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## ORIGINAL ARTICLE

# Comparison between the Effect of Nicotine Smoking in Tobacco and Electronic Cigarettes on Urinary Bladder in Adult Male Albino Rats: Biochemical and Histopathological Study

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### ABSTRACT

**Background:** Electronic cigarette (E-cig) is a device that instead of burning tobacco leaves evaporates a nicotine solution mixed with liquid tastes. E-cig has been readily available since its introduction in 2004, and its usage has expanded tremendously over the world. This work aimed to compare between the electronic cigarette consumption and tobacco cigarette smoking on urinary bladder, as regard the histopathological effect, oxidative, inflammatory, and apoptotic markers through biochemical and immunohistochemical methods. Forty rats were divided to 4 groups (fresh-air, E-Liquid, E-cigarette, and T-cigarette), after 8 weeks of exposure, rats were weighted and the urinary bladder of all rats were obtained for histological and biochemical analysis.

**Results:** both tobacco and electronic cigarette smoking resulted in a reduction in body weight with increase in urinary bladder weight, an increase in inflammatory marker activity (TNF- $\alpha$ , IL-6, and macrophage inflammatory protein levels), and an oxidant/antioxidant imbalance in rats (increase in ROS level, plus decrease in total antioxidant activity). Furthermore, cigarette smoking caused histological structural injury to the urinary bladder, with overexpression of Ki-67 and poor expression of E-cadherin, all results were more pronounced in tobacco cigarette smoking.

**Conclusions:** According to this study, even if electronic cigarettes have lesser pathological and biochemical effects on the urinary bladder than tobacco cigarettes, they still cause urinary bladder injury, hence it is strongly advised to avoid any devices that contain nicotine.

**Keywords :** Nicotine, electronic cigarette, tobacco, Ki-67, E-cadherin



### INTRODUCTION

Electronic cigarette (E-cig) is an electronic device that was developed by the Chinese pharmacist, Hon Lik, and appeared in the market since 2004 [1]. It was considered as a safer alternative to tobacco for delivering the stimulant nicotine, and their use is on the rise, especially among the younger generation [2]. Besides, it was often used as a steppingstone for cessation of tobacco smoking and was extensively promoted as being healthier, less expensive, and more

socially acceptable than traditional cigarettes [3, 4].

E-cigarettes have a rechargeable battery and a cartridge loaded with E-liquid that is heated by an atomizer to produce vapor that is inhaled through a mouthpiece [5, 6]. It uses E-liquid heating instead of tobacco combustion, encouraging some manufacturers to propose that using E-cig is less harmful than traditional smoking [4]. The metabolites of 1,3-butadiene, benzene, and acrylonitrile, among other carcinogens and

poisons, were significantly reduced when people switched from tobacco cigarettes (T-cig) to electronic cigarettes (E-cig), while nicotine exposure remained the same [7].

Assuming that E-cig and T-cig share some toxicity, considerable levels of acetaldehyde and formaldehyde were found in the vapor of E-cig, and trace quantities of acetone and acrolein were found at higher temperatures [8]. Additionally, flavoring components in E-cig may harm stem cells and gingival fibroblasts by releasing aldehydes and carbonyls into the vapor, which can damage DNA and cause protein carbonylation as well as speed up the ageing process in cells [9].

So, E-cig is not fully risk-free, and there have been concerns about the high levels of nicotine and its dependence as a result of its use [3]. Also, the poisonous, carcinogenic, and oxidative stress-inducing chemicals in its content leads into a negative impact on nearly all of the body's organs and increases the risk of chronic diseases [10]. The health effects from Electronic Nicotine Delivery Systems (ENDS), inaccurate component labels on E-cig liquid, a lack of international regulations for high-quality manufacture, and the fact that E-cigarettes can turn non-smokers into dual users [11, 12].

So, E-cig may share tobacco smoking for its known hazards as a risk factor for several diseases including cardiovascular and pulmonary diseases, respiratory tract infections, osteoporosis, gastrointestinal ulcers, reproductive disorders, and diabetes. Moreover, it is considered as a significant risk factor for a variety of malignancies including urothelial carcinoma of the bladder, kidney, pelvis, and ureters [13]. Cigarette smokers are at a higher risk of urinary bladder cancer approximately 3 times more than non-smokers [14]. Up to our knowledge, no researches have explored the potential impact of E-cigarettes on urinary bladder. So this study was conducted to compare between the histopathological effect of electronic cigarette consumption and tobacco cigarette smoking on urinary bladder, as well as to investigate oxidative, inflammatory, and apoptotic markers through biochemical and immunohistochemical methods.

## MATERIAL AND METHODS

### Chemicals

Nicotine-free E-liquid and E-cig liquid were purchased from an Egyptian market (Dollar blends comp., e.g.). Vegetable, glycerin, propylene glycol (PG), natural and artificial flavorings, and nicotine 18 mg/ml are all included in 1 mL of E-cig liquid. In addition to the

commercially available filtered tobacco cigarettes. An Egyptian bazaar sold a portable electric incense burner (home electric comp., China).

### Animals

Forty males mature (12 week) albino rats (body weight 253±27 gm) were procured from Zagazig University Laboratory Animal House-Egypt. The rats were housed at a temperature of 24°C and on a 12-hour light/dark cycle. The rats were provided full access to water and fed a conventional pelleted diet. They were adapted for two weeks before any experimental procedures to assure their physical well-being, exclude ill rats, and acclimate to their new environment. The National Institutes of Health (NIH) Animal Care Guidelines were followed for all rats, and the experiment was authorized by the Institutional Animal Care and Use Committee at Zagazig University in Egypt (ZU-IACUC/2/F/44/2022).

### Experimental design

The rats were allocated into four groups at random. Each group has 10 rats (fresh-air, E-Liquid, E-cigarette, and T-cigarette) as follows: **Fresh-air (F-air) group** was exposed to fresh air for 8 weeks and **E-Liquid (E-liq) group** was exposed to 1ml/day of nicotine-free E-liquid smoke vapor for 1 hour for 5 successive days/week for 8 weeks. **E-cigarette (E-cig) group** was exposed to 1ml/day of E-cig liquid smoke vapor for 1 hour for 5 successive days/week for 8 weeks [15]. **T-cigarette (T-cig) group** was exposed to 5 cigarettes smoke vapor for 1 hour for 5 successive days/week for 8 weeks. Rats were placed in an inhaling chamber that was 38 x 26.5 x 19 cm propylene box with 19-liter capacity. This chamber had 2 holes, one received E-liq, E-cig, or T-cig vapor puffing using a portable electric incense burner for 1 hour each day, while the other one was left for aeration. Two rats per chamber were used utilizing a whole-body exposure method. Then they were taken to a room that was entirely antiseptic.

Rats were examined for general health. Each animal's body weight was calculated by weighing it in a closed plastic container the day before and also at the last day of the experiment. The data were recorded in a book for each rat that had been given a unique identification number.

