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Research Article

Histomorphometric and Spermatogenic Evaluation of Musk Based-Incense Induced Testisculotoxicity in Adult Albino Rats

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Abstract: Male infertility is one of the major health problems in our society and the possibility that chemicals may disrupt the endocrine system in humans and animals has received considerable attention in the scientific and public community. This investigation was therefore set up to determine the testiculotoxic effect of musk-based incense (MBI) on adult albino rats, using histology and sperm parameters. The stereological evaluation of the rat testes showed that the tubular diameter, the cross sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the group B rats that were exposed to smoke from 2g of MBI approximated those of the control animals while rats that were exposed to 3g and 5g of MBI shows a statistically significant ($p < 0.05$) reduction when compared. The MBI exposed groups of rats demonstrated reduction of basal seminiferous epithelia cells, marked testicular atrophy, germinal aplasia and hypo-spermatozoa formation**.** The results evaluated from the sperm parameters shows a decrease in sperm motility, a decrease in sperm count, and an increase in abnormal sperm morphology. Animals in Group B (control) showed a non significant decrease ($p > 0.05$) in sperm motility, sperm count and sperm morphology when compared to Group A. Group C & D showed significant decrease ($p < 0.05$) in sperm count, sperm motility, and an increase in abnormal sperm morphology. The histological examinations of the MBI exposed groups of rats showed a derangement of the basal semineferous epithelia, testicular atrophy, germinal aplasia and hypospermatozoa in the lumen.
Keywords: Male infertility, MBI, hypospermatozoa, seminiferous tubules.

INTRODUCTION

Male infertility is one of the major health problems in our society and the possibility that chemicals may disrupt the endocrine systems in humans and animals has received considerable attention in the scientific and public community [1]. Many environmental xenobiotic chemicals such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloro ethane (DDT), dioxin, rhodinol, insecticides, herbicides and some pesticides have been discovered to have estrogenic effects [2-3]. The gonad is also considered the main target for environmental toxins [4]. This organ has membrane structures rich in polyunsaturated fatty acids. Membrane polyunsaturated fatty acids are highly sensitive to oxidative stress manifested through lipid peroxidation which usually results in loss of membrane integrity [4-5]. The testes and other male accessory glands play key roles in reproduction, however, Raj incense represents one of the most widely used classes of incense with high potential for human exposure in both rural and urban environment, yet little is known about the biochemical and cytoarchitectural effect in humans [4]. Musk is an active ingredient of incense called *Raj*® Individuals are most likely to get exposed to this through whole body inhalation, during manufacture and formation and application of this incense [5]. The present study therefore was designed to investigate the effect of musk-based incense on the testicular histology and sperm parameters in the rat testis. The fragrance of musk-based incense (MBI) is widely used in African countries including Nigeria. The fragrance is used as a gesture of hospitality during special occasions like religious ceremonies in churches and prayer houses for creating spiritual atmosphere and by Muslim clerics during prayers [6]. While the use of incense has increased in the last decade, there have been reports on their toxic effects on various organs such as; lungs, skin, liver [7-8]. Although, there are various studies on the cytotoxic effects of various types if incense on the

several other organs, there is a dearth of information in the literature on the testicolotoxic effect of MBI. The general intent of this research is to evaluate the effect of MBI on the testes of adult male albino rats

MATERIALS AND METHODS

Materials

Incense

A commonly used brand of incense '*Raj*®' (1 g and 23 cm) containing 0.9% w/w musk, 0.2% w/w sandal, and inert ingredients 0.1% w/w was purchased from Goodies supermarket Ado-Ekiti, Ekiti state.

Animals

Twenty male Albino rats (8 to 10 weeks old) weighing 165-180 g were obtained from the Animal House of AfeBabalola University Ado Ekiti. They were allowed to acclimatize for 2 weeks and were fed freely on standard commercial mouse cubes from Abuad Farms. Relatively constant environmental condition were maintained with proper aeration and good source of light (12h light-12h dark and 24 degree C ± 30 degree C). Food and water were provided *ad libitum*. The weighing and observations were done before the rats were exposed to Musk-Based Incense (MBI) respectively. The weights of the animals were estimated at procurement, during acclimatization, at commencement of the experiments and twice within a week throughout the duration of the experiment, using an electronic analytical and precision balance (BA210S, d= 0.0001 g) (Satorius GA, Goettingen, Germany).

