RESEARCH ARTICLE

Relationship of ozone application and time in rats with hypoxic-ischemic brain injury

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Abstract

Background: Despite the important advances in pregnancy and newborn follow-up, hypoxic-ischemic encephalopathy is still one of the prominent causes of newborn mortality and disability worldwide, and there is no sufficiently effective treatment for it yet. This study aimed to investigate whether the ozone injection, administered in a single-dose as a preconditioning agent before the hypoxia and in single and repeated doses on different days following the hypoxia, would affect the spatial memory performance of the rats in the Morris water maze test or on their apoptotic cell numbers.

Methods: The study consisted of 102 seven-day-old male Wistar baby rats randomly divided into five groups. Rats in all groups were induced with hypoxic-ischemic brain injury (HIBI) except for the Sham group, and 1.2 mg/kg ozone was administered intraperitoneally. For the apoptosis evaluation, eight rats from each of the first four groups were decapitated by cervical dislocation. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was used for immunohistochemical quantification of apoptosis in the excised brains. Blood malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured in the blood samples collected through cardiac puncture. Fourteen-week-old rats underwent the Morris water maze test to test their long-term spatial memory.

Results: On apoptotic quantification in the right hemisphere using the TUNEL assay, the numbers of apoptotic neurons in the ozone preconditioning group (Group 3) and the group given ozone on the day of hypoxia (Group 4) were found to be significantly higher than the Sham group (Group 1), but significantly lower than the non-treatment group (Group 2) ($p < 0.001$; $p < 0.001$, respectively). Group 3 rats had the highest mean MDA level and SOD activity. Considering the platform finding times in the first four days of the tests, Group 4 had the shortest times after Group 1; and on Day 4, Group 4 found the platforms significantly sooner than Groups 2, 3, and 5 ($p < 0.001$). Comparison of Groups 1 and 4 revealed significantly shorter times for Group 1 for each day except for Day 2.

Conclusions: Other studies have shown that controlled application of ozone would result in oxidative preconditioning and reduce the damage induced by reactive oxygen species through enabling adaptation to oxidative stress. Our study obtained remarkable and encouraging findings for ozone administration in HIBI by examining Group 4's performance in the first four days and the difference in its platform finding times between Day 1 and Day 4. HIPPOKRATIA 2021, 25 (2):56-62.

Keywords: Hypoxic-ischemic brain injury, ozone, preconditioning, Morris water maze, behavior

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Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE), affecting an estimated 15 per 10.000 live births, is considered one of the most common causes of neurological deficit in children. Several studies have shown that therapeutic hypothermia reduced mortality and became a standard treatment protocol for most newborn centers in developed countries[1]. Besides therapeutic hypothermia, there are other methods and agents which have been used for the treatment of hypoxic-ischemic brain injury (HIBI), such as erythropoietin administration, stem cell transplant, neural growth hormone therapy, phospholipase A2 inhibitors, cyclooxygenase inhibitors, lipoxygenase inhibitors, xanthine oxidase inhibitors, suppression of nitric oxide synthase, selenium, vitamin E, N-acetylcysteine, and ascorbic acid[2-6]. In HIBI, ischemia plays a more critical role. The reperfusion period following hypoxia and ischemia is the critical phase of heavy

injury and could be possible to reduce or prevent HIBI with the administration of treatments prior to this phase[7].

Ozone is one of the most important gases found in the stratosphere. Though its mechanism of effect is yet to be fully understood, ozone is known to cause the formation of oxygen radicals and lipid peroxidation[8]. Therefore, it has been suggested to stimulate the antioxidant system, thus improving its efficiency[9]. Based on recent pharmacological studies, ozone could be regarded as a prodrug. In specific non-toxic doses, it may induce biochemical pathways' remodeling and activate the secondary messengers in most systems. Currently, it has been shown that antioxidant enzymes, nitric acid pathways, and other cellular activities can be reorganized with a low dose of ozone[10]. Recently, ozone has been used in treating fibromyalgia and osteoarthritis, wound healing, cancer treatment, and SARS-CoV-2/COVID-19 studies[11-14]. Other studies have shown that ozone treatment did not have any side effects, and it increased the life quality of patients with peripheral arterial disease[15]. A study reported that ozone administration protected against ischemia-reperfusion injury in the liver[16]. Another study with rats concluded that ozone showed a neuroprotective effect in the brain ischemia model[17]. In light of this information, the present study aimed to show the effects of ozone administered at different times on HIBI.

