

# Beneficial Effects of Ozone Therapy on Oxidative Stress, Cardiac Functions and Clinical Findings in Patients with Heart Failure Reduced Ejection Fraction

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Published online: 17 January 2017

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**Abstract** The aim of study was to determine the effects of ozone therapy on the oxidative stress, cardiac functions and clinical findings in patients with heart failure reduced ejection fraction (HFrEF). A total of 40 patients with New York Heart Association 2 and 3 HF with left ventricular ejection fraction (LVEF) <35%, and 40 subjects without HF as control group were included in the study. Patients with HFrEF were given additional ozone therapy of major and minor administrations along with conventional HF treatment for 5 weeks. Before and after ozone therapy, left ventricular end-systolic and end-diastolic volumes (LVESV, LVEDV) and the 6 minute walk distance (6MWD) and blood levels of the superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSHPx), malondialdehyde (MDA), nitric oxide (NO) and N-terminal pro-brain natriuretic peptide (NT-proBNP) were measured. Ozone therapy significantly reduced the serum levels of NO and MDA ( $p < 0.001$ , respectively) and significantly increased the levels of SOD, CAT, GSH and GSHPx ( $p < 0.001$ , respectively). LVEDV and LVESV were found to be significantly reduced; however, LVEF was not found to be significantly increased ( $p = 0.567$ ). As the biochemical improvement marker of HF, NT-proBNP was

significantly reduced ( $p < 0.001$ ). The clinical HF improvement marker of 6 minute walk distance was also modestly increased ( $p < 0.001$ ). Ozone therapy might be beneficial in terms of activating antioxidant system and merit further therapeutic potential to conventional HF treatment in patients with HFrEF.

**Keywords** Heart failure · Ozone therapy · Oxidative stress · Antioxidant system

## Introduction

Experimental and animal models suggest that oxidative stress, which is characterized by excessive production of reactive oxygen species and reduction of antioxidant defense capacity, might play an important role in the pathophysiology of heart failure (HF) [1]. In HF patients, high serum oxidative stress (OS) levels correlate with advanced disease and known markers of poor prognosis [2]. Recent experimental studies have clearly demonstrated the therapeutic effects of antioxidants on progression of HF [1, 3]. Consequently, antioxidant therapy has been identified as a promising intervention to attenuate the oxidative damage and prevent the progression of HF. Ozone therapy (O<sub>3</sub>T) is a kind of treatment involving the administration of a certain amount of ozone/oxygen mixture into body cavities or the circulation system [4]. The result of repeated ozone applications is development of resistance to oxidative stress by stimulation of the antioxidant system. Ozone therapy can be used as an assisting treatment method especially for physiological situations involving the inflammatory system and suppressed the immune system [5, 6]. Some such situations include wound healing, macular degeneration secondary to advanced age, and ischemic

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and infectious diseases. Many experimental studies about these applications have shown that oxidative stress is reduced and the antioxidant system is increased [7]. In this context, the present study aimed to demonstrate the possible effects of O<sub>3</sub>T on oxidative stress, cardiac functions and clinical findings in patients with heart failure reduced ejection fraction (HFrEF).

## Patients and Methods

### Study Population

Forty patients who were admitted to Erzincan University Cardiology Department with left ventricle ejection fraction (LVEF) under 35% on echocardiography examination, resistant to traditional HF treatment, and with class 2 and 3 heart failure according to New York Heart Association (NYHA), and 40 subjects without HF as control group were enrolled in this study. Exclusion criteria were as follows: patients with; (1) hyperthyroidism, (2) hemorrhagic disorders, (3) glucose-6-phosphate dehydrogenase deficiency, (4) history of acute coronary syndrome within the past 21 days, (5) acute infectious, and (6) immunosuppressive or antioxidant medication usage. A total of 80 subjects fulfilled with these criteria were included in the study. Patients with study group were taking the standard heart failure therapy in the last 3 months. All patients gave written informed consent, and the study protocol was approved by the “Institutional Review Board.”

### Ozone Administration Protocol

The ozone/oxygen mixture can be applied by intravenous (major treatment, M-O<sub>3</sub>T), intramuscular (minor treatment, m-O<sub>3</sub>T), intraarticular, intrapleural, intrarectal, intradiscal and topical routes. The most common ozone administration form is the intravenous or systemic method [8].

