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Research Article

Maleficent effects of chronic tobacco *Shisha* smoke exposure on sperm DNA fragmentation, count, motility and morphology in adult male wistar rats

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Keywords:
Shisha smoke, malondialdehyde, testosterone, sperm DNA fragmentation, sperm count, sperm motility and sperm morphology

ABSTRACT

BACKGROUND: Currently there are no putative empirical data on the effect of *Shisha* smoking on sperm DNA integrity and some of the available data on the adverse effects of *Shisha* smoking on conventional semen characteristics: sperm count, sperm motility and sperm morphology are contradictory. Despite the well-known deleterious reproductive effects of cigarette smoking, it is relatively unclear whether or not *Shisha* smoking has the same effect on male reproductive parameters. The present study was aimed at determining the effect of chronic *Shisha* smoke exposure on semen parameters and sperm DNA integrity in adult male Wistar rats. **METHODS:** Twenty-one adult male Wistar rats between the ages of 8-12 weeks, weighing between 160 -180 g were divided randomly into three groups containing 7 rats per group. Group I rats were kept for 30 minutes daily in the nose-only exposure chamber for a period 13 weeks without exposure to *Shisha* smoke; group II (with water in the *Shisha* jar) was exposed to bonged shisha smoke (BSS) and group III (without water in the shisha jar) was exposed to unbonged *Shisha* smoke (UBSS), respectively for 7 seconds first and fresh air later for 53 seconds, alternatively for 30 minutes daily for a period of 13 weeks. The *Shisha* smoke was drawn from the *Shisha* apparatus outlet by a vacuum compressor at a pressure of 300 kPa into the nose-only exposure chamber where the rats were kept. At the end of the exposure, five animals from each group were randomly selected and anaesthetised with 0.4 mL/100g of combined ketamine and diazepam and blood samples were obtained through cardiac puncture. **RESULTS:** The result obtained showed that chronic exposure to *Shisha* smoke revealed a significant increase in testicular malondialdehyde (MDA) level, high sperm DNA fragmentation and abnormal cell morphology, marked reduction in serum testosterone concentration, sperm count and progressive motility.

INTRODUCTION

Sperm viability is determining in terms of its ability in a given population of spermatozoa to fertilize an ovum, in the same vein to equally support the development of a normal healthy embryo that will develop into a normal blastocyst, nidate into the uterus, establish a placenta, and differentiate into an apparently healthy human (Aitken and De luliis, 2007). *Shisha* also known as Hookah smoking or water-pipe tobacco smoking, is apparently healthy human (Aitken and De luliis, 2007).*Shisha* also known as Hookah smoking or water-pipe tobacco smoking, is generally viewed as a social activity and a relatively inexpensive way to get together and have fun is generally viewed as a social activity and a relatively inexpensive way to get together and have fun (American Lung Association, 2007). Often, the group shares one pipe and tries different flavours such as apple, bubble gum, chocolate, mint, orange soda, root beer and grape (Primack *et al*., 2009). It is widely perceived to be less harmful than other forms of tobacco use. The reproductive adverse effects of cigarette smoking have been studied with conflicting and unsatisfactory results (Mostafa, 2010), but there is

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no available data on effects of *Shisha* smoking on the reproductive system.

Water-pipe smoking (WPS) started in the Middle East and has gained popularity in several Western countries. This old practice has recently sprung up rapidly around bars in college campuses in the United States, Europe and Africa (Caroline *et al*., 2011; Martinasek *et al*., 2013), and has become a practice of concern among students of University campuses in Nigeria (Abraham *et al*., 2019). Originally created as a theoretically less harmful method of tobacco use, it was suggested that smoke should first be passed through a small receptacle of water so that it would be rendered harmless. It has been reported that more than 100 million people worldwide indulge in water pipe smoking (WPS) daily (Fahed *et al*., 2011)

Over the last decade, many studies have confirmed that sperm DNA damage has strong association with every early embryonic developmental problems. These include impaired fertilization, slow early embryo development, reduced implantation, high rate of miscarriage and birth defects in the offspring. Childhood cancers have also been associated with oxidative damage to sperm DNA as a consequence of paternal smoking (Sheena *et al*., 2013). There are many possible causes of such DNA damage, including abortive apoptosis, oxidative stress associated with male genital tract infection, exposure to redox cycling chemicals, and defects of spermiogenesis associated with the retention of excess residual cytoplasm

The DNA fragmentation occurs when there are changes to the bases or a physical break in one or both of the DNA strands which may result from oxidative stress induced by smoke. Sperm DNA fragmentation is the physical breaking of one or both DNA strands in sperm chromosomes. If this occurs within a gene in a sperm that fertilized the egg, the consequence may be the death of the resulting embryo or miscarriage (Sheena *et al*., 2013).

