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ORGINAL ARTICLE

Effect of Electronic Cigarette Smoking on Thoracic Aorta of Adult Male Albino Rat and the Possible Protective Role of Melatonin

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ABSTRACT

Background: Electric cigarette smoking (E-smoking) has a growing popularity especially among youth. Its effects on cardiovascular system are not well clarified. Melatonin has beneficial effects in prevention and improving cardiovascular risk factors. So we aim to study E-smoking effects on the thoracic aorta and investigate the possible protection provided by melatonin.

Methods: 40 male albino rats were divided equally into four groups; control group: Exposed to no experimental interventions. Melatonin group: Received 5mg/kg/day melatonin orally for 4 weeks. E-smoking group: Exposed to 1ml/day of e-liquid smoke vapor for 1 hour 5consecutive days/week for 4 weeks. E-smoking + melatonin group: Exposed to 1ml/day of e-liquid smoke vapor for 1 hour for 5consecutive days/week and treated with 5mg/kg/day melatonin orally for 4 weeks. Histological sections were stained with hematoxylin and eosin, orcein and masson trichrome stains, and immunostained for Cluster of Differentiation 34 (CD34) and Metalloproteinase-2 (MMP-2).

Results: E-smoking group showed decreased tunica media thickness, vacuolated smooth muscles cells, fragmented, atrophied and irregular elastic fibers with pale orcein staining, Strong CD34 and MMP-2 immunoexpressions, also Significant($p<0.001$) increment in serum malondialdehyde (MDA) and decrement in glutathione peroxidase (GPx) as compared to control group. In E-smoking+melatonin group, histological structure was mostly preserved with moderate CD34 and negative MMP-2 immunoexpression. Significant decrement in the MDA and increment in GPx serum levels were recorded.

Conclusion: E-smoking for 1 month in albino rat is sufficient to initiate aneurysmal pathological changes in the aortic wall. Melatonin provided a protection through inhibition of MMP-2 immunoexpression, its antioxidant and anti-inflammatory actions.

Key word: E-smoking; Aorta; Rat; Melatonin

INTRODUCTION

The electronic cigarette is a nicotine dispensing tool that has been considered to have less harm than traditional tobacco smoking; as the damaging effects of tobacco is produced through its compusion which is not present in electric cigarette. The electronic device is filled with electric cigarette liquid (e-liquid) which is heated by atomiser to produce smoke vapor to be inhaled **[1]**. It was stated that heating process results in decomposition of chemicals with production of new hazardous ones **[2].** The electronic cigarette vapor contains in addition to nicotine; formaldehyde, acetaldehyde, acrolein, acetone and other compounds that have negative impact and harmful effects on human health **[3].** With regard to nicotine content, electric cigarette is considered an unregulated source of nicotine delivery as nicotine amount varies in the e-liquid according to the manufacture and even the concentration may not be labeled on the liquid package and so the smoker can be exposed to fatal

nicotine amounts **[4].** Electric cigarette smoking (E-smoking) was reported to have cytotoxic and inflammatory effects **[5]** and was recorded also to increase airway resistance and diastolic pressure **[6].**

Melatonin, also known as N-acetyl-5-methoxytryptamine, is a hormone secreted by the pineal gland and also extrapineal sites e.g. kidney, liver, thymus. It mainly functions to regulate circadian rhythm. In recent years, melatonin has been reported to play an important role in regulating the function of various organs, being involved in maintaining organs homeostasis and protecting the functional activity under exposure to unfavorable environmental conditions **[7].** Also melatonin was found to have beneficial effects in prevention and improving cardiovascular risk factors and was proposed strongly as a treatment of cardiovasular diseases **[8].** Blood Melatonin concentration was recorded to decrease in smokers **[9].**

As E-smoking has a growing popularity especially among youth **[10],** it is important to investigate its effects on health. Also the harmful effects of E-smoking on lung have been well clarified **[11]** while it is not the case with regard to vascular system. it was declared the need for future studies to evaluate impact of E-smoking on human cardiovascular system **[12].** So in this work we aimed to study effects of E-smoking on the thoracic aorta and investigate the possible protection provided by melatonin against such effect.

METHODS

Equipments

Incense electric Burner (home electric comp., china).

120-L organic glass box

Chemicals:

Electronic cigarette liquid: **(play red bull, Egypt)**. the liquid contains 3 mg/ml nicotine in a 55/45 ratio of propylene glycol and vegetable glycerin with natural and artificial flavoring agents.

Melatonin: 10 mg rapid released capsules (11741, Puritan Pride, Inc. USA).

Animals:

This study was carried out on the animal house of the Faculty of veterinary Medicine, Zagazig University. 40 adult male albino rats weighing 200-250 gm were used. The rats were housed in ventilated wide polypropylene cages under hygienic conditions; Standard food and tap water were freely allowed. Temperature was maintained at 23±2°C. Rats were let for acclimatization to the laboratory conditions for two weeks before the start of the experiment. Animals were handled in accordance with the standard guide for the care and use of the Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC) with a reference number (Zu-IACUC/3/F/178/2022). All animal experiments comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals

Experimental design

The animals were divided equally into four groups (each contains 10 rats) as follows:

- **Control group:** Rats were not exposed to any experimental interventions.
- **Melatonin group:** Rats were treated orally with melatonin in a dose of 5 mg/kg/day dissolved in drinking water for 4 weeks **[13].**
- **E-smoking group:** Rats were exposed to 1ml/day of e-liquid smoke vapor for 1 hour for 5 consecutive days/ week for 4 weeks **[14].**
- **E-smoking + melatonin group:** Rats were exposed to 1ml/day of e-liquid smoke vapor for 1 hour for 5 consecutive days/ week and treated orally with melatonin in a dose of 5 mg/kg/day dissolved in drinking water for 4 weeks.