Our procedures for O<sub>3</sub>T application in HF conform to international guidelines of the “Madrid Declaration on Ozone Therapy” [9]. M-O<sub>3</sub>T was carried out as follows: 90 mL of blood were drawn by vacuum from an antecubital vein into a sterile glass bottle (Ozonosan, Iffezeim, Germany) in which 10 mL of 3.8% Na citrate solution (Galenica Senese Industries, Siena, Italy) as an anticoagulant had been previously added so that the blood/citrate volume ratio was 9:1. After blood withdrawal, the bottle was momentarily disconnected leaving the venous access open by a saline infusion [10]. A corresponding volume (90 mL) of gas was immediately added with an O<sub>3</sub> concentration of 20–50 micrograms/mL gas. Ozone was produced by an Medozon compact generator (Herrmann Apparatebau GmbH, Germany), in which O<sub>3</sub> concentration

was measured photometrically in real time and checked by iodometric titration according to the rules established by the International Ozone Association.

The gas was immediately and continuously mixed with the blood in the bottle for at least 5 min and with gentle rotating movement to avoid foaming. Due to the blood viscosity, the gas mixture does not instantaneously come into contact with the whole blood mass, thus this mixing time is necessary. During these 5 min of mixing, the ozone totally reacted with both the potent antioxidants of plasma and the unsaturated lipids bound to albumin, generating a small amount of hydrogen peroxide and alkenals. These two messengers were responsible for eliciting crucial biochemical reactions on both erythrocytes and within cells when the hyper-oxygenated ozonated blood was re-infused into the patient. At this point, the hyper-oxygenated ozonated blood was re-infused by promptly substituting the saline infusion with it. Reinfusion was accomplished in about 15–20 min, and the whole procedure was carried out in approximately 40 min. The m-O<sub>3</sub>-AHT is also precise, and it consists in treating usually 5 mL of blood with an equal volume of gas mixture with an ozone concentration of 80–100 mg/L of gas.

In this study, O<sub>3</sub>T was in the form of three M-O<sub>3</sub>T and one m-O<sub>3</sub>T treatments per week for 5 weeks. A moderate dose of ozone (20–40 mcg/mL) was administered [11]. We followed the concept “*start low, go slow*” [12]. Before each treatment, the symptoms of “healing crisis” were inquired about and dose regulation was made accordingly. The patient used no medication for at least 2 h before each treatment (especially anticoagulant, antihypertensive and antidiabetic medications). Five patients who could not tolerate an ozone dose of 20 mcg/mL or more were excluded from the study.

### Biochemical Analyses

Before and after the ozone treatment, serum samples were obtained from patients with HF in anticoagulant tubes. After a certain amount of the sample was removed for glutathione (GSH), glutathione peroxidase (GSHPx) and hemoglobin measurements, the remaining portion was centrifuged at 3500 rpm for 10 min. The separated plasma was investigated for malondialdehyde (MDA), nitric oxide (NO) and N-terminal pro-brain natriuretic peptide (NT-proBNP), while superoxide dismutase (SOD) and catalase (CAT) activity were identified in erythrocytes. Other biochemical parameters were studied routinely in our biochemistry laboratory.

The total NO detection kit (Enzo Life Science) is a complete kit for the quantitative determination of total NO (indirect) in plasma. The kit involves the enzymatic conversion of nitrate to nitrite, using the enzyme nitrate

reductase, followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm.

Malondialdehyde (MDA) levels (as a marker of lipid peroxidation) in the plasma were measured using thiobarbituric acid reaction substance method [13]. Values are expressed as MDA equivalents in nmol/mL-1 plasma.

Whole blood GSHPx (EC 1.11.1.9) activity was assayed using the method of Lawrence and Burk [14] and is expressed as unit/gram/hemoglobin (U/g Hb). GSHPx activity was determined in the presence of GSH and cumenhydroperoxide substrates, using an end-point direct assay. The activity was expressed as loss of reduced GSH/min.

The generation of superoxide radicals produced by xanthine and xanthine oxidase, following the reaction of nitro blue tetrazolium and the formation of formazan dye, was used to measure SOD activity in erythrocytes [15]. SOD activity is expressed as U/g Hb.

The GSH content of whole blood was measured at 412 nm according to the method of Sedlak and Lindsay and is expressed as nmol/g of kidney tissue [16].

Erythrocyte CAT (EC 1.11.1.6) activity was determined according to the method of AEBI and is expressed as katalaz/gram/hemoglobin [17]. The principle of the assay is based on the determination of the constant rate or the H<sub>2</sub>O<sub>2</sub> decomposition rate at 240 nm. Results are expressed as k (rate constant)/g Hb protein.

