The influence of ozonated autohemotherapy on oxidative stress in hemodialyzed patients with atherosclerotic ischemia of lower limbs

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ABSTRACT: Ozonated autohemotherapy is used as a complementary medical approach in the treatment of vascular disorders. One of the greatest problems concerning an application of ozone in medicine is its induction of oxidative stress. The standards of ozonotherapy were elaborated recently making this treatment useful and probably non toxic. The aim of the present study was to investigate the influence of ozonated autohemotherapy on the oxidative stress extent in hemodialyzed patients, known to be particularly exposed to generation and deleterious effects of free radicals. Twelve continuously hemodialyzed subjects with atherosclerotic ischemia of the lower limbs were examined in a prospective, controlled, single blind study. Autohemotherapy with blood exposure to oxygen served as a control. The protein and lipid peroxidation products, the reduced glutathione level in red blood cells and free hemoglobin plasma concentration were measured. The study showed that ozonated autohemotherapy with ozone concentration 50 µg/ml per gram of blood induced a significant decrease in glutathione level after 9 sessions of this procedure. Therapy did not cause either the enhancement of protein and lipid peroxidation, or erythrocytes damage. It seems likely that the antioxidant defense system, part of which is glutathione, neutralizes oxidative properties of ozone in this concentration and protects against oxidative cell damage. (Int J Artif Organs 2003; 26: 297-303)

KEY WORDS: Ozone, Autohemotherapy, Atherosclerosis, Hemodialysis, Renal failure, Oxidative stress, Free radicals, Hemolysis
indicated that treatment with ozone was useful in the therapy of AILL (3, 4). The mechanisms involved in the beneficial activity of this therapy, lead to the improvement of blood flow in hypoxic areas and the attenuation of ischemic symptoms (4).

One of the greatest problems concerning ozonotherapy is its potential toxicity. Ozone is known as a highly reactive oxidant that in inhalatory form may be hazardous to the respiratory system (5). Oxidative injury has been considered as one of the possible side effects of O3-AHT since the role of oxidative stress has been proven in the pathogenesis of many disorders (6, 7). It is obvious that ozone in contact with blood can induce the generation of reactive oxygen species (ROS) (8). However, this effect is detrimental only when high a concentration of ozone is used or in a case of a severely impaired antioxidant defense system (1). The safe range of ozone concentrations in O3-AHT is currently known (1). ROS generation during O3-AHT, applied in dosages from this range, is believed to be almost completely quenched by multifactorial antioxidant systems present in plasma and blood cells (1, 8).

The recommendations concerning ozone dosage are based on the studies and therapeutic experience derived from the treatment of subjects with preserved renal function. This problem has not yet been studied in hemodialyzed (HD) patients, who have an impaired antioxidant system and therefore are particularly threatened by deleterious effects of free radicals (9). In this context the safety of O3-AHT, a therapeutic intervention with a potential to generate ROS is of particular importance. We performed a prospective, controlled, single blind study to find whether O3-AHT causes oxidative stress in patients with ESRD treated chronically with HD. Effects of O3-AHT, applied in the therapeutic dose of ozone - 50µg/ml on the level of lipid and protein peroxidation, the extent of red blood cell hemolysis and the reduced glutathione (GSH) concentration in red blood cells were determined.

MATERIALS AND METHODS

Subjects

The group of patients comprised 12 subjects (8 male, 4 female), aged 64.8±7.6 (range 50-75) years with ESRD treated with hemodialysis. Diabetic nephropathy was diagnosed as primary renal disease and cause of renal replacement therapy in 3 patients, polycystic kidney disease in 5 and chronic glomerulonephritis in one. In four remaining individuals the cause of ESRD was not established. All patients underwent regular bicarbonate hemodialysis treatment, three times per week for more than one year (average 4.5±3.1 years). Low-flux, polysulfone dialysers were used in all subjects. All of them suffered from symptomatic AILL (stage II-IV according to Fontain) which was the main reason for the implementation of ozonotherapy.

The hemodialysis prescription, namely dialyser type, HD session length, rate of dialysis solution and blood flows were unchanged during the study. Any permanent pharmacological treatment known to influence redox status including hypotensive and lipid-lowering therapy remained unchanged throughout the study. No new drugs were administered. All patients received supplementation of vitamin C (400 mg/per week) as an antioxidant. The individuals with active chronic inflammatory disease and with symptomatic or asymptomatic (as confirmed by laboratory tests: C-reactive protein evaluation) acute infection were excluded from the study.

