# The dose-dependent effect of ozonated physiological solution on arterial vasodilation

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## Abstract

Preliminary researches have shown that reinfusion of ozonated blood may induce vasodilation in ischemic areas and reduce hypoxia. On the other hand ozone is associated with increased cardiovascular events and short-term inhalation of ozone at concentrations that occur in the urban environment causes acute conduit artery vasoconstriction. To help explain the mechanism behind these observations, we investigated the dose-dependent effect of ozonated solution on vascular function of central artery and microcirculatory system. MATERIAL AND METHODS: Experiments were carried out on Wistar male rats with the weight of 180-200 g. The animals received single or chronic injections of ozonated physiological solution or physiological solution (control group). Ozone concentration in solution was from 0.3 to 2.4 mcg/ml. Antioxidant enzyme activity - the superoxide dismutase and catalase were determined. Nitric oxide production was determined by measuring serum concentrations of its stable metabolites, nitrate and nitrite. Effects of ozonated physiological solution on arterial function were determined in central artery (carotid artery) and microcirculatory system of brain and heart *in vivo.* Except that dose-dependent effect on coronary flow was determined in the isolated Langendorff-perfused rat heart. Ozone concentration in Krebs-Henseleit ozonated solution was from 0.05 to 6.0 mcg/ml. Capillaries structure was studied by the method of transmission microscopy. RESULTS: Single injection of low concentration of ozone to intact rats did not change lipid peroxidation and superoxide dismutase activity in blood, an endothelium-dependent relaxation of the central vessels and improved microcirculation in the heart and brain. In group with chronic administration of the same dose lipid peroxidation was slightly increased, superoxide dismutase activity increased. Morphological and electron-microscopic methods revealed the increase in endothelial permeability. In contrast, chronic administration of high ozone concentrations did not induce augmentation of the superoxide dismutase activity, lead to the slight intensification of lipid peroxidation, gradually reduction plasma nitrites/nitrates. The highest ozone concentrations reduced of a blood flow in the coronary arteries in the isolated Langendorff-perfused rat heart. In this case reaction was irreversible. CONCLUSION: It was shown, that low concentrations of ozone induce vasodilation, and high concentrations of ozone considerably increase permeability of vessels and damage this artery reaction and microcirculatory system of brain and heart. The mechanism of vasodilation is dose-dependent and $H_2O_2$-dependent, but independent on nitric oxide production. Endothelial damage is connected with decrease of superoxide dismutase activity, injury of endothelial layer integrity due to alteration of membranes permeability. All this leads to disturbance of vasodilation as affect on total structure of a vascular wall.

## Introduction

Ozone as a substance is capable to influence on the metabolic processes in different organs and in the whole organism through ozonolysis of organic substrates. Ozone can act as a bioregulator [2] and to encourage controlled clinical investigations to evaluate definitely the validity of ozonetherapy. Among various types for the administration of ozone, the autohemotransfusion procedure, consisting in exposing blood to ozone [22], and direct injection of ozonated solution into a vein [13], i.e. to a calculated and brief oxidative stress, appears safe, simple, inexpensive and amenable to be adjusted to different pathological states. Ozone is highly effective in peripheral vascular disease, in cardiovascular and cerebrovascular disease, arteriosclerosis and hypercholesterolemia, and promptly restores circulation, relieves angina pain and improves brain function.

In these cases ozone or products of ozonolysis should act on an endothelium and actively change its function that should be reflected in a vessel function. The study [20] demonstrated that ozonated autohemotherapy with ozone concentration of 50 mcg/ml did not affect deleteriously the endothelium in patients with chronic renal failure on maintenance hemodialysis. Studies on the biological effects of human ozonated serum on Human endothelial cells in culture [21] were shown that the treatment with ozonated serum yields a dose dependent increase of thiobarbituric acid reactive substances (TBARS) and of hydrogen peroxide ($H_2O_2$) and a decrease of protein thiol groups (PTG). It appears that ozonation enhances IL-8, inhibits E-selectin and hardly modifies ET-1 production. Authors have assumed that reinfusion of ozonated blood, by enhancing release of NO, may induce vasodilation in ischemic areas and reduce hypoxia. Estimation an influence of medical ozone in vitro on the tone of normal and atherosclerotic vascular wall was shown, that medical ozone induces relaxation of normal rabbit aorta in vitro [6]. This effect of ozone was dose dependent and endothelium independent. It was also shown that in the presence of catalase ozone had no vasorelaxant activity. Except that ozone did not disturb of endothelium function under experimental conditions, using acetylocholine test.

