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ORIGINAL ARTICLE

Impact of Electronic Cigarette Exposure on the Testes of Adult Male Albino Rat and the Role of Cessation: Histopathological and Biochemical study

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ABSTRACT

Background: Electronic cigarettes (E-cigs) are proposed as almost risk-free efforts to quit smoking despite limited data on their effects on human health especially reproductive function. The study aimed to detect impact of E-cigs on testes of adult male albino rats and the recovering value of its withdrawal. **Methods:** 30 rats were divided equally into 3 groups; Control (C) group was exposed only to room air, E-cigs-exposed (Ec) group rats were exposed to 1 ml/day of burnt E-cigs liquid for 5 consecutive days/week for 4 weeks, and withdrawal (W) group in which rats were exposed to 1 ml /day of burnt E-cigs liquid for 5 consecutive days for 4 weeks and then left without exposure for the following 4 weeks.
Results: E-cigs exposure caused reduction of serum testosterone level, testicular SOD, LDH, 17β-hydroxysteroid dehydrogenase, VEGF and antiapoptotic marker Bcl2 with increase of testicular MDA, TNF-α, proapoptotic marker Bax, and Bax/Bcl2 ratio. Moreover, there was a disturbance of the testicular histological architecture in the form of spermatogenic cells' vacuolation, pyknosis and karyolysis, few sperm bundle, thickened congested blood vessels, thickened basement membrane, and strong positive immunoreactivity for Caspase 3. Cessation of E-cigs exposure was accompanied by an improvement in biochemical and histological outcomes but they did not return totally to the normal values which reflected the alarming health hazard of vaping and the need for a longer duration to recover from their toxic insults, especially on the gonadal functions.
Conclusion: E-cigs cannot be considered a healthy alternative to classical tobacco smoking.
Keywords: electronic cigarettes, smoking, testis, toxicity, withdrawal.

INTRODUCTION

A rapid increase in the use of electronic cigarettes (E-cigs), as devices that provide nicotine in a vaporized mixture instead of smoking with tobacco burning, is seen as a relatively safe alternative to traditional cigs and even as lifestyle-choice consumables especially among the youth [1].The significantly lower concentrations of the known hazardous chemicals in E-cigs aerosol compared with tobacco smoke, e.g. polycyclic aromatic hydrocarbons;tobacco-specific nitrosamines; and volatile organic compounds, have led to the speculation that E-cigs cause substantially less hazard [2]. Despite the role of E-cigs in helping smoking cessation, most of the quitters did not stop using them after they quit smoking with no available accurate safety data for its prolonged use [3]. E-cigs are, in fact, hazardous to vapers as shown by several short-term randomized clinical studies [4, 5]. The exhaled aerosol contains propylene glycol, glycerol, nicotine, acetone, formaldehyde, acetaldehyde [6], propanol, in addition to considerable levels of toluene and xylene [7], and metals such as silver, iron, nickel, aluminium, silicate, lead, tin, and cadmium [8]. Flavors that are usually added to vapour liquid are significant sources of toxic aldehydes produced during the thermal decomposition of e-liquid constituents [9].

The health implications of chronic or lifelong E-cigs usage in vapers might need several years to identify. There is an increasing tendency of inducing these possible toxicities in experimental animals, particularly rodents, resulting in a balanced assessment of both the value and limitations of E-cigs. Animal studies have demonstrated that E-cigs are potentially harmful but still less severe than cigarette smoke [10]. However, these hazards involve many body systems including the pulmonary [11], cardiovascular [12], hepatic and metabolic [13], and central nervous systems [14]. E-cigs also harm experimental pregnant and lactating females and their developing embryos and neonatal offspring, respectively [15, 16]. The reported toxic effects of E-cigs include pro-inflammatory, pro-fibrotic, and oxidative stress markers rise, with DNA damage accompanied by DNA repair inhibition and antioxidant enzymes down regulation [17].

Despite reproductive toxicity of isolated components of E-cigs was individually investigated [18], only limited research in literature is available on mixtures of these compounds evaluating their male gonadotoxicity (impaired testicular function) and carcinogenesis [1, 6, 19]. These studies, however, lack a proper histopathological toxicity assessment that has been later assessed in a paucity of studies [20, 21]. The study aimed to evaluate both the toxic histopathological changes and the functional impairment of the testicular tissue due to exposure to a commercial mixture of nicotine-containing E-cigs refill liquid demonstrating the proposed underlying pathogenesis. In this study, we focus on the recovering role of E-cigs cessation, we hypothesized that withdrawal of E-cigs may improve these toxic effects in a trial to stop the increasing universal consumption of such a subtle poison as an alternative to a blatant one (conventional cigarettes).

METHODES

Chemicals and devices:

1- Electronic cigarette (E-cigs) liquid (Dollar blends company, Egypt): was obtained from local retail, 20 mL solution composed of: propylene glycol, vegetable glycerine, flavours and nicotine (18 mg/mL).

2- Portable Electric Incense Burner (Home electric comp., China**).**

Animals: This study was carried out on 30 adult male Wistar albino rats weighing 200-240 grams. They were obtained from Breeding Animal House of Faculty of Medicine, Zagazig University, left to acclimatize for 2 weeks before the experiment, and housed in separate well-ventilated cages with free access to standard diet and water ad libtium. Rats were randomly divided into 3 equal groups (10 rats each) as follows: control group (C), E-cigs exposed group (Ec), and the withdrawal group (**W**).

Experimental design: This study protocol received endorsement from the Institutional Animal Care and Use Committee (IACUC)-Zagazig University No. ZU-IACUC/3/ F/149/2021. Control group (C) was exposed only to room air while E-cigs exposed group (Ec) was exposed to about 1 ml burnt/day of E-cigs liquid for 5 consecutive days/ week for 4 weeks [1] They were put in an inhalation chamber consisted of a propylene box (38 × 26.5 × 19 cm) with a capacity of 19 liters. E-cigs vapour was puffed on one hole using a Portable Electric Incense Burner for one hour/day and the other was left for aeration. Rats were exposed using a whole-body mode (two rats/ chamber). Then they were moved to a completely sterile room. In withdrawal group (W), rats were exposed to 1 ml burnt /day of E-cigs liquid by the previously described technique for 5 consecutive days/ week for 4 weeks and then left without exposure for the following 4 weeks.