During exposure to the smoke vapor, rats were place in the organic glass box with the e-liquid applied to the incense electric burner. Also for more caution we placed the groups exposed to smoke vapor; E-smoking and E-smoking + melatonin **groups**, **in** a common chamber separate from the control and melatonin groups.

24 hours after the last exposure, all animals were anesthetized by intra-peritoneal injection of thiopental 75mg/kg **[15]** then blood samples were obtained from the retro-orbital venous plexuses using micro-capillary glass tubes **[16].** The chest wall of the anaesthetized rats were opened and dissected, the thoracic aorta was removed for further histological preparation.

Histological study:

Light microscopic examination

The removed aortic Samples were immediately fixed in 10% buffered neutral formalin. Fixation was maintained for 24 hours. The aortic tissue was dehydrated in ascending grades of alcohol followed by clearing in xylene **[17]**. Serial 5 micrometer (µm) thick sections were obtained and stained with hematoxylin and eosin (H&E) **[18]**, orcein and masson trichrome (MT) stains **[19].**

Immunohistochemical examination

For examination of metalloproteinase 2 (MMP-2) and cluster of differentiation 34 (CD34) immunoexpression, the tissue sections were deparaffinized, Endogenous peroxidases and

unspecific-binding sites were separately blocked with 0.3% H2O2 in methanol for 20 minutes and with 5% BSA for 20 minutes. The sections were incubated in a humidified chamber at 4ºC overnight with either one of the rabbit polyclonal antibody against MMP-2 (Wuhan Borsd Biological Engineering Co Ltd, Wuhan, China) diluted 1:25 and mouse monoclonal against CD34 (DAKO, Carpinteria, USA) diluted 1:50 and were exposed to secondary goat-anti-rabbit antibody, following by incubation with the streptavidin-biotin-peroxidase complex. The reaction products of peroxidase were visualized by incubation with DAB. Sections were counterstained with Mayer hematoxylin **[20].**

The histological slides were examined by the light microscopy (The Leica DM500 microscope, Leica ICC50 W Camera Module) at the Anatomy and Embryology Department, Faculty of Medicine, Zagazig University, Egypt.

Serological study

2mL Blood samples were collected in test tubes, centrifugation of blood was done at 3000 rpm for 10 minutes to separate the serum which was stored at -20° C **[21].** The serum sample was subjected to detection of the following biological markers:

Estimation of Serum nitric oxide level: The serum concentration of nitric oxide (NO) was measured by spectrophotometery according to **Montgomery and Dymock [22]** using Rat total NO Elisa kit (MBS723386, mybiosource.com).

Estimation of Malondialdehyde serum level

The serum concentration of malondialdehyde (MDA) was measured by spectrophotometery according to **Ohkhawa et al. [23]** using rat MDA Elisa kit (MBS268427, mybiosource.com).

Estimation of glutathione peroxidase serum level

The serum concentration of glutathione peroxidase (GPx) was measured by spectrophotometery according to **Paglia and Valentine [24]** using rat GPx1 Elisa kit (MBS3809170, mybiosource.com).

Morphometrical study

Tunica media thickness, optical density of orcein staining, area percentage (%) of collagen fibers and optical density of CD34 and MMP-2 immunoexpressions was measured using Image J analyzing software.

Statistical analysis

Statistical analysis of the collected data from morphological and serological results was assessed by analysis of variance test (ANOVA) and least significant difference (LSD) post hoc test using Spss software version 19. Significance was considered when $P<0.05$. Data were displayed in the form of mean ± standard deviation (SD).

RESULTS

Histological study

The histological structure of the control and melatonin groups was similar in the present study and the statistical analysis of the studied parameters revealed non-significant difference between the two groups **(table 1).** So we displayed the histological photographs of one group of them the control group.

Light microscopic examination

Hematoxyin and Eosin Stained Sections

In the control group examination of hematoxylin and eosin stained sections of the aorta of adult male albino rats of the control group showed the tunica intima formed of single layer of flat endothelial cells. Tunica media formed of parallel and regularly arranged elastic fibers interposed between spindle shaped smooth muscle cells with oval to flat nucleus. Adventitia was formed of loose connective tissue **(Fig.1A).**

In the E-smoking group, the tunica media was markedly decreased in thickness with few irregularly arranged elastic fibers. Vacuolated smooth muscles of the tunica media with pyknotic darkly stained nuclei were seen. The intima exhibited focal thickened areas **(Fig.1B).**

In the E-smoking+melatonin group, histological structure was mostly preserved with regularly arranged elastic fibers in the tunica media. The smooth muscle cells were normal with oval to flat nuclei, but some with vacuolations and pyknotic nuclei were observed. Also still the tunica intima showed thickening. The adventitia appeared normal formed of loose connective tissue. **(Fig.1C).**