In other experiment [15] exposure to 3 and 4 ppm $O_3$ for 3 h resulted in a significant increase in bronchial artery flow without affecting any of the other cardiopulmonary parameters measured. These results indicate that $O_3$ induces a dose dependent increase in artery flow which is the result of a vasodilation of the bronchial vasculature which is not dependent upon changes in blood gases or upstream driving pressure. On the other hand ozone is associated with increased cardiovascular events and short-term inhalation of ozone at concentrations that occur in the urban environment causes acute conduit artery vasoconstriction [4]. Mechanisms of ozone action on the vessels remain unclear.

To help explain the mechanism behind these observations, we investigated the dose-dependent effect of ozone-oxygen mixture on vascular function of central artery and microcirculatory system.

## Material and methods

Experiments were carried out on Wistar male rats with the weight of 180-200 g. Animal care and treatment were conducted in accordance with the “Guide for the Care and use of Laboratory animals”. The animals received single or chronic injections during 6 days of 1 ml ozonated physiological solution or physiological solution (control group). Ozone concentration in ozonated solution was from 0.3 to 2.4 mcg/ml. After last injection rats were anesthetized with sodium pentobarbital (35 mg/kg) and the heart and brain were removed for morphological and electron-microscopic researches. Blood was obtained during decapitation using heparin.

Effects of ozonated physiological solution on arterial function were determined in central artery by direct measurement of arterial pressure in carotid artery after introduction of ozonated physiological solution in a vein and carotid artery and by morphological researches of microcirculatory vessels. Structural cell modifications are determined by the method of transmission microscopy on the electronic microscope Morgagni 268D. Morphometrical analysis was made with the program “AnalySIS”. Endothelium-dependent relaxation of the central vessels determine by stimulation with acetycholine (Ach).

Plasma nitrate/nitrite concentration was measured spectrophotometrically by color Griess reaction. Nitrates were converted into nitrites using copper-coated cadmium granules. For the calibration curve, $KNO_3$ was used.

Intensity of lipid peroxidation processes was estimated by measurement of malondialdehyde in plasma. Antioxidant enzyme activity - superoxide dismutase and catalase was determined. Thiobarbituric acid reactive substances (TBARS) were measured by reaction with thiobarbituric acid. Superoxide dismutase (SOD) activity was determined by the method of Nischikimi M. et al. [12]. with nitrotetrazolium blue chloride and N-Methylphenazonium methyl sulfate. Catalase activity (CAT) was determined by the method of Aebi H. [1]. All reagents were from Sigma, Fluka or Biochemica.

A model of isolated perfusion rat heart by the Langendorff technique was used to show that ozone effect on the coronary blood low variation has a dose-dependent character. After 30 min adapt period 5 min perfusion with ozonated Krebs-Henseleit solution was followed. Ozone concentration in Krebs-Henseleit solution was from 0.05 to 6.0 mcg/ml.

Data were analyzed by Student's test (unpaired) with use of Statistica 5.5. A probability value $<0.05$ was accepted as being statistically significant.

## Results and discussion

Studying of arterial pressure changes in carotid artery after introduction of an ozonated physiological solution has shown an artery decrease of arterial pressure from 115±1.7 mm Hg to 98±2.15 mm Hg ($p<0.05$). Difference between ozonized physiological solutions with various ozone concentrations was statistically insignificant. Reaction of decrease has been more expressed at intracarotid introduction of the ozonated solution (from 115±1.7 mm Hg to 92±1.12 mm Hg, $p<0.01$). This reaction has been comparable to reaction to Ach introduction (from 121±1.6 mm Hg to 88±1.25 mm Hg, $p<0.01$).

