DOI: 10.4149/BLL_2018_015

Bratisl Med J 2018; 119 (2)
81 – 85

EXPERIMENTAL STUDY

The efficacy of ozone therapy in neonatal rats with hypoxic ischemic brain injury

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ABSTRACT
OBJECTIVES: This study is aimed to determine the effect of ozone therapy in neonatal rats with experimentally induced hypoxic ischemic brain injury (HIBI).
METHODS: The study included 7-d-old male Wistar rats that were randomized to the sham, control, ozone 1, and ozone 2 groups. All rats except those in the sham group were kept in a hypoxia chamber, and then the rats in the control group were given 0.5 mL of saline. Those in the ozone 1 group were given ozone 1 mg kg^{-1} intraperitoneally, and those in the ozone 2 group were given ozone 2 mg kg^{-1} intraperitoneally.
RESULTS: There were significantly fewer apoptotic neurons in the right hemispheres of the rats in the ozone 1 and ozone 2 groups than in the control group ($p < 0.001$ and $p < 0.001$, respectively). There were significantly fewer apoptotic neurons in the right hemispheres of the rats in the ozone 2 group than in the ozone 1 group ($p < 0.001$). Morris Water Maze (MWM) test results were similar in the ozone 2 and sham groups.
CONCLUSIONS: The present study's findings show that ozone therapy reduced neuronal apoptosis and improved cognitive function in neonatal rats with experimentally induced HIBI *(Tab. 2, Ref. 30)*. Text in PDF *www.elis.sk.*
KEY WORDS: hypoxic ischemic brain injury, neuroprotection, ozone.

Introduction

Moderate to severe hypoxic ischemic encephalopathy (HIE) occurs in approximately 1–3/1000 live births, and is a major cause of neonatal death and disability (1). Recent multicenter randomized controlled studies have shown that therapeutic hypothermia (TH) decreases mortality and disability in infants with HIE (2); however, TH is not always sufficient, especially in cases of severe HIE, as very high number of infants die or suffer from disability despite receiving TH (3). As such, more efficient treatment methods that can be used with or without TH are needed for patients with HIE.

Ozone (O_3) is a cyclic gas composed of 3 oxygen atoms (4). Recently, medical ozone therapy has been shown to be a safe, economic, and effective treatment for many conditions, including advanced ischemic lesions, peritonitis, infected wounds, chronic skin ulcers, initial gangrene, and burns (5). Some animal studies reported that ozone inhibited the apoptosis and exhibited an anti-inflammatory effect in ischemia-reperfusion (I/R) injury experimentally induced in several organs (5, 6). In addition, studies have shown that ozone reinforced the endogenous antioxidant systems and protected organ functions by ensuring adaptation against transient oxidative stress (5, 7). A recent in vitro rat study reported that ozone exerted a neuroprotective effect in a brain ischemia model (8, 9). It was suggested that ozone exerts its neuroprotective activity via a kind of preconditioning effect by initiating repair on one hand and increasing the defensive capacity through a hormesis effect on the other (9). To the best of our knowledge, literature does not include any studies on the effect of ozone in a neonatal hypoxic ischemic brain injury (HIBI) model; therefore, the present study is aimed to determine the effect of ozone therapy in neonatal rats with experimentally induced HIBI.

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Material and methods

Experimental procedures

The study was conducted by the Pediatrics, Physiology, and Pathology Departments of Mersin University School of Medicine, at the Experimental Animals Research Laboratory and Physiology Behavior Laboratory. The Mersin University Animal Experiments Ethics Committee approved the study protocol. 7-d-old male Wistar rats (n = 64) were used in the present study. They were randomized into 4 groups:

Sham group (n = 16): Following neck dissection, the right carotid artery was located, but not tied. Hypoxia was not induced;

Control group (n = 16): Immediately following induction of HIBI, 0.4 mL of saline was administered via the intraperitoneal route;

Ozone 1 group (n = 16): Immediately following induction of HIBI, ozone 1 mg kg^{-1} was administered via the intraperitoneal route;

Ozone 2 group (n = 16): Immediately following induction of HIBI, ozone 2 mg kg^{-1} was administered via the intraperitoneal route.

All rats were administered inhalation anesthesia for <5 min, and then a midline neck incision was performed and the right carotid artery was located via microscopic guidance. In all rats, except those in the sham group, the right carotid artery was tied with 6.0 silk suture and the rats were placed in a hypoxia chamber containing 8% oxygen for 2 h. Next, the rats were removed from the chamber and administered treatment according to group, and then placed with their mothers for a 2-h recovery period (10).

Following 2 h of recovery, 8 rats from each group were euthanized via cervical dislocation and were decapitated. In order to observe neuronal apoptosis, the rats' brains were removed, and then examined via the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method. The remaining rats underwent Morris water maze (MWM) testing at age 14 weeks.

