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OZONATION OF HUMAN BLOOD INCREASES SPHINGOSINE-1-PHOSPHATE IN PLASMA

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Ozonated blood therapy is used in the treatment of several diseases, including superficial infections, burns, dental and intestinal conditions. Except that, the possibility of using ozone to sterilize blood supplies is under promising investigation. However, still little is known regarding the impact of blood ozonation, especially on biologically active serum sphingolipids. In the present work we sought to investigate the contents of sphingolipids, such as sphingosine, sphingosine-1-phosphate (S-1-P), sphinganine, and ceramide (CER) in the plasma, after immediate and prolonged (1 h) ozonation of human whole blood. For the measurements liquid chromatography hyphenated with the mass spectrometry was applied. We demonstrated that only the content of sphingosine-1-phosphate in the plasma was increased significantly, possibly exerting its beneficial effect for various physiological and clinical events.

Key words: *blood ozonation, sphingosine-1-phosphate, ceramide, sphingosine, sphinganine, liquid chromatography, mass spectrometry*

INTRODUCTION

Ozone therapy is still a matter of unfinished debate since many studies have shown rather vague and unsuitable effects of blood ozonation (1). However, other data present its great usefulness especially in the management of pain or infections, due to its immune-stimulating, antimicrobial and analgesic effects (2-5). Some other beneficial properties of ozonated blood autotransfusions were recently related to the possibility of ozone-dependent lowering plasma cholesterol as well as low density lipoproteins (LDL) levels (6). Recently, the clinical aspects of blood ozonation have been extensively reviewed, especially in regard to the biological and therapeutic effects as well as safety of ozone treatments (2).

Based on the contraries presented above, it seems that, still very little is known regarding the ozone action(s) on blood components. Ozone performance arises from generation of highly reactive oxygen species and subsequent activation of antioxidant enzymes (7). These oxidative properties of ozone show dose- and time- dependence until reestablishment of disrupted homeostasis, although higher dose (over 80 µg/ml) is usually accompanied by a number of side-effects (8).

Whether the consequences of blood ozonation include changes in sphingolipids behavior is currently not known. Sphingolipids are widespread constituents of human blood, present in blood cells as well as in plasma components. In health, they are important components of all cell membranes including erythrocytes, trombocytes and endothelial cells, providing not only structural support, but also serving as important intracellular signaling molecules (9-11). Much less is known regarding its extracellular role. Physiologically, in plasma, concentration of

ceramide (CER) is relatively higher comparing to sphingosine-1-phosphate (S-1-P) and therefore even a small change in CER can substantially change S-1-P or sphingosine (SPH) levels. Furthermore, the content of plasma sphingoid base compounds is regulated by binding capacity of lipoproteins (esp. HDL) and albumins (12). In healthy humans 70% of total plasma S-1-P is bound in the HDL fraction, although recently, S-1-P was shown as an integrative component of microvesicles or exosomes, that circulate in blood stream. It was also shown, that S-1-P present in these microvesicles might be responsible for several biological effects such as stimulation of angiogenesis (13). Based on this finding several data strongly implicate S-1-P involvement in development of cancer (14). In contrary, serum S-1-P has been also shown to have protective action(s) on cardiovascular system (15) as well as anti-inflammatory properties (16).

Nonetheless, sphingolipids are a complex and diverse group of lipids that recently has gained much attention as signaling molecules involved in many biological processes. Considering the involvement of extracellular ozone on different signaling pathways, we examined the influence of ozonation on sphingolipid content in human plasma.

MATERIAL AND METHODS

Subjects

The present study conforms with the principles outlined in the Declaration of Helsinki and was approved by the Ethical Committee for Human Studies of the Medical University of Bialystok and prior to the study all participants gave their

informed consent in the study. The selected group of 35 healthy volunteers was highly homogenic, since only men were recruited to the study, age ranged 25–35, with no indications of drug(s)/supplementation(s) usage, with similar basic blood morphology (RBC, WBC, PLC, hemoglobin levels as well as MHC meeting the criteria required for healthy donors). There was no history of internal disease (hypertension, diabetes, or other endocrine disease). The selected group of donors is advantageous as highly homogenic group of healthy subjects allowed us to exclude age, gender, hormonal and other *in vivo* factors and fully examine the influence of ozonation as well as aeration on the content of sphingolipids.

