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Comparison of the effects of hyperbaric oxygen and normobaric oxygen on sepsis in rats

Ahmet Ozturk[1], Levent Yamanel[2], Salim Ozenc[3], Mehmet Ince[4], Kemal Simsek[5], Bilgin Comert[6], Bulent Uysal[7], Halil Erturk[8], Umit Kaldirim[4], Turgut Topal[9], Serdar Sadir[9], Kenan Saglam[8]

ABSTRACT

Introduction: Several studies have been done on sepsis and many therapeutic agents have been developed. All agents were tested on animals prior to trials in humans. In this study, our aim was to investigate the healing effects of hyperbaric oxygen (HBO) and normobaric oxygen (NBO) due to pro-inflammatory cytokines and oxidative stress parameters, and the advantages of each other in an experimental model of sepsis.

Material and methods: The rats were randomized into four groups: (1) Sham group (n = 10), intraperitoneal saline-injected group; (2) Control group (n = 10), which were only treated with CEF after induction of sepsis; (3) HBO group (n = 10), treated with HBO after sepsis induction; (4) NBO group (n= 10), treated with NBO after sepsis induction. In all groups, serum TNF-α, as well as parameters of oxidative stress such as glutathione peroxidase, superoxide dismutase and malondialdehyde levels in the lung tissue, were measured.

Results: Our study revealed that treatment with HBO and NBO significantly cured the increased oxidative stress and tissue membrane injury following E.coli induced experimental sepsis (p=0,001). Overall, the NBO and HBO treatments were similar. However, the HBO treatment was more efficient than the NBO treatment with respect to the TNF-α levels (p=0,001).

Conclusion: HBO or NBO should be used as an agent for the adjuvant treatment of sepsis. It can be concluded that applying HBO therapy as an adjuvant will be more useful for the patients meeting the criteria of sepsis. Advanced studies are required to understand the mechanism of treatment and to investigate the usability and efficiency.

Key words: *Sepsis, oxidative stress, proinflammatory cytokines, hyperbaric oxygen, normobaric oxygen*

Introduction

Sepsis, leading to particular hemodynamic changes, progresses to widespread organ failure that could result in death [1]. It has been defined as "Poison of Blood" due to the detection of microorganisms in the blood of patients [2]. The promising results but modest success of the studies designed with great hopes have shown that the effective treatment of septic shock patients and the decrease of the mortality rate are yet to be achieved [3, 4]. Despite the rapid developments in all the field of research and medicine, the new strategies should be focused on the treatment of sepsis having quite a high rate of the deadly consequences. We could not find in the literature a study comparing the treatment

Author affiliations : *[1]Department of Geriatrics Medicine, [2]Department of Intensive Care, [4]Department of Emergency Medicine, [5]Department of Undersea and Hyperbaric Medicine, [6]Department of Biochemistry, [7]Department of Pathology, [8]Department of Internal Medicine, [9]Department of Physiology, Gulhane Military Medical Academy, Ankara, Turkey, [3]Department of Family Medicine, Diyarbakir Military Hospital, Diyarbakir, Turkey*

Correspondence : *Mehmet Ince, Department of Emergency Medicine, Gulhane Military Medical Academy, Ankara, Turkey. e-mail: drmince1@hotmail.com*

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of hyperbaric oxygen (HBO) and normobaric oxygen (NBO) for the treatment of sepsis. For this reason, we aimed to compare in this study the therapeutic effects of HBO and NBO treatment on clinical and laboratory markers of sepsis.

Materials and Methods

This study was carried out in Laboratories of Gulhane Military Medical Research, Development, and Physiology Department, with the approval of the Board of Ethics in Animal Experimentation of Gulhane Military Medical Academy Command and complying to rules of ethics for animal study of the Helsinki declaration.

Animals and Study Groups: In this study, 40 adult male Wistar-Albino rats weighing from 250 to 350 grams, obtained from Gulhane School of Medicine Research Center, Ankara, Turkey, were used. The rats were weighed individually, and they had similar characteristics and weight in each group. Animals were kept at constant room temperature in a 12-h light-dark cycle with free access to water and standard rat chow at least 1 week before the animals were randomly divided into four groups, namely:

- Group I (Sham group), in which intraperitoneal saline was administered.
- Group II (Control Group), in which only CEF after induction of sepsis was administered.
- Group III (NBO Group), in which CEF plus NBO after induction of sepsis was administered.
- Group IV (HBO group), in which CEF plus HBO after induction of sepsis was administred.