A 2008 report by the American Society for Reproductive Medicine (ASRM) states that, while routine DNA integrity testing in the evaluation of infertility is not done, sperm DNA damage is more common in infertile men and may affect reproductive outcomes in selected couples, including those with recurrent spontaneous miscarriage or idiopathic infertility (Zhang *et al*., 2014). Male infertility is the commonest cause of infertility (Sheena, 2013). It contributes nearly 50% of infertility in couples of reproductive age. This occurs as a result of population ageing and adverse changes in lifestyles, including smoking (De Mouzon *et al*., 2006).

The Sperm Chromatin dispersion test measures the presence of DNA damage. The test determines the percentage of sperm with fragmented DNA and the degree of DNA damage and provides a DNA Fragmentation Index (DFI) score to indicate the likelihood of sperm contributing to infertility problems.

The aim of this study was to determine the effect of chronic *Shisha* smoke exposure on testicular parameters (testicular MDA level, serum testosterone levels, sperm count, sperm motility and sperm morphology) and sperm DNA integrity in adult male Wistar rats.

MATERIALS AND METHODS

Experimental Animals

Twenty-one adult male Wistar rats between the ages of 8-12 weeks, weighing from 160 -180 g were used for this research. The animals were obtained from the Department of Human Physiology, Ahmadu Bello University, Zaria. The rats were housed in the Animal House of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria. The animals were kept in vivarium plastic cages (Plate I) under normal environmental temperature and fed with standard pellet diet and water given *ad libitum* except during exposure time. The rats were grouped into three groups of seven rats each and the exposure groups were exposed to *Shisha* smoke in a nose-only exposure chamber. All the animals were weighed once weekly during the 13 weeks duration of the study to determine weight changes.

Determination of sample size

The number of animals for laboratory experiment that would yield good power of statistics is six; from resource equation method for sample size, E= (6x3)-3, E=15, E lies between 10-20 (Jaykaran and Kantharia, 2013). To avoid attrition and unforeseen death, 2 rats were added to each group. 6- number of rats in each group that would be statistically significant, 3- represent number of groups (one control group and two experimental groups).

Chemicals and Drugs

Shisha grape flavour packet obtained from Kowa store, Samara, Zaria, ketamine sodium, diazepam and other chemicals of analytical grade were purchased from