Table 1 demonstrates changes in the plasma level of nitrite/nitrate and activity of Superoxide dismutase and Catalase. It is seen that ozonated solution introduction induce a gradual decrease in nitrite/nitrate corresponding to the increase of SOD activity. Activity of CAT does not change.

Table 1

Ozonation Effect on the levels of Thiobarbituric acid reactive substances, activity of Superoxide dismutase, Catalase and Plasma nitrate/nitrite concentration (M±SEM)

| Experimental series | TBARS nmol/g | SOD U/g | CAT U/g | $NO_2/NO_3$ (μM) |
|---|---|---|---|---|
| Unitary introduction | | | | |
| 1. Control (n=8) | 0.136±0,021 | 12.4±0,51 | 0.492±0.027 | 22.5±1,21 |
| 2. 0.3 mcg/ml $O_3$ (n=7) | 0.105±0,070 | 13.6±0,62 | 0.454±0.018 | 23.3±3,05 |
| 3. 0.7 mcg/ml $O_3$ (n=6) | 0.102±0,065 | 14.1±0,39 | 0.462±0.031 | 21.8±1,56 |
| 4. 1.7 mcg/ml $O_3$ (n=8) | 0.096±0,007 | 18.1±0,19 $p_{4-1}<0.05$ | 0.461±0.026 | 16.5±1,02 $p_{4-1}<0.05$ |
| 5. 2.4 mcg/ml $O_3$ (n=8) | 0.086±0,006 | 22.5±0,21 $p_{5-1}<0.01$ | 0.472±0.025 | 14.3±0,8 $p_{5-1}<0.01$ |
| Repeated introduction | | | | |
| 6. Control (n=8) | 0.14±0,011 | 12.6±0,38 | 0.465±0.024 | 21.8±1.52 |
| 7. 1.7 mcg/ml $O_3$ (n=8) | 0.067±0,013 | 10.35±0,15 | 0.501±0.022 | 17.8±1.23 |
| 8. 2.4 mcg/ml $O_3$ (n=8) | 0.062±0,011 | 9.58±0.18 $p_{8-6}<0.05$ | 0.568±0.022 | 12.3±1.56 $p_{8-6}<0.01$ |

Research of arteriole structure and capillaries in the heart and brain tissues has revealed the increased diameter arteriole and capillaries. Around of small vessels an edema most expressed around of large vessels (Fig. 1) was marked. The degree of the edema depends on a doze of ozone in an ozonated physiological solution.

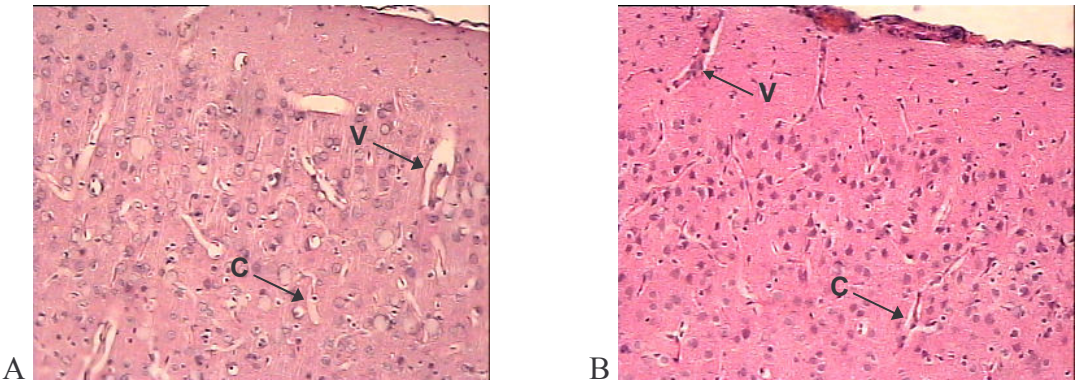


Fig. 1. Influence of an ozonated physiological solution with concentration of ozone 1.7 mcg/ml on structure of a brain tissue (cortex level). A - 15 minutes after introduction an ozonated physiological solution, B - 14th day after introduction an ozonated physiological solution. x 200.
V – small arterioles, C – capillaries.

