IJHS

Original Article

Ameliorative effects of African walnut on nicotine-induced reproductive toxicity in rat model

David Chibuike Ikwuka[1,2], Bond Ugochukwu Anyaehie[1], Ed Nwobodo[2], Emmanuel I. Umegbolu[3], Choice Chinaemerem Nworgu[1]

[1]Department of Physiology, College of Medicine, University of Nigeria, Enugu, Nigeria, [2]Department of Human Physiology, Faculty of Basic Medical Science, Nnamdi Azikiwe University Nnewi Campus, Nigeria, [3]Department of General Outpatient, District Hospital Awgu, Enugu State, Nigeria

Address for correspondence:
David Chibuike Ikwuka, Department of Physiology, University of Nigeria Enugu Campus, Enugu State, Nigeria.
Phone: +2348037147603.
E-mail: davidikwuka@gmail.com

WEBSITE: ijhs.org.sa
ISSN: 1658-3639
PUBLISHER: Qassim University

ABSTRACT

Objective: Walnuts are widely consumed nut by men in Nigeria and it has been connected to improving male reproductive health. This study evaluated the effect of African walnut on sperm parameters and testicular architecture of nicotine (NIC)-induced reproductive toxicity in male Wistar rats.

Methods: Wistar rats were randomly assigned into four groups, that is, GN_0 (1 ml/day normal saline and normal rat chow), GN_1 (1 ml/day NIC and normal rat chow), and GN_1W_6 and GN_1W_{12} (1 ml/day of NIC daily fed with 6% and 12% walnut-rich feed), respectively. This continued for 28 days. The animals were euthanized and their sperm was collected and its parameters were analyzed. The testis was harvested and prepared for histological examination.

Results: NIC significantly reduced sperm motility ($P = 0.0006$) and sperm count ($P = 0.0001$), induced mild apoptosis of Leydig cells and caused moderate spermatogenic arrest in GN_1. However, walnut-supplemented diet significantly increased the NIC-induced reduction in sperm motility ($P = 0.04$) and sperm count ($P = 0.0001$) and its consumption was effective in attenuating testicular damage caused by NIC administration in GN_1W_6 and GN_1W_{12}.

Conclusion: African walnut could exert therapeutic effect in the reduction of the adverse effect of NIC on the sperm motility, sperm count, and testicular architecture. It is worthwhile to consider it as a useful and affordable supplement to be added to the diet of males with infertility problems.

Keywords: Juglans, nicotine, oxidative stress, testis, tobacco

Introduction

Smoking is a critical public health problem that has adverse effects on human life,[1] and the interaction between lifestyle and reproductive health is a major source of concern in the world today.[2] Several studies from different parts of the world have identified that cigarette smoking has an adverse effect on the semen quality, especially in those who are heavy smokers or those who have been smoking for several years.[3-6]

Nicotine (NIC) is the most abundant alkaloid in tobacco leave and cigarette smoke, it sustains tobacco addiction and is suspected to be responsible for the deleterious effects cigarette smoking has on the reproductive system.[7] Studies have shown the adverse effect of NIC on semen parameters.[8-14] Interestingly, it has been observed that the concentration of NIC in seminal fluid is high in men passively exposed to cigarette smoke.[14,15] NIC increases oxidative stress that leads to the production of reactive oxygen species (ROS) which can predispose the patient to not only damage to the sperm chromatin compaction process but also cause an increase in sperm DNA fragmentation.[14]

Sperm motility and quality were shown to be increased following the addition of oral antioxidants to men diagnosed with oligoasthenoteratozoospermia.[16] African walnut is an edible nut that is widely consumed traditionally by men.[17] Studies have shown that African walnuts are rich in antioxidants such as saponins, phenols, flavonoids, and alkaloids.[18-20] It has been reported that walnuts possess the ameliorative effects against oxidative damage induced by hyperglycemia,[19] exhibited hepatoprotective activity against induced liver damage,[21] offer dose-dependent wound healing activity,[22] and reduce the oxidant and inflammatory load on brain cells.[23] Walnuts, when administered to men placed on a Western style diet, were also identified to, respectively, increase sperm motility, vitality, and normal morphology significantly.[24] Till date, there appears to be no study on the effects of walnut-enriched diet on sperm parameters conducted in an animal model induced with reproductive toxicity using NIC, hence this study.

