



RESEARCH ARTICLE

Hyperbaric Oxygen Therapy, a Possible New Approach in the Treatment of Retinal Damage in Methanol Poisoning in Rats

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ABSTRACT

It has been established that peroxidation, inflammatory processes, and organ damage are involved in the pathogenesis of methanol intoxication. Research has shown that using hyperbaric oxygen reduces oxidative stress, inflammation, and tissue damage. In this research, it was investigated how hyperbaric oxygen therapy affected methanol-induced peroxidation and retinal damage in rats. The animals were classified into six categories ($n = 8$): control group (C), nitrous oxide (N₂O) treated group, hyperbaric oxygen (HBO) treated group, methanol (MeOH) treated group, N₂O+methanol treated group (N₂O+MeOH) and N₂O+methanol+hyperbaric oxygen (N₂O+MeOH+HBO) treated group. Folate deficiency was started with N₂O, four hours before the starting dose (4g/kg) of methanol. The rats were given a methanol maintenance dose (2g/kg, i.p) 24h and 48h after the starting dose. After each methanol administration, rats were given HBO (2 ATA) for 1 hour. Methanol intoxication decreased plasma folic acid levels while increasing formate levels and oxidative stress index and it caused a decrease in the amount of the retinal ganglion cells and thinned the total retinal thickness in folate-deficient mice. HBO treatment reduced the oxidative stress index but did not adequately improve retinal healing. Our findings indicate that the HBO treatment used to treat retinal damage in methanol poisoning was ineffective. However, increasing the duration of HBO therapy may be effective in preventing methanol-induced retinal damage.

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INTRODUCTION

The appearance and odor of methanol are pretty similar to ethanol. Therefore, incidental or deliberate exposure to methanol causes intoxication. Like ethanol, methanol is metabolized in the liver, and the final products are converted to formaldehyde and formic acid. Formic acid blocks the mitochondrial cytochrome oxidase complex, interrupting oxidative phosphorylation, electron transport, and energy production. It is known that in methanol intoxication, formic acid, but not methanol, is fully accountable for retinal damage (Pohanka, 2016). Retinal abnormalities due to methanol intoxication in humans and other experimental animals have been documented by an

ophthalmologist and other researchers (Sweeting *et al.*, 2010; Liberski *et al.*, 2022). Visual alterations might vary from hazy vision and reduced visual field to complete blindness. As per existing theories of etiology of visual impairment in methanol poisoning are optic disc edema, impaired vision, and specific myelin loss of the retrolaminar optic nerve (Desai *et al.*, 2013).

Previous studies have tested the effects of various treatment protocols against methanol intoxication in rodents, rabbits, and monkeys (Sweeting *et al.*, 2010). In rats, formate is converted to carbon dioxide through a folate-dependent single-carbon pathway after ingesting methanol, therefore does not create acidosis. Thus, if folate deficiency is induced in rats, methanol poisoning can be

modelled experimentally. Furthermore, nitrous oxide inhalation can inactivate methionine synthase in rats and thus increases urinary excretion of formic acid and formiminoglutamic acid. This reduces the levels of tetrahydrofolate, the active form of folic acid, which is required to convert formic acid into carbon dioxide. As a result, high folic acid levels, which prevent methanol intoxication in rats, can be eliminated by nitrous oxide inhalation, and this experimental model is frequently used by researchers (Black *et al.*, 1985). Another reason for formate-mediated retinal damage in humans and experimental animals is the augmentation in oxidative stress (Sandhir and Kaur, 2006).

Hyperbaric oxygen (HBO) therapy is a suitable model for studying oxidative stress in humans and experimental animals (Schottlender *et al.*, 2021). Hyperbaric oxygen (HBO) therapy refers to the intermittent breathing of 100% oxygen at higher pressure of normal atmosphere pressure (1 ATA). HBO can exert both therapeutic and toxic effects, with the physiological effects of hyperoxia and the mechanical effects of increased pressure. Hyperbaric oxygen therapy is a helpful technique for treating a variety of conditions, such as inflammation, oxidative stress, and tissue repair (Lindenmann *et al.*, 2021; De Wolde *et al.*, 2021). However, studies show that HBO causes peroxidation and tissue injury by increasing the generation of free oxygen radicals (Korpinar and Uzun, 2019).

