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Original Article

Gastroprotective effect of Oxygen-Ozone therapy in the model of indomethacin induced acute gastric ulcer in rats

Senol Bicer[1], Cebrail Gursul[2], Ilhan Demiryilmaz[3], Ismail Demiryilmaz[4], Necla Aydin Peker[5], Huseyin Eken[4], Orhan Cimen[4], Levent Demirtas[6], Secil Cakarli[1]

Departments of [1]Pediatric Surgery, [2]Physiology, [4]General Surgery, [5]Pediatrics, [6]Internal Medicine, Erzincan University, Faculty of Medicine, Erzincan, Turkey; [3]Department of Orthopedics, IbniSina Hospital, Kayseri, Turkey

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Abstract: We evaluated the effects of a panel of pharmaceutical interventions in an experimental model of gastric ulcer. The aim of this study is to investigate the protective effect of ozone therapy in an experimental rat model of acute gastric ulcer induced by indomethacin. Twenty-eight Wistar-albino male rats were used in this study. The rats were divided into four groups of seven animals in group as follows: Group 1 (control), Group 2 (sham), Group 3 (ozone), and Group 4 (lansoprazole). After 24 hours of fasting, 5 ml ozone-oxygen mixture was administered to Group 3, 30 mg/kg lansoprazole-5 ml distilled water mixture was administered to Group 4, and 5 ml distilled water was administered to Group 2 by gavage. Five minutes later, 25 mg/kg of indomethacin was administered to stomachs of all rats by gavage except for the control group. After six hours, all animals were anesthetized and stomach tissue was extracted. Ulcers on the gastric surface were measured at the time of collection. Myeloperoxidase (MPO), malondialdehyde (MDA), catalase (CAT), total glutathione (tGSH), superoxide dismutase (SOD), and 8-hydroxy-deoxyguanosine (8-OHdG) were measured in the stomach tissue. Administration of ozone and lansoprazole led to decreased macroscopic gastric ulcer size induced by indomethacin. Ulcer area was elevated in the sham group (71.47 ± 17.08 mm^2) relative to the treatment groups. Ulcer area was 20.79 ± 12.88 mm^2 in ozone group, significantly lower than sham group but significantly greater than the lansoprazole group. MPO, MDA, CAT, and 8-OHdG levels after ozone administration were significantly reduced and tGSH and SOD levels were higher relative to the lansoprazole group ($P<0.01$). Ozone therapy has a protective effect on acute gastric ulcer induced by non-steroidal anti-inflammatory drugs through an antioxidative mechanism.

Keywords: Ozone therapy, gastric ulcer, oxidative stress, protective effect, lansoprazole

Introduction

Gastric ulcer is a multifactorial disease. Endogenous factors (leukotrienes, gastric acid, pepsin), bacterial infections, excessive use of alcohol, and non-steroidal anti-inflammatory drugs (NSAIDs) may cause gastric injuries. On the other hand, prostaglandins, bicarbonate, mucus, and mucosal blood flow are known gastroprotective factors [1, 2]. Proton pump inhibitors (omeprazole, lansoprazole), H_2 receptor blockers (ranitidine, famotidine) and herbal medicines are widely used for the prevention and treatment of gastric ulcers [3, 4]. NSAIDs lead to oxidative damage to the gastric mucosa with the effect of reactive oxygen products [5]. Lansoprazole blocks the release of gastric acid by inhibiting the proton pump and reduces oxidative stress on the gastric mucosa [6].

Ozone is a gaseous molecule consisting of three oxygen atoms. The oxygen molecule (O_2) is stable, but ozone (O_3) is an unstable structure. The use of ozone-oxygen gas mixture in treatment is known as ozone therapy [7]. The biological and therapeutic effects of ozone, a powerful antioxidant, originate from the generation of hydrogen peroxide. An innate defense system including antioxidant enzymes is stimulated as a result of reduced antioxidant levels and the generation of hydrogen peroxide, resulting in increased resistance to oxidative injury [8]. Numerous studies have shown that ozone therapy is useful in the treatment of toxic muco-

sal injuries, burns, circulatory diseases, and the prevention of ischemia-reperfusion injury [9-12].

