Evaluation Of Reproductive Toxicity Induced By Nicotine Free E-Cigarette Smoke In Pre-Pubertal And Adult Male Rats

Mai Taha[1], Heba Allah Abd El Rahman[1], Mona El Harouny[1], Mona Y.Y.Abd Allah[2], Laila Mohammed Ahmad El-Zalabany[1]

[1]Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura University
[2]Department of pathology, Faculty of Medicine, Mansoura University
**Corresponding author: Mai Taha [1], E-mail: Dr.Nassz@ymail.com*

KEYWORDS
Nicotine, E-Cigarette Smoke, Male Rats, Oxidative Stress Markers, Blood testes barrier.

ABSTRACT
Background: The three main components of electronic cigarettes (EC) are a cartridge (filled with electronic liquid), atomizer (vaporization chamber), and a rechargeable lithium battery (powerhouse). Variations in e-liquids can be found in nicotine doses as well as types of added flavors. There is growing evidence linking the use of EC to dangerous health consequences, including effects on male and female reproduction.
Aim: To evaluate the male reproductive toxicity of nicotine free E-cigarette smoke on pre-pubertal and adult male Sprague Dawley Albino rats as regard semen analysis, oxidative stress, endocrine disruptive effect and histological changes including effects on blood testes barrier affection.
Methods: To experiment, thirty-two male rats sixteen prepubertal rats and sixteen adult rats were divided into four groups each group consists of eight rats, Group I (Adult control group) and group II (Prepubertal control group) were subjected to inhalation-related stress by exposing them. Group III (Adult EC exposed group) and group IV (Prepubertal EC exposed group) were exposed to vapor generated from EC by using Electrical pump in the inhalation Propylene box (whole body exposure) 3 hours daily for 28 days. The rats were weighed at the start and the end of the experiment. At the end of the 28 days, blood samples were collected to detect the serum levels of testosterone and luteinizing hormone (LH). Oxidative stress markers (SOD and MDA) were assessed. Immunohistochemistry examination included detection of proteins specific for blood testes barrier (occludin and connexin 43).
Results: Testicular weight was significantly increased in the prepubertal, and adult control groups compared to the prepubertal and adult exposed groups respectively. Prepubertal EC exposed group had a significantly lower percentage of sperm motility, and a significantly increased percentage of abnormal sperm morphology compared to the adult EC exposed group. The testosterone level was significantly lower in prepubertal and adult exposed groups with significant increase in LH level. A significant decrease in SOD activity as antioxidant defense mechanism was observed in EC exposed groups. Prepubertal and adult EC exposed groups showed significant increased expression of connexin in line with decreased occludin immune staining compared to controls.
Conclusion: The present study confirmed the reproductive toxic effect of nicotine free EC vapor on pre-pubertal and adult male Sprague Dawley rats as

regard endocrine disruptive effects, semen abnormalities, testicular histological changes and testicular oxidative stress. The study also clarified the toxic effect of EC smoking on proteins of blood testes barrier particularly, (occludin and connexin 43).

Introduction

Vape pens, e-cigars, and vaping devices are other names for electronic cigarettes, or e-cigarettes. The vaporization chamber (atomizer), cartridge (filled with electronic liquid), and rechargeable lithium battery (powerhouse) comprise the common working system of e-cigarettes. The electronic liquid's (e-liquid) igniting process is started by suction through the mouthpiece [1].

Different battery voltages, the amount of e-liquids fired per product (per puff), and temperature modulation during vaping are among the operating parameters variations for e-cigarettes. However, there are differences in e-liquids' flavors and nicotine doses. The two primary inhaling aerosolized humectants found in e-liquids are often glycerol (vegetable glycerin) and propylene glycol (PG) [2].

When it comes to e-cigarettes, passive smoking is thought to be riskier than with traditional cigarettes. This is because e-cigarettes produce liquid droplets and inhaled particles that are smaller and evaporate more quickly. The notion that e-cigarettes constitute a source of pollution for the environment [3].

Reproductive abnormalities and male infertility are becoming global issues. Male reproductive toxicity has been linked to smoking and exposure to environmental contaminants [4]. Numerous harmful mechanisms related to reproduction are involved, including disrupted spermatogenesis, modifications to the blood–testis barrier (BTB), hormonal fluctuations, damage to DNA, oxidative stress, and inflammation. The BTB is a crucial testicular ultrastructure. The BTB serves as an immune barrier that keeps the growth of germ cells apart from the bloodstream [5].

Numerous protein complexes, including N-cadherin in (ES), connexin43 in (GJ), and occludin in (TJ), are seen in BTB [6]. There is mounting evidence linking the use of e-cigarettes to dangerous health consequences, including effects on male and female reproduction [7]. So, our study aimed to evaluate the male reproductive toxicity of E-cigarette smoke on pre-pubertal and adult rats as regard histological changes, oxidative stress variations and endocrine disruptive effect and to evaluate potential role of blood testes barrier affection in male reproductive toxicity by E-cigarette.

MATERIALS AND METHODS

This subacute experimental study was conducted at animal house, Urology and Nephrology Center, Mansoura University. The present study was ethically approved by the IRB of Faculty of Medicine, Mansoura University, code number (MD.21.05.469-12/07/2021).

Chemicals and Kits

Propylene glycol (PG) was obtained as analytic grade at a concentration of 93% in liquid form. Vegetable glycerin (VG) was obtained as analytic grade at concentration 92% in liquid form (PIOCHIM Company for laboratory chemicals aera 69A, 6th of October, Egypt). Phosphate buffer saline (PBS) was prepared by chemicals purchased from Sigma-Aldrich Company, Saint Louis, MO, USA. Testosterone hormone detection kits were purchased (as rat testosterone Radio Immuno Assay kit, Immuno Tech, USA, catalog No. IB79174). Luteinizing hormone (LH) detection kits were purchased from Mindray company (Cairo, Egypt, catalog NO. LH111). Superoxide dismutase (SOD) detection kits were purchased from BIODIAG-NOSTIC diagnostic and research reagents, Giza, Egypt, catalog No. SD25 21). Malondialdehyde (MDA)

detection kits were purchased from (BIODIAG-NOSTIC diagnostic and research reagents, Giza, Egypt, catalog No. MD25 29). Connexin 43 polyclonal antibody kits were purchased from (Abbkine Company, Georgia, USA, catalog NO. ABP0075). Occludin Rabbit polyclonal antibody kits were purchased from (AB clonal Company, Massachusetts, USA, catalog NO. A2601). Diaminobenzidine kits (Poly HRP DAB kit) were purchased from Sakura USA (Cat. No. 54-0117). Other chemicals used in the study as Sodium bicarbonate (NaHCO3), normal saline and Formaldehyde 40% were purchased as analytic grade from Al–Gomhoria Company for medicines (Cairo Egypt).

