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Research Article

Impact of hyperbaric oxygenation on oxidative stress in diabetic patients

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ABSTRACT

Taking into consideration that high concentration of oxygen can express toxic effects due to production of reactive oxygen species (ROS), the aim of our investigation was to establish the influence of hyperbaric oxygenation on oxidative stress parameters and antioxidant enzymes in patients with diabetes mellitus (DM) type 2. Investigation included 50 patients with DM type 2 divided into two groups. The first group consisted of 25 patients, mean age 70 years, mean duration of illness 12 years and without manifest peripheral vascular complications (Wagner 0). The second group consisted of 25 patients, mean age 74 years, mean duration of illness 17 years and with manifest peripheral vascular complications (Wagner 1-5).

All patients underwent the same therapeutic protocol, which included 10 hyperbaric oxygenation therapies, once a day for a duration of 60 minutes, with an average partial oxygen pressure of 1.7 atmospheres absolute (ATA). In blood samples the following parameters of redox balance were determined: levels of nitrites (NO_2-), index of lipid peroxidation (TBARS), superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2) and antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT).

Our results clearly show that hyperbaric oxygen (HBO_2) therapy does not have a pro-oxidative effect. Additionally, it seems that this procedure strongly mobilized the antioxidant enzyme system, thus improving defense from oxidative damage. All significant data are marked as $P<0.05$. Our results have shown that in terms of ROS production, HBO_2 can be safe to use in patients suffering from DM type 2 with or without vascular complications.

INTRODUCTION

Diabetes mellitus (DM) is a progressive metabolic disorder characterized by hyperglycemia resulting from lack of insulin secretion or action. This in turn leads to disorders of lipid, protein and carbohydrate metabolism. According to absolute or relative deficiency of insulin, diabetes is classified as type 1 and type 2 [1]. DM is associated with cardiovascular complications, nephropathy, neuropathy and retinopathy due to glucose uptake regardless of the presence of insulin. In developed countries, this disease is an important source of mortality and morbidity [2]. The World Health Organization (WHO) has assessed that 346 million people have diabetes; this number is predicted to double by 2030 [3].

It has been proposed that one of the crucial events in the pathogenesis of diabetes mellitus is the extent of release of ROS (reactive oxygen species). There is also additional evidence that damage caused by free radicals is possibly involved in beta-cell destruction [4]. Persistent hyperglycemia is present in both types of diabetes and causes increased production of oxygen free radicals. Mechanisms responsible for production of free radicals imply autoxidation of glucose, glycosylation of protein and increased lipid peroxidation. Production of free radicals leads to oxidative stress, which is associated with several health complications [5]. Reduction of glutathione concentration leads to disruptions in cellular signaling and upregulation of NF-kB trans-

KEYWORDS: diabetes mellitus; hyperbaric oxygen treatment; redox status

cription, leading to oxidative conditions [6]. In cases of catalase deficiency, β-cells undergo oxidative stress by producing excess ROS, which leads to β-cells dysfunction and ultimately diabetes. The antioxidant enzymes also play an important protective role in the pathogenesis of diabetes mellitus [4].

Hyperbaric oxygen (HBO_2) therapy is a therapeutic modality which provides extra oxygen to tissues. HBO_2 has been suggested as valuable addition to conventional treatment for many indications (e.g., decompression sickness, gas or air embolism, carbon monoxide poisoning, clostridial myositis and myonecrosis, arterial insufficiencies, osteomyelitis, diabetic foot ulcer) [7]. During HBO_2 therapy a person breathes pure oxygen (100%) at pressures greater than 1 atmosphere, usually 2 to 3 atmospheres absolute (ATA). By increasing blood oxygen content, HBO_2 creates a favorable gradient for the diffusion of oxygen into the tissues. In hypoxic tissues, the enhanced oxygen supply has multiple effects: a direct antihypoxic effect, a reduction in plasma viscosity, an increase in elasticity of erythrocytes, and a reduction of platelet aggregation. Furthermore, this treatment stimulates neocapillarization by increasing capillary penetrance of oxygen to about three times, and allows for nitric oxide (NO) production, thus supporting normal endothelium function [8]. HBO_2 is usually used in DM for the treatment of diabetic foot and peripheral vascular disease [9].