Ozone generation and administration

Evozone basic Plus (Reutlingen, Germany) was used for ozone generation (flow rate: 10 mL s^{-1}; concentration range: 0–80 μg mL^{-1}). Ozone at the concentration of 25 μg mL^{-1} was used for the study. Ozone was collected from the generator in ozone-resistant injectors and was immediately administered to the rats intraperitoneally at 1 mg kg^{-1} or 2 mg kg^{-1}.

Evaluation of apoptosis

In order to evaluate apoptosis, two samples from the parietal cortex, hippocampus, and subthalamic nucleus were obtained from each rat. A pathologist that was blinded to the groups and to which carotid artery was tied examined the rat brains. DNA fragmentation in neurons was observed by using the TUNEL method (*in situ* apoptosis detection kit, Biogen, catalog no. S7101). Firstly, deparaffinized coronal brain sections (5 mm thick) were treated with alcohol, after that they were incubated with protein kinase K at room temperature for 15 min. Next, endogen peroxidase activity was extinguished using 2 % H_2O_2, and then slices were incubated at 37 °C for 60 min in a moist chamber with 50 μL of TdT buffer. Following incubation, the reaction was made visible using a streptavidin-biotin-peroxidase complex and diaminobenzidine. 1 % methyl green was used to counter stain TUNEL-labeled slides. In order to evaluate numeric density, total TUNEL-positive neurons were enumerated in 5 high-power fields (400´) via light microscopy. Samples from the subthalamic nucleus, hippocampus, and parietal cortex of both the right and left hemispheres were used to enumerate apoptotic neurons (11).

MWM test

The rats were placed in the behavioral experiments laboratory 2 days prior to MVM testing for habituation. MWM testing was initiated at age 14 weeks and was performed for 5 days. Water (22 °C) was used to fill a 42-cm deep tank. The same investigator placed the rats in the tank during each MVM test. All rats underwent MWM testing between 0900 and 1400. The tank's image was transferred to a screen of a computer and this image was divided into 4 equal quadrants: north, south, east, and west.

In the first 4 days of testing, a 15-cm diameter platform was placed in the middle of the east quadrant at a height of 40 cm, so it could not be observed from outside of the 42-cm deep tank. On day 1 of testing, all rats were placed in each quadrant (beginning in the west quadrant and proceeding clockwise) with their heads turned toward the wall of the water maze. On days 2–4, the rats were placed first in the north, east and south quadrants first, respectively, proceeding in clockwise fashion to the other quadrants, as on day 1.

After being dropped into the water the rats were expected to find the hidden platform within 60 s. Rats that did not locate the platform within 60 s were guided by hand to the platform and were held there for 15 s. For each d/quadrant platform finding time (PFT) of the rats was recorded. On the 5^{th} day of the experiment, the hidden platform in the east quadrant was removed. After that, all rats were dropped into the water in the west quadrant and stayed in the water for 60 s. Time (s) that the rats spent in the east quadrant (which previously contained the platform) was recorded (12).

Statistical analysis

Statistical analysis was performed using SPSS v.11.5 for Windows. The normality of the distribution of continuous data was determined using the Shapiro–Wilk test. Variance analysis was used to identify differences between measurements on days 1, 2, 3, and 4 in each group, as well as differences between groups according to days. When variance was homogenous one-way ANOVA was used and the Welch test was used when it was not homogenous. Homogeneity of the variances was determined via the Levene test. Tukey's and Games–Howell tests were used for paired comparisons. In addition, inter-group differences in time spent in the east quadrant on day 5 were analyzed using one-way ANOVA and the Welch test. Differences between left and right hemisphere apoptotic neuron counts in each group were determined using the paired samples t test. Inter-group differences between right and left hemisphere measurements were determined using one-way ANOVA and the Welch test. Descriptive statistics are shown as mean ± SD. P values of < 0.05 were accepted as statistically significant.

Results

Two of the 64 rats included in the study died while inducing HIBI. Among the remaining 62 rats, 32 were decapitated for neuronal apoptosis assessment and MWM testing was performed with the 30 rats that remained at age 14 weeks (sham group: n = 8; control group: n = 8; ozone 1 group: n= 7; ozone 2 group: n = 7).

Neuronal apoptosis

Except the sham group, right hemispheres were containing significantly more apoptotic neurons than left hemispheres in all

Tab. 1. The number of TUNEL-positive apoptotic neurons according to group.