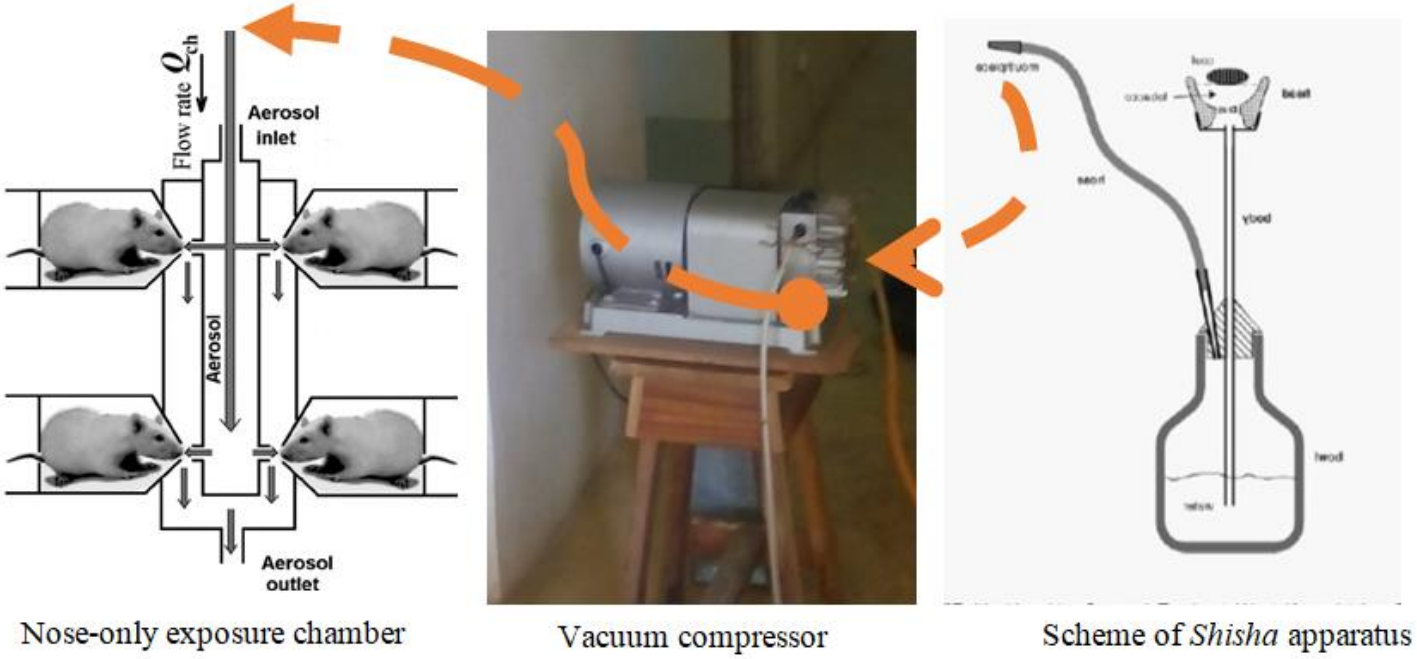

Fig. 1: Scheme for process of *Shisha* smoke production and administration into nose-only exposure chamber

reputable chemical and pharmaceutical stores within Zaria.

Experimental Protocol
Animal Grouping
Group I (n = 7 rats)
Group I served as control. The animals were kept in the nose-only exposure chamber for 30 minutes each day without *Shisha* smoke administration for thirteen weeks.
Group II (n = 7 rats)
Group II were exposed to *Shisha* smoke in the nose-only exposure chamber with water in the water-pipe smoke jar for 30 minutes daily for thirteen weeks.
Group III (n = 7 rats)
Group III were exposed to *Shisha* smoke in the nose-only exposure chamber without water in the water jar for 30 minutes daily for thirteen weeks.

Shisha Smoke Administration
Smoke produced by the *Shisha* apparatus was driven into a nose-only chamber containing the experimental rats through a vacuum compressor at a pressure of 300 KPa, as shown in Fig. 3.1 and Plate VI below for 7 seconds. Thereafter, fresh air was allowed into the chamber through the air-inlet for 53 seconds as described by Foroutanjazi *et al*. (2014). This was repeated for a duration of 30 minutes daily for a period of 13 weeks.

Fig. 2: Photograph of the apparatuses for processing Shisha smoke production and its administration into nose-only exposure chamber during the experimental period

Sample Collection
At the end of the experiment, five animals from each group were anaesthetized with 0.4 mL/100g of combined (ration 1:1) ketamine and diazepam anaesthesia (Lijuan *et al*., 2016), 24 h after last day of administration. Blood samples were collected by cardiac puncture and placed in plain bottle (EDTA-free bottle) for serum collection using suction pipette after

spontaneous sedimentation. Thereafter, the animals were dissected and the testes were harvested. The epididymis was resected from the right testis and carefully drained of its semen.

The left testis of each rat labeled according to the groups were removed from the scrotum. The testis was homogenised in 10 ml phosphate-buffered solution (pH 7.4) using a pestle and mortar. The Homogenates were turned into test tubes labeled according the group and number of each rat. The resultant homogenates were centrifuged at 2,000 revolutions per minute for 10 minutes at 4°C. The supernatant were collected and used for MDA assays.