Serum testosterone evaluation:

Just before sacrification, the rats were anesthetized by intraperitoneal injection of thiopental 50 mg/kg [22], blood was drained from the orbital sinus by placing a microhematocrit blood tube into the corner of the eye socket beneath the eyeball until blood revokes from the retro-orbital veins [23, 24]. Samples were left to clot, centrifuged for 15 minutes at 2500 RPM and serum samples were collected for assessment of testosterone level by rat testosterone ELISA kit (BioVendor Research and Diagnostic Products, Czechia).

Homogenate tissue analysis:

Preparation of testicular homogenate:

Rats' testes were isolated and freed from any adherent fatty tissues. The testes were split into two parts: one for histopathological assessment preserved in 10% neutral buffered formalin, and another one for total RNA extraction washed with ice-cold physiological saline.

For testicular homogenate preparation, all tissues were maintained at 4 ºC throughout the preparation. Testicular tissues were homogenized with 10 ml cold buffer (i.e. 100 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA) per gram tissue using Potter-Elvehjem type glass-Teflon homogenizer as the previously described technique [25]. The homogenates were

centrifuged (15 minutes at 5000 RPM) then the clear upper supernatants were collected and stored at -80°C for later analyses.

Oxidative stress and tissue damage markers

In the testicular homogenate, the oxidative stress marker malondialdehyde (MDA) and the antioxidant enzyme superoxide dismutase (SOD) levels were measured using ELISA specific experimental kits purchased from (MyBioSource, Inc. San Diego) [26], and testicular lactate dehydrogenase (LDH) level was assessed by spectrophotometry as the change in absorbance at 340 nm for 1 min (LDH-LQ, SPINREACT, S.A./S.A.U.Ctra. Santa Coloma, SPAIN) [27].

Gene expression studies by real-time PCR (qPCR)

Total RNA was extracted from rat testis using Trizol reagent (Thermo Fisher Scientific; Waltham, MA, United States) followed by a two-step real-time PCR to assess gene expression as explained previously [28]. We used a Hi Sen Script™ RH (-) cDNA Synthesis Kit (iNtRON Biotechnology Co., South Korea) for cDNA synthesis and qPCR involved mixture 5X HOT FIRE Pol Eva Green qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and specific primers (Table 1).The primer was checked with NCBI primer blast [29].

The reaction was carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) with cycling conditions comprising initial denaturation at 95°C for 12 min, followed by additional 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60 °C for 60 seconds, and then extension at 72 °C for 60 sec. The relative expression level of the tested genes was normalized to that of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the proportional fold changes in gene expression were calculated based on the $2^{-\Delta\Delta}$CT comparative method [30].

Gene transcript (mRNA) quantification by SYBR Green RT-PCR assays was performed for evaluation of testicular steroidogenesis key enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD), the microcirculation and spermatogenesis factor; vascular endothelial growth factor (VEGF), the pro-inflammatory cytokine; tumor necrosis factor-α (TNF-α), the pro-apoptotic marker; Bcl2-associated X protein (BAX), and the antiapoptotic marker; B-cell lymphoma 2 (Bcl2).

Histological and morphometrical analysis:

1. Hematoxylin and Eosin (H&E) staining:

The testicular tissue samples were fixated in 10% formalin, rinsed with tap water, dehydrated in a graduated ethanol solution (70–100%), and immersed in paraffin (Merck Millipore, Darmstadt, Germany). Serial 4-5 µm sections were cut and stained with H&E [31].

2. Periodic acid–Schiff (PAS) staining:

Prepared sections of 4-5 µm were deparaffinized and brought down to water. They were oxidized for 5 minutes with 1 percent periodic acid and then it is rinsed in distilled water after being washed in running water for 5 minutes. After 15 minutes in Schiff's reagent, the sections were rinsed under running tap water for 10 minutes. After that, the sections were dehydrated in progressively higher concentrations of alcohol, cleaned in xylol, and mounted in Canada balsam. PAS-positive substances appeared as magenta-red [32].

3. Caspase 3 Immunohistochemical examination:4-5µm thick testicular sections were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval. After blocking endogenous peroxidase activity with 3% (v/v) hydrogen peroxide for 10 mins, samples were incubated for 2 hours with a primary rabbit-polyclonal Caspase-3 antibody (1:500 concentration) (Cat. No. PAI29157, Thermo Fisher Scientific Co., USA). After that, they were washed in phosphate-buffered saline and treated for 10 min. at room temperature with biotinylated goat antipolyvalent. After 15 min. of stained with chromogen positive substrate, the slides were titrated for 1 min. with Mayer's hematoxylin, washed with tap water, then dehydrated [33]. All slides were examined by the light electric microscope (LEICA ICC50 W), Human Anatomy and Embryology Department, Faculty of Medicine, Zagazig University.

Quantitative morphometrical analysis: ImageJ software was used for measuring seminiferous tubular diameter and tubular epithelial height in H&E sections [34]. And, for Caspase 3 immunoreactivity, the apoptotic index was assessed: apoptotic (brown) cells expressed as a fraction of the total number of cells in a section [35]. 5 slides were examined/group, with 5 non overlapped different fields /slide (a total number of 25 fields X 400/group).

Statistical analysis: The results were presented as a mean ± standard deviation (SD). The Statistical Package for Social Science version 27 was used to analyze the obtained data. One-way analysis of variance (ANOVA) was used to measure variation between groups, and the Tukey post hoc test was used to make comparisons. The p-values of <0.05 were considered statistically significant and p-values of <0.001 were considered highly

significant, while p-values of >0.05 were statistically non-significant.

RESULTS

Serum testosterone: The present study revealed a highly significant reduction of serum testosterone level in rats exposed to E-cigs (Ec) compared with the C group. After 4 weeks of exposure cessation, the W group showed a significantly higher level of serum testosterone than the Ec group, however, it was still significantly lower than the control levels (Table 2, Figure 1a).