Considering the standpoint that high concentrations of oxygen can cause toxic effects due to production of ROS, the aim of our investigation was to establish the influence of hyperbaric oxygenation on oxidative stress parameters and antioxidant enzymes in patients with diabetes mellitus type 2 (DM2).

MATERIALS AND METHODS

Subjects

The investigation included 50 patients with DM2 who were divided into two groups classified according to the Wagner scale. The first group consisted of 25 patients, mean age 70 years, mean duration of illness 12 years and without manifest peripheral vascular complications (Wagner 0). The second group included 25 patients, mean age 74 years, mean duration of illness 17 years and with manifest peripheral vascular complications (Wagner 1-5). The study protocol was approved by the Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, and it was conducted in accordance with the principles of the Declaration of Helsinki. All the participants were informed about the research protocol before giving their written consent to participate in the study.

Hyperbaric oxygen therapy protocol

Hyperbaric treatment was performed at HBO Medical Center in Belgrade. All patients underwent the same therapeutic protocol, which included 10 hyperbaric oxygenation treatments, once daily with a duration of 60 minutes, with an average partial pressure of oxygen of 1.7 ATA. Therapy was conducted in monoplace hyperbaric chambers, BKL-S 303.

Sample collection protocol

Blood samples for biochemical analysis were collected before treatment and then on the third, fifth, seventh and tenth day of treatment. After centrifugation of heparinized venous blood, plasma and erythrocytes were separated. Routine laboratory analyses were measured pre- and post-HBO_2, including: blood glucose levels (BGL), glycosylated hemoglobin (HbA1c), urea, creatinine, creatine kinase and electrolytes such as potassium chloride and sodium chloride. These parameters of redox balance were then determined: levels of nitrites (NO_2-), index of lipid peroxidation (TBARS), superoxide anion radical (O_2-), hydrogen peroxide (H_2O2), and antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT).

Biochemical assays

Blood samples were drawn from an antecubital vein into a vacutainer test tube containing sodium citrate anticoagulant. Blood samples were analyzed immediately. Blood was centrifuged to separate plasma and red blood cells (RBCs). Biochemical parameters were measured spectrophotometrically, using a UV-1800 Shimadzu UV Spectrophotometer.

Superoxide anion radical determination

The level of superoxide anion radical (O_2-) was measured using nitro blue tetrazolium (NBT) reaction in TRIS-buffer combined with plasma samples and read at 530 nm [10].

Hydrogen peroxide determination

The protocol for measurement of hydrogen peroxide (H_2O_2) is based on oxidation of phenol red in the presence of horseradish peroxidase [11]. A 200-µl sample with 800 µl of PRS (phenol red solution) and 10 µl of POD (horseradish peroxidase) were combined (1:20). The level of H_2O_2 was measured at 610 nm.

Nitric oxide determination

NO decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite (NO_2-) was determined as an index of nitric oxide production with Griess reagent [12]. Then 0.1 mL 3 N PCA (perchloric acid), 0.4 mL 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 mL plasma were put on ice for 15 minutes, then centrifuged 15 minutes at 6,000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe.

Index of lipid peroxidation: thiobarbituric acid reactive substances

The degree of lipid peroxidation in plasma was estimated by measuring thiobarbituric acid reactive substances (TBARS) using 1% thiobarbituric acid (TBA) in 0.05 NaOH, incubated with plasma at 100°C for 15 minutes and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 mL of plasma and 0.4 mL trichloroacetic acid (TCA), then samples were put on ice for 10 min, and centrifuged for 15 minutes at 6,000 rpm. This method was described previously [13].

Determination of antioxidant enzymes SOD and CAT

Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/L NaCl, and hemolysates containing about 50 g Hb/L (prepared according to McCord and Fridovich [14] were used for the determination of CAT activity. CAT activity was determined according to Beutler [15]. Then 50 µl of CAT buffer, 100 µl hemolysate, and 1 mL 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. Distilled water was used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich [16]. A 100-µl hemolysate and 1-mL carbonate buffer were mixed, and then 100 µl of epinephrine were added. Detection was performed at 470 nm.

