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Original Article

The Effects of Ozone Oxidative Preconditioning on Subarachnoid Hemorrhage via Rat Cerebral Vasospasm Model

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ABSTRACT

Objective: Cerebral vasospasm after subarachnoid hemorrhage (SAH) is a major cause of morbidity and mortality. Inflammation is the major molecular mechanism observed in vasospastic SAH. Ozone (O_3) has been used as a therapeutic agent in the treatment of various conditions and diseases for years. The aim of this study was to evaluate the anti-inflammatory effect of ozone oxidative preconditioning (OOP) in a rat model of SAH in order to assess the therapeutic potential of O_3 in SAH therapy. **Materials and Methods:** In the presented study, an experimental *in vivo* SAH rat model that provided constriction of large cerebral arteries was used. The inflammatory response of cerebral vasospasm after SAH and the effects of OOP were evaluated by comparing the mRNA levels of inflammatory molecules (tumor necrosis factor-α, interleukin-1β, and intercellular adhesion molecule-1) in the serum samples of rats. **Results:** The level of inflammatory molecules increased in vasospasm at 12 h, 24 h, and 48 h in the posttreatment groups. However, intraperitoneal OOP decreased the level of inflammatory molecules dramatically. **Conclusions:** Our study indicated that O_3 treatment has potential in the management of inflammation created in a rat SAH model. These findings may inform further studies investigating possible uses of O_3 in the treatment of vasospasm.

Keywords: *Cerebral aneurysm, inflammation, ozone oxidative preconditioning, subarachnoid hemorrhage, vasospasm model*

Introduction

Although there have been many improvements in mortality rates and morbidity outcomes in patients with subarachnoid hemorrhage (SAH) over the last three decades, vasospasm following SAH is still a major cause of death and cerebral ischemia.[1-5] To ameliorate symptoms after SAH and present a solution, the pathogenesis of SAH needs to be understood. Inflammation is one of the most common molecular mechanisms seen after SAH, and a blood clot in the subarachnoid space causes an acute response with an increase in inflammatory cytokines in the cerebral vessels. Although the role of inflammation in the cerebral vasospasm process after SAH has been studied extensively, its importance and molecular basis were only recently clarified. The consensus is that vasospasm causes cerebral ischemia and is accompanied by inflammation,[6] the innate immune system is activated upon SAH.[7] In brief, the detail of the process is as follows: in the acute phases of SAH, intra- and extra-cellular adhesion molecule (ICAM-1 and vascular cell adhesion molecule [VCAM]),[8,9] selectin (L-, E-, and P-selectins),[10] and integrin (Mac1)[11] levels first increase in the local area. Subsequently, inflammatory cells (lymphocytes, plasma cells, and mast cells) and their cytokines (interleukin [IL]-1, IL-6, and tumor necrosis factor [TNF]-α) activate the inflammatory process, causing augmentation of these molecules in tissue, blood, or cerebrospinal fluid (CSF) samples.[12-14]

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Using anti-inflammatory molecules to reduce the effect of inflammation, we can enhance the management of vasospasm caused by SAH. Ozone (O_3) is an inorganic compound, used widely for decades in alternative medicine applications for the treatment of many diseases.[15,16] O_3 is a reactive form of oxygen that prepares the host to face physiopathologic events.[17] The application of O_3 is called ozone oxidative preconditioning (OOP). In respect of vasospasm and its pathologic effects, OOP offers an alternative method of reducing inflammation and the effects of vasospasm. The aim of this study was to evaluate the anti-inflammatory effect of OOP in a SAH model in rats to assess the therapeutic potential of O_3 treatment. To this end, a vasospasm model was created and the blood mRNA levels of major inflammatory molecules (TNF-α, IL-1β, and ICAM-1) were evaluated using reverse transcriptase-polymerase chain reaction (RT-PCR). The rationale for this is that previous studies indicated that the correlation of mRNA levels with protein levels of TNF-α, IL-1β, and ICAM-1 was consistent in serum and various tissue samples.[18,19]

