Hyperbaric oxygen, oxidative stress, NO bioavailability and ulcer oxygenation in diabetic patients.

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The Institute of Hyperbaric Medicine and Wound Care Clinic1, Nephrology Division2, Research & Development Unit3, Department of Vascular Surgery4, Assaf Harofeh Medical Center, Zerifin, Israel. Affiliated to Sackler School of Medicine, Tel-Aviv University, Israel.

Efrati S, Gall N, Bergan J, Fishlev G, Bass A, Berman S, Abu-Hamad R, Feigenzon M, Weissgarten J. Hyperbaric oxygen, oxidative stress, NO bioavailability and ulcer oxygenation in diabetic patients. Undersea Hyperb Med 2009; 36(1):000-000. Background: Hyperbaric oxygen therapy (HBO2) increases tissue oxygenation, thus serving as an adjunct therapy for diabetic wounds. However, in some patients there is insufficient increase in tissue O2. Aims: To investigate the pathophysiology of insufficient HBO2 and the possible role of N-acetylcysteine (NAC). Methods: Prospective, randomized, cross-over trial included 50 diabetic patients with non-healing ulcers. Each patient received two treatments with 100% oxygen/2ATA. NAC was administered i.v. at one of the two treatments. Basal and post-treatment peri-wound transcutaneous O2 (TcPO2) pressure, malondialdehyde (MDA), total anti-oxidant status (TAOS) and nitric oxide (NO) were assessed. An ulcer oxygenation increase above 200mmHg was accepted as sufficient. Results: During HBO2, 17 patients (34%) demonstrated insufficient increase in TcPO2. Concomitantly, their TAOS and NO decreased, while MDA increased. NAC administration attenuated these parameters, thus improving the HBO2 outcome. In those affected by NAC, the cure rate was 75%. By contrast, in 66% of patients with sufficient increase in TcPO2, TAOS was increased and MDA decreased irrespective of NAC administration. The cure rate in this subgroup was 82%. Conclusions: Insufficient increase of ulcer oxygenation during HBO2 results from exaggerated oxidative stress and decreased NO bioavailability. NAC administration-induced modulation of both parameters and may improve ulcer oxygenation during HBO2.

INTRODUCTION

Foot ulcers in diabetic patients are a major cause of morbidity and mortality, accounting for approximately two-thirds of all non-traumatic amputations performed in the United States (1, 2). The lifetime risk of a foot ulcer for diabetic patients is approximately 15%, the prognosis being based on progression of their limb ischemia and/or neurologic disease (3). Hyperbaric oxygen therapy (HBO2) has been used as an adjunct to antibiotics, debridement and revascularization for therapy of chronic, non-healing foot ulcers in diabetic patients (4-12).

Successful tissue oxygenation has always been the best predictor of wound healing following HBO2 therapy (13, 14). Therefore, the HBO2-induced increase in the peri-wound transcutaneous O2 pressure (TcPO2) within the trophic lesion area above 200 mmHg should be considered a powerful contribution to the conventional wound treatment (14). Most patients demonstrate enormous increase in blood O2 levels during HBO2. Unfortunately, some of these diabetic patients fail to achieve sufficient TcPO2 increase, and a small percentage even show a paradoxical reduction in tissue
O₂ concentrations. It has been hypothesized that increase in TcPO₂ is insufficient due to concomitant vasoconstriction.