Instruments

The electronic cigarette was purchased as a commercially available variable voltage EC 4th generation (Vapopresso designed in California and made in China). The used EC product size was: 47.8×29.5×146mm with charging current 2.5Amber, power range: 5-220watt and resistance range: 0.03-5 Ohm. The EC tank capacity was 2ml. The voltage was settled on 3.5 Volt and the device was equipped with a 1.5 Ohm resistance. Rechargeable lithium battery was used to supply power to the EC system. The tank was loaded with a nicotine-free liquid composed of propylene glycol (PG) and vegetable glycerin (VG) with volume ratio 1:1 [8].

The exposure in test groups was by using a whole-body mode. The inhalation chamber is composed of a propylene box (38×26.5×19 cm) with a capacity of nineteen Liters made in Egypt. An opening for a fresh air supply was connected in the whole-body exposure chamber [9]. Electrical pump (0.18 kilo Watt; 1.4/1.6 Amber; 230 Volt; 50/60 Hertz) was manufactured by China. The Electrical pump was installed on one side of the propylene box where it was used for generation of EC smoke [10].

We used the following; standard light microscopy (bioclure, OPTICA, Germany) supplied with a digital camera (Canon five megapixels, 3.2x optical zoom) (Tokyo, Japan), Cobas e 411 analyzer for immunoassay test Roche Diagnostics GmbH D-68298, Mannheim, Germany), Mindray Hormone biochemistry autoanalyzer (Mindray BS-120, Lotus pathology in NASHIk, India), Robinik biochemistry analyzer (A-374, TTC, Mahape, Nav Mumbai-400 710, India) for SOD and MDA analysis, and Neubauer's hemocytometer (Burari, Delhi, Indi catalog #68052-14,68052-15).

Animals

This study was conducted on thirty-two Sprague Dawley Albino male rats sixteen prepubertal rats (3 weeks old) with average weight (40-50gm) and sixteen adult rats (10 weeks old) with average weight (150-170gm). Rats were purchased from animal research facility at urology and nephrology center and were housed under standard conditions (12-h light-dark cycle, 22 °C and 60% humidity). Rats have free access to water and food throughout the experiment. Following seven of acclimatization, rats were haphazardly assigned to the experimental units.

Rats were divided into four groups, each group consisted of 8 rats, group I (Adult control group) was subjected to inhalation-related stress by exposing them, in the inhalation Propylene box, to air and noise produced by the Electrical pump for 10 min daily for 28 days, group II (Prepubertal control group) was subjected to inhalation-related stress by exposing them, in the inhalation Propylene box, to air and noise produced by the Electrical pump for 10 min daily for 28 days, group III (Adult EC exposed group) was exposed to vapor generated from EC by using Electrical pump in the inhalation Propylene box (whole body exposure) 3 hours daily for 28 days, each three hours consisted of 11 cycles of exposure, each cycle consisted of two puffs (6 seconds on; 5 seconds off; 6 seconds on), and it was followed by 20 min of recovery and group IV (Prepubertal EC exposed group) was exposed to vapor generated from EC by using Electrical pump in the inhalation Propylene box (whole body exposure) 3 hours daily for four weeks, each three hours consisted of 11 cycles of exposure, each cycle consisted of two puffs (six seconds on; five seconds off; six seconds on), and it was followed by twenty minutes of recovery.

Methods

The initial body weight (BW) was assessed at the beginning of the study and the final BW was assessed at the end of study duration for all rats. Blood samples were collected from the tail vein for testosterone and LH analysis after 24 hours from the last exposure at the end of the study. Immunoassay analysis was used to identify and detect the concentration of testosterone in blood samples, by usually using an antibody as a reagent. Chemiluminescent Immunoassay technique was used for the quantitative determination of luteinizing hormone (LH) in the serum or plasma.

Rats were sacrificed after twenty four hours from the last exposure and euthanized by intraperitoneal injection of Sodium thiopental (Sigmatic, United Kingdom) at dose of 800mg/kg. Testicles were removed and right Testicles were used for histological as well as histopathological examination, while as, the left Testicles were rapidly frozen at -800C till used for oxidative stress markers detection. The weight of both testicles was recorded at the moment of the removal. The gonadal relative weight in relation to body weight was measured by using the gonadosomatic index (GSI index) which was measured as: GSI = (testis weight (grams) / body weight (grams)) x 100.

Sperms were collected as follows: the epididymis of the right testis was removed, trimmed free of fat, and placed in physiological saline at 37°C. Regarding sperm count, gentle squeezing of the right cauda epididymis was performed to obtain epididymal fluid on a slide, which was suspended in phosphate buffer saline (PBS) and was drawn up to the '0.5' mark of the white blood cells (WBCs) pipette. The sperm count was done with the high-power objective (40×). Haematoxylin and eosin-stained seminal smears were utilized to detect the percentage of abnormal sperm. Measurement of sperm motility was conducted after the diluted semen sample, which was incubated in a CO2 incubator for 30 min.

Super oxide dismutase (SOD) and Malondialdehyde (MDA) levels were measured in testicular tissue homogenate by colorimetric method which depends on detecting the concentration of a compound in a solution with the aid of a colour reagent.

The histopathological analysis included the evaluation of germ cell vacuolization, blood vessels congestion, separation of seminiferous tubules, separation of germ cells from basement membrane and degenerative changes using Olympus digital microscope and digital camera. Immunohistochemistry evaluation of the dissected testicles was done for detection of two proteins in the blood testes barrier; occludin (tight junction protein) and connexin 43(gap junction protein).

Statistical Analysis

The collected data were analysed by using SPSS (Chicago, Illinois USA) for windows version 20.0 variables were tested for normality with the Kolmogorov- Smirnov test. Normally distributed continuous data was presented as mean±SD. ANOVA test was utilized to measure variation between groups, and the Tukey test was used to make comparisons. The p-values of <0.05 were considered significant.

RESULTS

Table (1) shows that there was a highly significant difference between the studied groups regarding the initial and final BWs ($p< 0.001$). The final body weight was significantly increased in the prepubertal control group compared with the prepubertal EC exposed groups ($p<0.001$). The final body weight was significant lower in adult EC exposed group compared control group ($p< 0.001$). As regards initial body weight, no significant difference was found between adult control group and adult EC exposed group ($p=0.0989$). Also, no significant difference was reported between initial body weights of prepubertal control group and prepubertal EC exposed group ($p=0.0887$). The highest percentage of weight change was shown in the prepubertal control group followed by prepubertal EC exposed group then the adult control group and finally the adult EC exposed group.