Material and methods

The present study was conducted by the Departments of Physiology, Pediatrics, Pathology, and Medical Biochemistry, Faculty of Medicine, Mersin University. It was carried out in the Laboratory Animal Research Center of the Faculty of Medicine and Behavioral Laboratory of the Department of Physiology of Mersin University. Mersin University's Local Ethics Committee for Animal Experiments approved the present study (decision No 2010/38). The study consisted of 102 seven-day-old male Wistar baby rats, randomized into five groups. In rats of all groups except for the Sham surgery group HIBI was induced.

Group 1 (Sham surgery) (n =26): Neck dissection was performed following anesthesia and the right carotid arteries were found but not ligated, and no hypoxia was induced either.

Group 2 (n =22): Physiological serum (PS) was administered intraperitoneally following the induction of hypoxia.

Group 3 (n =21): Ozone was administered intraperitoneally (1.2 mg/kg) one day before the induction of hypoxia.

Group 4 (n =21): Ozone was administered intraperitoneally (1.2 mg/kg) straight after the procedure of hypoxia induction on Day 7.

Group 5 (n =12): Ozone was administered intraperitoneally (1.2 mg/kg) following hypoxia on Days 96, 97, and 98 right before the Morris water maze test (Table 1).

Anesthesia was induced using the inhalation anesthetic agent isoflurane in the seven-day-old baby rats included in the study. Under the microscope, the right carotid arteries were identified following medial neck dissection and ligated for all rats except those in the Sham group. Following this ligation procedure, all the rats except the Sham Group were taken to the hypoxic chambers[18]. Temperature and oxygen levels in the hypoxic chambers were constantly monitored; the temperature was maintained at 33.5 ± 0.5 °C, and the oxygen level at 8 %. In order to show the induced HIBI and evaluate the neuronal apoptosis, eight rats from each of the first four groups were decapitated using cervical dislocation (their weights were calculated as 11,884 ± 0,961, 11,772 ± 1,017, 11,880 ± 0,956, 11,890 ± 1,284 (gr), respectively). One or two samples representing the subthalamic nuclei, the hippocampus, and the parietal cortex from the excised brains, were studied.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (In Situ Apoptosis Detection Kit, catalog no. S7101, Biogen, Cambridge, Massachusetts, USA) was used for immunohistochemi-

Table 1: The procedure schedule for all five groups included in the study, comprised in total 102 seven-day-old male Wistar baby rats.

Name of the Procedure	Time					
	Day 6	Day 7	Day 96	Day 97	Day 98	Week 16
Ozone preconditioning (Gr.3)	x					
HIBI induction (Gr.2,3,4,5)		x				
Ozone application (Gr.4)		x				
Decapitation for apoptotic quantification (Gr.1,2,3,4) (8 rats per group)		x				
Blood sample collection for SOD and MDA (Gr.1,2,3,4)		x				
Ozone application (Gr.5)			x	x	x	
Behavior experiments (Gr. 1,2,3,4,5)						x

Gr.: Group, Group 1: Sham surgery, Group 2: Physiological serum administered intraperitoneally following hypoxia, Group 3: Ozone administered intraperitoneally before hypoxia, Group 4: Ozone administered intraperitoneally straight after hypoxia, Group 5: Ozone administered intraperitoneally following hypoxia on Days 96, 97, and 98, HIBI: hypoxic-ischemic brain injury, SOD: superoxide dismutase, MDA: malondialdehyde.

cal quantification of apoptosis[19,20]. Levels of malondialdehyde (MDA), a by-product of lipid peroxidation, were measured in the blood samples collected through cardiac puncture from decapitated rats on Day 7 after birth. The presence of MDA was identified by incubating the samples with thiobarbituric acid (TBA) at pH 3.4 and 95 ºC under aerobic conditions and superoxide dismutase (SOD) enzyme activity assay was performed as well[21,22]. Evozone Basic Plus Ozone Unit was used as the ozone generator. This device runs at a flow rate of 10 ml/s and a concentration range of 0-80 µg/ml. The present study used an ozone concentration of 25 µg/ml. Ozone was administered intraperitoneally (1.2 mg/kg). Anti-ozone injectors with special coating were used for ozone injection.