At the end of experiment, animals were thoroughly anaesthetized intraperitoneally with thiopental (50 mg/kg) and slaughtered. The abdomens of rats were dissected thoroughly, and urinary bladder samples were obtained. Each bladder was emptied from its contents and weighed by special electrical scale then labeled with a code number for blind testing. The data

were recorded in a book for each bladder and given a unique identification number.

Half of each bladder was immediately frozen on dry ice and kept at  $-80^{\circ}\text{C}$  for tissue homogenate analysis, while the other half of each bladder was submerged and fixed in buffered formalin 10% for 24 hours for histological examination.

#### **Biochemical study of homogenate bladder tissue**

Throughout the preparation, all the urinary bladder tissues were kept at  $+4^{\circ}\text{C}$ , and were homogenized using 5–10 ml cold buffer (i.e., 100 mM potassium phosphate, pH 7.0, including 2 mM EDTA) per gramme tissue. After centrifuging the homogenates for 15 minutes at 50,000 rpm, the clear upper supernatants were collected and kept at  $-80^{\circ}\text{C}$  for analysis. This preserved homogenate was used for colorimetric assessment of:

- **Oxidative stress parameter assay:** Determination the reactive oxygen species (ROS) content according to ROS assay kit (Beyotime, China). ROS formation was quantified, and the results are given in pmol/min/mg.
- **Total Antioxidant Capacity (TAC) Assay:** The OxiSelect™ Assay Kit from Cell Biolabs was used to measure total antioxidant capacity. The data are presented in units of ng/mg.
- **The inflammatory markers:** Levels of tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) were tested and reported in pg/ml in accordance with the manufacturer's protocol using commercially available enzyme-linked immunosorbent assay (ELISA) kits from Abcam (TNF- $\alpha$ : ab46070; IL-6: ab100772, UK) and MIP-2 ELISA kits from Biosource International, Camarillo, USA.
- **The apoptotic marker assay:** Rat Tumor Protein p53 (P53) was assessed by ELISA specific experimental kits (MyBioSource, ELISA Test Kits). The results were given in pg/g tissue.

#### **Histological and morphometrical analysis**

Rat bladder tissue samples were processed and embedded in paraffin blocks after being instantly fixed in formalin, dried in graded ethanol and cleaned in xylene. A microtome was used to chop up samples of urinary bladder tissues into 4–5  $\mu\text{m}$  sections. To analyze general tissue histology and collagen fibers distribution, sections were stained with hematoxylin and eosin (H&E) and Mallory's trichrome as described by [16, 17]. From five slides, ten non-overlapping fields for each rat in each group were coded, allowing for blind assessment and evaluation, using light

microscope (LEICA ICC50 W) and ImageJ software.

- H&E Sections were assessed **1)** At 100 magnifications for the bladder wall thickness in millimeter, and urothelium thicknesses in micrometer [18]. **2)** At 100 magnifications for relative areas of bladder detrusor smooth muscle (DSM) [19].
- At 100, and 400 magnifications, urinary bladder tissues in different groups were evaluated for edema, bleeding, and inflammation and graded on a scale from 0 (normal) to 3 (severe changes). The severity of mucosal ulceration was graded as 0, 1, 2, and 3 (normal, epithelial denuding, localized ulceration and widespread epithelial ulceration respectively) [20]. Overall pathological score was calculated as sum of all previous conditions and blindly assessed.
  - Mallory's trichrome sections were assessed at 400 magnifications for the mean area percentages of blue-stained collagen fiber (CF) and red-counter stained smooth muscle (SM) within the musculosa and the CF/ SM % was calculated [18].

#### **Immunohistochemical and morphometrical analysis**

Paraffin-embedded rat urinary bladder sections were cut into 5  $\mu\text{m}$  sections and treated with rabbit monoclonal anti-Ki-67 (Zymed, San Francisco, CA, USA). and the primary antibody anti-E-cadherin (Abcam plc, Cambridge, UK) incubated overnight at  $4^{\circ}\text{C}$ . After applying the secondary antibody, a chromogen was then added (3,30-diaminobenzidine). Finally, Mayer's hematoxylin stain was used as a counterstain on tissue slices. In order to facilitate blind assessment and evaluation, ten fields from five different slides for each rat in each group were coded utilizing light microscope and ImageJ software.

- Ki-67 immunostained sections were assessed at 400 magnifications for calculation the proliferation index (PI), by dividing the number of positive cells (brown cells) to total number of cells (brown + blue)% [21].
  - E-cadherin immunostained sections were assessed at 400 magnifications for calculation E-cadherin score by adding: **1)** I light microscope and ImageJ software mmunohistochemical expression intensity, 0: faint, 1: mild, 2: moderate, and 3: intense [22]. **2)** Area % immunoreactivity, 1: 0-25%, 2: 25-50%, and 3: 50-75%, 4:75-100%.

#### **Statistical analysis**

The SPSS 18.0 programme was used to statistically analyze all the data. The Shapiro-Wilk test was used to examine if the data had a normal

distribution. The mean values of various groups were compared using a one-way ANOVA, and multiple comparisons were evaluated using the Tukey HSD Post-hoc Test. Normal distributed data were reported as mean and standard deviation (SD) (Statistical Package for Social Science). To demonstrate the difference in pathological score between the E-cig and T-cig groups, an unpaired T-test was utilized. Kruskal-Wallis H tests were used to compare the median values of the groups in non-normally distributed data, multiple comparisons were evaluated using the Mann-Whitney test, and non-normally distributed data were displayed as median (range). A value of P less than 0.05 or less than 0.001 considered to be statistically significant or highly significant respectively. While a value of P more than 0.05 considered to be non-statistically significant.

## RESULTS

### **[I] Effect of E-cigarette/ T-cigarette on body and UB weight**

The F-air group and E-Liq group showed no significant difference regarding to body or UB weights. When compared to the F-air and E-Liq groups: E-cig group showed non-significant decrease of body weight (-15.4%) and non-significant increase of UB weight (+33.3%). While T-cig exposure resulted in a significant decrease in body weight (-18.9%), and highly significant increase in UB weight (+61.9%). Comparing of both E-cig and T-cig groups, T-cig exposure resulted in a significant increase in UB weight than E-cigarette (table 1, fig. 1).

### **[II] Biochemical Study**

#### **1- Effect of E-cigarette/ T-cigarette on oxidative and antioxidant markers of the examined UB**

When compared to the F-air and E-Liq groups: E-cig inhalation led to a significant increase in ROS production (+ 59.1%) and highly significant decrease in TAC (-59.3%). While both ROS and TAC were highly significant difference in T-cig group (+149%, -76.6% respectively). Moreover, T-cig group showed also highly significant difference in both ROS and TAC when comparing with E-cig group (table 1, fig. 1).