There was a high significant difference between the study groups regarding the testes weight ($p< 0.001$). The testes weight was significantly increased in the adult control group compared to the adult EC exposed group ($p< 0.001$). Furthermore, the testes weight was significantly increased in the prepubertal control group compared to the prepubertal EC exposed groups ($p< 0.001$). There was also a significant difference between adult EC exposed group and prepubertal EC exposed group as regard testes weight ($p< 0.001$). There was also significant difference between adult EC control group and prepubertal EC control group as regard testes weight ($p< 0.001$).

There was insignificant difference between the studied groups regarding the Gonadosomatic index (GSI) (%). There was significant difference between the study groups regarding the testosterone level ($p< 0.001$). Adult EC exposed group to EC vapor had significant low testosterone level compared to adult control group ($p< 0.001$). Also, prepubertal EC exposed group had significantly low testosterone level compared to prepubertal control group ($p< 0.001$). Adult groups (Group I and Group III) had significantly increased testosterone levels compared to prepubertal groups (Group II and Group VI), respectively.

A significant difference was detected among all the studied groups regarding the LH level ($p< 0.001$). The LH level was significant higher in the adult EC exposed group compared to the adult control group ($p< 0.001$). Also, the LH level was significant higher in the prepubertal EC exposed group compared to prepubertal control group ($p=0.002$). The LH was higher in adult EC exposed group compared to prepubertal EC exposed group ($p< 0.001$). However, there was insignificant difference between adult control and prepubertal control groups ($p= 0.061$).

A significant difference was detected among all the studied groups regarding the sperm count ($p< 0.001$). Adult control group had significantly increased sperm count compared to adult EC exposed group ($p< 0.001$). The sperm count was significantly increased in the prepubertal control group compared to the prepubertal EC exposed group ($p < 0.001$). Additionally, prepubertal groups (II & VI) had a significant lower sperm count compared to adult groups (I, III) respectively ($p< 0.001$).

Concerning sperm motility, there was a highly significant difference among the studied groups ($p < 0.001$). The percentage of sperm motility was significantly increased in the adult control group compared to the adult EC exposed group ($p= 0.005$). In addition, the percentage of sperm motility was significantly increased in the prepubertal control group compared to the prepubertal EC exposed group ($p< 0.001$). The prepubertal EC exposed group had significantly lower percentage of sperm motility compared to adult EC exposed group ($p=0.003$). In addition, no significant difference was detected between the adult and prepubertal control group regarding the sperm motility ($p= 0.111$).

Concerning sperm morphological abnormalities, the percentage of sperm morphological abnormalities demonstrated a highly significant difference among the studied groups ($p < 0.001$). The percentage of sperm morphological abnormalities was significant higher in the prepubertal EC exposed group compared to the prepubertal control group ($p< 0.001$). Also, the percentage of sperm morphological abnormalities was significant higher in the adult EC exposed group compared to adult control group ($p< 0.001$). A significantly increased percentage of sperm morphological abnormalities was found in prepubertal EC exposed group compared to adult EC exposed group ($p= 0.028$). In addition, no significant difference was reported between the adult and prepubertal control groups as regard sperm morphological abnormalities ($p= 0.171$)

Table (1): Analysis of body weight, Testes weight, Gonadosomatic index, Testosterone, LH, Sperm count, motility, and morphology in all studied groups (N =32)

Body weight (gm)	**Group I (Adult control group) (N=8)**	**Group II (Prepubertal control group) (N=8)**	**Group III (Adult EC exposed group) (N=8)**	**Group IV (Prepubertal EC exposed group) (N=8)**	**Test of significance**
Initial body weight (gm)					
Mean ± SD	168.25 ± 1.54	45.88 ± 6.22	163.50 ± 11.61	44.50 ± 5.50	F= 115.578
p1		0.001**	0.0989	0.001**	p < 0.001**
p2			0.001**	0.0887	
P3				0.046*	
Final body weight					
Mean ± SD	278.13 ± 2.49	133.63 ± 8.83	205.13 ± 10.71	77.88 ± 6.06	F= 627.564
p1		< 0.001**	< 0.001**	<0.001**	p < 0.001**
p2				< 0.001**	
P3				< 0.001**	
Percent of weight change					
Mean ± SD	65.94 ± 12.98	194.61 ± 1.29	26.01 ± 11.02	78.01 ± 30.54	F= 115.578
p1		< 0.001**	0.024*	0.989	p < 0.001**
p2			< 0.001**	< 0.001**	
P3				0.046*	
Testes weight (gm)					
Mean ± SD	1.94 ± 0.13	1.02 ± 0.09	1.46 ± 0.20	0.49 ± 0.12	F= 149.420
p1		< 0.001**	< 0.001**	< 0.001**	p < 0.001**
p2			< 0.001**	< 0.001**	
P3				< 0.001**	
Gonadosomatic index (GSI) (%)					
Mean ± SD	0.70 ± 0.06	0.76 ± 0.05	0.72 ± 0.12	0.63 ± 0.16	F= 2.184
p1		0.904	0.986	0.597	P = 0.117
p2			0.802	0.081	
p3				0.396	
Testosterone (ng/ml)					
Mean ± SD	1.01 ± 0.09	0.42 ± 0.04	0.54 ± 0.10	0.23 ± 0.05	F= 164.884
p1		< 0.001**	< 0.001**	< 0.001**	p < 0.001**
p2			0.015*	< 0.001**	
P3				< 0.001**	
LH (mIU/ml)					
Mean ± SD	0.21 ± 0.03	0.15 ± 0.03	0.35 ± 0.06	0.24 ± 0.07	F= 24.739
p1		0.061	< 0.001**	0.529	p < 0.001**
p2			< 0.001**	0.002*	
p3				0.001**	
Sperm count (10^6/ml)					
Mean ± SD	119.67 ± 8.60	80.49 ± 8.07	82.58 ± 12.01	49.21 ± 10.82	F= 66.407
p1		< 0.001**	< 0.001**	< 0.001**	p < 0.001**
p2			0.975	< 0.001**	
P3				< 0.001**	

Sperm motility (%)					
Mean ± SD	93.13 ± 2.59	85.63 ± 4.17	81.25 ± 7.44	68.75 ± 9.16	F= 20.246
p1		0.111	0.005*	< 0.001**	p < 0.001**
p2			0.528	< 0.001**	
p3				0.003*	
Morphology (%)					
Mean ± SD	3 ± 1.20	8.25 ± 1.83	18.13 ± 7.51	25.50 ± 6.07	F= 32.963
p1		0.171	< 0.001**	< 0.001**	p < 0.001**
p2			0.002*	< 0.001**	
p3				0.028*	

EC: electronic cigarette, N: number, SD: standard deviation, F for ANOVA test
∗Significant if p ≤0.05
∗∗Highly significant result if p ≤0.001
p1: comparison with adult control group
p2: comparison with Prepubertal control group
p3: comparison with adult EC exposed group

Table (2) shows that there was a highly significant difference between the study groups regarding the SOD (p< 0.001). The SOD was significant higher in the adult control group compared to the adult EC exposed group (p< 0.001). Also, the SOD was significantly increased in the prepubertal control group compared to prepubertal EC exposed group (p< 0.001). Additionally, adult control group had significant higher SOD activity compared to prepubertal control group (p=0.003). However, there was insignificant difference between SOD in both EC exposed groups; the adult and the prepubertal group (p=0.997).