Determination of Serum Testosterone Level

Serum testosterone concentration was determined based on enzyme immunoassay competitive binding principle as described by Finecare™ D-Dimer Rapid Quantitative Immunoassay test kit-. Testosterone present in the sample competed with enzyme-labeled testosterone for binding with anti-testosterone antibody immobilised on the microwell surface. The amount of conjugate that bound to the micro-well surface was decreased in proportion to the concentration of testosterone in the sample. The testosterone concentration in the sample and the control were determined from a standard curve using method of Wilkle and Utley (1987) as follows:

(i) The average absorbance value (A. 450) for each reference standard, control and test sample were calculated.
(ii) A standard curve was prepared by plotting the average absorbance (A.450) versus the corresponding concentration of the standards on a log graph paper.
(iii) The absorbance (A. 450) value for each test sample was used to determine the corresponding concentration of testosterone in ng/ml from the standard curve (Wilkle and Utley, 1987).

Analysis of Sperm Parameters

The testes from each rat were carefully exposed and removed. They were trimmed free of the epididymal and adjoining tissues. From each separated epididymis, the caudal part was removed and placed in a beaker containing 1 ml physiological saline solution. Each section was quickly macerated with a pair of sharp scissors and was left for a few minutes to liberate its spermatozoa into the saline solution (Saleem *et al.*, 2013). Subsequently, the following parameters were determined.

Sperm motility

After sacrificing the animals, the caudae epididymes were removed and placed in a Petri-plate containing 1 ml of physiological saline solution (PSS) at room temperature. The epididymis were homogenised to allow the sperms to swim out. Sample of the preparation was placed on a glass slide and covered with cover-slip. The motility of epididymal sperm was evaluated microscopically within 2–4 min of their isolation from the cauda epididymis and the data expressed as percentages of progressively motile, none-progressively motile and non-motile spermatozoa. The percentage of motility was evaluated visually at x40 magnification. Motility estimations were performed from 3 different fields in each sample (Sonmez *et al.*, 2007).

Sperm count

The spermatozoa were counted by haemocytometer using the improved Neubauer (0.0025 mm^2, Marienfeld, Germany) chamber. The total epididymal sperm per ml was calculated as follows (Saleem *et al*, 2013):

[Total epididymal sperm /ml = *(Average number of sperm per chamber) x* 10^3 *x (Dilution Factor)]*.

Sperm morphology assessment by World Health Organisation criteria

The Epididymis were minced in 1 ml physiological saline solution (PSS) to make a suspension. Sperm morphology was determined by the method of WHO (1992). Following liquefaction, 10 µL of semen were spread onto a glass slide and allowed to air-dry at room temperature. The smears were stained with Giemsa stain and sperm morphology were assessed according to WHO criteria. 200 cells *per* smear were counted using brightfield illumination at final magnification of 1000X and oil immersion. According to WHO criteria, a morphologically normal spermatozoon has an oval head and an acrosome covering 40%–70% of the head area. A normal spermatozoon has no neck, midpiece, tail abnormalities nor cytoplasmic droplets larger than 50% of the sperm head. The abnormal spermatozoa were classified into two categories: (i) spermatozoa with defective heads (amorphous, hook less, banana shaped, double head, microcephaly and cephalocaudal junction defects), and (ii) spermatozoa with defective tails (two-tailed, coiled/bent tails); Vijay *et al.*, 2013.

Determination of Sperm DNA Fragmentation

The assessment of DNA damage was measured using an improved version of the sperm chromatin dispersion test (Halosperm kit; Halotech DNA S.L., Madrid, Spain). Samples were prepared for analysis according to the protocol described by Fernandez *et al*. (2003).

Exactly 60 µL of a prepared semen with phosphate-buffered solution (pH 6.88) was added into a thawed agarose solution in a tube at room temperature for five

minutes. 15 µL of the aliquot was dropped on a glass slide and covered gently a 22 x 22 mm^2 cover slip and kept inside DNA warmer at 8^0C for 8 minutes. A staining step was conducted to prepare the slides for counting processes. The samples were stained with Diff-Quik solution, each slide was immersed in 8 mL Diff-Quik solution I (eosinophilic) and Diff-Quik solution II (basophilic) for 20 minutes each in a DNA immersion staining tray. Thereafter, the slide was immersed in 10 mL of distilled water. The slide was placed into a tray and covered with 70% ethanol for two minutes, followed by 90% ethanol for two minutes, and, finally, 100% ethanol for two minutes. The slide was left to dry at room temperature and stained by Wright stain solution.