Blood collection

Twenty five ml of whole blood were taken from healthy subjects and anticoagulated with citrate phosphate dextrose (CPD) solution (0.12% final concentration). Blood was mixed with anticoagulant in blood bag on Laboratory Shaker 358S (ELPAN, Poland).

Ozone/oxygen mixture preparation

The oxygen-ozone gas mixture was freshly prepared from medical-grade oxygen using ATO-3 MINI ozone generator (CryoFlex, Poland). The concentration of ozone in the gas mixture generated for this study was 15–30 µg/ml. The mixture was kept at normal atmospheric pressure.

Blood ozonation

The ozonation procedure used in our study is highly comparable to standard ozonation protocols. Briefly, ozonation/aeration was performed immediately after donation. From each bag 5 ml of blood was collected and centrifuged at $2000 \times g$ for 10 min to obtain non-treated samples. The remaining 20 ml of blood were treated with a single dose of 10 or 20 ml of oxygene-ozone gas mixture or the same volume of air. Final concentrations of gaseous ozone added to blood were 15 or 30 µg/ml, respectively. Gas mixtures were transferred into the blood using syringes made of ozone resistant material containing antibacterial filter. Infusion pump operated syringes in order to maintain a constant flow of 1 ml/min. During ozonation/aeration blood bags were placed on laboratory shaker to ensure mixing of gas with blood. Immediately and 1 hour after the treatment blood samples were taken from the blood bag and centrifuged at $2000 \times g$ for 10 min to obtain plasma samples. All samples of plasma were stored in aliquots at -80°C until the day of analysis.

Analysis of plasma sphingolipids: sphinganine, ceramide, sphingosine and sphingosine-1-phosphate by UHPLC/MS/MS

The content of sphingolipids was measured using a UHPLC/MS/MS approach according to (17). Briefly, each plasma sample was spiked with internal standard mixture containing: 2 ng of 17C-sphingosine, 2 ng of 17C-S1P, and 20 ng of 17C16:0-Cer (Avanti Polar Lipids) and immediately afterwards sphingolipids were extracted by the use of the extraction mixture composed of isopropanol: ethyl acetate (15: 85; v:v). The mixture was vortexed, sonicated and then centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a new tube and pellet was re-extracted. After centrifugation supernatants were combined and evaporated under nitrogen. The dried sample was reconstituted in 100 µl of LC Solvent A for LC/MS/MS analysis. The following sphingolipids were quantified: Sph, dhSph, S1P, C14:0-Cer, C16:0-Cer, C18:1-Cer, C-18:0-Cer, C20:0-Cer, C22:0-Cer,

C24:1-Cer, C24:0-Cer. Sphingolipids were analyzed by means of an Agilent 6460 triple quadrupole mass spectrometer using positive ion electrospray ionization (ESI) source with multiple reaction monitoring (MRM). The chromatographic separation was performed using an Agilent 1290 infinity ultra performance liquid chromatography (UHPLC). The analytical column was a reverse-phase Zorbax SB-C8 column 2.1×150 mm, 1.8 µm. Chromatographic separation was conducted in binary gradient using 2 mM ammonium formate, 0.15% formic acid in methanol Sigma-Aldrich (St. Louis, MO) as solvent A and 1.5 mM ammonium formate, 0.1% formic acid in water Sigma-Aldrich (St. Louis, MO) as solvent B at the flow rate of 0.4 ml/min.

Statistical analysis

Prior to statistical analysis, normal distribution and homogeneity assumptions were checked. Untransformed data are presented as means \pm S.E. Statistical significance was assessed using one-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of ozone/air treatment on the plasma sphingolipids

In the pilot study we used low (15 µg) and high (30 µg) dose of ozone, however due to visible hemolytic blood degradation, observed after higher dose of ozone, we choose to examine lower ozone concentration pair-matched with aeration of the samples (data not shown).

Sphinganine (SPA)

Exposure of the whole blood to ozone had no significant effect on SPA content (*Fig. 1A*), although a trend towards an increased levels of plasma sphinganine was observed either immediately and 1 hour after exposure (+35% and +37%, $P > 0.05$, respectively, *Fig. 1A*). Interestingly, aeration of the blood also resulted in increased content in plasma, reaching significance after prolonged exposure (+58%, $P < 0.05$, *Fig. 1B*).