#### **2- Effect of E-cigarette/ T-cigarette on inflammatory and apoptotic markers of the examined UB**

Compared to the F-air and E-Liq groups: The E-cig group had a highly significant elevation of all the inflammatory markers; TNF- $\alpha$ , IL-6, MIP-2, and P53 by more than 50%. An extremely substantial rise of all of them by more than 100% was also seen in the T-cig group. Additionally, as compared to the E-cig group, the T-cig group

revealed a very significant rise in TNF- $\alpha$  and MIP-2 as well as a substantial increase in MIP-2 and P53 (tab. 1, fig. 1).

### **[III] Histological Study**

#### **1- Effect of E-cigarette/ T-cigarette on UB by using of H&E stain**

In the bladder sections of both F-air group and E-Liq group, a typical transitional epithelium (urothelium) was visible all the way to the lumen. Layers of smooth muscle and connective tissue both suggested a typical histological appearance. The bladder wall consists of mucosa, submucosa and muscosa. The mucosa was thrown into numerous thick folds and consisted of transitional epithelium. The urothelium was made up of three layers: "umbrella" cells on the surface, intermediate cells, and basal cells. The deepest layer of urothelium was made up of superficial cells, which were larger than other urothelial cells and were organized in an umbrella-like pattern horizontally over intermediate cell. The thickness of the urothelium ranged from 2 to 7 layers. Oval or somewhat elongated intermediate cells were perpendicular to umbrella cells and polarized neatly vertically toward the surface. Basal cells are cuboidal-shaped, smaller than intermediate cells, and found on the basement membrane. The submucosa (lamina propria), a loose layer of connective tissue containing many vessels, lymphatics, sensory nerve terminals, and some elastic fibers is located just under the urothelial basement membrane. The smooth muscle that made up the muscularis mucosa was dispersed erratically and came in various sizes. Additionally, there were vessels that tightly connected to the muscularis mucosa fibers and ran the length of the lamina propria, either constantly or occasionally. The morphology and distribution of the smooth muscles were the same in all the studied bladder areas (fig. 2 A-D).

H&E-stained bladder tissue sections from E-cig group showed various histological abnormalities; signs of inflammation of the lining urothelium, small focal regions of epithelial degradation, either areas of mild focal thinning and denudation or areas of mild urothelial thickening and hyperplasia. Some urothelial cells were pyknotic with dark stained nuclei. Also, there were small areas of cystitis cystica and areas of edema, besides thick-walled congested blood vessels (fig. 2 E-H).

H&E-stained sections of T-cig group exhibited severe histopathological alterations in form of significant distortion of the lining urothelium. Moreover, extensive areas of exfoliation and thickening leading to hyperplasia

with cystitis cystica and other areas with severe ulceration and necrosis. Many urothelial cells were pyknotic with dark stained nuclei and other cells were vacuolated. There were extensive areas of edema and congested lamina propria. Thick walled congested blood vessels and even ruptured blood vessel with bleeding in the lamina propria were noticeable. Also, cellular infiltration with inflammatory cells were visible. The musculosa was distorted in arrangement and showed wide separation of the muscle bundles (fig. 3 A-F)

## 2- Effect of E-cigarette/ T-cigarette on UB by using of Mallory's trichrome stain

In Mallory's trichrome-stained sections of urinary bladders of rats of both F-air and E-Liq groups, there were thin sheets of collagen fibers (blue bundles) distributed in between thick regular muscle bundles (red sheets) (fig. 4 A-B). While in both E-cig and T-cig groups, there were excess amounts of collagen fibers in between the interrupted muscle bundles (fig. 4 C-D).

## 3- Effect of E-cigarette/ T-cigarette on UB for Ki-67 & E-cadherin immunoreactivity

The urothelial cells of F-air and E-Liq groups had a little amount of positive nuclear immunoreactivity for Ki-67 (fig. 5 A-B). While the urothelial cells of both E-cig and T-cig groups had a strong positive nuclear immunoreactivity for Ki-67 (fig. 5 C-D)

The urothelial cells of F-air and E-Liq groups had strong positive cytoplasmic immune expression for E-cadherin. While the cytoplasm of the urothelium in both E-cig and T-cig groups had weak immune expression for E-cadherin (fig. 5 F-I).

### [IV] Morphometric Study

## 1- Histopathological changes by using of H&E stain

Pathological score of the control animals (F-air and E-liq groups) were "0" for all four pathological parameters, edema, bleeding, inflammation, and ulceration. While the mean of total pathological score in cigarette groups (6.8, and 9.6 in E-cig and T-cig groups respectively) exhibited highly significant difference, there were highly significant differences between both groups in edema and inflammation and no significant difference in both hemorrhage and ulceration (tab. 2).

In both the E-cig and T-cig groups, there was a highly significant increase in bladder wall thickness (+61.3% and +83.2%, respectively) as compared to F-air group. Furthermore, there was a significant increase in relative areas of DSM (+19% and +28.9%, respectively) in both the E-cig and T-cig groups. Furthermore, in terms of

urothelium thicknesses, there was a significant increase in the median of urothelium thicknesses (+48.2% and +80.5%, respectively) in both the E-cig and T-cig groups. In line to histological findings, there was a decrease in lowest value in the E-cig and T-cig groups (12.7, and 7.8 respectively) vs 18.9 in the F-air group and an increase in highest value in the E-cig and T-cig groups (70.4, and 105.4 respectively) vs 18.9 in the F-air group, resulting in the widest range in the T-cig group (97.6), followed by E-cig group (57.7) vs 26.8 in F-air group. In all morphometrical data, the E-liq group showed no statistically significant difference as compared to the F-air group (tab.3, fig 3 G-I).

## 2- Histopathological changes by using of Mallory's trichrome stain

The percentages of collagen fiber to smooth muscle contents were highly significant increase more than 5 folds (554.9%) in T-cig group and more than 2 folds (231.4%) in E-cig group. While, there was no statistical difference between F-air and E-liq groups (tab.3, fig. 4 E).

## 3- Ki-67 & E-cadherin immunoreactivity

proliferation index (PI) was calculated by dividing the number of positive Ki-67 cells (brown cells) to total number of cells, PI were highly significant increase more than 7 folds (701.9%) in E-cig group and more than 11 folds (1140%) in T-cig group comparing with F-air group. However, there was no statistical difference between F-air and E-liq groups (tab.3, fig. 5 E).

Positive cytoplasmic immune expression of urothelium for E-cadherin was highly significant decrease by -46.3% in E-cig group and -74.1% in T-cig group comparing with F-air group. However, there was no statistical difference between F-air and E-liq groups (tab.3, fig. 5 J).