Reduction-oxidation (redox) reactions generate reactive oxygen species (ROS) acting as inter- and intracellular mediators of signal transduction in physiologic and pathophysiologic processes. In vascular tissue, ROS have been shown to modulate vascular tone and structure. ·O₂ and H₂O₂ have been demonstrated to induce vascular contractions (15-19), whereas NO was shown to play a pivotal role in endothelium-dependent relaxation (20). In the brain, HBO₂ was also shown to induce vasoconstriction by increasing oxidative stress and decreasing NO bioavailability (21, 22). These effects may be mediated directly, via ROS-induced increase in cytosolic Ca²⁺ concentrations (23, 24), or indirectly via preventing vasorelaxation by reduction of NO concentrations via increased ·O₂ quenching, to form ONOO⁻ (25). Oxygen is a core substrate for ROS generation, so HBO₂ failure may result from exposure of the patient to oxidative stress (26-29). In particular, the increase in ROS accompanied by decreased NO may be deleterious for diabetic patients in whom the basal parameters of oxidative stress as well as NO bioavailability are already impaired (30, 31). This combination of augmented ROS and reduced NO may lead to peripheral vasoconstriction which would prevent the expected increase in HBO₂-induced tissue O₂ concentration.

N-Acetylcysteine (NAC), a thiol-containing antioxidant, has been demonstrated to decrease ROS and to increase NO bioavailability in a number of tissues (32, 33). The aim of the present study was two-fold: first, to test the hypothesis that augmented oxidative stress and the drop in NO bioavailability are responsible for the failure of HBO₂ to improve tissue oxygenation in diabetic patients suffering from foot ulcers; second, to investigate the possible role of N-acetylcysteine (NAC) in improvement of HBO₂ effect on tissue oxygenation, by modulating ROS accumulation and NO bioavailability.

SUBJECTS, MATERIALS AND METHODS

Study population
Fifty type II diabetic patients, aged 18 years or older, who were admitted to the Institute of Hyperbaric Medicine and Wound Care Clinic at Assaf Harofeh Medical Center (Israel) with non-healing foot ulcers were enrolled in this study. The study was designed as a prospective, randomized, cross-over trial. All the participants underwent a complete physical examination, and their medical history and medications were recorded. Their ulcers were defined as non-healing if proven unresponsive to the established medical therapy following 8 weeks of treatment. Patients were excluded from the study if they had macrovascular disease amenable for revascularization with more than 70% obstruction in femoral or popliteal arteries (evaluated by US doppler), a documented allergy to NAC, liver cirrhosis, chest pathology incompatible with pressure changes, or inner ear disease. Subjects suffering from claustrophobia were also excluded as were those who had been treated with NAC during the 3 months prior to the study enrollment. The protocol was approved by the local Ethics Committee for Human Studies, and all patients signed an informed consent form before enrolling them in the study.

Study protocol
The HBO₂ procedures were performed in a hyperbaric chamber at Assaf Harofeh Medical Center, Israel. Each patient enrolled in the study received two HBO₂ treatments, 90min each, within a 5 day-interval. The participants were randomly divided into two groups, A and B, destined “to be or not to be” treated with NAC (Siran 600, Temmler Pharma Germany).
To a part of group A, NAC was administered at the first HBO$_2$ session, while the other part received NAC at the second HBO$_2$ session. In both cases, NAC treatment was started 5 days before HBO$_2$, at a daily dose of 1200 mg (600mg twice a day). On the day of the 90min-HBO$_2$ session, 600mg of NAC were added at the beginning of the HBO$_2$ and every 30 minutes during the HBO$_2$ in addition to the usual 1200mg/day dose.

The following protocol was applied for both HBO$_2$ sessions:

a. 30 min- exposure to room air oxygen concentration (~20% oxygen).

b. 30 min- exposure to 100% oxygen, at 1 atmosphere of absolute pressure (ATA).

c. 30 min- exposure to 100% oxygen, at 2 ATA.

A flow chart of the study protocol is summarized in figure 1.

At the beginning and during each HBO$_2$ session, tissue oxygenation levels were evaluated by measurements of peri-wound transcutaneous pressure of O$_2$ (TCpO$_2$) using Novametrix 840 (Novametrix Medical Systems Inc. Wallingford, Connecticut, U.S.A. 06492) (34). TCpO$_2$ was evaluated as closely as possible to the trophic area. The TCpO$_2$ evaluations at 30 minute-exposure to the 2 ATA/100% oxygen were performed within the hyperbaric chamber. In accordance with commonly accepted clinical experience, TCpO$_2$ <200mmHg during HBO$_2$ was considered insufficient for wound healing (13, 14).

