The biochemical aspects of ozone therapy

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Abstract

- The recent years of ozonotherapy have been marked by growing recognition of low doses of ozone administered parenterally. These doses launch or activate a whole cascade of biochemical processes. It can be seen in activation of antioxidant defense system, reinforcement of circulation, improvement of trophic processes in organs and tissues and of rheological blood properties, increasing the immunomodelling effect and detoxication.

- For 25 years using different experimental model we have investigated possible ways to use ozone as (1) regulator of energetic processes, (2) control over ion and cation transport, (3) protolytic system of the organism, (4) c-AMP and c-GMP being secondary messengers.
The present work contains the experimental results that make it possible to attach ozone quite a number of regulatory effects.
• The first group of experiments was done using the model of hypovolemic hypotensia in dogs. Hypovolemia was resulted from free bloodletting from femoral artery until the pressure lowed down to 40 mm Hg. The volume of blood loss was 31-33 ml/kg. The pressure was kept for an hour according to Wiggers’s method. Then the bloodloss was compensated by saline perfusion. Two hours later (restoration period) reinfusion of the discharged blood was done. Starting from the 15th minute extracorporeal blood ozonation was performed in oxygenator connected with arterio-venous bypass (100ml of blood was barbotaged in oxygenator with ozone/oxygen mixture for 5 minutes, ozone concentration being 48 mcg/l).
The model of hypovolemic hypotension in dogs

1. Promedol
   10 mg/kg
2. Nembutal
   30 mg/kg

Ozonated saline
40 mm Hg

Wiggers’s method (1 hour)
• The concentration was received in earlier experiments on whole blood of intact animals and the blood taken in post-resuscitation period according to ozone influence on pro- and antioxidant balance.

• Then the blood was replaced into the vein. The procedure of blood treatment lasted 60 minutes.

• There were used 80 dogs.

• Myocardium of intact animal was analyzed at the height of hypoxia at 120th minute of restoration period.
The received samples were used to define the activity of pro- and antioxidant systems with analysis of the levels of lipid peroxidation products:

- Dien conjugates - DC
- Malone dialdehyde – MDA
- Activity of catalase and SOD

Levels of ATP, ADP, AMP and kreatine-phosphate.

Activity of ATP-dependent enzymes:
- protonic H⁺ –ATP-ase in mitochondria
- Total Ca²⁺-ATP-ase activity,
• In lipids taken from homogenate of myocardium tissue the DC and MDA levels were found to be increased at the hypoxia height.

• In control series by the end of restoration period DC levels were increased and MDA decreased. However in experimental series blood ozonation caused the decrease in DC level and even more pronounced compared with the controls in MDA.
DC Levels Change in Myocardium Tissue of a Dog in Experiment

DC (nmol/g)

Intact  hypoxia  restoration

control

O₃
MDA Levels Change in Myocardium Tissue of a Dog in Experiment

- Intact
- Hypoxia
- Restoration

MDA (nmol/g)

control

$O_3$
At the same time it was noted activation of antioxidant enzymes: superoxide dismutase and catalase. The most pronounced was catalase activation (3 times as much).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Intact</th>
<th>Hypoxia</th>
<th>Restoration period Control</th>
<th>Restoration period Ozone</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>14,30± 1,10</td>
<td>18,40±0,60*</td>
<td>16,50± 1,30</td>
<td>18,20± 1,30*</td>
</tr>
<tr>
<td>Catalase</td>
<td>0,29± 0,14</td>
<td>2,13±0,19*</td>
<td>0,38± 0,08</td>
<td>0,98± 0,10*</td>
</tr>
</tbody>
</table>

Note: * Valid differences concerning intact series (P<0,05).
• Infusions of ozonated blood in the first experimental series resulted in increase of $H^+$ – ATP-ase activity in mitochondrias of cardiomyocytes, exceeding the initial value. It is caused by correction of aerobic oxidation connected with oxidative phosphorilation and generation of $\Delta\mu H^+$.