A statistical significant ($P<0.001$) decrement in the mean values of the tunica media thickness was reported in the E-smoking group in comparison to the control group. The E-smoking +melatonin group which showed a relative increment in the mean values that was significantly different from the E-smoking ($P<0.001$) and control ($P<0.05$) groups **(Fig.1D & table 1).**

Orcein Stained Sections

Orcein stained section of the thoracic aorta revealed in the control group well defined parallel elastic fibers with dense staining in the tunica media **(Fig.2A).** In E-smoking group, widely separated irregular elastic fibers with relative pale staining were observed in tunica media, also fragmented and atrophied fibers were seen

(Fig.2B). The E-smoking+melatonin group showed less separated well defined parallel elastic fibers with moderate staining, but still some fragmented fibers were observed **(Fig.2C).**

The optical density mean values of orcein staining showed a statistical significant (P<0.001) decrement in the E-smoking group as compared to control group. In the E-smoking+melatonin group an increment in the mean values was reported and found to be significantly different from both E-smoking (P<0.01) and control (P<0.05) groups **(Fig.2D & table 1).**

MT stained Sections

The control group MT-stained sections showed normal amount and distribution of the collagen fibers surrounding well defined elastic lamellae of tunica media **(Fig.3A).** In the E-smoking group excess collagen was observed surrounding poorly defined elastic lamellae of tunica media **(Fig.2B).** The E-smoking+melatonin group showed normal amount of collagen fibers surrounding a somewhat well defined elastic lamellae of tunica media **(Fig.3C).**

Statistical significant increment of the percentage of collagen fibers a (p<0.001) was observed in the E-smoking group as compared to control group. The mean values in the E-smoking +melatonin group showed a decrement that was significantly different (p<0.01) from the smoking group and non significantly different from the control group(p>0.05) **(Fig.3D& table 1).**

Immunohistochemical examination

CD34 immunostained Sections

Examination of the CD34 immunostained sections revealed a negative immunoexpression of CD34 in the adventitia of the control group **(Fig.4A).** In the E-smoking group, a Strong cytoplasmic CD34 immunoexpression was seen in the adventitial cells **(Fig.4B).** While In the E-smoking+melatonin group, there was moderate cytoplasmic immunoexpression in the adventitial cells **(Fig.4C).**

Statistical significant increment of the optical density of CD34 immunoexpression (p<0.001) was observed in the E-smoking group as compared to control group. The mean values in the E-smoking+melatonin group showed a decrement that was significantly different (p<0.001) from the smoking group and non-significantly different from the control group (p>0.05) **(Fig.4D& table 1).**

MMP-2 immunostained Sections

MMP-2 immunostained sections of the control group revealed negative MMP-2 immunoexpression in the nuclei of the smooth muscle cells and weak nuclear MMP-2 immunoexpression in the intimal cells **(Fig.5A).** In the E-smoking group, a strong nuclear MMP-2 immunoexpression was observed in the smooth muscle and intimal cells **(Fig.5B).** While In the E-smoking+melatonin group negative nuclear MMP-2 immunoexpression in the smooth muscle cells and moderate nuclear MMP-2 immunoexpression in the intimal cells were seen**(Fig.5C).**

Statistical significant increment of the optical density of MMP-2 immunoexpression (p<0.001) was observed in the E-smoking group as compared to control group. The mean values in the E-smoking+melatonin group showed a decrement that was significantly different from both the smoking group (p<0.01) and the control group(p<0.05) **(Fig.5D& table 1).**

Serological study

NO serum level showed a statistical significant increment in the E-smoking group (p<0.001) as compared to control group. A decrement in the serum level was observed in the E-smoking + melatonin group that was significantly different (p<0.001) from the smoking group and non significantly different from the control group (p>0.05). **(Table 1)**

A statistical significant (p<0.001) increment in MDA and decrement in GPx serum levels was observed in E-smoking group in comparison to control group. An increment in the serum level of GPx was observed in the E-smoking + melatonin group that was significantly different from both the E-smoking group(p<0.001) and control group(p<0.01). **(Table 1)**

Table (1) Statistical comparison by by ANOVA and LSD post hoc test of the of tunica media thickness (µm), optical density of orcein staining area% of collagen fibers and, optical density of CD34 & MMP-2 immunoexpression

Parameters	Control (mean±SD)	Melatonin (mean±SD)	E-smoking (mean±SD)	E-smoking+melatonin (mean±SD)	F	P
Tunica media thickness(um)	112.14±5.17	111.36±4.91	61.23±5.82 ***	102.17±7.01 */###	104.35	<0.001
Optical density of orcein staining	0.74±0.07	0.73±0.07*ns	0.45±0.11 ***	0.61±0.02 */##	20.82	<0.001