Laboratory scored for 400 spermatozoa for each slide under the ×100 objective of the bright-field microscope according to the patterns established by Fernandez *et al*. (2005). Conserved spermatozoa with integrity of intact DNA, a peripheral halo of DNA loops around a central core was observed. Spermatozoa with fragmented DNA produced very small halos or no halos at all. The classification of DNA fragmentation index (%DFI: i.e., % sperm cells containing damaged DNA) was calculated as follows (Osman *et al*., 2014):

(i) ≤ 15% DFI = Excellent to good sperm DNA integrity
(ii) > 15 to < 25% DFI = Good to fair sperm DNA integrity
(iii) > 25 to < 50% DFI = Fair to poor sperm DNA integrity
(iv) ≥ 50% DFI = Very poor sperm DNA integrity

Determination of Testicular Malondialdehyde Concentration

Testicular hydroperoxide level was evaluated using an analytical system. The test is a colorimetric test that takes advantage of the ability of hydroperoxide to generate free radicals after reacting with transitional metals. When buffered chromogenic substance was added to the reaction, a colored complex appeared. This complex was measured spectrophotometrically. Lipid peroxidation level in the testis were measured by method of Ohkawa *et al*. (1979) as thiobarbituric acid reactive substances (TBARS). Testis was homogenised in ice cold 0.15 M KCl (10%) and the concentration of TBARS was expressed as µmol of MDA per mg tissue using 1,1,3,3-tetramethoxypropane as standard. The absorbance was read at 532 nm using spectrophotometer.

Data Analyses

Data obtained from the study were expressed as mean ± SEM. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Values of $P < 0.05$ were considered statistically significant. Statistical Package for Social Sciences (SPSS) version 20 was used for the analysis.

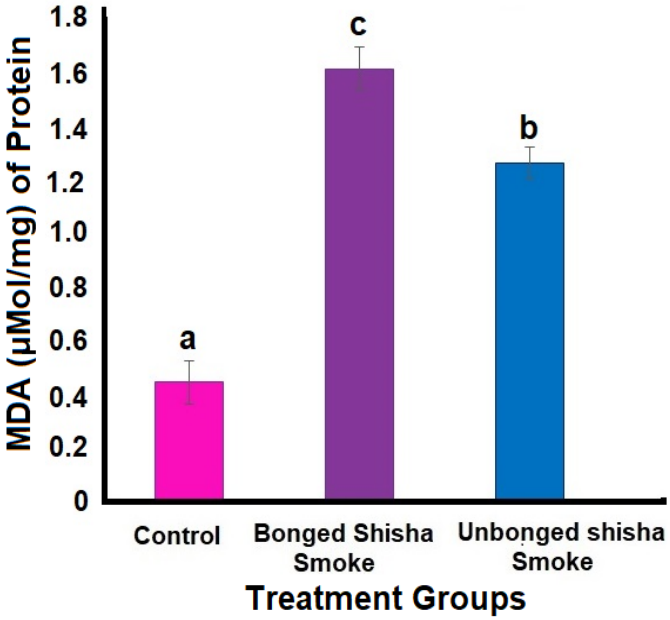

Fig. 3: Effects of *Shisha* smoke exposure on testicular malondialdehyde level in adult male Wistar rats. [a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

