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Effect of Formaldehyde on Rat Testis Structure

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ABSTRACT

Background: Formaldehyde is found in different kinds of medicine and industrial products, cigarette smoke, and even numerous vegetables, fruits and seafood that have been illegally preserved with formalin. The aim of this study was to investigate the effects of formaldehyde on adult albino rat testes through light microscopic examination, immunohistochemistry, biochemistry, morphometry and statistical analysis. **Methods:** Thirty adult male albino rats were utilized in this work. These animals were equally divided into three main groups, with 10 animals each. Group I (control group): The animals were injected with intraperitoneal sterile physiological saline. Group II animals were injected intraperitoneally with a daily dose of formaldehyde (5 mg/kg body weight "BW") for 30 days. Group III animals were injected intraperitoneally with a daily dose of formaldehyde (10 mg/kg BW) for 30 days. By the end of the experiment, blood samples were collected for biochemical study of testosterone; and all animals were anaesthetized and testis specimens were dissected out and weighted then subjected to histological, immunohistochemistry, and morphometric examination. **Results:** Administration of formaldehyde at a dose of 5 and 10 mg/kg caused a decrease in serum testosterone. Moreover, it caused a decrease in the testis weights and induced several histopathological changes in the testis of adult male albino rats such as atrophy of seminiferous tubules with absence of sperm bundles and degeneration of germ. It also increased the collagen fibers deposition in testis as evidenced by Masson`s trichrome staining. The testis showed a positive immune reaction to 8-hydroxy-2'-deoxyguanosine. **Conclusion:** Exposure to formaldehyde is suggested to result in some testicular damage. It is suggested that special precautions might be taken to limit the occupational and environmental formaldehyde exposure; and to prevent or even minimize its contamination with food and water.

Keywords: *Formaldehyde, Testis, Structure, Light microscopy, Rat.*

INTRODUCTION

Formaldehyde (FA) is a colorless and highly water-soluble aldehyde. Its intake occurs through the topical, oral, injection and mostly via the respiratory system. It can be inhaled with smokes due to the combustion of burning fossil fuels and in the fumes of paints and in cigarette smoke.[1] It can be ingested in fresh water, food and drugs. In food, it can occur naturally or through contamination as it can be added as a preservative or disinfectant agent. It can also result from cooking or smoking of foods.[2] Even in infancy, the babies might be exposed by injection to FA present in diphtheria, polio and tetanus vaccine preparations as a result of the manufacturing process.[3] Several malignancy treating drugs are formulated with FA which is required for drug activation.[4] Some cosmetics especially hair smoothing products containing FA or methylene glycol which require the use of heat that leads to volatilization of both FA gas as well as methylene glycol vapors. This increases the potential for hair stylist and consumer exposure to FA from methylene glycol formulated in keratin products.[5]

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Formaldehyde is an organic carbon compound which receives an increasing attention as pollutants with potential adverse health effects.[6] It induces oxidative stress which has been reported to be the mechanism of

FA toxicity in multiple tissues of the exposed animals, in liver, lymphocytes, heart, brain, lung and gonads.[7-11] It has been widely proved that 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a critical biomarker of oxidative stress.[12]

The aim of this study was to study possible hazard effects of FA on the structure of adult albino rat testes after intraperitoneal injection using light microscopic examination and 8-OHdG immunohistochemistry; and to confirm such effects by some biochemical and sperm investigations.

MATERIALS ANDMETHODS

Materials

- **Chemicals**
  1- FA was obtained in the form of formalin liquid 37% from El Gomhoria Company for Chemical and Medical Trading, Zagazig, Egypt.
  2- Kits for immunohistochemistry were supplied by (DAKO life trade Egypt).

- **Animals**

Thirty healthy adult male albino rats weighing 180-200 gm were used in the present study. The animals were obtained from Zagazig scientific and medical research center at the Faculty of Medicine, Zagazig University All animals were kept under hygienic conditions. Standard food and water ad-libitium were allowed. All rats were handled in accordance to the standard guides for the care and use of laboratory animals.[13]

They were divided into three groups:

Group I (Control): It included 10 rats; and received a daily dose of a physiological saline.

Group II: It included 10 rats; and they received a daily dose of formaldehyde (5mg/kg BW) by intraperitoneal injection for 30 days.

Group III: This group included 10 rats. They received a daily dose of intraperitoneal injection of formaldehyde (10 mg/kg BW) for 30 days.[14]

By the end of the experiment (30 days), all animals were anesthetized and weighed; then venous blood samples were collected by means of micro-capillary glass tubes from the retro-orbital plexus for assessment of testosterone.[15] The abdominopelvic cavities were opened; and the testes were dissected out. Epididymal content of each rat was obtained immediately by cutting the tail of epididymis and squeezing it gently to obtain the fresh undiluted semen in a clean Petri dish. The testis' specimens were rinsed in a phosphate buffer saline, then weighed and finally dissected and examined for the histological, immunohistochemistry and morphometric examination.