Experimental procedures involving the animals and their care were conducted in conformity with International, National and institutional guidelines for the care of laboratory animals in Biomedical Research and Use of Laboratory Animals in Biomedical Research as promulgated by the Canadian Council of Animal Care [9]. Further, the animal experimental models used conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals [10].

ANIMAL GROUPINGS AND MUSK-BASED INCENSE (MBI) EXPOSURE.

Four groups of rats (A, B, C and D) consisting of 5 animals each was housed separately in four undisturbed cages of size $5m^3$ with cross ventilation to avoid the cross exposure to incense smoke [5]. Group A serves as the control and were exposed to natural fresh air every day for 4 weeks consecutively, Group B rats were exposed to smoke emanating from the burning of 2 g of MBI material [3,5,11], Group C were exposed to the smoke emanating from the burning of 3 g of MBI and Group D rats were exposed to smoke emanating from the burning of 5 g of MBI material. All exposure is via whole body inhalation for 45-50 mins every day and for a period of 4 weeks.

ANIMAL SACRIFICE AND SAMPLE COLLECTION

The rats at the time of sacrifice were first weighed and then anaesthetized by placing them in a closed jar containing cotton wool soaked in chloroform. The abdominopelvic cavity was opened up through a midline abdominal incision to expose the reproductive organs. Then the testes and epididymis were excised. The weight of the testes of each animal was evaluated .The testes were weighed with an electronic analytical and precision balance (BA 210S, d = 0.0001-Sartoriusen GA, Goettingen, Germany). The volume of each testis was measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. One of the testes of each animal was fixed in 10% formol-saline for histological examination.

DETERMINATION OF MORPHOMETRIC PARAMETERS

Histological slides were prepared from the formol-saline fixed testes. However, prior to embedding, it was ensured that the sections were orientated perpendicular to their long axes, and designated as "vertical sections". For each testis, five vertical sections from the polar and the equatorial regions were sampled [12] and an unbiased numerical estimation of the following morphometric parameters was determined using a systematic random scheme [13].

Diameter (*D*) of seminiferous tubules

The diameter of seminiferous tubules with profiles that were round or nearly round were measured for each animal and a mean, *D*, was determined by taking the average of two diameters, *D1* and *D2* (Perpendicular to one another). *D1* and *D2* were taken only when $D1/D2 \geq 0.85$.

Cross-sectional area (*AC*) of the seminiferous tubules

The cross-sectional areas of the seminiferous tubules were determined from the formula $AC = \pi D2/4$, (where π isequivalent to 3.142 and *D* the mean diameter of the seminiferous tubules).

Number of profiles of seminiferous tubules in a unit area of testis (*NA*)

The Number of profiles of seminiferous tubules per unit area was determined by using the unbiased counting frame proposed by Gundersen [14]. Using this frame, in addition to counting profiles completely inside the frame we counted all profiles with any part inside the frame provided they do not touch or intersect the forbidden line (fulldrawn line) or exclusion edges or their extension.

Numerical Density (*NV*) of seminiferous tubules

This is the number of profiles per unit volume and was determined by using the modified Floderus equation: $NV = NA/(D + T)$ [15] where, *NA* is the number of profiles per unit area, *D* is the diameter and *T* the average thickness of the section.

The evaluation of the diameter was done with calibrated eyepiece and stage grids mounted on a light research microscope. Estimation of volume density of testicular components and number of seminiferous tubules were done on a computer monitor onto whom a graph sheet was superimposed and on which slides were projected from a research light microscope (Model N - 400ME, CEL-TECH Diagnostics, Hamburg, Germany).