There was significant difference between the studied groups regarding the MDA (p< 0.001). The MDA was significant higher in the adult EC exposed group compared to the adult control group (p< 0.001). Also, the MDA was significant higher in the prepubertal EC exposed group compared to prepubertal control group (p< 0.001). Adult EC exposed group had significantly increased MDA compared to prepubertal EC exposed group (p< 0.001). There was insignificant difference between adult control and prepubertal control groups (p=0.921).

There was a highly significant difference between the studied groups regarding the percentage area of connexin immunostaining (p< 0.001). The percentage area of connexin positive staining was significantly increased in the adult EC exposed group compared to the adult control group (p< 0.001). Furthermore, the percentage area of connexin positive staining was statistically significantly increased in the prepubertal EC exposed group compared to the prepubertal control group (p< 0.001). The adult EC exposed group had significantly stronger connexin expression compared to the prepubertal EC exposed group (p< 0.001). Also, the adult control group had significantly increased connexin expression compared to the prepubertal control group (p< 0.001).

There was significant difference between the studied groups (p< 0.001). The adult EC exposed group had significant lower percentage area of occludin immune staining compared to the adult control group (p< 0.001). Additionally, the prepubertal EC exposed group had significant lower occludin expression compared to the prepubertal control group (p< 0.001). There was insignificant difference in occludin expression between the adult control and prepubertal control groups (p= 0.999). No significant difference was found between the adult EC exposed and prepubertal EC exposed groups as regard occludin expression (p= 0.997).

Table (2): Analysis of SOD (U/g.t), MDA (nmol/g.t), of percentage area of positive connexin immune staining and positive occludin immune staining in all studied groups (N =32)

	Group I (Adult control group) (N=8)	**Group II (Prepubertal control group) (N=8)**	**Group III (Adult EC exposed group) (N=8)**	**Group IV (Prepubertal EC exposed group) (N=8)**	**Test of significance**
SOD (U/g.t)					
Mean ± SD	200.20 ± 23.81	155.06 ± 21.95	83.75 ± 18.44	81.31 ± 27.85	F= 49.613
p1		0.003*	< 0.001**	< 0.001**	p < 0.001**
p2			< 0.001**	< 0.001**	
P3				0.997	
MDA (nmol/g.t)					
Mean ± SD	11.71 ± 2.08	8.80 ± 1.60	55.70 ± 15.46	29.45 ± 9.74	F= 43.633
p1		0.921	< 0.001**	0.003*	p <0.001**
p2			< 0.001**	0.001**	
p3				< 0.001**	
Percentage area of connexin immunostaining					
Mean ± SD	6.59 ± 0.73	1.57 ± 0.70	35.61 ± 1.53	18.75 ± 1.46	F= 834.519
p1		< 0.001**	< 0.001**	< 0.001**	p < 0.001**
p2			< 0.001**	< 0.001**	
p3				< 0.001**	
Mean ± SD	6.35 ± 0.95	6.34 ± 0.73	2.64 ± 0.71	2.56 ± 0.61	F= 64.494
p1		0.999	< 0.001**	< 0.001**	p < 0.001**
p2			< 0.001**	< 0.001**	
p3				0.997	

EC: electronic cigarette, N: number, SD: standard deviation, F for ANOVA test
∗Significant if p ≤0.05
∗∗Highly significant result if p ≤0.001
p1: comparison with adult control group
p2: comparison with Prepubertal control group
p3: comparison with adult EC exposed group

Figure (1) shows that adult control group (Fig. 1- A) and prepubertal control group (Fig. 1-B) had normal histological architecture as seminiferous tubules were filled with germ cells and no separation of germ cells from basement membrane. In addition, prepubertal control group did not show germ cell vacuolization, separation of seminiferous tubules and congested blood vessels (Fig. 1-B). The adult control group showed minimal physiological non-degenerative changes such as (basal germ cell vacuolization, separation of seminiferous tubules and mildly congested blood vessels) (Fig. 1-A). On the other hand, the adult EC exposed group showed separation of germ cells from basement membrane with degenerative changes of seminiferous tubules. In addition, extensive germ cell vacuolization, marked congested blood vessels and mild focal hyperplasia of leydig cells were observed in adult EC exposed group (Fig. 1-C). Prepubertal EC exposed group revealed germ cell vacuolization with separation and degeneration of seminiferous tubules. However, there was an absence of congested blood vessels, separation of germ cells from basement membrane and hyperplasia of leydig cells in the prepubertal EC exposed group (Fig. 1-D).

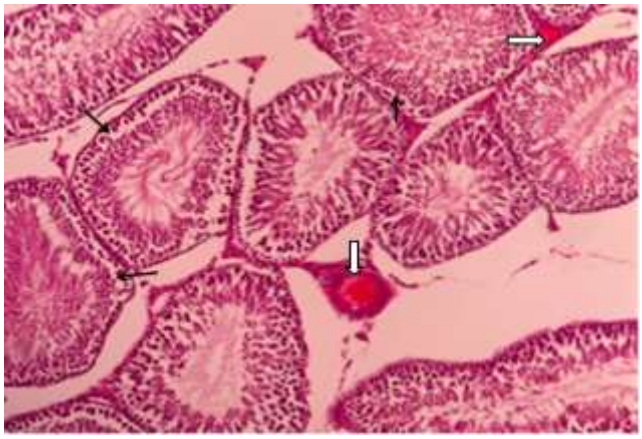


Figure (1-A): photomicrograph picture of the adult control group, H&E stained slides (x100) revealed basal germ cell vacuolization (black arrows) with separation of seminiferous tubules, congested blood vessels (white arrows), no separation of germ cells from basement membrane with filling of seminiferous tubules by germ cells (no degenerative changes).