Oxidative stress and tissue damage markers: E-cigs exposure significantly showed highly significant rise of lipid peroxidation product MDA and decrease in the antioxidant enzyme SOD in Ec group as compared with C group. Despite the significant decrease of MDA level in W group in comparison to the Ec, the increase in testicular SOD after vapour cessation (W versus Ec groups) was insignificant. The improvement of oxidative stress markers (MDA and SOD) in the W group was still significantly different from the normal values of the C group (table 2, Figure 1b&c). The testicular concentration of the LDH showed a highly significant reduction with E-cigs exposure compared with both C and W groups, as well, LDH rise in the W group is significantly lower than that of C group (P<0.001) (Table 2, Figure 1d).

Gene expression studies by qPCR: The testicular gene transcript of the steroidogenesis key enzyme 17β-HSD showed a highly significant reduction in the Ec group compared with those of the C and the W groups, and despite its significant rise in W, it was still significantly lower than the control values. There was a highly significant decline of the microcirculation and the spermatogenesis factor, VEGF, in both Ec and W groups in comparison with the C group, however, it showed a significantly higher gene expression in W group than Ec group (Table 3, Figure 2a&b). Our results also demonstrated a highly significant increase in the inflammatory mediator TNF-α in Ec rats versus the C rats. TNF-α was significantly lower in the W group compared with the Ec but significantly higher than the C group values (Table 3, Figure 2c).A highly significant difference was observed in the gene expression of the proapoptotic marker Bax among the studied groups (P < 0.001), being highest in the Ec group and lowest in the C group with a marked reduction in the W group, however, it is still incomparable with the control levels. The antiapoptotic marker Bcl2 showed a significant reduction on exposure to E-cigs (Ec group) compared with the C group. The amount of transcript was significantly increased on cessation of E-cigs exposure (W) to reach comparable levels to those found in the C group. The Bax/Bcl2 ratio was found the highest in testicular tissue of Ec rats when compared with C and W rats' testes. This ratio improved in the W rats but remained significantly higher than that in the C rats (P <0.001) (Table 3, Figure 2 d,e,f).

H&E histological: H&E stained sections of the control group **(C)** showed normal testicular histology; consisting of rounded or oval seminiferous tubules filled with sperm bundles and separated by interstitial spaces (Figure 3: a1). Seminiferous tubules showed normal spermatogenic series and Sertoli cells in between. They were lined with thin basement membranes which consisted of flat myoid cells. Spermatogenic series was found in many layers, namely; spermatogonia with rounded nuclei along with pyramidal elongated Sertoli cells with oval nuclei in between. Towards the tubular lumen, spermatocytes appeared as rounded cells with dark nuclei and coarse chromatin granules and then spermatids appeared by their rounded and elongated types. Sperm bundles filled the tubular lumen. Narrow interstitial spaces showed a moderate amount of loose connective tissue containing thin walled blood vessels and the interstitial cells of Leydig (Figure 3: a2). H&E stained sections of E-cigs exposed group **(Ec)** showed some pathological features; the seminiferous tubules were distorted and relatively small in diameter with few sperm bundles in their lumen and wide interstitial spaces containing acidophilic material (Figure 3: b1). Also, there were thickened congested blood vessels (Figure 3: b1&b2). Moreover, the lining tubular basement membrane was thick. Spermatogenic cells showed different pathological pattern in the form of; vacuolation, separation and detachment from the basement membrane, pyknotic dark stained nuclei and karyolysis (Figure 3: b2).H&E stained sections of withdrawal group **(W)** showed some degree of testicular improvement. The tubules were relatively larger than Ec group, some regained normal arrangement of spermatogenic cells and others showed some degenerative changes. Spermatogenesis increased relative to Ec group, some tubular lumens contained sperm bundles while others did not (Figure 3: c1). Interstitial spaces between tubules showed neither acidophilic material nor congested blood vessels (Figure3: c1&c2). Some spermatogenic cells were normally arranged while others were still disarranged and showed vacuolation (Figure 3: c2).

The morphometric analysis of seminiferous tubular diameter revealed a high statistical significant difference between the studied groups (Table 4& Figure 3: d1). In terms of tubular epithelial height, there was a high statistical significant difference between different groups (Table 4& Figure 3: d2). **Periodic acid–Schiff histological:** C group showed PAS reaction in the thin regular basement membrane of the seminiferous tubules, abundant sperm bundles in tubular lumen and the Leydig cells in narrow interstitial spaces (Figure 4: a). While Ec group showed intense PAS reaction in the corrugated thickened basement membrane and the Leydig cells in the wide interstitial spaces with absence of sperm bundles in tubular lumen (Figure 4: b). In W group, there was PAS reaction in the relatively thin basement membrane of the seminiferous tubules and sperm bundles in tubular lumen (Figure 4: c). **Caspase 3 immunoreactivity:** In control group, there was a negative immunoreactivity for Caspase 3 in the germinal epithelium (Figure 5: a). While in Ec group, there was a strong positive immunoreactivity for Caspase 3 in the germinal epithelium (Figure 5: b). Moreover, W group showed weak immunoreactivity for Caspase 3 (Figure 5: c).

The apoptotic index of Caspase 3 immunostaining revealed a statistical significant difference between different groups. There was a high significant difference between control and Ec group also between Ec group and W group and significant difference between control and W group (Table 4& Figure 5: d).

Table (1): Primers Sequences used for real-time PCR:

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession No	Product size
GAPDH	GGCACAGTCAAGGCTGAGAAT-G	ATGGTGGTGAAGACGCCAGTA	NM_017008.4	143
17β-HSD	AGTGTGTGAGGTTCTCCCG-GTACCT	TACAACATTGAGTCCATGT-CTGGCCAG	NM_054007.1	161
VEGF	CCCAGCCACCAGAAGAAAGT	GGCACGAGTTGTTTGACCAC	NM_053549.1	125
TNF-α	AGGGTCTGGGCCATAGAAC	CCACCACGCTCTTCTGTCTAC	NM_012675.3	103
BAX	CGAATTGGCGATGAACTGGA	CAAACATGTCAGCTGCCACAC	NM_017059.2	109
BCL2	GACTGAGTACCTGAACC-GGCATC	CTGAGCAGCGTCTTCAGAGACA	NM_016993.1	135