The objective of this study was to investigate the effects of hyperbaric oxygen administration on the chronic methanol-induced retinal damage and oxidative stress in rats consuming nitrous oxide (N₂O) and folate.

MATERIALS AND METHODS

Study design and animal grouping: The experiment was carried out on 48 adult male Sprague-Dawley rats weighing 150-180g. All animals were given *ad libitum* food and water and kept on a 12-hour light/dark cycle. Animals were managed with the permission of the Animal Ethics Committee (Kayseri, Turkey, No:19/188). The goal was to create a rodent animal model that exhibited features of human methanol toxicity, such as visual dysfunction caused by formate accumulation.

The rats were separated into six groups randomly (8 rats /group). Healthy control (C) animals received only saline (% 0,9 NaCl, i.p), Nitrous oxide (N₂O, Gazsan, Turkey) group exposed via inhalation N₂O: O₂ (1:1, flow rate 2 L/min) for 72 hours, in an airtight cage. Hyperbaric oxygen group (HBO, Gazsan, Turkey) rats were administered 2 atm of 100% oxygen for 1 hour for three days, in a hyperbaric chamber. Methanol group (MeOH) rats were given 4 mg/kg of methanol as an initial dose and 2 mg/kg as a maintenance dose at the 48th and 72nd hours. In N₂O+MeOH group, Nitrous oxide was administered to rats for 72 hours. The first dose of 4 g/kg methanol was given intraperitoneally four hours after the nitrous oxide was given. At the 24th and 72nd hours, a maintenance dose of 2 g/kg of methanol (i.p.) was given out. N₂O+MeOH+HBO group, 2 ATA 100% oxygen therapy was applied for 1 hour after each methanol injection in rats given nitrous oxide and methanol with the protocol as mentioned above. At the conclusion of the treatment protocol, the animals were euthanized and Intracardiac

blood samples were centrifuged at 5000 rpm. for 10 minutes with EDTA and preserved at -20°C and tissue samples were preserved at -80°C till further analysis.

ELISA analysis: Plasma folic acid levels were determined using a commercial ELISA kit according to the protocol of manufacturers (BT Laboratory, Shanghai, China).

Formate assay: The formate assay kit (EnzyChrom™ BioAssay Systems, USA.) is based on the oxidation of formate catalyzed by formate dehydrogenase, which produces carbon dioxide and NADH, which reduces a formazan (MTT) dye. The lowered MTT fluorescence at 565 nm is related to the formic acid content in plasma samples.

TAS-TOS analysis: A commercial kit was used to evaluate the total antioxidant status (TAS) levels in plasma samples (Rel Assay Kit Diagnostics, Turkey, catalog number RL 0017) in accordance with the kit protocol. The results are given in mmol Trolox equiv./L. Total oxidant status (TOS) was determined using an assay kit (Rel Assay Kit Diagnostics, Turkey, catalog number RL 0024) in accordance with the kit protocol. Results are expressed as μmol H₂O₂ equiv./L. The oxidative stress index (OSI) was calculated as the ratio of TOS to TAS levels.

Histological analysis: All animals were administered xylazine/ketamine anesthesia and euthanized humanely by dislocation of the cervical spine at the end of the experiment. Each eye was removed and preserved in a 10% formalin solution before being dehydrated in a graded alcohol concentration, lodged in paraffin wax, and sectioning was done through microtome. 5 μm sections were mounted on glass slides and stained with both hematoxylin and eosin for typical histological evaluation. A light photomicroscope (Olympus BX51, Japan) was used to examine and microphotography was performed for all sections.

The retinal ganglion cells were counted in the sections. In five different visual fields, the thickness of the inner nuclear layer (INL), the outer nuclear layer (ONL) and the total retinal thickness (TRT) were measured. Retinal histomorphometric measurements and cell counts were defined and calculated by an expert histologist using the image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis: The Sigma Plot 12.0 application was used for data analysis. The change between groups was compared with *ANOVA test*. If the p-value was less than 0.05, it was considered to be statistically significant.