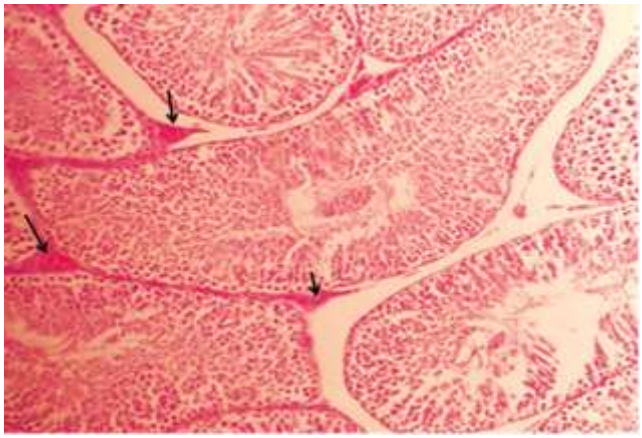
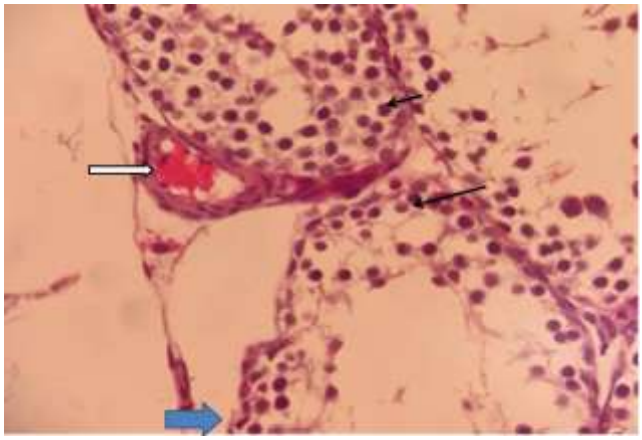
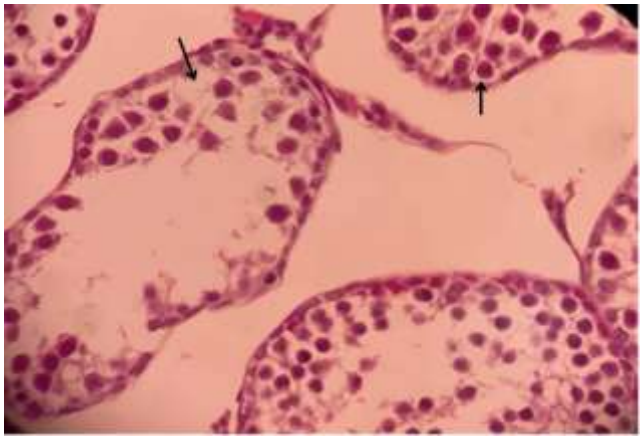


Figure (1-B): photomicrograph picture of the prepubertal control group, H&E stained slides (x100) revealed no germ cell vacuolization with no separation of seminiferous tubules, blood vessels (black arrows), no separation of germ cells from basement membrane with filling of seminiferous tubules by germ cells (no degenerative changes).



(Figure 1-C): photomicrograph picture of the adult EC exposed group, H&E stained slides (x200) revealed extensive germ cell vacuolization (black arrows) with separation of seminiferous tubules, congested blood vessels (white arrows), separation of germ cells from basement membrane (blue arrows) with degenerative changes of seminiferous tubules.



(Figure 1-D): photomicrograph picture of the prepubertal EC exposed group, H&E stained slides (x200) revealed germ cell vacuolization (black arrows) with separation of seminiferous tubules, no congested blood vessels, no separation of germ cells from basement membrane with degenerative changes of seminiferous tubules

Figure (1): Representative photomicrographs of H&E stained slides of the study groups

Figure (2) illustrates representative photomicrograph pictures of connexin staining in the four studied groups. The adult control group had week expression of connexin staining appeared only in about (5%) of cells (Fig. 2-A). In addition, the prepubertal control group revealed minimal expression of connexin immune staining (Fig. 2-B). The strongest expression of connexin (brownish staining) was observed in adult EC exposed group as appeared in about (>75%) of cells (Fig. 2-C). The prepubertal EC exposed group had moderate connexin expression in about (50%) of cells (Fig. 2-D).

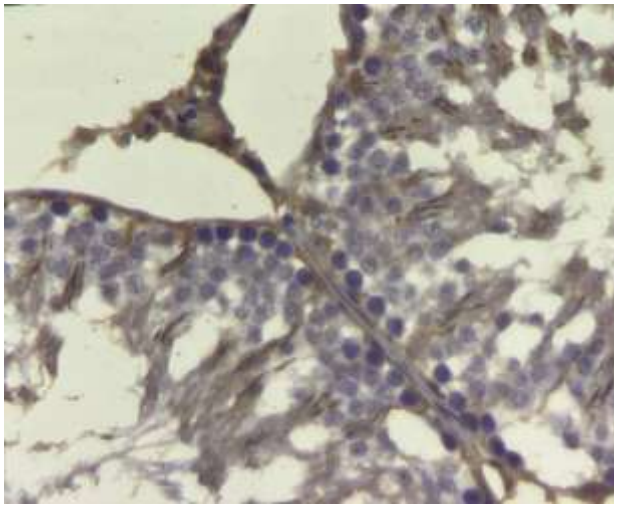


Figure (2-A): photomicrograph picture of the adult control group, connexin immune stained (x200) revealed weak staining in about (5%) of cells.

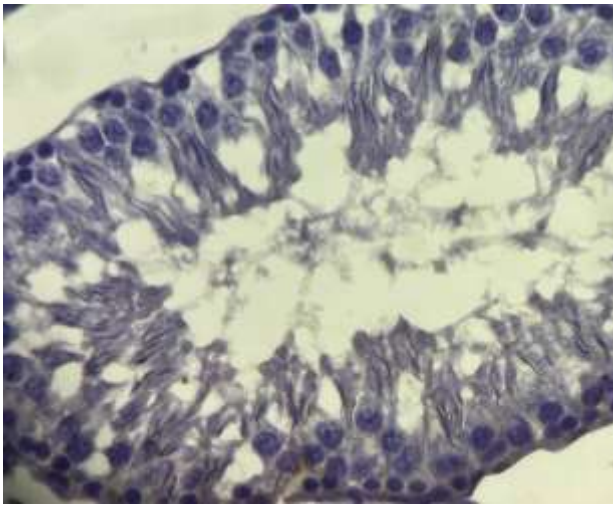


Figure (2-B): photomicrograph picture of the prepubertal control group, connexin immune stained slides (x200) revealed minimal staining.