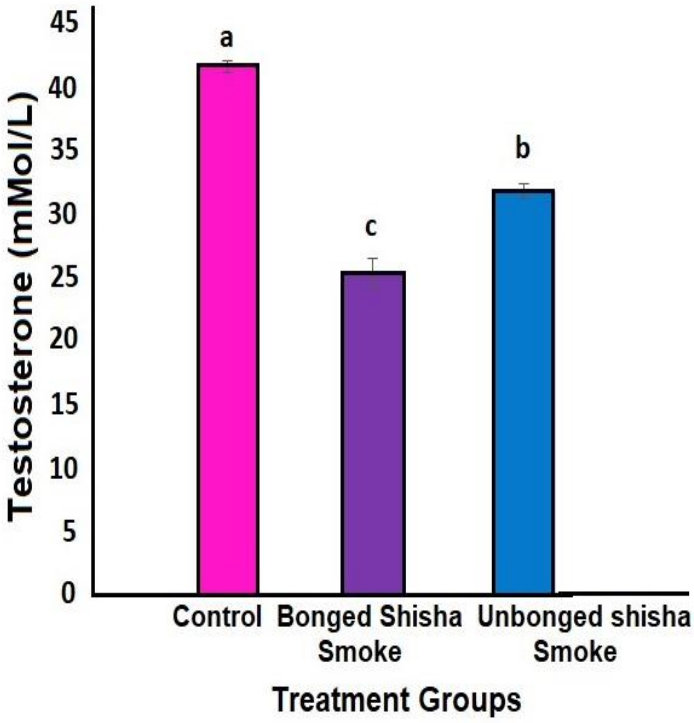

Fig. 4: Effects of *Shisha* smoke exposure on serum testosterone concentration in adult male Wistar rats. [a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

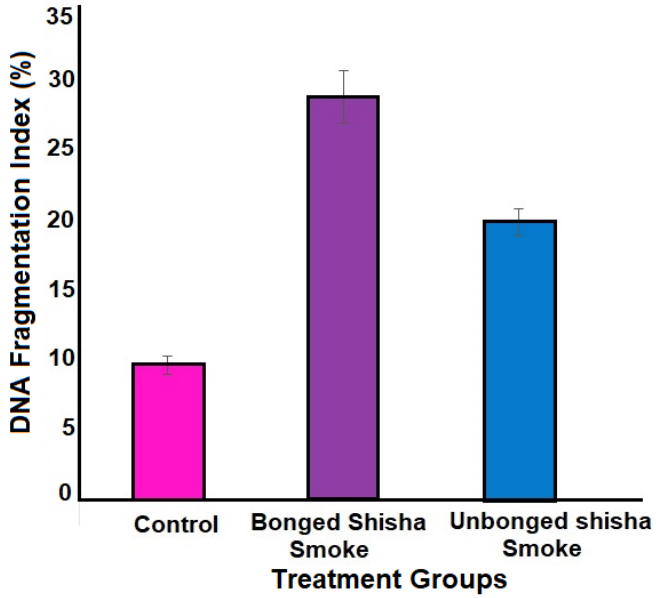

Fig. 5: Effects of Shisha smoke exposure on sperm DNA fragmentation index in adult male Wistar rats. [a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

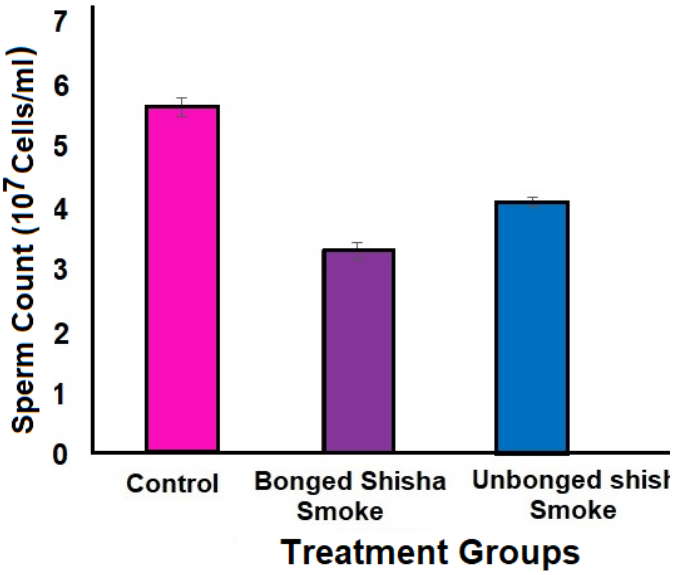

Fig. 6: Effects of Shisha smoke exposure on epididymal sperm count in adult male Wistar rats. [a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

DISCUSSION

The present study observed significant increase in testicular MDA level in both bonged and un-bonged shisha smoke group (with water and without water in the shisha apparatus bowl respectively) as compared to control group, however, the MDA level is more in the bonged shisha smoke group. This finding of the present study is consistent with the result obtained by Safyudin and Subondrate (2016). They observed significant increase in serum MDA level of smokers as compared to non-smokers. An increase in MDA level in the exposed groups in this study indicates an increase in oxidative stress production in the rat's testis; since MDA level determination is a measure of the amount of lipid peroxidation. Shisha smoke generate free radical that consequently result in lipid peroxidation in the testicular tissues that leads to increased level of MDA in these groups as compared to control group.