Group	Right hemisphere	Left hemisphere	P_0
Sham	1.60±0.84	1.50±0.85	0.823
Control	10.40±2.07	3.90±1.20	**<0.001**
Ozone1	5.70±0.95	2.40± 1.43	**<0.001**
Ozone2	3.40±0.52	1.80±0.79	**<0.001**
P_1	<0.001	<0.001	
P_2	<0.001	0.451	
P_3	<0.001	1.000	
P_4	<0.001	0.025	
P_5	<0.001	0.001	
P_6	<0.001	1.000	

Data given are mean ± SD, P_0: Comparison of the number of apoptotic neurons in the left and right hemispheres, P_1: Comparison of the number of apoptotic neurons in the sham and control groups, P_2: Comparison of the number of apoptotic neurons in the sham and ozone1 groups, P_3: Comparison of the number of apoptotic neurons in the sham and ozone2 groups, P_4: Comparison of the number of apoptotic neurons in the control and ozone1 groups, P_5: Comparison of the number of apoptotic neurons in the control and ozone2 groups, P_6: Comparison of the number of apoptotic neurons in the ozone1 and ozone2 groups

Tab. 2. Mean MWM PFT on days 1–4.

Groups	d 1 (s)	d 2 (s)	d 3 (s)	d 4 (s)	P_0
Sham	41.39±12.08	19.34±11.76	9.64±5.87	11.03±7.51	*0.001*
Control	51.85±10.13	41.78±18.75	41.59±25.48	34.68±23.66	0.260
Ozone1	47.54±11.84	41.89±15.28	31.61±18.75	28.51±17.48	*0.152*
Ozone2	45.29±7.49	30.88±9.13	27.22±15.39	15.33±9.31	*<0.001*
P_1	0.313	0.014	0.037	0.042	
P_2	1.000	0.010	0.033	0.077	
P_3	1.000	0.353	0.048	0.731	
P_4	1.000	1.000	0.800	0.929	
P_5	1.000	0.428	0.543	0.207	
P_6	1.000	0.394	0.951	0.250	

MWM; Morris water maze, PFT; platform finding time, data given are mean ± SD, P_0: Indicates the difference in PFT from day 1 to day 4 within each group, P_1, P_2, P_3, P_4, P_5, and P_6 indicates the difference in PFT between the sham and control groups, between the sham and ozone1 groups, between the sham and ozone2 groups, between the control and ozone1 groups, between the control and ozone2 groups, and between the ozone1 and ozone2 groups, respectively

groups (Tab. 1). The numbers of right hemisphere apoptotic neurons in the sham, ozone 1, and ozone 2 groups were significantly lower than in the control group ($p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). There were significantly fewer right hemisphere apoptotic neurons in the sham group than in the ozone 1 and ozone 2 groups ($p < 0.001$ and $p < 0.001$, respectively). The number of right hemisphere apoptotic neurons in the ozone 2 group was significantly lower than in the ozone 1 group ($p < 0.001$).

The numbers of left hemisphere apoptotic neurons in the sham, ozone 1, and ozone 2 groups were significantly lower than in the control group ($p < 0.001$, $p = 0.025$, and $p = 0.001$, respectively). The sham, ozone 1, and ozone 2 groups did not differ significantly in terms of left hemisphere apoptotic neurons ($p > 0.05$).

MWM

PFT was significantly shorter in the sham and ozone 2 groups on day 4 than on day 1 ($p = 0.001$ and $p < 0.001$, respectively), whereas in the control and ozone 1 groups PFT values did not change on day 4 from those on day 1 ($p = 0.152$ and $p = 0.260$, respectively) (Tab. 2). On day 5 of MWM testing, the rats in the control group spent 18.45 ± 8.86 s in the east quadrant (where on days 1–4 the hidden platform was located), versus 26.02 ± 5.91 s in the sham group, 18.44 ± 5.39 s in the ozone 1 group, and 24.18 ± 2.88 s in the ozone 2 group. On day 5, the rats in the sham group spent significantly more time in the east quadrant than the rats in the control and ozone 1 groups ($p = 0.026$ and $p = 0.016$, respectively). There were no significant differences in time spent in the east quadrant on day 5 between the sham and ozone 2 groups ($p = 0.538$).

Discussion

Perinatal asphyxia occurs due to insufficient levels of oxygen and blood supplied to the tissues and organs of fetuses and neonates. Although the precise mechanism of cellular damage following hypoxia and ischemia is not known, energy deficiency, and excessive increases in excitatory neurotransmitters, free oxygen radicals, and lipid peroxidation lead to the cascade of injury events (13, 14). Free oxygen radicals are a serious threat to vital organs and tissues, and nucleic acids and polyunsaturated fatty acids in cell walls (1, 15). Polyunsaturated fatty acids are negatively affected to the greatest extent from this oxidative damage, which is referred to as lipid peroxidation.