Parameters	Control (mean±SD)	Melatonin (mean±SD)	E-smoking (mean±SD)	E-smoking+melatonin (mean±SD)	F	P
Area % of collagen fibers	39.60±1.69	40.09±2.71^{*ns}	50.23±4.67 ***	41.82±6.70 $^{*ns/\#\#}$	7.56	<0.001
Optical density of CD34 immunoexpression	0.18± .05	0.19±0.04^{*ns}	0.37±0.06***	0.22 ±0.04$^{*ns/\#\#\#}$	20.37	<0.001
Optical density of MMP-2 immunoexpression	0.19±0.02	0.17±0.01$^{* ns}$	0.30±0.06***	0.23±0.05$^{*/\#\#}$	14.19	<0.001
NO serum level (µmol/l)	46.97± 7.67	44.51±8.97^{*ns}	72.23±6.27***	42.24±9.57$^{*ns/\#\#\#}$	17.31	<0.001
MDA srum level (nmol /ml)	0.61± 0.12	0.63±0.11^{*ns}	1.58±0.27***	0.90±0.07$^{**/\#\#\#}$	47.80	<0.001
GPx serum level (U/ml)	181.25±31.34	175.50±25.30 ^{*ns}	68.34±13.68***	128.87±10.52$^{**/\#\#\#}$	34.12	<0.001

(*)comparison in relation to control group, (#)comparison in relation to smoking group. *ns: P>0.05, *: sig<0.05, **: sig<0.01 ***: sig<0.001, ##: P<0.01,###: P<0.001.

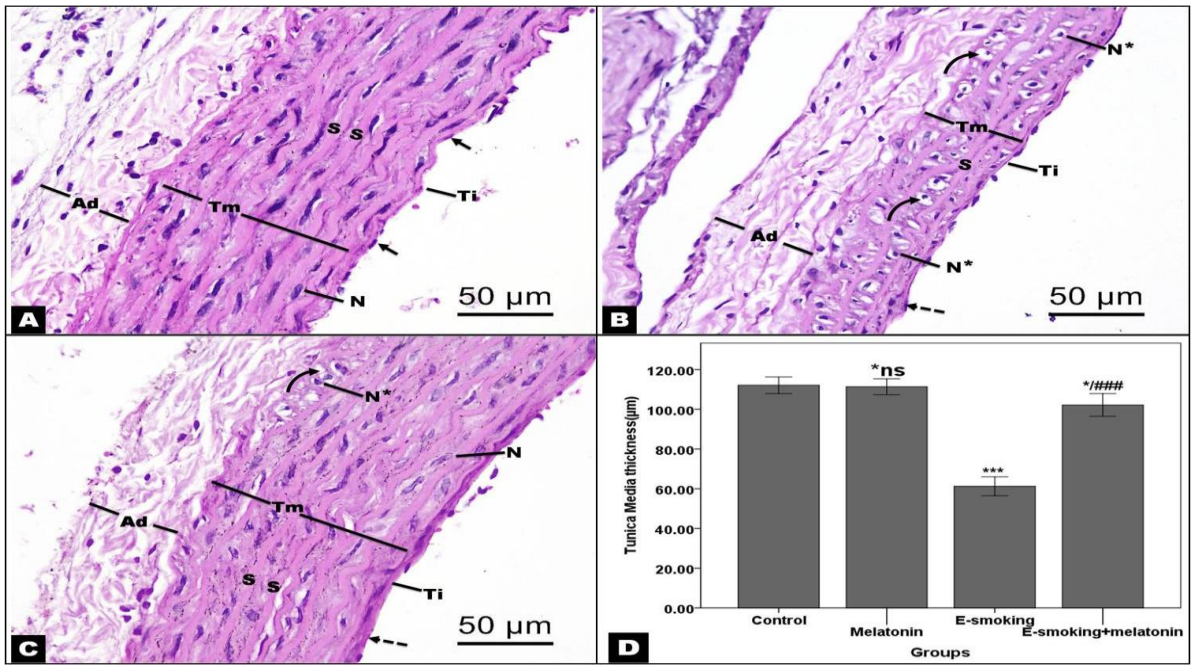

Figure 1. A-C: H&E stained section (x400, scale bar 50µm) of male albino rat thoracic aorta from different studied groups. **A: Control group** showing tunica intima (Ti) formed of single layer of flat endothelial cells (arrow). Tunica media (Tm) formed of parallel and regularly arranged elastic fibers(S) interposed between spindle shaped smooth muscle cells with oval to flat nucleus (N). Adventitia (Ad) is formed of loose connective tissue. **B: E-smoking group** showing tunica media (Tm) markedly decreased in thickness with few irregularly arranged elastic fibers (S). Vacuolated Smooth muscles (curved arrow) with pyknotic darkly stained nuclei (N*) are seen. Tunica intima (Ti) exhibited focal thickened areas (dotted arrow). Adventitia (Ad) formed of loose connective tissue. **C: E-smoking + melatonin group** mostly showing preserved histological structure; regularly arranged elastic fibers(S)in the tunica media(Tm). The smooth muscle cells are normal with oval to flat nuclei (N), but some with vacuolations (curved arrow) and pyknotic nuclei (N*) are still observed. The tunica intima (Ti) shows thickening (dotted arrow). Adventitia (Ad) formed of loose connective tissue. **D:** Bar chart showing statistical analysis by ANOVA and LSD post hoc test of the tunica media thickness means values among studied groups. (*): Comparison in relation to control group, (#):Comparison in relation to smoking group.*ns: P>0.05, *: sig<0.05, ***: sig<0.001, ###: P<0.001.