The diagrams in Fig.2 demonstrate the dependence between a degree of the edema and concentration of ozone. Supervision was carried out in 15 minutes after introduction, in 1, 14 day.

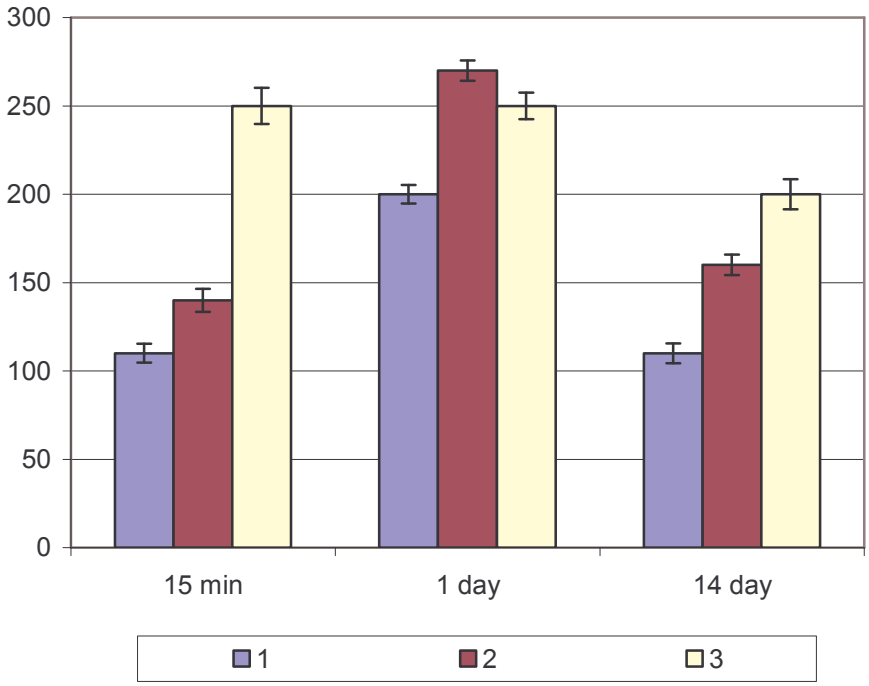


Fig. 2. Effect of an ozonated physiological solution on a degree of the edema around of arterioles and capillaries in rat brain tissue (percentages of the control value). 1 – 0.7 mcg/ml $O_3$; 2 - 1.7 mcg/ml $O_3$; 3 - 2.4 mcg/ml $O_3$.

Electron-microscopic researches (Fig. 3) through 15 mines after introduction of an ozonated physiological solution have revealed wide gleams of capillaries (>3.60 $\mu^2$), most part of them contained erythrocytes and gentle osmiofilic flake material, identifying plasma fibers. The

endothelium is slightly hydropic, in some cases with small quantity of vacuoles and hydropic cellular membranes on a significant extent. Basal and luminal membranes are slightly hydropic too. Similar reaction was followed in rat heart too.