Statistical analysis

IBM SPSS Statistics 20 was used for statistical analysis. Values were expressed as mean ± SE. The Student's t-test and analysis of variance (ANOVA) were used for comparison between groups and inside groups. P-values lower than 0.05 were considered to be significant.

RESULTS

There were no statistically significant changes in BGL, Hb1Ac, urea, creatinine, creatine kinase and levels of electrolytes after HBO_2 compared to baseline values (Table 1).

Levels of nitrites

During the observed 10-day period of HBO_2 therapy, there were statistically significant changes in NO_2- between both groups. In the group with vascular complications a significant increase in this parameter was noticed on the third day at the beginning of therapy. Values of NO_2- decreased statistically significantly (P-values lower than 0.05 were considered to be significant) at the fifth day compared to the third day. Values of NO_2- in the group without vascular complications were increasing until the end of treatment. A statistically significant increase was seen in values of NO_2- measured on the 10th day versus its values measured on the seventh day (Figure 1A).

Levels of index of lipid peroxidation measured as thiobarbituric acid reactive substances

During the observed 10-day period of HBO_2 therapy, there were no statistically significant changes of TBARS values between both groups. However, values of TBARS in the group without vascular complications were lower compared to the other group. On the other hand, in the group with vascular complications, values of TBARS significantly differed between the fifth and seventh days, when values of this parameter increased (Figure 1B).

Levels of superoxide anion radical

Values of superoxide anion radical were statistically significantly increased in diabetic patients with vascular complications at 10th day compared to baseline values. However the highest values of this parameter were noticed on 3rd day of therapy. We have also found that levels of O_2- were decreased after HBOT compared to baseline values in group without vascular complications (Figure 1C).

TABLE 1. The effects of HBO_2 on laboratory parameters in patients with DM type 2 with and without vascular complications

laboratory parameter	Wagner 0		Wagner 1-5	
	before HBO_2	after HBO_2	before HBO_2	after HBO_2
glucose [mmol/L] (ref. values: 4.1 – 5.9)	5.1	5.0	5.4	4.9
urea [mmol/L] (ref. values: 2.5 – 7.5)	10.2	10.0	10.3	10.1
creatinine [μmol/L] (ref. values: 62 – 115)	113	109	110	108
potassium [mmol/L] (ref. values: 3.5 – 5.1)	4.9	4.8	5.1	5.0
sodium [mmol/L] (ref. values: 136 – 148)	145	144	142	144
chlorides [mmol/L] (ref. values: 98 – 111)	101	95	98	105
creatine kinase [U/L] (ref. values: 32 – 300)	416	401	420	396
HbA1c [mmol/mol] (ref. values: 24 – 43)	50	45	57	49

Levels of hydrogen peroxide

During the observed 10-day period of HBO_2 therapy, there were no statistically significant change in H_2O_2 between groups. Baseline values in the group without vascular complications were significantly higher than values during 10 days of HBO_2. We saw a significant decrease of this parameter on the third day and a significant increase on the seventh day of therapy in the group without vascular complications. In the group with vascular complications, values of this parameter showed a continuous decrease (Figure 1D).

Superoxide dismutase activity

During the observed 10-day period of HBO_2 therapy, there were no statistically significant changes in SOD values between groups. In the group without vascular complications the values of SOD were high until the fifth day and then began decreasing until the end of HBO_2 treatment. However SOD activity in the group with vascular complications were lowest at the seventh day in comparison to values on the other days (Figure 2A).

Catalase activity

During the observed 10-day period of HBO_2 therapy, there were statistically significant changes of CAT values between groups. In the group without vascular complications, values of this parameter increased statistically significantly on the third, fifth and 10th day from the beginning of treatment. On the other hand, continuous increase of CAT was noticed in the group with vascular complications (Figure 2B).