Methods

Preparation of walnut meal

African walnut was obtained and a specimen of the walnut was identified and authenticated by taxonomists from the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, with herbarium voucher specimen number UNH711a. The nuts were gotten by breaking the pods. The nuts were washed and boiled at 100°C for 2 h. It was then allowed to cool. The shells were removed and the milky colored nuts were air dried. The dried nuts were made into powder with mechanical grinder. The powdered walnut granules were stored in an air-tight container. The powder was formulated into feed with grower's mash in 6% and 12% weight of feed intake concentration. The procedure for its preparation is in accordance with Ghorbani *et al*.[25] The phytochemical qualitative analysis of the powdered walnuts was evaluated.

The mix ratio was calculated thus:

The percentage:(100-The percentage) =6%:(100–6%) = 6:94 =Walnut:feed.

=12%:(100–12%) = 12:88 = Walnut:feed.

$$\therefore \text{Weight of walnut mixed with feed} = \frac{\text{\% Walnut of feed intake} \times \text{Weight of feed to be mixed with walnut}}{100 - \text{\% Walnut of feed intake}}$$

Drug

NIC solution was prepared by mixing NIC hydrogentartrate (Sigma Chemical Company, St. Louis) at a concentration of 6.84 mg/ml (as the salt) into a 0.9 saline solution.[26]

Study design and animals

The study is an experimental study carried out from May to July 2018. Twenty-four sexually mature male Wistar rats (*Rattus norvegicus*) (200–220 g) obtained from the animal house of the Faculty of Basic Medical, University of Nigeria, Enugu, Nigeria. The animals were randomly assigned into four groups (n = 6/each) and were maintained in standard cages in a well-ventilated animal house of the Department of Physiology, University of Nigeria, Enugu Campus, Nigeria, with a 12:12 h light/dark cycle at room temperature. All efforts were used to reduce the number of rats used in the experiments.

Group 1 (GN_0) received 1 ml/kg normal saline and normal rat chow daily, Group 2 (GN_1) received 1 ml/kg NIC and normal rat chow daily, Group 3 (GN_1W_6) received 1 ml/kg NIC and 6% walnut-rich feed daily, and Group 4 (GN_1W_{12}) received 1 ml/kg NIC and 12% walnut-rich feed daily.

Ethical approval

The study was approved by College of Medicine Research Ethics Committee University of Nigeria Enugu Campus and it is coherent with the experimental guidelines of the U.S. National Institute of Health (NIH) and Institutional Animal Ethics Committee (IAEC) on the care and use of laboratory animals.

Sperm and tissue recovery

The total body weights of Wistar rats were taken before and after the experiment duration. Following the 4 weeks (28 days) experimental period, the rats were anesthetized with diethyl ether and sacrificed. Orchidectomy was performed by open castration method. A pre-scrotal incision was made and the testicles were milked out of the incision site and weighed with OHAUS electric weighing balance (OHAUS Corporation Parsippany, New Jersey, United States). The left testis was removed along with its epididymis. The caudal epididymis was removed and placed in 400 µl pre-warmed (to 37°C) human tubal fluid (with HEPES, Cat. # 2002, *in vitro* care, Frederick, MD), a capacitating medium used previously.[27,28] The samples were analyzed immediately after collection.

The testes were dewax in xylene for 30 min, xylene removed by rinsing in absolute alcohol, 90%, 70%, and 50% alcohol for 2 s each. It was wash in two changes of water, stained in hematoxylin for 20 min, washed in water, and differentiated in 1% acid. It was blue in tap water, washed in water, and counter stained in eosin for 5 min, washed in water, dried, and cleared in xylene before mounted in D.P.X and dry for micrograph and interpretation.