Behavioral experiments were conducted in a room measuring 2.9 m wide, 3.9 m long, and 3.1 m high. The windows of this room were insulated against light and sound. Room temperature was maintained at a constant 23 ºC. Furthermore, during the experiments, an automatic dimmer was used to obtain a lighting rhythm of 12-hour light and 12-hour dark with four dimmable halogen lamps. All the experimental phases were recorded using the software Noldus Ethovision XT. Rats in the relevant group to be tested were taken to the behavioral laboratory two days before each experiment to ensure that all the rats included in the study were exposed to the same stress factors and they all spent the same amount of time in the experiment area for habituation. Spatial memory was assessed when the rats were 14 weeks old. The Morris water maze test was performed using a 150 cm diameter and 60 cm deep temperature-controlled stainless steel vessel. Visible and fixed visual clues were placed around the vessel. Tests were performed from 09.00 to 13.00 every day. The vessel was filled with water at 22 ± 1 ºC up to 42 cm. For the first four days of the experiment, a platform of 15 cm diameter, reaching up to 40 cm from the bottom, was submerged and fixed in the middle of the eastern quadrant. On Day 1, all the rats were put in the water facing the vessel wall once in each quadrant, beginning from the western quadrant and going clockwise, meaning they were released into the water four times in total. On Days 2, 3, and 4, it was ensured that the first release each day would be from a different quadrant by following a clockwise pattern. We allowed rats to swim for 60 seconds at each release, and those getting on top of the platform and standing there for five seconds within those 60 seconds were considered to have completed this phase of the test. Times to find the platform were recorded at each release for four days. On Day 5, the platform was removed from the vessel, and the rats were released from the western quadrant only once and recorded for 60 seconds. Upon completion of these 60 seconds, the mean time spent in the eastern quadrant (where the platform had previously been), the swimming speed, and the distance covered swimming were recorded in seconds, cm/s, and cm respectively[23,24].

Statistical analysis

We performed the statistical analysis using the SPSS for Windows, Version 11.5 (SPSS Inc., Chicago, IL, USA) software. The normality of continuous variables' distribution was determined using the Shapiro-Wilk test, revealing that the data had a normal distribution. We used a one-way analysis of variance (ANOVA) to test the differences between the groups for SOD enzyme activity and MDA levels. We used Levene's test to determine the homogeneity of variances. For multiple comparisons in one-way ANOVA, we used Tukey's honestly significant difference test, while for the quantification of difference between left and right hemispheres apoptotic cells in the brain within each group, the paired sample t-test was used. To determine the differences between the groups on the left and right hemispheres measurements, we utilized the one-way ANOVA. Considering the comparisons relating to Days 1, 2, 3, and 4 of the long-term spatial memory test, differences between groups and the days pertaining to each group were analyzed using a two-factor analysis of variance (two-way repeated-measures ANOVA). We used repeated-measures ANOVA for the analysis of the differences between days 1, 2, 3, and 4 in each group. We used one-way ANOVA and Welch tests to test differences between the groups within each day. One-way ANOVA was used when variance was homogenous, whereas we used the Welch test when it was not homogenous. Adjusting for multiple comparisons, we used the Bonferroni test in one-way ANOVA while in Welch test statistics the Games-Howell test. Also, we used repeated-measures ANOVA to analyze the differences between the northern, southern, eastern, and western quadrants on Day 5 of the long-term spatial memory test. Descriptive statistics are shown as the mean ± standard deviation. The level of statistical significance was set at $p < 0.05$.

Results

Of the 102 seven-day-old rats included in the study, 32 were decapitated as planned. Upon the death of two rats for unknown reasons, learning and memory tests proceeded with 68 rats.