Methods

1- Determination of plasma testosterone

Blood was collected from retro orbital venous plexuses of rats then poured into lab tubes. Serum was separated by centrifugation at 3000xg for 15 minutes, then collected and stored at -205C until analysis. Serum testosterone levels were measured by Chemiluminescent Enzyme Immunoassay.

2- Light microscopic examination

A-Histological and histochemical study

Samples from testis were rapidly fixed in 10% formol saline for 48 hours, dehydrated through graded alcohols and embedded in paraffin.[16] Transverse sections of 5µm thickness were obtained from all specimens stained with hematoxylin and eosin (Hx&E) and other sections stained with Masson trichrome stains.[17]

B-Immunohistochemical study for 8-OHdG

Sections of 5 µm in thickness were prepared from paraffin embedded tissues. After deparaffization, endogenous peroxidase was quenched with 3% H2O2 in deionized water for 5–10 min. Nonspecific binding sites were blocked by incubating the sections in 10% normal rabbit serum for 10–15 min. The sections were then incubated with polyclonal rabbit anti-iNOS (dilution 1:25) overnight at 4 °C, followed by incubation with biotinylated goat-antirabbit IgG at room temperature for 10–15 min. After phosphate buffered saline (PBS) rinses of 3 × 3 min, the horse radish peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10–15 min. The antibody binding sites were visualized by incubation with a diaminobenzidine–H2O2 solution. The sections incubated with PBS instead of the primary antibody were used as negative controls. Brown-yellow granules in cytoplasm or nuclei were recognized as positive staining for 8-OHdG. Activity of 8-OHdG tissues was shown by commercial 8-OHdG immunohistochemical stain kits (DAKO life trade Egypt). Streptavidin–biotin complex was used. Cytoplasmic staining with 8-OHdG was revealed to be positive.

3- Morphometric study

The morphometric study was done using image analyzer software (Leica Qwin 500 Image Analyzer, England). The total images per animal were 15 images. According to this method, we used an optical magnification of 400 for assessment of seminiferous tubules diameter (STD) and seminiferous epithelial height (SEH).

4- Statistical analysis

All the achieved data were statistically evaluated with SPSS, version 18.0 software.[18] Testing methods included one-way analysis of variance (ANOVA) for comparisons between more than two groups in

normally distributed data; and Kruskal Wallis test was used for comparison between more than two groups in not normally distributed data followed by least significant difference (LSD) test for comparison between two groups. The *p*-values of ≤0.05 were considered to indicate statistical significance, while *p*-values of <0.01 indicate highly significant results. All the results were expressed as mean ±SD**.**

RESULTS

1- Biochemical results

The obtained data showed that there was a decrease in mean of values of testosterone between group I and FA-treated groups [groups II and III]. This decrease was highly significant (p <0.001) [Table 1].

2- Testis weight

There was a decrease in the mean values of testes' weights. This decrease was highly significant (p <0.001) in the mean values of weights between group I and FA-treated groups [Table 1].

3- Light microscopic examination

A. Histopathological results

Examination of Hx&E-stained sections of this group revealed the testis was surrounded by a capsule, which was composed mainly of dense collagenous fibrous connective tissue. The parenchyma of the testis was consisted of small multiple rounded seminiferous tubules. The structural components of the testis were the seminiferous tubules and interstitial tissues [Figure 1].

The seminiferous tubules were lined with two types of cells, the Sertoli cells and the spermatogenic cells. The cells rested on a thin basement membrane. The spermatogenic cells were found in many layers, namely, the spermatogonia, spermatocytes; spermatids and finally mature spermatozoa. The spermatogonia were rounded cells with rounded nuclei; and Sertoli cells were tall cells with oval nuclei. The interstitial tissues were narrow and showed clusters of Leydig cells [Figures 2,3].

In the FA-treated groups, there was a thickening of capsule with no sperms in the lumen. The testis showed irregular seminiferous tubules and tubular atrophy. There were disrupted seminiferous tubules and loss of normal architecture. The testis also showed a degeneration of Sertoli cells and an increase in the spaces between tubules with marked degeneration of Leydig cells. The tubules had vacuoles; and there was no acidophilic materials and replaced by casting (hyaline material) in the lumens. The tubules also had cellular debris and giant cells. The basement membrane was thickened. The interstitial tissues were wide with congested blood vessels [Figures 4-9].