Materials and Methods

Induction of vasospasm

Animal studies were approved by the Institutional Animal Care and Use Committee of Yeditepe University School of Medicine (Date July 14, 2010, No.:124). The study design was based on the measurement of large cerebral artery diameters at various time points after experimental SAH formation. A total of 36 male Wistar rats (Hilltop Labs) weighing ~240 g were used in this study. Anesthesia was achieved through intramuscular injection of chloral hydrate (350 mg/kg i.p.) and xylazine (4 mg/kg i.p.). To produce the SAH model, the right femoral artery of the rats was first exposed through surgery and cannulated using a polyethylene-10 catheter. Autologous blood (100 μL) was injected into the cisterna magna to produce SAH and vasospasm as previously described.[20] A total of 36 rats, divided into six groups, were subjected to the SAH procedure. Three different study groups and three different control groups were used, each containing six rats.

O_3 was generated from a medical-grade oxygen device, and the concentration was measured using 254-nm ultraviolet spectrophotometry. The O_3 dose is the product of the O_3 concentration. In the study groups, 1 mL O_3 with a concentration of 50 μg/mL was injected into the intraperitoneal space at the same time as the injection of blood into the cisterna magna. In the control groups, cerebral vasospasms were created, but instead of O_3, an equivalent volume of isotonic saline was injected intraperitoneally. A total of 12 rats (six from the study group and six from the control group) were sacrificed by administering a lethal dose of intraperitoneal ketamine 12 h after the procedure. A further 12 rats were culled 24 h after the procedure, and the remaining 12 rats were culled 48 h after the procedure.

Perfusion-fixation

Perfusion-fixation was performed as described elsewhere. Briefly, the thoracic left ventricle was cannulated using a 23G butterfly needle and descended by clamping, the right atrium was opened, and the circulation of the bloodstream through this line was tested first using anticoagulant containing phosphate-buffered saline buffer. Perfusion was performed with 10% formalin (pH 7.4 at 37°C) at a flow rate of 5.5 mL/min for 2 min.

Histologic evaluation

Six rats from each group were analyzed at different time points, 12 h, 24 h, and 48 h post-SAH, to assess histologic changes in MCA. Cross-sections (0.5 mm) of arteries were cut using an ultramicrotome, mounted on slides with a mounting medium, and stained with hematoxylin and eosin (H and E). Images were used to calculate lumen area and vessel diameter. Sections were placed in 10 mM citrate buffer (pH 6) and boiled for 5 min in a microwave. Sections were subsequently incubated at 37°C for 30 min in a destaining buffer.

Measurements of vessel diameters and statistical analyses

Three major cerebral arteries were photographed using a microscope (Leica DM2500) at different magnifications (×4, ×10, and ×20). Vessel diameters were measured using an image analysis system on microscope software (Leica LAS). Arterial segments were analyzed by two independent researchers who were blinded to the study. Basilar artery (BA) diameter was determined at three locations, and the mean value was calculated and used the BA diameter value. The anterior cerebral arteries and middle cerebral arteries were measured at points 100 μm distal to the bifurcation of the internal carotid artery. Data were collected and reported as mean and standard deviation, and groups were compared using GraphPad Prism software and classic statistical approaches (one-way analysis of variance with Fisher's least significant difference *post hoc* test). Significance was considered as $P < 0.05$.

Evaluation of mRNA levels

RNA isolation was performed on the blood samples of each rat. Homogenization was performed using a bead homogenizer (Benchmark Bead Blaster), RNA isolation was performed from tissue lysate using an

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M and N RNA isolation kit, and cDNA synthesis was performed using a GeneALL HyperScript first-strand cDNA synthesis kit. All protocols were performed according to the manufacturer's protocols. RT-PCR was performed. The reaction conditions were as follows: initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 10 s, 60°C for 30 s, 72°C for 1 s, and final cooling at 40°C for 30 s. Data were normalized to a GAPDH reference gene. RT-PCR was performed in triplicate. The primer sequences for the genes were as follows: TNF-α: F: CGAGTGACAAGCCCGTAG; R: GGATGAACACGCCAGTCG; 469 bp. ICAM-1: F: CGGTAGACACAA GCA AGAGA; R: GCAGGGATTGACCATAATTT; 517 bp. IL-1β: F: CCAGGATGAGGACCCAAGCA; R: TCCCGACCATTGCTG TTTCC; 519 bp. GAPDH: F: ACCACAGTCCATGCCATCAC; R: TCCACCACCCTGTTGCTGTA, 452 bp.