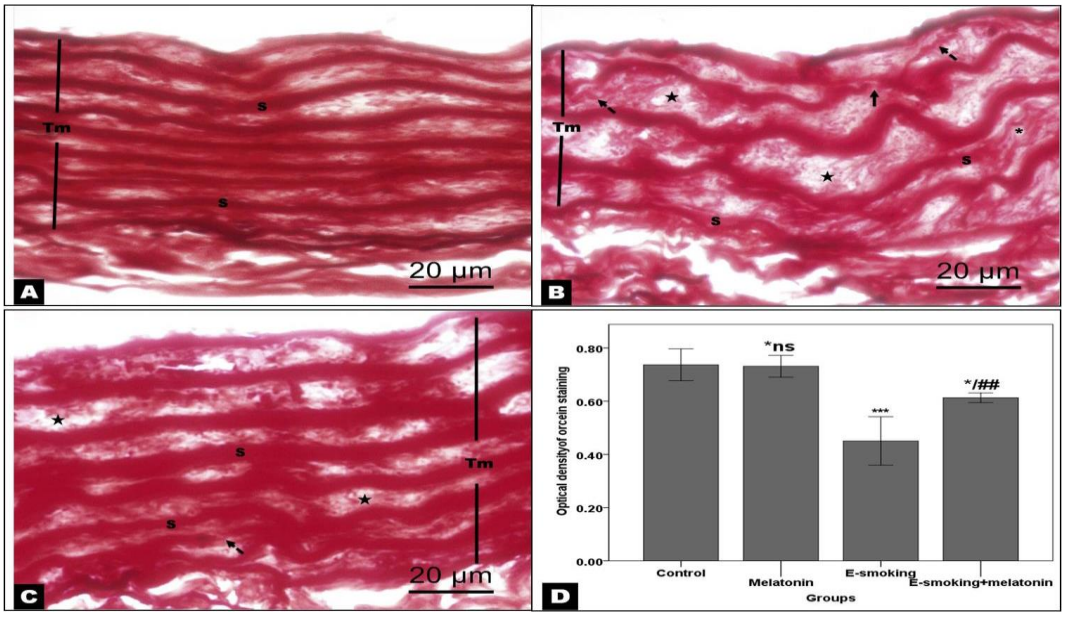

Figure 2. A-C: Orcein stained (x1000, scale, bar 20µm) section of male albino rat thoracic aorta from different studied groups. **A: Control group** showing well defined parallel elastic fibers (S) with dense staining in the tunica media (Tm). **B: E-smoking group** showing widely separated (star) irregular elastic fibers (S) and relative pale staining in the tunica media (Tm), fragmented (arrow) and atrophied (dotted arrow) fibers. **C:** E-**smoking+melatonin group** showing less separated (star), well defined parallel elastic fibers (S) with moderate staining in the tunica media (Tm), still some fragmented fibers (dotted arrow) are observed. **D: Bar chart** showing statistical analysis by ANOVA and LSD post hoc test of the orcein staining optical density mean values among studied groups. (*): Comparison in relation to control group, (#):Comparison in relation to smoking group.*ns: P>0.05, *: sig<0.05, ***: sig<0.001, ##: P<0.01.

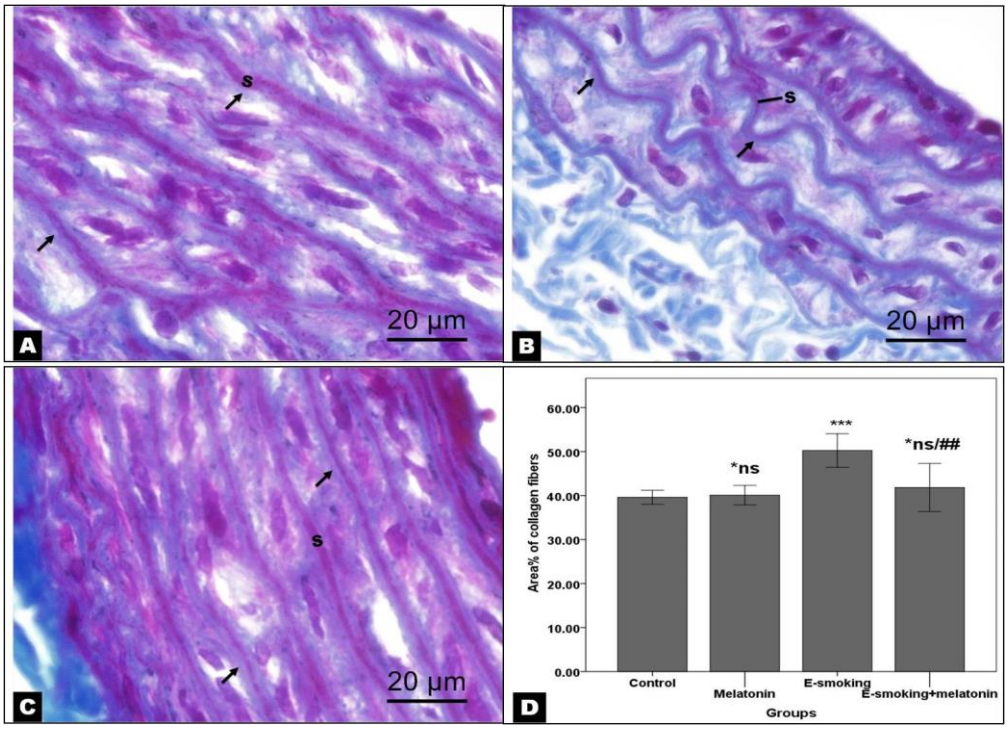

Figure 3. A-C: MT- stained section (x1000, scale bar 20µm) of male albino rat thoracic aorta from different studied groups. **A: control group** showing normal amount and distribution of the collagen fibers (arrow) surrounding well defined elastic lamellae (S). **B: E-Smoking group showing** excess collagen (arrow) surrounding poorly defined elastic lamellae (S). **C: E-smoking+melatonin group showing** normal amount of collagen fibers (arrow) surrounding a somewhat well defined elastic lamellae (S). **D: Bar chart** showing statistical analysis of the area % of collagen fibers by ANOVA and LSD post hoc test. (*):Comparison in relation to control group, (#):Comparison in relation to smoking group.*ns: P>0.05, ***: sig<0.001, ##: P<0.01.