B. Masson's trichrome stain

Masson's trichrome-stained sections of FA-treated groups revealed an increase in the collagen fibers of the capsule and around the blood vessels [Figures 10-12].

C. Histochemical results

The examination of 8-OHdG slides of the FA-treated group showed a positive immune reaction for 8-OHdG protein by detecting brown granules in the cytoplasm of the seminiferous epithelium [Figures 13-15].

The examination of 8-OHdG slides of the high treated group (10 mg/kg) showed a strong positive immune reaction for 8-OHdG [Figure 15].

D. Epididymal spermatozoa examination

Control group showed normal living sperms with normal heads and tails. FA-treated showed dead sperms and abnormal forms of sperms in form of kinked tail, banana heads and tailless [Figures 16-18].

Morphometric study

1- The diameter of the seminiferous tubules: It was decreased in groups II and III in comparison to the diameter of group I. This decrease was highly significant (p <0.001) [Table 1].

2- The height of seminiferous epithelium: There was also a decrease of the epithelium in groups II and III in comparison to the height of group I (236.56 ± 103.91). This decrease was a highly significant (p <0.001) [Table 1].

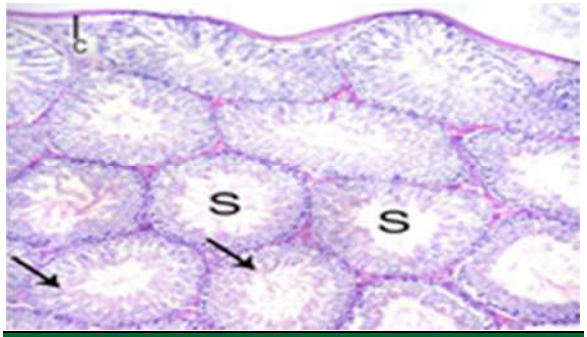

Figure 1: Photomicrograph of a section of the testis of an adult male albino rat of control group (I) showing normal capsule (C), multiple seminiferous tubules (S) lined by many layers of cells (arrows).
(Hx&E x100)

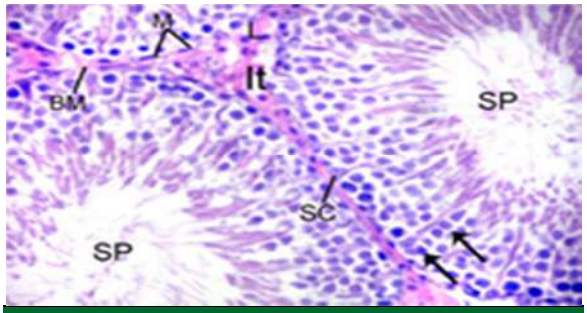

Figure 2: Photomicrograph of a section of the testis of an adult male albino rat of control group (I) showing seminiferous tubules lined by spermatogonic cells (arrows)

and Sertoli cells (SC). The cells rest on thin basement membrane (BM) which consists of a single layer of myoid cell (M). The interstitial spaces (It) between tubules are narrow with clusters Leydig cells (L). There are acidophilic materials of sperm bundles (SP) in the lumen of seminiferous tubules.

(Hx&E x400)

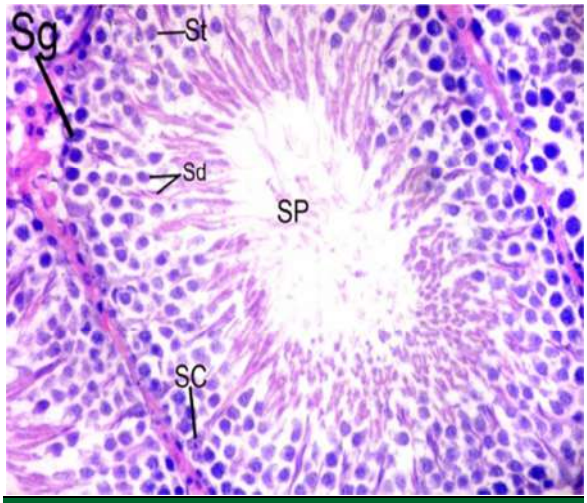

Figure 3: Photomicrograph of a section of the testis of an adult male albino rat of control group (I) showing seminiferous tubules lined by stratified germinal epithelium with spermatogonia (Sg), spermatocytes (St), round and elongated spermatids (Sd) and Sertoli cells (SC). There are acidophilic materials of sperms bundles (SP) in the lumen of tubules.

