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Original article

Evaluation of the Genotoxic Effects of Patients Undergoing Hyperbaric Oxygen (HBO) Therapy

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Hyperbaric oxygen (HBO) therapy is successfully applied for a wide variety of diseases. However, recent studies in humans undergoing hyperbaric oxygen (HBO) therapy have revealed that HBO is able to induce genotoxic effects especially in lymphocytes while the biological significance of this outcome is still not clear. HBO mediated genotoxicity in lymphocytes has been determined by using the in vitro Cytokinesis Block Micronucleus (MN) test in patients undergoing HBO therapy. Blood samples were obtained from 100 voluntary patients and were drawn by venipuncture before and immediately after the first session of HBO treatment. MN frequencies were significantly increased after the first session of HBO treatment. According to the MN test results, it is observed that HBO treatment can increase the clastogenic effects.

Key words: Hyperbaric oxygen therapy, Genotoxicity, Micronucleus test

Hiperbarik Oksijen Tedavisinin (HBOT) Sebep Olduğu Genotoksik Etkilerin İncelenmesi

HBOT günümüzde pek çok farklı alanda uygulama alanı bulabilen modern bir tedavi yöntemi olarak karşımıza çıkmaktadır. Son yıllarda bu tedavi yöntemi ile ilgili olarak yapılan HBOT'nin özellikle lenfositlerde sebep olduğu genotoksik etkilerin araştırıldığı pek çok çalışma bulunmaktadır. Bu çalışmanın amacı HBOT uygulandıktan sonra oluşabilecek genotoksik hasarın Sitokinezi Bloke Edilmiş Mikroçekirdek (MN) testi kullanılarak değerlendirilmesidir. Bu amaçla HBO tedavisi uygulanan 100 gönüllü hastadan tedaviye başlamadan önce ve ilk tedavi seansının hemen sonrasında kan numunesi alınmıştır. HBO tedavisinin ilk seansının mikroçekirdek frekanslarını artırdığı gözlenmiştir. MN testi sonuçlarına göre, HBO tedavisinin hastalarda klastojenik etkileri arttırabildiği görülmüştür.

Anahtar kelimeler: Hiperbarik oksijen tedavisi, Genotoksisite, Mikroçekirdek testi

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INTRODUCTION

Hyperbaric oxygen (HBO) therapy is increasingly used in a number of areas of medical practice with symptoms caused by lack of oxygen in the target tissues. HBO implies the inhalation of 100% oxygen under a pressure greater than sea level (1 atmosphere absolute, ATA) in a

hyperbaric chamber for a total of 3x20 min periods, interspersed with 5 min of air breathing (1).

The approved indications for HBO therapy are air-gas embolism, decompression sickness, acute carbon monoxide intoxication, soft tissue infections, radiation necrosis, chronic osteomyelitis, skin graft and flaps, several acute ischemic conditions, gaseous gangrene, sudden hearing loss and impaired wound healing (such as diabetic wounds) (2). However, it is known that exposure to oxygen at high ambient pressure can cause damage to mammalian cells. Exposure to HBO leads to an increase in the amount of dissolved oxygen and therefore reactive oxygen species (ROS) in the blood (3, 4). An increase in free radicals in the blood from persons undergoing HBO exposure was directly demonstrated by electron spin resonance spectroscopy (5,6).

When antioxidant defenses are not completely efficient, increased free radical formation in the body is likely to increase damage. The term "oxidative stress" is generally used to refer to this effect. Oter et al. (7) revealed that, after 2h of HBO exposure at 3 ATA, the levels of the oxidative stress markers, thiobarbituric acid reactive substances (TBARS) and total superoxide dismutases (SOD) were elevated in the lung, brain and erythrocytes and glutathione peroxidase (GPx) and nitrate/nitrite (NOx) activities were found to be elevated in the brain of rats.

In previous studies the HBO-mediated DNA damage was shown with the alkaline version of the comet assay (8, 9). Also we have demonstrated the HBO induced oxidative DNA damage with the same labour team by using alkaline version of the comet assay (10).

The Cytokinesis Block Micronucleus (CBMN) test with human lymphocytes is a well established and sensitive test for the evaluation of chromosome breakage in humans. The aim of this study is to evaluate the mutagenic effects in lymphocytes of patients (n=100) undergoing HBO therapy by using the micronucleus test.

EXPERIMENTAL

Test subjects and HBO treatment

This study was approved by the Ethical Committee of the Gülhane Military Medical Academy (83, 26.12.2006). One hundred volunteer patients provided informed consent to participate in this study.