Table (2): Comparison of mean different studied testicular markers among the studied groups:

Group	Control ($\bar{x}$ ± SD)	E-cigs ($\bar{x}$ ± SD)	Withdrawal ($\bar{x}$ ± SD)	F	P	Tukey post hoc test
Testosterone: (ng/ml)	6±1.1	2.32±0.56	3.07±0.70	**56.8**	**<0.001****	**<0.001**^{1} <0.001**^{2} 0.02*^{3}**
MDA (nmol/mg)	1.13±0.20	7.49±0.37	4.65±1.68	**101.54**	**<0.001****	**<0.001**^{1} <0.001**^{2} <0.001**^{3}**
SOD (U/mg)	342.3±6.82	102.3±25.7	130±36.89	**250.06**	**<0.001****	**<0.001**^{1} <0.001**^{2} 0.06 NS^3**
LDH (U/L)	247±5.08	63.15±1.77	103.61±24.45	**446.78**	**<0.001****	**<0.001**^{1} <0.001**^{2} <0.001**^{3}**

Sd: Standard deviation F: ANOVA test
NS: Non-significant (P>0.05) *: Significant (P<0.05) **: highly significant (P<0.001)
P1: Control versus E-cigs, P2: Control versus withdrawal, P3: E-cigs versus withdrawal

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Table (3): Comparison of the mean values of gene expression of functional, inflammatory, and apoptotic parameters among the studied groups:

Group	Control (C) ($\bar{x}$ ± SD)	E-cigs (Ec) ($\bar{x}$ ± SD)	Withdrawal (W) ($\bar{x}$ ± SD)	F	P	Tukey post hoc test
17β-HSD	4.79±1.06	1.00±0.16	1.61±0.53	**86.88**	**<0.001****	**<0.001**[1] **<0.001**[2] **0.003***[3]
VEGF	64.52±13	10.51±0.26	41.23±9.62	**85.66**	**<0.001****	**<0.001**[1] **<0.001**[2] **<0.001**[3]
TNF-α	0.57±0.19	1.04±0.20	0.79±0.23	**11.59**	**<0.001****	**<0.001**[1] **0.04***[2] **0.02***[3]
BAX	1.03±0.16	13.72±3.42	6.61±2.53	**66.97**	**<0.001****	**<0.001**[1] **<0.001**[2] **<0.001**[3]
BCL2	1.09±0.35	0.64±0.13	1.01±0.33	**6.96**	**0.004***	**0.004***[1] 0.61 NS[2] **0.02***[3]
BAX/BCL2 Median (IQR)	0.99±0.17 1.02 (0.81-1.21)	17.01±6.83 18.38 (9.78-25.28)	11.20±9.96 5.46 (3.32-22.16)	**NO** **20.47**	**<0.001****	**<0.001**[1] **<0.001**[2] **<0.001**[3]

Sd: Standard deviation F: ANOVA test
NS: Non-significant (P>0.05) *: Significant (P<0.05) **: highly significant (P<0.001)
P1: Control versus E-cigs, P2: Control versus withdrawal, P3: E-cigs versus withdrawal

Table (4): **Morphometric analysis for H&E and immune-histological examination in different study groups:**

Group	Control ($\bar{x}$ ± SD)	E-cigs ($\bar{x}$ ± SD)	Withdrawal ($\bar{x}$ ± SD)	F	P	Tukey post hoc test
Seminiferous tubular diameter	375.5±78.7	140.6±25.2	243.1±46.1	**46.47**	**<0.001***	**<0.001**[1] **<0.001**[2] **<0.001**[3]
Epithelial height of seminiferous tubules	67.3±8.4	27.11±4.6	49.69±1.3	**130.35**	**<0.001****	**<0.001**[1] **<0.001**[2] **<0.001**[3]
Apoptotic index of Caspase 3 immunostaining	11.23±2.6	55.47±10.2	26.9±7.6	**89.5**	**<0.001****	**<0.001**[1] **0.002***[2] **<0.001**[3]

Sd: Standard deviation F: ANOVA test
NS: Non-significant (P>0.05) *: Significant (P<0.05) **: highly significant (P<0.001)
P1: Control versus E-cigs, P2: Control versus withdrawal, P3: E-cigs versus withdrawal

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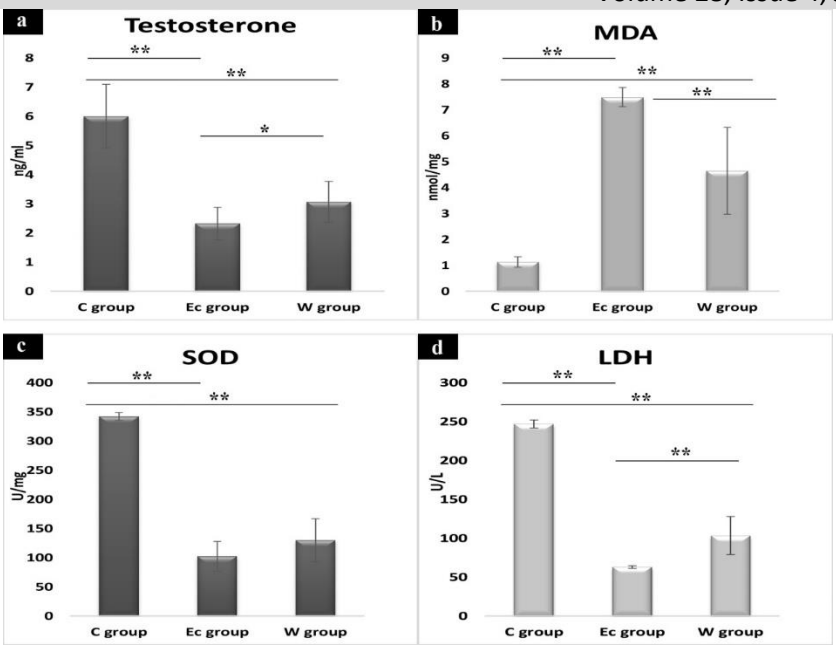

Figure (1): a. serum testosterone, **b**. testicular MDA, **c**. testicular SOD, and **d**. testicular LDH levels among different groups: Control (C), E-cigs (Ec), and withdrawal (W). *: significant difference, **: highly significant difference.

