Ozone as a Regulator of Physiological Processes in the Organism

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Abstract

An attempt to explain therapeutic efficacy of low doses of ozone due to its trigger properties has been made in the present article. The model of hypoxia in dogs has been used to reveal H^+-ATP-ase activation This enzyme provides the conjugation of respiration and oxidative phosphorylation. There has been demonstrated activation of K^+ -Na^+ -ATP-ase which is responsible for concentration gradient of K^+ and Na^+ in intra- and intercellular space and, hence, for electric potential at rest. Analysis of proteolytic system in rats showed moderate activation of trypsin, chemotripsin and elastase in the intestine and in blood but not in the pancreas. Activation of kallikrein-kinin system triggers a number of metabolic processes and of blood coagulation system in particular. Correction of secondary messengers -cAMP and cGMP was found with the use of oncogenesis model in rats.

Introduction

The recent years of ozonetherapy 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 15 years using different experimental model we have investigared possible ways to use ozone as (1) regulator of energetic processes, (2) control over ion and cation transpot, (3) proteolytic system of the organism, (4) -cAMP and cGMP being secondary messengers.

Materials and Methods

The first group of experiments (1) 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 15^{th} minute extracorporeal bood ozonation was performed in oxyginator connected with arterio-venous bypass (100ml of blood was barbotaged in oxyginator with ozone/oxygen mixture for 5 minutes, ozone concentration being 48 mcg/l). 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 ATP-dependent enzymes: 1) total Ca^{2+}-ATP-ase activity, that included enzyme of cytoplasmotic membrane, mitochondria and sarcoplasmotic reticulum, was assessed on the whole homogenate from myocardium tissue; 2) K^+ –Na^+ –ATP-ase activity was analyzed in cytoplasmotic fraction; 3) protonic H^+ –ATP-ase was estimated in mitochondria.

These fractions were defined by differential centrifugation. Incubation media to calculate Ca^{2+} -ATP-ase activity included: ATP (2 mm); $MgSO_4$ (2mm); tris-HCI (3mm); $CaCI_2$ (1,5mm). Incubation time was 20 minute at 37ºC.

Incubation media for K^+ –Na^+ –ATP-ase contained NaCI – 100 мМ, 3 мМ of ATP-Na_2 solution per 20 мМ KCL, 5 mM $MgSO_4$, 50 mM tric-HCL buffer (pH 7,4).Incubation time was 10 minutes at 37 ºC. Anzyme activity was calculated according to the variety of its action without oubain and with oubain (by adding 0,1 мМ into the media.)

Incubation to define protone ATP-ase was done for 20 minutes at 37 ºC in the media, that consisted of ATP (2 мМ), $MgCl_2$ (2 мМ), tric-HCL (3 мМ). Hydrolysis was stopped by adding $HCLO_4$. ATP-ase activity was measured according to the amount of non-organic phosphorus accumulated as a result of ATP hydrolysis with ATP-ase influence, and expressed in P mM/ protein/ hour.

The second group of experiments (2) 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 (36) were used as control ones; 2- experimental rats (205).

The animals of the experimental subgroup received 1ml of ozonated saline 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 controled in gaseous phase by spectrophotometer with the wavelength of 254 nm.. Ozone consentration in saline was calculated by unified method of iodometric titration. Single ozone doses were: 0,005; 0,027; 0,053; 0,505mcg. To study cumulative effect of ozone doses of 0,184; 0,505; 1000 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 and of small intestine.

The examined tissues were analysed for:

1.trypsin-like ptoteinase activity by Erlanger method (1961). The method is based on trypsin capacity to lyse synthetic substrate N-α-benzoyl-arginin-paranitroanilin with formation of benzoyl-L arginin and yellow-coloured paranitroaniline. Saline light absorption was measured with spectrophotometer with wavelength of 410 nm in experimental tubes opposed to control ones.

2.Chymotripsine-like proteinase activity was measured with the use of Glp-Phe-pNa substrate with pH 7,7. The initial concentration of substrate in dimethylformamide was 20 mg/ml.

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.

The activity of kinin-destroying enzyme was studied with the help of peptic substrate Glp-Ala-Ala-Leu-pNa. Its activity in plasma and in homogenates of the pancreas and of the small intestine was studied and revealed with Leu-pNa substrate.

5.To calculate the quantity of total protein a set of ready-made liquid reagents by “Dia Sis”(Germany) was used as well as Statfax» (Awareness Technology INC) device.

6.A complex method was used to measure the activity of tripsin-like proteinase, α1- antitripsin and α2- macroglobulin in blood plasma (Карягина, 1990).

The third group of experiments (3) 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 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 experimenal series. Citrated plasma was studied both rich and poor in thrombocytes. Besides, washed erythrocytes were used. The condition of hemostasis was aseessed according to plasmotic and thrombocytic hemostasis.

The extended coagulogramme included:
1) Indices of the 1st phase of coagulation - partially activated thromboplastin time, activated recalcification time;
2) Indeces of the 2nd phase of coagulation- protrombin index, trombin time;
3) The main indices of the final stage of coagulation – fibrinogen and factor XIII-fibrin-stabilizing concentrations.