The beneficial effects of drugs in the treatment of gastric ulcers can be assessed by animal models of ulcer. The aim of this study is to investigate the protective effects of ozone therapy on gastric ulcer induced by indomethacin, a non-steroidal anti-inflammatory drug, in an experimental rat model.

Materials and methods

This study was approved by the Ethical Committee for Animal Experimentation of the Atatürk University, Erzurum, Turkey (27.05.2015-111).

Animals and experimental groups

Twenty-eight Wistar-albino male rats weighing 250-335 g were randomly allocated into four groups of seven animals in each. The rats used in the experiments were obtained from the Atatürk University Medical Practice and Research Center and were housed in aerated, plastic, breeding cages at a constant temperature of 21 ± 1°C with a cycle of twelve hours of darkness followed by twelve hours of light. The rats were divided into four groups of seven animals as follows: Group 1 (control), Group 2 (sham), Group 3 (ozone), and Group 4 (lansoprazole). Animals were fasted for twenty-four hours prior to the study. After the fasting period, no further treatment was administered in Group 1. The remaining groups received experimental treatments by oral gavage. Group 2 received 5 ml distilled water. Group 3 received 5 ml ozone-oxygen mixture. Group 4 was treated with 30 mg/kg lansoprazole in 5 ml distilled water. Five minutes later, 25 mg/kg of indomethacin in 5 ml distilled water solution was administered by oral gavage to all animals in Groups 2-4.

Ozone production

Ozone (O_3) was created using an ozone generator (OZONO-SAN Photonik 1014, Hansler GmbH; Nordring&Iffezheim, Germany). The ozone flow rate was kept constant at 60 mg/ml concentration, 97% oxygen + 3% ozone gas mixture at 3 L/min.

Surgery and sample collection

Each rat was anesthetized using ketamine hydrochloride (80 mg/kg) and xylasine hydrochloride (10 mg/kg) delivered intraperitoneally 6 hours after the administration of indomethacin. Ulcers on the gastric surface were measured. Stomach tissue was stored at -80 °C for later biochemical examination after measurement of the ulcers.

Measurement of ulcer area

The stomachs of all experimental animals were extracted and the ulcer foci on the gastric surface were assessed macroscopically. The width of the ulcer area on the gastric surface was measured using a mm^2 scale [13, 14].

Biochemical evaluation

Tissue samples procedure

Tissues were rinsed in ice cold phosphate buffered saline (PBS) (pH=7.2-7.4) and weighed before homogenization. The tissues were minced to small pieces in PBS (pH=7.2-7.4). Afterwards, the tissues were homogenized using tissue grinders and the homogenates were centrifuged for 20 minutes at 2,000-3,000 rpm. The supernatant was removed, aliquoted, and stored at -20°C for ELISA.

ELISA assays

Tissue CAT, SOD, MPO, MDA, GSH and 8-OHDG levels were quantified using an ELISA kit (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, China). All assay procedures were carried out according to the manufacturer's instructions. The limit of detection for the CAT, SOD, MPO, MDA, GSH and 8-OHDG assays was 0.52 ng/ml, 0.016 ng/ml, 0.25 ng/ml, 0.024 nmol/ml, 10.25 mg/L and 0.027 ng/ml, respectively. Absorbance was measured at 450 nm wavelength using an Epoch spectrophotometer for both standards and samples (BioTek Instruments, Inc., Winooski, VT, USA). A standard curve was plotted with concentration on the x-axis and absorbance on the y-axis to determine the levels of CAT (ng/ml), SOD (ng/ml), MPO (ng/ml), MDA (nmol/ml), GSH (mg/L) and 8-OHDG (ng/ml) in the samples.

