

in the course of treatment. However, the result obtained in this study indicates the need for further detailed investigation of the behavior of ET in dialysate in the future.

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## Ozonated Autohemotherapy in Patients on Maintenance Hemodialysis: Influence on Lipid Profile and Endothelium

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**Abstract:** Ozonated autohemotherapy (O3-AHT) is used in the treatment of atherosclerotic ischemia of lower limbs (AILL). The impact of ozone on serum lipids and endothelium injury is of particular interest since these factors are important in the development of atherosclerotic lesions. To evaluate this issue, a prospective, placebo-controlled study was designed. Twelve hemodialyzed subjects with AILL received autohemotherapy with oxygen as a control followed by O3-AHT with ozone concentration of 50  $\mu\text{g/ml}$ . Serum lipids and plasma activity of von Willebrand factor (vWF) were measured. After O3-AHT, total cholesterol significantly decreased compared to the baseline (-8.34%) [ $P < 0.01$ ]. LDL cholesterol was also significantly lower than the initial value (-17.71%) [ $P < 0.001$ ]. No significant changes in the activity of vWF were found after the first session of O3-AHT and after all nine sessions of O3-AHT. The study demonstrated that O3-AHT did not affect deleteriously the endothelium in patients with chronic renal failure on maintenance hemodialysis. It may stimulate beneficial changes in serum lipid profile manifesting as a decrease in the total- and LDL-cholesterol levels. **Key words:** Ozone—Autohemotherapy—Lipids—Endothelium—Hemodialysis—Renal failure.

Ozonated autohemotherapy (O3-AHT) has been used in the treatment of atherosclerotic ischemia of lower limbs (AILL) for many years (1). In our previous report, the beneficial impact of O3-AHT on the clinical signs of AILL was shown in dialyzed subjects (2). Quite recently, comparatively favorable effects with regard to prolongation of intermittent claudication distance were observed in our placebo-controlled study (3). Exposure to ozone has also been suggested as inducing beneficial alterations in serum lipid profile (4,5). Furthermore, ozone given intra-arterially has been shown to inhibit the progres-

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sion of atherosclerotic lesions in a rabbit model of atherosclerosis (6). This issue is, however, controversial since the deleterious effects of ozone exposure are also known. Ozone may induce oxidative stress (7), which in turn can cause endothelial injury (8), the first and principal step in the development of atherosclerosis. Oxidized forms of lipids are considered another important risk factor of this process. Their generation after O3-AHT was noted in some studies (9) but not in all (5). In view of these conflicting results, it is difficult to conclude whether ozonotherapy promotes atherosclerosis or rather attenuates its development. This problem is of particular interest with regard to patients with end-stage renal disease (ESRD) on maintenance dialysis who manifest complications of atherosclerosis very often. To gain a better insight into this issue, a prospective oxygen controlled study was performed to evaluate the influence of O3-AHT on the endothelium and serum lipid profile.

## MATERIALS AND METHODS

### Subjects

Twelve chronic hemodialysis (HD) subjects (eight male, four female), aged  $64.8 \pm 7.6$  (range 50–75) years manifesting symptomatic AILL were recruited. They underwent regular bicarbonate HD treatment, three times per week for more than one year (average  $4.5 \pm 3.1$  years). The HD prescription, namely, dialyzer type, HD session length, rate of dialysis solution and blood flows remained unchanged during the study. No pharmacological treatment was either changed or newly administered.

### Study design

Subjects received 9 sessions of autohemotherapy connected with the blood exposure to medical oxygen (AHT) as a control, followed by 9 sessions of autohemotherapy along with exposure to an oxygen-ozone mixture with ozone concentration of  $50 \mu\text{g/ml}$  (O3-AHT). The details of O3-AHT were described

previously (2). The sessions were performed three times a week just before the hemodialysis session in a single blind manner. The plasma activity of von Willebrand factor (vWF) and the serum lipid levels were measured. Blood samples were collected before the first AHT, after the ninth AHT and after the ninth O3-AHT. To evaluate the effects of the first exposure to ozone blood was also withdrawn before and 20 min after the first O3-AHT.

### Biochemical analysis

Serum levels of total cholesterol (TCH), HDL cholesterol (HDL-CH), and triglycerides (TG) were measured calorimetrically using standard methods. LDL cholesterol (LDL-CH) was calculated according to Friedewald's formula (none of the patients had triglyceride levels higher than 400 mg/dl). vWF plasma activity was analyzed using an ELISA kit (vWF200, Axis-Shield Diagnostic, Dundee, UK) according to the kit-provider's recommendations.

### Statistics

Data are expressed as mean  $\pm$  SD. Differences of variables measured more than twice were assessed by the analysis of variance for repeated measurements or Friedman's test; otherwise by Student's *t*-test for paired comparison or the Wilcoxon test. Data were evaluated using STATISTICA (version 5.1, StatSoft Inc.).

## RESULTS

The effects of O3-AHT on serum lipid profile are shown in Table 1. After O3-AHT, the TCH level was significantly lower compared both to baseline ( $-8.34\%$ ) [ $P < 0.01$ ], and to the AHT (control) [ $P < 0.01$ ]. LDL-CH level was also significantly lower than the initial value ( $-17.71\%$ ) [ $P < 0.001$ ], and lower than the level after AHT (control) [ $P < 0.001$ ]. Table 1 also presents the effects of O3-AHT and AHT on the plasma activity of vWF. No significant changes were found after the first and the ninth O3-AHT.