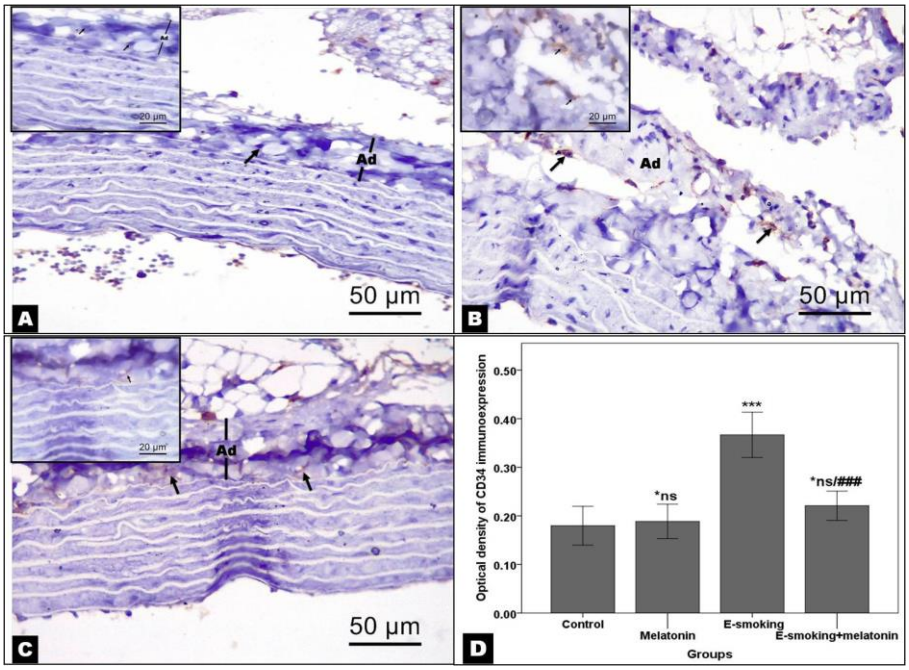

Figure 4. A-C: CD34- immunostained section (x400, scale bar 50µm; inset photos showing a higher magnifications x 1000, scale bar 20µm) of male albino rat thoracic aorta from different studied groups. **A: Control group** showing negative immunoexpression of CD34 (arrow) in the adventitia (Ad). **B: E-Smoking group** showing a Strong cytoplasmic CD34 immunoexpression in the adventitial cells (arrow), adventitia (Ad). **C: E-smoking+melatonin group** showing moderate cytoplasmic CD34 immunoexpression in the adventitial cells (arrow), adventitia (Ad). **D: Bar chart** showing statistical analysis of the optical density of CD34 immunoexpression by ANOVA and LSD post hoc test: (*): Comparison in relation to control group, (#): Comparison in relation to smoking group.*ns: P>0.05, ***: sig<0.001, ###: P<0.001.