Induction of Sepsis: Experimental sepsis was created using a controlled inoculation model with a standard strain of *E. coli* (2.1×10^9 cfu/ml) as a single bolus dose and administered intraperitoneally (ip) to rats [5]. Sepsis was diagnosed by increased leukocyte counts (white blood cells were measured from the 1 ml blood that was taken from the tail vein), rectal temperature, respiratory rate, and tachycardia. After the induction of sepsis, Cefepime HCl (CEF) treatment (50 mg/kg ip.) was applied to all groups except sham [5].

Hyperbaric Oxygen Therapy: For the application of hyperbaric oxygen, a cylindrical hyperbaric chamber was specially designed and manufactured in the Turkish Army Force, 800 Main Warehouse and Factory Command (Etimesgut / Ankara). It has a diameter of 40 cm, and a length of 60 cm, and it is made of a chromium, nickel and steel mixture body and had been tested resistance to 10 ATA pressure was used. The pressure in the chamber was continuously controlled with a manometer mounted on the chamber and a gauge automatically released extra gas. A 1.5-2 lt / min flow rate of oxygen input was applied into the chamber provided by tubes that contained pure oxygen under high pressure [6, 7]. After placing the animals in the chamber, the atmosphere was washed with oxygen rapidly (flushing). Meanwhile, vary proportions of absorbent granules, namely soda lime including sodium hydroxide, calcium dioxide and a calcium hydroxide mixture, were introduced in the chamber for CO2 might be accumulated due to respiration of animals in the environment [8]. At a 12 hours interval, HBO therapy was applied as 2 times, 2.8 ATA/per day as suggested by Pedoto for a period of 90 minutes as proposed by Oter et al [5, 9].

Normobaric Oxygen Application: The same chamber was used for NBO therapy, the input and output of the chamber were automatically adjusted to 5 lt/min., and the pressure was adjusted to 1 ATA for the period of 90 min [10]. After the induction of sepsis, CEF, HBO (90 min - 2.8 ATA), and NBO treatments were applied to rats 2 sessions/per day.

Sacrification and Sampling: At the end of the fifth day after induction of sepsis, the rats were anesthetized with general anesthesia and access to the lungs was achieved by opening the chest cavities. When the blood samples were taken from the vena cava inferior, SF was injected to right ventricula. When fading of the color of the lungs was visible and the change between blood and SF reached an adequate level, the lungs were removed. The extracted tissue of the lungs was put into a tank of liquid nitrogen to be stored at -80 ° C. All these procedures were performed as quickly as possible to avoid and additional cause of stress on the lung tissue, especially after anesthesia. Therefore, the time spent on each animal did not pass 3-4 minutes. In addition, the time used to dissect the animals that did not receive HBO therapy was adjusted to the same time used in the HBO groups, their equivalents.

Tissue Homogenization: The tissue was removed from the freezer to be prepared for dissolution analy-

sis. After that it was homogenized with Retsch brand "Mixer Mill MM 400" model homogenizer after mixing phosphate buffer as 1/9 volume ratio. A portion of the resulting homogenate was reserved for Malondialdehyde (MDA) and protein measurements. The supernatants were taken from another portion of the homogenate and they were put in the Eppendorf tubes to be used for the measurement of glutathione peroxidase (GSH-Px) and Superoxide dismutase (SOD), after they were centrifuged as 700 rpm for 10 min. Phosphate buffer (50 mM) was prepared by adding 6.8 g / l KH2PO4 solution in distilled water until pH became 7.4 and 7.1 g/l Na2HPO4 solution in distilled water.

Biochemical Analysis of Serum and Tissue Samples: TNF-α levels of blood samples and MDA, SOD, and GSH-Px levels of lung tissue were studied. The global levels of tissue protein were also measured to be used as standards for the results to be obtained by analysis of oxidant and antioxidant parameters in lung tissue. TNF-α was analyzed using a kit of 'BioSource rat TNF-α Solid-phase-sandwich enzyme-linked-immunosorbent-assay (ELISA) [11]. MDA measurement was made by the method described by Okhawa et al [12]. SOD activity was measured by the method described by Sun et al [13]. The level of GSH-Px activity was measured by the method described by Paglia [14]. The level of proteins from homogenates, extract and supernatant for MDA, SOD and GSH-Px respectively was measured using the method described by Lowry [15].