The subjects were 12-79 years of age and categorized according to their gender, age, smoking habits, coffee consumption, alcohol consumption and diseases, shown in Table 1. The patients were exposed to 10 consecutive HBO treatments (1 session/day), according to a routine therapy protocol. The treatment consisted of exposure to 100% oxygen at a pressure of 2,5 ATA in a hyperbaric chamber (for 12 patients) for 3x20 min periods, interspersed with 5 min periods of breathing air. Heparinized venous blood samples were taken before the first session of HBO treatment and immediately upon exit

Table. 1. The evaluation of the patients participated to this study by their general characteristics.

		n
Gender	Male	77
	Female	23
Age mean: 41 range: 12-79	<30	28
	30-45	34
	45-60	25
	>60	13
Smoking habits	Non smoker	72
	Smoker	28
Coffee drinking	Non drinking	69
	Drinking	31
Previous exposure to radiation	Yes	34
	No	66
Alcohol consuming	Non consumer	88
	Consumer	12
Diseases	diabetic foot	23
	CO intoxication	2
	sudden audition loss	37
	burger disease	4
	freezing	4
	torsion of testis	4
	osteomyelitis	3
	ulcerative colitis	3
	gun injury	2
	necrosis	2
	other diseases	16

from the chamber after the first session. Therefore, the patients acted as their own controls. The blood samples were kept at 4°C and processed within 1h.

Blood samples

From each of the 100 patients, 5 mL of heparinized blood samples was collected. The MN test was performed in the lymphocytes from the 100 patients before and after the first therapy of HBO. For each sample, 10^6 cells/slide were used in the assay.

Cytokinesis Block Micronucleus (CBMN) test

In the assay 10 ng/mL, 20 ng/mL, 40 ng/mL, and 60 ng/mL Mitomycin-C (MMC) were used as positive controls. To determine the frequencies of lymphocytes with CBMN test, 10^6 cells of isolated lymhocytes inoculated into 1.5 mL culture medium (RPMI 1640, L-Glutamine, Strept./Penicilin, heat inactivated fatal calf serum). PHA-L (Sigma) was added as a mitotic agent for initiation of growth. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. At the 48 h of post-cultivation 4.5 µg/mL Cytochalasin-B (Sigma) was added in to the medium in order to block the cytokinesis. The cells were harvested by a cytospin centrifuge (Rotina 38, Hettich). After drying, slides were stained by Giemsa (Merck). Micronucleus was scored in 1.000 binucleated cells using a light microscope. To avoid the cytoyoxic effects on the MN frequencies, the proportion of mononucleated (MI), binucleated (MII), trinucleated (MIII), and tetranucleated (MIV) cells per 500 cells were scored in order to calculate the NDI (Nuclear Division Index) by using the given formula; NDI = [M1 + 2(M2)+ 3(M3) + 4(M4)] / 500 (15).

SPSS for Windows Release 20.0 was used for all data analysis.

Statistical procedures

SPSS for Windows Release 20.0 was used for all data analysis. All results were expressed as mean± standard deviations. The significance of increase or decrease in MN frequencies and NDI values in lymphocytes between the control (pre-treatment) and exposed (after treatment) groups were compared by using the two tailed Student's *t* test. The limit for statistical significance was fixed as $p<0.05$.

RESULTS

The results of the MN frequencies and NDI values of positive controls at 10 ng/mL, 20 ng/mL, 40 ng/mL, and 60 ng/mL concentrations of MMC were shown in Table 2 and also in Figure 1.

Table 2. The MN frequencies and NDI values for the positive controls of MMC (10-60 ng/mL). BNMN: Binucleated micronucleus; NDI: Nuclear Division Index; MMC: Mitomycin C. Statistical comparisons were performed with two tailed Student's *t* test vs. control, $p<0.05$.

	BNMN	NDI
Kontrol	4,5	1,7676
MMC 10 ng/mL	10*	1,736
MMC 20 ng/mL	14*	1,678
MMC 40 ng/mL	20*	1,648
MMC 60 ng/mL	36*	1,61

Figure 2 summarizes the MN test results in lymphocytes taken from 100 patients, before (BT) and immediately after (AT) the first session of HBO therapy. As shown in the figure, the BNMN numbersin the AT patients were significantly higher than BT patients.

DISCUSSION

HBO therapy is applied for a wide variety of diseases with symptoms caused by lack of oxygen in the target tissues. However, exposure to high concentrations of oxygencan causeto an increase in the amount of dissolved oxygen and also reactive oxygen species in the blood (8, 11). It is thought that the toxicity of HBO can be mediated by the production of oxygen free radicals, which lead to lipid peroxidation and tissue damage (12). Eken et al. (13) investigated the effects of HBO on oxidative stress and genotoxicity. Their study revealed that HBO treatment did not cause significant changes in erythrocyte antioxidant capacity and lipid peroxidaton, however it could induce genotoxicity.