Results

Ozone oxidative preconditioning improves the morphologic features of vasospastic vessels

The extent of hemorrhage observed at various time points differed for OOP-treated and untreated rats. However, OOP-treated rat brains showed more recovery than untreated rats with regard to blood remnants on the surface of the brain [Figure 1]. H and E staining was used to examine the morphology and distinct structure of vessels in both the control and O_3-treated groups [Figure 2]. Both the groups showed a significant reduction in the thickness of the BA wall (P = 0.023) [Figure 3] and lumen area (P = 0.018) [Figure 4]. Severe degrees of vasospasm and thickening of vessel walls were observed in the post-SAH 12-h group, regardless of whether they were O_3 treated. However, at 24 h of SAH, the O_3-treated group showed less vasospasm, with more recovery of the vessel wall. These morphologic recoveries were most obvious in the 48-h groups [Figures 3 and 4]. In the 48-h groups, there was a statistically less thickened vessel wall in the O_3-treated group compared with the vasospasm-only group. In 12-h post-SAH BA images, endothelial cells detached and swelled, the internal elastic lamina was severely contracted, the smooth muscle layer was smooth, and luminal stenosis was observed. These pathologic changes were seen to diminish at 24 h and 48 h after the SAH, but more recovery was observed in the OOP groups.

Ozone oxidative preconditioning decreases the expression of inflammatory molecules

RT-PCR studies showed less inflammatory cytokines in the OOP groups compared with the control groups. The mRNA levels of ICAM-1, IL-1β, and TNF-α were

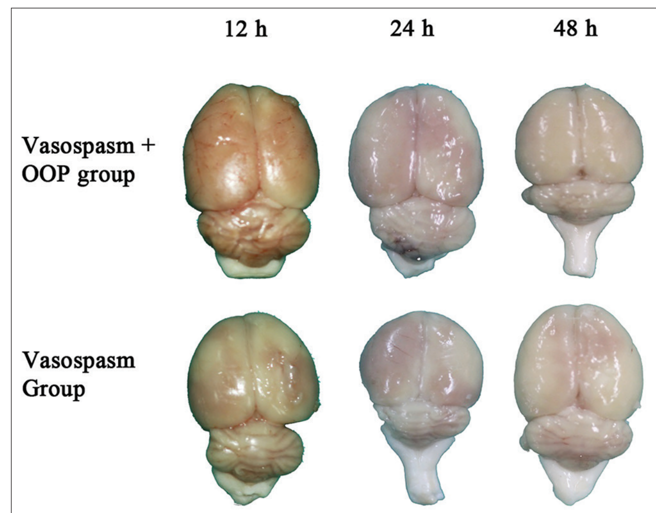

Figure 1: Photographs of postsubarachnoid hemorrhage rat brains. Different time points show the differing extents of hemorrhage in ozone oxidative preconditioning-treated and untreated rats. Ozone oxidative preconditioning-treated rat brains showed more recovery than untreated rat brains