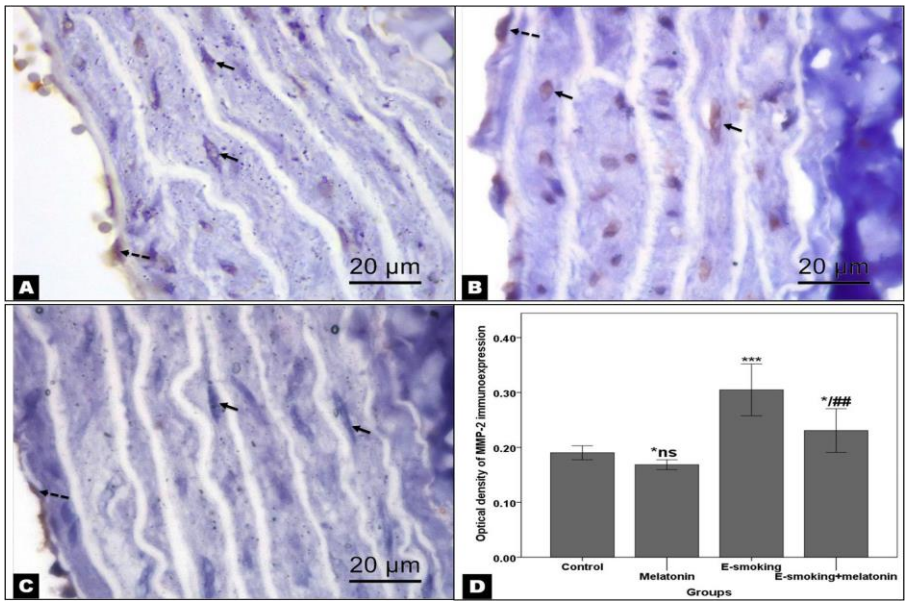

Figure 5. A-C: MMP-2- immunostained section (x1000, scale bar 20µm) of male albino rat thoracic aorta from different studied groups. **A: Control group** showing negative MMP-2 immunoexpression in the nuclei of the smooth muscle cells (arrow) and weak MMP-2 nuclear immunoexpression in the intimal cells (dotted arrow). **B: E-Smoking group** showing a strong nuclear MMP-2 immunoexpression in the smooth muscle (arrow) and intimal cells (dotted arrow). **C: E-smoking+melatonin group** showing negative MMP-2 immunoexpression in the smooth muscle cells (arrow) and moderate nuclear MMP-2 immunoexpression in the intimal cells (dotted arrrow). **D: Bar chart** showing statistical analysis of the optical density of MMP-2 immunoexpression by ANOVA and LSD post hoc test: (*):Comparison in relation to control group, (#):Comparison in relation to smoking group.*ns: P>0.05, *: sig<0.05, ***: sig<0.001, ##: P<0.01.

DISCUSSION

The present work studied the possible protection provided by melatonin against the effects of E-smoking on thoracic aorta of albino rats. We used male rats as the nicotine sensitivity was reported by several studies to show sex differences being more in females **[25],** also the period of the study was 30 days which corresponds to 3 years of human exposure **[26].**

The current histological study revealed in the E-smoking group vacuolations of the smooth muscle cells irregularly arranged elastic fibers with marked decrease in the tunica media thickness that was proved to be significantly different from the control group. Our results goes with **Barão et al. [27]** who observed that cigarette smoking induced degenerative effects in the tunica media in the form of cellular loss and degraded elastic fibers.

The pale orcein stained, fragmented and atrophied elastic fibers observed in the E-smoking group suggests marked decrease in elastin that was confirmed by statistical comparison of the optical density of orcein staining among different groups. Though smoking period in this study does not provide a chronic exposure, the results goes with that of **Wagenhäuser et al. [28]** who stated that chronic exposure to nicotine induces aortic stiffness, elastic fibers fragmentation and decrease in total elastin content predisposing to development of aneurysm. **Ikonomidis et al. [29]** reported that electronic smoking increased arterial stiffness in acute and chronic exposure but to less extent than traditional smoking. According to **Barão et al. [27]** the first changes that specify vascular wall degeneration in aneurysmal dilatation is the degradation of elastin with subsequent disturbance of the normal structural integrity and development of aneurysm. Also elastic fiber disruption and fragmentation is a pathological finding in aortic aneurysm**[30]**

We observed in the E-smoking+melatonin group that most of the histological structure was preserved. Melatonin was reported to decrease aortic stiffness, inhibit elastin breakdown and macrophage infiltration, prevent smooth muscle loss thus improving aortic morphology and decreasing incidence of aneurysmal dilatation **[31].**

We observed in the control group a normal amount and distribution of the collagen fibers surrounding well defined elastic lamellae of tunica media. Collagen is an important component of the extracellular matrix together with elastin they determine the aorta tensile strength. Its amount has been found to change by age and pathological conditions such as aortic aneurysm **[32].**

Our results revealed in the E-smoking group a relative excess collagen in tunica media of the aortic wall. **Al Hariri et al.[33]** reported that cigarette smoking induced inflammatory reaction might stimulate markers of fibrosis, but **Kugo et al.[34]** stated that nicotine promoted the destruction of elastin and collagen in aorta inducing aneurysmal changes**.** It was reported that collagen amount is increased in aortic aneurysm while its concentration is decreased**[35].** According to **Carmo et al. [36]** the relative increase in collagen proportion can be due to the decrease in elastin content. This go with our results as we observed the excess collagen surrounding poorly defined elastic lamina in the E-smoking group. Also the area % of collagen fibers showed statistical significant increment in relation to control group.

little collagen was observed surrounding a somewhat well defined elastic lamellae in the E-smoking+melatonin group, this is in accordance with **Tang et al. [37]** who reported that melatonin decreased collagen deposition in the vessel wall. **Repová-Bednárová et al. [38]** stated that the antifibrotic effects of melatonin are suggested to reduce stiffness of the aortic wall and the small arteries with subsequent beneficial effects on the pulse wave and peripheral vascular resistance. Melatonin was found to inhibit fibrotic reactions in response to smoking by down regulating transforming growth factor β1 (TGF-β1) **[39].**

Strong CD34 immunoexpression in adventitial cells was observed in the E-smoking group with significant increment in the optical density of CD34 immunoexpression as compared to control group. **Sakata et al. [40]** stated that CD34 is expressed excessively in inflammatory aortic aneurysm with a distribution confined to the adventitia and its surrounding tissue. Vascular CD34 has the ability to stimulate recruitment of inflammatory cells into peripheral tissue [**41].** Our results suggest an inflammatory state**.**