DISCUSSION

Numerous studies have underlined the controversy that exists regarding the effect of HBO_2 on oxidative stress and enzymes of antioxidative defense in several pathophysiological models. Using healthy rats, a study by Ay and co-authors has shown a pro-oxidative activity of HBO_2 as a result of an increased index of lipid peroxidation (TBARS) and activity by SOD and CAT enzymes. In this study one HBO_2 treatment was applied for 90 minutes [17]. Otec and co-workers found that the pro-oxidative effect of HBO_2 is time-dependent [18]. Furthermore, HBO_2 reduced infarct size and

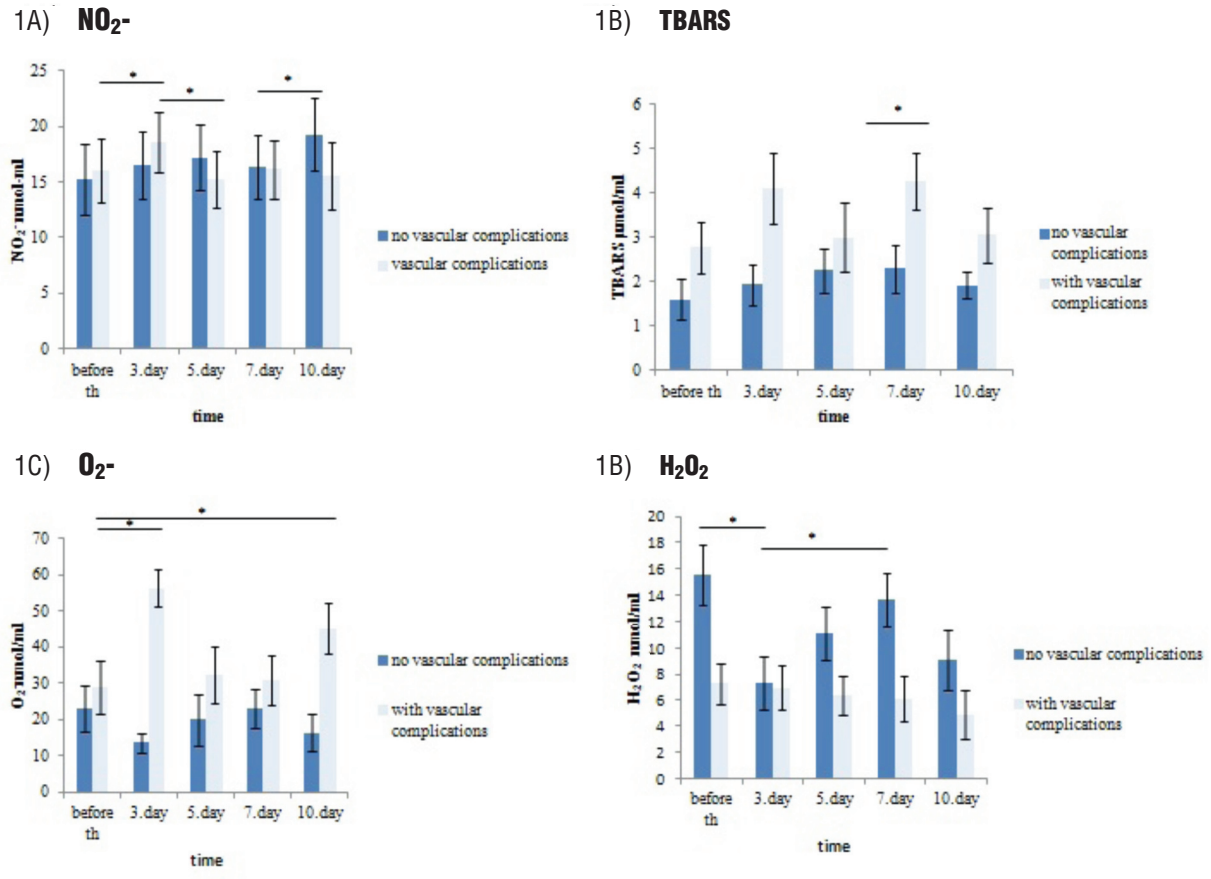

FIGURE 1. The effects of HBO_2 therapy on oxidative stress parameters in patients with diabetes mellitus type 2 with and without vascular complications. (th)-therapy * statistical significance at the level of $P < 0.05$