Table 1: Effect of E-cigarette/ T-cigarette on Weights, Oxidative, Inflammatory, and Apoptotic Markers in Different Studied Groups					
	F-air group (n10)	E-Liq group (n10)	E-cig group (n10)	T-cig group (n10)	P value
<b>Body and urinary bladder weights</b>					
<b>Body wt. *</b>	237.3±41.2	240.4±24.6	200.7±18.7	192.4±22.5 <sup>a</sup>	<0.001
% Of dif. #		+1.3%	-15.4%	-18.9%	
<b>UB wt.</b>	0.063±0.009	0.072±0.011	0.084±0.021	0.102±0.014 <sup>bc</sup>	<0.001
% Of dif. #		+14.2%	+33.3%	+61.9%	
<b>Oxidative Stress Markers</b>					
<b>ROS</b>	86.6±12.5	93.2±14.1	137.8±32.3 <sup>a</sup>	215.6±54.1 <sup>bd</sup>	<0.001
% Of dif. #		+7.6%	+59.1%	+149%	
<b>TAC</b>	414.9 ± 46.5	327.9 ± 36.4	168.4 ± 24.2 <sup>b</sup>	97.1 ± 17.6 <sup>bd</sup>	<0.001
% Of dif. #		-21%	-59.3%	-76.6%	
<b>Inflammatory Markers</b>					
<b>TNF-α</b>	83.4±11.2	92.5±5.5	134.7±10.3 <sup>b</sup>	217.3±22.4 <sup>bd</sup>	< 0.001
% Of dif. #		+10.9%	+61.5%	+160.6%	
<b>IL-6</b>	52.7±8.3	56.4±7.2	93.4±21.3 <sup>b</sup>	112.4±18.3 <sup>bc</sup>	< 0.001
% Of dif. #		+7%	+77.2%	+113.3%	
<b>MIP-2</b>	203.2±23.6	224.2±14.7	325.1±11.9 <sup>b</sup>	417.7±59.2 <sup>bd</sup>	< 0.001
% Of dif. #		+10.3%	+60%	+105.6%	
<b>Apoptotic marker</b>					
<b>P53</b>	119.7±14.5	121.8±17.3	203.2±25.1 <sup>b</sup>	238.4±39.2 <sup>bc</sup>	< 0.001
% Of dif. #		+1.8%	+69.8%	+99.2%	
* At last day, # % Of difference= mean of (E/T-liq/cig) group - mean of F-air group)/ mean of F-air group %. UB: urinary bladder, TNF-α: Tumor Necrosis Factor Alpha (pg/ml), IL-6: Interleukin-6 (pg/g tissue), MIP-2: macrophage inflammatory protein-2 (pg/ml), ROS: reactive oxygen species (pmol/min/mg), TAC: total Antioxidant Capacity (ng/mg units), p53: Rat Tumor Protein 53 (pg/g tissue).					
One-way ANOVA, and Tukey HSD Post-hoc Test, P > 0.05: no significant differences, P < 0.05: significant differences, P < 0.001: highly significant differences. <sup>a</sup> significant vs F-air group, <sup>b</sup> highly significant vs F-air group. <sup>c</sup> significant vs E-cig group, <sup>d</sup> highly significant vs E-cig group					

Table 2: Pathological score calculation in different studied groups					
	F-air group (n10)	E-Liq group (n10)	E-cig group (n10)	T-cig group (n10)	P value
<b>Oedema</b>	0	0	1.3±0.4	2.4±0.6 <sup>d</sup>	< 0.001
<b>Haemorrhage</b>	0	0	1.8±0.3	2.1±0.4	0.0739
<b>Inflammation</b>	0	0	1.5±0.2	2.3±0.5 <sup>d</sup>	< 0.001
<b>Ulceration</b>	0	0	1.6±0.5	2.1±0.7	0.0826

**Table 2: Pathological score calculation in different studied groups**

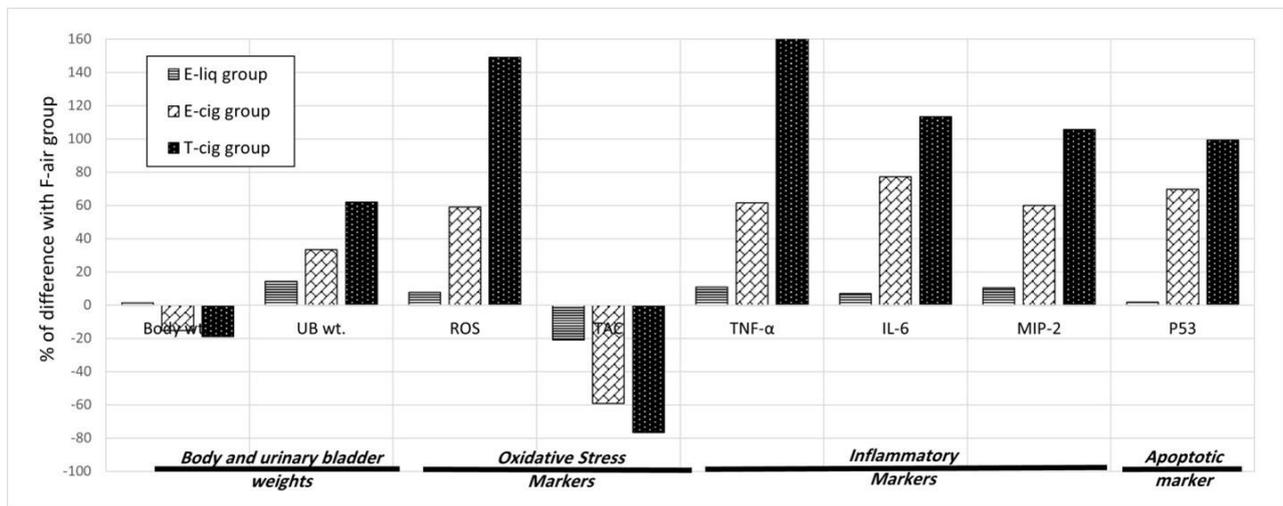
<b>Total pathological score</b>	0	0	6.8±1.3	9.6±1.4 <sup>d</sup>	< 0.001
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t- test to compare mean ± SD E-cig vs T-cig.  
P > 0.05: no significant differences, <sup>c</sup>P < 0.05: significant differences, <sup>d</sup>P < 0.001: highly significant differences.