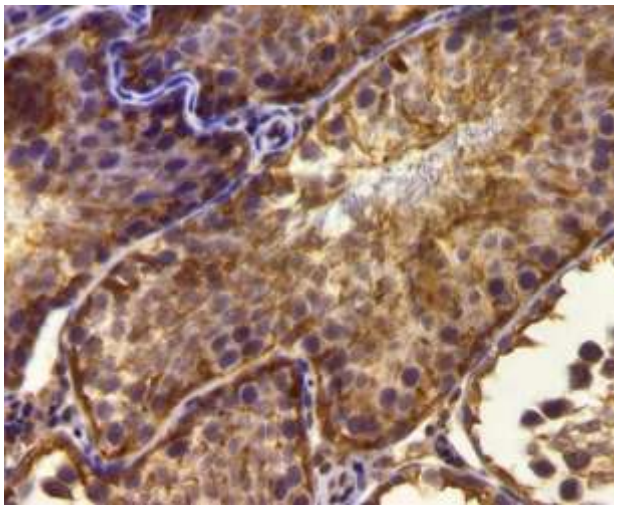


Figure (2-C): photomicrograph picture of the adult EC exposed group, connexin immune stained slides (x200) revealed the strongest staining (brownish staining) in (>75%) of cells.

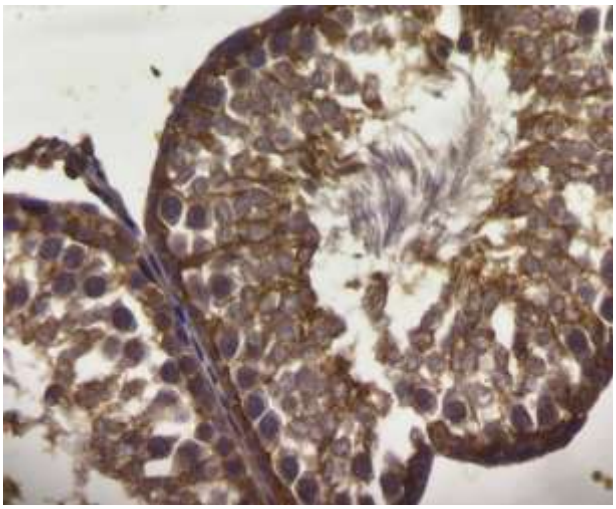


Figure (2-D): photomicrograph picture of the prepubertal EC exposed group, connexin immune stained slides (x200) revealed moderate staining in about (50%) of cells.

Figure (2): Representative photomicrographs of Connexin immune stained of the study groups

Figure (3) illustrated representative photomicrograph pictures of occludin staining in the four studied groups. Strong expression of occludin immune staining (brownish staining) was observed in adult control group (Fig. 3-A) and in prepubertal control group (Fig. 3-B). On the other hand, adult EC exposed group (Fig. 3-C) and prepubertal EC exposed group (Fig. 3-D) had week expression of occludin immune staining. The occludin staining was mainly found in the basally located inter-Sertoli junctional area and in the cytoplasm of Sertoli cells. In the interstitial tissue, occludin immune reactivity was found in blood capillaries.

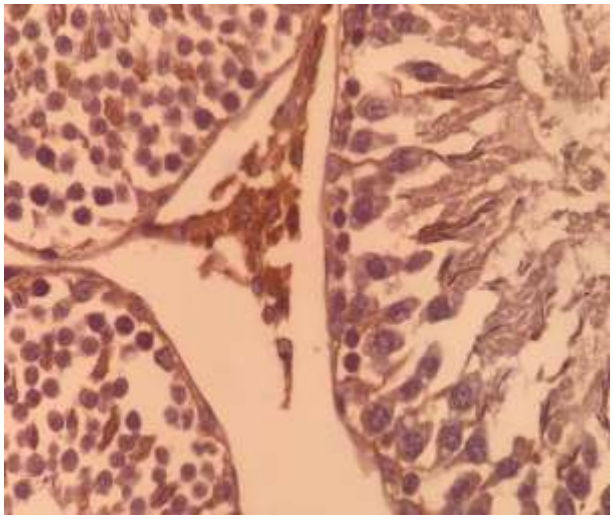


Figure (3-a): photomicrograph picture of the adult control group had strong expression of occludin immune staining (brownish staining) (x100).

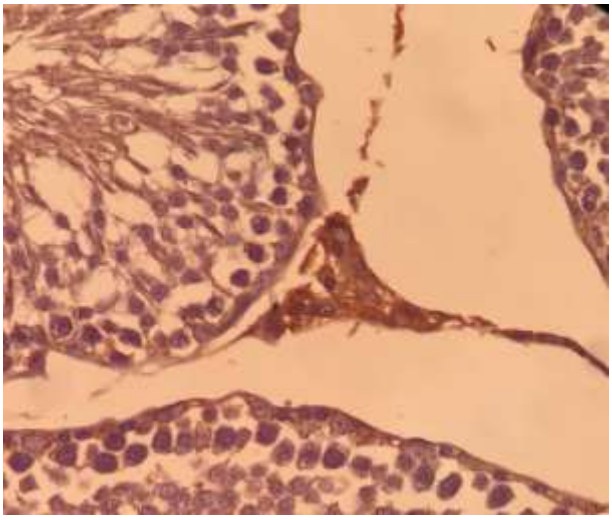


Figure (3-b): photomicrograph picture of the prepubertal control group had strong expression of occludin immune staining (x100).

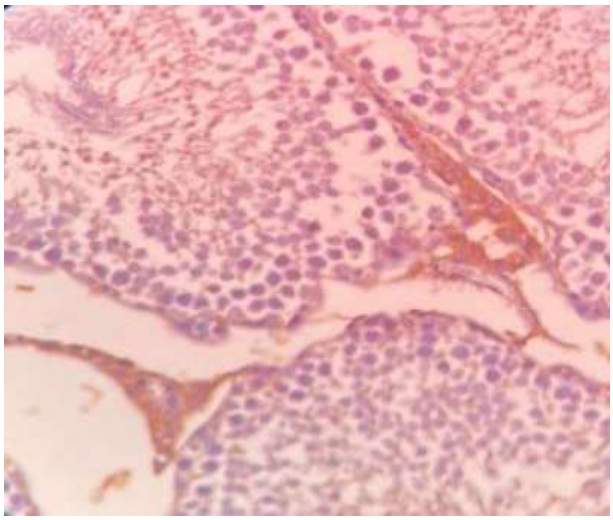


Figure (3-C): photomicrograph picture of the adult EC exposed group, revealed week occludin expression (x200).