Sperm characteristics analysis

Sperm motility was immediately assessed. The sperm was squeezed on a pre-warmed slide, two drops of warm 2.9% sodium citrate were added, covered with a cover slip, examined, and scored under the microscope using ×40 objective lens with reduced light.[29] To determine sperm morphology, an portion of sperm was fixed, mounted on slides, and stained with Coomassie Brilliant Blue G-250 in 60% methanol-acetic acid for 10 min. Sperms were analyzed for morphological abnormalities according to the World Health Organization[30] standards. The prepared slides were afterward examined under the Nikon Eclipse E100 Biological microscope using oil immersion with ×100 objective lens. Sperm count was done with the aid of the improved Neubauer counting chamber (Hawksley Oxford Street, London) viewed under a light microscope (Nikon Eclipse E100). The counting was done in five Thoma chambers.[31]

Statistical Analysis of data

Data entry and analysis were performed using IBM SPSS version 22.0 (SPSS Inc., Chicago, IL., USA) for Windows.

Data analysis was done using descriptive statistics of proportions (mean ± standard deviation). Independent samples t-test was used to analyze the difference among groups and $P < 0.05$ was considered to be statistically significant.

Results

In Table 1, the phytochemical screening of the mass of dried extracted walnuts meal indicated the presence of carbohydrates, reducing sugars, amino acids, oils, saponins, glycosides, alkaloids, and terpenoids. However, no tannins, flavonoids, phenols, nor resins were detected.

In Figure 1, the body mass of rats fed with 6% and 12% walnut-enriched diet did not differ significantly in comparison to rats fed the standard (control) diet over a 4 weeks period. The walnut-enriched diet had no effect on body weight. Body weight is a function of the number of weeks on the diet. Data expressed as mean (±SD) for GN_0, GN_1, GN_1W_6, and GN_1W_{12}. This result is parallel to the report of Nwaoguikpe *et al*.[18]

Table 2 shows a significant decrease ($P = 0.0006$) in sperm motility of GN_1 (55.0%) following the administration of NIC as compared to GN_0 (78.3%). There was, however, a significant increase ($P = 0.04$, $P = 0.03$) in sperm motility of GN_1W_6 and GN_1W_{12} (70%, 68.3%) relative to GN_1 (55.0%) following the addition of walnut at 6% and 12%, respectively, to the diet of the groups. Addition of walnut at 6% and 12% does not show to significantly increase ($P = 0.2$, $P = 0.08$) sperm motility in GN_1W_6 and GN_1W_{12} (70%, 68.3%) when compared to GN_0 (78.3%).

The result data also revealed that there was a significant decrease ($P < 0.0001$) in sperm count of GN_1 (49.0 Million/ml) relative to that of GN_0 (78.7 Million/ml). The sperm count values for GN_1W_6 and GN_1W_{12} (73.3 Million/ml and 81.3 Million/ml) are not significantly different ($P = 0.2$, $P = 0.4$) from GN_0 (78.7 Million/ml). However, there was a progressive significant increase ($P < 0.0001$) in sperm count of GN_1W_6 and GN_1W_{12} (73.3 Million/ml and 81.3 Million/ml) when compared to sperm count of GN_1 (49.0 Million/ml).

Table 2 result further reveals that there was no significant difference ($P = 0.51$) in the mean percentages between GN_0 (90.0%) and GN_1 (89.0%), no significant difference ($P = 0.47$, 0.51) between the mean percentage of GN_0 (90.0%) and GN_1W_6 and GN_1W_{12} (91.0 and 89.0), and no significant difference ($P = 0.19$) between the mean percentage of GN_1 (89.0%) as relative to GN_1W_6 and GN_1W_{12} (91.0 and 89.0). This implies that sperm morphology was not significantly affected by the administration of NIC and the addition of walnut to diet.

Table 1: Phytochemical screening of the mass of dried walnut extract

Extract	Aqueous extract	Ethanolic extract
Carbohydrates	++	++
Reducing sugar	+	−
Amino acid	+	++
Oil	+	−
Saponins	+++	−
Flavonoids	−	−
Glycosides	++	−
Alkaloids	++	+
Steroids	+	++
Terpenoids	+	+
Phenol	−	−

(−): Absence, (+): Less presence, (++): Moderate presence, (+++): High presence

Table 2: Sperm parameters (Mean±SD) of the positive control (GN_0), negative control (GN_1), 6% walnut (GN_1W_6), and 12% walnut (GN_1W_{12}), respectively

