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Original Article

The effect of ozone on blood pressure in DOCA-salt-induced hypertensive rats

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Abstract: Background: Hypertension is a risk factor for the cardiovascular diseases. Ozone as a therapeutic agent for the treatment of several disorders. We aimed to observe the effects of ozone on the blood pressure in DOCA-salt hypertensive rats. Methods: Twenty three young Sprague Dawley male rats were divided into three groups; Control (C), Hypertension (H) and Hypertension + Ozone (HO). Hypertension was induced by injection of DOCA-salt (25 mg/kg, s.c.) twice weekly, 4 weeks, whereas intraperitoneal ozone was administered (1.1 mg/kg) for 10 days. Serum endothelin-1, nitric oxide and renin levels were measured with ELISA. Blood pressures were monitored using a tail cuff system. Endothelin-1, ET receptor A and ET receptor B mRNA expression in heart and vascular tissue were assessed by quantitative reverse transcription polymerase chain reaction. Results: Blood pressure, serum endothelin-1 and ET receptor A mRNA expression levels were increased in H group, whereas serum renin, nitric oxide and ET receptor B mRNA expression levels in the heart and vascular tissue decreased compared with C and HO groups, which were counteracted by ozone treatment. Conclusion: Ozone treatment decreases blood pressure and is effective in preventing the progression of hypertensive disease, the mechanisms of which are associated with anti-vasoconstrictor effects through reducing the levels of serum endothelin-1 and ET receptor A mRNA expression in the heart and vascular tissue.

Keywords: DOCA-salt, hypertension, ozone, blood pressure, endothelin-1

Introduction

Cardiovascular disease is a major health problem across the world, accounting for 30% of all deaths Hypertension is a major risk factor for cardiovascular diseases and a majör healthcare problem [1], the mechanisms for the progression of higher blood pressure are still not completely clarified. Deoxycorticosterone acetate (DOCA) is an agent commonly used to induce hypertension in experimental animals. The DOCA-salt hypertensive rat model shows a markedly depressed renin angiotensin system and thus has been regarded as an angiotensin-independent model with decreased circulating plasma renin concentrations [2].

Ozone as a therapeutic agent for the treatment of several disorders [3]. Ozone therapy stimulates the antioxidant response in cardiomyopathy patients [4]. Ozone has been shown to reduce blood pressure in a diabetic population [5]. Ozone exposure may influence autonomic regulation of the heart rate and has been shown to alter circulating inflammatory and fibrinolytic markers in healthy persons [6, 7].

The effect of ozone application on blood pressure in the hypertensive rats has not been explored. Ozone has not been studied in hypertension rats with DOCA-salt hypertension model before. Therefore, the main purpose of this work is to determine the role of ozone administration in ameliorating blood pressure in DOCA-salt-induced hypertensive rats so as to establish its potential use in the strategy for the treatment of hypertensive patients.

Methods

Animals

Twenty three adult male Sprague Dawley rats (Dumlupınar University Experimental Animal Laboratory, Kütahya, Turkey) weighing 250-300

Table 1. Primers used for PCR reactions

Probe	Orientation	Sequence	Length (bp)
β-actin	Forward	5'-CTATCGGCAATGAGCGGTTCC-3'	147
	Reverse	5'-TGTGTTGGCATAGAGGTCTTTACG-3'	
ET-1	Forward	5'-GCTCCTGCTCCTCCTTGATG-3'	500
	Reverse	5'-CTCGCTCTATGTAAGTCATGG-3'	
ETR-A	Forward	5'-CCTCATGACCTGTGAGATGCTC-3'	448
	Reverse	5'-CATGCTGTCCTTGTGGCTGC-3'	
ETR-B	Forward	5'-CAGGAGCAAGCTGCAACATGC-3'	546
	Reverse	5'-CCAGCTTGCACATCTCAGCTCC-3'	

Table 2. The levels of plasma ET-1, renin and NO in Control (C), Hypertension (H) and Hypertension + Ozone (HO) groups