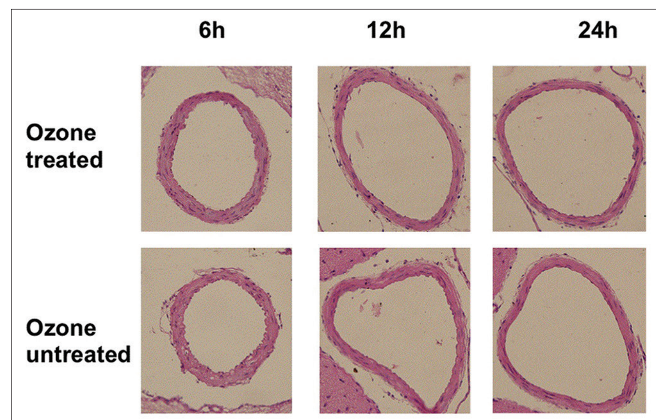

Figure 2: Histochemistry of basilar arteries, showing thickened vessel wall and vasospastic lumen area. Increase in lumen area began after postsubarachnoid hemorrhage 12 h; ozone oxidative preconditioning groups showed more increase compared with the untreated group. The decrease in vessel wall thickness began after postsubarachnoid hemorrhage 12 h; the ozone oxidative preconditioning groups showed more decrease compared with the untreated group

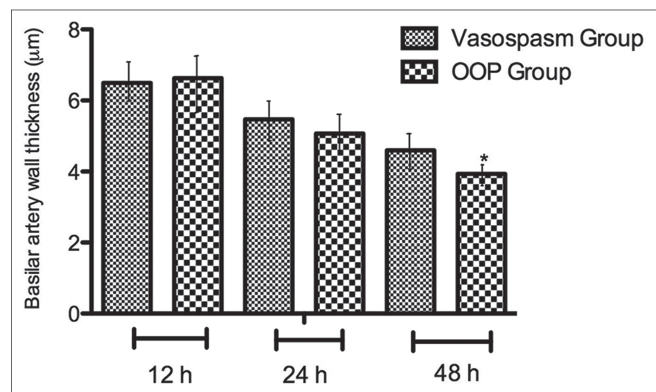

Figure 3: Comparison of basilar artery walls. Vessel wall thickness significantly decreased at 48 h. Statistical significance considered as $P < 0.05$

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significantly greater in the O_3-untreated group than in the treated group at 24 h and 48 h post-SAH [Figure 5]. The density of mRNA bands on agarose gels was compared by evaluating the target mRNA/GAPDH mRNA level densities [Figure 6]. The relative expression of the TNF-α gene's mRNA/GAPDH mRNA was 0.1 ± 0.005 in the control group and 0.09 ± 0.005 in the OOP group at 12 h post-SAH. This expression was 0.28 ± 0.08 and 0.16 ± 0.04 in the control and OOP groups at 24 h post-SAH and 0.44 ± 0.047 and 0.3 ± 0.058, respectively, at 48 h post-SAH. The relative expression of the IL-1β gene's mRNA/GAPDH mRNA was 0.15 ± 0.02 in the control group and 0.13 ± 0.05 in the OOP group at 12 h post-SAH. This expression was 0.4 ± 0.08 and 0.24 ± 0.081 in the control and OOP groups at 24 h post-SAH and 0.51 ± 0.12 and 0.29 ± 0.11, respectively, at 48 h post-SAH. The relative expression of the ICAM-1 gene's mRNA/GAPDH mRNA was 0.17 ± 0.05 in the control group and 0.15 ± 0.051 in the OOP group at 12 h post-SAH. This expression was 0.36 ± 0.066 and 0.21 ± 0.062 in the control and OOP groups at 24 h post-SAH and 0.44 ± 0.11 and 0.3 ± 0.12, respectively, at 48 h post-SAH. Comparisons of each target gene's mRNA/GAPDH mRNA ratios are shown in Figure 6. In summary, the mRNA levels of all genes were seen to increase over time. However, the OOP groups' mRNA levels for all genes had decreased by comparison with control rats at the same time points. These differences were significant at 24 h and 48 h after oxidative O_3 preconditioning for all genes [Table 1].

Discussion

Experimental cerebral vasospasm results through progressive narrowing of the cerebral vessels, involving both thickening of the blood vessel wall and reversible vasoconstriction.[21,22] The pathogenesis of post-SAH cerebral vasospasm in humans is unclear and is probably complex and multifactorial.