Quantification of apoptotic cells in the brain via TUNEL assay

Within-group comparison of the number of apoptotic cells in the right and left hemispheres of the brain was determined using the TUNEL assay, and revealed that the hypoxia model used created a significant increase in the number of apoptotic cells in the right hemisphere compared to the left hemisphere, except for the Sham surgery group (Table 2). Statistical analysis of the number of apoptotic neurons identified in the right hemisphere of the brain did not find a significant difference only between Groups 3 and 4 ($p = 0.803$), whereas there was a significant difference between all other groups ($p < 0.001$). In conclusion, the numbers of apoptotic neurons in the right hemisphere of the group preconditioned with ozone (Group 3) and the group administered ozone on the

Table 2: Quantification of apoptotic neurons via terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay.

Group	Right Hemisphere	Left Hemisphere	p-value
Group 1 (n =8)	2.900 ± 0.737	2.800 ± 0.632	0.678
Group 2 (n =8)	10.900 ± 1.728	5.000 ± 1.632	0.0001
Group 3 (n =8)	6.400 ± 1.173	2.800 ± 1.032	0.0001
Group 4 (n =8)	5.888 ± 1.054	3.333 ± 0.707	0.0001

Values are given as mean ± standard deviation, n: number.

day of hypoxia (Group 4) were significantly higher than the Sham group (Group 1) but significantly lower than the non-treatment group (Group 2).

Plasma malondialdehyde level

On examination of the blood samples collected, the highest mean MDA levels were detected in the plasmas of the rats in Group 3. Plasma MDA levels were found to be 11.720 ± 7.486 nmol/ml in Group 1, 19.705 ± 6.163 nmol/ml in Group 2, 24.775 ± 9.488 nmol/ml in Group 3, and 24.166 ± 10.966 nmol/ml in Group 4. Between-group comparison of the plasma MDA levels revealed significant differences in the pairs Group 1 vs Group 3 and Group 1 vs Group 4 (p =0.008 and p =0,018, respectively); however, no significant difference was detected between other groups.

Superoxide dismutase enzyme activity assay

The highest mean SOD enzyme activity was detected in the blood samples collected from the rats in Group 3. SOD levels were 2.857 ± 0.987 U/ml in Group 1, 2.444 ± 1.206 U/ml in Group 2, 3.519 ± 1.426 U/ml in Group 3, and 3.060 ± 1.162 U/ml in Group 4. A between-group comparison of the SOD enzyme activity revealed no significant difference (p =0.253).

Morris water maze learning and memory tests

When we examined the times to find the platform for each group on Day 1, Group 1 had the shortest times, and there was a significant difference between Group 1 and Group 3 (p =0.035). No significant difference on Day 1 was found between the other groups regarding times finding the platform. Group 1 rats also found the platform in the shortest time on Day 2. Group 4 scored the second-best results, and there was no significant difference between Group 1 and Group 4 (p =0.483). Group 1 had significantly shorter times to find the platform than the other three groups (p <0.001). Times finding the platform in Group 4 rats were significantly shorter than only Group 3 (p <0.001). On Day 3, Group 1 rats came first again with the shortest times finding the platform, which were significantly shorter than those of each of the other three groups (p <0.001). No significant difference was found between the other groups. On Day 4, again, Group 1 rats found the platform in the shortest time, significantly shorter from those of the other groups (p <0.001 between Group 1 and Groups 2, 3 and 5; p =0.04 between Group 1 and Group 4). Group 4 had the shortest mean time after Group 1. Group 4 rats, too, found the platform in a significantly shorter time than Groups 2, 3, and 5 (p <0.001). Comparison of the difference in times finding the platform between Day 1 and Day 4 revealed no significance between Group 1 and Group 4, the groups which shortened the time to find the platform the most and scored as the best learners. There were significant differences between these groups and Groups 2, 3, and 5 (p <0.001 between Group 1 and other groups; p =0.015 between Group 4 and Group 2; p =0.001 between Group 4 and Group 3; p =0.037 between Group 4 and Group 5) (Table 3). On Day 5 of the learning and memory tests, the platform was removed, and the periods spent by the rats in the eastern quadrant (where the platform had previously been) were examined. It was found out that Group 1 and Group 4 spent the most time in the eastern quadrant. The times spent in the eastern quadrant of these two groups were significantly longer than the times they spent in the other quadrants (p <0.001). There was no significant difference for the other groups (Group 2, p =0.577: Group 3, p =0.382; Group 5, p =0.111).