Statistical Methods: All calculations were made with the help of micro-processor and the commercial statistical software package (SPSS PC, Ver.12.0, SPSS Inc, USA). Numeric values were stated as mean ± standard error of the mean (mean ± SD). First, Kruskal-Wallis test was done among all groups, and then Mann-Whitney U test, which is a non-parametric method, was done for comparisons between groups for which significant results were found, in accordance to the number of subjects. $p < 0.05$ was considered significant.

Results

A pre-defined rat model of sepsis was established to using *E. coli* for sepsis in the three groups (Group 2, Group 3, and Group 4). Mean white blood cell count, the mean rectal temperature, the average heart rate, and mean respiratory rate were 20600 ± 3400 /mm^3, 38.9 ± 1.5 ^{0}C, 154 ± 15 beats/min, and 100 ± 16 times/min, respectively.

Average of the mean values and standard errors (mean ± SD), with statistically significant differences in the values, are presented in table 1. In the NBO and HBO groups, the SOD levels (an indirect indicator of antioxidant capacity in blood samples of the sham) were statistically higher than in the control group (p<0,001). SOD levels were also significantly higher in the sham group than in the HBO and NBO groups. However, there was no significant difference between the HBO and NBO groups (Table 1). This means that the anti-oxidant capacity of the tissue in rats with sepsis is low. Those groups receiving oxygen (HBO-NBO) could not reach normal levels of oxygen but they were better than the groups not receiving oxygen.

In addition, the MDA level, which is considered to be an indirect indicator of tissue damage in the cell membrane, had statistically significant difference between sham, HBO and NBO groups. There was no statistically significant difference between the HBO and NBO therapies on the level of MDA (Table 1).

The level of GSH-Px, an indirect indicator of tissue of the antioxidant capacity, in the sham group was significantly higher than in the control, HBO and

Table 1. Oxidant-antioxidant status in lung tissue and serum levels of SOD, GSH-PX and TNF-α.

	Grup 1 (Sham)	Grup 2 (Control)	Grup 3 (Cef+NBO)	Grup 4 (Cef+HBO)	P value*
MDA (mmol/gprotein)	0,34±0,1	1,22±0,2	0,39±0,2	0,44 ±0,17	<0,001 a,b,e
SOD (U/gprotein)	136,38±25,8	49,41±12,2	83,92±14,7	94,76 ±14,55	<0,001 a,b,c,d
GSH-Px (U/gprotein)	17,64±4,5	7,36±2,6	7,53 ±4,2	9,28 ±2,8	<0,001 a,b,c
TNF-α (pg/ml)	67,36±34,5	281,06±165,1	206,83±49	242,07 ±73,24	<0,001 a,b,c,f

* Kruskal Wallis test According to Bonferroni Corrected Mann Whitney U test. Statistically significant differences between **a:** group 1 and 2, **b:** group 1 and 3, **c:** group 1 and 4, **d:** group 2 and 3, **e:** group 2 and 4, **f:** group 3 and 4.

NBO groups (p <0,001). However, it was significantly increased in the HBO group compared with the control group (Table 1). In other words, HBO and NBO therapy had significantly increased oxidative stress and damage of tissue membrane caused by induction of experimental *E. coli* sepsis but there were no differences between the two therapies. TNF-α level in the sham was significantly lower than in control, HBO and NBO groups (p <0,001).

In spite of all these results, it has not been possible to determine the effects of systemic therapy of HBO and NBO on the course of sepsis, because due to its design this is not a survival study.

Discussion

The results of our study are original since it is the first time that the antioxidant, anti-ischemic and anti-inflammatory effects of NBO and HBO treatments were examined on an experimental rat model of sepsis induced by *E. coli*. It was shown that differences between these treatments were not very meaningful. There was no obvious superiority of HBO, which was more difficult to be applied and tolerated, compared to the NBO treatment.

In our study as well as in many studies, the most commonly used experimental model of sepsis model, the Wistar-Albino rat [5], was selected. Male gender was selected with the aim of preventing false positive and negative results due to possible changes in hormonal functions that might affect the findings. In our study, the *E. coli* sepsis model was selected. A single dose of material prepared in culture and emulsified was applied as bolus. The inoculation models, in accordance with bacteremia models, create a realistic abdominal microenvironment with ongoing infection and inflammation. There is a risk of early death due to intoxication in this model. And antibiotics cannot reduce the risk of death [16, 17]. Consistent with the literature, in our preliminary study, when bacteria were inoculated into the peritoneal cavity by the controlled inoculation method all rats (12 rats) were killed after intoxication.