(Hx&E x400)

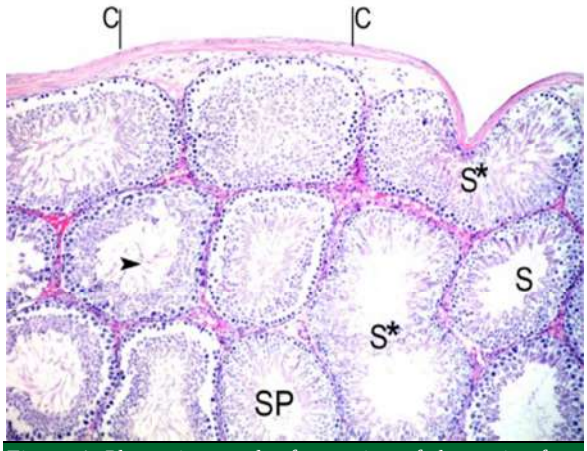

Figure 4: Photomicrograph of a section of the testis of an adult male albino rat (group II) showing thickening of capsule (C). There are nearly normal seminiferous tubules(S) with sperm bundles (SP) in the lumens of the tubules. There are also disturbed tubules (S*) with loss of their normal architectures. Some tubules show little amount of sperm bundles (arrow heads).

(Hx&E x100)

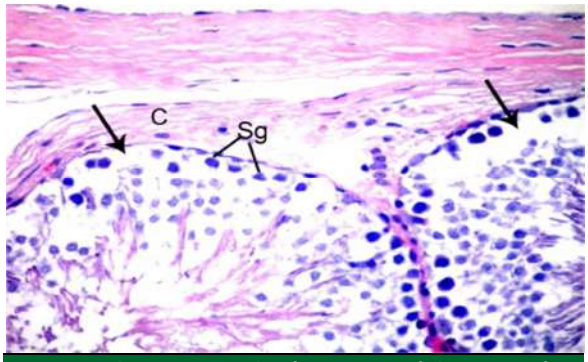

Figure 5: Photomicrograph of a section of the testis of an adult male albino rat (group II) showing thickening of the capsule (C). Spermatogonia (Sg) rest on a basement membrane. There is degeneration of Sertoli cells (arrows).

(Hx&E x400)

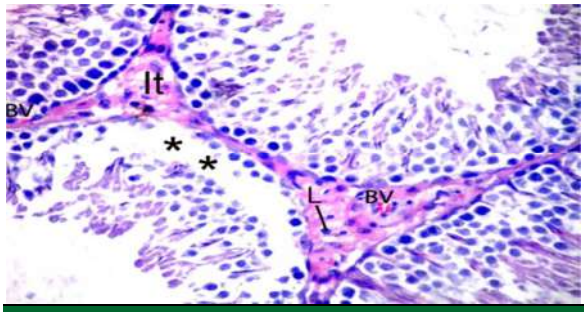

Figure 6: Photomicrograph of a section of the testis of an adult male albino rat (group II) showing degeneration of germ cells and separation from their basement membrane (asterisk). There are thick congested blood vessels (BV) and normal Leydig cells (L) in the wide intersitium (It).

(Hx &E x400)

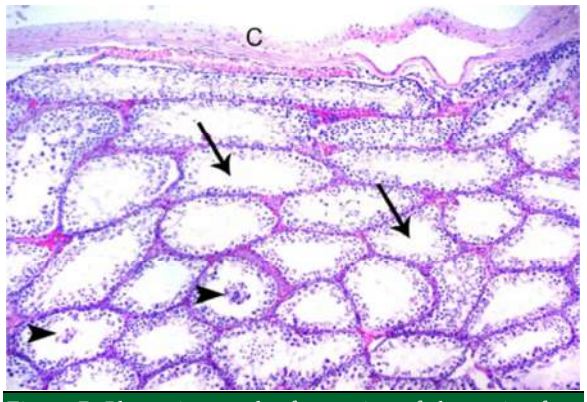

Figure 7: Photomicrograph of a section of the testis of an adult male albino rat (group III) showing thickened irregular capsule(C). The tubules have no sperm bundles (arrows) indicates arrest of spermatogenesis. There is cellular debris (arrow heads) in the lumen of tubules due to immature spermatogenetic cells.