Statistical analysis

Descriptive statistics of continuous variables are shown as mean ± standard deviation and frequency (%). Variables were evaluated after evaluation of normality and variance homogeneity (Shapiro Wilk and Levene test). In the data

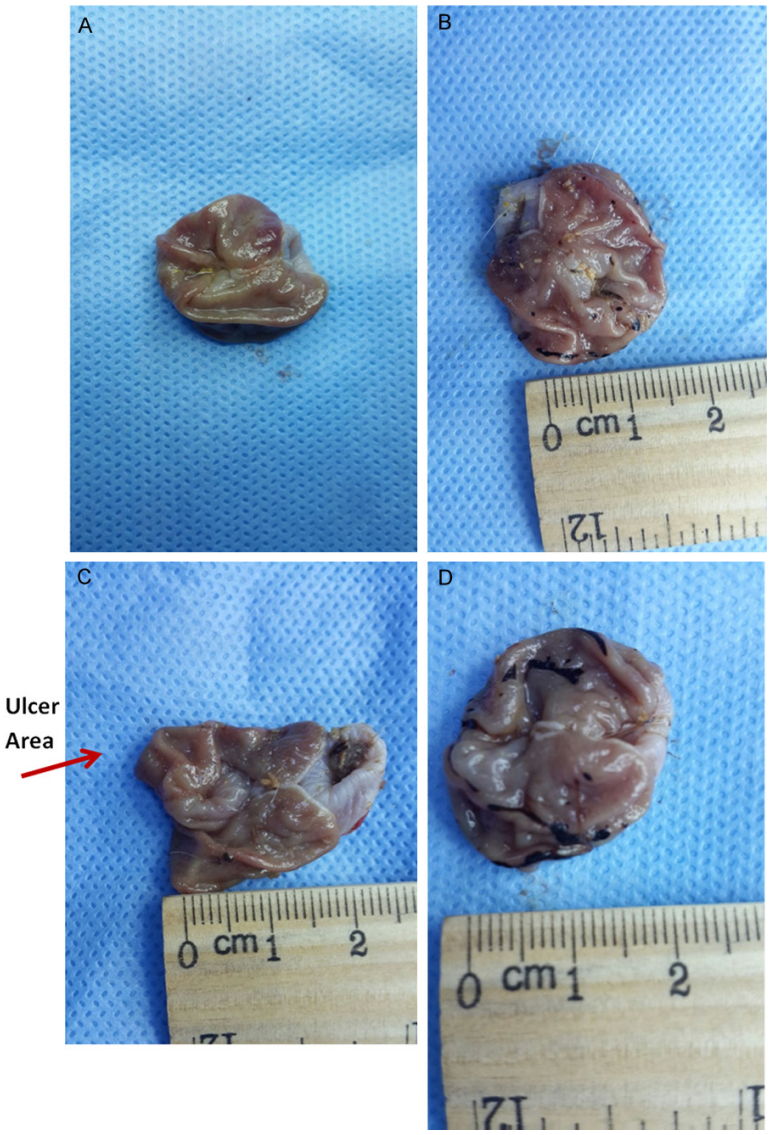

Figure 1. Macroscopic views of gastric mucosa. A. The Gastric mucosa was normal in the control group. B. moderate ulcers were apparent in the Ozone therapy group. C. ulcer area was minimal in the Lansoprazole treatment group. D. and severe ulcers were apparent on the gastric surface in the Sham group.

analysis, one-way Analysis of Variance and Tukey HSD test of multiple comparison tests were used for comparison of three or more groups, and the Kruskal-Wallis and Bonferroni-Dunn test for multiple comparisons were used to evaluate variables that did not conform to the normal distribution. The level of statistical significance was accepted as α=0.01. All data were evaluated using SPSS, Chicago IL, Version 17 software. SPSS Statistics 17.0 is a comprehensive system for analyzing data. SPSS Statistics can take data from almost any type of file and use them to generate tabulated reports, charts, and plots of distributions and trends, descriptive statistics, and complex statistical analyses. SPSS Statistics makes statistical analysis more accessible for the beginner and more convenient for the experienced user. Simple menus and dialog box selections make it possible to perform complex analyses without typing a single line of command syntax. The Data Editor offers a simple and efficient spreadsheet-like facility for entering data and browsing the working data file.

Results

The Macroscopic appearance of the stomach is shown for all groups in **Figure 1**. Normal gastric mucosa was observed in the control group (**Figure 1A**). Moderate ulcer area was observed in the Ozone therapy group (**Figure 1B**), minimal ulcers were observed in the Lansoprazole treatment group (**Figure 1C**), and severe ulcer occurred on the gastric surface in the Sham group (Group 2) in which no treatment was given (**Figure 1D**).