Anticoagulation activity was estimated by antitrombin III (AT-III) activity index**.** Fibrinilytic 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 dessiminated intravascular coagulation (DIVC). Analysis of the throbocytic part included calculation of thrombocytes, their aggregation capacity, induced with ADP, adrenalin, ristomicin with the use of aggregometer «Trimlite».

The fourth group of experiments (4) concerned experimental oncogenesis. Sarcoma-45 was used as the main model. Its strain was delivered from the Institute of Experimental Oncology (Oncological Research Center named after N.N, Blokhin, Moscow). The model of neoplasia was done by reinoculation of the tumour strain to rats. 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. During the experiment repeated reinoculations were done from tumour-inoculated animals on the 10-14th day of sarcoma-45 development (10 animals).

The work also presents estimation of the levels of cyclic nucleotides in liver tissues. Cyclic nucleotides cAMP and cGMP participate in regulation of biochemical and physiological processes in cells. The contents of cyclic nucleotides was measured by radioisotopic method with the use of cGMP [^{125}I], cAMP [^{125}I] Amersham" sets at the laboratory of Nizhni Novgorod Diagnostic center.

The results received in the course of the experiment were assessed with the methods of variational statistics with calculation of average arythmetics value (M), average mistake of the average arythmetics value (m), Student criterium (t) and significance level (p) done with the help of Microsoft Excel on a personal computer Celeron 466.

Results and discussion

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 restores carriers. Thus, saturation with oxygen, being more intensive compared with control series without ozone, provides the conditions to generate a greater value of $\Delta \mu H^+$. $\Delta \mu H^+$ 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). (Fig.1).

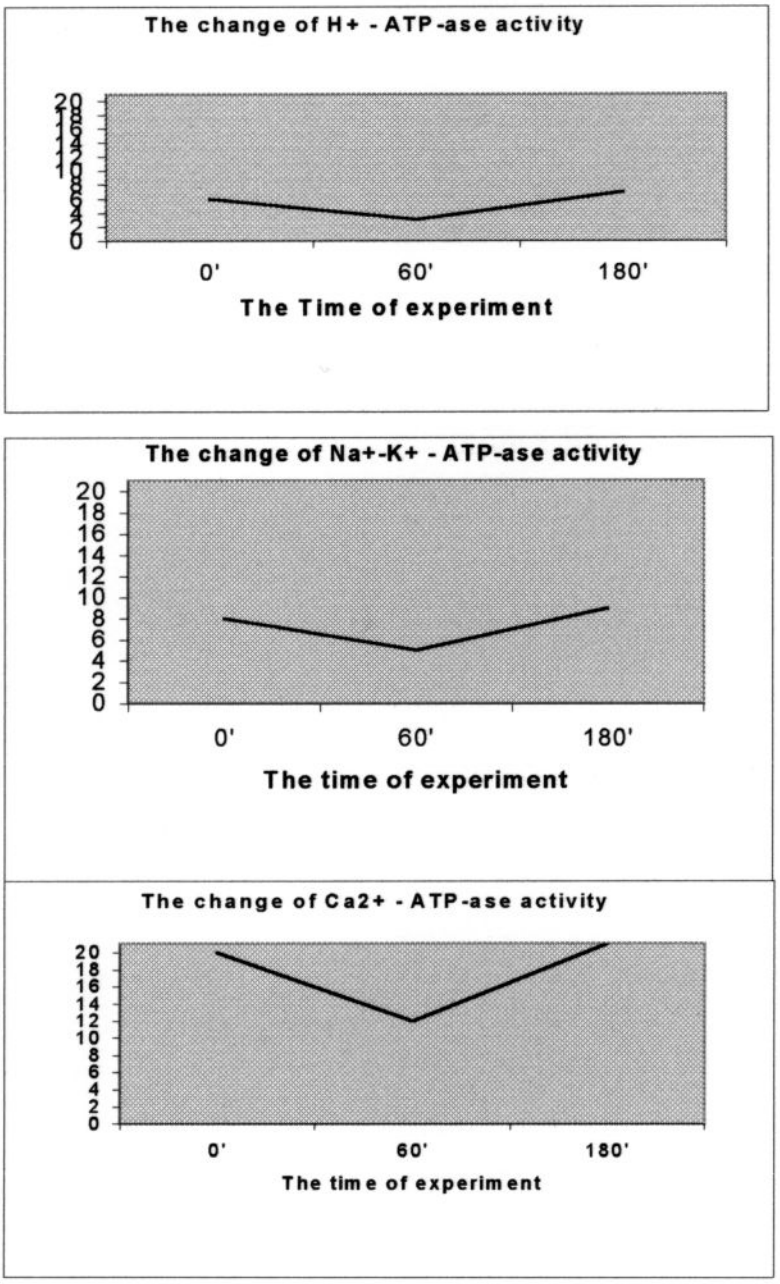

Fig.1. Changes in ATP-ase activity in experiments with hypovolemic hypotension (mcM P/ g of protein)

Increase in H^+ –ATP-ase activity can satisfy the miocardium need in ATP to maintain the cantractive activity and synthetic processes, damaged by hypoxia, induced by hemorrhagis shock. Correlation coefficient of H^+ –ATP-ase activity/ATP level was 0,76 ($p<0,001$).

