





## Comparing the Efficacy of Ozone Therapy and Medical Treatment on Serum Levels of TNF- $\alpha$ and HS-CRP and Improvement of Neurological Symptoms in the Rehabilitation Phase of Patients With Ischemic Stroke

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### Abstract

**Background:** Stroke is a leading cause of disability and mortality worldwide; therefore, finding efficient alternative treatments to control the disease and improve patients' neurological symptoms is of paramount importance. The current study aimed to investigate the efficacy of adding ozone therapy to classic treatment in improving neurological symptoms and serum levels of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) as well as high-sensitivity C-reactive protein (HS-CRP) in the rehabilitation phase of patients with ischemic stroke.

**Materials and Methods:** This interventional study was performed on 72 patients with stroke. Using Random Allocation Software, the participants were divided into two groups: control (medical treatment and physiotherapy,  $n=36$ ) and intervention (ozone therapy plus medical treatment and physiotherapy,  $n=36$ ). Ozone therapy was performed by major autohemotherapy using the standard protocol. Disease severity was determined by the National Institutes of Health Stroke Scale (NIHSS) and Modified Rankin Scale (MRS) at the beginning and end of the study. TNF- $\alpha$  and HS-CRP serum levels were determined by the enzyme-linked immunosorbent assay (ELISA). This research project was registered in the Iranian Registry of Clinical Trials (IRCT20200202046342N1).

**Results:** The results of the study showed a significant decrease in the NIHSS score and HS-CRP as well as TNF- $\alpha$  serum levels in the patients receiving both classic and ozone treatments compared to those receiving only classic treatment ( $P<0.001$ ).

**Conclusion:** According to the study results, ozone therapy combined with medical treatment improved neurological symptoms in the rehabilitation phase of patients with ischemic stroke.

**Keywords:** Ozone therapy, Ischemic stroke, NIHSS, MRS, HS-CRP, TNF- $\alpha$

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### Introduction

A stroke occurs when insufficient blood flows to the brain to meet the metabolic demand (1). There are two types of ischemic (85%) and hemorrhagic (15%) strokes (2). Stroke is the second leading cause of mortality worldwide, following cardiovascular diseases. In addition, stroke is a significant cause of long-term disabilities (3). Following a decrease in blood flow and glucose, several cellular and molecular mechanisms are activated in the ischemic zone (4), where leukocytes become activated and inflammatory mediators and free radicals begin to secrete, ultimately leading to the damage of neurons (5).

Inflammatory responses in ischemic stroke are

initiated by necrotic cells, reactive oxygen species, and inflammatory cytokines (6). Such responses activate microglia, which in turn produce more cytotoxic compounds such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1  $\beta$ ), IL-6, inducible nitric oxide synthase (iNOS) (6), and C-reactive protein (CRP), which are recognized as acute phase reactants produced by hepatocytes (7) and risk factors for ischemic stroke (8). TNF- $\alpha$ , a chemotactic reactant against leukocytes, triggers inflammation in brain events and increases the thrombogenic property of stroke by increasing the tissue factor of plasminogen-activating inhibitor and platelet-activating factor (9).

Given the importance of strokes and their high incidence rate, finding more efficient methods to control the disease and improve the patient's neurological symptoms is of paramount importance. In this regard, previous studies have indicated the applicability of ozone therapy in treating some aspects of strokes (10-14). In this technique, ozone gas is used as a systemic treatment in which blood is taken, mixed with a specific ozone dose, and re-infused (15). Such a mixture has shown systemic effects on the correction of hypoxia, tissue toxification, metabolic disturbances, and dysregulated immune responses (16-20), which are seen in ischemic stroke.

In this study, we aimed to evaluate the therapeutic effects of ozone therapy plus medical treatment on the neurologic improvement of stroke according to the National Institutes of Health Stroke Scale (NIHSS) as well Modified Rankin Scale (MRS) and serum levels of inflammatory markers of high-sensitivity C-reactive protein (HS-CRP) and TNF- $\alpha$  in the rehabilitation phase of ischemic stroke.

## Materials and Methods

### Participants and Design

This interventional study was performed on patients with ischemic stroke who were referred to the emergency department of Ayatollah Yasrebi Hospital, Kashan, Iran, in 2018. The sample size was calculated to be 72 (36 individuals per group) using G Power based on a study by Wu et al (12). The participants were selected by simple random sampling.

### Inclusion and Exclusion Criteria

Inclusion criteria were as follows: being in the rehabilitation phase (at least two months after the attack) of an ischemic stroke (NIHSS < 15, MRS < 5), age of 18-80 years, blood pressure of  $\leq$  180/110 mm Hg, and absence of cerebral haemorrhage on computed tomography of the brain. The exclusion criteria were a lack of hemorrhagic stroke, cancer, active infection, pregnancy or lactation, favism, hyperthyroidism, and severe psychiatric disorders.