Discussion

HIBI and complications secondary to HIBI are among the most significant causes of newborn mortality and morbidity in developed and developing countries. Despite the recent developments in maternal and newborn follow-up, there has not been a substantial difference in their incidence, and there is no sufficiently effective treatment modality yet[25]. It is known that late treatment after HIBI development is not effective enough. Thus, researchers have focused on early treatment to reduce injury. While no other treatment than supportive care has been implemented until recently, many treatment options have been proposed based on a better understanding of HIE physiopathology. These treatment options include allopurinol, magnesium sulfide, erythropoietin, melatonin, xenon, free radical scavengers, nitric oxide synthase suppressors, calcium channel blockers, hypothermia treatment, stem cell transplant, and growth factors therapy[26].

Ozone is known to cause the formation of oxygen radicals and lipid peroxidation[8]. Therefore, it has been suggested that it stimulates the antioxidant system, thus improving its efficiency[9]. Furthermore, ozone increases 2,3-DPG, which results in an elevated oxygen release from hemoglobin to the tissues[27]. It has been suggested that ozone increases tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin (IL)-2, and IL-8, triggers vascular prostacyclin (PGI_2) synthesis, and is an immunomodulator[28,29]. A study reported that ozone

Table 3: Average platform finding times of the groups by day and the difference in platform finding times between Days 1 and 4 for each group in the learning and memory tests.

	Platform Finding Time (sec.)				Difference in platform finding time Between Days 1 and 4 (sec.)
	Day 1	Day 2	Day 3	Day 4	
Group 1	48.82 ± 20.64	32.08 ± 21.72	18.52 ± 16.88	12.28 ± 12.21	38.97 ± 19.53
Group 2	54.75 ± 15.11	48.80 ± 20.03	41.74 ± 22.88	40.25 ± 22.59	19.23 ± 22.22
Group 3	57.37 ± 10.68	55.29 ± 11.94	43.35 ± 23.28	43.67 ± 21.23	15.24 ± 20.54
Group 4	49.60 ± 19.53	38.91 ± 23.57	35.06 ± 23.62	22.27 ± 22.54	32.50 ± 22.42
Group 5	53.75 ± 17.29	48.41 ± 21.86	40.44 ± 21.63	40.72 ± 23.31	19.90 ± 22.96
p_1	0.352	<0.001	<0.001	<0.001	
p_2	0.035	<0.001	<0.001	<0.001	
p_3	1.000	0.483	<0.001	0.040	
p_4	0.631	0.001	<0.001	<0.001	
p_5	0.842	0.256	0.997	0.931	
p_6	0.548	0.141	0.572	0.001	
p_7	0.998	1.000	0.998	1.000	
p_8	0.102	<0.001	0.400	<0.001	
p_9	0.733	0.319	0.969	0.967	
p_{10}	0.791	0.233	0.759	0.001	

Between-group comparisons for difference in platform finding times are shown by P values (P_1 - P_{10}) for each set of groups. Group 1: Sham surgery, Group 2: Physiological serum administered intraperitoneally following hypoxia, Group 3: Ozone administered intraperitoneally before hypoxia, Group 4: Ozone administered intraperitoneally straight after hypoxia, Group 5: Ozone administered intraperitoneally following hypoxia on Days 96, 97, and 98. The first set from P_1 to P_4 indicates the comparison of Group 1 to Groups 2, 3, 4, and 5, respectively. The second set from P_5 to P_7 indicates the comparison of Group 2 to Groups 3, 4, and 5, respectively. The third set from P_8 to P_9 indicates the comparison of Group 3 to Groups 4, and 5, respectively. Lastly, the fourth set P_{10} indicates the comparison of Group 4 to Group 5.

administration protected against ischemia-reperfusion injury in the liver[16]. Likewise, the ozone administration was observed to reduce the apoptotic cell number in our study. Another study used ozone for preconditioning in brain ischemia and compared its effects with hyperbaric oxygen therapy[30]. It was first suggested in 1996 that ozone induced adaptation to chronic oxidative stress[31]. Since then, other studies have shown that controlled ozone administration resulted in preconditioning and could reduce the reactive oxygen species-induced injury by enabling adaptation to oxidative stress[32].

