BACKGROUND: Ozone as a strong oxidant may induce an inflammatory response.

Aim: The hypothesis was verified as to whether ozonated autohemotherapy using an ozone dose in therapeutic range changes the plasma concentration of C-reactive protein and interleukin-6, markers of inflammation.

Methods: In a controlled, single-blind, cross-over study, 12 chronically hemodialyzed patients with peripheral arterial disease were exposed to nine sessions of autohemotherapy with blood exposure to oxygen as a control followed by nine sessions of ozonated autohemotherapy with an ozone concentration of 50 µg/ml.

Results: There was no statistical difference between C-reactive protein levels at baseline (1.53 ± 1.01 mg/l), after nine sessions of control autohemotherapy (1.48 ± 0.96 mg/l), and after nine sessions of ozonated autohemotherapy (1.55 ± 0.84 mg/l). There was also no statistical difference between the interleukin-6 serum concentration at baseline (438 ± 118 pg/ml), after nine sessions of control autohemotherapy (444 ± 120 pg/ml), and after nine sessions of ozonated autohemotherapy (466 ± 152 pg/ml).

Conclusion: The results of this study suggest that ozonated autohemotherapy using an ozone concentration of 50 µg/ml does not induce an inflammatory response.

Key words: Ozone, Ozonated autohemotherapy, Inflammation, C-reactive protein, Interleukin-6, Hemodialysis, Peripheral arterial disease

No effects of ozonated autohemotherapy on inflammation response in hemodialyzed patients

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Introduction

Major ozonated autohemotherapy constitutes a complementary medical approach in the treatment of different disease states (reviewed in 1, 2). The main criticism concerning this controversial therapy is associated with the oxidative properties of ozone and its potentially harmful influence on cells and biological processes.[3] To gain more light into this issue, we commenced the clinical project aimed to check the safety of ozonated autohemotherapy (O3-AHT) in the treatment of ischemic complaints in hemodialyzed (HD) patients with peripheral arterial disease. O3-AHT applied in the therapeutic dose range according to Bocci's recommendations has been found not to cause either protein or lipid oxidative modification.[4] Intravascular hemolysis, another possible side effect, was also not observed in the same study. We have also demonstrated that O3-AHT does not have any deleterious effects on the endothelium nor promotes platelet aggregation.[4,5] Other authors did not find any negative effects of ozone on white blood cell or red blood cell function.[6,7]

In the present study, the influence of O3-AHT on the inflammation process was evaluated in subjects on dialysis maintenance. These patients are particularly threatened by the development of inflammation. Renal failure *per se* may contribute to the inflammation response, which enhances as renal function declines, and is greatest still in patients on dialysis (reviewed in 8, 9). Inflammation is considered an important non-traditional cardiovascular risk factor. Given that HD patients are at increased risk for cardiovascular complications, one has no doubt that any intervention that may potentially induce inflammatory response in these subjects needs particular attention.

Patients and methods

Subjects

Twelve chronically HD patients were enrolled to the study. They underwent regular bicarbonate HD treatment, three times per week. Their detailed characteristic have been presented elsewhere.[10,11] All of them suffered from symptomatic peripheral

arterial disease (stage II–III according to Fontain), which was the main reason for the implementation of O3-AHT. The HD prescription, namely dialyzer type, HD session length, rate of dialysis solution and blood flow, as well as any pharmacological treatment, were unchanged during the study and 2 months prior to the study commencement. Individuals with active chronic inflammatory disease and those with symptomatic or asymptomatic (as confirmed by laboratory tests; C-reactive protein [CRP] evaluation) acute infection were excluded from the study.

Study design

At the beginning subjects received nine sessions of autohemotherapy with blood exposure to medical oxygen (AHT) as a control, followed by nine sessions of O3-AHT. The procedures were performed three times a week in the early morning, just before the HD session, in a single blind manner. An ozone generator (ATO-3; Kriometrum, Warsaw, Poland) attested by the Polish Health Ministry was used in the study. The procedure of O3-AHT has been described elsewhere in detail.[4,10,11] During O3-AHT sessions, 250 ml of the patients' blood was exposed *ex vivo* to an oxygen–ozone mixture with an ozone concentration of 50 μg/ml. Three blood samples were collected from all subjects at the following time-points: before the first session of AHT (first collection), after the ninth session of AHT (second collection — control) and after the ninth session of O3-AHT (third collection).

Interleukin-6 bioassay

Interleukin (IL)-6 levels in the sera were determined using the B9 hybrydoma proliferation assay.[12] In this assay, 10×10^3 B9 cells (a gift from Dr L. Aarden, Netherland Red Cross, Amsterdam, The Netherlands) were cultured on a 96-well plate (NUNC™; Roskilde, Denmark) with 50 times diluted samples of sera or supernatants in the final volume of 100 μl of RPMI

1640 (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA), supplemented with 5×10^{-5} M of 2-mercaptoethanol (Sigma, Mannheim, Germany), 10% fetal calf serum (Gibco BRL, Life Technologies, Inc.), 100U/ml of penicilin and 100 μg/ml of streptomycin (Sigma). All samples were put on a 96-well plate in triplicate. After 48 h of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, 20 μl of MTT (Sigma) were added to each well of the plate. The plate was incubated for the next 4 h in the aforementioned conditions, and after this time 100 μl of isopropanol (Sigma) were added. The optical density was read at 570 nm on the automated plate reader (Bio-Tek FL600; Bio-Tek Instruments, Inc. Winooski, Vermont, USA). A standard curve relating cell proliferation to doses of recombinant human IL-6 (Sigma) was used to quantify IL-6 activity in the samples.