Plasma NT-proBNP levels were spectrophotometrically measured using an ELISA kit (Uscn Life Science, Product No: SEA485Hu). The test principle applied in this kit is sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to NT-proBNP. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to NT-proBNP. Next, avidin conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain NT-proBNP, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulfuric acid solution, and the color change is measured spectrophotometrically at a wavelength of 450 ± 10 nm. The concentration of NT-proBNP in the samples is then determined by comparing the O.D. of the samples to the standard curve.

### Two-Dimensional Doppler Echocardiographic Examination

Echocardiographic examinations were conducted before ozone treatment and at the end of the 5-week period using

Vivid S5 Dimension® (GE Vingmed Ultrasound AS N-3190 Horten, Norway), while echocardiography was performed with a 2.5-MHz transducer. All standard measurements were taken using the criteria of the American Society of Echocardiography in left lateral decubitus position [18]. Echocardiographic analyses were performed by an experienced echocardiography specialist who was blind to all data. Left ventricular ejection fraction was calculated using the modified Simpson's method.

### 6-Min Walk Test (6MWT)

The 6MWT followed the recommendations of the American Thoracic Society guidelines [19]. The 6MWT were conducted before ozone treatment and at the end of the 5-week period. Patients were given standardized instructions to walk as fast as possible for 6 min. The 6 minute walk distance (6MWD) was recorded after walking.

### Statistical Analysis

The data were analyzed using a statistical software package. Normal distribution was assessed using the Kolmogorov–Smirnov one sample test. Numerical variables are expressed as mean ± SD and median (min–max). Paired samples t test and Wilcoxon test were used for comparisons. Statistical significance was set at  $p < 0.05$ .

## Results

### Baseline Characteristics

A total of 40 patients with HFrEF and 40 subjects without HF as control group were included in the present study. Baseline characteristics of HFrEF patients and subjects without HF were shown in Table 1. There were no statistical difference between the HFrEF patients and subjects without HF in terms of age, gender, smoking, presence of chronic obstructive pulmonary disease, primary hypertension, diabetes mellitus type-2, atrial fibrillation, and coronary artery disease, aspirin, angiotensin receptor blocker and anticoagulant usage ( $p > 0.05$ , for all).

Mean age of patients with HFrEF was 65.4 ± 6.8 years. Left ventricle EF was 26.6 ± 7.9%. In the etiology of HF, 55% were ischemic while 45% were non-ischemic dilated cardiomyopathy. Functional classifications were 62.5% NYHA class 2 and 37.5% NYHA class 3. More than 75% of patients were receiving optimal HF treatment at tolerable doses. 12.5% of patients had AF rhythm, and these patients were additionally receiving anticoagulant therapy.

Clinical, biochemical and echocardiographic characteristics of the patients with HFrEF and subjects without HF

**Table 1** Baseline characteristics of patients with heart failure with reduced ejection fraction (HFrEF) patients and subjects without HF

Characteristics	Patients with HFrEF ( <i>n</i> = 40)	Subjects without HF ( <i>n</i> = 40)	<i>p</i> value
Age (years)	65.4 ± 6.8	64.7 ± 7.8	0.26
Male sex (%)	32 (80%)	33 (82.5%)	0.775
Hypertension (%)	16 (40%)	16 (40%)	1.000
DM type-2 (%)	16 (40%)	14 (35%)	0.644
CAD (%)	22 (55%)	21 (52.5%)	0.823
COPD (%)	10 (25%)	11 (27.5%)	0.799
Smoking (%)	16 (40%)	14 (35%)	0.644
AF rhythm (%)	5 (12.5%)	5 (12.5%)	1.000
Aspirin usage (%)	22 (55%)	21 (52.5%)	0.823
Beta blocker usage (%)	31 (77.5%)	20 (50%)	<0.001
ACEI usage (%)	31 (77.5%)	14 (35%)	<0.001
ARB usage (%)	7 (17.5%)	7 (17.5%)	1.000
Spirolactone usage (%)	32 (80%)	0	<0.001
Furosemide usage (%)	37 (92.5%)	0	<0.001
Ívabradine usage (%)	1 (2.5%)	0	<0.001
Anticoagulant usage (%)	5 (12.5%)	5 (12.5%)	1.000

Values are presented as mean ± SD or *n* (%)

DM diabetes mellitus, HFrEF heart failure reduced ejection fraction, DCMP dilated cardiomyopathy, NYHA New York Heart Association, CAD coronary artery disease, COPD chronic obstructive pulmonary disease, AF atrial fibrillation, ACEI angiotensin-converting enzyme inhibitor, ARB angiotensin receptor blocker

were shown in Table 2. There was no statistically significant difference regarding SBP, DBP, HR, urea, creatinine, AST, ALT and parameters of hemogram between patients with HFrEF and healthy subjects ( $p > 0.05$ , for all). According to echocardiography findings, patients with HFrEF had significantly higher LVEDD and LVEDV and had significantly lower LVEF compared with healthy subjects ( $p < 0.001$ , for all).