There are studies on utilizing ozone as a preconditioning agent against ischemia-reperfusion injury in the literature. Ajamieh et al compared the effects of ischemic preconditioning and ozone preconditioning in the hepatic ischemia-reperfusion model they induced in rats. Another study showed that ozone therapy could reduce the renal injury in the rats with ischemia-reperfusion injury model by inhibiting the oxidative stress and inflammation in the early phase and preventing myofibroblast activation and renal interstitial fibrosis up to the late phase[33]. In their study, Cai et al showed that ozone therapy helped maintain redox homeostasis by increasing SOD activity and reducing MDA levels in ischemia-reperfusion injury[34].

Ischemic preconditioning is a potent and endogenous mechanism that is believed to provide protection against repeated short-term ischemia-reperfusion and the subsequent ischemia-reperfusion injuries. Besides, low doses of ozone have been shown to create oxidative preconditioning by enabling the protection and activation of the endogenous antioxidant systems[10]. Stadlbauer et al administered intraperitoneal ozone to cardiac graft donors and recipients before transplantation and showed, histologically and biochemically, its effect of reducing ischemia-reperfusion injury developing during transplantation[35]. In light of the data from these studies, our study used a preconditioning group. Although histopathological findings on apoptotic neuron number yielded more favorable results for the preconditioning group compared to the HIBI group, behavioral experiments gave unfavorable results. For more favorable results in behavioral experiments with the preconditioning group, increasing the ozone dose and repeated administration on consecutive days may be recommended in further studies. Reinfusion of the blood mixed with ozone results in vasodilatation, SOD activation, and decreased glutathione levels in the site of ischemia. It was shown that oxidative stress was reduced as a result of these effects[36]. Compliant with these previous studies, the present study found that SOD activity decreased in the HIBI group and increased in the ozone groups. It was thought that this SOD activity increase in the ozone groups could, in turn, reduce oxidative stress.

It was shown that the neurons in the subthalamic nuclei, hippocampus, and the parietal cortex are more susceptible to HIBI, and it was recommended that these regions be examined for the histopathological evaluation of apoptosis[8,37]. The present study examined these brain regions in particular. Studies have shown that the number of apoptotic neurons in neonatal rats increased in both brain hemispheres, only more in the hemisphere where the carotid artery was bound, following ischemia and a

one to three-hour hypoxia[38]. Considering an increase in the number of apoptotic neurons will result in a high severity of HIBI, the finding that quantification of apoptotic neurons via TUNEL assay revealed significantly lower numbers of apoptotic neurons for Groups 3 and 4 compared to HIBI Groups suggests that ozone administration may affect the number of neurons undergoing apoptosis. Our conclusion is also supported by the macroscopic images and weights of the excised brains. Previous studies in the literature have reported that the Morris water maze test is used to assess spatial learning in particular, and this skill is associated with hippocampal functions[39]. It was found out that rats with HIBI had longer times finding the hidden platform than the control group[40]. Investigating the times the study groups' rats found the platform in the first four days, our study showed that these times decreased significantly as days passed for all groups. When the parameters of the times to find the platform in the first four days, the difference in the times to find the platform between Day 1 and Day 4, and the time spent in the eastern quadrant are considered together, the group which was administered ozone immediately after HIBI induction (Group 4) encourages us to carry out further studies on the use of ozone in the treatment of HIBI.

Conflict of interest

Authors have no conflicts of interest to disclose.