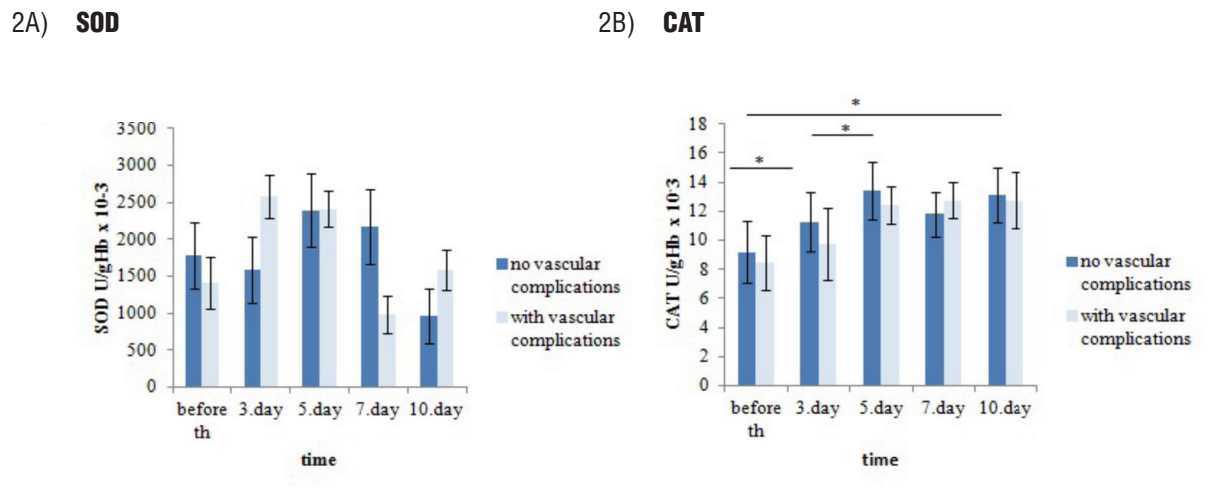

FIGURE 2. The effects of HBO_2 on enzyme of antioxidant protection in patients with DM2 with and without vascular complications. (th)-therapy * statistical significance at the level of $P < 0.05$

increased catalytic activity of CAT in an experimental infarct of the myocardium [19]. As regards clinical studies, some of them support HBO_2 treatment in different pathophysiological states while others not [20-24].

Chronic vascular complications are the main causes of mortality and morbidity in diabetic patients [25]. The main role in the development of chronic vascular complication is endothelial dysfunction, which leads to production of vasoconstrictors via oxidative degradation of NO and decreased synthesis of NO. Increased production of O_2- interacts with NO, producing one of the most harmful free radicals, peroxynitrite (ONOO-), which leads to vasoconstriction and hypoxia [26].

First, we examined the effects of HBO_2 on levels of pro-oxidants, such as NO_2- and TBARS in patients with DM2 without and with vascular complications. Our study has shown continuous increase of NO_2- values in diabetic patients without vascular complications. Also, values of this parameter were higher at the end of the therapy in comparison to the beginning of HBO_2 treatment.

On the other hand, in the group with vascular complications values of NO_2- were lower at the end of the HBO_2 in comparison to the beginning. Studies dealing with influence of HBO_2 on NO bioavailability show controversial results. Some of them have shown that HBO_2 acts via activation of nitric oxide synthase (NOS) [27]. Other studies have shown opposite effects of HBO_2 in the reduction of NO bioavailability [28].

An investigation of Chen and co-workers found that patients with DM2 had significantly increased levels of NO after HBO_2. Our results as well as those of aforementioned studies could be consequence of high insulin levels, which might have a main role in NO production [29].

We found that the values of TBARS in the group without vascular complication slightly increased from the beginning of HBO_2 treatment until the seventh day. However, in a group with vascular complications the values of this parameter statistically increased on the third day. Literature data showed that indices of lipid peroxidation (TBARS) were higher in diabetic patients than in healthy patients who received HBO_2 therapy. As regards diabetic patients with vascular complications the levels of this parameter and allene oxide synthase (AOS) enzyme were much higher than in patients without vascular complications [30-31]. Similar results were noticed in a study by Grudol and co-authors, where values of the index of lipid peroxidation in diabetic patients with terminal complications were high at the beginning of therapy and then unchanged 15 days after therapy [32].