Serum MDA, NO synthase and NT-proBNP were found to be significantly increased, whereas SOD, CAT, GSH, GSHPx and 6MWT were found to be significantly decreased in patients with HFrEF compared with healthy subjects ( $p < 0.001$ , for all).

### Parameters Before and After Ozone Treatment

Clinical and echocardiographic characteristics in patients of HFrEF at before study versus after study were shown in Table 3. There was no significant difference in terms of SBP and DBP; however, there was a significant reduction regarding HR, LVEDD, LVESD, LVESV, LVEDV before and after the ozone treatment ( $p < 0.001$ , for all). However, there was no significant increase in LVEF ( $p = 0.567$ ).

Biochemical and the six-min walk test characteristics of patients with HFrEF before and after ozone treatment were shown in Table 4. There were no significant differences in terms of hemogram, liver and renal function tests before and

after the ozone treatment ( $p > 0.05$ , for all). The oxidant parameters including NO and MDA significantly decreased ( $77.2 \pm 2.7$  vs  $20.6 \pm 3.3$  and  $5.6 \pm 1.3$  vs  $4.1 \pm 0.7$ , respectively,  $p < 0.001$ ); however, the antioxidant parameters such as SOD, CAT, GSH and GSHPx significantly increased ( $442.2 \pm 19.2$  vs  $503.2 \pm 23.8$ ,  $23.4 \pm 1.7$  vs  $28.3 \pm 3.3$ ,  $15.9 \pm 2.1$  vs  $20.3 \pm 2.9$ ,  $41.4 \pm 1.7$  vs  $50.1 \pm 2.5$ , respectively,  $p < 0.001$ ) after the ozone treatment in patients with HFrEF. The high NT-proBNP which is a marker of HF was significantly diminished, and the 6MWD was also significantly increased after ozone treatment ( $1214.3 \pm 274.4$  vs  $675.6 \pm 177.1$ ,  $66.6 \pm 5.9$  vs  $69.3 \pm 5.9$ , respectively,  $p < 0.001$ ).

### Discussion

In the current study, we demonstrated that the OS markers significantly diminished, while the antioxidant system markers significantly increased after ozone administration to patients with HFrEF. In addition, after ozone administration 6MWD, indicating functional capacity, significantly increased.

Heart failure is a syndrome affecting many systems. The balance of the oxidant and antioxidant systems is one of the systems affected. The antioxidant system is suppressed while the oxidant system, or the amount of reactive oxygen species (ROS), increases causing oxidative stress. The most

**Table 2** Clinical, biochemical and echocardiographic characteristics of heart failure with reduced ejection fraction (HFrEF) patients and subjects without HF

Characteristics	Patients with HFrEF ( <i>n</i> = 40)	Subjects without HF ( <i>n</i> = 40)	<i>p</i> value
Systolic BP (mmHg)	118.4 ± 7.6	119.1 ± 7.6	0.676
Diastolic BP (mmHg)	73.4 ± 7.0	72.9 ± 6.9	0.715
Heart rate (pulse/min)	83.6 ± 13.1	81.1 ± 10.7	0.354
LVEDD (mm)	63.0 ± 6.3	47.1 ± 3.5	<0.001
LVESD (mm)	51.4 ± 5.7	21 ± 2.5	<0.001
LVEDV (ml)	231.9 ± 40.8	71.9 ± 7.2	<0.001
LVESV (ml)	168.9 ± 31.2	24.4 ± 1.0	<0.001
LVEF (%)	26.6 ± 7.9	66.1 ± 4.4	<0.001
Hemoglobin (g/dL)	14.1 ± 1.8	14.1 ± 1.8	0.890
Platelet count (×10 <sup>3</sup> /mm <sup>3</sup> )	234.0 ± 75.5	228.6 ± 74.8	0.748
White blood cell (×10 <sup>3</sup> /mm <sup>3</sup> )	8.6 ± 2.9	8.5 ± 2.8	0.917
Urea (mg/dL) [median/(min–max)]	46.0/(24.0–127.0)	45.0/(25.0–55.0)	0.139
Creatinine (mg/dL) [median/(min–max)]	1.04/(0.6–2.7)	1.0/(0.6–1.7)	0.954
AST (U/L)	22.6 ± 5.3	22.1 ± 6.4	0.708
ALT (U/L)	21.7 ± 11.8	21.0 ± 9.9	0.767
Sodium (mmol/L)	137.9 ± 3.1	136.5 ± 1.7	0.014
Potassium (mmol/L)	4.3 ± 0.4	4.2 ± 0.4	0.344
MDA (nmol/ml)	5.6 ± 1.3	3.0 ± 0.8	<0.001
NO (mcmol/L)	77.2 ± 2.7	10.1 ± 2.0	<0.001
SOD (U/g Hb)	442.2 ± 19.2	602.0 ± 24.3	<0.001
CAT (k/g Hb)	23.4 ± 1.7	37.6 ± 3.9	<0.001
GSH (nmol/L)	15.9 ± 2.1	29.3 ± 3.1	<0.001
GSHPx (U/g Hb)	41.4 ± 1.7	59.6 ± 2.6	<0.001
NT-proBNP (pg/ml)	1214.3 ± 274.4	174.8 ± 42.8	<0.001
6MWD (m)	66.6 ± 5.9	173.8 ± 28.4	<0.001