• Besides, in case of hypoxia there accumulates a great amount of restored carriers.
The Activity of $H^+\text{--ATP-ase}$

Note: * Valid differences concerning intact series ($P<0.05$).
• It is well seen in the table, ATP level at hypoxia height falls down 1.7 times the height and kreatine phosphate as low as 2.2 times, while in control group the rise is seen which does not reach the original value. Under the influence of ozone there takes place a complete restoration of ATP levels and more pronounced of kreatine phosphate. The major part of energy is consumed by ATP-dependent enzyme transport activity.
• Thus, saturation with oxygen, being more intensive compared with control series without ozone, provides the conditions to generate a greater value of $\Delta \mu \text{H}^+$. $\Delta \mu \text{H}^+$ can support all energy-consuming processes and in particular, chemical work (synthesis of ATP and pyrophosphate, electrons backward transfer along redox chain, including transhydrogenase reaction, osmotic work (transport of ions and of non-charged metabolites opposed to their concentration gradient).

• Increase in $\text{H}^+ – \text{ATP}$-ase activity can satisfy the myocardium need in ATP to maintain the contractive activity and synthetic processes, damaged by hypoxia, induced by hemorrhagic shock. Correlation coefficient of $\text{H}^+ – \text{ATP}$-ase activity/ATP level was 0,76 ($p<0,001$).

• Normalization of oxidative phosphorylation due to ozone contributed to normalization of ATP level.
## The Level of Macroenergy Substance in Myocardium

<table>
<thead>
<tr>
<th></th>
<th>ATP, mmol/g</th>
<th>ADP, mmol/g</th>
<th>AMP, mmol/g</th>
<th>CP, mmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>7.07 ± 0.49</td>
<td>1.92 ± 0.23</td>
<td>1.46 ± 0.11</td>
<td>8.13 ± 0.70</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>3.89 ± 0.26*</td>
<td>1.96 ± 0.25</td>
<td>2.52 ± 0.54*</td>
<td>3.94 ± 0.51*</td>
</tr>
<tr>
<td>Control</td>
<td>5.79 ± 0.29</td>
<td>1.99 ± 0.15</td>
<td>1.54 ± 0.24</td>
<td>4.74 ± 0.82</td>
</tr>
<tr>
<td>(O3)</td>
<td>7.09 ± 0.58**</td>
<td>2.19 ± 0.11</td>
<td>1.71 ± 0.19</td>
<td>6.57 ± 0.52*</td>
</tr>
</tbody>
</table>

Note: * Valid differences concerning intact series (P<0.05).
The Activity of Na\(^{+}\)-K\(^{+}\)—ATP-ase

Note: * Valid differences concerning intact series (P<0.05).
• In hypoxia the $\text{K}^+ - \text{Na}^+ - \text{ATP}$-ase activity had a 44.2% decrease compared with initial value of intact animal. In control group it tended to increase at the end of the experiment.

• The use of ozone led to enzyme activation with the activity exceeding the initial one.

• $\text{K-}\text{Na-ATP}$-ase defines the generation mechanism of difference of potentials of excitable membranes, which is essential for nerve impulse transfer.
The activity of Ca$^{2+}$-ATP-ase

Note: * Valid differences concerning intact series (P<0.05).
• Similar changes appeared to characterize Ca$^{2+}$ ATP-ase activity.

• In hypoxia Ca$^{2+}$-ATP-ase activity had 38% decrease, while in the control group Ca$^{2+}$-ATP-ase activity had 11% raise at the end of the experiment (p<0.05).

• Thus ozonation significantly increased the enzyme activity (69%-(p<0.001)), bringing its level to the original one.
• The **second group** of experiments was done on white non-linear male rats with body mass of 200-220g.