DETERMINATION OF EPIDIDYMAL SPERM PARAMETERS

Progressive sperm motility

This was done immediately after the semen collection. Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (27^{o}C) and two drops of warm 2.9% sodium citrate was added, the slide was then covered with a warm cover slip and examined under the microscope using X400 magnification. Ten fields of the microscope were randomly selected and the sperm motility of 10 sperms was assessed on each field. Therefore, the motility of 100 sperms was assessed randomly. Sperms were labeled as motile, sluggish, or immotile. The percentage of motile sperms was defined as the number of motile sperms divided by the total number of counted sperms (i.e. 100) [15].

Epididymal sperm concentration

Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi [17]. Briefly, the epididymis was minced with anatomic scissors in 5mL physiologic saline, placed in a rocker for 10 minutes, and allowed to incubate at room temperature for 2 minutes. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 mL formalin (35%). Total sperm number was determined by using the new improved Neuber`s counting chamber (haemocytometer). Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and was allowed to stand for 5 minutes. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five 16-celled squares. The sperm concentration was calculated, multiplied by 5 and expressed as [X] x 10^6 /ml, where [X] is the number of spermatozoa in a 16-celled square.

Sperm morphology

The sperm cells were evaluated with the aid of light microscope at X400 magnification. Caudal sperm were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada). Five hundred sperm from the sample were scored for morphological abnormalities [18]. Briefly, in wet preparations using phase-contrast optics, spermatozoa were categorized. In this study a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head and was expressed as a percentage of morphologically normal sperm.

TISSUE PREPARATION FOR LIGHT MICROSCOPY

This was done as essentially as described by Akpantah *et al.* [19]. The organs were cut in slabs of about 0.5 cm thick and fixed in Bouin's fluid for a day after which it was transferred to 70% alcohol for dehydration. The tissues were passed through 90 % alcohol and chloroform for different durations before they were transferred into two changes of molten paraffin wax for 20 min each in an oven at 57°C. Serial sections of 5 µm thick were obtained from a solid block of tissue and were stained with haematoxylin and eosin stains, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following clearance in xylene, the tissues were oven-dried. Light microscopy was used for the evaluations.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD for n = 5. The level of homogeneity among the groups was tested using Analysis of Variance (ANOVA) as done by Snedecor and Cochran [20]. Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test (DMRT). A value of $p < 0.05$ was considered to indicate a significant difference between groups (Duncan, 1957). Analysis of data was done using both electronic calculator and Statistical Package for Social Sciences (SPSS) / PC computer program (version 11.0 SPSS, Cary, NC, USA).

RESULT

Effects of Musk-Based Incense (MBI) on Gross Anatomical Parameters of Albino Rats

There was non-significant ($p > 0.05$) decrease in testis weight, testis weight/body weight ratio and testis volume in 2 g incense exposed group when compared to the control counterpart, whereas statistically significant ($p < 0.05$) decrease was observed in 3 g and 5 g incense exposed group compare to the control counterpart.

Table-1:-Effects of Musk-Based Incense (MBI) on Gross Anatomical Parameters of Albino Rats

Treatment Groups	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Diff. (g)	Testis Weight (g)	Testis Volume (mL)	Testis Weight/Body Weight Ratio
GROUP A	168.5 ± 2.0	175.1 ± 1.0	6.0	1.10 ± 2.8	1.10 ± 0.3	0.006
GROUP B	I70.5 ± 4.5	155.5 ± 1.4	15.0*	0.95 ± 1.0	0.90 ± 0.2	0.006
GROUP C	176.5 ± 2.9	146.1 ± 1.8	30.4*	0.60 ± 0.6*	0.60 ± 0.4*	0.004*
GROUP D	180.0 ± 5.0	120.2± 4.0	49.2*	0.32 ± 0.5*	0.33 ± 0.3*	0.002*

**** p< 0.05 significantly different from control. Values are expressed as mean ± SD for n = 5 in each group.***

Group A = fresh air for 4weeks (control), **Group B** = 2 g Incense exposure for 4 weeks**, Group C** = 3 g Incense exposure for 4 weeks**, Group D**= 5 g Incense exposure for 4 weeks

THE EFFECTS MUSK-BASED INCENSE (MBI) ON RAT TESTIS MORPHOMETRIC PARAMETERS

The stereological evaluation of the rat testes showed that the tubular diameter, the crosssectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the group B rats that were exposed to smoke from 2g of MBI approximated those of the control animals (Table 2).