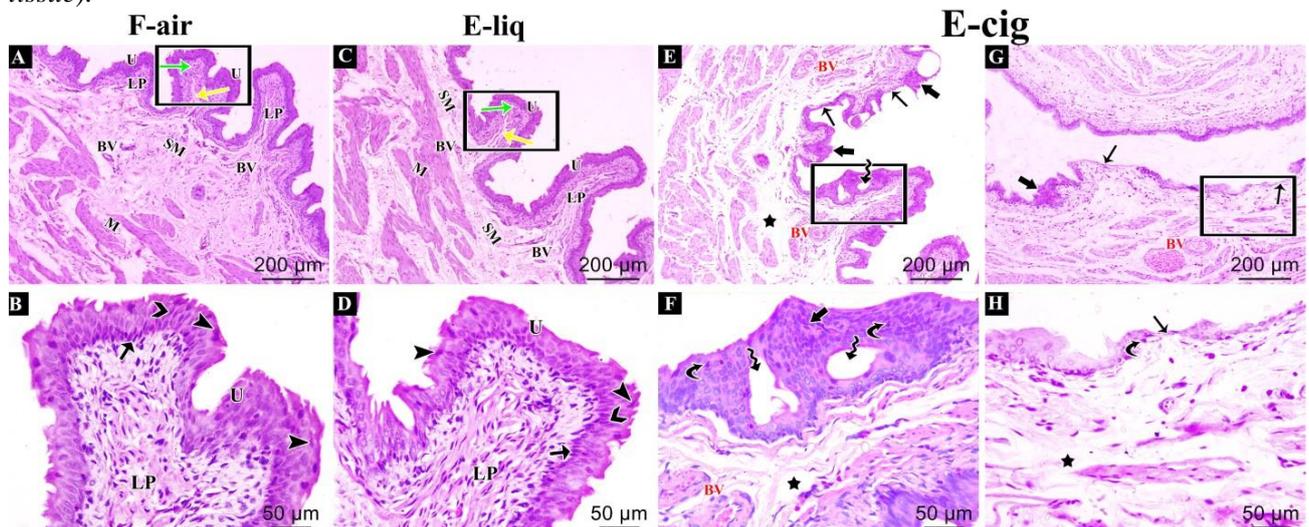
**Table 3: Effect of E-cigarette/ T-cigarette on histological and immunohistochemical morphometrical parameters of urinary bladder in different studied groups**

	<b>F-air group (n10)</b>	<b>E-liq group (n10)</b>	<b>E-cig group (n10)</b>	<b>T-cig group (n10)</b>	<b>P value</b>
<b>H&amp;E morphometry</b>					
<b>Bladder wall thickness<sup>1</sup></b>	1.19 ± 0.10	1.32 ± 0.11	1.92 ± 0.13 <sup>b</sup>	2.18 ± 0.15 <sup>bd</sup>	<0.001
% Of dif. #		+10.9%	+61.3%	+83.2%	
<b>Urothelium thicknesses<sup>2 @</sup></b>	32.5 (18.9-45.7)	30.7 (19.8-43.9)	67 (12.7-70.4) <sup>a</sup>	73.1 (7.8-105.4) <sup>b</sup>	0.034
% Of dif. in median		-11.1%	+48.2%	+80.5%	
<b>Relative areas of DSM</b>	72.6±9.3	74.2±7.7	86.4±11.8 <sup>a</sup>	93.6±12.5 <sup>b</sup>	<0.001
% Of dif. #		+2.2%	+19%	+28.9%	
<b>Mallory's trichrome morphometry</b>					
<b>CF area%</b>	16.4 ± 4.3	21.4 ± 7.1	36.2 ± 10.1 <sup>b</sup>	58.6 ± 6.9 <sup>bd</sup>	<0.001
% Of dif. #		+30.5%	+120.7%	+257.3%	
<b>SM area%</b>	81.6 ± 6.7	75.2 ± 8.3	51.2 ± 9.3 <sup>b</sup>	40.5 ± 9.7 <sup>bc</sup>	<0.001
% Of dif. #		-7.8%	-37.3%	-50.4%	
<b>CF/ SM %</b>	21.3± 3.5	28.4 ± 6.3	70.6 ± 6.9 <sup>b</sup>	139.5 ± 12.4 <sup>bd</sup>	<0.001
% Of dif. #		+33.3%	+231.4%	+554.9%	
<b>Immunohistochemical morphometry</b>					
<b>Proliferation index*</b>	5.3± 1.3	6.2± 1.7	42.5± 7.5 <sup>b</sup>	62.3± 10.2 <sup>bd</sup>	<0.001
% Of dif. #		+17%	+701.9%	+1140%	
<b>E-cadherin score</b>	5.4± 0.9	4.7± 1.2	2.9± 0.7 <sup>b</sup>	1.4± 0.5 <sup>bc</sup>	<0.001
% Of dif. #		-13%	-46.3%	-74.1%	

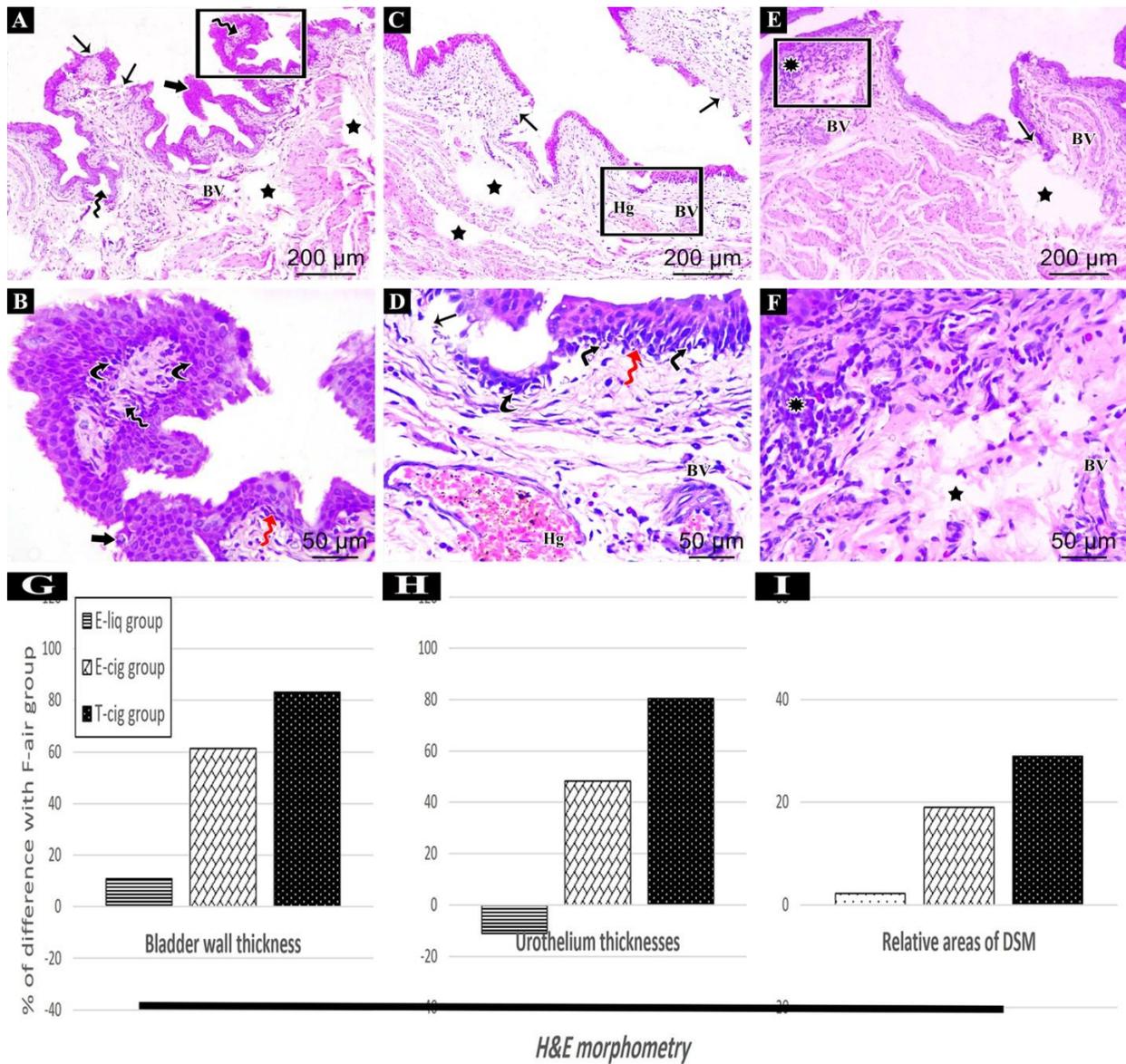
<sup>1</sup>: in mm, <sup>2</sup>: in µm \* In anti-Ki-67-stained sections, # % Of difference= mean of (E/T-liq/cig) group - mean of F-air group) / mean of F-air group %. DSM: bladder detrusor smooth muscle, CF/ SM ratio: percentages of blue-stained collagen fiber (CF) / red-counter stained smooth muscle (SM) within the musculosa.  
One-way ANOVA, and Tukey HSD Post-hoc Test to compare mean ± SD. <sup>@</sup>Kruskal Wallis H tests, and Mann–Whitney test to compare median. P > 0.05: no significant differences, P < 0.05: significant differences, P < 0.001: highly significant differences.  
<sup>a</sup> significant vs F-air group, <sup>b</sup> highly significant vs F-air group. <sup>c</sup> significant vs E-cig group, <sup>d</sup> highly significant vs E-cig group