RESULTS

Effect of Hyperbaric oxygen exposure on plasma folic acid levels in methanol intoxicated rats: Nitrous oxide significantly reduced plasma folic acid levels in rats. At the same time, exposure to methanol and nitrous oxide also significantly reduced plasma folic acid levels in rats. However, the application of methanol alone did not cause a significant change on folic acid levels. Hyperbaric oxygen administration increased plasma folic acid levels of methanol-intoxicated rats, but this increase was not statistically significant. (Fig. 1).

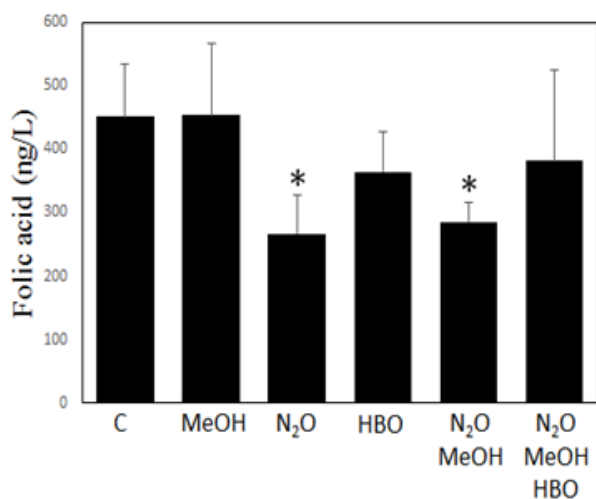


Fig. 1: Plasma folic acid levels in methanol intoxicated rats: Effects of hyperbaric oxygen exposure. n=8, *P<0.05 compared to the control group.

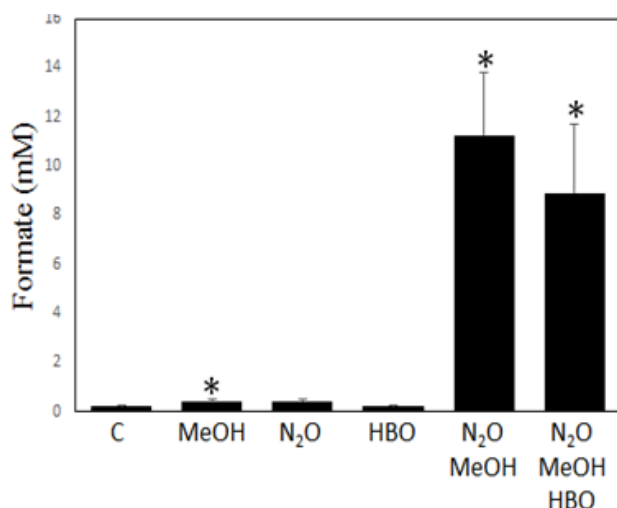


Fig. 2: Plasma formic acid levels in methanol intoxicated rats: Effects of hyperbaric oxygen exposure. n=8, *P<0.05 compared to the control group.

Effect of Hyperbaric oxygen exposure on plasma formic acid levels in methanol intoxicated rats: In particular, combined nitrous oxide and methanol exposure significantly increased plasma formic acid levels in rats. Hyperbaric oxygen exposure decreased plasma formic acid levels in methanol-intoxicated rats, but this alteration was not statistically significant (Fig. 2).

Effect of Hyperbaric oxygen exposure on plasma oxidative stress status in methanol intoxicated rats: Methanol administration alone and methanol administration together with nitrous oxide significantly increased plasma total oxidant status (TOS) in rats. Hyperbaric oxygen administration markedly reduced total oxidant status in methanol-intoxicated rats (Fig. 3A).

Interestingly, plasma total antioxidant status was significantly increased in rats treated with nitrous oxide and methanol in combination with nitrous oxide. However, again, hyperbaric oxygen exposure, significantly decreased the total antioxidant status (TAS) in methanol-intoxicated rats (Fig. 3B).