Patients in whom tissue oxygenation increased above 200mmHg, with or without NAC, were subjected to a complete treatment protocol consisting of administration of 100% O$_2$ for 90 minutes at a pressure of 2 ATA 5 times a week for at least 4 weeks.

Patients that positively responded to NAC administration by >200 mmHg increase in ulcer tissue oxygenation, proceeded with NAC therapy throughout the entire HBO$_2$ protocol. Where and when needed, the sessions also continued beyond the experimental protocol, until complete wound healing was achieved. The number of additionally prescribed treatments varied, dependent upon the clinical responsiveness of each given patient.

**Ulcer classification**

In order to evaluate the severity of the ulcer, we used the University of Texas Wound Classification System. The system is based on a matrix of wound grade (depth) and stage (infection and/or ischemia) in order to categorize the wounds by their severity. In brief, the wounds were graded by their depth

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**Fig. 1. Flow chart of the study protocol.**

NAC=N-acetylcysteine.

HBO$_2$=hyperbaric oxygen therapy
according to the following criteria: grade 0 - a pre- or post-ulcerative site that had healed; grade 1 - superficial wounds through the epidermis or epidermis and dermis that had not penetrated the tendon, capsule or bone; grade 2 - wounds that penetrated the tendon or capsule; and grade 3 - wounds that penetrated the bone or the joint. Within each wound grade, the following four stages were distinguished: (A) - clean wounds, (B) - non-ischemic infected wounds, (C) - ischemic non-infected wounds, and (D) - infected ischemic wounds. Complete healing was determined as grade 0, stage A. Partial healing was defined as improvement by at least one grade and one stage.

**Oxidative stress and nitric oxide evaluation**

At the beginning and at the end of each of the two HBO$_2$ sessions, 5ml of venous blood were drawn into test tubes containing potassium EDTA (Becton-Dickinson, UK). Following centrifugation, the separated plasma samples were immediately frozen at -80°C for further measurements of malondialdehyde (MDA), total anti-oxidant status (TAOS), and nitric oxide (NO).

**MDA** was measured on an HPLC device (Varian Tectron, Australia), using a Chromosystem 6700 reagent kit (Chromosystem GmbH, Münich, Germany), according to the manufacturer’s protocol.

Plasma TAOS was determined using a RANDOX® total antioxidant assay kit, (RANDOX Laboratories, LTD, U.K.). This assay is based on the capacity of the plasma to inhibit a peroxidase-mediated formation of 2,2-azino-bis-3-ethylbenthiazoline-6-sulfonic acid (ABTS+) radical. The relative inhibition of ABTS+ formation in the presence of plasma is proportional to the antioxidant capacity of the sample. The assay procedure and the resultant calculations were performed according to the manufacturer’s instructions.

Total NO content was assessed by an ELISA assay (R&D Systems Inc, USA), based on enzymatic conversion of the nitric oxide metabolites to nitrites using nitrate reductase as a converting enzyme. The total amount of nitrites was subsequently measured by a colorimetric method based on the Griess reaction.

**Statistical analysis**

Numerical measurements were expressed by their means ± standard deviations, while categorical measurements were expressed by their percentages. In order to examine baseline differences in percentages between the two groups, the chi-Square or Fisher's exact tests were used. The base-line differences in means were examined using t-test for independent groups.

As explained above, all of the patients were examined four times for each of the measurements (MDA, TAOS and NO) and the study design was a 2X2 repeated measurements design with yes/no NAC treatment and pre/during HBO$_2$. In addition, patients were divided into two independent sub-groups: the group of patients with the expected increase in TcPO$_2$ during HBO$_2$ (>200mmHg) and the group of patients with an insufficient increase in TcPO$_2$ during HBO$_2$ (<200mmHg). The resulting statistical model was ANOVA with 2 repeated measurements and one factor variable. Using this model enables us to examine the differences between the independent groups, the differences within the repetitions and the existence of all possible interactions.