Furthermore, other pro-oxidants, such as the superoxide anion radical and hydrogen peroxide were also significantly affected by HBO_2. We have found decreased levels of O_2- after HBO_2 compared to baseline values in group without vascular complications, while levels of this parameter were higher in group with vascular complication. This indicates that patients with vascular complications have a lower activity of antioxidant enzymes in comparison to patients without vascular complications. Decreased levels of H_2O_2 in the group with vascular complications might be due to increased CAT activity, which detoxifies hydrogen peroxide to water. Furthermore, one study revealed that levels of H_2O_2 reduced due to increased activity by SOD and CAT, which are the major scavenging enzymes [33].

It is thought that HBO_2 therapy may increase oxidative stress via the production of reactive oxygen species, but literature data indicate that although oxidative stress can occur with HBO_2 treatment, it appears to be less of a concern at hyperbaric pressures under 2.0 ATA [34]. Our results clearly show that HBO_2 does not have a pro-oxidative effect. Parameters of oxidative stress were smoothly decreased during the treatment, which is in compliance with our previous study [35].

In order to complete a picture of the influence of HBO_2 on redox status we examined the activity of antioxidant enzyme systems such as SOD and CAT. Considering the fact that SOD is an enzyme that catalyzes the transformation of the superoxide anion radical to hydrogen peroxide, we can note that these two parameters are in correlation in both groups. Increase of O_2- is followed by decrease in SOD, particularly in the group with vascular complications. In the group without vascular complications, SOD values were high until the fifth day and then decreased until the end of HBO_2 treatment. These findings may suggest that HBO_2 should be used from the fifth day in diabetic patients without vascular complications, while therapy for patients who have diabetes with vascular complications should last longer.

As regards values of CAT during 10 days of HBO_2

treatment, there was significant change in both investigated groups. Similar to SOD values, CAT levels in the group without vascular complications were high until the fifth day. High CAT values were followed by a drop in H_2O_2 values particularly in the group with vascular complications. These results are in agreement with the results of previously conducted research that showed that hyperbaric oxygen treatment below 2.0 ATA can increase the activity of antioxidant enzymes including SOD and CAT [35].

There were no statistically significant changes in BGL, Hb1Ac, urea, creatinine, creatine kinase and levels of electrolytes after HBO_2. This is not in accordance with the previously published papers in this field. Several studies suggest an increase in insulin production or modification of metabolism favorable to the diabetic, resulting in lower blood sugar. However, most of these studies have included diabetes mellitus type 1 (DM1) patients [36-38]. The possible mechanisms of hypoglycemia involve an increase in activity of insulin receptor sites and changes in insulin sensitivity due to upregulation of PPAR-γ signaling associated with hyperbaric oxygen [39]. There are data that show insulin sensitivity increased within three days of hyperbaric oxygen treatment and maintained for 30 sessions [40].

Few studies have revealed that hypoglycemia might be encountered after HBO_2 among DM1 and DM2 patients [41-43]. The possible reason for a discrepancy between our results and results of mentioned authors may be duration of HBO_2 treatment. The shorter period of exposure to hyperbaric oxygenation present in our study might have not been sufficient to cause changes in parameters. There are limited data regarding effects of HBO_2 on kidney function and levels of electrolytes on the human population. Our results are in agreement with literature data that HBO_2 does not cause renal impairment in a rat model [44,45], which reinforces the assumption that HBO_2 is safe in healthy rats.

Of course, better information about oxidative stress during HBO_2 treatment in such patients could be more scientifically significant if patients can be divided in several groups, especially those with vascular complications according to the Wagner scale.

A limitation of our study is a small sample size. Regarding that, we compared only effects in groups of patients with versus without general vascular complications.

CONCLUSION

Our study has shown that in terms of the production of reactive oxygen species, hyperbaric oxygen therapy can be safe to use in patients suffering from diabetes mellitus type 2 with or without vascular complications. Moreover, it seems that this procedure strongly mobilizes the antioxidant enzyme system, thus improving defense from oxidative damage. Based on our results, treatment for patients without vascular complications should last five days; for diabetics with vascular complications treatment should last longer than five days. However, more research is required to establish the complete mechanism by which HBO_2 therapy can modify oxidation-reduction reactions in patients with DM2 in order to become an additional routine therapeutic strategy in the treatment of diabetes. Keeping in mind that HBO_2 was not connected to the changes in BGL, its usage can be considered safe in terms of this parameter.

■

The authors declare that no conflicts of interest exist with this submission.

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