Hence, when the plasma oxidative stress status was evaluated, the oxidative stress index (OSI) increased significantly in rats treated with methanol and nitrous oxide with methanol. Hyperbaric oxygen exposure significantly reduced the oxidative stress index in methanol-intoxicated rats (Fig. 3C).

Histological results: Light microscopy was used to examine the impacts of hyperbaric oxygen exposure on retinal microstructure in methanol-intoxicated rats. Examination of sections in the retina of the Control (GCN: 23.5±1.3, TRT: 199±9.5), Nitrous oxide (GCN: 19.5±7.5, TRT: 195.5±12), Hyperbaric oxygen (GCN: 22.6±5.6, TRT: 213.5±21.5) and Methanol (GCN: 22±4.5, TRT: 198.5±18) groups showed similar results. Examination of retinal sections of the methanol with nitrous acid (GCN: 18±2.3, TRT: 166.7±11.5) group showed a significant reduction in the total thickness of the retina and the total ganglion cell counts. Hyperbaric oxygen exposure (GCN: 20±2, TRT: 171.7±15) did not adequately protect retinal tissue in methanol-intoxicated rats. Exposure to hyperbaric oxygen caused an increase in total retinal thickness (TRT) and ganglion cell number (GCN). However, this alteration was not statistically significant (Fig. 4). When the inner and outer nuclear layer thickness were evaluated, the results were similar to the ganglion cell count and total retinal thickness data.

DISCUSSION

This experimental study investigated the potential of hyperbaric oxygen (HBO) therapy in treating methanol-induced retinal degeneration. It was observed that HBO had a significant impact on lowering the oxidative stress index in rats with methanol toxicity. However, histopathological analyses of retinal ganglion cell survival do not look very promising in the treatment of methanol-induced retinal damage by HBO. As no published literature is available it will be a kind of preliminary research to examine the effect of HBO in methanol-induced retinal degeneration.

Methanol is a highly toxic organic solvent rapidly absorbed by the skin, oral mucosa, and gastrointestinal tract (Rietjens *et al.*, 2014). After exposure to methanol, it is metabolized in the liver to formaldehyde by alcohol dehydrogenase, which is converted by aldehyde dehydrogenase to formic acid. Eventually, formic acid appeared as the primary toxic substance in methanol poisoning. Abuse of methanol as a substitute for ethanol was, for the first time, reported in the early 1900s and its incidence increased rapidly. Methanol intoxication can cause several health problems, such as respiratory failures, gastrointestinal problems, metabolic acidosis, and even death. Considering the toxic effects of methanol, the most characteristic is retinal toxicity and acute blindness (McMartin *et al.*, 2016 ; Jones, 2021).

Formic acid buildup in organs causes mitochondrial malfunction, decreased oxygen consumption, and ATP depletion in injured cells, leading to histotoxic oxidative stress. Formic acid-induced histotoxic hypoxia exclusively affects retinal ganglion cells and neurons. Therefore, the death of retinal ganglion cells is a crucial factor in retinal ischemia induced by formic acid accumulation, which can lead to vision loss and blindness (Liberski *et al.*, 2022).