Group parameters	GN_0 Mean±SD	GN_1 Mean±SD	GN_1W_6 Mean±SD	GN_1W_{12} Mean±SD
Motility (%)	78.3±7.6	*55.0±8.7	*70.0±13.2	*68.3±10.4
Count (Million/ml)	78.7±7.8	49.0±1.7	73.3±6.7	81.3±4.5
Morphology (%)	90.0±2.0	89.3±1.5	91.0±2.6	89.3±1.5

Data presented in % ± SD, t-test statistics. *Represents significance difference as compared with control group ($P<0.05$)

Histological studies on testis

Figure 2 shows the histological analysis with ×400 which reveals that GN_0 has a well enhanced spermatogenesis. In GN_1, there was variation in the testicular architecture evidence by the mild apoptosis of interstitial cells of Leydig to moderate spermatogenic arrest due to the effect of NIC. In GN_1W_6, there was mild apoptosis of the interstitial cells of Leydig which is attenuated as compared to GN_1, and GN_1W_{12} showed a well-enhanced spermatogenesis as seen in GN_0.

Discussion

In the present study, the effects of walnut-supplemented diet on sperm parameters conducted in male Wistar rats induced with reproductive toxicity using NIC were investigated. From our investigation, NIC proves to affect the male reproductive system and threatens male fertility.

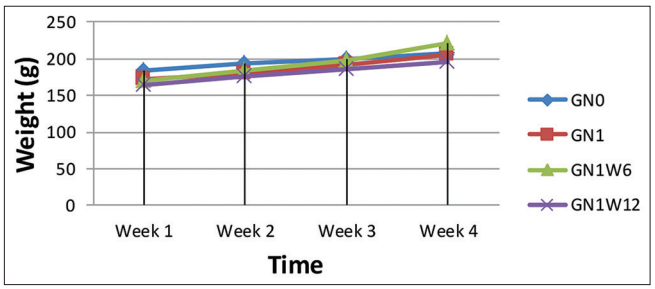

Figure 1: Weight over time chart for GN_0, GN_1, GN_1W_6, and GN_1W_{12} respectively

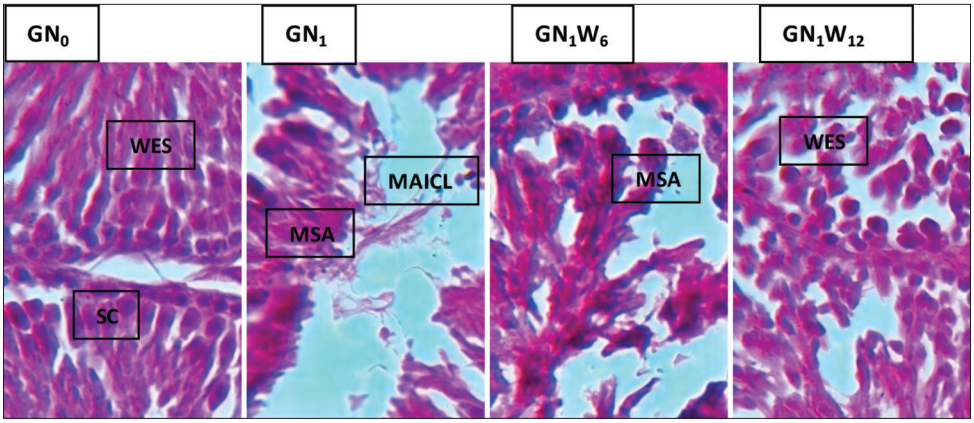

Figure 2: Photomicrograph of sections of testis for GN_0, GN_1, GN_1W_6, and GN_1W_{12} respectively
WES: Well-enhanced spermatogenesis, SC: Sertoli cells, MSA: Moderate spermatogenic arrest, MAICL: Mild apoptosis of interstitial cells of Leydig