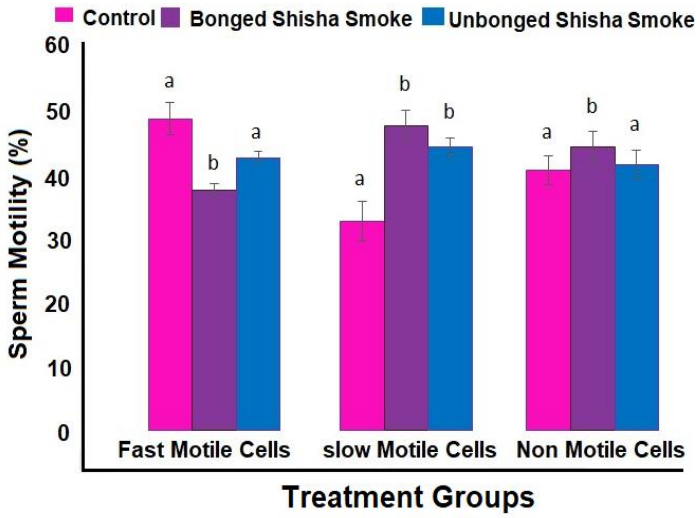

Fig. 7: Effects of Shisha smoke exposure on sperm motility in adult male Wistar rat. [a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

The present study showed that, serum testosterone level is considerably lower in the bonged shisha smoke (BSS) and un-bonged shisha smoke (UBSS) groups compared with control. Similar results were reported by Heidary *et al*. (2012), they found decreased serum testosterone level in male rats exposed to cigarette smoke. The reduction in serum testosterone level found in this study following shisha smoke exposure may be attributed to its nicotine content as previously reported by Jorsaraei *et al*. (2008). Also direct destructive effect of nicotine on reproductive system and sex hormone secretion through free radicals formation leading to extensive cellular deterioration following tobacco smoking has been reported by Heidary *et al*. (2012).

The results of the current study indicated that sperm DNA fragmentation index is significantly higher in BSS and UBSS groups compared to the control group. The results are in support by the findings of Taha *et al*. (2014), who reported a significant positive correlation between smoking and sperm DNA fragmentation in oligoasthenoterozoospermic men. Also, Sandrine *et al*.

Table 1: Effects of Shisha smoke exposure on sperm morphology in adult male Wistar rats

Sperm Parameters	Control	Bonged Shisha Smoke	Unbonged Shisha Smoke
Normal Morphology	191.0 ± 1.52[a]	184.0 ± 0.86[b]	188 ± 2.24[a]
Abnormal Head	0.60 ± 0.04[a]	3.00 ± 0.14[b]	1.60 ± 0.20[b]
Abnormal Tail	8.20 ± 1.10[a]	14.8 ± 0.86[b]	7.6 ± 0.50[a]
Total number of abnormal sperm	44	89	56
Total number of normal sperm	956	921	944
% of abnormal cells	**0.044**	**0.088**	**0.056**

(2006), found that smokers' spermatozoa have significant higher DNA fragmentation than non-smoker (32% versus 25.9%, P < 0.01). Shisha smoke has been reported to increase the amount of free radicals in the body of smokers through its CO, nicotine, tar, heavy metals etc. Free radicals consequently cause oxidative DNA and chromosomal damages (Theron *et al*., 2011).

The present study revealed a significant reduction in epididymal sperm count in the exposed *shisha* smoke groups compared to the control. This result is in accordance with the findings of Irene *et al*. (2011), they reported a significant positive correlation between tobacco shisha smoking and sperm count, and other semen parameters among Egyptian men who smoke shisha. The possible mechanism for reduction in sperm count in the groups exposed to shisha smoke may be attributed to the potentiated toxicants in shisha smoke, such as nicotine (a very toxic alkaloid) and its major metabolites like cotinine and trans-3-hydroxycotinine, which they have been known to cross the blood-testis barrier, and were implicated to mediate the deleterious effects of shisha smoke on spermatogenesis in the seminiferous tubules as evidently showed in the reeked testicular histology of the exposed shisha smoke groups. Furthermore, the concentrated and diverse toxin in shisha smoke may directly interact with, and may greatly affect the seminiferous tubules and the Leydig cells that essentially contribute its secretions (testosterone) to the development and maturation of the spermatogonia to spermatozoa.