Ceramide (CER)

Both treatments ozonation as well as aeration of the blood had no significant effect on ceramide levels in plasma (*Fig. 1C* and *1D*). Interestingly, a trend towards increased desaturation of FA moieties present in ceramide was noticed (*Table 1*).

Sphingosine (SPH)

Acute and prolonged treatment of the blood with O_3 had no significant effect on SPH content in plasma (+9%, and +6%, $P > 0.05$, *Fig. 2A*). However, prolonged aeration increased significantly sphingosine concentration in plasma (+38%, $P < 0.05$, *Fig. 2D*).

Sphingosine-1-phosphate (S-1-P)

Ozonation of the blood elucidated the most prominent changes in plasma S-1-P levels. Immediately after ozone exposure a trend towards increase was notice (+17%, $P > 0.05$, *Fig. 2C*), which reached significance level with prolonged exposition (+28%, $P < 0.05$, *Fig. 2C*). Aeration of the blood with the same volume of the air resulted in not significant plasma change in S-1-P levels (+5% and +13%, $P > 0.05$, *Fig. 2D*).

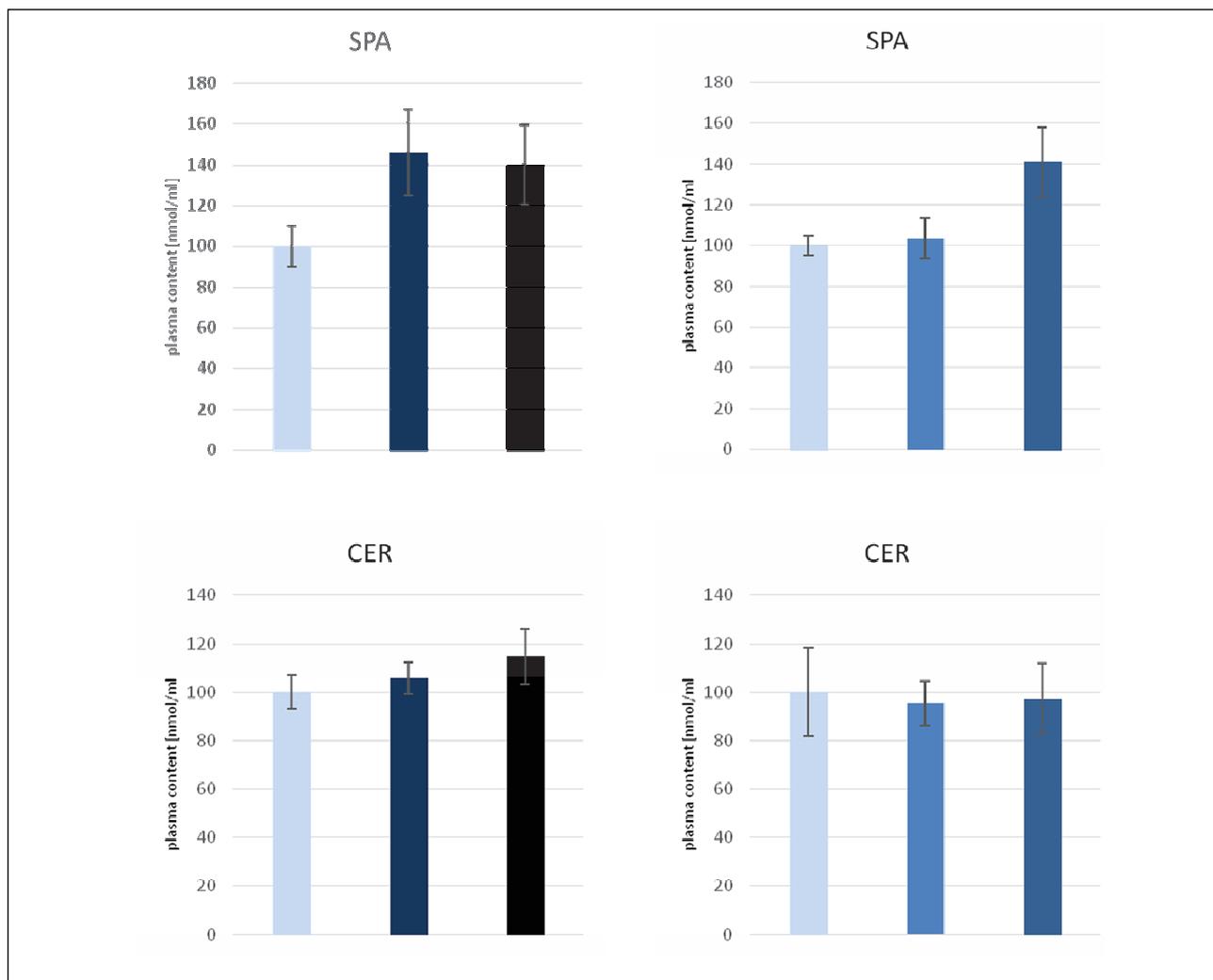


Fig. 1. The effects of ozone (A and C) and air (B and D) treatments (15 $\mu\text{g/ml}$ blood) on the contents of sphinganine (SPA) and Ceramide (CER) in human plasma. The asterisk represents statistically significant difference ($P < 0.05$) as compared with the Control; $n = 35$.

Ozone - acute ozonation of blood (15 $\mu\text{g/ml}$); Ozone 1 h - prolonged ozonation of blood (15 $\mu\text{g/ml}$); Air - acute aeration of blood (15 $\mu\text{g/ml}$); Air 1 h - prolonged aeration of blood (15 $\mu\text{g/ml}$).

Table 1. The effects of ozone on FA-Ceramide composition in human plasma ($n = 35$). Values were determined by UHPLC/MS/MS as described in Materials and Methods and expressed in pmol/ml (for each FA moieties). The asterisk represents statistically significant difference ($P < 0.05$) as compared with the Control; $n = 35$.