*HFrEF* heart failure reduced ejection fraction, *BP* blood pressure, *LVEDD* left ventricular end-diastolic diameter, *LVESD* left ventricular end-systolic diameter, *LVEDV* left ventricular end-diastolic volume, *LVESV* left ventricular end-systolic volume, *LVEF* left ventricular ejection fraction, *AST* aspartate aminotransferase, *ALT* alanine transaminase, *MDA* malondialdehyde, *NO* nitric oxide, *SOD* superoxide dismutase, *CAT* catalase; *GSH*, glutathione, *GSHPx* glutathione peroxidase, *NT-proBNP* N-terminal pro-brain natriuretic peptide, *6MWD* 6 minute walk distance

**Table 3** Clinical and echocardiographic characteristics of heart failure with reduced ejection fraction (HFrEF) patients before and after ozone treatment

Characteristics	Patients with HFrEF ( <i>n</i> = 40)		<i>p</i> value
	Before the ozone treatment	After the ozone treatment	
Systolic BP (mmHg)	118.4 ± 7.6	116.7 ± 7.3	0.267
Diastolic BP (mmHg)	73.4 ± 7.0	73.5 ± 7.0	0.160
Heart rate (pulse/min)	83.6 ± 13.1	80.6 ± 12.5	<0.001
LVEDD (mm)	63.0 ± 6.3	61.1 ± 6.4	<0.001
LVESD (mm)	51.4 ± 5.7	49.0 ± 5.3	<0.001
LVEDV (ml)	231.9 ± 40.8	221.1 ± 40.5	<0.001
LVESV (ml)	168.9 ± 31.2	161.0 ± 28.2	<0.001
LVEF (%)	26.6 ± 7.9	26.9 ± 7.2	0.567

Values are presented as mean ± SD

*BP* blood pressure, *LVEDD* left ventricular end-diastolic diameter, *LVESD* left ventricular end-systolic diameter, *LVEDV* left ventricular end-diastolic volume, *LVESV* left ventricular end-systolic volume, *LVEF* left ventricular ejection fraction

**Table 4** Biochemical and the six-min walk test characteristics of heart failure with reduced ejection fraction (HFrEF) patients before and after ozone treatment

Characteristic	Patients with HFrEF ( <i>n</i> = 40)		<i>p</i> value
	Before the study	After the study	
Hemoglobin (g/dl)	14.1 ± 1.8	14.0 ± 1.9	0.590
Platelet (10 <sup>3</sup> /mm <sup>3</sup> )	234.0 ± 75.5	231.4 ± 92.4	0.794
White blood cell (10 <sup>3</sup> /mm <sup>3</sup> )	8.6 ± 2.9	8.3 ± 2.4	0.492
Ürea (mg/dl) [median/(min–max)]	46.0/(24.0–127.0)	45.1/(23.0–127.0)	0.811
Creatinine (mg/dl) [median/(min–max)]	1.04/(0.6–2.7)	1.06/(0.4–3.8)	0.237
AST (U/L)	22.6 ± 5.3	24.2 ± 9.6	0.187
ALT (U/L)	21.7 ± 11.8	21.4 ± 14.8	0.880
Sodium (mmol/L)	137.9 ± 3.1	137.7 ± 4.5	0.812
Potassium (mmol/L)	4.3 ± 0.4	4.3 ± 0.5	0.530
MDA (nmol/ml)	5.6 ± 1.3	4.1 ± 0.7	<0.001
NO (mcmol/L)	77.2 ± 2.7	20.6 ± 3.3	<0.001
SOD (U/g Hb)	442.2 ± 19.2	503.2 ± 23.8	<0.001
CAT (k/g Hb)	23.4 ± 1.7	28.3 ± 3.3	<0.001
GSH (nmol/L)	15.9 ± 2.1	20.3 ± 2.9	<0.001
GSHPx (U/g Hb)	41.4 ± 1.7	50.1 ± 2.5	<0.001
NT-proBNP (pg/ml)	1214.3 ± 274.4	675.6 ± 177.1	<0.001
6MWD (m)	66.6 ± 5.9	69.3 ± 5.9	<0.001