The sham group had the largest ulcer area (71.47 ± 17.08 mm^2); this result differed significantly from the other groups. The ulcer area was 20.79 ± 12.88 mm^2 in ozone group and this was significantly lower than the sham group but significantly higher than the lansoprazole treatment and control groups. The ulcer area was 5.80 ± 3.85 mm^2 in the lansoprazole treatment group, which was significantly greater than the control group but significantly less than the ozone or sham treatment groups (**Table 1**).

The statistical analysis of MPO, MDA, CAT, tGSH, SOD, and 8-OHdG measurements are summarized in **Table 2**. MPO (36.34 ng/ml), MDA (2.78 nmol/ml), CAT (87.21 ng/ml), and

Table 1. Comparison of groups in terms of ulcer area

Group 1 (Control) mm^2	Group 2 (Sham) mm^2	Group 3 (Ozon) mm^2	Grup 4 (Lansoprazole) mm^2
1) 0	1) 92.8	1) 35.3	1) 2.7
2) 0	2) 70.6	2) 24.2	2) 5.3
3) 0	3) 88.3	3) 23.1	3) 4.5
4) 0	4) 78.4	4) 8.3	4) 3.7
5)0	5) 68.8	5) 13.7	5) 3.1
6) 0	6) 57.5	6) 37.2	6) 13.7
7) 0	7) 43.9	7) 3.7	7) 7.6
Mean: 0	71.4	20.7	5.8

	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum	p
Control	7	0	0	0	0	0	
Sham	7^{a}	71.47	17.08	6.45	43.90	92.80	
Ozone	$7^{a,b}$	20.79	12.88	4.87	3.70	37.20	0.001**
Lansoprazole	$7^{a,b,c}$	5.80	3.85	1.45	2.70	13.70	
Total	21	32.69	31.16	6.80	2.70	92.80	

**P<0.01; a: Different from Group 1; b: Different from Group 2; c: Different from Group 3.

8-OHdG (13.97 ng/ml) levels were elevated in the sham group but were significantly reduced in the treatment groups. The decrease in MPO, MDA, and 8-OHdG was greater in the ozone therapy group compared to the lansoprazole group (P=0.001). On the other hand, the levels of tGSH (210.68 mg/L) and SOD (2.74 ng/ml) reduced in the sham group but significantly elevated in the treatment groups. This increase was more significant in ozone therapy group compared to the lansoprazole group (P=0.001) (**Table 2**).

Discussion

Gastric ulcer, one of the most common diseases of gastrointestinal tract, is caused by increased gastric acid secretion and by degradation of gastric mucosal integrity. Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin are major causes of gastric ulcer formation. Numerous studies have evaluated a range of potential medical treatments for gastric ulcer [15]. The aim of this study is to investigate the protective effects of Ozone, a powerful antioxidant, on gastric ulcer induced by Indomethacin relative to Lansoprazole, a commonly used proton pump inhibitor with proven clinical efficacy.

Most of NSAIDs, including Indomethacin are polar molecules with high affinity for the lipophilic regions of cell membranes. The polar groups of these drugs can cause lipid peroxidation and membrane damage through the loss of structural phospholipids and membrane proteins [16]. Lansoprazole, a benzimidazole derivative, is a widely used proton pump inhibitor that inhibits gastric acid secretion. The efficacy of this agent has been demonstrated in the treatment of reflux esophagitis, active gastritis, duodenal ulcer, and the treatment of gastric and duodenal ulcers induced by non-steroidal anti-inflammatory drugs (NSAIDs). Lansoprazole selectively inhibits the membrane enzyme H+/K+ ATPase in gastric parietal cells. Lansoprazole is more effective than histamine (H_2)-receptor antagonists [17]. Lansoprazole confers additional protection to the gastric mucosa through the reduction of oxidative stress and increased production of antioxidants [6]. Ozone has multiple therapeutic effects in wound healing mediated by the release of nascent oxygen, which has bactericidal capabilities, and the stimulation of antioxidant enzymes. Previous clinical and experimental studies have demonstrated the beneficial effects of ozone therapy in wound healing and ulcer treatment [18-20].