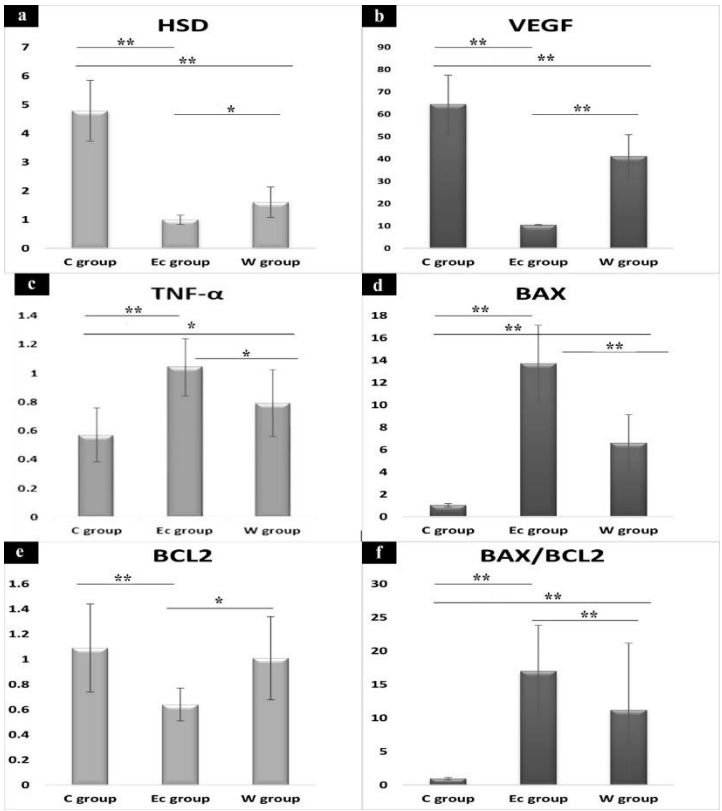

Figure (2): Comparison of the mean values of gene expression of functional (17β-HSD, VEGF), inflammatory (TNF-α), and apoptotic parameters (Bax, Bcl2 &Bax/Bcl2 ratio) among the studied groups. Control (C), E-cigs (Ec), and withdrawal (W). *: significant difference, **: highly significant difference.

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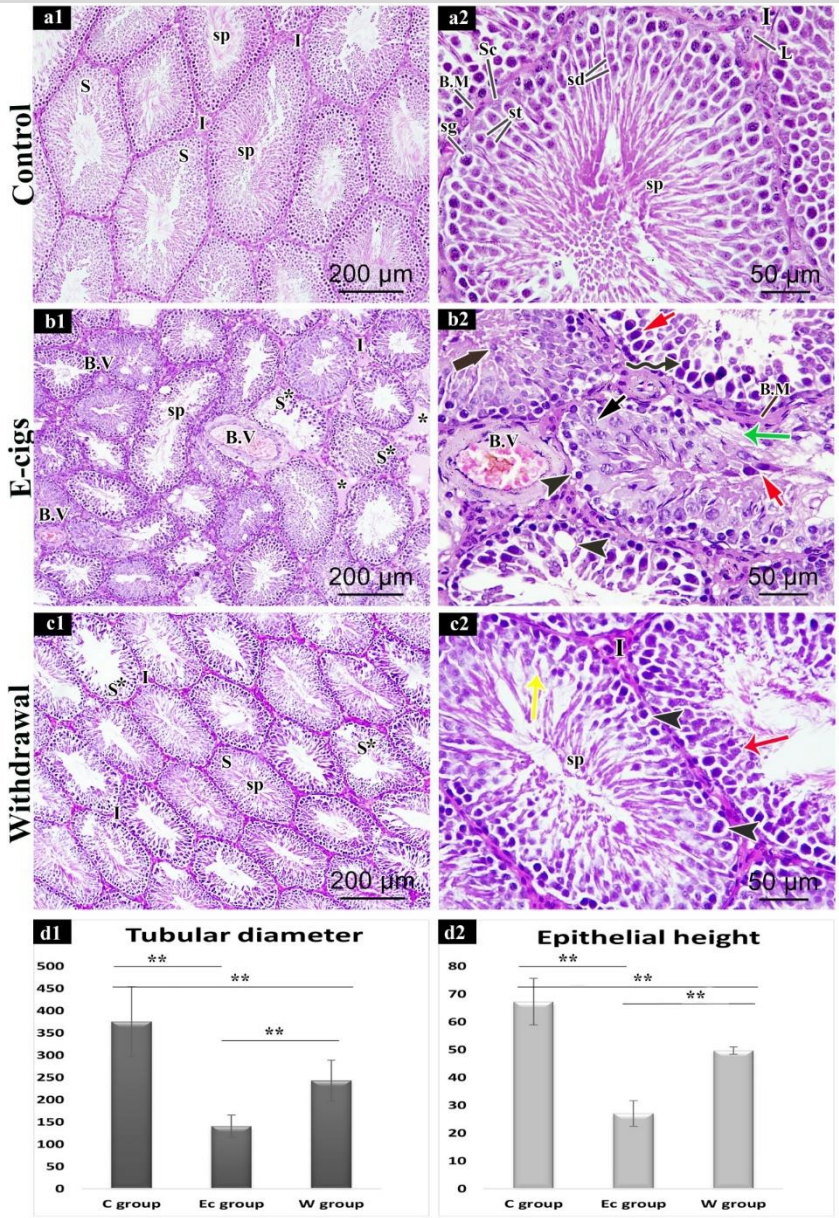