• According to experimental purposes the animals were subdivided into two subgroups: 1- intact rats (n = 36) were used as control ones; 2- experimental rats (n= 205).
• The animals of the experimental subgroup received 1ml of ozonated saline (0,9% NaCl) intraperitoneally.
• The saline was prepared by barbotage of 50 ml of sterile saline with ozone/oxygen mixture.
• Oxygen rate flow was 1 l/min.
• Ozone concentration was controlled in gaseous phase by spectrophotometer with the wavelength of 254 nm.
• Ozone concentration in saline was calculated by unified method of iodometric titration.
• Single ozone doses were: 0,027; 0,053; 0,505 mcg.
• To study cumulative effect of ozone doses of 0,184; 0,505 mcg ozonated saline was injected every other day 6 times with volume of 1 ml.
• At the end of the experiment the animals were decapitated under anesthesia.
• Biochemical analyses were made on blood plasma and homogenates of pancreas tissue.
• The examined tissues were analysed for:
  • 2. Chymotrypsine-like proteinase activity was measured with the use of Glp-Phe-pNa substrate with pH 7.7.
  • 3. Elastase activity was estimated with the use of Z-Gly-Ala-Ala-pNa substrate.
  • 4. Proteolytic kallikrein activity was assessed with Z-D-Ala-Leu-Arg-pNa substrate.
  • 5. α1- antitrypsin and α2- macroglobulin in blood plasma.
• The results of experiments show, that ozonated saline with single ozone doses of 0,027-0,053 mcg produce a positive effect on proteolytic system. Trypsin, chemotrypsin and elastase activity did not increase. In the same time we observed the rise of α1-antitrypsin and α2-macroglobulin.

• The marked elevation of the main proteolytic inhibitors testify the switching on of the compensatory mechanisms that launch physiological processes of adaptation.
### Inhibitors antiproteolytic activity of blood plasma (M±m)

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Intact rats</th>
<th>Doze ozone, mcg</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁-antitrypsin, mc mol/l</td>
<td>8,89±0,04</td>
<td>8,96±0,08</td>
</tr>
<tr>
<td>α₂-macroglobulin, mcmol/l</td>
<td>0,88±0,03</td>
<td>1,15±0,08*</td>
</tr>
</tbody>
</table>

Note: * Valid differences concerning intact series (P<0,05).
• Six doses of ozone (1,104-5,995 mcg) produce significant reinforcement of proteolytic activity in tissues of the experimental animals and decrease of α1-antitrypsin and α2-macroglobulin activity in blood plasma.

• Proteinase-inhibitory disbalance in multiple ozone introduction is caused by unlimited proteolysis leading to serious disorders in a number of important homeostasis regulation systems and to excess protein accumulation in liquid media of biologically active products of protein degradation.
The Activation of Proteolysis Systems in experiment

Tripsine  Himotrip  Elastase
Exper  Intact

*
• It can be proved by significantly enhanced activity of practically all investigated proteinases and, particularly, of homogenates of pancreatic tissues.

• Trypsin is known to activate all pancreatic enzymes. Trypsin 9-fold activation due to ozonated solution (ozone dose 3,033 mcg) in comparison with the intact animals makes it possible to assume the consequent activation of other enzymes.

• The revealed 6-fold chymotrypsin-like proteinases (P<0.001) and 1.8-fold elastase activation (P<0.001) confirm this assumption.
• The release of trypsin aggregation with blood activates kallikrein-kinin system.

• The latter is regarded as a functional mediator between blood coagulation systems and fibrinolysis and is capable to activate compliment system and renin-aldosteron-angiotensin system.

• Kallikreins, pertaining to the class of serine proteinase of trypsin-like action, are known to perform the role of allergic and inflammatory mediators and to participate in regulation of microcirculation, arterial pressure, coagulation, activation of complement system, intercellular interactions resulting in morphological changes of target organs.
• Ozone dose of 3,033 mcg increases proteolytic activity of the basic KKS enzyme – kallikrein by 4,6 times (p<0,001) in homogenated of pancreatic tissues.

• However, blood plasma does not show so evident activation (1,4 increase and p<0,001). At the same time, 4,3 increase of kininase (p<0,001), kinin-degradating enzyme, in plasma testifies the possibility to regulate KKS state with the dose of ozone.