(Hx&E x100)

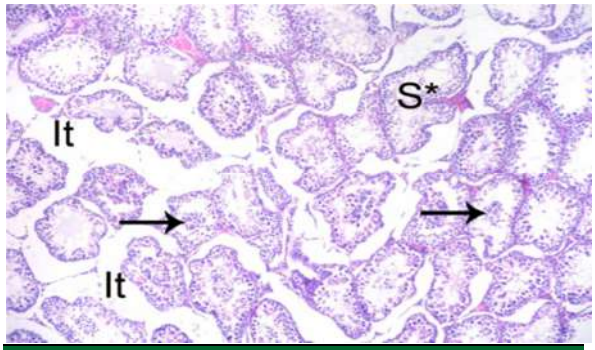

Figure 8: Photomicrograph of a section of the testis of an adult male albino rat (group III) showing irregular shrunken seminiferous tubules(S*) with loss of normal architecture and no sperm bundle in their lumens. There is widening of the interstitial tissues (It) with absence of Leydig cells. The tubules have cellular debris (arrow heads).
(Hx&E x100)

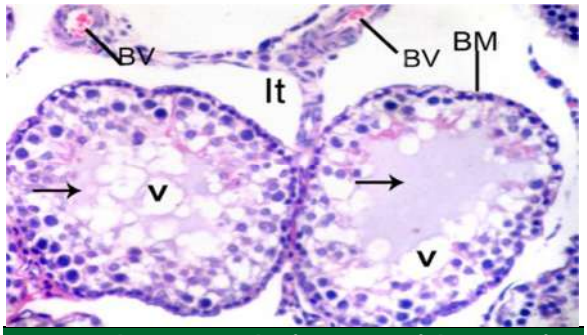

Figure 9: Photomicrograph of a section of the testis of an adult male albino rat (group III) showing vacuoles (V) and hyaline materials (arrows) in the lumen of the tubules. The spaces between tubules are wide (It) with congested blood vessels (BV). There is a thick basement membrane (BM).
(Hx&E x400)

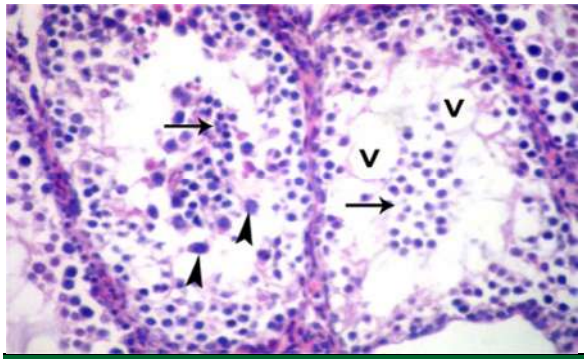

Figure 10: Photomicrograph of a section of the testis of an adult male albino rat (group III) showing exfoliation of cells (cellular debris) (arrows) and vacuoles (V) appear in the lumen of tubules. There are giant cells (arrow heads) with dark stained nuclei.
(Hx&E x400).

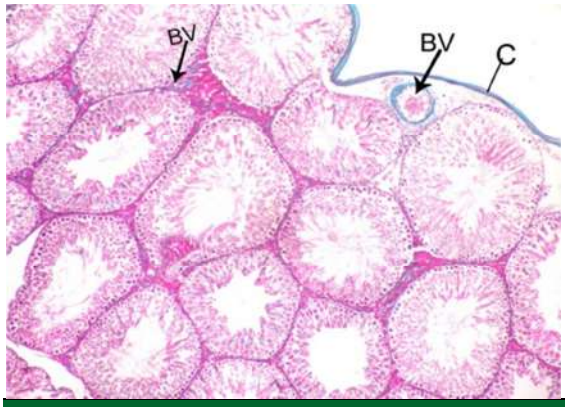

Figure 10: Photomicrograph of a section of the testis of group I showing normal distribution of collagen fibers in the capsule(C) and around blood vessels (BV).
(Masson trichrome x100)

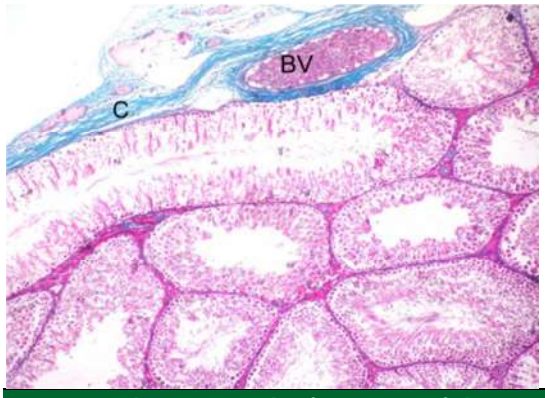

Figure 11: Photomicrograph of a section of the testis of group II showing moderate increase of collagen fibers in testis in the capsule (C) and around the blood vessels (BV).
(Masson trichrome x100)