Figure (3): H&E stained sections oftesticular tissues in different experimental groups; **control group** (**a1 X 100**): consists of seminiferous tubules (s) filled with sperm bundles (sp) and separated by interstitial spaces (I). (**a2 X 400**): showing normal seminiferous tubules lined with thin basement membranes (BM).Spermatogenic series is found in many layers, namely; spermatogonia (sg) and Sertoli cells (SC) in between them, spermatocytes (st) then spermatids (sd) and sperm bundles (sp) are filling the tubular lumen. Narrow interstitial spaces (I) containing the interstitial cells of Leydig (L). **E-cigs exposed group**; (**b1 X 100**): showing distorted small seminiferous tubules (S*) with few sperm bundles (sp) in their lumen and wide interstitial spaces (I) containing acidophilic material (*). **(b2 X 400):** showing thick tubular basement membrane (B.M). Spermatogenic cells showing vacuolation (arrow heads), separation and detachment from the basement membrane (green arrow), pyknotic dark stained nuclei (zigzag arrow), karyolysis (black short arrow) and exfoliation (thick arrow). There are many giant cells (red short arrows). Also, there are thickened congested blood vessels (B.V) (b1&b2).**Withdrawal group** (**c1*100**): showing relatively larger tubules, some showing degenerative changes (S*) and others, regaining normal arrangement of spermatogenic cells (S) and containing sperm bundles (sp). (**c2 X 400**): showing normally arranged spermatogenic cells (red arrow) while, others still disarranged (yellow arrow) and showing vacuolation (arrow heads). Normal Interstitial spaces (I) between tubules are present (c1&c2).
d1&d2: Morphometrical comparison of seminiferous tubular diameter and epithelial height among different groups Control (C), E-cigs (Ec), and withdrawal (W). *: significant difference, **: highly significant difference.

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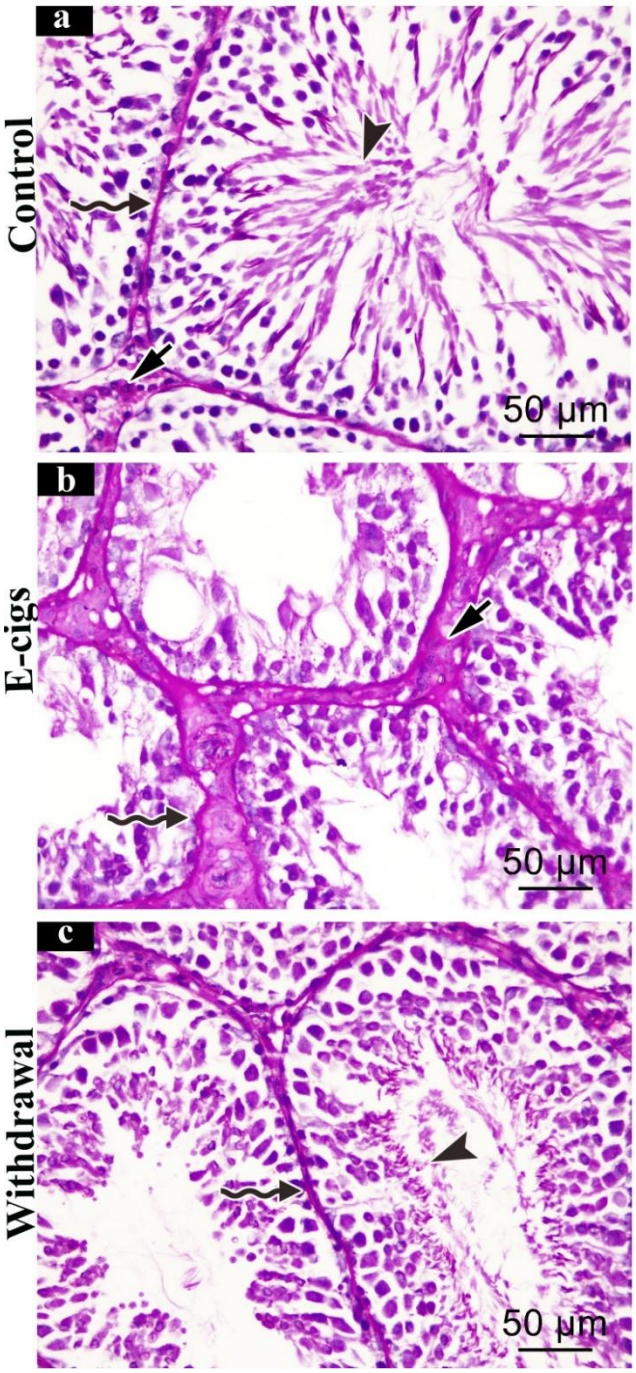

Figure (4): PAS reaction among different groups (**X** 400); basement membrane of the seminiferous tubules (zigzag arrow), sperm bundles in tubular lumen (arrow head) and the Leydig cells in interstitial spaces (short arrow). **a**: control group; **b**: E-cigs group; **c**: withdrawal (W) group.

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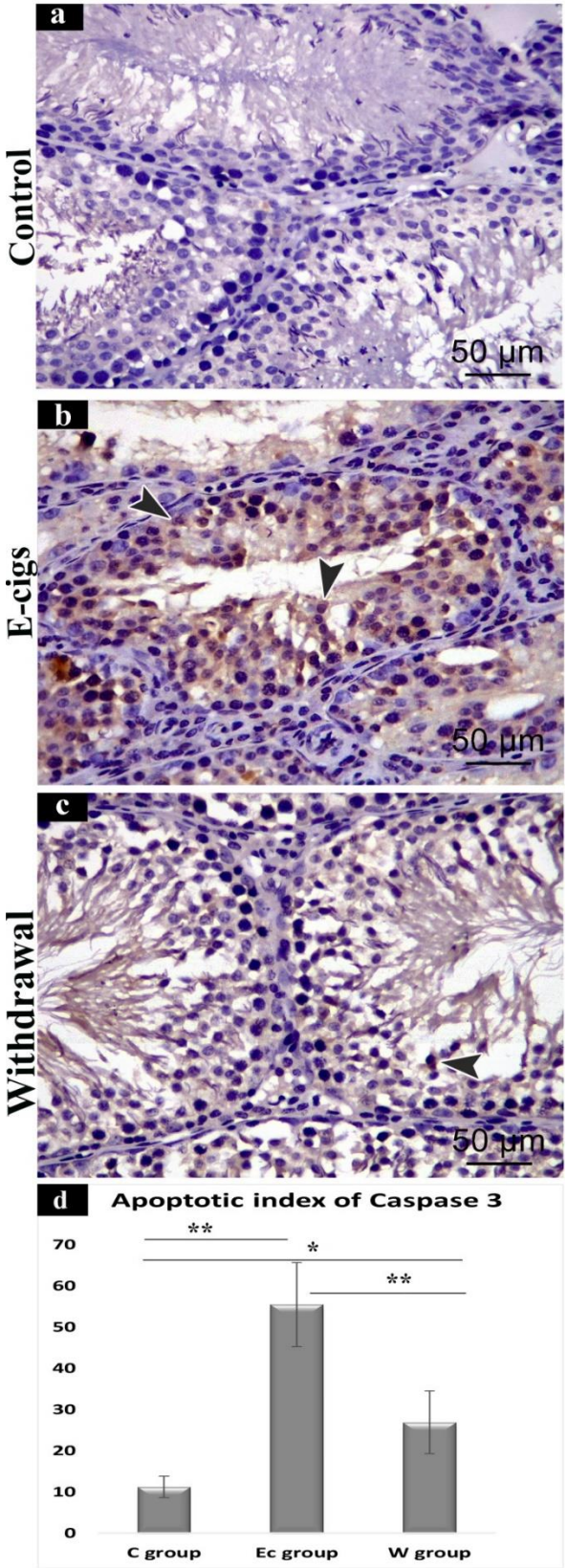