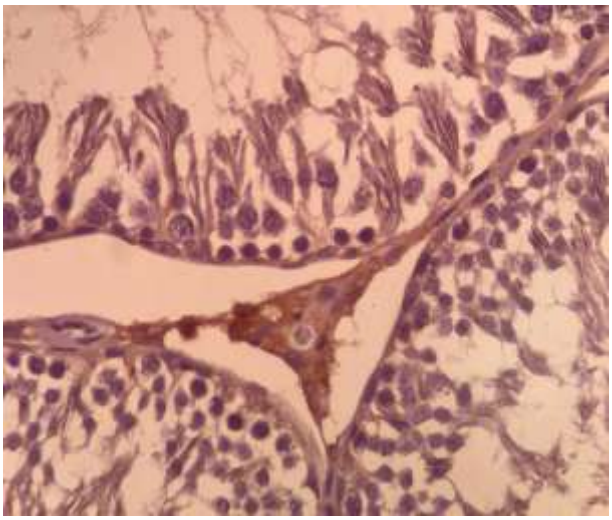


Figure (3-D): photomicrograph picture of the prepubertal EC exposed group revealed week occludin expression (x200).

Figure (3): Representative photomicrographs of occludin immune staining of the study groups.

DISCUSSION

According to the WHO, male infertility is a public health concern that affects 186 million individuals globally. Tobacco smoking is considered a main lifestyle factor that has adverse effects on reproductive system [11]. Initially; EC smoking was used as an aid in the cessation process of conventional cigarette smoking [12, 13].

Typically, e-liquid contains two primary inhaling aerosolized humectants: propylene glycol (PG) and vegetable glycerin (VG). Although PG and VG are known to be nontoxic, their oxidation and thermal decomposition in EC resulted in many toxic compounds (e.g., formaldehyde, acetaldehyde, acrolein, and benzaldehyde). The EC utilization through vaping is shown to have several negative effects on the lungs, immune system, and liver functions [14].

Hence, our study was conducted to assess the male reproductive toxicity of nicotine free EC vapor on pre-pubertal and adult rats as regard endocrine disruptive effects, semen abnormalities, testicular histological changes and testicular oxidative stress. Also, the study assessed for the first time the potential deleterious effect of EC smoking on proteins of blood testes barrier particularly (occludin and connexin 43).

Thirty-two male Sprague Dawley Albino rats were used in this study; sixteen were prepubertal rats, aged three weeks, and sixteen were adult rats, aged ten weeks. The rats were further divided into four groups, each with eight rats, based on the exposure pattern: Groups I and II (adult control and prepubertal control groups) respectively, Groups III (adult EC exposed group), and Group IV (prepubertal EC exposed group) had been exposed three hours daily to vaper generated from fourth generation EC via whole body exposure for 28 days.

According to the final body weight in this study, it was significantly elevated in the prepubertal control group and adult control group compared to the prepubertal EC exposed group and adult EC exposed group, respectively.

In harmony with current findings; Wetendorf et al., (2019) reported decreased body weight in prepubertal off springs of C57BL/6J mice which were exposed to EC vapor (PG, VG, 24 mg/mL nicotine) during pregnancy [15].

Contradictory to the present results, Vivarelli et al., (2019) reported insignificant difference in body weight of adult rats, inhaled nicotine free EC (PG, VG, flavor) vapor for 28 days, compared to controls [16]. This discrepancy from current data can be assumed to either the difference in strains, EC liquid composition or the nature of feeding during study.

In the current study, testicular weight was significantly higher in the prepubertal, and adult control groups compared to the prepubertal, and adult EC exposed groups, respectively. Similarly, Saygın et al., (2023) reported decreased testicular weights and gonadosomatic index in adult rats exposed to EC vapor (PG, VG, nicotine 0.6 mg/ml nicotine as the liquid mixture without any flavoring agent) compared to controls [17].

This decrement of testicular weight in EC exposed animal models could be assumed to toxic effects of e-liquid ignition products; as formaldehyde and acrolein. Yang et al., (2017) reported marked reduction in fetal body and testicular weights of Sprague-Dawley rats' premature pups after exposure to major dosages of acrolein intraperitoneal injection (5 mg/kg) in late pregnancy due to decreased fetal testosterone

synthesis as well as abnormal expression of StAR and 3β-HSD [18].

In the current work, the EC smoking inhalation had deleterious effects on semen parameters of both prepubertal and adult rats. Sperms count and motility were significantly increased in the prepubertal, and adults control rats compared to the prepubertal, and adult EC exposed groups, respectively. The percentage of sperm morphological abnormalities was significantly increased in the prepubertal, and adult EC exposed groups compared to the prepubertal and adult control groups; respectively. This agreed with El-Golli et al., (2016) who found decreased sperm viability and count in adult male Wistar rats exposed to intraperitoneal injection of either e-liquid with nicotine (PG, VG, nicotine 0.5 mg/kg) and e-liquid without nicotine (PG, VG) for four weeks [19]. The harmful effects of EC vaping on the semen parameters could be explained by the endocrine disruption and oxidative stress induction effects of PG and VG products of ignition e.g., Formaldehyde [20, 21].

Interestingly in the current work, sperm motility and percentage of sperm morphological abnormalities were worse in prepubertal EC exposed group compared to adult EC exposed group. The fact that declared the higher susceptibility of the prepubertal rats to EC exposure toxic effects compared to adult rats. This could be explained by immature metabolic detoxification in prepubertal rats compared to adults that makes them more sensitive to toxin exposure versus adult animals [22].

In the present work, both adult and prepubertal EC exposed rats had abnormal testicular histological architecture in the form of degenerative alterations in the seminiferous tubule as well as germ cells vacuolation and detachment from the basement membrane. The current histopathological changes in EC exposed groups reflected poor semen quality in these groups. Moreover, present histological findings came in harmony and explained by the concurrent hormonal changes and testicular oxidative stress in EC exposed groups [17].

In line with present findings, Abdel-Kareem et al., (2022) demonstrated seminiferous tubules distortion as well as vacuolated detached spermatogenic cells with pyknotic nuclei in adult rats exposed to EC vapor (VG, PG, flavor and nicotine 18mg/ml) five successive days/week for 28 days [23].

The present histological findings of EC groups were comparable with testicular changes that occurred in response to the toxicities of the products of e-liquid ignition as formaldehyde and acrolein. Khalil et al., (2017) reported impaired spermatogenesis in adult rats orally exposed to formaldehyde (25 mg/kg/day) for 65 consecutive days [24].