• The revealed simultaneous elevation of proteolytic kallikrein activity and of kininase gives evidences of “proportional” KKS activation that is characterized by maintenance of biochemical balance and can be regarded as compensatory reaction.
• Kininase was registered to have an 1,4 decrease (p<0,001) compared with intact animals findings.

• So we may assume that along with synthesis of kinin there develops inhibition of kinin-eliminating processes that might serve as additional mechanism to increase active kinin concentration in lymph and in blood.
• **The third group of experiments** was done to study ozone effect on hemostasis. To solve the set tasks a number of experiments were carried on blood in vitro. The blood was taken from healthy donors (coagulation parameters within the normal range –50 samples) and from patients with atherosclerosis (hypercoagulation – 55 samples).

• The range of the chosen ozone concentrations was determined by the concentrations widely used in clinical practice (90, 130, 270, 500 и 910 mcg/l).

• The control samples contained blood with addition of saline (25:1) to achieve the hemodelution level, corresponding to that in the experimental series. Citrated plasma was studied both rich and poor in thrombocytes. Besides, washed erythrocytes were used. The condition of hemostasis was assessed according to plasmotic and thrombocytic hemostasis.
• The extended coagulogramme included:
• Indices of the 1\textsuperscript{st} phase of coagulation - partially activated thromboplastin time, activated recalcification time;
• Indices of the 2\textsuperscript{nd} phase of coagulation - protrombin index, trombin time;
• The main indices of the final stage of coagulation – fibrinogen and factor XIII-fibrin-stabilizing concentrations.
• Anticoagulation activity was estimated by antitrombin 111 (AT-111) activity index.

• Fibrinolytic activity was defined by lysis time of euglobulin fraction. In addition to that, there was measured the concentration of soluble fibrin monomeric complexes (SFMC) and fibrin degradation products (FDP) in blood plasma, which are known to be the main markers for trombin generation and development of disseminated intravascular coagulation (DIVC).

• Analysis of the thrombocytic part included calculation of thrombocytes, their aggregation capacity, induced with ADP, adrenalin, ristomicin.
• The first experimental group contained blood samples of actually healthy persons and, thus the findings were within normal range of procoagulant, anticoagulant and fibrinolytic hemostatis aspect, along with normal trombocytic parameters.

• To estimate ozone effect on hemocoagulation and fibrinolysis parameters in hypercoagulated blood, the samples were taken from patients with atherosclerosis with the tendency to thrombogenesis.

• According to the performed experiments, ozonated saline with ozone concentrations of 90, 130, 270 mcg/l produced insignificant but statistically valid hypocoagulation changes.
• It is expressed in extent of activated partial thromboplastine time (APTT), activated time of recalcification (ACT), and trombin time (TT). Prothrombin index (PTI) had an insignificant raise but stayed within normal range.

• **Fibrinolytic blood activity appeared to be elevated.**

• Hypocoagulation ozone effect may be explained by increase of anticoagulant activity due to its influence. It is revealed in growing antithrombin III (AT-III) activity, that is known to be the main anticoagulant in plasma. There was revealed a definite dose-dependent ozone effect on hypocoagulation.

• Of great importance are the findings received on using high doses of ozone - 500 и 910 mcg/l. They were found to dramatically activate hemostasis with 1,5 shortening of APTT and of ACT, TT and evident increase of PTI.
• Ozone high concentrations were proved to stimulate thrombinogenesis that is manifested in 2-fold increase of TT in blood plasma, formation of fibrinogen-degradating products. It is connected with two main points – accumulation of great amount of fibrin-monomeric complexes that are easily lysed with plasmin and augmentation of plasmin in plasma.