However, there was a statistically significant ($p < 0.05$) reduction in the tubular diameter, the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the animals that were exposed to 3g and 5g of MBI (Table 2).

Table-2: The Effects Musk-Based Incense (MBI) on Rat Testis Morphometric Parameters

Treatment groups	**D (µm)**	**Ac (x 10^3µm3)**	**NA (x10^{-8} µm^{-2})**	**NV (x10^{-10}µm^{-2})**
GROUP A	157.21±4.21	28±7.33	25.12±9.12	12.88±9.31
GROUP B	150.64±3.21	26±6.21	23.74±5.12	10.10±5.23
GROUP C	112.21±5.23*	17±7.32*	15.22±5.50*	7.11±8.52*
GROUP D	101.32±5.41*	14±9.22*	14.74±8.12*	5.52±9.33*

P < 0.05 significantly different from control, Value are expressed as mean ± SD for n = 5 in each group

Group A = fresh air for 4 weeks (Control)**, *Group B*** = 2 g Incense exposure for 4 weeks**, Group C** = 3 g Incense exposure for 4 weeks**, Group D** = 5 g Incense exposure for 4 weeks

EFFECT OF MUSK-BASED INCENSE (MBI) ON THE HISTOLOGICAL PROFILES OF THE TESTIS

The sections of testes of group A rats showed normal testicular architecture with distinct seminiferous tubules, a normal cross sectional epithelial outline, numerous spermatozoa within their lumen, oval outlined with normal seminiferous epithelium and intact testicular Interstitium. However there was minimal damage in the testicular interstitium of group B rats. The seminiferous tubular outline was moderately reduced and there were few spermatozoa within their lumen. The seminiferous tubules of Group C rats showed degeneration in testicular architecture. The testicular interstitium were also reduced and were detached from the seminiferous tubules, isolating them from each other. Vacuolized interstitium were also observed. The rats in Group D showed total degeneration of testicular interstitium, significant reduction in the diameter of seminiferous tubules and lumens devoid of spermatozoa were observed. There was also a significant reduction of the basal seminiferous epithelial cells.

