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Ozone therapy and the activity of selected lysosomal enzymes in blood serum of patients with lower limb ischaemia associated with obliterative atheromatosis

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Background: The paper compares the effects of ozone therapy and conventional balneological methods on health condition of patients with obliterative atheromatosis and on serum activity of three lysosomal enzymes.

Material/Methods: Sixty-four patients with lower limb ischaemia in the course of obliterative atheromatosis (without diabetes) were enrolled in the study. Thirty-two patients were treated with ozone administered by intravenous infusions and 30-minute aerosol oxygen-ozone baths. A comparative group was formed of 32 patients treated with traditional balneology. There was also a control group made up of 30 healthy subjects. Ozone therapy as well as traditional balneology were administered daily for the period of 10 days, excluding Saturdays and Sundays. Blood for biochemical analysis was collected from elbow vein in the following time intervals: 24 hours before ozone therapy or classical balneology, one hour after therapy and on the 10th day of treatment. The activity of cathepsin D, acid phosphatase and arylsulphatase as well as the levels of α-1-antitrypsin (protease inhibitor) were determined in blood serum of patients with obliterative atheromatosis.

Results: In patients who received ozone therapy the activity of analysed lysosomal hydrolases returned to the values typical for healthy subjects. Patients' general condition also improved. The use of traditional balneological methods did not result in any significant change either in the activity of lysosomal hydrolases, the level of α-1-antitrypsin or general condition of patients.

Conclusions: Ozone therapy administered by intravenous infusions and aerosol oxygen-ozone baths of lower extremities yields much better therapeutic results in comparison with classical balneology.

key words: **obliterative atheromatosis • ozone therapy • arylsulphatase • acid phosphatase • cathepsin D • α-1-antitrypsin**

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BACKGROUND

Lower limb ischaemia is associated e.g. with obliterative atheromatosis, which just like other illnesses related to unhealthy life style belong to 'civilisation diseases'. Apart from main etiological factors in obliterative atheromatosis, risk factors include but are not limited to smoking, unhealthy diet, hyperlipidaemia, overweight, sitting position, arterial hypertension and prolonged stress.

Coexistence of several risk factors often contributes to early development of first symptoms of the disease. Pathologic changes are not limited to lower extremities; the atheromatous process takes place in all blood vessels [1,2].

The basic pathomorphological change observed in obliterative atheromatosis is so-called atheromatous plaque, which contributes to the formation of aggregated deposits of lipids, cholesterol and dead phagocytes. They are deposited in the intima of large and medium-size arteries. Atheromatous plaques stiffen vessel wall, making vascular lumen narrow down. These constrictions hinder blood flow and are conductive to the formation of thrombi [3]. When a thrombus grows, vascular lumen is narrowed or it may be even completely occluded [4,5]. Consequently, blood stream does not reach all the tissues [6,7] which results in the development of necrotic changes [8] and inflammation.

These processes alter physical and chemical conditions in a cell. Biological membranes, including lysosomal membranes, lose their continuity, which results in the release of acid hydrolases from lysosomes to cytoplasm. Released hydrolases initiate autolysis of cells, increasing the necrosis in hypoxaemic tissues [9,10].

Lysosomal enzymes play an important role in the process of intracellular digestion. Lysosomes phagocytize and digest dead cell components and bacteria. Hyperactivity of lysosomal hydrolases may be one of the symptoms of tissue necrosis resulting from insufficient oxygen supply [11,12]. The damage of lysosomal membranes and the release of hydrolases to cytoplasm are also related to increased activity of enzymes in blood [13].

The elimination of risk factors for obliterative atheromatosis ensures the improvement of general health condition only in patients with minor ischaemic changes. Conservative treatment does not always restore normal haemodynamic conditions [14,16]. High rate of failure in the treatment of obliterative atheromatosis of lower limbs makes investigators search for alternative methods of therapy. One of them, which has been known for a long time, but remained unpopular chiefly due to technical considerations is ozone therapy [17,19].