Groups	C (n=7)	H (n=8)	HO (n=8)	P
ET-1 (pg/mL)	$20.9 \pm 1.24^{a,b}$	$29.8 \pm 3.95^{a,c}$	$18.2 \pm 0.51^{b,c}$	0.000
Renin (mU/mL)	38.6 ± 1.44^{a}	$26.0 \pm 0.58^{a,b}$	38.7 ± 0.96^{b}	0.001
NO (nmol/μL)	1.97 ± 0.14^{a}	$1.12 \pm 0.25^{a,b}$	2.29 ± 0.32^{b}	0.040

p: Shows the differences between all groups (Kruskal Wallis test). a, b, c: In each line, the difference between the means with same letters are significant, $P \leq 0.05$ (Mann-Whitney U test). ET-1, Endothelin-1; NO, Nitric oxide.

g were used. All rats were kept under environmentally controlled conditions in an air-conditioned room at 21°C, with appropriate humidity and a 12 h:12 h light:dark cycle and were fed standard rat chow and water ad libitum. The animals were acclimatized to the laboratory condition five days prior to behavioral study and were maintained in the laboratory until the completion of the study. This project was approved by the Dumlupınar University Ethics Committee of Animal Care and Usage, Kütahya, Turkey.

Experimental design

The male animals were selected randomly and divided into three experimental groups: control (C, n=7), Hypertension (H, n=8), Hypertension + Ozone (HO, n=8). Hypertension was induced by DOCA-salt treatment as previously described [8]. DOCA was injected (25 mg/kg of body weight in 0.4 ml of dimethylformamide subcutaneously) twice weekly for 4 weeks, and tap water for drinking was replaced by 1% NaCl during the treatment period. In control groups, the same volume of serum physiologic was injected.

Ozone application

Ozone (O_3) generated by the ozone generator (Humazon® ProMedic-Humares GmbH, Germany). The O_3 flow rate was kept constant at 3 L/min representing concentration of 50 μg/ml and about 3% of the O_3/O_2 gas mixture. The rats of HO group were treated with O_3/O_2 mixture at 1.1 mg/kg BW via intraperitoneal route [9] for 10 days Tygon polymer tubes and single-use silicontreated polypropylene syringes (ozone resistant) were used throughout the reaction to ensure containment of O_3 and consistency of concentrations.

Blood pressure measurement

To confirm the induction of hypertension, blood pressures was measured in conscious rats by the indirect tail cuff method, using a Biopac Student Lab PRO 3.7 software (Model No. MP36, AD Instruments Co.) and pneumatic pulse transducer (MAY NIBP200-A, Ankara, Turkey) before DOCA treatment, on the 28th days of DOCA treatment and after ozone treatment. The rat was kept in plastic restrainer (Kursunluoglu Metal Co., Denizli, Turkey) and the tail cuff was applied on the tail of rat for determination of blood pressure. Normal blood pressures of all the rats were recorded as baseline blood pressure. Systolic blood pressure (SBP), Diastolic blood pressure (DBP) and mean arterial blood pressure (MAP) were estimated from recorded graphs. All measurements were performed without anaesthesia at room temperature in a silent room.

Tissue preparation and blood sampling

At the end of the experimental period, all of the animals were anesthetized with Ketamin/Xylazine HCl (75 mg/kg/10 mg/kg intraperitoneally). Blood sample was collected from the aorta in a glass tube to clot for 2 h at room temperature and centrifuged for 20 min to obtain serum samples for endothelin-1 (ET-1), renin and nitric oxide (NO) analysis. Serum were stored at -80°C. Heart and vascular tissue samples were then collected in liquid nitrogen for analysis of molecular biologic studies, and stored at -80°C until analysis.

Biochemical analyses

Serum concentrations of ET-1, renin were analyzed with rat ELISA assay kit using the chemiluminescence method (Boster, Valley Ave and