Figure (5): Immunoreactivity for Caspas 3 in testicular tissue (**X**400): positive immunoreactivity taking brown color (arrow head).**a**: control group; **b**: E-cigs group; **c**: withdrawal (W) group **d**: Morphometrical comparison of the apoptotic index of Caspas 3 immunostaning. Control (C), E-cigs (Ec), and withdrawal (W).*: significant difference, **: highly significant difference.

DISCUSSION

One of the growing concerns is the toxic effect of E-cigs refill liquids and/or their vapours on the reproductive system. Rodent exposure studies for human extrapolation (animal models) have been essential to provide plenty of information for E-cigs toxicological inhalation testing, especially for long-term exposure [36]. The designed model is intended to demonstrate toxic effects of E-cigs liquid cocktail vapour exposure, not to reproduce human vaping conditions [1].

The detected decrease in serum testosterone secondary to E-cigs refill liquid exposure in our study was reported also by El Golli et al. [19]. Nicotine had significant inhibitory effects on male reproductive hormones [37]. Consistent with our results, withdrawal improvement of serum testosterone was reported by Oyeyipo et al. [38] following one month of cessation. However, in a clinical study, serum samples showed higher testosterone levels in those who succeeded in smoking cessation for one year, but this rise was not statistically significant [39]. This is can be explained by several theories like higher levels of sex hormone-binding globulin in smokers [40], disrupted testicular cellular content, i.e. decreased Leydig cell, secreting testosterone, number and function [41], and impaired steroidogenesis as we found in the current study and as reported before [6, 19].

The distorted testicular redox homeostasis due to E-cigs exposure shown as elevated lipid peroxidation product MDA levels and decreased antioxidant enzyme SOD as described before by El Golli [19]. There is strong evidence of the association between E-cig exposure and oxidative stress induction in different tissues as well [42]. It was recognized that oxidative stress is a leading cause of testicular failure among smokers [43], and this can be attributed to inhibition of a major regulator of steroidogenesis; Steroid Acute Regulatory (StAR) protein, or the release of reactive oxygen species as a liquid thermal degradation by-product [44], or the activation of multiple intracellular apoptosis signalling by the oxidative stress [45]. It's noticeable, however, that despite significantly decreased MDA in the W group, and SOD didn't significantly increase after one-month cessation reflecting the slower and incomplete recovery of the cellular antioxidant mechanisms.

A decrease in testicular LDH due to exposure to E-liquid, as obtained in the current study, was reported by El Golli et al. [19]. This was explained by LDH adjacency to the plasma membrane thus any slight disruption to the cellular membrane will be accompanied by its release from the cell interior to the intercellular spaces [46]. This is in turn was associated with an increase in serum LDH activity due to this leakage [47]. The contrary increase in the activity of testicular LDH on E-cigs exposure observed in other studies [1, 6] may be a compensatory mechanism to distortion of the germinal epithelium [48].

We have proposed an impairment of 17β-HSD, as a key enzyme in the steroidogenesis pathway, on exposing rats to E-cigs which is consistent with previous studies [6, 19]. This was reported also due to nicotine exposure [49]. Inhibition of steroidogenic proteins impairs testosterone production and consequently other reproductive functions [50]. This is one explanation of the recorded decreased serum testosterone in Ec rats. Additionally, the significant rise in testicular 17β-HSD in W group explains the corresponding elevation in serum testosterone in the same group of rats. However, these improvements didn't reach the normal control levels after 4 weeks of abstinence

It was recognized long ago that vascular endothelial growth factor (VEGF) is not only a paracrine regulator in testis maintaining the vascular structure and the permeability of testicular microcirculation, but also stimulating the germ cell proliferation, meiosis, differentiation, and regulating undifferentiated spermatogonia reserve [51]. Moreover, VEGF can inhibit testicular cell apoptosis [52]. Del Vento and his collaborators [53] have recently shown that VEGF increased testicular vascular maturity both structurally and functionally. We reported a significant decrease in testicular VEGF in the Ec group even after E-cigs cessation in W group. Several studies reported that VEGF is a very sensitive marker that significantly decreases in response to different injuries such as testicular ischemia-reperfusion injury [54]

Our results reflected a significant increase in testicular inflammatory mediator TNF-α in response to E-cigs exposure denoting the involvement of the inflammatory mechanism in E-cigs-induced testicular damage. TNF-α is a major inflammatory and immune response cytokine [55]. This rise in TNF-α didn't show a substantial decline to reach the normal control values in W group.

The current study showed a significant rise of the proapoptotic Bax in Ec rats, and despite a significant decline in W rats, it didn't reach the C rats' values. On the other hand, diminished expression of the anti-apoptotic protein Bcl2

along with impaired testicular function and disrupted structure in Ec rats is in line with the results shown when nicotine was given intraperitoneally [56]. Interestingly, Bcl2 in W group rats restored a comparable value with that of the control. There were significant correlations between Bcl2 and Bax gene expression, and spermatogenic cells apoptosis in passive-smoking rats [57].