Values are presented as mean ± SD

AST aspartate aminotransferase, ALT alanine transaminase, MDA malondialdehyde, NO nitric oxide, SOD superoxide dismutase, CAT catalase, GSH glutathione, GSHPx glutathione peroxidase, NT-proBNP N-terminal pro-brain natriuretic peptide, 6MWD 6 minute walk distance

important mechanism causing suppression of the antioxidant system in HF is increased activity in the neurohumoral and sympathetic system and tissue damage caused by these [20, 21]. As a result, undesirable situations for HF-like worsened myocardial remodeling, increased apoptosis of myocytes, endothelial dysfunction and disrupted excitation–contraction occur. Many studies show that the antioxidant system is suppressed and oxidative stress increases in HF [22, 23]. A study by Belch et al. [24] observed that in HF patients, malondialdehyde levels increased, while plasma thiol levels decreased. Arai et al. [25] showed that heart failure subsequent to MI is associated with an antioxidant deficit as well as increased oxidative stress, first in the LV, followed by the RV. Furthermore, these changes correlate with the hemodynamic function in each of the ventricles, suggesting their role in the pathogenesis of ventricular dysfunction. In addition, Hill and Singal [22] suggested that heart failure subsequent to myocardial infarction may be associated with an antioxidant deficit, as well as increased myocardial oxidative stress.

Hydrogen peroxide forming as a result of oxidative stress and lipid oxidation during ozone administration behaves like a secondary messenger mediating the biological effects of O<sub>3</sub>T. The result of repeated ozone administration stimulates the antioxidant system developing resistance to oxidative stress. Additionally, a variety of cytokine levels increase

linked to oxidation of fatty acids found in cell membranes. The treatment of pathophysiological situations, especially those where the inflammatory process, is intensely experienced, and the immune system is at the forefront, by O<sub>3</sub>T is surprising. During the last decade, biological and clinical work has shown that a judiciously performed ozone therapy in still responsive patients is able to correct this abnormal situation by upregulating antioxidant enzymes such as SOD, GSH-peroxidases, reductases and transferases and glucose-6-phosphate dehydrogenase. This result, firstly demonstrated for SOD in 1996 [26], is due to the repetition of small and acute oxidative stresses induced by precise doses of well-calibrated ozone against the potent antioxidant capacity of human blood. Borrego et al. [27] reported that ozone pretreatment prevented the increase in serum creatinine levels, the glutathione depletion and the inhibition of superoxide dismutase, catalase and glutathione peroxidase activities induced by cisplatin in the rat kidney. Safwat et al. [28] reported the evidence for potentially positive effects of pre-aging O<sub>3</sub>T in neutralizing chronic oxidative stress associated with aging in rat liver and kidneys. In this study, there was a significant decrease in levels of oxidant parameters of NO and MDA after O<sub>3</sub>T. Again after O<sub>3</sub>T, a significant increase in the antioxidant parameters of SOD, CAT, GSH and GSHPx was identified.

In clinical trials where M-O<sub>3</sub>T is used, the dose and duration vary. Borrelli et al. [29] observed that M-O<sub>3</sub>T

could be a safe and effective therapeutic option for high-risk patients with dry age-related macular degeneration. M-O<sub>3</sub>T was carried out in an out-patient setting twice weekly for the first 7 weeks; twice monthly for a further 3 months and then monthly until the 12th month. Ozone (225 mL, 50 mg/L) was added. Wu et al. [30] suggest that M-O<sub>3</sub>T promotes recovery of neurological function in acute cerebral infarction patients by reducing remote injury and additionally, exhibits high safety. M-O<sub>3</sub>T was carried out once a day, for 10 ± 2 days as a course. Ozone (100 mL, 47 mg/L) was added. In this study, M-O<sub>3</sub>T was carried out three times a week, for 5 weeks. Ozone (100 mL, 20–50 mg/L) was added.