### Intervention

The participants were divided into two control (routine classic medical treatment and physiotherapy, n=36) and intervention (ozone therapy plus medical treatment and physiotherapy, n=36) groups using Random Allocation Software. Therefore, patients in the intervention group received their conventional and ozone therapy as complementary treatments. Ozone therapy was performed through major autohemotherapy by the standard protocol (21). The mean age of the patients in the control and intervention groups was found to be  $66.8 \pm 10.47$  and  $65.8 \pm 10.36$  years, respectively ( $P > 0.6$ ).

## Measurement

The severity of the disease was determined at the beginning and the end of the study using NIHSS and MRS.

The NIHSS is a tool that can determine whether the stroke is mild or severe and whether the effects improve or weaken over time. The NIHSS measures several aspects of brain function, including awareness, vision, emotion, movement, speech, and language. A certain number of points are presented for each of these physical and cognitive functions during a focused neurological examination. A maximum score of 42 indicates a severe and devastating stroke (stroke severity by NIHSS; 0=no stroke, 1-4=mild stroke, 5-15=moderate stroke, 15-20=moderate to severe stroke, 21-42=severe stroke).

The MRS is a commonly used scale for measuring the degree of disability or dependence in the daily activities of people who have suffered a stroke or other causes of neurological disability. It has become the most widely used clinical outcome measure for stroke clinical trials. The MRS scores range from 0 to 6, with 0 indicating perfect health without symptoms and 6 indicating death (0: no symptom, 1: no significant disability in which the patient can carry out all usual activities despite some symptoms, 2: slight disability in which the patient can look after own affairs without assistance but unable to carry out all previous activities, 3: moderate disability in which the patient requires some help but able to walk unassisted, 4: moderately severe disability in which the patient is unable to attend to own bodily needs and walk without assistance, 5: severe disability in which the patient requires constant nursing care and attention, bedridden, incontinent, 6: dead).

The serum levels of TNF- $\alpha$  were determined by the sandwich enzyme-linked immunosorbent assay (ELISA) using the kits manufactured by eBioscience Company. HS-CRP was measured using an ELISA kit.

### Statistical Analysis

Data analysis was performed in SPSS version 21.0. Analysis was done using the Kolmogorov-Smirnov test to determine the normality of the dependent variables and an independent *t* test to compare the groups regarding the effects of treatment on the changes in each variable. A paired *t* test was used to compare the quantitative variables before and after the treatment in each group. To remove the effect of pretreatment values of different variables, we used the analysis of covariance (ANCOVA). Pearson's correlation coefficient was used to evaluate the correlation between the variables after treatment. A *P* value of less than 0.05 was considered statistically significant.

## Results

The clinical and laboratory characteristics of the patients

are summarized in Table 1. There were 20 (50.6%) and 18 (50%) male patients in the control and intervention groups, respectively.

Comparing each group before and after the treatment, we found a significant decrease in the NIHSS and MRS scores after treatment in each study group ( $P < 0.001$ ). However, a significant decrease was observed in the serum levels of HS-CRP and TNF- $\alpha$  after treatment only in the group of patients receiving both medical treatment and ozone therapy ( $P < 0.001$ ) (Table 1).

Comparing the two groups with each other after the treatment, we found a significant decrease in the NIHSS score and HS-CRP as well as TNF- $\alpha$  serum levels in the patients receiving both classic and ozone treatments compared to those receiving only classic treatment ( $P < 0.001$ ) (Figure 1).

To remove the effect of pretreatment values of hs-CRP, TNF- $\alpha$ , NIHSS, and MRS, the analysis of covariance (ANCOVA) was used, the results of which showed that ozone therapy was effective in the changes of HS-CRP and TNF- $\alpha$  serum levels as well as NIHSS score ( $P < 0.001$ ). However, the treatment group could not significantly maintain the lower levels of the MRS severity index ( $P = 0.086$ ).