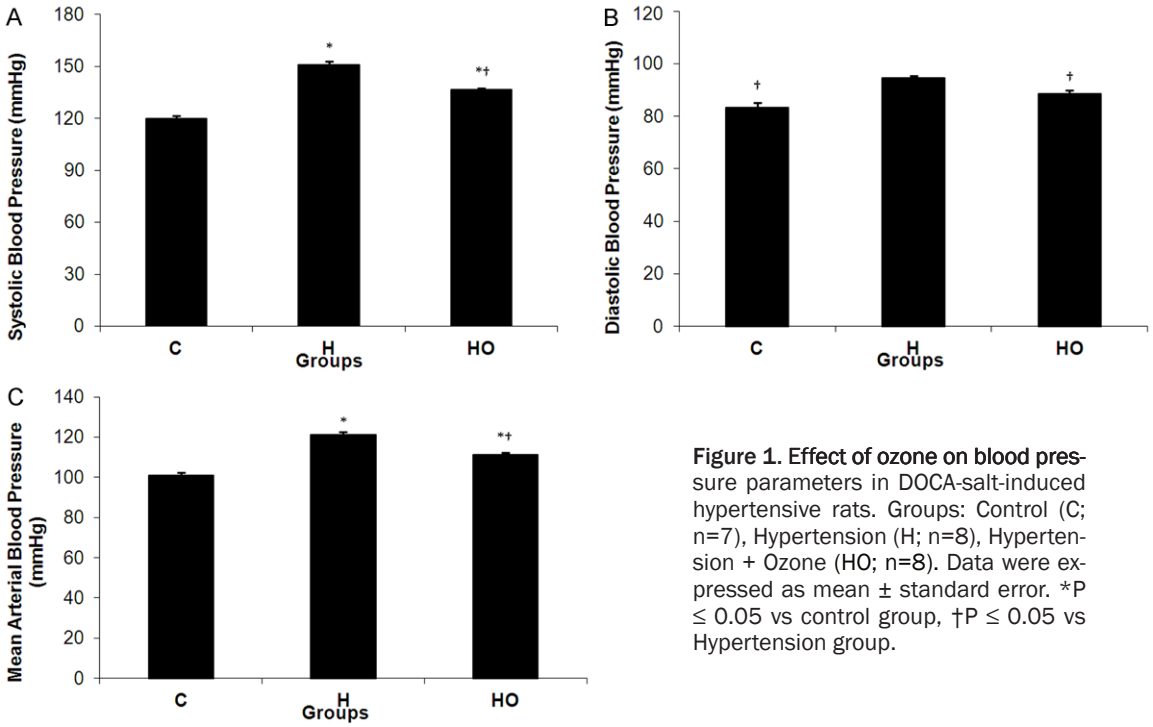

Figure 1. Effect of ozone on blood pressure parameters in DOCA-salt-induced hypertensive rats. Groups: Control (C; n=7), Hypertension (H; n=8), Hypertension + Ozone (HO; n=8). Data were expressed as mean ± standard error. *P ≤ 0.05 vs control group, †P ≤ 0.05 vs Hypertension group.

Cusabio, China). The level of NO in serum was evaluated by enzymatic colorimetric method using a commercial standard enzymatic assay kit (Biovision, USA) according to the instruction of manufacturer by an ELISA microplate reader (Thermo Multiscan GO, 1510, Finland).

Real-Time polymerase chain reaction (RT-PCR)

Total RNA isolation was extracted from heart and vascular tissue samples by the GeneJET RNA Purification Kit (Thermo, Cat No: #K0732) according to the manufacturer's protocol. Total mRNA concentrations were measured at 260 nm using a Maestro Nano Micro-Volume spectrophotometer (Maestrogen Inc., Las Vegas, NV). Samples were stored at -80°C until further analysis. Complementary DNAs (cDNA) were synthesized by EasyScript™ cDNA Synthesis Kit (abm) and were stored at -20°C until used in the real-time polymerase chain reaction.

A total of 20 µL of PCR mixture containing cDNA template (equivalent to 20 ng total RNA), PCR-grade H_2O, 10 µM each of forward primer, reverse primer set as mentioned in **Table 1** and EvaGreen 2X qPCR Master Mix (abm) was amplified by using an LightCycler 480 Real Time PCR System (Roche Diagnostics, Germany) with an initial melt at 95°C for 10 min followed by 35 cycles at 95°C for 15 s, 58°C for 60 s and 72°C for 20 s. The number of amplification steps required to reach an arbitrary intensity threshold (C_t) was computed. The relative gene expression was represented as 2 ($-\Delta C_t$), where ΔC_t=Cttarget-Ctβ-actin. Fold change for the treatment was calculated as $2^{-\Delta\Delta}C_t$, where $\Delta\Delta C_t$=ΔCttreatment-ΔCtvehicle.

Statistical analysis

Statistical analysis was done with SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) 16.0 pocket program. All results were given as means ± standard error (SE). Comparisons among multiple groups were done with Kruskal-Wallis test, and between two groups were with Mann-Whitney U test. Values smaller than P ≤ 0.05 were accepted as statistically significant.