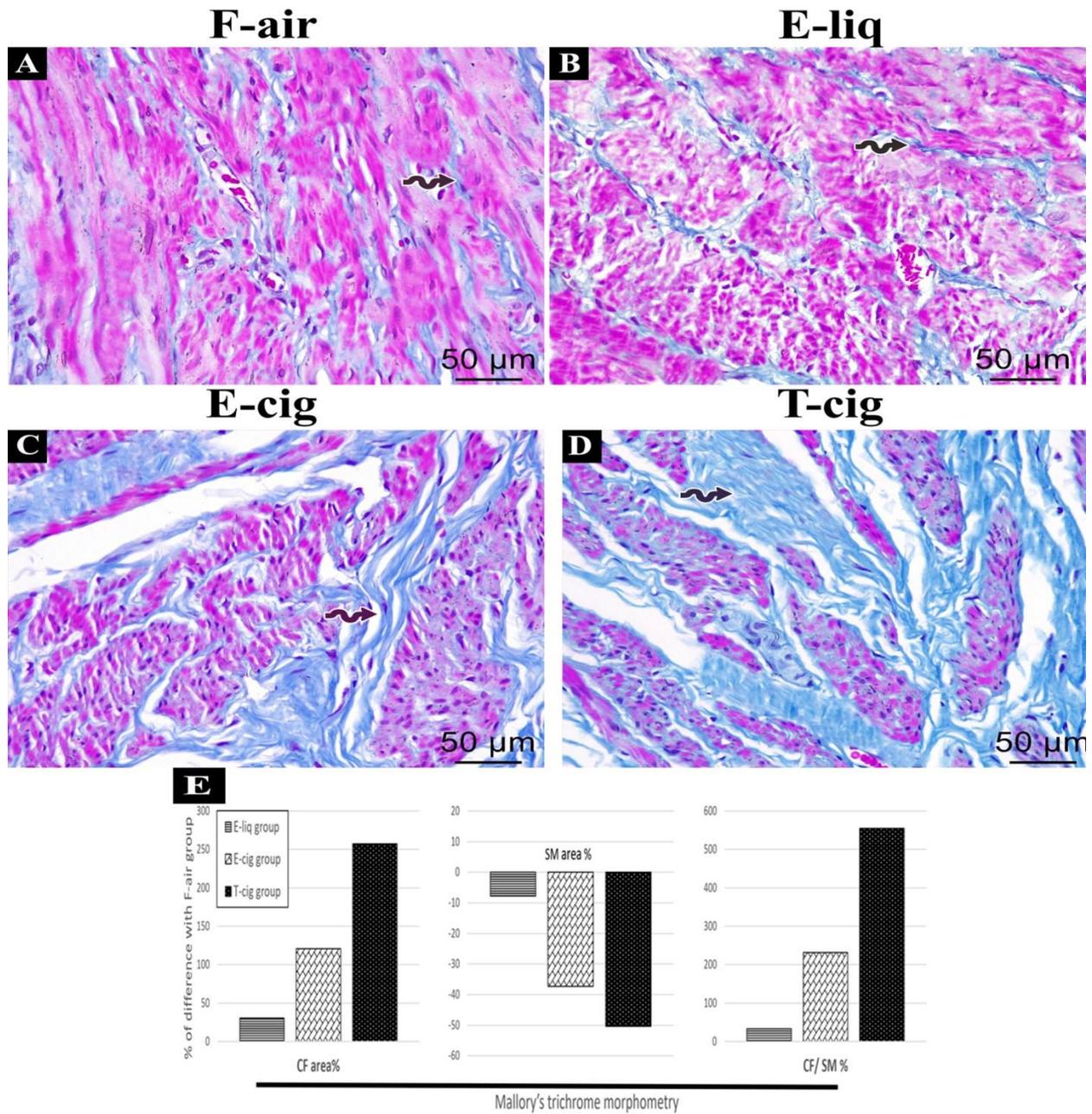
**Figure (1):** chart showing the percentage of difference between means of different study groups on weights, oxidative, inflammatory, and apoptotic markers (% Of difference= mean of (E/T-liq/cig) group - mean of F-air group)/ mean of F-air group %). UB: urinary bladder, TNF-α: Tumor Necrosis Factor Alpha (pg/ml), IL-6: Interleukin-6 (pg/g tissue), MIP-2: macrophage inflammatory protein (pg/ml), ROS: reactive oxygen species (pmol/min/mg), TAC: total Antioxidant Capacity (ng/mg units), p53: Rat Tumor Protein (pg/g tissue).