In this study, the mean ulcer area was 71.47 mm^2 in the gastric mucosa of sham-treated animals. The ulcer area was 20.79 mm^2 in the ozone therapy group, a significant reduction relative to the sham group. Lansoprazole had the greatest protective effect, with ulcer area comparable to the control group (5.80 mm^2). These findings support the conclusion that Lansoprazole, widely used in the treatment of gastric ulcer, has a strong protective effect against NSAID-induced stomach ulceration. Although Ozonated water was not as effective as Lansoprazole, it had a clear macroscopic protective effect on gastric ulcer formation induced by indomethacin.

Free oxygen radicals are produced during tissue damage, resulting in increased and oxidative stress. Lipid and protein oxidation products

Table 2. Statistical analysis of biochemical data

		N	Mean	Std. Deviation	Std. Error Mean	Minimum	Maksimum	P
MPO	Group 1	7	13.18	2.93	1.11	8.62	16.70	
	Group 2	7[a]	36.34	4.85	1.83	30.16	44.06	
	Group 3	7[a,b]	20.53	4.07	1.54	14.07	25.78	0.001**
	Group 4	7[a,b,c]	23.25	3.45	1.30	19.06	27.96	
	Total	28	23.32	9.28	1.75	8.62	44.06	
MDA	Group 1	7	0.68	0.18	0.07	0.40	0.92	
	Group 2	7[a]	2.78	0.93	0.35	1.57	4.02	
	Group 3	7[a,b]	1.07	0.22	0.08	0.83	1.41	0.001**
	Group 4	7[a,c]	2.02	0.26	0.10	1.81	2.55	
	Total	28	1.64	0.96	0.18	0.40	4.02	
CAT	Group 1	7	17.41	8.01	3.03	0.00	23.80	
	Group 2	7[a]	87.21	5.11	1.93	80.02	94.54	
	Group 3	7[a,b]	31.64	2.84	1.08	28.01	35.83	0.001**
	Group 4	7[a,b,c]	41.49	3.23	1.22	36.54	45.62	
	Total	28	44.44	27.07	5.11	0.00	94.54	
tGSH	Group 1	7	605.45	87.29	32.99	515.68	752.91	
	Group 2	7[a]	210.68	57.06	21.57	142.60	294.64	
	Group 3	7[a,b]	485.62	67.45	25.49	415.07	588.74	0.001**
	Group 4	7[a,b,c]	349.72	33.37	12.61	286.01	386.27	
	Total	28	412.87	162.16	30.65	142.60	752.91	
SOD	Group 1	7	9.80	1.21	0.46	8.21	11.53	
	Group 2	7[a]	2.74	0.61	0.23	2.01	3.65	
	Group 3	7[a,b]	6.47	0.60	0.23	5.71	7.49	0.001**
	Group 4	7[a,b,c]	4.66	0.46	0.17	4.11	5.55	
	Total	28	5.92	2.75	0.52	2.01	11.53	
8-OHdG	Group 1	7	5.77	0.53	0.20	5.12	6.67	
	Group 2	7[a]	13.97	0.82	0.31	12.84	15.12	
	Group 3	7[a,b]	6.53	0.43	0.16	6.11	7.08	0.001**
	Group 4	7[a,b,c]	7.94	0.64	0.24	6.95	8.78	
	Total	28	10.52	10.43	1.97	5.12	61.01	

**P<0.01; a: Different from group 1; b: Different from group 2; c: Different from group 3.

are elevated during oxidative stress and antioxidant enzymes are decreased. MDA, an end product of lipid peroxidation, causes destruction of cells. tGSH neutralizes free radicals and peroxides [21]. Ozone, an antioxidant agent, increases tGSH levels but decreases MDA concentrations [22]. Increased MDA in the gastric tissue following indomethacin administration is evidence of the presence of oxidative stress. Elevation of MDA in the sham group relative to the other groups indicates that oxidative damage was greatest in this group. After ozone administration, MDA was reduced and tGSH was elevated relative to animals that received Lansoprazole. This condition suggests that Ozonated water is a more powerful antioxidant than Lansoprazole. Reactive oxygen species (ROS) are superoxide radicals ($O_2 \cdot -$) formed in small amounts during normal oxygen metabolism and include hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•). Superoxide radical ($O_2 \cdot -$) occurs as a result of one-electron reduction of molecular oxygen (O_2) in almost all aerobic cells. The auto-oxidation of reduced transition metals can generate superoxide radicals. Superoxide radicals are more reactive at low pH. SOD is an enzyme that plays an important role in prevention of free radical damage. This enzyme converts superoxide into hydrogen peroxide. CAT is localized in peroxisomes and con-