Results

Serum ET-1

There were statistically significant differences in serum ET-1 levels among the groups of C, H and HO (P=0.000). The levels of serum ET-1 in the H group compared with the C and HO groups were found to be significantly higher, P=0.004 and P=0.000. In addition, the low levels of serum ET-1 were found in the HO groups compared with C group, P=0.040 (**Table 2**).

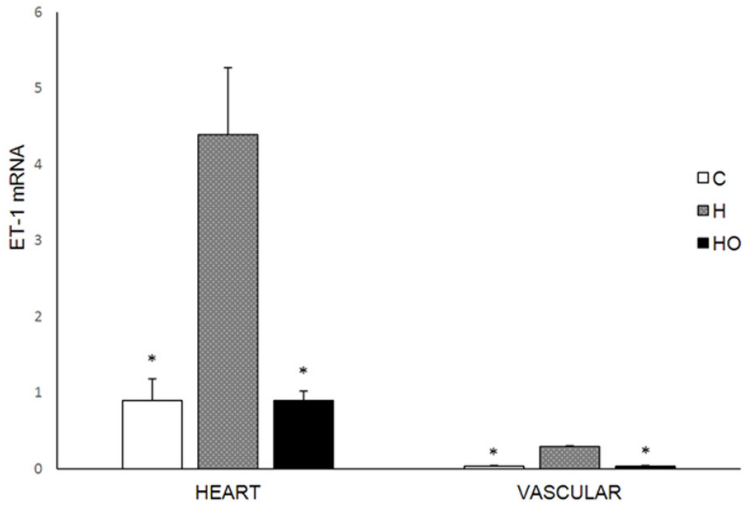

Figure 2. Effect of ozone on mRNA expression of ET-1 in the heart and vascular tissues of DOCA-salt-induced hypertensive rats. Groups: Control (C; n=6), Hypertension (H; n=6), Hypertension + Ozone (HO; n=6). Data were expressed as mean ± standard error. *P ≤ 0.05 vs Hypertension group.

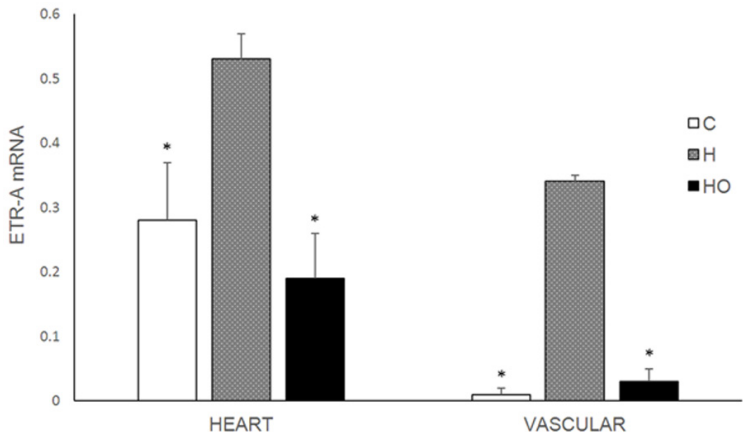

Figure 3. Effect of ozone on mRNA expression of ETR-A in the heart and vascular tissues of DOCA-salt-induced hypertensive rats. Groups: Control (C; n=6), Hypertension (H; n=6), Hypertension + Ozone (HO; n=6). Data were expressed as mean ± standard error. *P ≤ 0.05 vs Hypertension group.

Serum renin

Significant differences were observed in the levels of serum renin among the groups of C, H and HO (P=0.001). It was observed that decreased in serum renin level of the H group compared with the C and HO groups was statistically significant (P=0.000 and P=0.000) (**Table 2**).

Serum no

Serum NO levels differed a significantly among the groups of C, H and HO (P=0.040). The low levels of serum NO were found in H group compared with C and HO groups, P=0.029 and P=0.038 (**Table 2**).

Systolic blood pressures (SBP), Diastolic blood pressures (DBP) and mean arterial blood pressures (MAP)

In this study, any statistically significant difference in systolic blood pressures, diastolic blood pressures and mean arterial blood pressures of rats in all the groups before DOCA treatment could not be observed (data not shown).