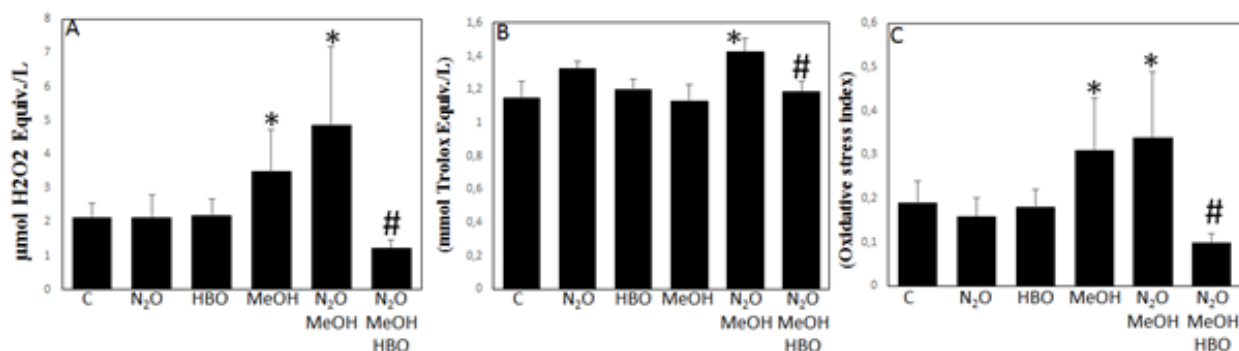


Fig. 3: (A) Plasma total oxidant status (TOS), (B) total antioxidant status (TAS), and (C) oxidative stress index (OSI) in methanol intoxicated rats: Effects of hyperbaric oxygen exposure. n=8, *p<0.05 compared to the control group.

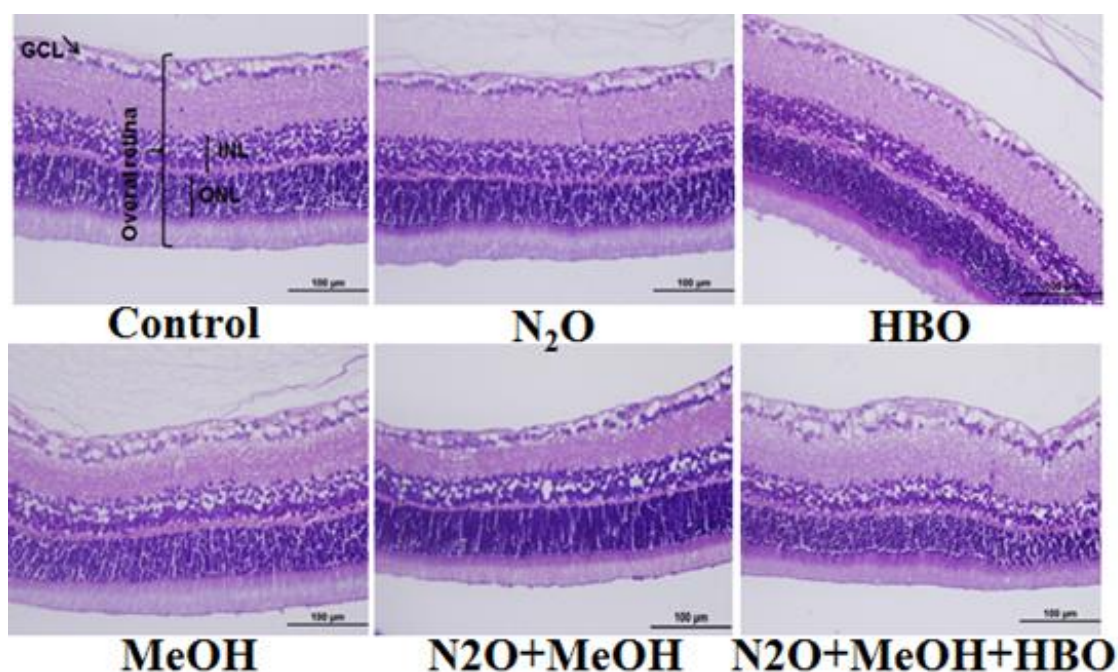


Fig. 4: The histological retinal sections of the Control, Nitrous oxide (N₂O), Hyperbaric oxygen (HBO), Methanol (MeOH), Nitrous oxide with Methanol (N₂O+MeOH), and Hyperbaric oxygen exposure to Nitrous oxide with Methanol (N₂O+MeOH+HBO) groups. Decreased total retinal thickness and ganglion cell number was observed in the N₂O+MeOH group compared to the control group. HBO treatment did not protect cell count and retinal thickness in methanol-intoxicated rats. GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

