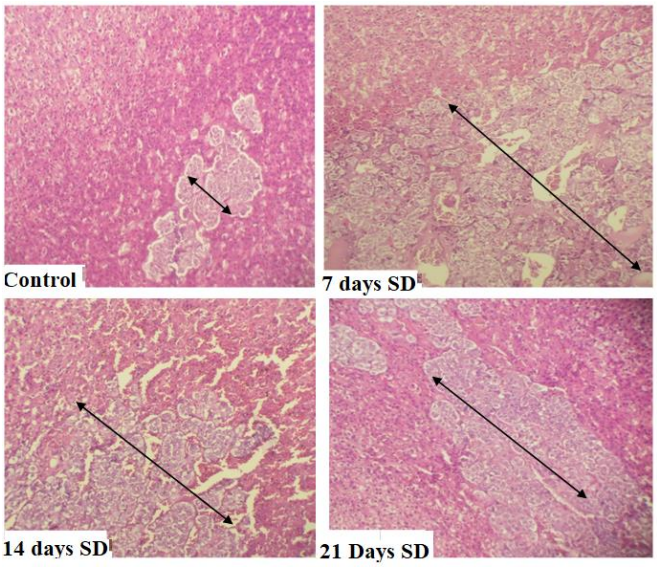

Fig. 6: Photomicrographs of adrenal gland sections from control and sleep deprived rats showing normal adrenal medulla (black spanned arrow) in control and adrenal medulla hyperplasia (black spanned arrow) in 7 days SD, 14 days SD and 21 days SD. Tissue sections were stained with H&E and presented at x100 magnification.

Effects of sleep deprivation were already evident as early as the 7th day. Contrary to the adaptation exhibited towards weight loss in the sleep deprived group, the disruption in pattern of oestrous cycles were further intensified as the duration of sleep deprivation extended. This suggests a possible lack of adaptation to sleep deprivation by the physiological processes

associated with the control of reproductive functions. Since reproductive cycles are under the regulation of hormones of the hypothalamic-pituitary-gonadal axis, further disruption of the cycles may signify persistent alteration in any of these organs.

Sleep deprivation also caused adrenal medulla hyperplasia known as pheochromocytoma in humans and characterized by excessive production of catecholamines (Pacak, 2011). The inference made from this result is that sleep deprivation may cause an increase in the production of catecholamines. This corroborates the extrapolation made from a case report in which obstructive sleep apnea caused pseudo-pheochromocytoma and raised the level of catecholamines (Wakil & Atkin, 2008; Marmouch, et al., 2016). Additionally, cases of pheochromocytoma co-existing with amenorrhoea (Wakil & Atkin, 2008) or infertility have been reported. In the study by Wakil and Atkin (2008), it was well elucidated that the amenorrhoea developed secondary to the excessive production of catecholamines (Wakil & Atkin, 2008). Animal studies have shown that hypothalamic alpha adrenergic receptors play a role in controlling gonadotropin releasing hormone and luteinizing hormone (DeGroot et al., 2001) and stimulation of the ascending noradrenergic bundle partially or completely inhibits luteinizing hormone pulse pattern (Wakil & Atkin, 2008). Any obstruction in the secretion of these hormones will adversely affect reproduction, as these hormones control the female reproductive cycle.

CONCLUSION

The results of this study showed that partial sleep deprivation reduces the frequencies of oestrous cycle phases characterized by ovulation. Thus, adequate sleep will be highly beneficial for women, especially those seeking to get pregnant.

REFERENCES

AAP, C. on adolescence, ACOG, C. on A. H. care, Diaz, A., Laufer, M., & Breech, L. (2006). Menstruation in Girls and Adolescents: Using the Menstrual Cycle as a Vital Sign. *Pediatrics*, *118*(5), 2245–2250. https://doi.org/10.1542/peds.2006-2481

Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology*, *13*(1), 37. https://doi.org/10.1186/s12958-015-0032-1

Akindele, O. O., Kunle-Alabi, O. T., Adeyemi, D. H., Oghenetega, B. O., & Raji, Y. (2014). Effects of vitamin E and melatonin on serum testosterone level in sleep deprived Wistar rats. *African Journal of Medicine and Medical Sciences*, *43*(4), 295–304.