Glutathione peroxidase, catalase, and superoxide dismutase (SOD) are antioxidant enzymes that protect biological structures against free radical-mediated injury (13); however, neonates have insufficient antioxidant enzyme levels and activity (16). In addition, rich in unsaturated phospholipids, the brain is extremely susceptible to oxidative stress (17). Studies have shown that free oxygen radical levels are significantly elevated in cases of HIE and that antioxidant enzyme activity does not increase sufficiently (13). Once the importance of free radical damage in the pathogenesis of HIE was understood, researchers began to study the use of antioxidant agents for the treatment of HIE. It was reported that melatonin, a potent antioxidant, is useful for treating HIE (18), as was erythropoietin, which is known to have antioxidant effects (19). Moreover, it was suggested that the suppression of free radical activity and lipid peroxidation associated with TH contributes significantly to its neuroprotective activity (20). Based on these findings, we think that while free radical damage plays an important role in the pathogenesis of HIE, neonates with HIE have insufficient endogenous antioxidant capacity, and that limiting the free radical damage will be beneficial in the treatment of HIE. As such, we also think that ozone, which stimulates production of endogenous antioxidant enzymes, might also be beneficial in the treatment of HIE. In fact, earlier studies reported that ozone stimulated the production of antioxidant enzymes, including glutathione peroxidase, catalase, and SOD (7).

In recent years, clinically successful utilization of ozone therapy for such conditions as disc hernia, diabetic foot, and peripheral artery disease have been reported (21–23). Furthermore, ozone therapy was reported to limit the effects of experimentally induced I/R injury in multiple organs (24, 25). Xing et al (26) reported that tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, in-

tercellular adhesion molecule 1 (ICAM-1), and monocyte chemo attractant protein-1 (MCP-1) mRNA levels were significantly suppressed, and that toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF-κB) expression was inhibited in response to ozone oxidative preconditioning (ozoneOP) in rats with experimentally induced renal I/R. Based on these findings, Xing et al (26) suggested that ozone OP exerted a potent anti-inflammatory effect via modulation of TLR4 and NF-kappa.

Wang et al (6) reported a significant decrease in renal fibrosis in response to ozone OP in rats with experimentally induced renal I/R. Chen et al (24) reported that ozone OP inhibited the inflammation and apoptosis in rats with renal I/R injury. Peralta et al (25) observed a decrease in hepatic damage with ozone treatment in an experimental I/R model. Güven et al (27) reported that antioxidant levels increased, TNF-α level decreased, and histopathologically observed intestinal damage decreased following administration of intraperitoneal ozone to rats with experimentally induced necrotizing enterocolitis. Sukhotnik et al (5) reported that ozone therapy administered intraperitoneally in adult rats increased intestinal cell production and decreased apoptosis. One study reported that intraperitoneally administered ozone decreased the TNF-α level and increased the SOD level in a rat model of experimental peritonitis (4). Bakkal et al repeatedly administered low doses of intraperitoneal ozone to rats with experimentally induced radiation lung injury, and reported a decrease in TNF-α and IL-1β levels, an increase in SOD activity, and a reduction in lung damage (28).

Zhang et al (29) reported a significant decrease in wound healing time in response to ozone-oxygen therapy in patients with diabetic foot ulcers. Zhang et al (29) also observed a significant increase in the levels of vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF- β), and platelet-derived growth factor (PDGF) following ozone-oxygen therapy. Based on these findings, Zhang et al (29) suggested that the positive effects of ozone-oxygen therapy on wound healing might be due to an increase in endogenous growth factors. Shehata et al (30) reported that ozone therapy can correct the biochemical changes in the cerebral cortex of rats that occur due to aging. Recently, Frosini et al (9) reported that ischemic brain injury was reduced in response to ozone therapy in an in vitro rat model. To the best of our knowledge the present study is the first to show that intraperitoneal ozone therapy administered to neonatal rats with experimentally induced HIBI decreased neuronal apoptosis and improved cognitive functions; these positive effects of ozone were more pronounced when administered at 2 mg kg^{-1} than 1 mg kg^{-1}.

An important limitation of the present study is that it did not determine the mechanism by which ozone exerted its neuroprotective activity in neonatal HIBI; however, earlier studies suggest that ozone stimulates production of antioxidant enzymes, suppresses inflammation, stimulates immune and neuroendocrine system activity, and promotes release of growth factors via preconditioning- and hormesis-like effects (7, 9, 21), which might have been elucidated in the present study if antioxidant enzyme levels, the levels of several growth factors, or the levels of several inflammatory biomarkers had been investigated. In conclusion, the present findings show that ozone therapy had a neuroprotective effect in neonatal rats with experimentally induced HIBI; however, additional research is needed to understand how ozone exerts its neuroprotective effect.

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Received September 28, 2017.
Accepted October 30, 2017.