As usual, for all statistical analyses a p-value less than 0.05 was considered to be a significant result. The statistical analyses were performed using SPSS version 14.01.
RESULTS

Fifty two patients were initially randomized for this study. Two of them were subsequently excluded, one due to gastrointestinal discomfort following NAC administration, and the other due to a problem in the ear. Baseline patients’ clinical characteristics are summarized in Table 1.

The mean baseline TCpO \(_2\) at room air was 29±5 mmHg. Twenty one patients (42%) had a baseline TCpO \(_2\) <25 mmHg. After a 30 minute-exposure to 100% oxygen at 2 ATA, 33 patients (66%) demonstrated sufficient increase in their TCpO \(_2\), far beyond 200mmHg (444±83mmHg), while in 17 patients (34%) there was an insufficient increase in TCpO \(_2\), <200mmHg (78±16mmHg). The TCpO \(_2\) data are summarized in Table 2 and in Figures 2 and 3. As detailed in the Methods section, patients with macrovascular disease amenable for revascularization with more than 70% obstruction in femoral or popliteal arteries (evaluated by US Doppler) were excluded from the study. 50% stenosis in the femoral artery was found in 3 patients (9%) from the group with the expected increase in TCpO \(_2\) compared to 2 patients (11.7%) in the group with the insufficient increase in TCpO \(_2\), p=0.77.

In the group of patients with insufficient oxygenation during HBO\(_2\), addition of NAC resulted in a significant increase in TCpO \(_2\) from 78±16mmHg to 334±67mmHg, p<0.001 (Figure 3). In 12 out of these 17 patients, administration of NAC resulted in TCpO \(_2\) increase above 200mmHg. No additional improvement in TCpO \(_2\) was detected when NAC treatment was applied to the other 33 patients, 444±83mmHg vs. 406±91mmHg, p=0.64, (Figure 3). All patients demonstrating TCpO \(_2\) increase above 200 mmHg, completed the HBO\(_2\) protocol (at least 4 weeks of treatment, mean of 6.3±1.8 weeks). The cure rates were 82% (27/33) in those patients who had a sufficient increase in TCpO \(_2\) (> 200mmHg) without NAC and 75% (9/12) in those who needed NAC in order to achieve a sufficient increase in TCpO \(_2\) (Figure 2). Clinical characteristics of the patients demonstrating sufficient (>200mmHg) or insufficient (<200mmHg) increases in TCpO \(_2\) were not significantly different prior to starting the study and remained unchanged at the end of the study (Table 1).

The results of MDA, TAOS and NO measurements are summarized in Table 2 and in Figures 4- 6. In the group of patients with the expected increase in TCpO \(_2\) to above 200 mmHg (n=33), HBO\(_2\) had significant effect on TAOS, p<0.001. These results are controlled for the confounding effect of NAC treatment. As detailed in table 2 and figures 4-6, in the group of patients with the expected increase in TCpO \(_2\), HBO\(_2\) had increased TAOS and decreased MDA. Other important findings are the significant 2X2 interactions between Pre/ During HBO and NAC +/- treatment for all three measurements with all p-values less than 0.001. By examining the four means of each measurement (see table 2) one can notice that: (1) as for TAOS, treatment with NAC increased the baseline TAOS and the TAOS during HBO\(_2\); (2) as for MDA, NAC significantly decreased the baseline MDA and the MDA during HBO\(_2\); (3) as for NO, the levels of NO with NAC were not higher than the levels of NO without NAC.