Study design

At the beginning subjects received 9 sessions of autohemotherapy connected with the blood exposure to medical oxygen (AHT) as a control, followed by 9 sessions of autohemotherapy connected with exposure to ozone (O3-AHT). Procedures were performed three times a week in the early morning, just before the hemodialysis session in a single blind manner. Ozone generator (ATO-3, Kriometrum, Warsaw, Poland) attested by the Polish Health Ministry was used in the study. The procedure was as follows: 250 ml of patient blood was drawn into a sterile, transparent, glass bottle. Sodium citrate was used as an anticoagulant. The bottle was then connected to the ozone generator and this way blood was exposed to gas: medical oxygen in the control part of the study and oxygen-ozone mixture during the O3-AHT. Gas was applied in four programmed cycles which lasted altogether 5 minutes. There was a precisely measured volume of gas, equal to the blood volume (1 to 1 relationship). In order to avoid blood foaming, bottle was continuously and gently shaken. Afterwards, the blood was slowly reinfused into the donor via arterio-venous dialysis needle. During O3-AHT sessions blood was exposed to oxygen-ozone
mixture with ozone concentration of 50 µg/ml per gram of blood.

The markers of lipid peroxidation (plasma levels of malonaldehyde and 4-hydroxyalkenals) (LPO), the marker of protein peroxidation (plasma level of carbonyl groups) (PP), the level of reduced glutathione in red blood cells (GSH) and the plasma concentration of free hemoglobin (FHP) were measured. Three blood samples were collected from all subjects at the following timepoints: before the first session of AHT, after 9 sessions of AHT (control), and after 9 sessions of O3-AHT. To evaluate the influence of the first exposure to ozone on the parameters measured, samples of blood were also withdrawn before and 20 minutes after the first session of O3-AHT.

**Biochemical analysis**

*LPO* - Samples of blood were collected in sterile EDTA tubes and centrifuged at 2500 g for 10 min at 4°C. Afterwards, supernatant was carefully collected and stored at -75°C until the assay. Samples were protected from light. Quantitative analysis of lipid peroxidation in the plasma was performed by colorimetric assay according to Esterbauer (10) using a commercially available kit (LPO-586, Oxis, Portland). Briefly, measurement was based on the reaction of chromogenic reagent, N-methyl-2-phenylimidole with malonaldehyde and 4-hydroxyalkenals, products of polyunsaturated fatty acid peroxidation. Reaction in the presence of the methanesulfonic acid was carried on for 60 minutes at 45°C. After centrifugation, absorbance was measured at 586 nm. All assays were performed in duplicate.

*GSH* - The level of reduced glutathione in red blood cells was measured colorimetrically using a commercial kit (GSH-400, Oxis, Portland). Samples of blood were collected into heparinized tubes. After 5 minutes centrifugation at 2500 g at 4°C, erythrocyte pellet was stored at -70°C until analysis, but no longer than 2 weeks. Analytical procedures were performed according to kit producer’s recommendations. Briefly, the method was based on a chemical reaction leading to formation of chromophoric thione. In the first step, reaction between all mercaptans from the sample and 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate led to the formation of thioethers. The second step was β-elimination reaction that took place under alkaline conditions, which specifically transformed thioethers obtained from glutathione into a thione (11). After 10 min incubation at 50°C absorbance was measured at 400 nm. Final results were expressed in micromoles GSH per gram hemoglobin (Hb). Hemoglobin was determined spectrophotometrically. All analyses were performed in duplicate.

*PP* - The level of carbonyl groups was measured according to a method described by Garibaldi et al (12). The method is based on a reaction of 2,4-dinitrophenylhydrazine with carbonyl groups of protein molecules. The product of this reaction was measured colorimetrically at 370 nm. Results were expressed in nmol/mg of protein.

*FHP* - The level of plasma free hemoglobin was analysed according to the method described by Watkins that utilizes fractional absorbance of oxyhemoglobin at 578 nm (13). Sample of blood was drawn into the heparinized tubes. Immediately after plasma separation, FHP was measured spectrophotometrically.

**Statistics**

Data are expressed as mean ± SD. Distribution of variables was evaluated using Shapiro-Wilk’s test. Differences of variables measured more than twice were assessed by analysis of variance for repeated measurements or Friedman’s test; otherwise by Student’s t-test for paired comparison or Wilcoxon test. Data were evaluated using STATISTICA (version 5.1, StatSoft Inc.)