TABLE 1. Lipid profile and activity of vWF at the baseline, after control AHT and after O3-AHT

	Baseline	After AHT(control)	After 1st O3-AHT	After 9th O3-AHT
TCH (mg/dl)	$211.0 \pm 46.1$	$215.0 \pm 49.5$		$193.4 \pm 53.4^a$
LDL-CH mg/dl)	$130.3 \pm 42.6$	$129.0 \pm 46.7$		$107.2 \pm 44.4^b$
HDL-CH (mg/dl)	$44.6 \pm 9.1$	$45.3 \pm 9.3$		$45.9 \pm 8.7$ NS
TG (mg/dl)	$171.5 \pm 96.2$	$167.4 \pm 92.8$		$164.4 \pm 92.8$ NS
vWF (%)	$72.6 \pm 55.7$	$61.3 \pm 53.8$	$78.7 \pm 50.4$	$83.4 \pm 65.9$ NS

<sup>a</sup> Significant difference ( $P < 0.01$ ): 9th O3-AHT vs. baseline, and 9th O3-AHT vs. AHT.

<sup>b</sup> Significant difference ( $P < 0.001$ ): 9th O3-AHT vs. baseline, and 9th O3-AHT vs. AHT.

TCH, total cholesterol; LDL-CH, low-density lipoprotein cholesterol; HDL-CH, high-density lipoprotein cholesterol; TG, triglycerides; vWF, von Willebrand factor; NS, nonsignificant difference.

## DISCUSSION

Abnormalities in lipid metabolism are common in patients with ESRD. In HD subjects further acceleration of lipid abnormalities is observed (10). It is suggested that ozonotherapy ameliorates these alterations. We demonstrated that O<sub>3</sub>-AHT caused a significant decrease in LDL-CH and TCH levels. The fall of LDL-CH was particularly evident. Our results are in close agreement with findings on subjects with normal renal function treated with O<sub>3</sub>-AHT (5). Ozone given intra-arterially has also been found to induce comparable results (4). None of the above-mentioned studies revealed any changes in TG and HDL-CH levels. Parallel conclusions were drawn from another study, where rabbits with dietary induced atherosclerosis displayed a decrease in TCH and LDL-CH after intravenous administration of ozone (11). The method of LDL-CH level evaluation used in the present study (Friedewald equation) limits the significance of our findings on this point. Direct measurement would be a better analytical tool in this regard.

The mechanism by which ozone causes a decrease in blood cholesterol is not clear. The theory involving oxysterols (OSTs) as a critical regulatory factor responsible for such ozone activity was proposed originally by Hernandez (5). OSTs, oxygenated derivatives of cholesterol, were shown to induce a suppressive effect on the synthesis of lipids both at transcriptional and posttranscriptional levels (12). It was suggested that ozone, when in contact with the blood during O<sub>3</sub>-AHT, reacts with polyunsaturated fatty acids producing organic radicals and hydrogen peroxide. These products induce oxidation of cholesterol when the blood is reinfused to a patient after ozonation. The oxidation of cholesterol may lead to its excretion with bile or further oxidation to water-soluble bile acids. It is likely that OSTs created in these processes are able to decrease the rate of cholesterol synthesis (5).

The possibility that ozone mediates the creation of oxidized LDL, resulting in increased uptake by macrophages and a subsequent decrease in LDL-CH level in plasma, can not be excluded. This could be, of course, harmful. Oxidized LDL was not measured in the present study. Conflicting data on this point exist in the literature (5,9). To clarify this issue in vitro studies and animal experiments are necessary.

HD patients are exposed to endothelial stimulation or even injury (13). Since ozone can induce the generation of free radicals, ozonotherapy may be

supposed to be additionally harmful for these patients as far as endothelial injury is concerned. To verify this hypothesis, the influence of O<sub>3</sub>-AHT on the plasma activity of vWF was examined. vWF is believed to serve as an index of endothelial injury, or more likely, of altered endothelial function (14). There was no change in vWF activity after the first and the ninth session of O<sub>3</sub>-AHT when compared to the baseline level. These results are in line with findings on subjects with normal renal function treated with an ozonated isotonic solution of NaCl given intravenously (15). In view of these reports, the deleterious effect of ozone in therapeutic dosages on endothelium appears unlikely. Since a clinical study to look at endothelial function/scarring has a limited scientific value, in vitro experiments should also be performed to confirm these findings. Considering that theoretically possible endothelial injury may reflect oxidative cell modification, the precise control of ozone dosage seems to play a crucial role. Oxidative properties of ozone have been shown to be dose-dependent and the therapeutic concentrations window was found to range between 20 and 80 µg/ml (1). On the basis of the results of another study performed in our center, it seems likely that O<sub>3</sub>-AHT with ozone dosage of 50 µg/ml does not cause oxidative injuries in HD patients (16). The antioxidant defense system is able to neutralize the oxidative properties of ozone and protect against oxidative cell damage. This study confirmed this thesis with respect to endothelial cells.

As was mentioned above, ozone has been shown to ameliorate atherosclerosis in an animal study (6). Unfortunately, there are no human studies evaluating this controversial issue. Only the impact of OSTs on the development of atherosclerosis was evaluated. Of 13 studies, six indicated the proatherogenic effect of OSTs, four indicated an antiatherogenic effect, whereas three showed no clear-cut activity (17). With the current knowledge it is not possible to draw firm conclusions as to whether ozonotherapy enhances atherosclerosis or rather protects against this process. Summarizing, the study demonstrated that O<sub>3</sub>-AHT with ozone concentration of 50 µg/ml, applied three times a week, did not affect deleteriously the endothelium in HD patients. It may stimulate potentially beneficial changes in serum lipid profile manifesting in a decrease of TCH and LDL-CH levels.

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