In the group of patients who had insufficient increase in TCpO \(_2\) (<200mmHg, n=17), HBO\(_2\) had also significantly affected all three parameters TAOS, NO and MDA. However, compared to the group of patients with the expected increase in TCpO \(_2\) the effect was in the opposite direction: the mean levels TAOS and NO had significantly decreased during HBO\(_2\) while MDA mean levels increased. The resulting p-values are: p=0.017, p<0.001 and p<0.001 (table 2, figures 4-6). As stated previously, these results are
### Table 1. Baseline characteristics of patients with the expected increase in peri-wound transcutaneous PO\(_2\) (TcPO\(_2\) >200mmHg) during hyperbaric therapy and in the patients with an insufficient increase peri-wound transcutaneous PO\(_2\).

<table>
<thead>
<tr>
<th></th>
<th>Expected increase in TcPO(_2) (n=33)</th>
<th>Insufficient increase in TcPO(_2), (n=17)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64.3±9</td>
<td>67.8±8</td>
<td>0.1</td>
</tr>
<tr>
<td>Male/Female</td>
<td>25 (76%)/8 (24%)</td>
<td>14 (82%)/3 (17%)</td>
<td>0.59</td>
</tr>
<tr>
<td>Smokers</td>
<td>17 (51%)</td>
<td>9 (53%)</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>Concomitant diseases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>18 (55%)</td>
<td>10 (59%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>14 (42%)</td>
<td>8 (47%)</td>
<td>0.75</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>3 (9%)</td>
<td>2 (11%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Hypertension</td>
<td>25 (76%)</td>
<td>13 (76%)</td>
<td>0.96</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>8 (24%)</td>
<td>5 (29%)</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>Concomitant medications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic antibiotics</td>
<td>27 (82%)</td>
<td>14 (82%)</td>
<td>0.65</td>
</tr>
<tr>
<td>ACE inhibitors/ ARB’s*</td>
<td>22(67%)</td>
<td>11 (65%)</td>
<td>0.94</td>
</tr>
<tr>
<td>Calcium channels blockers</td>
<td>15 (45%)</td>
<td>7 (41%)</td>
<td>0.93</td>
</tr>
<tr>
<td>β- Blockers</td>
<td>8 (24%)</td>
<td>5 (29%)</td>
<td>0.69</td>
</tr>
<tr>
<td>α- Blockers</td>
<td>3 (9%)</td>
<td>2 (11%)</td>
<td>0.77</td>
</tr>
<tr>
<td>Diuretics</td>
<td>25 (76%)</td>
<td>14 (82%)</td>
<td>0.59</td>
</tr>
<tr>
<td>Insulin</td>
<td>23 (70%)</td>
<td>13 (76%)</td>
<td>0.61</td>
</tr>
<tr>
<td>Aspirin/ Clopidogrel</td>
<td>30 (91%)</td>
<td>16 (94%)</td>
<td>0.69</td>
</tr>
<tr>
<td>Statins</td>
<td>24 (73%)</td>
<td>13 (76%)</td>
<td>0.96</td>
</tr>
<tr>
<td><strong>Classification ulcers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C</td>
<td>4 (12%)</td>
<td>2 (12%)</td>
<td>0.3</td>
</tr>
<tr>
<td>2D</td>
<td>11 (33%)</td>
<td>6 (35%)</td>
<td>0.89</td>
</tr>
<tr>
<td>3C</td>
<td>14 (42%)</td>
<td>8 (47%)</td>
<td>0.75</td>
</tr>
<tr>
<td>3D</td>
<td>4 (12%)</td>
<td>1 (1%)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

*ACE- Angiotensin Converting Enzyme, ARBs-Angiotensin receptor blockers
# The U of Texas Wound Classification System.

### Table 2. Changes in peri-wound transcutaneous PO\(_2\) (TcPO\(_2\)), malondialdehyde (MDA), total anti-oxidant status (TAOS) and nitric oxide (NO) in patients with the expected increase in ulcer oxygenation (>200mmHg) (n=33) and in patients with insufficient increase in ulcer oxygenation (n=17) during hyperbaric therapy.