At the end of the experiment, SBP, DBP and MAP levels were observed to be statistically significantly different among the groups of C, H and HO (P=0.000 and P=0.001). Four weeks after treatment with DOCA-salt, SBP was significantly higher in H groups given DOCA-salt (150.9 ± 1.96 mmHg) than C (119.9 ± 1.43 mmHg) and HO groups (136.4 ± 0.75 mmHg) (P=0.000). In addition, the high levels of SBP were found in HO group compared with C groups, P=0.000 (**Figure 1A**). Similarly, DBP was significantly higher in H group (94.7 ± 0.74 mmHg) than C (83.3 ± 1.92 mmHg) and HO groups (88.5 ± 1.38 mmHg) (P=0.001) (**Figure 1B**). MAP was significantly higher in H group (121.3 ± 1.11 mmHg) than C (101.0 ± 1.24 mmHg) and HO groups (111.1 ± 1.09 mmHg) (P ≤ 0.05). In addition, a higher level of MAP was found in the HO group than in the C group (P=0.001) (**Figure 1C**).

Levels of ET-1, ET receptor A (ETR-A) and ET receptor B (ETR-B) gene mRNA Expression in the heart and vascular tissue

ET-1, ETR-A and ETR-B gene mRNA expression levels were determined as the relative according to β-*actin* gene mRNA levels in the heart and vascular tissue samples of the groups in

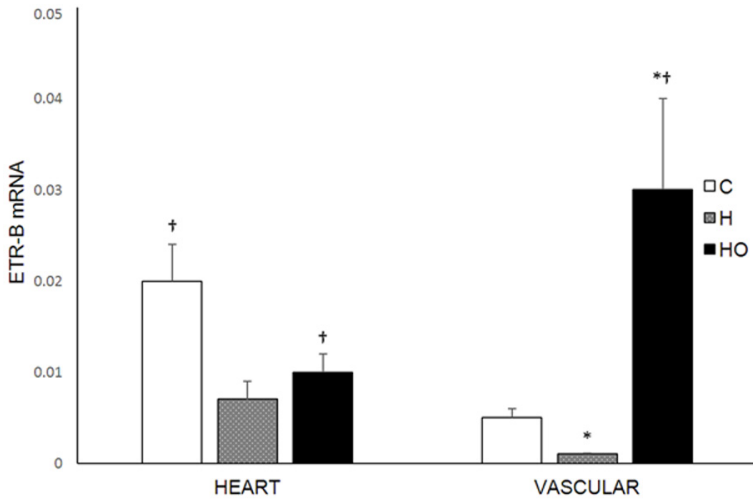

Figure 4. Effect of ozone on mRNA expression of ETR-B in the heart and vascular tissues of DOCA-salt-induced hypertensive rats. Groups: Control (C; n=6), Hypertension (H; n=6), Hypertension + Ozone (HO; n=6). Data were expressed as mean ± standard error. *P ≤ 0.05 vs control group, †P ≤ 0.05 vs Hypertension group.

this study. The differences in the *ET-1* gene mRNA expression levels in the heart tissue among C (0.89 ± 0.29), H (4.39 ± 0.88), HO (0.89 ± 0.13) groups and in the vascular tissue among C (0.03 ± 0.01), H (0.29 ± 0.009), HO (0.03 ± 0.01) groups were significant. (P=0.004 and P=0.003 respectively) In addition, the levels of *ET-1* gene mRNA expression in the H group were significantly higher than in C and HO groups in the heart and vascular tissues (P=0.004 and P=0.002) (**Figure 2**).

There were significant differences in *ETR-A* gene mRNA expression levels in the heart tissue among C (0.09 ± 0.005), H (0.06 ± 0.01), HO (0.03 ± 0.01) groups, (P=0.032) and in the vascular tissue among C (0.03 ± 0.01), H (0.29 ± 0.009), HO (0.03 ± 0.01) groups, (P=0.004). In addition, the levels of *ETR-A* gene mRNA expression in the H group were significantly higher than in C and HO groups in the heart and vascular tissues (P ≤ 0.05) (**Figure 3**).

There were significant differences in *ETR-B* gene mRNA expression levels in the heart tissue among C (0.02 ± 0.004), H (0.007 ± 0.002), HO (0.01 ± 0.002) groups, (P=0.039) and in the vascular tissue among C (0.005 ± 0.001), H (0.001 ± 0.0001), HO (0.03 ± 0.01) groups, (P=0.001). The levels of *ETR-B* gene mRNA expression in the H group were significantly lower than in C and HO groups in the heart and vascular tissues (P ≤ 0.05). In addition, the high levels of *ETR-B* gene mRNA expression in the vascular tissue were significant in the HO group compared with the C and H groups (P=0.002) (**Figure 4**).