Alvarenga, T. A., Hirotsu, C., Mazaro-Costa, R., Tufik, S., & Andersen, M. L. (2015). Impairment of male reproductive function after sleep deprivation. *Fertility and Sterility*, *103*(5), 1355–1362.e1. https://doi.org/10.1016/j.fertnstert.2015.02.002

Antunes, I. B., Andersen, M. L., Baracat, E. C., & Tufik, S. (2006). The effects of paradoxical sleep deprivation on estrous cycles of the female rats. *Hormones and Behavior*, *49*(4), 433–440. https://doi.org/10.1016/j.yhbeh.2005.09.005

Bullivant, S. B., Sellergren, S. A., Stern, K., Spencer, N. A., Jacob, S., Mennella, J. A., & McClintock, M. K. (2004). Women's sexual experience during the menstrual cycle: Identification of the sexual phase by noninvasive measurement of luteinizing hormone. *The Journal of Sexual Research*, *41*(1), 82–93. https://doi.org/10.1080/00224490409552216

Chang, J. J., Pien, G. W., Duntley, S. P., & George, A. (2011). Sleep Deprivation during Pregnancy and Maternal and Fetal Outcomes : Is There a Relationship ? *Sleep Medicine Reviews*, *14*(2), 107–114. https://doi.org/10.1016/j.smrv.2009.05.001.Sleep

DeGroot, L. J., Jameson, J. L., Burger, H. G., Loriaux, D. L., Marshall, J. C., Melmed, S., … Rubenstein, A. H. (2001). *DeGroot Endocrinology*. (L. J. DeGroot & J. L. Jameson, Eds.) (Fourth edi). Philadelphia: W.B. Saunders Company.

Deivendran, S., Marzook, K. H., & Radhakrishna Pillai, M. (2014). The role of inflammation in cervical cancer. *Advances in Experimental Medicine and Biology*. Retrieved from http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L608419692%5Cnhttp://dx.doi.org/10.1007/978-3-0348-0837-8_15%5Cnhttp://rug.on.worldcat.org/atoztitles/link/?sid=EMBASE&issn=22148019&id=doi:10.1007%2F978-3-0348-0837-8_15&atitle=The+

Ezenwanne, E. B. (2011). Current concepts in the neurophysiologic basis of sleep; A review. *Annals of Medical and Health Sciences Research*, *1*(2), 173. Retrieved from http://amhsr.org/article.asp?issn=2141-9248;year=2011;volume=1;issue=2;spage=173;epage=179;aulast=Ezenwanne

Goel, N., Basner, M., Rao, H., & Dinges, D. F. (2013). *Circadian rhythms, sleep deprivation, and human performance*. *Progress in Molecular Biology and Translational Science* (1st ed., Vol. 119). Elsevier Inc. https://doi.org/10.1016/B978-0-12-396971-2.00007-5

Hamid, H. Y., & Zakaria, Z. A. B. (2013). Reproductive characteristics of the female laboratory rat, *12*(19), 2510–2514. https://doi.org/10.5897/AJB12.2046

Hernández, J. L., Matorras, P., Riancho, J. A., & González-Macías, J. (2003). Involuntary weight loss without specific symptoms: A clinical prediction score for malignant neoplasm. *QJM - Monthly Journal of the Association of Physicians*, *96*(9), 649–655. https://doi.org/10.1093/qjmed/hcg107

Hirshfield, A. N. (1985). Comparison of granulosa cell proliferation in small follicles of hypophysectomized, prepubertal, and mature rats. *Biology of Reproduction*. https://doi.org/10.1095/biolreprod32.4.979

Hublin, C., Kaprio, J., Partinen, M., & Koskenvuo, M. (2001). Insufficient Sleep — A Population-Based Study in Adults. *Sleep*, *24*(4), 392–400. https://doi.org/10.1093/sleep/24.4.392