• All this results in reinforcement of fibrinolytic blood activity. Ozone concentration of 270 mcg/l in blood with original hypercoagulation potentiates thrombingeneration for blood plasma reveals drastin increase of soluble fibrin monomeric complex concentration, appearance of FDP, quick growing of fibrinolytic blood activity.
Changes in fibrinolytic activity in incubation of blood with normal and hypercoagulation with ozonated saline. Ozone concentrations are different.
It can be assumed that hemostasis and fibrinolysis activation have the same mechanisms due to ozone effect. The same picture was noted with the involvement of other hemostasis inductors – adrenaline, acydosis, hypoxia, etc.

The same regulations were observed in assessing ozone effect on thrombocytic hemostasis. Ozone concentrations of 90 - 270 mcg/l produce actual decrease in thrombocytes aggregation in healthy people, irrespective of the aggregation inductor used – ADP, adrenaline, ristomicin.

On the contrary, ozone high concentrations of 500 и 910 mcg/l produce significant activation of thrombocytes aggregation. In both cases the concentration of blood platelets did not decrease.
Dose-dependent ozone effect on induced aggregation of thrombocytes in blood with normal and hypercoagulation
• Completely different result was received when ozonated saline was added into the blood with hypercoagulation. Even insignificant ozone doses of 130 mcg/l do not decrease thrombocytes aggregation.

• Ozone in high concentrations results in significant hyperaggregation of thrombocytes. There was found valid reverse correlation between Shiff Bases level in erythrocytes and catalase activity.
• In our opinion, the damaging mechanisms leading to increase in thrombocytes aggregation due to ozone effect come into action under the influence of destabilization of structural and functional properties of cell membranes.

• Thrombocytes activation can be caused by hydroperoxides of polynonsaturated fatty acids generated free radical oxidation of phospholipids.
The fourth group of experiments concerned experimental oncogenesis. Sarcoma-45 was used as the main model.

The model of neoplasia was done by reinoculation of the tumour strain to rats \( n = 100 \).

The animals were subcutaneously injected of 1ml of the tumour suspension with Henks solution in the area of the right femur.

The size of the tumour particles did not extend 1mm.

The animals of the experimental subgroup received 1,5 ml of ozonated saline (0,9% NaCl) intraperitoneally 5 times day after day. The saline was prepared by barbotage the sterile saline with ozone/oxygen mixture with concentration 3000 mcg/l of g.

Further on we present the results revealed the contents of cyclic nucleotides in the tumour and in the liver of tumour-inoculated in experiments to study rats before and after ozonation.

Cyclic nucleotides c-AMP and c-GMP participate in regulation of biochemical and physiological processes in cells.

The contents of cyclic nucleotides was measured by radioisotopic method at the laboratory of Nizhniy Novgorod Diagnostic center.
The contents of cyclic nucleotides in the liver of tumour-inoculated rats pM/g of tissue (M±m)

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>c-AMP</th>
<th>c-GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>754.83±19.02</td>
<td>9.25±0.40</td>
</tr>
<tr>
<td>Sarcoma -45 20 days</td>
<td>1057.83±74.04*</td>
<td>10.31±0.78</td>
</tr>
<tr>
<td>Sarcoma – 45 20 days ozone</td>
<td>760.67±8.31**</td>
<td>9.86±0.55</td>
</tr>
</tbody>
</table>

Note: * - p<0.05 compared with intact; ** - p<0.05 – compared with controls.
The contents of cyclic nucleotides in tumour-tissue (M±m)

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>c-AMP, pM/g of tissue</th>
<th>c-GMP, pM/g of tissue</th>
<th>cAMP/cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>837,500±34,908</td>
<td>24,496±1,777</td>
<td>39,303±7,193</td>
</tr>
<tr>
<td>Sarcoma – 45 20 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoma-45 20 days ozone</td>
<td>1288,667±131,116 *</td>
<td>10,842±0,880*</td>
<td>126,448±21,749*</td>
</tr>
</tbody>
</table>

Note: * - p<0,05 compared with intact;
** - p<0,05 – compared with controls
• The liver of experimental animals with 20 days of tumour development reveal 40% increase in c-AMP contents (p>0.05) with no significant changes in c-GMP level.