Bax liberates cytochrome c from the mitochondria, which triggers the cell death series of events. Bcl2 blocks this action and prevents the consequent stimulation of the apoptotic mechanism. Thus, the ratio of the death agonist (Bax) to the antagonist (Bcl2) is important in determining the cell response to an apoptotic signal [58]. Our results showed an increased Bax/Bcl2 ratio in the Ec group which is in favour of activated cellular apoptosis, and this ratio, unfortunately, was still significantly higher in the W group than that in the C group denoting incomplete recovery from the induced apoptotic status. Our results are in agreement with several studies that reported the increased Bax/Bcl2 ratio as an index of induced testicular apoptosis in response to cadmium exposure [59, 60], one of the serious toxic ingredients of tobacco products; including E-cigs [61].

In the current study, seminiferous tubules of Ec rats demonstrated disruption of their cellular contents and peeling of germ cells with the development of cell fragments; same findings were also recorded by Mosbah et al. [62], and El Golli et al. [63], both explained that buildup of sloughed spermatogenic cells could be owing to Sertoli cells' failure to ingest desquamated germ cells in this context, our H&E results confirmed the foregoing by revealing giant cells in the seminiferous tubules of E-cigs exposed testes. These giant cells were also observed in an experiment conducted by Shojaeepour et al. [64] that investigate the effects of Cadmium (Cd) on rat testes.

In this study, the testes of Ec rats revealed numerous forms of tubular degeneration, including spermatogenic cell vacuolation, acidophilic material deposition between tubules, peritubular hemorrhage, and clogged blood arteries. All of these findings were improved to some extent 4 weeks after cessation of exposure, this was in harmony with Wawryk-Gawda et al. [65] who discovered similar pathological abnormalities in the testes and epididymis of rats exposed to E-cigs as well as traditional cigarettes. Meanwhile, as reported by the previous authors, these lesions improved 2 weeks after termination of exposure. However, Nicotine causes the peritubular extracellular matrix to disorganize. Because peritubular structures have been shown to play a role in the control of Sertoli cell morphology and function, abnormal changes to them may disrupt spermatogenesis and conception [66]. Also, Nesseim et al. [67] found these lesions in rats exposed to nicotine with different concentrations and recorded the nicotine amount and exposure period were proportionate to these alterations with the recovery of the injured seminiferous tubules after nicotine abstinence. In addition, Coşkun et al. [68] found vacuolation in tubular cells of mice treated with nicotine for 14-28 days.

Our H&E results in E-cigs exposed rats showed pyknotic dark-stained nuclei and karyolysis. Rahali et al. [69] said that E-liquid without nicotine may disrupt spermatogenesis or enhance sperm death rates by widespread apoptosis. Nicotine and its principal metabolite, cotinine, are also thought to be strong spermatogenesis inhibitors.

In the current study, Ec group showed intense PAS reaction in the corrugated thickened tubular basement membrane. This was explained by Aydos et al. [66] as the tubular basement membrane is considered a biological barrier for poisonous compounds; its thickening was to prevent hazardous substances from passing into the seminiferous tubular lumen. Moreover, nicotine was found to generate collagen fibrils and change myoid cell contractility.

In the current study, Ec-induced apoptosis was confirmed histologically by Caspase 3 labeling. The apoptotic index of Caspase 3 immunostaining was increased significantly in rats exposed to E-cigs more than in both other groups. This was consistent with Lopes [70], who suggested that nicotine exposure has a strong influence on sperm apoptosis and DNA stability, and explained it as a result of the spermatozoa's presence of nicotinic acetylcholine receptors. According to Mohammadi et al. [71], nicotine stimulates various intracellular death-related pathways by increasing the production of the activated form of Caspase 3 and the activity of Caspase 3 enzyme.

In this context, Mosadegh et al. [56] subjected rats to intraperitoneal nicotine and discovered high rates of P53 and Caspase 3 in seminiferous tubules, as well as downregulation of the anti-apoptotic protein Bcl2, demonstrating that nicotine may have anti- or pro-apoptotic effects on cells. However, according to Jalili et al. [72] nicotine stimulates apoptosis in the heart and

lungs but inhibits apoptosis in the kidneys and liver.

In the current study, compared to both control and withdrawal groups, E-cigs exposure produced a decrease in mean tubular diameter and epithelial height. This was in line with the findings of Mohamed and Abdelrahman [73], who found a substantial reduction in the diameter and surface area of the seminiferous tubule in the nicotine-treated group compared to the control group. On the other hand, En et al. [74] exposed rats to different concentrations of nicotine (0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, and 4.0 mg/kg) and stated that when compared to the control, the mean diameter of seminiferous tubules that got a nicotine treatment of 4.0 mg/kg exhibited a substantial decrease while treatment groups such as 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg demonstrated a negligible decrease. Based on certain investigations, the death of germinal cells causes the diameter of seminiferous tubules to decrease, resulting in a decrease in the epithelial thickness of seminiferous tubules. Moreover, apoptosis may cause a decline in the amount of seminiferous tubular cells, resulting in a decrease in both diameter and epithelial thickness.

Conclusion: E-cigs had deleterious effects on human reproductive health, with the need for a relatively long duration to restore the normal reproductive function after cessation.

As a limitation of the study, it didn't include direct sperm analysis as part of testing the testicular impairment, as we found the relatively unique and extensive histopathological evaluation is more informative and representative of the direct germ cell toxicological insult.

Abbreviations: E-cigs; electronic cigarettes, C; Control group, Ec; E-cigs-exposed group, W; Withdrawal, IACUC; Institutional Animal Care and Use Committee, MDA ;Malondialdehyde, SOD; Superoxide dismutase, LDH; lactate dehydrogenase, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, 17β-HSD; 17β-hydroxysteroid dehydrogenase, S VEGF; Vascular endothelial growth factor, TNF-α; Tumor necrosis factor-α, Bcl2; B-cell lymphoma 2, BAX; Bcl2-associated X protein, H&E; Hematoxylin and Eosin, PAS; Periodic acid–Schiff, SD; Standard deviation, ANOVA; One-way analysis of variance, StAR; Steroid Acute Regulatory, VEGF; Vascular endothelial growth factor.

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