| | C14-Cer | C16-Cer | C18-1-Cer | C18-Cer | C20-Cer | C22-Cer | C24-1-Cer | C24-Cer |
|-------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--------------------|-------------------|
| Control | 0.032 \pm 0.001 | 1.338 \pm 0.010 | 0.008 \pm 0.001 | 0.254 \pm 0.02 | 0.036 \pm 0.001 | 0.295 \pm 0.023 | 0.593 \pm 0.016 | 2.105 \pm 0.014 |
| Ozone | 0.031 \pm 0.001 | 1.307 \pm 0.02 | 0.006 \pm 0.001 | 0.254 \pm 0.023 | 0.042 \pm 0.001 | 0.315 \pm 0.027 | 0.583 \pm 0.0011 | 2.472 \pm 0.019 |
| Ozone (1 h) | 0.033 \pm 0.001 | 1.399 \pm 0.012 | 0.006 \pm 0.001 | 0.266 \pm 0.012 | 0.042 \pm 0.001 | 0.331 \pm 0.021 | 0.607 \pm 0.001 | 2.573 \pm 0.021 |
| Air | 0.030 \pm 0.001 | 1.070 \pm 0.013 | 0.010 \pm 0.001 | 0.182 \pm 0.021 | 0.040 \pm 0.001 | 0.287 \pm 0.022 | 0.562 \pm 0.0012 | 1.739 \pm 0.011 |
| Air (1 h) | 0.029 \pm 0.001 | 1.062 \pm 0.012 | 0.010 \pm 0.001 | 0.181 \pm 0.015 | 0.039 \pm 0.001 | 0.289 \pm 0.19 | 0.572 \pm 0.001 | 1.727 \pm 0.013 |

Ozone - acute ozonation of blood (15 $\mu\text{g/ml}$); Ozone 1 h - prolonged ozonation of blood (15 $\mu\text{g/ml}$); Air - acute aeration of blood (15 $\mu\text{g/ml}$); Air 1 h - prolonged aeration of blood (15 $\mu\text{g/ml}$).

DISCUSSION

Ozonated therapy is still poorly recognized in many countries or even prohibited (USA), although private medical services are using this therapy worldwide (1-5). Therefore, the ozone effect(s) on human blood is of great interest. As a consequence, the effects

of ozonation of human blood were subjected to several investigations, that established targets for ozone actions such as: uric acid, ascorbic acid, glutathione, albumin-thiol groups as well as polyunsaturated fatty acids (2, 18). It was also shown that ozonated therapy is able to induce several beneficial effects in serum lipid profile, including lowering total cholesterol as well as