<table>
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<th>TABLE I - DETAILED RESULTS OF THE STUDY</th>
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<td><strong>GSH µmol/g Hb</strong></td>
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<td>12.37 ± 10.88&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td><strong>PP nmol/mg protein</strong></td>
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<td><strong>FHP mg/dl</strong></td>
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<td><strong>LPO µmol/l</strong></td>
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<sup>a</sup> significant difference (p< 0.05): start level vs. level after 9 th O3-AHT
<sup>b</sup> borderline significance difference (0.05<p< 0.1): start level vs. level after AHT (control).
RESULTS

All twelve patients who entered the study completed the protocol. Table I shows detailed results of the study.

GSH concentration in red blood cells after O3-AHT (9 sessions) was statistically lower (p<0.05) when compared to the baseline (-64.51%). GSH concentration after AHT (control) was lower compared to baseline (-42.27%). Difference was in the borderline range of significance (p<0.07).

LPO level after O3-AHT (9 sessions) was higher by 33.6% compared to baseline and was not statistically significant. There were no significant differences in PP level between 3 collections. FHP level did not change during the study.

GSH concentration in red blood cells, LPO plasma level, PP plasma level and FHP concentration did not change after the first session of O3-AHT compared to the levels before this procedure.

DISCUSSION

Free radicals and other ROS are physiologically formed in the human body. Their generation may also result from exposure to toxic agents or from different disease processes. Antioxidant defense includes enzymatic and nonenzymatic components that are in balance with the generation of ROS. The imbalance in favor of the later is termed as oxidative stress. The subsequent oxidative injury of lipids, proteins and DNA may lead to serious cell damage (14).

The prooxidative state of HD patients implies several factors related to ESRD as well as HD procedure (9). Uremia, per se, is believed to cause overproduction of ROS and impair the antioxidant system (15, 16). Hemo-
and bioincompatibility of membranes and blood lines used in dialysis and the presence of trace amounts of endotoxins and/or pyrogens in the dialysate play a critical role in the production of ROS triggered by HD procedure (17, 18). Additionally, HD impairs the antioxidant defense by losses of hydrophilic unbound small-molecular-weight substances and trace elements such as vitamin C and selenium (19, 20). These abnormalities may be further exacerbated in older or diabetic individuals, the group still growing among patients on maintenance HD (21). There is no doubt that protection from the oxidative stress is of particular importance in HD patients. Evidence showed that oxidative stress is involved in several disease states, such as anemia, secondary amyloidosis and atherosclerosis, the major cause of mortality in this population (2, 7, 22-24).

The authors demonstrated previously that O3-AHT might attenuate clinical signs of lower limb ischemia in HD patients with AILL. In a prospective controlled study, a significant prolongation in intermittent claudication distance (by 30.5%) after O3-AHT was found (25). The question remains, however, whether this therapy is safe when oxidative stress is considered. Ozone is known to be a strong oxidant. When dissolved in the blood, it produces a number of ROS. A major part of ozone activity is inhibited by antioxidants but some react with polyunsaturated fatty acids (PUFA) and generate hydrogen peroxide and lipid oxidation products. The latter are postulated to be important messengers responsible for transmitting both beneficial and toxic effects of ozone. A small part of ozone decomposes directly to hydroxyl radical. Increased plasma level of hydrogen peroxide, a unionized molecule, is immediately transferred into intracytoplasmic water. The intracellular environment counteracts this potentially toxic process by quenching hydrogen peroxide with GSH (1, 8, 26, 27).

In the present study, a medium concentration of ozone in an oxygen-ozone mixture (50 µg/ml) was used. According to Bocci (1), oxidative effects of ozone are dose-dependent. The therapeutic window of ozone concentration for O3-AHT ranges between 20-80 µg/ml (0.42-1.66 mmol/L). In the healthy population, oxidative properties of ozone in dosages even up 100 µg/ml are almost completely handled by antioxidant forces (8, 26).

To examine the effects of O3-AHT on the antioxidant defense system, GSH concentration in red blood cells was evaluated. GSH is an important nonenzymatic player in the antioxidant defense and one of the first agents involved in the protection from oxidative stress due to ozone exposure (14, 28, 29) GSH concentration decreased nearly by 65 % after 9 sessions of O3-AHT. This result is in line with previous reports that showed increased consumption of GSH during O3-AHT (28, 30). Strikingly, the drop of GSH store, observed in the present study, was much higher than those reported in other studies performed in individuals with normal renal function. Bocci et al (31) used ozone in concentration even up to 80 µg/ml and found a decrease of GSH no higher than 15%. In another study, the total antioxidant
status of blood, measured by the Rice-Evans method, decreased by 20% after exposure to 80 µg ozone/ml (32). It seems likely that the high consumption of GSH due to increased ROS generation in HD patients, enhanced by O3-AHT achieved supremacy over the GSH regeneration process.