Discussion

Hypertension is a leading risk factor for the development and progression of chronic cardiovascular diseases [10]. The DOCA-salt model rapidly induces cardiovascular remodelling as in chronic hypertension in humans. Although elevated blood pressure may probably be a major effector of cardiac hypertrophy in the DOCA-salt hypertensive rats, neurohumoral factors such as endothelin, vasopressin and sympathetic nerves may play an important independent role in regulating cardiovascular remodelling in these rats [11]. In studies, effect of ozone on blood pressure in DOCA-salt rats are yet to be reported.

A significant increase was seen in the SBP, DBP and MAP levels in the DOCA-salt hypertensive rats compared with controls in this study. In the study performed by Priyadharsini [12], it was shown that the systolic, diastolic and mean arterial blood pressure started rising actively from the 3rd week and almost by 4th week the hypertensive rats [12]. Prahalathan et al. [13] observed that systolic and diastolic blood pressure was considerably increased in DOCA-salt hypertensive rats [13]. This study's results are compatible with the aforementioned study.

In this study, there were significant increases in serum ET-1 levels in the H group compared with the all groups. A study by Akcılar et al. [14] found an increase in the levels of plasma ET-1 in the DOCA-salt induced hypertensive rats [14]. Kimura et al. [15] demonstrated that renal ET-1 content in DOCA-salt hypertensive rats significantly increased after two weeks [15]. Earlier studies have shown that ET-1 contributes to the development of high blood pressure and vascular growth in DOCA-salt rats [16]. These findings suggest that the enhanced serum ET-1 has a role in the development of hypertension in rats.

There were significant decreases in serum NO levels in the H group compared with the C and HO groups. Allcock et al. [17] observed that an increased production of NO by the kidney in DOCA-salt hypertensive rats [17]. Contrary to this finding are other studies in which it was observed that endothelial-derived NO release stimulated by ET-1 or acetylcholine was decreased in the perfusate of isolated kidneys from DOCA-salt rats [18-20]. Since these experiments were presumably examining endothelial-derived NO release, it may be possible that NO production is increased in renal tubules at a time in which endothelial production is decreased. It has been well known that NO is a potent vasodilator molecule that accounts for an endothelium-derived relaxing factor, a decrease in NO results in an elevation of blood pressure [21]. Therefore, increasing serum ET-1 levels caused a decrease in NO levels due to increased blood pressure in the H group.

In this study, there were significant decreases in serum renin levels in the H group compared with the all groups. It has been reported that the administration of DOCA, in combination with a high salt diet and unilateral nephrectomy induces a low renin form of hypertension [22]. Clinical studies have shown primary aldosteronism or a decrease in renin to aldosterone ratio to be a significant cause of hypertension [23, 24]. In another study, it was demonstrated that the DOCA-salt hypertensive rat model shows a markedly depressed renin angiotensin system and thus has been regarded as an angiotensin-independent model with decreased circulating plasma renin concentrations [25]. Previous studies have shown that increasing the ET-1 level reduces renin secretion from the kidney [16, 26]. Therefore, increasing ET-1 levels caused a decrease in renin and an increase blood pressure levels in the H group in this study.

Physiological and pathophysiological responses to ET-1 in various tissues are mediated by interactions with ETR-A and ETR-B subtypes. ETR-A is expressed mainly in the vascular tissues, where it mediates vasoconstrictive and growth effects [27]. The function of ETR-B appears to be more complex, as it is located on a variety of cell types [28]. ETR-B on endothelial cells causes vasodilatation through the release of NO and prostacyclin [29]. In our study, while *ET-1 and ETR-A* mRNA expression increased in the heart and vascular tissue of the H group compared with the C and HO groups, a decrease was found in *ETR-B* mRNA expression. In the study performed by Pollock et al. [30] the authors found that the selective blockade of ETR-B resulted in increased blood pressure and increased sodium and water retention in DOCA-salt rats [30]. These results suggest that ETR-A has an important role in the pathogenesis of DOCA-salt hypertension. Therefore, increased *ETR-A*, and decreased *ETR-B* mRNA expression in DOCA-salt hypertensive rats may have leading to decreased serum NO levels and increased vasoconstrictor effect by ET-1. Consequently, in the light of these findings, blood pressure increased in the H group.