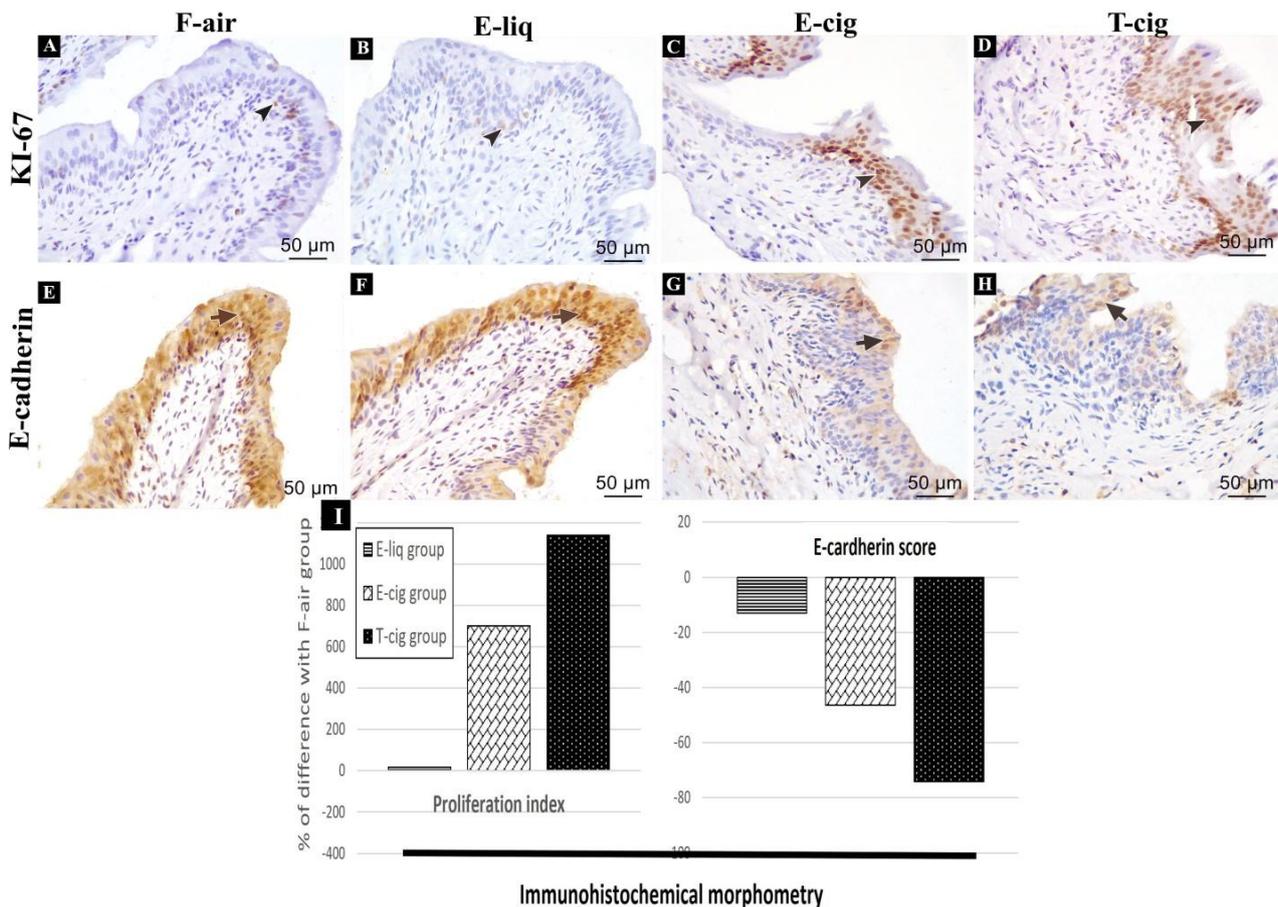
**Figure (2):** photomicrographs of H&E-stained sections of the urinary bladder of rats in the different groups. The fresh air group (F-air) (A&B) and E-Liquid group (E-Liq) (C&D) are showing mucosa, submucosa (SM) and musculosa (M) of the bladder wall. The mucosa is thrown into numerous folds and consists of urothelium (U) which is differentiated into superficial umbrella cells (arrow heads), intermediate cells (empty arrow head) and basal cell layer (short arrow). The lamina propria (LP) was divided into two zones: a thick zone on the surface (green arrow) with a lot of cells and a deep lighter zone (yellow arrow) with fewer cellular elements. Musculosa (M) is the thickest layer made of smooth muscle bundles that are oriented in different directions, with presence of normal blood vessels (BV). While the electronic cigarette (E-cig) group (E-H) are showing areas of mild focal thinning and denudation (thin arrows) and areas of mild urothelial thickening and hyperplasia (thick arrows). Some urothelial cells are pyknotic with dark stained nuclei (curved arrows). Also, there are small areas of cystitis cystica (zigzag arrows), areas of edema (star), and thick walled congested blood vessels (red BV). (Bar: A, C, E & G: 200 μm X 100 - B, D, F & H: 50 μm X 400).



**Figure (3):** Photomicrographs of H&E-stained sections of the urinary bladder of rats of the tobacco cigarette (T-cig) group are showing extensive histopathological changes. Apparent distortion of the urothelial cells (red zigzag arrow). There are extensive areas of exfoliation and hyperplasia (thick arrow) with cystitis cystica (zigzag arrow) and other areas of sever ulceration and necrosis (thin arrows). Many urothelial cells are pyknotic with dark stained nuclei (curved arrows) and other cells are vacuolated (right angled arrows). There are extensive areas of edema (stars) and cellular infiltration with inflammatory cells (asterisk), thick walled congested blood vessels (BV) and even ruptured blood vessel with hemorrhage (Hg). (Bar: A,C&E: 200 μm X 100 - B,D&F: 50 μm X 400).



**Figure (4):** Photomicrographs of Mallory's trichrome-stained sections of the urinary bladder of rats in the different groups. F-air and E-Liq groups (A& B) are showing thin sheets of collagen fibers (blue bundles, wavy arrow) distributed in between thick regular muscle bundles (red sheets). While E-cig and T-cig groups (C&D) are showing excess amounts of collagen fibers (blue bundles, wavy arrow) in between the interrupted muscle bundles. (Bar= 50  $\mu$ m X 400).



**Figure (5):** The urothelial cells of F-air and E-Liq groups (A&B) have a little amount of positive nuclear immunoreactivity for Ki-67 (arrow head). While the urothelial cells of both E-cig and T-cig groups (C&D) have a strong positive nuclear immunoreactivity for Ki67 (arrow head). The urothelial cells of F-air and E-Liq groups (E&F) have strong positive cytoplasmic immune expression for E-cadherin (short arrow). While the cytoplasm of the urothelium in both E-cig and T-cig groups (G&H) have extensive weak immune expression for E-cadherin (short arrow). (Bar= 50 μm X 400).

### DISCUSSION

E-cigarettes have been promoted as a healthy alternative, yet like T-cig, they have been linked to several health hazards. This study, focused on the effect of E-cig and T-cig smoking on urinary bladder. Results showed that both have detrimental effects that were lighter with E-cig. This agreed with Singh and Kathiresan [23] who reported that E-cigarettes release hazardous carbonyl chemicals because of heat decomposition reactions. These compounds can be harmful to human health, albeit to a lower extent than those found in T-cig smoke.

Comparatively to non-users, E-cig users showed considerably higher levels of many carcinogens that can be transformed into compounds linked to bladder cancer in their urine. Similar to cigarette smoking and its established connection to bladder cancer, the long-term implications of urothelial exposure to these toxicants are unclear but concerning [24]. Besides, Lee et al. [25] reported that ECS (E-cig smoke) is carcinogenic and that E-cig smokers

have a higher risk than nonsmokers to develop lung and bladder cancer and heart diseases.