The administration of oxygen-ozone mixture by intravenous infusion prevents the formation of further ischaemic changes in tissues, as oxygen availability in necrotic areas improves. Ozone makes cell membranes more permeable. Blood level of 2,3-DPG also increases which facilitates the penetration of oxygen from blood into tissues [20–22].

The present work compares the effect of ozone therapy and classical balneological methods on health condition of patients with obliterative atheromatosis and on serum activity of three lysosomal enzymes.

The assessment of the activity of lysosomal hydrolases in certain disease entities makes treatment more effective and may be also helpful in the diagnosis and monitoring of a given disease [23].

MATERIAL AND METHODS

The study group

Thirty-two patients (8 women and 24 men) with lower limb ischaemia in the course of obliterative atheromatosis of lower extremities received ozone therapy. Patients' mean age was 65.8±6.9 years. The patients have had the symptoms of the disease for the average of 4.5±3.0 years. Sixteen patients from the group on ozone therapy suffered from ischaemic heart disease, 10 – from arterial hypertension and there were 2 subjects with the history of stroke.

Along with ozone therapy the patients also received the drugs prescribed to them by their doctors (pentoxifylline – 24, acetylsalicylic acid – 10, anticoagulants – 2).

Thirty-two patients (8 women and 24 men) with lower limb ischaemia in the course of obliterative atheromatosis of lower extremities received traditional balneological treatment. Patients' mean age was 62±8 years. All the subjects from this group suffered from ischaemic heart disease and two of them also had arterial hypertension. Along with balneological therapy the patients also took medicines prescribed to them by their doctors (pentoxifylline and acetylsalicylic acid).

Control group consisted of 30 healthy subjects (15 women and 15 men) aged approximately 60 years.

Treatment

Ozone therapy as well as classical balneological treatment was administered daily for the period of ten days, excluding Saturdays and Sundays.

Ozone therapy was given as:

– intravenous infusions, and
– 30-minute aerosol oxygen-ozone baths.

500 mL of normal saline satiated wit ozone (60 μg O_3/mL) was administered by intravenous infusion within 1.5 hours. After the administration of half the volume of physiological saline it was replaced as the concentration of ozone drops down to 50% after approximately 40 minutes.

Normal saline was satiated with ozone for 10 minutes, using ATO_3 ozone generator.

During aerosol ozone baths of lower legs the patients assumed sitting position. Ozone concentration in a chamber was 19 μg/L.

Patients from comparative group received whirlpool massage therapy and carbonic acid baths. The study was approved by Bioethics Committee at Medical University in Bydgoszcz.

Collection of blood samples for biochemical analysis

Blood for biochemical analysis was withdrawn on empty stomach in the following time intervals: 24 hours before starting ozone therapy (1st collection), one hour after the end of ozone therapy (2nd collection) and on 10th day of ozone therapy (3rd collection). Blood from patients receiving classical balneological treatment (comparative group) was collected in the same time intervals. Patients from control group gave their blood samples only once.

The following were determined in blood serum: the activity of cathepsin D (E.C.3.4.4.23) with the use of Anson's method [24], the concentration of α-1-antitrypsin (protease inhibitor) [25], the activity of acid phosphatase (E.C.3.1.3.2.) according to Bessey [26] and the activity of arylsulphatase (E.C.3.1.6.1.) according to Roy's method modified by Błeszyński [27]. Plasma levels of total cholesterol, LDL and HDL cholesterol and triglycerides were also assessed.

Statistical analysis

The results obtained were analysed statistically with t-Student test for independent variables and Scheffe test for dependent variables.

RESULTS

There were no significant differences in the activity of lysosomal enzymes between male and female patients and hence the results obtained were averaged.