<table>
<thead>
<tr>
<th></th>
<th>Expected increase in TcPO(_2) (&gt;200mmHg), (n=33)</th>
<th>Insufficient increase in TcPO(_2) (&lt;200mmHg), (n=17)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>21% O(_2), 1 ATM</td>
<td>100% O(_2), 1 ATM</td>
<td></td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>21% O(_2), 2 ATM</td>
<td>100% O(_2), 2 ATM</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>21% O(_2), 1 ATM</td>
<td>100% O(_2), 2 ATM</td>
<td></td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>21% O(_2), 2 ATM</td>
<td>100% O(_2), 2 ATM</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>21% O(_2), 1 ATM</td>
<td>100% O(_2), 2 ATM</td>
<td></td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>21% O(_2), 2 ATM</td>
<td>100% O(_2), 2 ATM</td>
<td></td>
</tr>
<tr>
<td>TcPO(_2)(nmHg)</td>
<td>30±4</td>
<td>444±8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MDA(µg/L)</td>
<td>7.6±3.3</td>
<td>7.2±5.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TAOS (mmol/L)</td>
<td>1.6±.2</td>
<td>1.8±.4</td>
<td>0.001</td>
</tr>
<tr>
<td>NO (µmol/L)</td>
<td>10.1±4</td>
<td>9.0±4</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* ANOVA with repeated measurements model was used for comparison between the groups (the group with expected increase in TcPO\(_2\) vs. the group with insufficient increase in TcPO\(_2\)) while controlling for the confounding effects of pre/post HBO and +/- NAC treatment.

&\(p<0.05\) compared to pre-HBO in the same subgroup of patients.

#\(p<0.05\) compared to the same subgroup without NAC treatment.

TcPO\(_2\)=peri-wound transcutaneous pressure of O\(_2\); MDA= malondialdehyde; TAOS= total anti-oxidant status; NO= nitric oxide.
**Fig. 2.** Flow chart describing the ulcer oxygenation response and the cure rates.

**Fig. 3.** Tissue oxygenation in patients with the expected increase in peri-wound transcutaneous $\text{PO}_2$ ($\text{TcPO}_2 < 200 \text{mmHg}$) ($n=33$) and in patients with insufficient increase in ulcer oxygenation ($n=17$) during hyperbaric therapy.

**Fig. 4.** Malondialdehyde (MDA) levels in patients with the expected increase in peri-wound transcutaneous $\text{PO}_2$ ($\text{TcPO}_2 > 200 \text{mmHg}$) ($n=33$) and in patients with insufficient increase in ulcer oxygenation ($n=17$) during hyperbaric therapy.

**Fig. 5.** Total antioxidant status (TAOS) in patients with the expected increase in peri-wound transcutaneous $\text{PO}_2$ ($\text{TcPO}_2 > 200 \text{mmHg}$) ($n=33$) and in patients with insufficient increase in ulcer oxygenation ($n=17$) during hyperbaric therapy.

**Fig. 6.** Nitric oxide (NO) levels in patients with the expected increase in peri-wound transcutaneous $\text{PO}_2$ ($\text{TcPO}_2 > 200 \text{mmHg}$) ($n=33$) and in patients with insufficient increase in ulcer oxygenation ($n=17$) during hyperbaric therapy.

$\text{TcPO}_2$: Tissue transcutaneous $\text{O}_2$ pressure.

* $p<0.05$ compared to pre-hyperbaric oxygen
# $p<0.05$ compared to the same subgroup without N-acetylcysteine treatment.

NAC=N-acetylcysteine.
controlled for the confounding effect of NAC treatment. Other important findings are the significant 2X2 interactions between Pre/During HBO₂ and NAC +/- treatment for these three measurements with all p-values less than 0.001. By examining the four means of each measurement (see table 2) one can noticed that: (1) as for TAOS, treatment with NAC significantly increased the baseline TAOS and reversed the expected decrease in TAOS during HBO₂; (2) as for MDA, NAC significantly decreased the baseline MDA and reversed the expected increase in MDA during HBO₂; (3) as for NO, NAC treatment had significantly increase the baseline NO and had attenuated the expected decrease in NO during HBO₂.