The results of the present study also demonstrated a significant lower percentage values for progressive or fast sperm motility in the exposed shisha smoke groups compared to the control and significant higher percentage non-progressive motility and non-motile sperm cells in the group exposed to shisha smoke with water in the bowl as compared to any other group. This findings of the current study is in line with the report of Irene *et al*. (2011). The decrease in progressive sperm motility (an index of fertility possibility) seen in the exposed shisha smoke groups may be ascribed to the effects of shisha smoke toxicant substance disturbance in spermatogenesis or epididymal sperm maturation process secondary to secretory dysfunction at the level of the Leydig and Sertoli cells. Smoke toxins have detrimental effects on the various cyth structural modifications and biochemical changes that spermatozoa naturally undergo during epididymal maturation and may lead to decrease sperm motility (Irene *et al*., 2011). Also nicotine and other chemical in tobacco smoke possibly cause their damage to mitochondrial genome and mitochondrial enzymatic activity in the epididymal or seminal vesicle functions, thereby affecting sperm motility (Priyadarsini *et al*., 2014).

Significant increase in head and tail abnormalities were observed in the BSS group compared to any other groups in this study. This finding is in agreement with the work of Jason (2015), who reported significant increase in abnormal sperm morphology in male cigarette smokers.

Also, the present study revealed significant positive correlation between MDA level and DFI and PSA; testosterone concentration and sperm count and a significant negative correlation between MDA and testosterone; DFI and sperm count. This implies that the higher the level of MDA the lower the testosterone concentration, the higher the DFI and in effect, the lower the sperm count. This finding is consistent with the report of Abasalt *et al*. (2013), who found negative correlation between MDA production and sperm count in astheno- and oligoastheno- teratospermic men. Shisha smoke imposed oxidative stress in the exposed

groups through generation of reactive oxygen species. Lipid peroxidation is one of the deleterious effects of ROS and is considered as an indicator of membrane polyunsaturated fatty acid oxidation (Tremellen, 2008). Furthermore, it has been reported that ROS in addition to reaction with the polyunsaturated fatty acid, it also react with DNA nucleotides leading to base modification particularly 8-hydroxy-2'-deoxyguanosine (8-oxoguanine) formation and consequently DNA fragmentation (Nassira *et al*., 2011).

Tobacco smoke is made up of gas and organic compounds. Nicotine is the most abundant organic particle present in tobacco smoke, which is responsible for some of the deleterious effects on the various organs (Jorsaraei *et al*., 2008). Nicotine is been researched to be known as a very toxic alkaloid (Branian and Hansen, 2002). Most of the inhaled nicotine is quickly oxidized to its major metabolite cotinine which occurred mainly in vapourized state (Binnie *et al*., 2004). Cotinine has a longer half-life than nicotine which is about 20 hours against 2 hours for nicotine. This is probably the reason why this study observed more deleterious effects in the bonged shisha smoke group than the un-bonged shisha smoke group. The significant different in MDA level in the exposed shisha smoke groups is probably due to the ability of nicotine to freely dissolve in water and form un-protonated component – a higher percentage of nicotine forms, that is highly volatile which increase its rate of absorption in the respiratory tract (Henningfield *et al*., 2004). Additionally, tobacco smoke contains a mixture of several harmful substance like carbon monoxide, hydrogen cyanide, ammonia, volatile hydrocarbons, alcohol, aldehydes and ketones (Kitawaki *et al*., 2001) which could cause the harmful effects seen in both bonged and un-un-bonged shisha smoke groups in this study.

CONCLUSION

In conclusion, the present study has revealed significant harmful effects of *Shisha* smoking in the testis of the experimental animals after 13 weeks of *Shisha* smoke exposure which resulted in high serum MDA level and sperm DNA fragmentation, decreased testosterone concentration, sperm count and motility and high sperm morphological damage. These effects were higher in the bonged *Shisha* smoke group.

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