According to the study's findings, the T-cig group produced much more ROS than the E-cig group while also significantly reducing TAC. T-cig group had a highly significant rise in TNF-α and MIP-2 as well as a considerable increase in MIP-2 and P53 compared to E-cig group in terms of inflammatory and apoptotic markers. This agreed with Barnes [26] and Caramori et al. [27] who noticed increased release of the inflammatory cytokines TNF-α, IL-6 and IL-8 in smokers with early stages of COPD.

Moreover, it was supported by Allehaibi et al. [28] who mentioned that following nicotine treatment, intraepithelial lymphocytes were found in several areas of the urinary bladder, and the condition was diagnosed as a recurrent common reactive inflammatory illness as recorded by Grignon and Sakr [29] and Kagami et al. [30]. Besides, Al Dera [31] and Wang et al. [32] attributed the rise in TNF-α to the rupture of the cellular basement membrane, which caused a

number of inflammatory and immune cells to migrate to the site of the damage and release a variety of cytokines that exacerbated the inflammatory response. Additionally, these results cope with Suzuki et al. [33] who observed the occurrence of apoptosis, and oxidative stress in the urothelium of the urinary bladder and attributed the generation of some ROS in the urothelium to the cell proliferation and other intracellular and extracellular mechanisms. Moreover, it agreed with Allehaibi et al. [28] who found that injection of nicotine resulted in positive expression of p53 in some cells of lamina propria suggesting that it affects the proliferating epithelium mitogenically rather than cytotoxicity as mentioned before by Cohen [34] in human study and Apasov et al. [35] in animal study.

ROS initiate lipid peroxidation leading to damage of DNA strands, particularly mitochondrial DNA (mtDNA), results in mitochondrial damage and a decrease in ATP generation. Because ATP is required for the body's metabolic processes, cell death will occur if the amount of metabolic dysfunction decreases. Moreover, Protein oxidation is triggered by proteins that bind to ROS, causing damage to enzymes, transporters, and calcium homeostasis (Ca). Membrane disintegration and increased membrane permeability are caused by changes in membrane protein structure, which can lead to cell death [36].

Results of histological examination in T-cig group showed severe histopathological alterations in the form of distortion of the lining urothelium, extensive areas of exfoliation, hyperplasia with cystitis cystica and other areas with severe ulceration and necrosis. Pyknosis and vacuolation of urothelial cells, edema, and congestion of lamina propria, congested thick-walled blood vessels, cellular infiltration with inflammatory cells. distortion and wide separation of the muscle bundles of the musculosa. On the other hand, these histopathological alterations were slightly less prominent in E-cig group.

These outcomes supported by Dodmane et al. [37], Allehaibi et al. [28], Suzuki et al. [33], Suzuki et al. [38] and Suzuki et al. [39] who found hyperplasia of urinary bladder epithelium after oral administration of nicotine which indicate increased number of proliferating cells. Also agreed with Tang et al. [2] who found flat and/or papillary urothelial hyperplasia with increased mitotic activity in some E-cig exposed mice. On the other hand, a comparative study between E-cig and T-cig conducted by Reinikovaite et al. [40] recorded that both produce

very similar, damaging effects to the lungs. Theophilus et al. [41] on the other hand, found no abnormalities in the urine bladders, with the exception of amyloid accumulation in the submucosa of one rat, which they ascribed to the short length of the trial.

Hyperplasia of urinary bladder was further confirmed by immunohistochemical assay in this work which revealed highly significant increase of Ki-67 Proliferation index for more than 7 folds in E-cig group and more than 11 folds in T-cig group compared to the other two groups indicating that cigarette smoking stimulates proliferation of the urothelium. Whereas McGrath-Morrow et al. [42] noticed decreased expression of Ki-67 in the lung of nicotine exposed mice via E-cig indicating a significant inhibition of alveolar growth. Ki-67 is a non-histone nuclear protein with a short life, expressed by proliferating cells during certain phases of the cell cycle [43, 44]. It was clearly observed in our study that both apoptotic marker (P53) and proliferative marker immunoexpression Ki-67 were elevated, as pathological features of urinary bladder contained both areas of ulceration and other areas of proliferation.

On the other hand, it has been reported that collagen fibers and smooth muscles make up most of the bladder wall. Collagen fibers form a major part of the extracellular matrix of bladder that lack elasticity, while the smooth muscle controls bladder contractility [45, 46]. So, appropriate bladder contraction and relaxation depends on the ratio of smooth muscle to connective tissue and increased collagen fibers in between the wide muscle fascicles causes bladder wall stiffening. This occurs as one of the hazards of smoking where the results of the present work showed that percentages of collagen fiber to smooth muscle contents were highly significant increase more than 5 folds in T-cig group and more than 2 folds in E-cig group with no statistical difference between F-air and E-liq groups.

E-cadherin is a calcium-dependent adhesion molecule involved in epithelial cell histogenesis, differentiation, and stability [47]. E-cadherin expressed mainly by epithelial cells that helps to maintain the integrity of functional epithelium by stabilizing homotypic adhesion between adjacent epithelial cells mediate cell-to-cell adhesion [48, 49]. A significant decrease of urothelial immunoexpression for E-cadherin in T-cig more than E-cig group compared to the other two groups was noticed in this study. This was in agreement with Sun et al. [50] who noticed progressive decrease in the protein expression of

E-cadherin with increased concentration of cigarette smoke emissions. Aberrations in E-cadherin expression produce ruptures in cell-to-cell connections, allowing cells to migrate [51]. Moreover Bryan and Tselepis [52] and van der Horst et al. [53] concluded that these ruptures are caused by a decrease in E-cadherin expression, which leads to an epithelial-to-mesenchymal transition (EMT). EMT accelerates the growth and metastasis of many epithelial-derived carcinomas (including urinary bladder cancer); it also enhances the mesenchymal features of tumor cells, promoting their invasive qualities and motility.

In summary, E-cig induces inflammation, apoptosis, oxidative stress, and hyperplasia of urinary bladder to a slightly lesser extent than T-cig. So, it is important to evaluate the benefits of E-cig for populations undertaking smoking cessation, and further studies are recommended to study the long-term effects and dependency. Additionally, consumers should be aware that E-cigarettes are not completely harmless.

### CONCLUSION

According to this study, even if electronic cigarettes have slightly fewer pathological and biochemical effects on the urinary bladder than tobacco cigarettes, they still cause urinary bladder damage, hence it is strongly advised to avoid any devices that contain nicotine.

### Declarations

#### **Ethics approval and consent to participate:**

All studies and procedures involving rats were approved by Institutional Animal Care and Use Committee at Zagazig University in Egypt (ZU-IACUC/2/F/44/2022) and by the National Institute of Health (NIH) guidelines. Consent for publication the publication of this manuscript has been approved by all authors.

#### **Consent for publication:** Not applicable

**Availability of data and materials:** The data and datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

**Competing interests** There are no conflicts of interest declared by any of the authors.

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