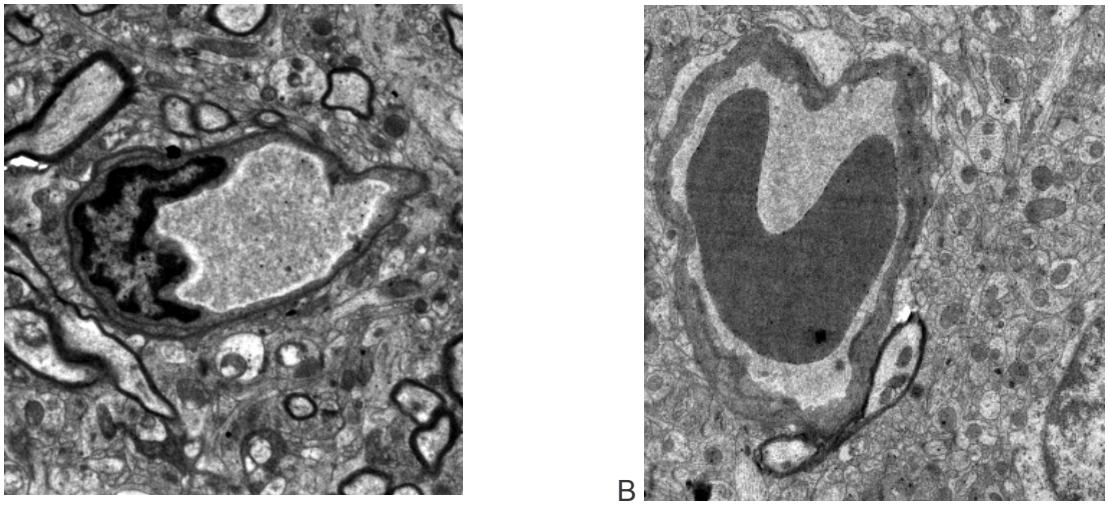


Fig. 3. Effect of an ozonated physiological solution on the capillaries structure. Electron-microscopic researches of cerebral cortex zone. A – control; x7100, B - in 15 minutes after introduction; x3500.

The diagrams in Fig.4 demonstrate the effect of an ozonated physiological solution on coronary blood flow. It is seen that the increase of ozone concentration evoke to blood flow reduction after the period of increase of a blood flow. Reduction of coronary blood flow is irreversible after application of concentration of ozone in solution 6.0 mcg/ml.