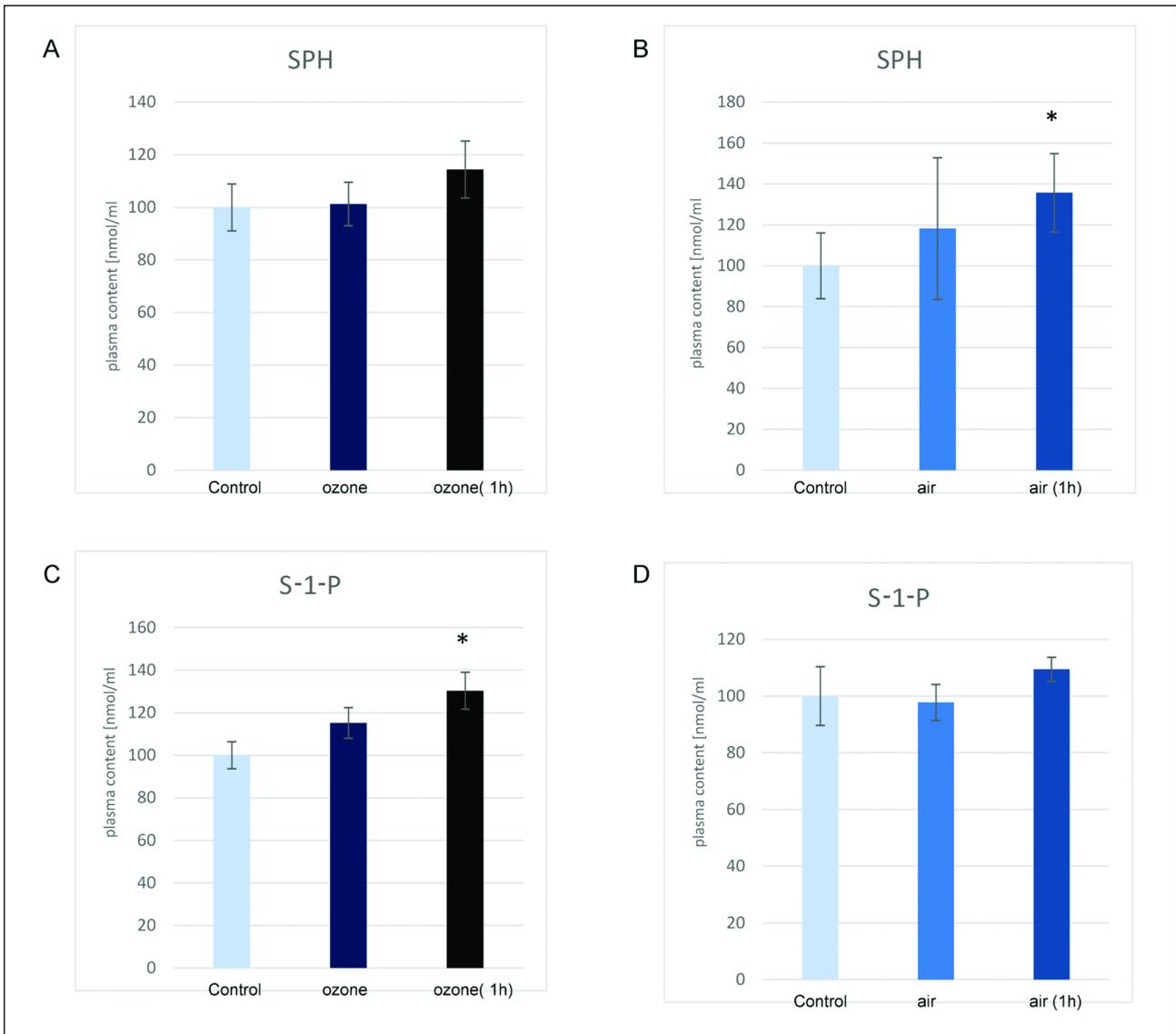


Fig. 2. The effects of ozone (A and C) and air (B and D) treatments (15 $\mu\text{g/ml}$ blood) on the contents of sphingosine (SPH) and sphingosine-1-phosphate (S-1-P) in human plasma. The asterisk represents statistically significant difference ($P < 0.05$) as compared with the Control; $n=5$.

Ozone - acute ozonation of blood (15 $\mu\text{g/ml}$); Ozone 1 h - prolonged ozonation of blood (15 $\mu\text{g/ml}$); Air - acute aeration of blood (15 $\mu\text{g/ml}$); Air 1 h - prolonged aeration of blood (15 $\mu\text{g/ml}$).