Inflammation in the vessel wall plays an important role in the pathology of vasospasm.[23,24] An increased number of lymphocytes and macrophages, inflammatory mediators, complement and adhesion molecules (ICAM-1) and VCAM-1, and selectins (E-, L-, and P-selectin) have been demonstrated in ruptured aneurysms.[24-26] Changes in cytokines, including TNF, endothelin-1 (ET-1), and IL-1, IL-6, and IL-8, have been noted in experimental and clinical cerebral vasospasms after SAH.[23] ET-1 has been studied extensively as a mediator of vasospasm.[13,27]

The use of anti-inflammatory agents such as cyclosporin A or others has been widely applied, with variable success, in cerebral vasospasm.[25,28,29] O_3 is a form of oxygen and is functional in glucose and adenosine triphosphate delivery to ischemic tissues. O_3 induces angiogenic cascade and may trigger hematopoietic stem cell activation, which can provide tissue regeneration.[17] Its protective effects are well established in cases of lung, liver, renal, and hepatic ischemia/reperfusion injury.[30-32]

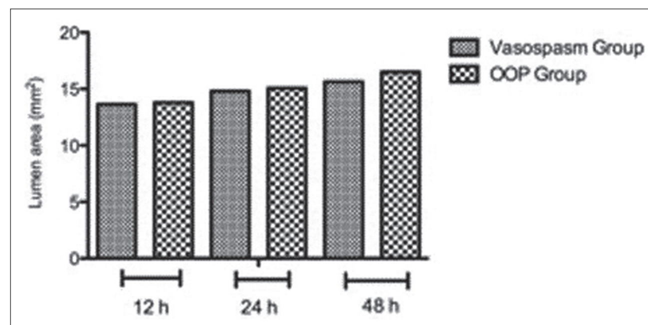

Figure 4: Lumen diameters in ozone oxidative preconditioning-treated and untreated rats. Statistical significance considered as $P < 0.05$

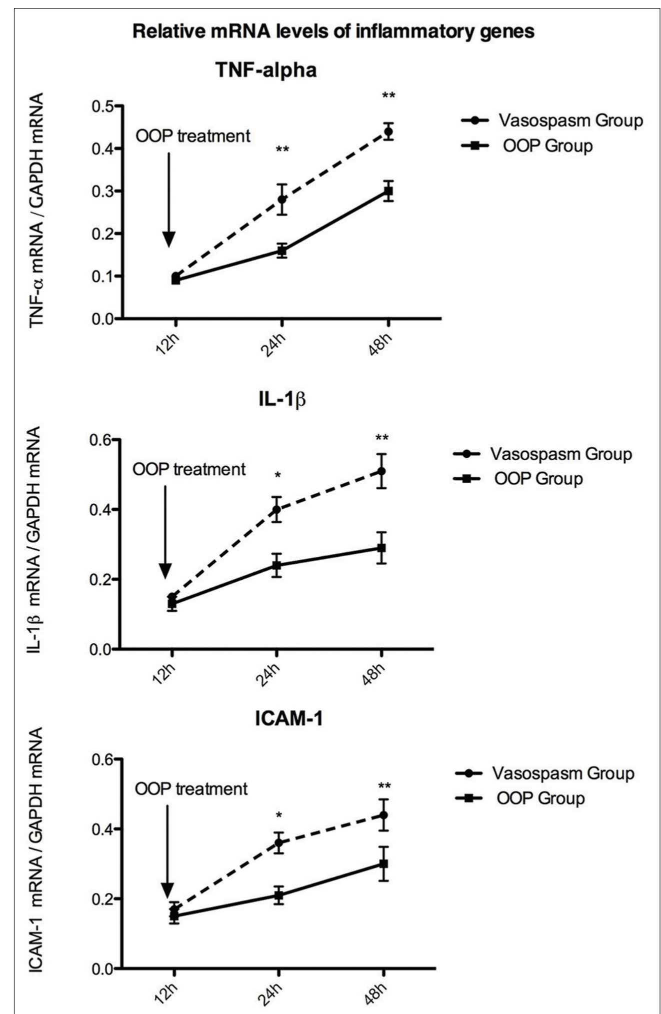

Figure 5: Relative mRNA levels of inflammatory molecules. Relative expression was evaluated by comparing agarose gel band densities proportional to GAPDH gene density. All targeted inflammatory molecules showed significant change at 24 h and 48 h. $*P < 0.05$; $**P < 0.001$