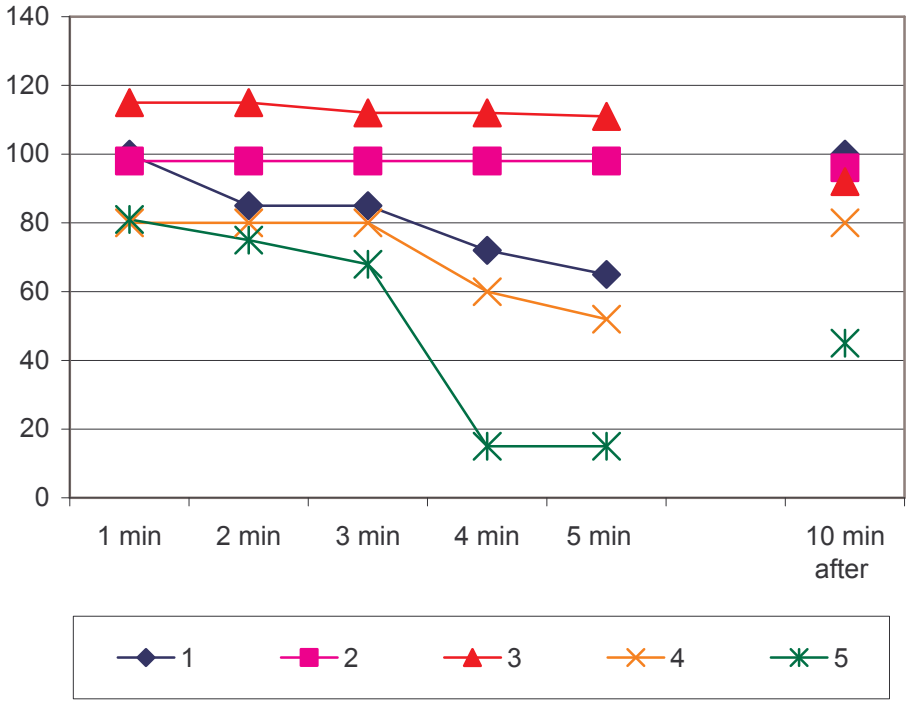


Fig. 4. Effect of an ozonated Krebs-Henseleit solution on a coronary blood flow in isolated perfusion rat heart by Langendorff-Fallen (percentages of the control value). 1 – oxygenated Krebs-Henseleit solution, 2 - ozonated Krebs-Henseleit solution, ozone concentration 0.3 mcg/ml; 3 - ozonated Krebs-Henseleit solution, ozone concentration 0.6 mcg/ml; 4 - ozonated Krebs-Henseleit solution, ozone concentration 2.4 mcg/ml; 5 - ozonated Krebs-Henseleit solution, ozone concentration 6.0 mcg/ml. 10 min after – time after changes of a ozonated solution on control Krebs-Henseleit solution with gas $O_2$:$CO_2$.

Recent evidence suggests that oxidant stress plays a major role in several aspects of vascular biology. Oxygen free radicals are implicated as important factors in signaling mechanisms leading to vascular pathologies such as postischemic reperfusion injury and atherosclerosis. In the endothelium, superoxides enhance and peroxides attenuate agonist-stimulated $Ca^{2+}$ responses, suggesting differential signaling mechanisms depending on radical species. In smooth muscle cells, both superoxides and peroxides disrupt the sarcoplasmic reticulum $Ca^{2+}$-ATPase, leading to both short- and long-term effects on smooth muscle $Ca^{2+}$ handling. [8]. Oxygen metabolites have been reported to produce vasoconstriction and/or vasodilation in a variety of in vitro or in vivo vascular preparations. Hydrogen peroxide can produce either vasodilation or constriction via stimulation of prostaglandins. The soluble form of guanylate cyclase in vascular smooth muscle, an enzyme which produces the intracellular mediator of relaxation cyclic GMP, is also a site of action of vasoactive $O_2$ metabolites. Guanylate cyclase is directly activated by nanomolar concentrations of nitric oxide (produced by endothelial cells or nitrovasodilator drugs) or $H_2O_2$ (via its metabolism by catalase) [23]. These findings [17] show that hydrogen peroxide increases the intracellular $Ca^{2+}$ concentration and stimulates the formation of L-citrulline from L-arginine coupled with nitric oxide synthesis in cultured endothelial cells.

Our date indicate that ozonated solution with low ozone concentration induces small oxidant stress and some activation of enzyme system of antioxidant protection as a result of response on release ozonolysis products. Active SOD transforms superoxide into hydrogen peroxide ($H_2O_2$) in this case. Increase of $H_2O_2$ may lead to vasodilation. Authores [10] have recently identified that endothelium-derived $H_2O_2$ is an EDHF in mesenteric arteries of mice and humans and in porcine coronary microvessels. However, the mechanism for the endothelial production of $H_2O_2$ as an EDHF remains to be elucidated. These results prove the novel concept that endothelial Cu,Zn-SOD plays an important role as an "EDHF synthase" in mice, in addition to its classical role to scavenge superoxide anions.

In other studies Matoba T et al [11] examined hypothesis that hydrogen peroxide derived from endothelial NO synthase (eNOS) is an EDHF. Catalase, which dismutates $H_2O_2$ to form water and oxygen, inhibited EDHF-mediated relaxation and hyperpolarization, but it did not affect endothelium-independent relaxation following treatment with the K+ channel opener levcromakalim. Exogenous $H_2O_2$ elicited similar relaxation and hyperpolarization in endothelium-stripped arteries. Finally, laser confocal microscopic examination with peroxide-sensitive fluorescence dye demonstrated that the endothelium produced $H_2O_2$ upon stimulation by ACh and that the $H_2O_2$ production was markedly reduced in eNOS-KO mice. These results indicate that $H_2O_2$ is an EDHF in mouse small mesenteric arteries and that eNOS is a major source of the reactive oxygen species.