Furthermore, in the current work adult EC exposed group showed mild focal hyperplasia of Leydig cells. Leydig cell hyperplasia (LCH) is an extremely uncommon but benign condition that is characterized by an increase in the number and size of Leydig cells of the testicles associated with high nucleoli count and smooth endoplasmic reticulum [25]. In accordance with our results, Gnanadeepam et al., (2018) illustrated defective spermatogenesis and focal tubular necrosis, focal hyperplasia of interstitial Leydig cells and Sertoli cell crowding were also observed in adult inhaled formaldehyde for either seven or twelve weeks [26]. In contrast, Kanwal et al., (2016) recorded significant decrease in Leydig cell count in mice exposed to nicotine intraperitoneally (1.5ml/kg) for 15 days [27].

In the present results, adult control rats had physiologically significantly increased testosterone level compared to prepubertal control rats. It can be explained by the LH surge that occurred at onset of puberty with subsequent increased testicular testosterone synthesis [28].

In the current study, hormonal disruption induced by EC exposure occurred in the form of significant decreased testosterone levels in prepubertal and adult EC exposed groups compared to the

prepubertal and adult control groups, respectively. In addition, the LH level was significantly increased in the prepubertal, and adult EC exposed group compared with the prepubertal and adult control groups respectively.

The present results came in agreement with; Abdel-Kareem et al., (2022) who revealed significant reduction of serum testosterone levels in adult rats exposed to EC vapor (VG, PG, flavor and nicotine 18mg/ml) compared to the controls after four weeks. They emphasized their results by recovery of serum testosterone levels to control levels in the withdrawal group after further four weeks of EC exposure cessation [23].

El-Golli et al., (2016) showed that intraperitoneal injection of e-liquid with or without nicotine led to reduction in testicular dehydrogenase enzyme which involved in the biosynthesis of active steroid and testosterone hormones activity [19].

Our results were in the same line with Zulkarnain et al., (2023) who recorded Leydig cells hyperplasia associated with impaired testosterone synthesis in adult rats exposed to nicotine contained EC vapor. The findings emphasized the decreased testosterone levels due to impaired testosterone synthesis process [29].

As regards oxidative stress markers in the current study, a significant reduction in SOD activity as antioxidant defense mechanism was observed in prepubertal and adult EC exposed groups compared to prepubertal and adult control groups, respectively. This was in line with a significant increase in the marker of lipoperoxidation MDA in prepubertal and adult EC exposed groups compared to prepubertal and adult control groups, respectively.

The current results were agreed with, Vivarelli et al., (2019) who found marked increased levels of testicular carbonyl protein and MDA in adult rats exposed to nicotine free EC vaping, in line with decline pattern with decreases that overcome 60% antioxidant enzyme activities (SOD, Catalase (CAT), and Glutathione Peroxidase (GPx)) [8]. Also, Abdel-Kareem et al., (2022), reported similar patterns of oxidative stress markers (increased MDA and decreased SOD) in adult rats exposed to nicotine EC vaping (e-liquid contained: VG, PG, flavor and nicotine 18mg/ml) compared to controls for four weeks. Studying the EC vaping withdrawal effect on testicular stress markers revealed reversibility of MDA to control levels, while SOD recovery was insignificant compared to controls [23].

Mohasseb et al., (2011) reported decreased SOD with subsequent increased MDA in testicular tissues of adult rats exposed to formaldehyde by inhalation at a concentration of 10 mg/m3 for 2 weeks [30]. Also, Mao et al., (2023) founded that acrolein showed to increased testicular ROS generation, protein oxidation, P38 activation and apoptosis [31].

Although, there was insignificant difference in SOD values between adult and prepubertal EC exposed groups in the current work. The current adult EC exposed group had a significantly increased MDA compared to prepubertal EC exposed group. This fact could be explained by the slower and incomplete recovery of the cellular antioxidant mechanisms in adult compared to prepubertal [23]. Oxidative stress is described as one of the major causes of infertility which occurred due to imbalance between ROS production and the antioxidant defense mechanisms. Oxidative stress is usually a preliminary step in the induction of several harmful pathways such as inflammation, autophagy and apoptosis [32].

In the present results, prepubertal and adult EC exposed groups showed significantly increased expression of connexin 43 compared to prepubertal and adult controls, respectively. There is proof that a variety of environmental pollutants, including smoking, can modify testicular connexin 43 through

induction of oxidative stress with subsequent alteration of cell membrane integrity and cellular junctions [4].

In consistent with our results, most reported toxins were associated with decreased expression of testicular connexin 43. For example, Dolati et al., (2020) reported decreased connexin 43 expression in testes of lead exposed rats compared to controls [33]. Additionally, Liang et al., (2024) found that glyphosate, herbicide, exposure was associated with down-regulation of Connexin 43 gene expression in rooster testes in line with enhanced autophagic destruction of Connexin 43 protein [34].

In the current study, the prepubertal and adult EC exposed rats had statistically lower percentage area of occludin immune staining compared to the prepubertal and adult control rats, respectively. The result which came in harmony with current impairment of spermatogenesis in EC exposed groups. Accordingly, loss of occludin expression leads to impairment of BTB permeability and so leads to the male infertility [5]. In addition, occludin knockout mice were noticed to be sterile, and their seminiferous tubules atrophied and lacked of germ cells [35].

Similar results were recorded in BTB and other tissues cellular junction of animal models exposed to products of EC ignition e.g., acrolein and formaldehyde. Chen et al., (2017) found downregulation and/or redistribution of three tight junction proteins in male C57BL/6J mice (aged ten weeks) orally exposed to acrolein (5 mg/kg Bw) [36]. Arican et al., (2009) recorded deceased expressions of cadherin-1, occludin, gamma-catenin proteins in intercellular junctional complexes of the nasal mucosa of female rats exposed to formaldehyde inhalation [37].

In our study and in the above-mentioned similar studies, the decreased occludin expression in association with induced oxidative stress could be explained by that hydrogen peroxide quickly increased the tyrosine kinase-dependent phosphorylation of adherens junction and tight junction proteins such β-catenin, ZO-1, occludin, and E-cadherin. Hence, this abnormal phosphorylation of BTB proteins results in their dissociation and lose of their interaction with the cytoskeleton [38].

CONCLUSION

The present study confirmed the reproductive toxic effect of nicotine free EC vapor on pre-pubertal and adult male Sprague Dawley rats as regard endocrine disruptive effects, semen abnormalities, testicular histological changes and testicular oxidative stress. The study also clarified the toxic effect of EC smoking on proteins of blood testes barrier particularly occludin and connexin 43.

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