## Discussion

This study showed the clinical efficacy of adding ozone therapy, as a complementary treatment, to conventional medical treatment in lowering the severity of the ischemic stroke and serum levels of TNF- $\alpha$  and HS-CRP as inflammatory markers of this disease. The primary mechanism by which ozone could manifest such effects may be that it improves blood circulation in ischemic tissues (22), reduces platelet aggregation, and prevents thrombus formation (23). Medical ozone also increases blood adenosine triphosphate (ATP) concentration, penetrating brain tissues and treating cerebral oedema by regulating the ATP-dependent sodium and potassium ion channels (24). It also strengthens the antioxidant system through the induction of superoxide dismutase, catalase, and glutathione peroxidase antioxidant enzymes (16-20). Free radicals and reactive oxygen species are increased in the ischemic brain (25). Hoffmann and Viebahn stated that medical ozone activates the pentose phosphate pathway in erythrocytes to increase 2,3-bisphosphoglycerate levels, thereby improving

oxygen delivery. Furthermore, it may block the release of bradykinin and inflammatory prostaglandins, by which it reabsorbs oedema (26). Modifying the immune response, medical ozone increases the release of soluble receptors of inflammatory cytokines such as IL-1 and IL-6 (which trap these cytokines) and endogenous proteins of inflammatory cytokine antagonists such as IL-1, IL-8, IL-12, IL-15, and TNF- $\alpha$  (27, 28).

In line with our study, few studies have shown that ozone therapy may contribute to the recovery of the brain after a stroke. For example, a study showed the significant impact of ozone therapy on the rate of neural regeneration and recovery of clinical function after ischemic stroke in patients receiving routine medical treatment plus ozone therapy compared to those receiving only routine medical treatment. Additionally, no hypersensitivity reaction to ozone was seen in any subjects (29). In such conditions of ischemic stroke, ozone improves cerebral blood flow (30), thereby reverting damage to brain tissues (31).

Inflammatory markers such as CRP and cytokines have been reported to play significant roles in all steps of atherogenesis. Additionally, they are among the most relevant molecules expressed during acute and chronic inflammatory processes of ischemic stroke (32, 33). Given the significant reduction in serum CRP and TNF levels in the ozone therapy group, we may conclude that medical ozone could decrease the destructive inflammation present at an ischemic stroke. One possible mechanism for this reduction is through the modulation of oxidative stress. Buyuklu et al found that ozone therapy can effectively reduce oxidative stress factors leading to inflammation (34). Furthermore, Wu et al showed that ozone therapy could reduce oxidative stress and brain injury in animal models of cerebral ischemia (35). Such effect has also been reported in myocardial (36) and skeletal (37) infarction, improving their microcirculation during both phases of ischemia and reperfusion injury (38). Mechanistically, oxidative stress caused by the initial ischemic event activates microglia and astrocytes, which secrete some inflammatory cytokines such as TNF- $\alpha$  that lead to an upregulation of cell adhesion molecules on endothelial cells, allowing blood-derived inflammatory cells, mainly neutrophils, to infiltrate the ischemic brain area. Neutrophils themselves also secrete cytokines which cause further activation of glial cells. These processes all result in neuronal cell death and enhance damage to the

**Table 1.** Comparison of the Clinical and Laboratory Indices in the Intervention Groups

| Variables             | Medical treatment |                 |              |         | Medical treatment + ozone therapy |                 |             |         | ANCOVA Test |
|-----------------------|-------------------|-----------------|--------------|---------|-----------------------------------|-----------------|-------------|---------|-------------|
|                       | Before Treatment  | After Treatment | Difference   | P Value | Before Treatment                  | After Treatment | Difference  | P Value |             |
| HS-CRP (mg/L)         | 3.91 ± 1.32       | 3.85 ± 1.23     | 0.068 ± 0.62 | 0.520   | 3.65 ± 1.11                       | 2.54 ± 1.10     | 1.11 ± 0.82 | <0.001  | <0.001      |
| TNF- $\alpha$ (pg/mL) | 59.09 ± 9.87      | 56.49 ± 13.93   | 2.59 ± 8.6   | 0.079   | 55.96 ± 13.30                     | 39.73 ± 19.14   | 16.2 ± 18.2 | <0.001  | <0.001      |
| NIHSS                 | 10.19 ± 2.21      | 8.19 ± 2.49     | 2 ± 1.24     | <0.001  | 10.19 ± 2.56                      | 7.19 ± 2.77     | 3 ± 1.31    | <0.001  | 0.001       |
| MRS                   | 3.28 ± 0.57       | 2.83 ± 0.70     | 0.44 ± 0.5   | <0.001  | 3.33 ± 0.76                       | 2.46 ± 0.87     | 0.69 ± 0.67 | <0.001  | 0.086       |