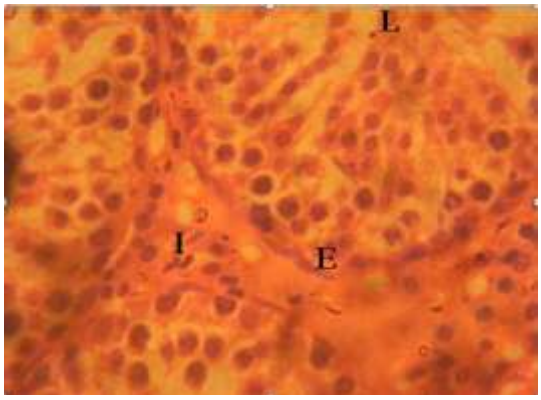

Fig-1: Cross-section of the testis of Group A

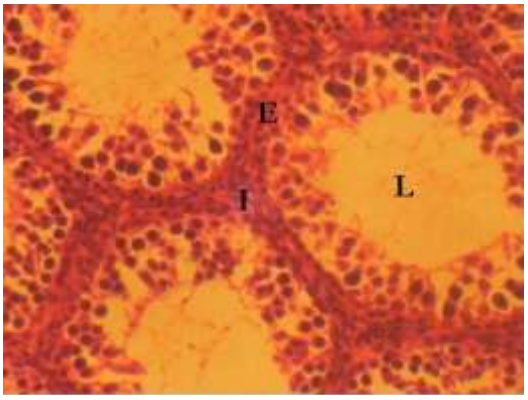

Fig-2: Cross-section of the testis of Group B

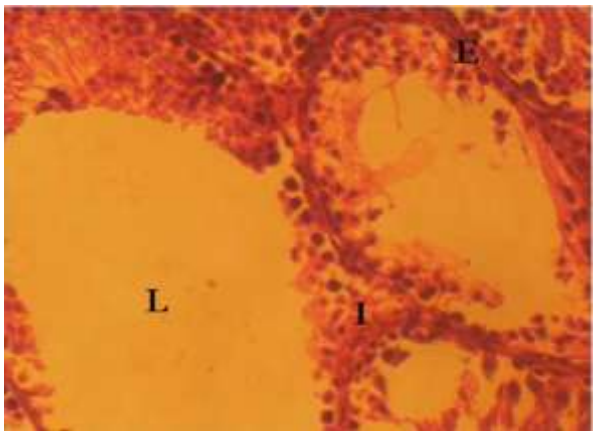

Fig-3: Cross-section of the testis of Group C rats

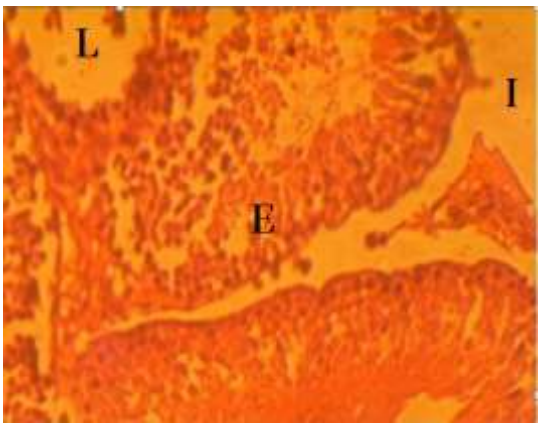

Fig-4: Cross-section of the testis of Group D

Stain: Haematoxylin and Eosin
Magnification: x 400
E = Seminiferous epithelium
L = lumen of seminiferous tubule
I = Testicular Interstitium

EFFECT OF MUSK-BASED INCENSE (MBI) ON THE SPERM PARAMETERS OF ADULT MALE ALBINO RATS

Sperm Count

The group of Rats exposed to 2 g of incense showed non-significant ($p > 0.05$) decrease in sperm concentration (105.22 ± 2.4 x10^{-6}/mL) compared to the control group (120.62 ± 2.9 x10^{-6}/mL), 3 g incense exposed group provoked significantly ($p < 0.05$) decreased sperm concentration (87.72 ± 1.9 x10^{-6}/mL) and 5 g incense exposed group showed marked oligospermia (69.33 ± 1.4 x10^{-6}/mL) with their sperm concentration being significantly lower ($p < 0.05$) compared to the control group.

Sperm Motility

Although the sperm motility of 2 g incense exposed group showed a lower non-significantly ($p > 0.05$) (60.34 ± 4.8 %) compared to the control group (82.06 ± 6.2 %). However, the 3 g and 5 g incense exposed groups still had significantly lower ($p < 0.05$) (44.30 ± 3.5 %) and (30.22 ± 2.0 %) value compared to the control counterpart.

Sperm Morphology

The 2 g incense group showed evidence of non-significantly ($p > 0.05$) decrease in normal sperm morphology (60.2 ± 7.6 %) and non-significantly ($p > 0.05$) increased in abnormal sperm morphology (20.2 ± 6.1 %) compared to the control group (80.3 ± 9.2 %, 17.2 ± 5.8 %) respectively, The 3 g incense group however, showed a significant ($p < 0.05$) decrease in normal sperm morphology (51.0 ± 2.2 %) and a significant ($p < 0.05$) increase in abnormal sperm morphology (23.7 ± 1.7 %) when compared to the control group. Moreover, the rats exposed to 3 g incense also had significant ($p < 0.05$) (42.1 ± 1.8 %) decrease in normal sperm morphology and a significant increase in abnormal sperm morphology ($p < 0.05$) (40.01 ± 3.8 %) when compared to the control group.