References

1. Lee CYZ, Chakranon P, Lee SWH. Comparative Efficacy and Safety of Neuroprotective Therapies for Neonates With Hypoxic Ischemic Encephalopathy: A Network Meta-Analysis. Front Pharmacol. 2019; 10: 1221.
2. Resitoglu B, Celik Y, Komur M, Polat A, Erdogan S, Arslankoylu AE, et al. The efficacy of ozone therapy in neonatal rats with hypoxic ischemic brain injury. Bratisl Lek Listy. 2018; 119: 81-85.
3. Celik Y, Resitoglu B, Komur M, Polat A, Arslankoylu AE, Okuyaz C, et al. Is levetiracetam neuroprotective in neonatal rats with hypoxic ischemic brain injury?. Bratisl Lek Listy. 2016; 117: 730-733.
4. Qin X, Cheng J, Zhong Y, Mahgoup OK, Akter F, Fan Y, et al. Mechanism and Treatment Related to Oxidative Stress in Neonatal Hypoxic-Ischemic Encephalopathy. Front Mol Neurosci. 2019; 12: 88.
5. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med. 2005; 353: 1574-1584.
6. Farooqui AA, Ong WY, Horrocks LA. Inhibitors of brain phospholipase A2 activity: their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders. Pharmacol Rev. 2006; 58: 591-620.
7. Hossain MA. Molecular mediators of hypoxic-ischemic injury and implications for epilepsy in the developing brain. Epilepsy Behav. 2005; 7: 204-213.
8. Guevara-Guzmán R, Arriaga V, Kendrick KM, Bernal C, Vega X, Mercado-Gómez OF, et al. Estradiol prevents ozone-induced increases in brain lipid peroxidation and impaired social recognition memory in female rats. Neuroscience. 2009; 159: 940–950.
9. Guven A, Gundogdu G, Vurucu S, Uysal B, Oztas E, Ozturk H, et al. Medical ozone therapy reduces oxidative stress and intestinal damage in an experimental model of necrotizing enterocolitis in neonatal rats. J Pediatr Surg. 2009; 44: 1730-1735.
10. Re L, Mawsouf MN, Menéndez S, León OS, Sánchez GM, Hernández F. Ozone therapy: clinical and basic evidence of its therapeutic potential. Arch Med Res. 2008; 39: 17-26.
11. Tirelli U, Cirrito C, Pavanello M, Piasentin C, Lleshi A, Taibi R. Ozone therapy in 65 patients with fibromyalgia: an effective therapy. Eur Rev Med Pharmacol Sci. 2019; 23: 1786-1788.
12. Sconza C, Respizzi S, Virelli L, Vandenbulcke F, Iacono F, Kon E, et al. Oxygen-Ozone Therapy for the Treatment of Knee Osteoarthritis: A Systematic Review of Randomized Controlled Trials. Arthroscopy. 2020; 36: 277-286.
13. Izadi M, Kheirjou R, Mohammadpour R, Aliyoldhasi MH, Moghadam SJ, Khorvash F, et al. Efficacy of comprehensive ozone therapy in diabetic foot ulcer healing. Diabetes Metab Syndr. 2019; 13: 822-825.
14. Martínez-Sánchez G, Schwartz A, Donna VD. Potential Cytoprotective Activity of Ozone Therapy in SARS-CoV-2/COVID-19. Antioxidants (Basel). 2020; 9: 389.
15. Juchniewicz H, Lubkowska A. Oxygen-Ozone (O_2-O_3) Therapy in Peripheral Arterial Disease (PAD): A Review Study. Ther Clin Risk Manag. 2020; 16: 579-594.
16. Peralta C, León OS, Xaus C, Prats N, Jalil EC, Planell ES, et al. Protective effect of ozone treatment on the injury associated with hepatic ischemia-reperfusion: antioxidant-prooxidant balance. Free Radic Res. 1999; 31: 191-196.
17. Zhang S, Jiang W, Ma L, Liu Y, Zhang X, Wang S. Nrf2 transfection enhances the efficacy of human amniotic mesenchymal stem cells to repair lung injury induced by lipopolysaccharide. J Cell Biochem. 2018; 119: 1627-1636.
18. Bratek E, Ziembowicz A, Salinska E. *N*-Acetylaspartylglutamate (NAAG) Pretreatment Reduces Hypoxic-Ischemic Brain Damage and Oxidative Stress in Neonatal Rats. Antioxidants (Basel). 2020; 9: 877.
19. Zhu C, Wang X, Cheng X, Qiu L, Xu F, Simruner G, et al. Post-ischemic hypothermia-induced tissue protection and diminished apoptosis after neonatal cerebral hypoxia-ischemia. Brain Res. 2004; 996: 67-75.
20. Atici A, Bozlu G, Turhan AH, Polat A, Nayci A, Okuyaz C, Taskinlar H. The role of trapidil on neuronal apoptosis in neonatal rat model of hypoxic ischemic brain injury. Early Hum Dev. 2008; 84: 243-247.
21. Yagi K. Armstrong D. Radicals in Diagnostic Medicine. Plenum Press, New York, 1994, 1-15.
22. Durak I, Canbolat O, Kavutçu M, Oztürk HS, Yurtarslani Z. Activities of total, cytoplasmic, and mitochondrial superoxide dismutase enzymes in sera and pleural fluids from patients with lung cancer. J Clin Lab Anal. 1996; 10: 17-20.
23. Alcalá JA, Callejas-Aguilera JE, Nelson JB, Rosas JM. Reversal training facilitates acquisition of new learning in a Morris water maze. Learn Behav. 2020; 48: 208-220
24. Vannucci RC. Hypoxic-ischemic encephalopathy. Am J Perinatol. 2000; 17: 113-120.
25. Wilkinson DJ. Cool heads: ethical issues associated with therapeutic hypothermia for newborns. Acta Paediatr. 2009; 98: 217-220.
26. Kelen D, Robertson NJ. Experimental treatments for hypoxic ischaemic encephalopathy. Early Hum Dev. 2010; 86: 369-377.
27. Di Mauro R, Cantarella G, Bernardini R, Di Rosa M, Barbagallo I, Distefano A, et al. The Biochemical and Pharmacological Properties of Ozone: The Smell of Protection in Acute and Chronic Diseases. Int J Mol Sci. 2019; 20: 634.
28. Valacchi G, Bocci V. Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets. Mediators Inflamm. 1999; 8: 205-209.
29. Valacchi G, Bocci V. Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells. Mediators Inflamm. 2000; 9: 271-276.
30. Oter S, Seyrek M, Sadir S. Effectiveness of hyperbaric oxygen and medical ozone pretreatment against oxidative stress after global cerebral ischemia in rats. J Cereb Blood Flow Metab. 2009; 29: 413-414.
31. Bocci V. Does ozone therapy normalize the cellular redox bal-