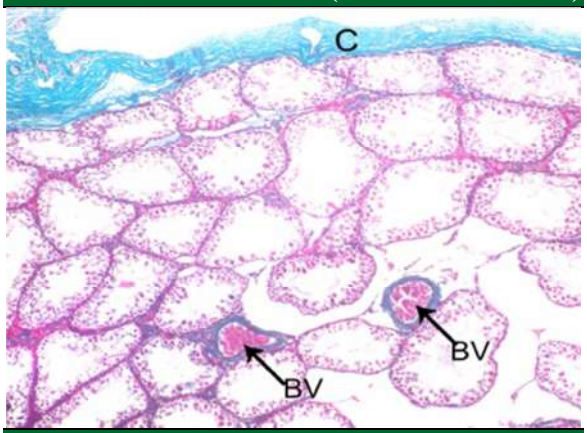

Figure 12: Photomicrograph of a section of the testis of an adult male albino rat (group III) showing marked increase of collagen fibers deposition in the wavy capsule (C) and around the congested blood vessels (BV) in the intersititum.
(Masson trichrome x100)

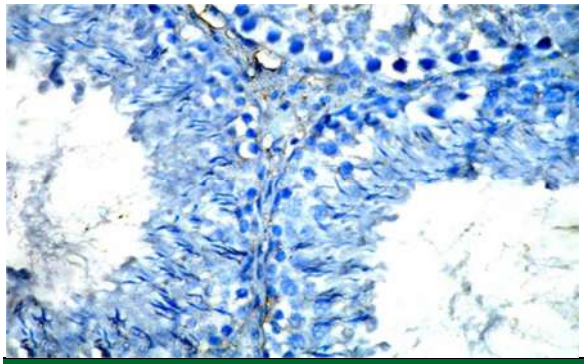

Figure 13: Photomicrograph of a section of the testis of an adult male albino rat of control group (group I) showing negative immune reaction for 8-OHdG.
(8-OHdG immunohistochemical X400)

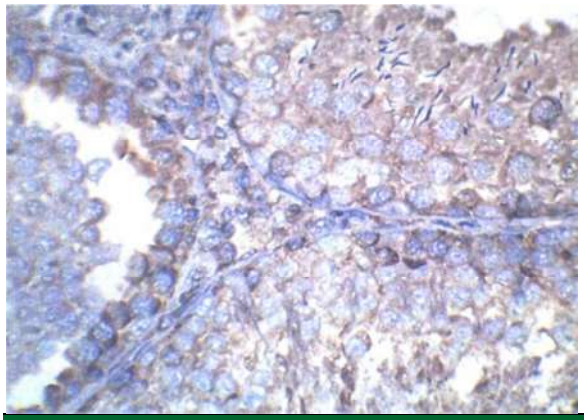

Figure 14: Photomicrograph of a section of the testis of an adult male albino rat of group II showing a weak positive immune reaction for 8-OHdG which appears as brown granules in the cytoplasm of seminiferous epithelium.
(8-OHdG immunohistochemical X400)

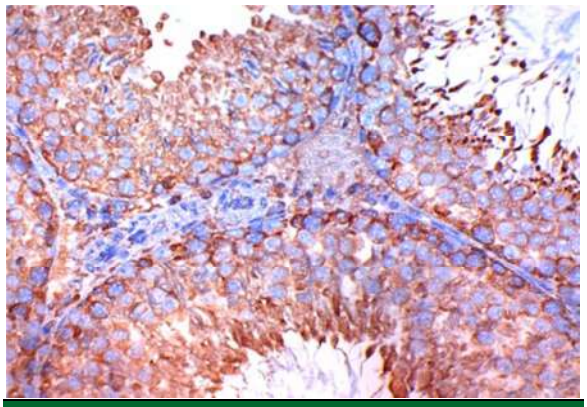

Figure 15: Photomicrograph of a section of the testis of an adult male albino rat of group III showing a strong positive immune reaction for 8-OHdG protein which appears as brown granules in the cytoplasm of seminiferous epithelium.
(8-OHdG immunohistochemical X400)

Table 1: Comparisons between mean values of testesterone, testis weight, diameter and height of seminiferous tubules in the different studied group using ANOVA (analysis of variance) test.