There have been studies using several exogenous antioxidants for oxidative stress in heart failure. Ghatak et al. [31] observed that the superoxide anion and MDA levels were significantly higher in patients with heart failure in the pretreatment state, compared to those in post-treatment state. Conversely CAT, GSH-reductase and SOD were higher in the post-treatment period compared to their values before treatment. Keith et al. [32] observed that supplementation with vitamin E did not result in any significant improvements in prognostic or functional indices of heart failure or in the quality of life of patients with advanced heart failure. In addition pooled analysis suggests that the use of coenzyme Q10 has no clear effect on left ventricular ejection fraction or exercise capacity [33]. This study observed a beneficial effect on the oxidant/antioxidant system. Again clinically there was a significant increase in 6MWD and biochemically a significant reduction in NT-proBNP identified with O<sub>3</sub>T.

Our study has several limitations. First, this study had a single-center design and the sample size was relatively small. Second, all of the HF patients and control individuals enrolled in the study were Turkish. One should consider that our results cannot be applied to all HF patients because of the differences between them in terms of their nationalities. Third, follow-up period was also relatively short to evaluate the effects of ozone on mortality and other cardiovascular events in our study cohort. And finally, our control group did not have heart failure. In this context, a new placebo-controlled study can be designed to show the independent effects of ozone therapy.

## Conclusion

Ozone therapy might have beneficial effects on the oxidant/antioxidant system and clinical parameters in patients with HF rEF. According to the results of the present study, we believe that administration of O<sub>3</sub>T might be an additional treatment option to the current therapy of patients with HF rEF.

**Funding** This study was supported by the Scientific Research Fund of Erzincan University.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that there is no conflict of interest.

## References

1. Sawyer, D. B. (2011). Oxidative stress in heart failure: what are we missing? *The American Journal of the Medical Sciences*, *342*, 120–124.
2. Amir, O., Paz, H., Rogowski, O., Barshai, M., Sagiv, M., Shnizer, S., et al. (2009). Serum oxidative stress level correlates with clinical parameters in chronic systolic heart failure patients. *Clinical Cardiology*, *32*, 199–203.
3. Nain, S., Wojnarowicz, C., Laarveld, B., & Olkowski, A. A. (2008). Effects of dietary vitamin E and C supplementation on heart failure in fast growing commercial broiler chickens. *British Poultry Science*, *49*, 697–704.
4. Bocci, V. (2006). Scientific and medical aspects of ozone therapy state of the art. *Archives of Medical Research*, *37*, 425–435.
5. Stübinger, S., Sader, R., & Filippi, A. (2006). The use of ozone in dentistry and maxillofacial surgery: a review. *Quintessence International*, *37*, 353–359.
6. Nogales, C. G., Ferrari, P. H., Kantorovich, E. O., & Lage-Marques, J. L. (2008). Ozone therapy in medicine and dentistry. *The Journal of Contemporary Dental Practice*, *9*, 75–84.
7. Martínez-Sánchez, G., Al-Dalain, S. M., Menéndez, S., Re, L., Giuliani, A., Candelario-Jalil, E., et al. (2005). Therapeutic efficacy of ozone in patients with diabetic foot. *European Journal of Pharmacology*, *523*, 151–161.
8. Di Paolo, N., Gaggiotti, E., & Galli, F. (2005). Extracorporeal blood oxygenation and ozonation: clinical and biological implications of ozone therapy. *Redox Report*, *10*, 121–130.
9. The International Scientific Committee of Ozone Therapy, “Madrid declaration on ozone therapy,” [http://www.aepromo.org/declaracion/madrid/Madrid declaration.pdf](http://www.aepromo.org/declaracion/madrid/Madrid%20declaration.pdf).
10. Bocci, V., & Oxygen-ozone therapy. (2002). *A critical evaluation* (pp. 1–427). Dordrecht, The Netherlands: Kluwer Academic Publishers.
11. Bocci, V. A., Zanardi, I., & Travagli, V. (2011). Ozone acting on human blood yields a hormetic dose-response relationship. *Journal of Translational Medicine*, *17*, 66.
12. Bocci, V., Zanardi, I., Huijberts, M. S., & Travagli, V. (2011). Diabetes and chronic oxidative stress. A perspective based on the possible usefulness of ozone therapy. *Diabetes and Metabolic Syndrome*, *5*(1), 45–49.
13. Placer, Z. A., Chusman, L., & Johnson, B. C. (1966). Estimation of products of lipid peroxidation in biological fluids. *Analytical Biochemistry*, *16*, 359–364.
14. Lawrence, R. A., & Burk, R. F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and Biophysical Research Communications*, *71*, 952–958.
15. Sun, Y., Larry, W. O., & Ying, L. (1988). A simple method for clinical assay of superoxide dismutase. *Clinical Chemistry*, *34*, 497–500.
16. Sedlak, J., & Lindsay, R. H. C. (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman’s reagent. *Analytical Biochemistry*, *25*, 192–205.
17. Aebi, H. E. (1987). Catalase. In H. U. Bergmeyer (Ed.), *Methods of enzymatic analysis* (3rd ed., pp. 273–286). Florida: Verlag Chemie, Weinheim.