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Table 1: Relative expression ratios of all genes. Relative expression values are expressed as the mean of band density±standard deviation. Relative expressions were calculated using the density of target gene mRNA/GAPDH gene mRNA

	TNF-α				IL1-β				ICAM-1			
	Control Group		OOP Group		Control Group		OOP Group		Control Group		OOP Group	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
12h	0.10	0.005	0.09	0.005	0.15	0.02	0.13	0.050	0.17	0.050	0.15	0.051
24h	0.28	0.080	0.16	0.040	0.40	0.08	0.24	0.081	0.36	0.066	0.21	0.062
48h	0.44	0.047	0.30	0.058	0.51	0.12	0.29	0.110	0.44	0.110	0.30	0.120

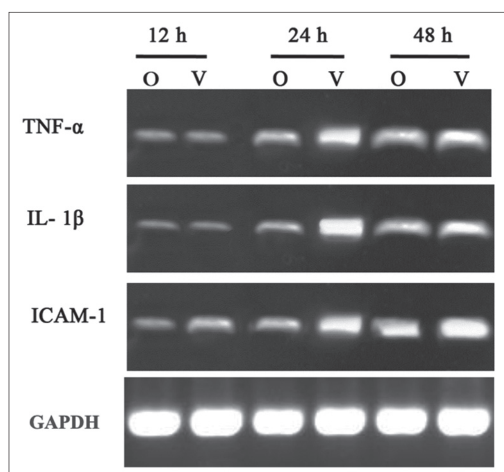

Figure 6: Agarose gel images of polymerase chain reaction products. "O" indicates ozone oxidative preconditioning-treated vasospasm group; "V" indicates untreated vasospasm group

OOP is a well-known, simple method that offers a new approach to protecting organs from ischemia injury caused by various mechanisms such as vasospasm. However, it remains unclear as to whether the protective effects of OOP are associated with repression of inflammation. Chen *et al*. confirmed that OOP ameliorated renal dysfunction and reduced inflammation. Other studies have shown the various effects of OOP, including how OOP increases blood flow in CCA and MCA with prolonged effects.[33,34] We hypothesized that O_3 was a potential anti-inflammatory agent capable of inhibiting the inflammation created by post-SAH vasospasm.

Ozone oxidative preconditioning ameliorated the subarachnoid hemorrhage-caused morphologic deteriorations

With the OOP treatment, we provided morphologic assessment data for post-SAH vasospasm in a rat model. We observed that the hemorrhage on the surface of rat brains caused by vasospasm rapidly recovered upon OOP treatment at 48 h. However, the vasospasm-only group still contained hemorrhage remnants on their surfaces, indicating a prolonged effect of inflammation. Furthermore, BA thickness significantly decreased and the lumen area significantly increased in the OOP-treated group when compared with the vasospasm-only group at 48 h. These morphologic evaluations are the first signs of healed inflammation with OOP treatment. We needed further molecular evaluations to compare inflammatory markers at the mRNA level, and therefore, we measured the gene expression levels.

Ozone oxidative treatment decreases the gene expression level of inflammatory molecules

TNF is produced by mononuclear phagocytes and T-cells, activating neutrophils and endothelial cells, producing acute-phase reactants, and shifting the body toward catabolism.[35] Hemolysis, which is an unavoidable consequence of hemorrhage, induces a significant increase in TNF-α in both blood and vascular tissues. Vecchione *et al*. inhibited TNF-α in an *in vivo* model of SAH and observed that acute cerebral vasoconstriction was resolved and vasospasm was prevented.[36] The elevation of TNF-α and other inflammatory cytokines in the blood is also correlated with poor clinical outcomes.[27] The TNF-α result in the present study aligns with the findings of previous studies. We observed that TNF-α levels were elevated in the vasospasm groups, incrementally correlated with the time elapsed after SAH. TNF-α levels reached a maximum at 48 h, which was our final sampling point. Administration of OOP led to a relative decrease in TNF-α levels. A comparison of the SAH and SAH + OOP groups showed a significant difference at 24 h and 48 h. Here, we provide an association of OOP and TNF-α in the rat vasospasm model.