ANOVA with a repeated measurements model was used for comparison of TAOS, MDA and NO between the groups (the group with expected increase in TcPO₂ vs. the group with insufficient increase in TcPO₂) while controlling for the confounding effects of pre/post HBO₂ and +/- NAC treatment. The following are the results of the three parameters evaluated: (1) The mean level of TAOS was significantly higher in the group of patients with the expected increase in TcPO₂, p=0.001. (2) The mean level of MDA was significantly lower in the group of patients with the expected increase in TcPO₂, p<0.001. (3) The mean level of NO was significantly higher in the group of patients with the expected increase in TcPO₂, p=0.001.

**DISCUSSION**

The aim of the present study was twofold: first, to test the hypothesis that augmented oxidative stress and a drop in NO bioavailability might be responsible for the failure of HBO₂ to improve tissue oxygenation in diabetic patients suffering from foot ulcers, and second, to investigate the possible role of N-acetylcysteine (NAC) in the improvement of HBO₂ effect on tissue oxygenation by modulating ROS accumulation and NO bioavailability. As a part of the HBO₂ protocol for treating their foot ulcers, all patients were exposed to equally high levels of oxygen, which is the core substrate for ROS generation. However, their responses to HBO₂ markedly differed. 34% of the enrolled population demonstrated an insufficient increase in ulcer oxygenation (TcPO₂<200mmHg) when exposed to 2 atmospheres of 100% O₂. This group of patients had significantly lower TAOS, higher MDA and lower NO levels as compared to the group of patients who had increased their TcPO₂>200 mmHg. Most importantly, NAC therapy administered prior and during HBO₂, significantly improved tissue oxygenation, apparently via reducing oxidative stress (increased TAOS, reduced MDA) and increasing NO bioavailability.

Long-term vascular complications represent the main cause of morbidity and mortality in diabetic patients (1-3, 35). Endothelial dysfunction, the most common complication in this patient category, plays a main role in pathogenesis of atherosclerosis, hypertension and the resultant ischemic vascular diseases (36). Decreased NO bioavailability is notorious for promoting vascular constriction and tissue ischemia. Indeed, the mechanisms of diabetes induced endothelial dysfunction include, along with augmented production of prostanoidvasoconstrictors, increased oxidative degradation of NO (30, 31, 37). Thus far, two main factors have been considered responsible for decreased bioavailability of the latter in diabetes: the above mentioned increased oxidative degradation of the existing NO and, concomitantly, the reduced synthesis of new NO molecules (30, 31, 38). ROS reduces NO concentrations via increased O₂⁻ quenching, thus forming ONOO⁻ (peroxynitrites), which possess vasoconstrictor properties. The final
net result is a shift from vasodilatation to vasoconstriction and, resultanty, tissue hypoxia (39). In the present study, 17 out of 50 patients (34%) failed to demonstrate the expected increase in TePO₂ following exposure to oxygen, the core substrate for ROS generation. In these 17 patients HBO₂ induced a significant augmentation of oxidative stress, as represented by increased MDA and decreased TAOS, and a decline in NO bioavailability. One could suggest that the decreased NO bioavailability may lead to vasoconstriction, thus preventing the expected increase in wounded tissue oxygenation. These results are in accordance with a number of previous experimental studies reporting the relationship between oxidative stress, NO and vasoconstriction (15-17, 30, 31, 39). However, to the best of our knowledge, this is the first study conducted on diabetic subjects, which demonstrates a tight straightforward relationship between HBO₂, oxidative stress levels, NO bioavailability and TePO₂ in diabetic patients.