The use of ozone given by intravenous infusions and aerosol baths of lower legs increased the activity of lysosomal hydrolases to the values found in healthy subjects (Table 1) and improved patients' general condition (Table 2). Ozone therapy resulted in greater activity of arylsulphatase and cathepsin D and in the decrease of the activity of acid phosphatase and serum levels of α-1-antitrypsin to the values observed in healthy subjects. There was a negative correlation between cathepsin D activity and the concentration of α-1-antitrypsin, one of protease inhibitors ($r=-0.65$; $p<0.001$)

The use of whirlpool massages and carbonic acid baths applied to lower legs of patients from the comparative group did not bring about any significant changes in the

Table 1. Activity of selected lysosome enzymes and serum levels of α-1-antitrypsin in patients (men and women) with lower limb ischaemia associated with obliterative atheromatosis. Values classified with reference to therapy applied.

Enzyme	Control group	Therapy applied					
		Ozone therapy			Conventional balneology		
		Analysis					
		I	II	III	I	II	III
Arylsulphatase (10^{-3}nM 4NC/mg protein)	3.20±0.53	1.71±0.40*+	1.88±0.42*	2.71±0.68*	1.64±0.34*	1.73±0.30*	1.98±0.42*
Acid phosphatase (10^{-3}nM p-nitrophenol/mg protein)	8.60±1.40	14.11±2.56*+	12.21±1.9*	9.97±1.48*	14.08±2.26*	14.09±2.70*	14.14±2.16*
Cathepsin D (10^{-2}nM tyrosine/mg protein)	7.25±1.60	1.75±0.38*+	3.41±0.69*	5.39±1.19**	1.89±0.54*	2.75±0.61*	2.59±0.65*
α-1-antitrypsin (mg tyrosine)	1.07±0.12	1.62±0.22*+	1.39±0.22*	1.19±0.15	1.63±1.13*	1.64±0.14*	1.63±0.16*

Statistically significant differences – relative to control group: *$p<0.0001$; ** $p<0.001$; between analysis I and II+$p<0.05$

Table 2. Effect of ozone therapy and conventional balneology on general condition of patients with lower limb ischaemia associated with obliterative atheromatosis.

Index	Ozone therapy	Conventional balneology
BMI	Reduced by 2.4%	Reduced by 3.2%
Ankle arm index (right extremity)	Improved by 13.8%	Improved by 2.8%
Ankle arm index (left extremity)	Improved by 14.9%	Improved by 5.7%
Intermittent claudication distance in the corridor	Improved by 34.2%	Improved by 22.0%
Intermittent claudication distance on a track	Improved by 50.6%	Improved by 22.0%
Total cholesterol	Reduced by 5.0%	Reduced by 7.0%
HDL	No change	Elevated by 4.8%
LDL	Reduced by 7.2%	Reduced by 8.3%
Triglycerides	Reduced by 11.1%	Reduced by 20.0%

activity of analysed lysosomal hydrolases or in the concentration of α-1-antitrypsin. As opposed to ozone therapy, conventional balneological treatment did not result in a satisfactory enhancement in patients' general condition. After ozone therapy, the distance of intermittent claudication in the corridor and on a track improved by the average of 40%, while an ankle arm index got better by 14%. On the other hand, no statistically significant change was observed in the levels of total cholesterol, LDL and HDL cholesterol and Body Mass Index (BMI).

DISCUSSION

Despite the fact that some authors reported harmful effects of ozone on tissues in animal studies [28,29], ozone therapy plays an important role in the treatment of lower limb hypoxia associated with obliterative atheromatosis. Animal studies showed that excessive amounts of ozone may be harmful e.g. by the formation of many peroxides and hydroxylic radicals.

Ozone supplied to human body reacts unlike that in laboratory conditions [30]. Numerous papers, however, report improved oxygenation of blood and tissues, reduced blood viscosity and coagulability, and lower aggregation of blood platelets [31]. Oxygen-ozone mixture administered by intravenous infusions to patients with obliterative atheromatosis does not damage endothelium [8].

Being an allotropic variety of oxygen, classified as a reactive form of oxygen, ozone damages cell structures. Its reactivity is reflected in strong oxidation, bactericidal, virucidal, fungicidal and protozoicidal capacities. It is extremely efficient in topical treatment of infections caused by anaerobes. Topical application of ozone spray helps to clear wounds [32].