Our finding on the negative effects of NIC on the male reproductive system is in line with the reports and findings of other researchers.[7,10,13,14,31,32] Condorelli *et al*.[14] posited that NIC increases oxidative stress leading to the production of ROS which can cause not only damage to the sperm chromatin compaction process but also increase sperm DNA fragmentation; it is of note that damage to sperm chromatin compaction process and increases sperm DNA fragmentation leads to defective sperm function which is identified as the most common cause of male fertility. Although physiologically controlled concentration level of ROS plays important roles in physiological processes such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion to facilitate fertilization,[32] the excess production of ROS disrupts the delicate balance between ROS and antioxidants leading to lipid peroxidation, DNA damage, and sperm apoptosis.[33] ROS is produced endogenously in leucocytes, immature spermatozoa, and varicocele, and exogenously by radiation, toxins, smoking, and alcohol consumption.[33] However, in healthy men, there exists an effective scavenging system made up of various endogenous antioxidants which belong to both enzymatic and non-enzymatic groups that can remove the excess physiological ROS and prevent oxidative stress.[34] However, the effect of this scavenging system is attenuated with increased level of NIC in seminal fluid.[33]

In the present study, the supplementation of diet where walnuts composed of 6% and 12% of caloric intake for a 4-week period, compared to that in the study with humans,[24] caused no significant change in the body wight. However, the findings of this study are not consistent with the findings of Ting *et al*. and Rock *et al*.[35,36] who recorded that walnut-enriched diet promotes weight loss. This result may be probably due to the lower percentage of walnut added to the diet of the experimental animals.

There was significant improvement in the sperm motility and sperm count of rats supplemented with 6% and 12% walnut in diet but there was no clear observable increase in abnormal sperm morphology across the groups. The significant improvement in sperm motility is parallel to that reported in humans.[24] However, the report on human males also showed significant improvement in their sperm morphology following the addition of walnut to their diet.[24] The action of walnut in the improvement of sperm motility and count may be attributed to the antioxidants such as saponins, glycosides, and alkaloids present in walnut which is able to inhibit the production of malondealdehyde which is the end product of lipid peroxidation caused by the hydroxyl and superoxide radicals generated by NIC. Saponins in earlier studies have been associated with improvement in sperm parameters.[37]

Testicular histology damage by NIC corresponds with the report of Baker and Aitken[38] who posited that NIC linked oxidative stress is known to cause peroxidative damage in sperm plasma membranes. However, there was dose-dependent improvement in the testicular architecture of rats fed with 6% and 12% walnut-supplemented diet.

Finally, from our findings, it is worthy of note that NIC adversely affects sperm parameters and induces pathological changes in the interstitial cells of Leydig. However, walnut supplement improved sperm parameters such as motility and count and also attenuated the testicular damage caused by NIC.

Conclusion

Our study confirmed that NIC affects the male reproductive system through its adverse effects on the interstitial cells of Leydig, seminiferous tubules, and spermatogenesis. African walnut has demonstrated potential to attenuate the adverse effect of NIC on the sperm motility, sperm count testicular architecture. This effect which has been shown to be dose dependent did not have any significant effect on the morphology of sperm and weight of the rats. It is, therefore, worthwhile to consider walnut as a potential safe, useful, and

affordable supplement to be added to the diet of males with infertility problems.

Authors' Declaration Statements

Ethical approval

The study was approved by College of Medicine Research Ethics Committee University of Nigeria Enugu Campus and it is coherent with the experimental guidelines of the U.S. NIH and IAEC on the care and use of laboratory animals.

Data availability

The authors confirm that the data supporting the findings of the study are available within the article.

Competing interest

The authors declare that there are no commercial or financial relationships that could constitute as potential conflicts of interest in the conduct of the research.

Funding statement

The authors declare that this study received no form of financial support or funding from any institution.

Authors' Contributions

The study arose from an original idea from DCI and BUA. All authors contributed to the study's design. DCI wrote the first draft. EIU and CCN carried out sample analysis and data analysis. DCI, EN, EIU, and CCN are responsible for the preparation of the manuscript. BUA and EN revised and guided all the stages of the experiment and writing of the manuscript.

Acknowledgments

The authors would like to acknowledge Dr. M. Ezenwali and the technical staff of Brain Phosphorylation Laboratory, Enugu, for their assistance in conducting experimental sessions.