Kim, H., Son, J., Yoo, H., Kim, H., Oh, J., Han, D., … Njdf, G. (2016). Effects of the Female Estrous Cycle on the Sexual Behaviors and Ultrasonic Vocalizations of Male C57BL / 6 and Autistic BTBR T + tf / J Mice. *Experimental Neurobiology*, *25*(4), 156–162. https://doi.org/10.5607/en.2016.25.4.156

Koban, M., Sita, L. V, Le, W. W., & Hoffman, G. E. (2008). Sleep deprivation of rats: the hyperphagic response is real. *Sleep*, *31*(7), 927–33. from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2491509&tool=pmcentrez&rendertype=abstract

Li, J., Kim, J. S., Abejuela, V. A., Lamano, J. B., Klein, N. J., & Christian, C. A. (2016). Disrupted female estrous cyclicity in the intrahippocampal kainic acid mouse model of temporal lobe epilepsy. *Epilepsia Open*, 1–9. https://doi.org/10.1002/epi4.12026

Luyster, F. S., Strollo, P. J., Zee, P. C., & Walsh, J. K. (2012). Sleep: A Health Imperative. *Sleep*, *35*(6), 727–734. https://doi.org/10.5665/sleep.1846

Macciò, A., & Madeddu, C. (2011). Inflammation and Ovarian Cancer.

Marcondes, F. K., Bianchi, F. ., & Tanno, A. . (2002). Determination of The Estrous Cycle Phases of Rats: Some Helpful Considerations. *Brazilian Journal Biology*, *62*(4), 609–614. https://doi.org/10.1590/S1519-69842002000400008

Marmouch, H., Arfa, S., Graja, S., Slim, T., & Khochtali, I. (2016). Obstructive sleep apnea presenting as pseudopheochromocytoma. *The Pan Africa Medical Journal*. https://doi.org/10.11604/pamj.2016.23.75.8979

Micevych, P., Sinchak, K., Mills, R. H., Tao, L., LaPolt, P., & Lu, J. K. H. (2003). The luteinizing hormone surge is preceded by an estrogen-induced increase of hypothalamic progesterone in ovariectomized and adrenalectomized rats. *Neuroendocrinology*, *78*(1), 29–35. https://doi.org/10.1159/000071703

NIH. (1996). *Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals. NIH Publication revised*.

Orzeł-Gryglewska, J. (2010). Consequences of sleep deprivation. *International Journal of Occupational Medicine and Environmental Health*, *23*(1), 95–114. https://doi.org/10.2478/v10001-010-0004-9

Pacak, K. (2011). Phaeochromocytoma: A catecholamine and oxidative stress disorder. *Endocrine Regulations*, *45*(2), 65–90. https://doi.org/10.4149/endo_2011_02_65

Pereda, J., Gómez-Cambronero, L., Alberola, a, Fabregat, G., Cerdá, M., Escobar, J., … Sastre, J. (2006). Co-administration of pentoxifylline and thiopental causes death by acute pulmonary oedema in rats. *British Journal of Pharmacology*, *149*(4), 450–455. https://doi.org/10.1038/sj.bjp.0706871

Plas-Roser, S., Hassani, M., & Aron, C. (1977). Follicle-stimulating hormone. *Acta Endocrinologica*, *85*(1), 151–157.

Suchecki, D., & Tufik, S. (2000). Social stability attenuates the stress in the modified multiple platform method for paradoxical sleep deprivation in the rat. *Physiology and Behavior*, *68*(3), 309–316. https://doi.org/10.1016/S0031-9384(99)00181-X

Tufik, S., Andersen, M. L., Bittencourt, L. R. A., & de Mello, M. T. (2009). Paradoxical sleep deprivation: Neurochemical, hormonal and behavioral alterations. Evidence from 30 years of research. *Anais Da Academia Brasileira de Ciencias*, *81*(3), 521–538. https://doi.org/10.1590/S0001-37652009000300016

Wakil, A., & Atkin, S. (2008). Secondary amenorrhoea due to pheochromocytoma: a case report. *Cases.J.*, *1*(1757–1626 (Electronic) LA–eng PT–Journal Article), 30. https://doi.org/10.1186/1757-1626-1-30