• ATP and GTP content in liver of experimental animals was found to be increased after 5 injections of ozonated saline.

• Activity of proliferative processes in tissues of Sarcoma-45 was decreasing. The fact being supported by decreased c-GMP levels and increased c-AMP/ c-GMP ratio.
• Levels of c AMP and c GMP can indirectly show the tendency and activity of cells physiological processes. In extreme conditions c-AMP contents in the cells of different organs increases in response to activation of hypothalamo-hypophys-adrenal system.

• In liver cells the cyclic nucleotide acts as an agent to mobilize internal potential of cells in order to provide active reactions of synthesis and energy generation.
• Correction of c-AMP contents in the liver of tumour-inoculated rats to the level of intact animals after 5 injections of ozonated saline can be explained by slowing down of compensatory reactions at the early stages of tumour development.

• It might be connected with sarcoma-45 weakening toxic influence on the organism and liver cells metabolism.

• Then in the process of growing tumour effect and the increase of c-AMP contents.
Conclusion

- Ozone can act trigger-like launching the main regulatory mechanisms:
  - 1. Optimization of pro- and antioxidant balance resulting in normalization of structure and function of cellular membranes and, hence, the activity of membranebuilt enzymes, receptors, canals and other protein structures.
  - 2. Activate H⁺-ATP-ase, to provide ATP important energy-dependent processes.
  - 3. Bring K⁺-Na⁺ and Ca²⁺-ATP-ase to normal range and to regulate distribution processes in inter- and intracellular space.
  - 4. Maintain proteolysis system at the level necessary to utilize nutritional proteins and dying tissues and to keep hemostasis system at an active level.
• Intraperitoneal injection of ozonated saline was characterized with dose-dependent response of proteolytic system, inhibitory antiproteolytic plasma potential.

• Doses of ozone (3,033 – 5,995 mcg), used in the course of 6 procedures done every other day, induce free radicals processes and decline antioxidant activity of the animal that is revealed in elevated activity of α1-antitrypsin and α2-macroglobulin, compensatory activation of kallikrein-kinin system. Increase of proteolytic activity in blood plasma, disbalance of kallikrein-kinin system and decline of α1-antitrypsin and α2-macroglobulin due to 6 ozone doses of 5,995 mcg become unfavourable prognostic sign, testifying of homeostatic disbalance in the organism.
• Estimation of proteolytic enzymes (trypsin-like and chymothripsin-like proteinases, elastase, kallikrein, kininase) can be used as a sensitive criterion to control efficacy and safety of ozonated saline.

• Experiments established ozone concentrations of 90 – 270 mcg/l of ozonated saline to have hypocoagulation effect, that is confirmed by longer period of coagulation, increase of anticoagulant and fibrinolytic activity. Concentration of 270mcg/l appeared to be a threshold between hypo – and hypercoagulative state.

• Ozonated saline with concentrations of 500 – 910 mcg/l was found to have pronounced procoagulation properties and results in acceleration of blood coagulation with simultaneous abrupt inactivation of anticoagulant activity.

• Ozone has its influence on secondary messengers that can be seen in c-AMP and c-GMP changes in the liver and tumour of sarcoma-45 inoculated animals.
• Blood incubation with ozonated saline, ozone concentrations being 130-270 mcg/l cause changes in the parameters of thrombocytic hemostasis by lowering the degree of thrombocytes induced aggregation.

• Ozonated saline with ozone concentrations of 500-910 mcg/l on the contrary, enhances aggregating capacity of thrombocytes.

• Hemostatic estimation in blood with hypercoagulation (patients with cardiac ischemic disease) done after the course of ozonetherapy (ozone concentration in ozone/oxygen gas mixture being 2000 mcg/l, that is equivalent to 270 mcg/l of saline) demonstrated normalization or plasma and thrombocyte parameters that correlated with the decrease in lipid peroxidation intensity.
Thank for attention!
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