ance? Implications for therapy of human immunodeficiency virus infection and several other diseases. Med Hypotheses. 1996; 46: 150-154.
32. León OS, Menéndez S, Merino N, Castillo R, Sam S, Pérez L, et al. Ozone oxidative preconditioning: a protection against cellular damage by free radicals. Mediat Inflamm. 1998; 7: 289-294.
33. Jiang B, Su Y, Chen Q, Dong L, Zhou W, Li H, et al. Protective Effects of Ozone Oxidative Postconditioning on Long-term Injury After Renal Ischemia/Reperfusion in Rat. Transplant Proc. 2020; 52: 365-372.
34. Cai HA, Tao X, Zheng LJ, Huang L, Peng Y, Liao RY, et al. Ozone alleviates ischemia/reperfusion injury by inhibiting mitochondrion-mediated apoptosis pathway in SH-SY5Y cells. Cell Biol Int. 2020; 44: 975-984.
35. Stadlbauer TH, Eisele A, Heidt MC, Tillmanns HH, Schulz S. Preconditioning with ozone abrogates acute rejection and prolongs cardiac allograft survival in rats. Transplant Proc. 2008; 40: 974-977.
36. Ajamieh HH, Menéndez S, Martínez-Sánchez G, Candelario-Jalil E, Re L, Giuliani A, et al. Effects of ozone oxidative preconditioning on nitric oxide generation and cellular redox balance in a rat model of hepatic ischaemia-reperfusion. Liver Int. 2004; 24: 55-62.
37. Komur M, Okuyaz C, Celik Y, Resitoglu B, Polat A, Balci S, et al. Neuroprotective effect of levetiracetam on hypoxic ischemic brain injury in neonatal rats. Childs Nerv Syst. 2014; 30: 1001-1009.
38. Bozlu G, Atici A, Turhan AH, Polat A, Nayci A, Okuyaz C, et al. Platelet-activating factor antagonist (ABT-491) decreases neuronal apoptosis in neonatal rat model of hypoxic ischemic brain injury. Brain Res. 2007; 1143: 193-198.
39. Morris RGM. Spatial localization dose not require the presence of local cues. Learn Motiv. 1981; 12: 239-260.
40. Ikeda T, Mishima K, Yoshikawa T, Iwasaki K, Fujiwara M, Xia YX, et al. Selective and long-term learning impairment following neonatal hypoxic-ischemic brain insult in rats. Behav Brain Res. 2001; 118: 17-25.