To confirm the specificity of the test, the monoclonal anti-IL-6 antibody (Genzyme, Rüsselsheim, Germany) was added (1:10, 1:20, 1:50) to the samples. The anti-IL-6 monoclonal antibody completely inhibited the ability of the B9 cells to proliferate. This assay had a detection limit of 1 pg/ml. The inter-assay coefficient of variation ranged between 9.5% and 10.9%. The inter-assay coefficient of variation ranged between 15.2% and 17.7%.

Serum concentrations of IL-6 were assessed using a specific sandwich enzyme-linked immunosorbent assay by R&D Systems, Minneapolis, MN, USA. All samples were analyzed in triplicate. The amount of cross-reactivity was assessed by comparison with the concentration yielding a 50% inhibition of binding. Sensitivity levels were between 0.3 and 3000 pg/ml (IL-6); intra-assay and inter-assay precision variability was < 3% (controls, data not shown). Enzyme-linked immunosorbent assay antibodies against IL-6 were used to absorb the cytokines from cell culture supernatants or sera.

CRP assay

CRP plasma level analysis was performed immuno-turbidimetrically using a commercially available kit (Tina-quant CRP; Boehringer Mannheim, Germany) according to the manufacturer's instruction.

Statistical analysis

Data are expressed as means ± standard deviations. Differences of variables were assessed by analysis of variance for repeated measurements. Data were evaluated using the STATISTICA software package (version 6.0; StatSoft Inc., Tulsa, OK, USA).
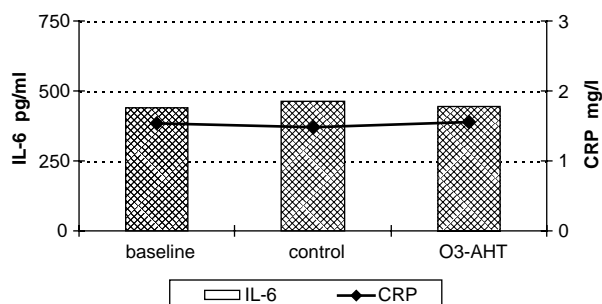

FIG. 1. CRP and IL-6 plasma concentrations after control autohemotherapy and after ozonated autohemotherapy (O3-AHT).

Results

All of 12 patients who entered the study completed the protocol. During the study no patient demonstrated symptoms of acute infectious disease. The CRP serum level, analyzed as a marker of inflammation, was in the normal range throughout the study in all patients (0–9 mg/l). There were no statistical differences between CRP levels at baseline (1.53±1.01 mg/l), after nine sessions of control AHT (1.48±0.96 mg/l), and after nine sessions of O3-AHT (1.55±0.84 mg/l). There was also no statistical difference between IL-6 serum concentrations at baseline (438 +118 pg/ml), after nine sessions of control AHT (444±120 pg/ml), and after nine sessions of O3-AHT (466±152 pg/ml).

Discussion

The investigation of systemic inflammation has been intensive in recent years, as it has been clearly shown that even low-level inflammation may be responsible for increased risk for cardiovascular disease in individuals with as well as without renal failure.[13] Markers of inflammation such as CRP and IL-6 powerfully predict death from all causes and from cardiovascular disease.[14] The underlying mechanisms of such deleterious consequences of inflammation involve vascular injury and malnutrition. It has been clearly demonstrated that inflammation, by suppressing synthesis or increasing catabolic rates of albumin, transferrin, and other negative acute-phase proteins, either combines with modest malnutrition or mimics malnutrition. More importantly, inflammation alters protein composition and endothelial structure and function so as to promote vascular disease.[8,9]

The generation of oxygen free radicals is known to be involved in the development of the systemic inflammatory response. In addition to their actions as noxious mediators generated by inflammatory cells, these molecules also play a crucial role contributing to the onset and progression of inflammation in distant organs. In the early stages of the inflammation process, free radicals exert their actions via activation of nuclear factors, such as nuclear factor-kappa-β or activator protein-1, that induce the synthesis of cytokines.[15] The fact that ozone dissolving in blood generates the production of free radicals such as hydrogen peroxide[3] forms the background for the hypothesis that O3-AHT may also promote the inflammation.

The previous *in vitro* studies demonstrated that ozone induced secretion of proinflammatory cytokine IL-6 from human macrophages and lymphocytes.[16,17] Otherwise, there is no clinical studies on this point with respect to therapeutic use of ozone. To examine the effects of O3-AHT on *in vivo* inflammatory response, the CRP and IL-6 plasma concentrations were measured before and after nine sessions of O3-AHT in the study, using autohemotherapy along with oxygen exposure as a control. CRP, a short-lived acute-phase protein, was used as a non-specific but very sensitive marker of inflammation. We also analyzed plasma level of IL-6, a pleiotropic proinflammary cytokine that induces the synthesis of CRP in hepatocytes.[14] The study clearly demonstrated that neither the CRP nor IL-6 levels changed after O3-AHT. The plasma concentration of CRP and IL-6 measured after nine sessions of O3-AHT did not differ from the CRP and IL-6 levels assessed after control AHT (Fig. 1). To our best knowledge, this is the first clinical study concerning the influence of O3-AHT on markers of inflammation. Given our results, one may conclude that O3-AHT in a dose of 50 μg of ozone/ml of ozone–oxygen mixture did not induce an inflammatory response. One may hypothesize that it might be a consequence of the relatively low dose of ozone, which has previously been shown not to cause oxidative injuries in HD patients.[11] The possibility cannot be excluded that ozone-induced inflammation is attenuated with multiday exposure.[18] The results of this study seem to further confirm the safety of O3-AHT treatment in patients chronically treated with HD.

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