LDL levels (19, 20). Here, we report that ozone treatment of the human blood increases the content of sphingosine-1-phosphate in plasma, concomitantly not affecting ceramide levels, which might be highly desired and beneficial. We may speculate so, since plasma S-1-P levels were found to be highly cardio-protective (15, 21) and others have also shown an interdependence of relatively lower S-1-P plasma levels with acute myocardial infarction (15, 22) or chronic heart failure (15, 23) or stable coronary artery disease (15, 24). In contrary, increased levels of plasma ceramide were usually associated with increased risk of type 2 diabetes and obesity (25).

As far, most of the investigations were done on human blood in autohemotherapy (AHT), to generate a controlled stress response of possible therapeutic relevance in some immune dysfunctions and in the management of pain or infections (19). In this set up, the initial disruption of redox status is eventually reestablished by antioxidant enzymes, when this blood is infused

into the patient (26). However, the design of our study was different and relayed on rapid and prolonged ozonation of blood with subsequent estimation of sphingolipids in plasma. Ozone instantaneously dissolves in the plasma and rapidly reacts with hydrophilic antioxidants as well as unsaturated fatty acids (mainly albumin-bound PUFA) although the capacity of blood cells to regenerate dehydroascorbate and GSH disulfide levels requires prolonged time (26, 27).

In our study a very homogenous group of donors was chosen, to avoid age, hormonal, blood-born (HCT, Hb, MHC levels) or gender related influences. We focused on relatively low dose of ozone comparing to other studies since with higher dose of ozone we have noticed the incidence of hemolysis, although the treatment of the whole blood with 10 up to 80 $\mu\text{g/ml}$ was reported to render no injury of any cellular elements (27, 28). We measured chosen sphingolipids concentration in plasma, since serum S-1-P concentration

might be substantially amplified by activated platelets during preparation of samples (29).

As mentioned above, human S-1-P concentration in plasma is highly regulated and depends on platelets activation and distribution between RBCs and plasma protein compartments (esp. HDL, albumin and microvesicles). It's widely accepted that S-1-P plasma levels are buffered mainly by RBCs, which can take up and/or release S-1-P, but not degrade since RBCs S-1-P enzymes activities are either undetectable (30) or very low (31). There is a matter of debate whether S-1-P is actively exported/transported *via* protein-mediated mechanism(s) in or out of the cell (32). Once formed and present in plasma, S-1-P may be dephosphorylated or degraded (33). Summing up, biological effects of circulating S-1-P depend on many factors and the mechanism(s) regulating its levels in plasma are of critical importance for evaluating functional consequences that are traditionally ascribed to beneficial role for HDL fraction. Likely, the effects of blood ozonation are not directly related to erythrocytes or platelets activation, since it was shown that membrane sulfhydryls and ATP-ases are major targets and ozone does not react with membrane lipids (34, 35). In contrary, ozonation of blood might result in change of serum FA profiling as others reported that polyunsaturated fatty acids (PUFA) are potential targets for ozone and peroxydation of PUFAs may lead to increased formation of lipid oxidation products (2, 6). Importantly, PUFAs are highly involved in protection of cardiovascular system against injury (36), although elevation of selected ceramide FA species have been reported in plasma from type 2 patients and were correlated with the severity of insulin resistance (25). Based on that, we checked the effects of ozone treatment on FA composition in ceramide fraction and found no significant effects of ozonation on FA saturation status. Lack of distinct changes in total amount as well as selected ceramide FA species may indicate the safety of our ozonation procedure, although others have reported oxidation of double bounds (37) or shortening of the FA at the sn-2 position in phospholipids (38).

On the other hand, important, but unsolved and still promising issue of using ozone to sterilize blood supplies is under intense investigation (39). Obviously, the ozone treatment might be effectively used to sterilize blood supplies and even more since there are reports showing ozone-related inhibition of red blood cells deformation *in vitro* (34).

Abbreviations: LC/MS, liquid chromatography with the mass spectrometry; UPLC-MS/MS, ultra performance liquid chromatography - tandem mass spectrometer; S-1-P, sphingosine-1-phosphate; CER, ceramide; SPH, sphingosine; SPA, sphinganine.

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Conflict of interest: None declared.

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