Mekala et al. [42] recorded that $CD34^+$ cells were preferentially found within the vascular adventitia. In fact the adventitia is full of various types of progenitor and stem cells including CD34 positive cells that can form endothelial and smooth muscle cells **[43].** In case of vascular injury, adventitial CD34 positive progenitor cells were found by **Shen et al. [44]** to undergo

differentiation into smooth muscle like cells that were added to the outer layer of tunica media. Smoking was reported by **Burke and Fitzgerald [45]** to induce vascular injury through oxidative stress. CD34 is a marker of vascular injury**[46].** We suggest that the adventitial cells with positive CD34 immunoexpression observed in E-smoking group are to compensate for the structural changes observed in tunica media of the E-smoking group.
Weak CD34 immunoexpression was observed in the E-smoking+melatonin group. Melatonin was reported to provide protection against both smoking induced vascular injury **[47]** and nicotine related aortic aneurysm **[48].**
We observed MMP-2 immmunoexpression in the control group to be negative in the smooth muscle cells and weak in the intimal cells. MMP-2 is a member of the Metalloproteinases (MMPs) family which are proteolytic enzymes that function to degrade extracellular components **[49].** MMP-2 is expressed by cardiomyocytes, endothelial cells, vascular smooth muscle cells, macrophages, and fibroblasts **[50].** It was found also to be expressed by resident mesenchymal cells even in normal aortic tissue **[51].**
In the present study strong MMP-2 immunoexpression was seen in smooth muscles and endothelial cells of the E-smoking group. The optical density of the immunoexpression showed significant increment as compared to control group. **Wagenhäuser et al. [28]** reported that smoking increased activity of MMPs**.** They recorded that Smokers have higher levels of MMP-2 than non smokers. High MMPs levels caused degradation of the elastic fibers and weakness of the vessel wall with resultant changes in the biomechanical properties of the vascular wall leading to aneurysmal dilatation. In fact, Smoking has a strong association with aortic aneurysm**[52]** and MMP-2 is critical for aneurysm pathogenesis **[51].** In contrary, **Kugo et al. [34]** reported no change in MMP-2 level with nicotine administration. However **Ning et al. [53]** reported that Cigarette Smoke was found to Stimulates production and Activity of MMP-2 involving a transcription factor: early growth response 1 (EGR-1) in human lung fibroblasts. According to **Mulorz et al. [54]** e-cigarette vapor alone without nicotine produced acceleration of aortic aneurysm in mice and upregulation of involved gens in aneurysm pathology including MMP-2 and interleukin-6. Also MMPs expression and activity increased in cases of tissue injury and inflammation **[55].** Inflammatory state was recorded in our work manifested by increased CD34 immunoexpression.
We observed moderate MMP-2 immunoexpression in the E-smoking+melatonin group with a significant decrement in the optical density of MMP-2 immunoexpression as compared to E-smoking group. This is in accordance with **Duan et al. [48]** who reported that melatonin reversed protein expression induced by nicotine with susbsequent protection against development of aortic aneurysm. Melatonin was found to suppress EGR-1 expression **[56].**
In the present work a statistical significant increment in NO serum levels was reported in the E-smoking group. this is in accordance with **Ghasemi et al. [57]** who reported a higher level of No in serum of active smokers as compared to non smokers. NO is a highly reactive molecule that is involved in regulation of several vascular processes such as vessel wall relaxation, vessel tone and blood pressure in addition to its antioxidant and antiinflammatory actions. Unfortunately beside these beneficial actions, no one can have toxic effects depending on the concentration. Its elevation can cause proinflammatory and prooxidative effects, tissue damage and apoptosis **[58].** In addition NO was found to induce development and progression of vascular aneurysm **[59].**
In the E-smoking+melatonin group a significant decrement was reported in NO serum level as compared to E-smoking group. Melatonin decreases NO production by endothelial cells involving the inhibition of intracellular calcium mobilization **[60].** Low levels of melatonin are reported to be associated with increased nitric oxide production **[61].**
We observed a decrement in GPx and increment in MDA serum levels in the E-smoking group suggesting a state of oxidative stress. This is in accordance with the results **Mandour et al. [62]. Safyudin and Subandrate [63]** stated that smoking is associated with oxidative stress induction increasing local and system levels of MDA and decreasing GPx levels. In addition oxidative stress is linked to pathogenesis of aortic aneurysm; it contributes to inflammatory process and upregulation of MMPs with resultant degradation of extracellular matrix and Induction of smooth muscle apoptosis **[42].** High MDA serum levels **[64]** and decreased activity of GPx **[65]** were recorded in aortic aneurysm patients**;** in fact MDA level is considered a risk and a predictor of aneurysm formation **[66].**
A significant increment in GPx and decrement in MDA serum levels were observed in the E-smoking + melatonin group as compared to the E-

smoking group revealing an improvement of the oxidative status. Melatonin can scavenge free radicals, stimulates antioxidative enzymes and augments antioxidants efficiency **[67].** In fact melatonin level has positive correlation with the antioxidant capacity and it was found to provide protection against nicotine related aortic aneurysm [48].

Our results revealed histological, immuohistochemical and biochemical changes that are part of aortic aneurysm pathology. These go with **Hage et al. [68]** who reported that the histological effects induced by E-smoking were similar to that of aneurymsal changes. In our study melatonin provided a protection against such effects preserving normal histological structure.

We can conclude that E-smoking for 1 month in albino rat is sufficient to initiate aneurysmal pathological changes in the aortic wall. Melatonin provided a great protection interfering with such changes through its inhibition of MMP-2 immunoexpression, its antioxidant and anti-inflammatory actions.

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