In monkeys and humans, methanol toxicity is characterized by retinal neurodegeneration and blindness, which occur with an accumulation of formic acid in the blood. In contrast, formic acid does not accumulate after methanol administration in the blood of rats and mice. Therefore, methanol toxicity has been observed in humans and primates but not in mice or rats. Formic acid is eliminated by one-carbon pathway, and the rate of folate oxidation to carbon dioxide is linked to folate (folate) content in the liver and the activity of folate-dependent enzymes. It has been demonstrated that total folate levels are 60% lower in the human liver compared to the rat liver. In addition, 10-Formyltetrahydrofolate dehydrogenase activity, the enzyme that catalyzes the last stage of formate oxidation to carbon dioxide, has been shown to be significantly lower in the human liver. Therefore, low hepatic total folate levels and decreased hepatic 10-formyltetrahydrofolate dehydrogenase activity are important parameters explaining the rapid development of

toxicity in species susceptible to methanol poisoning. Furthermore, folic acid deficiency significantly increases susceptibility to methanol toxicity in rats. Therefore, the subanesthetic dose of N₂O (Nitrous oxide) or methotrexate injection is used to create a folic acid deficiency in rats and mice (Varela-Moreiras and Selhub, 1992; Sweeting *et al.*, 2010; Singh *et al.*, 2015).

Methanol is a powerful oxidant agent that triggers oxidative stress in plasma and tissues. Cells and tissues are damaged as a result of oxidative stress (Ijaz *et al.*, 2021). It is suggested by Kozak *et al.* (2020) that methanol exposure caused a significant elevation in total oxidant status and oxidative stress index in the plasma of folic acid-deficient rats. According to the results of a previous study, methanol exposure increased the total oxidant status and decreased the total antioxidant status in the retina of rats (Sahin *et al.*, 2013). Moreover, MeOH exposure increased free radical formation and oxidative protein damage in rats' retina and optic nerves (Skrzydłowska *et al.*, 2000). In parallel to

these reports, MeOH exposure induced oxidative stress in the plasma of rats in this study. It has been demonstrated by Rothfuss and Speit (2002) that HBO causes an adaptive response that protects human blood cells from oxidative stress. It is noteworthy that HBO may be a potential therapy option for reducing ageing because of its capacity to boost natural antioxidant enzymes that decrease ROS-induced cellular damage (Godman *et al.*, 2010). Similarly, in this study, HBO treatment prevented oxidative stress in MeOH-intoxicated rats by reducing plasma total oxidant status and oxidative stress index.

When hypoxia causes cell, tissue, and organ damage, the first thought that comes to mind is providing more oxygen to the patient to compensate for the oxygen deficiency. Hyperbaric oxygen therapy is being evaluated in treating various diseases with symptoms caused by oxygen deficiency in target tissues (Ortega *et al.*, 2021). The accumulation of formic acid in retinal tissue causes hypoxia-mediated ganglion cell loss, which causes vision loss in patients with methanol poisoning (Liberski *et al.*, 2022).

The results of the present study demonstrated that in rats with folate deficiency, methanol intoxication caused retinal ganglion cell damage and decreased total retinal thickness. The use of hyperbaric oxygen therapy for one hour once a day for three days did not help to repair retinal damage. Extending the duration of hyperbaric oxygen therapy may help to repair retinal damage. There has been limited research on the effect of hyperbaric oxygen therapy on retinal damage caused by methanol poisoning. In a study conducted by Marinov *et al.* (2016) visual damage was observed as a result of methanol poisoning in 4 out of 39 cases (16.0%), and among them two cases progressed to complete blindness. While some types of eye damage are irreversible, partial vision regeneration has been observed in cases treated with 2 to 3 cycles of hyperbaric oxygen therapy (HBOT) during recovery.

Conclusions: Methanol poisoning combined with folate deficiency reduced folic acid levels and increased formate formation in rats. However, there is evidence of increased oxidative stress and retinal tissue damage. These pathophysiological changes indicated that methanol poisoning in rats is adequately modelled. Although hyperbaric oxygen therapy reduced oxidative stress, it was ineffective in repairing retinal damage as per therapeutic protocol used in this experimental model.

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Authors contribution: MAB devised the idea and supervised the research work. MAB and AC (Cumaoglu) executed the research and wrote the manuscript. AY and PAS performed histological analyzes and evaluated the

results. AA surgically removed the eyes. AC (Cumaoglu) and AC (Cetin) carried out the biochemical analyzes.

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