18. Schiller, N. B., Shah, P. M., Crawford, M., DeMaria, A., Devereux, R., Feigenbaum, H., et al. (1989). Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. American Society of Echocardiography Committee on Standards, Subcommittee on Quantitation of Two Dimensional Echocardiograms. *Journal of the American Society of Echocardiography*, 2, 358–367.
19. ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories. (2002). ATS statement: guidelines for the six-minute walk test. *American Journal of Respiratory and Critical Care Medicine*, 166, 111–117.
20. Pimentel, D. R., Amin, J. K., Xiao, L., Miller, T., Viereck, J., Oliver-Krasinski, J., et al. (2001). Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. *Circulation Research*, 89, 453–460.
21. Arstall, M. A., Sawyer, D. B., Fukazawa, R., & Kelly, R. A. (1999). Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. *Circulation Research*, 85, 829–840.
22. Hill, M. F., & Singal, P. K. (1996). Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. *American Journal of Pathology*, 148, 291–300.
23. Hill, M. F., & Singal, P. K. (1997). Right and left myocardial antioxidant responses during heart failure subsequent to myocardial infarction. *Circulation*, 96, 2414–2420.
24. Belch, J. J., Bridges, A. B., Scott, N., & Chopra, M. (1991). Oxygen free radicals and congestive heart failure. *British Heart Journal*, 65, 245–248.
25. Arai, M., Alpert, N. R., MacLennan, D. H., Barton, P., & Periasamy, M. (1993). Alterations in sarcoplasmic reticulum gene expression in human heart failure. A possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. *Circulation Research*, 72, 463–469.
26. Bocci, V. (1996). Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. *Medical Hypotheses*, 46(2), 150–154.
27. Borrego, A., Zamora, Z. B., González, R., Romay, C., Menéndez, S., Hernández, F., et al. (2004). Protection by ozone preconditioning is mediated by the antioxidant system in cisplatin-induced nephrotoxicity in rats. *Mediators of Inflammation*, 1, 13–19.
28. Safwat, M. H., El-Sawalhi, M. M., Mausouf, M. N., & Shaheen, A. A. (2014). Ozone ameliorates age-related oxidative stress changes in rat liver and kidney: Effects of pre- and post-ageing administration. *Biochemistry*, 5, 450–458.
29. Borrelli, E., Diadori, A., Zalaffi, A., & Bocci, V. (2012). Effects of major ozonated autohemotherapy in the treatment of dry age related macular degeneration: a randomized controlled clinical study. *International Journal of Ophthalmology*, 5(6), 708–713.
30. Wu, X. N., Zhang, T., Wang, J., Liu, X. Y., Li, Z. S., Xiang, W., et al. (2016). Magnetic resonance diffusion tensor imaging following major ozonated autohemotherapy for treatment of acute cerebral infarction. *Neural Regeneration Research*, 11(7), 1115–1121.
31. Ghatak, A., Brar, M. J., Agarwal, A., Goel, N., Rastogi, A. K., Vaish, A. K., et al. (1996). Oxy free radical system in heart failure and therapeutic role of oral vitamin E. *International Journal of Cardiology*, 57, 119–127.
32. Keith, M. E., Jeejeebhoy, K. N., Langer, A., Kurian, R., Barr, A., O’Kelly, B., et al. (2001). A controlled clinical trial of vitamin E supplementation in patients with congestive heart failure. *The American Journal of Clinical Nutrition*, 73, 219–224.
33. Ishiyama, T., Morita, Y., Toyama, S., Yamagami, T., & Tsukamoto, N. (1976). A clinical study of the effect of coenzyme Q on congestive heart failure. *Japanese Heart Journal*, 17, 32–42.