There are several indications suggesting that HBO therapy alone or in conjunction with another treatment is useful [18]. Muth et al. reported that the administration of the combined treatment of CEF and HBO to rats with sepsis induced by intraperitoneal injection increased the levels of anti-oxidative enzymes and regulated the concentration of growth factors and cytokines [19]. These effects of breathing pure oxygen at supra atmospheric pressures led to researchers to inquire the effects of HBO in carbon monoxide (CO) poisoning and ischemia/reperfusion injury . The fact that HBO therapy may be an adjuvant therapy and that it could be a useful strategy combined with antibiotic therapy in rats with sepsis was emphasized in this study . We found changes indicating a healing effect at both the level of cytokines as well as the antioxidant / oxidant parameters in the groups treated with a combination of CEF and HBO/ NBO.

HBO has already been widely used as adjunct therapy in the inflammatory condition and ischemic tissue damage. Luongo et al. showed that in animals HBO reduces symptoms, increases TNF-α and NO levels and prolongs survival in bacterial and non-endotoxic shock generated by Zymosan (a wall component of *Saccharomyces cerevisiae*) [20]. Recent studies found that HBO reduces the pro-inflammatory cytokines of monocyte-macrophages. Moreover, it was shown that HBO is effective on the levels of SOD and GSH-Px in a study conducted on a rat model of experimental pancreatitis [21]. In our study, the levels of SOD increased but there were not significant changes on the GSH-Px levels compared to the control group treated with antibiotics, after HBO and NBO treatment of the experimental model of sepsis in rats.

Yucel et al. obtained positive results with NBO therapy in their experimental model of sepsis induced by peritonitis in rats, especially when antibiotics were added [10]. The treatment of NBO has been accepted as part of the classic treatment on the guide of 2008 Surviving Sepsis Campaign. In our study, when compared to the control group, we found significant improvement when NBO was given to the rats that had experimental sepsis, at the levels of both cytokines and antioxidant enzyme SOD, as the result of radical toxicity and MDA as the oxidative stress parameter; however, compared to the HBO treatment, in spite of the effect to oxidative stress and free radicals, there was no difference.

A positive correlation was found between TNF-α levels and sepsis by Bayar et al., i.e., TNF-α levels were

higher in sepsis [22]. Budak et al. found that HBO therapy in sepsis decreased the pro-inflammatory response and increased the inflammatory response. Therefore, they concluded that HBO therapy can be used an effective adjuvant therapy in sepsis [23].

In our study, compared to the control group, HBO and NBO treatments significantly decreased the TNF-α level, which is an indicator of the proinflammatory response; however, the HBO therapy and had significantly higher effect than the NBO treatment. This situation can be interpreted as a decrease in the severity of sepsis parallel to the increase of oxygen pressure.

On the other hand, it was seen that HBO might be suppressed in many different pathological conditions associated with increased oxidative stress such as cystitis [24], sepsis [5], nephritis [25], colitis [26] and ototoxicity [27].

HBO application forms the oxidative stress in the lung and the brain affected by oxygen at the primary level; on the other hand, as a result of effecting pathology in the reverse direction with the oxygen produced by itself, it reduces oxidative stress coming up in the pathological situations [11,12]. We gave 2.8 ATA /90 minutes of HBO as mentioned in the study of Pedoto et al. NBO was applied at a pressure of 1 ATA/90 min, after setting the input of the chamber [9]. HBO significantly reduced MDA levels, which are a marker of oxidative stress formed after experimental sepsis, compared to the level of control, but the healing effect was not observed. Since there was no difference between HBO and NBO treatments, it can be further concluded that pure oxygen breathing is responsible for more than high pressure exposed. This study is important due to the possibility of going away from the idea of the necessity of use in adjuvant therapy in combination with antibiotic therapy if the superiority of HBO therapy is not demonstrated compared to NBO treatment.

Conclusions

HBO or NBO should be used as an agent for the adjuvant treatment of sepsis. If the conditions are satisfied, applying HBO therapy as an adjuvant will be more useful for the patients meeting the criteria of sepsis. However, more detailed and comprehensive studies with different methods showing the effects of both treatments are needed.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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