Table-3: Effects of Musk-Based Incense (MBI) on the sperm parameters Adult Male Albino Rat

Treatment Groups	Sperm Count (x10^6/mL)	Sperm Motility (%)	Sperm Normal (%)	Morphology Abnormal (%)
GROUP A	120.62 ± 2.9	82.06 ± 6.2	80.3 ± 9.2	17.2 ± 5.8
GROUP B	105.22 ± 2.4	53.34 ± 4.8	60.2 ±7.6	20.2 ± 6.1
GROUP C	87.72 ± 1.9*	44.30 ±3.5*	51.0 ± 2.2*	23.7 ± 1.7*
GROUP D	69.33 ± 1.4*	30.22± 2.0*	42.1 ± 1.8*	40.01 ± 3.8*

P < 0.05 significantly different from control, Value are expressed as mean ± SD for n = 5 in each group
Group A = fresh air for 4 weeks (Control)**, *Group B*** = 2 g Incense exposure for 4 weeks**, Group C** = 3 g Incense exposure for 4 weeks**, Group D** = 5 g Incense exposure for 4 weeks

DISCUSSION

Many environmental xenobiotic chemicals such as chormated biphenyls (PCB'S), dichlorodioxyphenyl-trichloro ethane (DDT), dioxin, insecticides, herbicides, and some pesticides have been discussed to have estrogenic and other toxic effects [2]. The gonad is also considered the main target for environmental toxins [4]. There is a dramatic increase in the use of incense with the trade name Raj. Furthermore, because of its widespread use by both Christian and Muslim clerics; musk has enjoyed a

considerable attention on account of its potential health hazards [21].

In this study it was observed that the control group of animal model had a significant increase in gross anatomical parameters. The improved values of body weight of the control group of animals could mean they were still in their active growth phase during the study [22]. The finding from this study showed a significant decrease in the testes, body weight, testis volume in rats exposed to incense smoke when compared to the control groups. This is in concordance with the report of Muktar *et al.,* [5] and Akingbade *et al.,* [23] which also investigated incense exposure in animal models. The decrease in body and testicular weight of animals that were exposed to musk in the experiment could be attributed to severe parenchyma atrophy in the seminiferous tubule following musk challenge. The decrease in the gross anatomical parameters can also be explained by the fact that the energy derived from feed consumed was mostly used in the detoxification mechanism such as metabolism, degradation and elimination of musk out of the system [24].

The stereological evaluation of the rat testes showed that the tubular diameter, the cross sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the group B rats that were exposed to smoke from 2g of MBI approximated those of the control animals while rats that were exposed to 3g and 5g of MBI shows a statistically significant ($p < 0.05$) reduction when compared with the control rats. This result agree with that of Akunna *et al.*,[25]. The histological evidences in this study showed degenerative changes characterized by vacuolization of the interstitum, reduced luminal spermatozoa and devoid spermatozoa in cross section of the seminiferous tubules of rats exposed to various concentration of incense (2 g, 3 g and 5 g). This is in agreement with several other previous reports on male infertility studies in animal models involving cytotoxic chemicals [4, 26].

Sperm motility has been reported to be highest in the caudal epididymis within the reproductive tract before spermatozoa are ejaculated [27]. Several studies accessing sperm parameters therefore utilize the caudal epididymis [26, 28, 29]. In this present study, the incense exposed rats showed significant reductions in spermatozoa concentration, sperm motility and normal sperm morphology when compared to the control groups. These results are found to be in conformity with several other reports on incense exposure [3, 30]. Previous studies have also shown that incense exposure in animal models has led to decrease in testicular sperm count, increase in percentage number of abnormal sperm and decrease in normal sperm morphology [31]. Musk-based incense (MBI) induce testicular toxicity is mainly as a result of oxidative stress [32]. The increase oxidative stress damages the sperm membrane protein and DNA [33].

This could explain the derangement in the sperm parameters in the animals that were exposed to MBI.

RECOMMENDATION

Despite these well-established toxic effects of MBI on the rat testis, there is a need for further investigations in humans especially the Christians and Muslim clerics that are more exposed to incense, to determine its lethal dose. This is even more pertinent when one consider reports that natural substances are selectively toxic to the mammalian tissue while sparing the lower animals such as rodents because they process more efficient xenobiotic biotransformation system than man.

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