In the present study, the clastogenic effect of HBO in lymphocytes was investigated by the Cytokinesis Block Micronucleus (MN) test. The CBMN is a well established genotoxicity test that is frequently used in human biomonitoring.

Figure 1 shows that the positive controls of 10 ng/mL, 20 ng/mL, 40 ng/mL, and 60 ng/mL MMC statistically induce the BNMN numbers in each concentrations in MN test. The results from the MN performed in parallel with the same 100 blood samples are shown in Figure 2. In each of the subjects tested, the HBO-induced frequency of micronuclei was higher after the 1st HBO therapy than it was before. Our results revealed that HBO treatment increased significantly the genotoxic effects in peripheral blood lymphocytes after the first session of therapy.

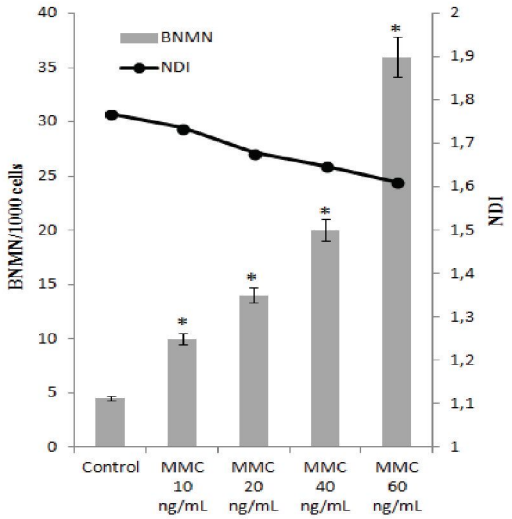

Figure 1. The MN frequencies and NDI values for the positive controls of 10 ng/mL, 20 ng/mL, 40 ng/mL, and 60 ng/mL of MMC in CBMN test. BNMN: Binucleated micronucleus; NDI: Nuclear Division Index; MMC: Mitomycin C. * $p<0.05$ vs.control (two tailed Student's *t* test).

Consequently, the studies conducted in mammalian cells have revealed that the HBO induced DNA damage can lead gross genetic alterations and chromosome aberrations (4, 14, 15). On the other hand, mutations have not been reported in humans undergoing HBO therapy so far. However, it is important to note that the available human studies were conducted in healthy humans. Therefore the mutagenic potential of HBO exposure should be studied further in patients undergoing HBO therapy concerning its approved indications. There is only one study (10) conducted by us with the same labour team showing the DNA damaging effects of HBO treatment performed in the lymphocytes of the patients undergoing HBO with different diseases. The results of a previous study suggest that HBO-induced 8-oxodG does not significantly lead to point mutations (15). They found negative results in the *hprt* test, however, they showed that HBO efficiently induces mutations in the mouse lymphoma assay (MLA). But the mutagenic effect in MLA was solely due to the strong increase of small colony mutants, so it was suggested that HBO causes mutations by induction of chromosomal alterations.

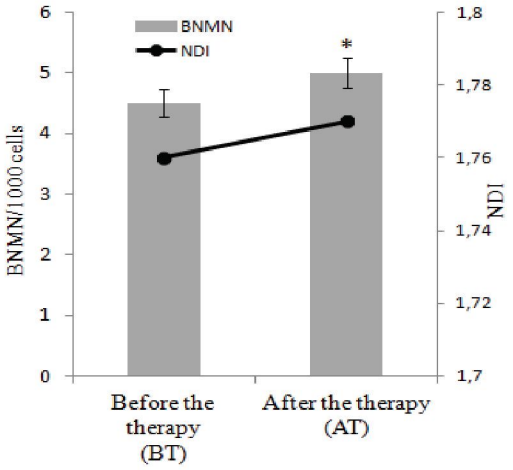

Figure 2. The results of the MN test performed in the 100 blood samples. BNMN: Binucleated micronucleus; NDI: Nuclear Division Index. * $p<0.05$ vs.before therapy (two tailed Student's *t* test).

Our results of MN test have supported this findings of the study. From this point of few this is the first and the largest study which shows the increases in the frequencies of MN in the lymphocytes from patients undergoing HBO therapy.

CONCLUSION

Taken together our results indicate that HBO treatment leads to an increase of genotoxic effects in human lymphocytes after the first therapy session. Accordind to our results, it can be said that the genotoxic effect of HBO treatment is mainly based on a clastogenic mechanism.

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