	Group I *(Mean ±SD)*	**Group II *(Mean ±SD)***	**Group III *(Mean ±SD)***	**F**	***p***
Testoste-rone (ng/ml)	10.5 ±3.03	1.81 ±1.31	1.66 ±0.67	67.02	<0.001 *
Testis weight (mg)	1140 ±61.46	748 ±164.9	545.2 ±201.5	38.33	<0.001 **
Diameter (µm)	660.67 ±293.06	612.02 ±95.29	367.91 ±72.87	5.49	0.01 *
Height (µm)	236.59 ±103.91	153.21 ±22.26	70.9 ±14.72	13.79	<0.001 **

**: Highly significant (P <0.001); SD: Standard deviation

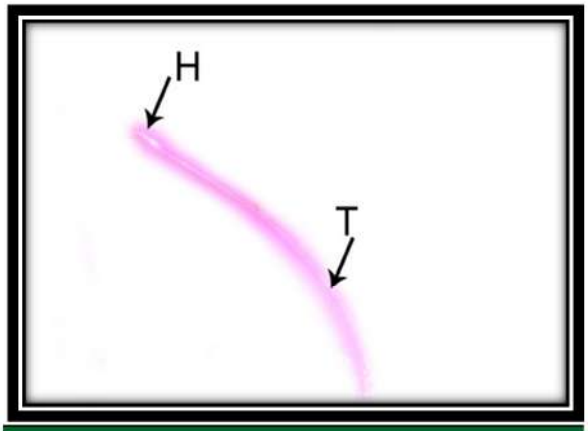

Figure 16: Photomicrograph of a normal living sperm from group I, showing head (H) and tail (T). The sperm appears hollow.
(Eosin-Nigrosin X400)

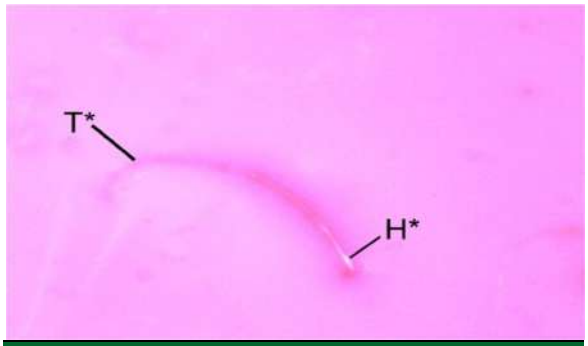

Figure 17: Photomicrograph of a living sperm from group I, showing banana head (H*) and kinked tail (T*).
(Eosin-Nigrosin X400)

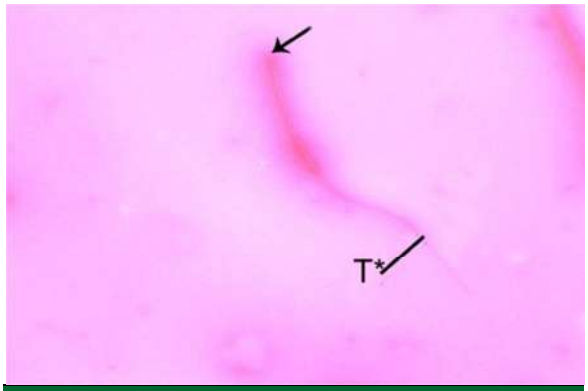

Figure 18: Photomicrograph of a dead sperm from group III, showing kinked tail (T*) and headless (arrow).
(Eosin-Nigrosin X400)

DISCUSSION

Adult albino rats were chosen in this work as they could be housed, bred and handled without difficulties. Also, they have long life span and they are relatively disease free.[19] Male animals were preferred because they have constant hormone levels in comparison to females who have variable ones. This variability should not be ignored as hormones can play a role in many inflammatory responses. There are significant differences in mitochondrial injury, nuclear condensation, ER (endoplasmic reticulum) status and plasma membrane permeability between sexes presenting female cells as being more sensitive, at certain exposure times.[20]

In the present study, testis weight was statistically decreased in the FA-treated groups when compared to the control groups. Also, there was no statistically difference between formaldehyde treated groups. Diminution in testicular weights of FA-treated groups is due to histological changes of testis in form of seminiferous tubules trophy and morphometric changes in form of decrease diameters of tubules. These result agree with other studies who observed that testicular weight was significantly decrease in rat exposed to formaldehyde as FA caused regressive histological changes in seminiferous tubules.[21,22] In accordance with the current study, Chowdhury et al reported that there was a gradual diminution in testicular weights in rats which were subjected to intraperitoneal injection of formaldehyde daily at doses 5, 10 and 15 mg/kg BW over a period 30 days and also they observed that testicular weight was lower at the doses of 10 and 15mg/kg formaldehyde.[14] These findings disagrees with those of Zahra et al who studied mice that were administrated FA (2.5, 5, 7.5 and 10 mg/kg) for 40 days by intraperitoneal injection.[9] At the end of exposure period, there were no significant differences observed between the experimental and control groups in the testis weights.