References

1. Kim HJ, Park KK, Chung WY, Lee SK, Kim KR. Protective effect of white-fleshed peach (*Prunus persica* (L.) Batsch) on chronic nicotine-inducedtoxicity. J Cancer Prev 2017;22:22-32.
2. Durairajanayagam D. Lifestyle causes of male infertility. Arab J Urol 2018;16:10-20.
3. Gaur DS, Talekar M, Pathak VP. Effect of cigarette smoking on semen quality of infertile men. Singapore Med J 2007;48:119-23.
4. Calogero A, Polosa R, Perdichizzi A, Guarino F, La Vignera S, Scarfia A, *et al*. Cigarette smoke extract immobilizes human spermatozoa and induces sperm apoptosis. Reprod Biomed Online 2009;19:564-71.
5. Chohan KR, Badawy SZ. Cigarette smoking impairs sperm bioenergetics. Int Braz J Urol 2010;36:60-5.
6. El-Melegy NT, Ali ME. Apoptotic markers in semen of infertile men: Association with cigarette smoking. Int Braz J Urol 2011;37:495-506.
7. Egesie UG, Galam NZ, Gambo IM, Ayodeji AA, Simji GS, Kwekyes LS, *et al*. Evaluation of andrological indices and testicular histology following administration of varied doses of nicotine. J Biol Agric Healthc 2013;3:83-94.
8. Arabi M, Shareghi B. Anti-fertility effect of nicotine. Zhonghua Nan Ke Xue 2005;11:323-30.
9. Brand JS, Chan MF, Dowsett M, Folkerd E, Wareham NJ, Luben RN. Cigarette smoking and endogenous sex hormones in postmenopausal women. J Clin Endocrinol Metab 2011;96:3184-92.
10. Oyeyipo IP, Raji Y, Emikpe BO, Bolarinwa AF. Effects of nicotine on sperm characteristics and fertility profile in adult male rats: A possible role of cessation. J Reprod Infertil 2011;12:201-7.
11. Tweed JO, Hsia SH, Lutfy K, Friedman TC. The endocrine effects of nicotine and cigarette smoke. Trends Endocrinol Metab 2012;23:334-42.
12. Condorelli RA, La Vignera S, Giacone F, Iacoviello L, Vicari E, Mongioì L, et al. *In vitro* effects of nicotine on sperm motility and bio-functional flow cytometry sperm parameters. Int J Immunopathol Pharmacol 2013;26:739-46.
13. Al-Turki HA. Effect of smoking on reproductive hormones and semen parameters of infertile Saudi Arabians. Urol Ann 2015;7:63-6.
14. Condorelli RA, La Vignera S, Giacone F, Iacoviello L, Mongioì LM, Li Volti G, *et al*. Nicotine effects and receptor expression on human spermatozoa: Possible neuroendocrine mechanism. Front Physiol 2017;8:177.
15. Pacifici R, Altieri I, Gandini L, Lenzi A, Passa AR, Pichini S, *et al*. Environmental tobacco smoke: Nicotine and cotinine concentration in semen. Environ Res 1995;68:69-72.
16. Wirleitner B, Vanderzwalmen P, Stecher A, Spitzer D, Schuff M, Schwerda D, *et al*. Dietary supplementation of antioxidants improves sperm quality of IVF patients in terms of motility, sperm count, and nuclear vacuolization. Int J Vitam Nutr Res 2012;82:391-8.
17. Okonkwo CO, Ozoude UJ. The impact of processing on the nutrient content, vitamin and mineral composition of African Walnut (*Tetracarpidium conophorum*). Int J Nov Res Life Sci 2014;1:10-6.
18. Nwaoguikpe RN, Ujowundu CO, Wesley B. Phytochemical and biochemical compositions of African walnut (*Tetracarpidium conophorum*). J Pharm Biomed Sci 2012;20:1-5.
19. Ajilore BS, Olorunnisola OS, Owoade OA. *Tetracarpidium conophorum* (African walnut) seeds protects against diabetes-induced liver damage in rats treated with streptozotocin. Rom J Diabetes Nutr Metab Dis 2020;27:135-45.
20. Udedi SC, Ani ON, Anajekwu BN, Ononamadu CJ, Igwilo IO, Ibeabuchi CG, *et al*. Nutritional compositionand antioxidant activity of African Walnut, *Tetracarpidium conophorum*. J Appl Biochem 2014;107:170-80.
21. Oriakhi K, Uadia PO. Hepatoprotective fractions from methanol extracts of *Tetracarpidium conophorum* (African walnut) seeds. Arch Bas Appl Med 2020;8:45-8.
22. Ezealisiji KM, Omotosho AE, Udoh R, Agbo MO. Wound healing activity of n-hexane and methanol extracts of *Tetracarpidium conophorum* (mull. Arg.) Hutch (African walnut) in Wistar rats. Malays J Pharm Sci 2014;12:79-88.
23. Poulose SM, Miller MG, Shukitt-Hale B. Role of walnuts in maintaining brain health with age. J Nutr 2014;144:561S-6.
24. Robbins WA, Xun L, FitzGerald LZ, Esguerra S, Henning SM, Carpenter CL. Walnuts improve semen quality in men consuming a Western-style diet: Randomized control dietary intervention trial. Biol Reprod 2012;87:1-8.
25. Ghorbani R, Mokhtari T, Khazaei M, Salahshoor MR, Jalili C, Bakhtiari M. The effect of walnut on the weight, blood glucose and sex