Lately, a growing amount of data is available in the literature on beneficial effects of NAC in several models of toxic and ischemic injuries. Previous animal and human studies have demonstrated that NAC administration improves endothelial dysfunction and induces NO-dependent and NO-independent vasodilatation (32, 33, 40-43). The possible underlying mechanisms may be decreased oxidative stress and consequent degradation of NO (32, 33), increase NO production (32, 33, 44), improved NO tolerance (40), amplification of NO vasodilatatory effect by stimulation of calcitonin gene-related peptide release (41), increase PGE₂ synthesis (42) and activation of K<sub>ATP</sub> channels as well as low-conductance Ca<sup>2+</sup> and K<sup>+</sup> channels (43). In the present study, we evaluated the combined effects of NAC and HBO₂ therapies on oxidative stress parameters, NO bioavailability and tissue oxygenation in diabetic patients. NAC treatment attenuated the expected increase in oxidative stress and decrease in NO concentrations in patients with insufficient increase in TePO₂. Consequently, only in these patients treatment with NAC prior and during HBO₂ improved TePO₂ levels. These differential physiologic effects may prove that NAC therapy is applicable for a variety of high-risk patients with augmented oxidative stress and severe endothelial dysfunction. A recent study reporting that NAC administration reduces the incidence of cardiovascular events in a cohort of patients with end stage renal disease maintained on chronic hemodialysis, supports this hypothesis (45).

Thus far, reports on the effects of HBO₂ on oxidative stress and NO metabolism have been controversial. In general, HBO₂ is considered to be both an inducer of ROS generation and an up-regulator of antioxidant enzymatic activities (21, 22, 26, 27, 46, 47). The final net result depends on the baseline characteristics of the investigated population, etiology of the disease, and specificity of the HBO₂ protocol (21, 22, 26, 27, 46, 47). In some studies, HBO₂ was shown to increase NO bioavailability, via activation of nitric oxide synthase (48). However, in a number of different studies, HBO₂ was shown to reduce endothelium-derived NO bioavailability via pathways either dependent or independent of oxidative stress (21, 49). This diversity of effects on NO bioavailability might explain the non-uniformity of responses to HBO₂ demonstrated by the patients participating in the present study. Thus, in 66% of the patients, HBO₂ increased the antioxidant capacity, as measured by the TAOS, while in 34% of the patients HBO₂ induced a significant decrease in TAOS and increase in MDA. In turn, such non-uniformity of responsiveness to HBO₂ would also explain the heterogeneity in TePO₂.

This study has several limitations. Mainly, possible parallel changes in systemic parameters, such as blood pressure, systemic
vascular resistance and other reference sites for TcPO\(_2\) measurements, were not concomitantly evaluated during the HBO\(_2\). These limitations are notable, since blood samples for assessment of oxidative stress and NO bioavailability were drawn from peripheral veins, thus reflecting the overall systemic changes. It would be of great interest to investigate whether the local effects observed close to the ulcer area are in correlation with concomitantly ongoing systemic alterations of these parameters. However, the limitations taken into consideration, from the clinical point of view the most important outcome of the present study is uncovering the contributory role of NAC to successful treatment of diabetic patients with non-healing foot ulcers via improving tissue oxygenation.

CONCLUSIONS

1. Diabetic patients suffering from foot ulcers are in fact, a heterogeneous group differentially responding to HBO\(_2\) with respect to peri-wound transcutaneous PO\(_2\), oxidative stress and NO. The possible mechanism responsible for the insufficient peri-wound transcutaneous PO\(_2\) following exposure to 2 ATA of 100% O\(_2\), involves increased oxidative stress and decreased NO bioavailability.

2. NAC administration prior to and concomitantly with HBO\(_2\), may improve insufficient peri-wound transcutaneous PO\(_2\), apparently via attenuation of oxidative stress and increase NO bioavailability.

3. The relevance of these observations for non-diabetic patients with oxidative stress and decreased NO availability is a matter for further investigations.

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