It is noteworthy that a decrease in the GSH level (42.27%, borderline significance) was also observed after control AHT among exposure to oxygen, known not to cause induction of oxidative stress (32). Thus, it may be suggested that some part of oxidative stimulation after O3-AHT may result not only from ozone exposure per se but also from the procedures associated solely with the autohemotherapy. It is well known that blood manipulation, contact of blood with drains and exposure to the energy of light may be the factors which can induce ROS production (33, 34).

To examine whether this shift of the redox balance in favor of the prooxidative state induced any serious injury, the levels of protein and lipid peroxidation as well as hemolysis extent were evaluated. Protein peroxidation is one of the most serious disturbances in oxidatively injured cells (35). We did not find any changes in the PP level either after AHT or O3-AHT (9 sessions). Hemolysis, an index of erythrocyte damage, is thought to be the most critical deleterious effect of ozone on the blood cells (36). There is evidence that the influence of ozone on erythrocytes is closely dose-dependent. Hemolysis during exposure to ozone in concentrations even up to 100 µg/ml is practically negligible in the general population (37, 38). In the present study, we did not observe the enhancement of erythrocyte damage either. The FHP level was stable after AHT and O3-AHT (9 sessions) as compared to the baseline results.

The lipid peroxidation is another manifestation of oxidative cell injury. We measured concentration of the final products of this process: malonaldehyde and 4-hydroxyalkenals (LPO) and found only a nonsignificant increase LPO level after 9 sessions of O3-AHT. Giunta et al (39) observed a significant increase in malonaldehyde level after O3-AHT. In contrast, other authors reported an increase in LPO level only in the samples of blood directly exposed to ozone, i.e. taken from the bottle (28). In conditions of oxidative stress derived from ozone exposure, interpretation of these results is a little difficult. Lipid oxidation products may originate both from oxidatively injured cells as well as from the reaction between ozone and PUFA incorporated in plasma lipoproteins; the process occurs in the bottle where blood is exposed to ozone. Given these findings and the results on PP and FHP, discussed previously, oxidative cell injury after exposure to ozone in the concentration of 50 µg/ml seems unlikely.

A similar conclusion may be drawn analyzing the results from the second part of study, where early changes in the parameters measured, immediately after the first exposure to ozone were determined. Ozone dissolves in the blood almost immediately. Free radicals disappear over several hours (37, 40). The changes in the antioxidant system may also proceed very quickly (32). Based on these facts, the evaluation of LPO, PP, FHP and GSH levels just after the first O3-AHT was performed. No significant changes were observed. GSH levels 20 minutes after the first O3-AHT procedure did not differ compared to the concentrations found just before this session. No changes in LPO and PP levels were found. No increase in FHP concentration was observed either.

In summary, this study demonstrated that O3-AHT with an ozone concentration of 50 µg/ml, applied three times a week, did not induce oxidative cell injury in patients with ESRD on maintenance HD. Nine sessions of O3-AHT caused, however, significant depletion in the GSH level in erythrocytes, most possibly, associated with its increased consumption under oxidative conditions. Nevertheless, the antioxidant defense system was able to protect the cells against oxidative damaging processes. Thus, it may be concluded that O3-AHT is a safe method and may be used as an adjunct medical approach in HD patients when oxidative injury is considered.

Given the present data showing a significant decrease in GSH level and a little increment in lipid peroxidation extent, as well as the fact that a lower dose of ozone (i.e. 35 µg/ml) may also provide beneficial clinical effects in HD patients (reported previously (4)), one may recommend the use of ozone in doses a little lower than those applied in the present study. The therapeutic doses defined for the general population are somewhat high for HD patients who are under constant oxidative stress. To achieve a who better adaptation of the organism to such therapy, beginning O3-AHT with very low doses of ozone, and titration during following sessions up to the final dosage may be advised. Antioxidant defense may be intensified by the administration of acetylcysteine, precursor of glutathion, and by increasing vitamin C dosage supplementation during O3-AHT.
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