Ozone therapy is most often administered by the satiation of venous blood with ozone using autohaemotransfusion [33], the administration of oxygen-ozone mixture in physiologic saline to arteries or veins [34].

It is believed that the products of ozone reactions with organic compounds in the cells have therapeutic properties [35]. Hydroxyperoxides formed during osmolysis have different properties than lipid peroxidation products synthesized in the course of radical reactions [19,36]. These products are hydrophilic and are capable of removing harmful peroxides and free oxygen radicals. They also activate enzyme systems of cell clearance [37,38].

Therapeutic effects of ozone therapy in patients with lower limb ischaemia associated with obliterative atheromatosis are probably the result of specific ozone properties. This claim seems to be supported both by subjective and objective improvement in patients' general condition and the changes in the activity of lysosome hydrolases.

After ten intravenous infusions of ozone and aerosol baths of lower limbs, the distance of intermittent claudication on a track improved by about 50%, while the distance of intermittent claudication in the corridor improved by 34% in comparison to the status before admission to hospital. Classical balneological treatment resulted only in 22% improvement.

The enhancement of patients' clinical status and objective improvement of ankle arm index as well as longer distance of intermittent claudication after ozone therapy administered to patients with lower limb ischaemia are consistent with the findings reported by Sroczyński et al. [39].

The change in the activity of lysosomal enzymes towards normal values and the improvement of general condition of patients with lower limb ischaemia have also been discussed by other authors [40], while Włodarczyk [41] reported a change in the activity of antioxidation enzymes.

It is presumed that ozone administered by intravenous infusion or externally in various forms protects tissues not only against hypoxia, but also against revascularisation once the oxygenation conditions are changed [42]. The improvement of blood supply usually means that there is a sudden inflow of large amounts of oxygen that ischaemic tissues are not prepared for [43].

Better blood supply ensures access to the wound for macrophages, neutrophils and lymphocytes. These cells phagocytize damaged or dead structures and microorganisms. Phagocytizing cells are particularly rich in lysosomes. In the state of hypoxia macrophages phagocytize damaged tissues and help to eliminate local inflammations [44,45]. Changes in the activity of lysosome enzymes are also observed in the course of other diseases [11,12].

Low cathepsin D activity in blood serum of patients with lower limb ischaemia may be the consequence of inhibiting enzyme activity by inhibitors. All the patients with obliterative atheromatosis demonstrate high levels of α-1-antitrypsin, one of protease inhibitors, compared with the values observed in healthy subjects ($p<0.0001$). Greater secretion of this antitrypsin to plasma probably allows to maintain homeostasis between high cathepsin D activity and autolysis of hypoxic tissues.

Sloane et al. [46] found high activity of cathepsin B and a simultaneous low concentration of endogenous protease inhibitors in blood serum of patients with neoplastic disease. It is believed that protease inhibitors play much more important role than proteases themselves in the pathology of certain diseases [47].

Higher activity of acid phosphatase in patients with lower limb ischaemia associated with diabetic macroangiopathy has been reported by e.g. Marques et al. [48] and Drewa et al. [40]. Their observations are consistent with our findings. Patients with lower limb ischaemia in the course of obliterative atheromatosis also show high activity of acid phosphatase. Conventional balneological treatment does not change acid phosphatase activity. Ozone limits the increase in the activity of acid phosphatase probably

through the stabilisation of lysosome membranes and delimitation of the phagocytosis of monocytes and macrophages, also preventing cell autolysis and tissue damage [19]. Lower activity of arylsulphatase among patients with the hypoxia of tissues in lower limbs may follow from excessive amount of their inhibitors such as sulphates, sulphites and phosphates [49].

CONCLUSIONS

Ozone properties discussed above alter the activity of three lysosomal enzymes analysed in this work to the activity found in the plasma of healthy subjects and they also improve patients' clinical status. Ozone therapy administered by intravenous infusions and in the form of aerosol oxygen-ozone baths of lower extremities yields much better therapeutic results in comparison with classical balneology (whirlpool massage and carbonic acid baths).

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