According to our results ozonated solution evoke to decrease in plasma nitrate/nitrite concentration. Very likely SOD inhibits eNOS. Brennan LA, Wedgwood S, Black SM. [3] indicate that increased levels of $H_2O_2$ may be involved in the inhibition of eNOS by NO and that the scavenging of $H_2O_2$ may be useful to prevent eNOS inhibition during treatments that involve inhaled NO or NO donors.

Except that we were shown that catalase activity remain constant if we apply low ozone concentration. It is likely that endogenous catalase plays an important role in the protection of vascular reactivity of rat aorta against oxidant damage by high (1 mM) but not lower (0.1 mM) concentrations of hydrogen peroxide. The data are consistent with the promotion of non-selective damage to the vascular smooth muscle cells by hydrogen peroxide, but endothelial damage may also be sustained [9]. Catalase did not affect the vasodilation produced by Ach [19], but $H_2O_2$ induced endothelium-independent relaxation which was abolished by catalase [7].

Mechanism of $H_2O_2$-dependent vasodilation remains unclear. It was [5] conclude that myoendothelial gap junctions underpin smooth muscle hyperpolarizations evoked by Ca2+ ionophore A23187 and ACh, but that A23187-induced relaxation is dominated by extracellular

release of $H_2O_2$. Endothelium-derived $H_2O_2$ may thus be regarded as a relaxing factor, but not a hyperpolarizing factor, in rabbit arteries. Some observations [24] suggest that at suitable pathophysiological concentrations, $H_2O_2$ could induce release of an endothelium-derived relaxing factor (EDRF), probably nitric oxide (NO), from endothelial cells of the canine cerebral artery. The $H_2O_2$ relaxant effects are clearly Ca2+-dependent, require formation of cyclic guanosine monophosphate (cGMP), and may be associated with release of endogenous acetylcholine (ACh). Thus, stimulation of NO synthesis induced by $H_2O_2$ may involve the mechanisms other than the increases in intracellular $Ca^{2+}$ in endothelial cells. In the particulate fraction from cultured endothelial cells, addition of exogenous $H_2O_2$ (1 mM) or catalase (100 U/ml) did not affect L-citrulline formation. However, co-administration of v and catalase stimulated L-citrulline formation. These findings suggested that not only the increases in intracellular $Ca^{2+}$ but also the products by the reaction with $H_2O_2$ and catalase are likely to be involved in the stimulation of NO synthesis induced by $H_2O_2$ [16]. In addition, $H_2O_2$ generated in the endothelium seems to regulate the vascular response and also act as a mediator to release other vasoactive substances [18].

However, our date indicate that vasodilation combine with slightly edema around arteriole and capillaries. This fact may be responsible for membrane permeability damage. It is very likely that the increase of $H_2O_2$ promote both vasodilation and endothelium permeability.

Ozonated solution with high ozone concentration induces injury to endothelium and cessation of blood flow may be developed as a result of total edema of endothelium in pathophysiological situations (e.g. inflammation, ischemia/reperfusion, etc.).

## Conclusion

In such a way the endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors, including prostacyclin, NO, and endothelium-derived hyperpolarizing factor (EDHF). Oxygen metabolites have been reported to produce vasoconstriction and/or vasodilation and it was recently identified that endothelium-derived $H_2O_2$ is an EDHF in mesenteric arteries of mice and humans and in porcine coronary microvessels. Mechanism of $H_2O_2$-dependent vasodilation remains unclear. Our date indicate that ozonated solution with low ozone concentration induces small oxidant stress and some activation of enzyme system of antioxidant protection as a result of response on release ozonolysis products. Active SOD transforms superoxide into hydrogen peroxide in this case. It is very likely that the increase of $H_2O_2$ may be reason of ozone-dependent vasodilation. High ozone concentration induces injury to endothelium and cessation of blood flow as a result of total edema of endothelium. The increase of endothelium permeability is additional negative reaction but reversible if low ozone concentrations are applied.

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