In the current study, there was a statistically significant decrease in the testosterone level in the FA-treated groups, when compared with that the control groups. Also, there was a non-significant statistical difference between FA-treated groups. This was consistent with those of Chowdhury et al who suggested that there was a significant decline of serum testosterone through intraperitoneal injection of 10 and 15 mg/kg doses in rat.[14] This decrease was explained by deformation of Leydig cells after administering formaldehyde caused diminution testosterone biosynthesis. Moreover, high lipid accumulation in the Leydig cell region suggests the non-utilization of lipids towards testosterone biosynthesis. This finding disagrees with Zahra et al who reported that there were no significant differences in testosterone levels between control and FA-treated groups which are administrated by intraperitoneal injection at doses (2.5, 5, 7.5 and 10 mg/kg) for 40 days.[9]

In the present work, light microscopic examination of the testis of the control albino rats group revealed that the normal histological pattern as the parenchyma of the testis was consisted of small multiple rounded seminiferous tubules. The structural components of the testis were the seminiferous tubules and interstitial tissues. The seminiferous tubules were lined by many layers of cells. The centers of seminiferous tubules are occupied by sperm bundles. These results were in agreement with those described by Vosoughi et al.[23]

In the present work, the testis of the group III treated with a 10 mg/kg intraperitoneal dose of FA revealed that there was a thickening of basement membrane. There was also seminiferous tubules atrophy as well as increasing in the spaces between germ cells. These results were in accordance with Golalipour et al who reported that the experimental animals exposed to FA vapor (10 mg/m3 for two weeks) showed atrophy of the seminiferous tubules, a decrease in the number of spermatogenic cells and disorganization of the seminiferous epithelial cells.[24] Hegazy et al attributed some cases of decreased rate of spermatogenesis to apoptosis. High rates of such apoptosis might lead to azoospermia.[25] Additionally observations demonstrated that the high proportion of spermatogenic cells was dissociated from basement membrane of seminiferous tubules. This finding is in accordance with Razi et al who also explained presence of giants cells located close to basement membrane, suggesting karyokinesis in FA-exposed rats.[26] Moreover, group III revealed an absence of Leydig cells. These results were in general agreement with other studies.[14,27]

In the current study, group III showed that there were

no acidophilic materials inside the lumen of seminiferous tubules. These results were in agreement with the experimental study of Shah et al indicating that the spermatogenesis was arrested.[28] Also, there was vacuolization of seminiferous epithelium in this group. This is consistent with that of Vosoughi et al.[23] The seminiferous tubules of group III showed presence of cellular debris in the lumens. This finding is explained by D`souza that seminiferous epithelial sloughing was usually the result of interruption of intercellular bridge.[29]

Masson's trichrome-stained sections in the FA-treated animals showed an increase of collagen fibers as prominent deposition of collagen fibers in the testicular capsule and in the interstitial tissues in-between the seminiferous tubules. This finding was attributed to the oxidative stress of formaldehyde. This explanation was supported by the results of Bancon and Britton who reported that formation of hydroxyl radical and other highly reactive oxidizing molecules in biological system led to lipid peroxidation.[30] The latter causes oxidative damage to proteins and nucleic acids. The end results of these reactions lead to the increase in collagen and ground substance formation.

The examination of 8-hydroxy-2-deoxyguanosine (8OHdG) slides of the groups II and III showed positive immune reaction for 8-OHdG protein. To explain the immunohistochemical study of the present study, reactive oxygen species can be produced through exposure to a variety of chemical and physical agents. ROS-initiated oxidative stress caused by FA can be regulated by cell defense mechanisms, including super-oxide dismutase (SOD), catalase (CAT), and glutathione (GSH). One major pathway of ROS-induced DNA damage involves a reaction at the C-8 position of 2-deoxyguanosine to form 8-OHdG.[32]

The morphometric findings of present study were in accordance with the histopathological results which showed increasing distances between seminiferous tubules and decreasing the mean of seminiferous tubules diameters and seminiferous epithelial height. The morphometric findings obtained in the present study were consistent with the findings of Golalipour et al that showed that exposure to FA vapor for 18 weeks in the rat induced histological changes in seminiferous tubules and decreased the mean of seminiferous tubular diameters.[24] Also, Ozen et al revealed that exposure to FA for 91 days caused significant reductions in tubular diameters.[27]

FA-treated groups showed abnormal forms of sperms in form of headless and kinked tails sperms. This finding agrees with Tang et al.[11] The authors added that such changes in sperms indicate the genotoxicity of formaldehyde.

CONCLUSION

Administration of formaldehyde might adversely affect the reproductive system in males.

Recommendations

More future studies are required using other animals; as well as subjects exposed to FA are recommended to be followed up for early discovery of its adverse effects. Much more attention should be paid for limiting the occupational and environmental exposure to FA. Special precautions might be taken to limit the level of the environmental, water and food contamination by FA; and many alternatives should be developed to avoid its possible hazards.

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