verts hydrogen peroxide into water and oxygen [23]. In this study, one explanation for the remarkable efficacy of Lansoprazole may be that Lansoprazole reduced the reactivity of superoxide radicals by decreasing pH through inhibition of the proton pump. Boyacıoglu *et al*. pointed out that increased CAT activity following indomethacin treatment of the rat stomach was reduced by lansoprazole and lycopene, an antioxidant substance; however, SOD levels were increased under these conditions [24]. Our study produced similar results. Indomethacin administered to gastric mucosa after ozonated water and lansoprazole administration reduced CAT activity but increased SOD activity. Since CAT levels in ozone group were lower than in the Lansoprazole group and SOD activity was higher in ozone group, we propose that ozone is a more powerful antioxidant than lansoprazole. MPO activity is an acute inflammation marker that reflects polymorphonuclear cell infiltration. MPO activity can also be used to assess the degree of ulceration, and MPO increases in the gastric tissue of rats treated with indomethacin [25]. In this study, MPO activity was increased by indomethacin administration, but inhibited by Ozone and Lansoprazole treatment. The decrease in MPO activity was more significant in the Ozone group compared to the Lansoprazole group ($P = 0.001$). The antioxidant effects of ozonated water may be more effective in limiting ROS generation after indomethacin treatment in comparison to lansoprazole.

2'-deoxyguanosine is oxidized to 8-hydroxy-2'-deoxyguanosine (8-OHdG) by ROS in DNA. 8-OHdG is a substrate for several DNA-based excision repair systems and is released from cells after DNA repair. Because of this feature, 8-OHdG is used as a biomarker of oxidative DNA damage [26]. It has been used in some studies to measure gastric DNA damage caused by NSAIDs and to evaluate the efficacy of protective agents [27]. Increase in 8-OHdG levels following indomethacin treatment indicates that DNA damage. 8-OHdG level decreased after ozone and lansoprazole administration, and this decrease was more significant in ozone group. This demonstrates that both lansoprazole and ozone prevent DNA damage through an antioxidative effect. DNA damage decreased more significantly following ozone administration due to the antioxidant effect of ozonated water.

Zamora *et al.* [28] evaluated Ozonated sunflower oil and Cimetidine in ulcer treatment in an experimental study of indomethacin-induced gastric mucosa damage. We first administered Lansoprazole and Ozone and then administered indomethacin in our study in order to investigate protective effect of Oxygen-Ozone therapy on acute gastric ulcer formation. Freshly ozonated water is widely used than olive or sunflower oils. Ozone production time is between 5-20 minutes in water ozonation. Ozonation of either olive or sunflower oils requires a much longer time and the procedure needs to be well-standardized in for gas-flow, O_3 concentration, oil volume, and temperature. In addition, water ozonation is much less expensive in comparison to ozonated oils [29]. The high efficacy of ozonated sunflower oil relative to Cimetidine reported in previous studies is not consistent with the findings of the present study. Our study evaluated Lansoprazole, a more potent antiulcer agent in comparison to Cimetidine. Thus, the clinical differences between Cimetidine and Lanopazole may be more significant than the apparent differences between ozone-based therapies. There may be clinical differences between the protective and therapeutic effects of these agents.

In summary, ozonated water is a potent antioxidant that protects against acute gastric ulcer formation induced by indomethacin in a rat model. Ozone therapy has a more significant antioxidative effect than lansoprazole. However, the gastroprotective effect of ozonated water is macroscopically much weaker than lansoprazole, a potent proton pump inhibitor.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Senol Bicer, Department of Pediatric Surgery, Faculty of Medicine, Erzincan University, Erzincan 24000, Turkey. Tel: +90.446.212 2215; +90.505.6943470; Fax: +90.446.212 2218; E-mail: drsenolbicer@gmail.com

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