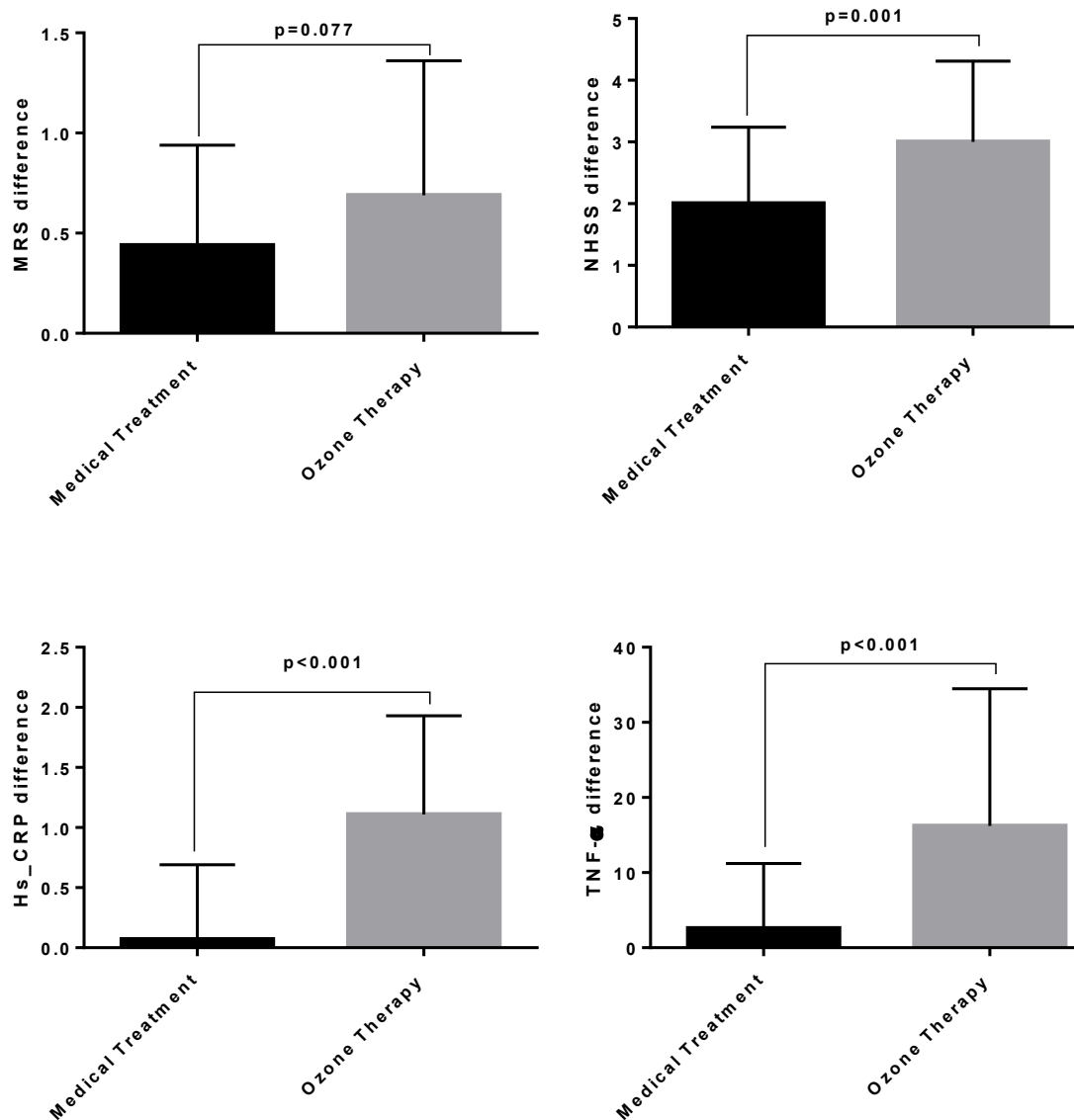


Figure 1. Comparison of the Clinical and Laboratory Indices between the Intervention Groups

ischemic brain (2).

Our study had limitations as well. We did not perform functional assays that provide complementary information on the immunomodulatory mechanisms of ozone. We also did not perform longitudinal assays. The present study is limited in having a small number of participants, which should be considered when interpreting its findings. Therefore, further research on this topic is warranted.

### Conclusion

According to the results of the current study, ozone therapy combined with medical treatment improved neurological symptoms in the rehabilitation phase of patients with ischemic stroke, maybe by reducing inflammatory mediators.

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### Authors' Contribution

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### Competing Interests

The authors declare no conflict of interest.

### Consent for Publication

Consent for publication was obtained from all participants.

### Data Availability Statement

The data sets used during the current study are available from the corresponding author upon reasonable request.

### Ethical Approval

The study was approved by the research council (9793) and ethics committee (IR.KAUMS.MEDNT.REC.1397.057) of Kashan University of the Medical Sciences. Moreover, the research project was registered at the Iranian Registry of Clinical Trials website (identifier: IRCT20200202046342N1; <https://www.irct.ir/trial/45578>).

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