A significant decrease was seen in the SBP, DBP and MAP levels in the HO groups compared with other groups in this study. The evidence for a direct cardiovascular effect of ozone, however, is more limited. In addition effects of intraperitoneal ozone administration have not been studied on blood pressure in hypertensive rats using DOCA-salt hypertension model before. Therefore, there has not been any study in the literature between intraperitoneal ozone, blood pressure and hypertension that can be compared with the results of this study. Previous studies have demonstrated that exposure to ozone and concentrated ambient particles together caused arterial vasoconstriction and increased blood pressure [31] and indeed ozone has been shown to reduce blood pressure in a diabetic population at the same time as particulate air pollution increased blood pressure [5]. Ozone exposure may influence autonomic regulation of the heart rate and has been shown to alter circulating inflammatory and fibrinolytic markers in healthy persons [32, 33]. In the study performed by Dong et al. [34], the authors found that ozone and carbon monoxide are associated with increased arterial blood pressure and hypertension among the children. Wang et al. [35] demonstrated that heart rate only reduced significantly in ozone combined fine particulate matter (PM2.5) groups. Blood pressure rose significantly in combined groups and high-dose PM2.5 alone exposure [35]. In another study, it was shown that ozone (0.5 ppm) exposure for 8 h increased atrial natriuretic factor (ANF) in the lungs, heart, and circulatory system, suggesting that ANF may mediate the decreased blood

pressure and pulmonary edema observed with ozone exposure [36]. In this study and previous studies on this subject, the dose and type of administration of ozone differ leading to the differences in the results.

In this study the low levels of serum ET-1 and the high levels of serum renin and NO were statistically significant in the HO group compared with the H group. According to these findings, differences in these parameters may contribute to fall of blood pressure levels in the HO group. These results suggest that ozone can be effective in regulating blood pressure but, could not lower to the completely normative level. Experimental a study has suggested that intraperitoneally oxygen/ozone can upregulate endothelial nitric oxide synthase, and prevent ischaemic injury in an ischaemia-reperfusion model [37]. In a study by Mogel et al. [38], it was shown that ozone increases NO production at vascular tissues [38]. Therefore, increasing serum NO levels caused a decrease in serum ET-1 levels in this study. Wiley et al. [39] shown that NO decreases *ET-1* mRNA and also the expression of *ET receptors* [39]. This study's results are compatible with the aforementioned study. So serum NO responsible for the decrease in the level of ET-1. In addition, decreasing ET-1 can cause an increase in the levels of serum renin by increasing the NO secretion in the HO group.

In this study, while *ET-1* and *ETR-A* mRNA expression reduced in the heart and vascular tissue of the HO group compared with the H group, a increase was found in *ETR-B* mRNA expression. Another reason for the decrease in blood pressure in the HO group was increased *ETR-B* mRNA expression in heart and vascular tissue, leading to increased levels of serum NO. There has not been any study in the litarature between ozone's intraperitonel application and *ET-1, ETR-A, ETR-B* mRNA expression that can be compared with the results of this study. Thomson et al. [40] reported that ozone inhalation significantly increased *preproET-1* mRNA level in the cerebral hemisphere, pituitary and so ozone rapidly modulate the expression of genes involved in key vasoregulatory pathways in the brain and pituitary [40]. In a study by Kodavanti et al. [41], it was observed that expression of *ET-1* and *ETR-A* was enhanced after 16 weeks of exposure to ozone, whereas *ETR-B* increased only with ozone plus diesel exhaust particles in the aorta, but not in the heart [41]. In another study, it was shown that expression of lung *ETR-A* mRNA was reduced by ozone immediately after 4 h inhalation exposure and after 24 h recovery in clean air. In contrast, expression of lung *ETR-B* mRNA increased immediately after exposure to ozone [42].

As a result, the low levels of serum NO may play a role in the interaction of ET-1 with the ETR-A mRNA expression rather than ETR-B mRNA expression in the H group, leading to increased vasoconstrictor effect and consequently, blood pressure increased in the H group. Ozone significantly attenuated DOCA-salt hypertension-induced increases in serum ET-1 and ETR-A mRNA expression in the heart and vascular tissue ($P \leq 0.05$). These results suggest that the antihypertensive effects of ozone were associated with increased ETR-B mRNA expression in heart and vascular tissue, leading to increased levels of serum NO and ozone could be a novel endogenous antihypertension factor.

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Disclosure of conflict of interest

None.

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