hormones of diabetic male rats. Int J Morphol 2014;32:833-41.

26. Mendez IA, Carcoba L, Wellman PJ, Cepeda-Benito A. High-fat diet meal patterns during and after continuous nicotine treatment in male rats. Exp Clin Psychopharmacol 2016;24:477-84.
27. Aravindan RG, Fomin VP, Naik UP, Modelski MJ, Naik MU, Galileo DS, *et al*. CASK interacts with PMCA4b and JAM-A on the mouse sperm flagellum to regulate Ca^{2+} homeostasis and motility. J Cell Physiol 2012;227:3138-50.
28. Andrews RE, Galileo DS, Martin-DeLeon PA. Plasma membrane Ca^{2+}-ATPase 4: Interaction with constitutive nitric oxide synthases in human sperm and prostasomes which carry Ca^{2+}/CaM-dependent serine kinase. Mol Hum Reprod 2015;21:832-43.
29. Morrissey RE, Schwetz BA, Lamb JC, Ross MD, Teague JL, Morris RW. Evaluation of rodent sperm, vaginal cytology, and reproductive organ weight data from national toxicology program 13-week studies. Fundam Appl Toxicol 1988;11:343-58.
30. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010. p. 56-8.
31. Freund M, Carol B. Factors affecting haemocytometer count of sperm concentration in human semen. J Reprod Fertil 1964;8:149-55.
32. Oyeyipo IP, Raji Y, Emikpe BO, Bolarinwa AF. Nitric oxide synthase inhibition ameliorates nicotine-induced sperm function decline in male rats. Asian Pac J Reprod 2015;4:212-6.
33. Agarwal A, Virk G, Ong C, Plessis SS. Effect of oxidative stress on male reproduction. World J Mens Health 2014;32:1-17.
34. Mortazavi M, Salehi I, Alizadeh Z, Vahabian M, Mohammadi RA. Protective effects of antioxidants on sperm parameters and seminiferous tubules epithelium in high fat-fed rats. J Reprod Infertil 2014;15:22-8.
35. Ting L, Omar MG, Allysa A, Jill HR, Debra KS, Jason MK, *et al*. Consumption of walnuts in combination with other whole foods produces physiologic, metabolic, and gene expression changes in obese C57BL/6J high-fat-fed male mice. J Nutr 2016;146:1641-50.
36. Rock CL, Flatt SW, Barkai HS, Pakiz B, Heath DD. Walnut consumption in a weight reduction intervention: Effects on body weight, biological measures, blood pressure and satiety. Nutr J 2017;6:1-10.
37. Peiris LD, Dhanushka MA, Jayathilake TA. Evaluation of aqueous leaf extract of *Cardiospermum halicacabum* (L.) on fertility of male rats. Biomed Res Int 2015;2015:175726.
38. Baker MA, Aitken RJ. Reactive oxygen species in spermatozoa: Methods for monitoring and significance for the origins of genetic disease and infertility. Reprod Biol Endocrinol 2005;3:67.