IL-1 is a family of 11 cytokines, among which IL-1α and IL-1β are the most studied members. IL-1β is produced by activated macrophages and mediates the inflammatory response. In patients who underwent surgical treatment after aneurysmal SAH, IL-1β levels in CSF were elevated during the first 15 days.[35] Our study demonstrated an increased level of IL-1β mRNA in blood samples of post-SAH rats, regardless of OOP treatment. However, OOP-treated rats, showing less vasospasm at 24 h and 48 h, also showed a lesser increase of IL-1β mRNA compared with the untreated SAH controls. The difference between the vasospasm and vasospasm + OOP-treated groups was significant in rats at 24 h and 48 h. It may be concluded that OOP

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reduced the expression of IL-1β by neutralizing it by an unknown mechanism in the same way as the other proinflammatory cytokines.

ICAM-1 is an immunoglobulin superfamily protein with an important function in cell signaling. Along with the Mac-1 and LFA-1 integrins, ICAM-1 facilitates the transmigration of leukocytes across vascular endothelia in response to inflammation. The signal-transducing functions of ICAM-1 seem to be associated primarily with proinflammatory pathways. Levels of ICAM-1, VCAM-1, and ET-1 decayed over time in a manner that suggested a correlation with the pathogenesis of cerebral vasospasm. In clinical studies, the levels of ICAM-1 in CSF of patients with SAH were increased in comparison with patients with unruptured and normal controls.[26] Previous *in vivo* rat experiments demonstrated increased levels of ICAM-1 in post-SAH rats after 2 days.[37] Administration of monoclonal antibodies targeted against ICAM-1 is known to reduce vasospasm in rabbit and rat BA models, as well as in a rodent femoral artery model of SAH.[38-40] In our study, the level of ICAM-1 also increased in the post-SAH vasospasm rats. However, the vasospasm + OOP groups showed a lesser increase compared with OOP-untreated vasospasm controls. This difference was significant at 24 h and 48 h. These results are also compatible with the macroscopic observations of the rat brains. Based on the present results, it may be hypothesized that the administration of O_3 inhibited the expression of ICAM-1.

In summary, we created a model and measured cytokines (IL-1, IL-6, and TNF-α) in an experimental SAH + vasospasm model in rats. We found that the amount of cytokines (IL-1, IL-6, and TNF-α) was less in the SAH + vasospasm + OOP-treated group than in the SAH + vasospasm + OOP-untreated group. It can be considered that cytokines (IL-1, IL-6, and TNF-α) may play an important role in the genesis of vasospasm and OOP may slow the production of cytokines (IL-1, IL-6, and TNF-α), and by doing so, it may prevent vasospasm. Our model is important because it shows the anti-inflammatory effect of O_3 in the prevention of post-SAH vasospasm, and it may open a way in research for the treatment of vasospasm.

As a final remark, our study confirms the role of O_3 treatment for post-SAH vasospasm, demonstrating for the first time the anti-inflammatory effect of O_3 on morphologic and molecular levels. O_3 treatment in humans is only possible via intravascular administration; in the present study, O_3 treatment was performed with intraperitoneal administration. Intraperitoneal administration of O_3 was used as in previous studies because there is no easy or standard method of intravascular injection of oxidative O_3. Little is known about the adverse effects of OOP.

Conclusions

The present study demonstrates that intraperitoneal OOP ameliorates the morphologic changes caused by vasospasm by repressing the activity of inflammatory molecules created by SAH. The level of TNF-α, IL-1β, and ICAM-1 increased post-SAH at 12 h, 24 h, and 48 h. However, OOP significantly decreased the level of inflammatory molecules at 24 h and 48 h, indicating the anti-inflammatory potential of O_3 treatment for post-SAH vasospasm. Further studies are needed to achieve a fuller understanding of the mode of action of OOP. The level of other proinflammatory cytokines should be evaluated during OOP treatment.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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