Normalization of oxidative phosphorylation due to ozone contributed to normalization of ATP level, and hence, to ATP-ase transport activity. Correlation coefficients between K^+ –Na^+ – ATP-ase and Ca^{2+} ATP-ase are valid at the stage of experiment ($p<0,001$*).*

Figure 1 shows the change in the K^+ –Na^+ –ATP-ase activity of cytoplasmotic membranes in vivo. In hypoxia the K^+ –Na^+ –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. 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 oryginal one.

The results of the second group of experiments done on rats show, that ozonated saline with single ozone doses of 0,027-0,053 mcg produce a positive effect on proteolytic system. The marked elevation of the main proteolytic inhibitors testify the switching on of the compensatory mechanisms that launch physiological processes of adaptation (Table I). Six doses of ozone (1,104-5,995 mcg) produce significant reinforcement of proteolytic activity in the experimental animals and decrease of α1-antitrypsin and α2-macroglobulin activity in blood plasma.

Table I.Inhibitors antiproteolytic activity of blood plasma (M±m)

Observation dynamics	Doses of ozone Mcg	α1-antitrypsin	α2-macroglobulin
Intact	0,027	8,89±0,04	0,88±0,03
2 hours		8,96±0,08	1,15±0,06*
24 hours		9,34±0,08*	1,10±0,05*
48 hours		8,78±0,07	0,87±0,06
Intact	0,053	8,63±0,09	0,88±0,05
2 hours		9,18±0,10*	1,35±0,09*
24 hours		8,95±0,06*	0,69±0,07
48 hours		8,88±0,12	0,87±0,06

Note: * Valid differences concerning intact series ($P<0,05$).

Proteinas-inhibitory disbalance in multiple ozone introduction is caused by unlimited proteolysis leading to serios disorders in a number of important homeostasis regulation systems and to excess protein accumulation in liquid media of biologically active products of protein degradation. 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 1,6-fold trypsin-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 (RAAS). 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 series of experiments to study ozone effect on homeostasis gave the following results.

The first expeimental group contained blood samples of actually healthy personsand, 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 artherosclerosis 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 extention 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 thrombingenesis that is manifested in 2-fold increase of TT in blood plasma, formation of fibrinogen-degradating products (FDP). It is connected with two main points – accumulation of great amount of fibrin-monomeric complexes that are easily lysed with plasmin and augmention of plasmin in plasma. All this results in reinforcement of fibrinolytic blood activity. Ozone concentration of 270 мcg/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. 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 throbocytic hemostasis. Ozone concentrations of90 - 270 мсg/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 мсg/l produse significantactivation of thrombocytes aggregation. In both cases the concentration of blood platelets did not decrease.

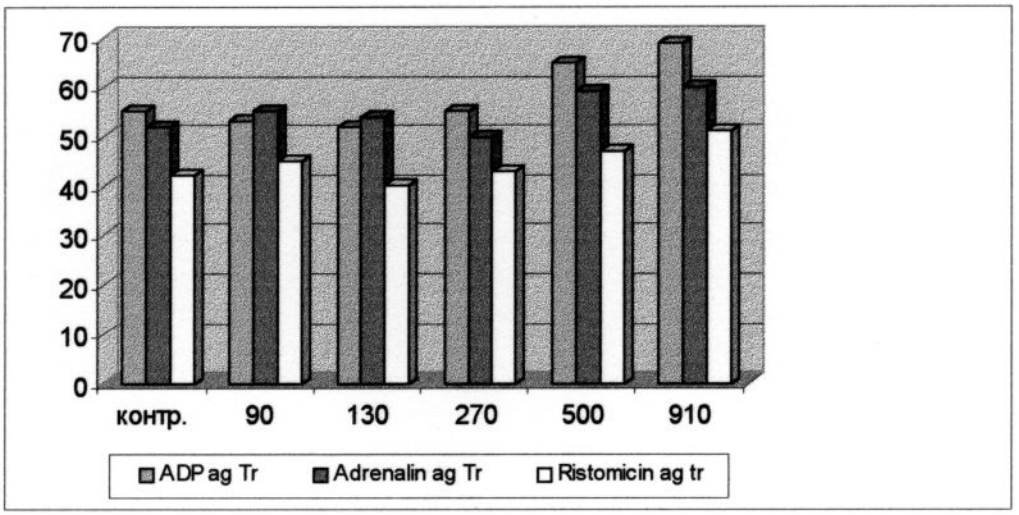

Fig.2 Dose-dependent ozone effect on induced aggregation of thrombocytes in blood with normal and hypercoagulation.

Completely different result was received when ozonated saline was added into the blood with hypercoagulation. Even insigficant ozone doses of 130 мсg/l do not decrease thrombocytes aggregation. Ozone in high concentrations results in significant hyperaggregation of thrombocytes.There was found valid reverse correlation between SB level in erithrocytes and catalase activity.

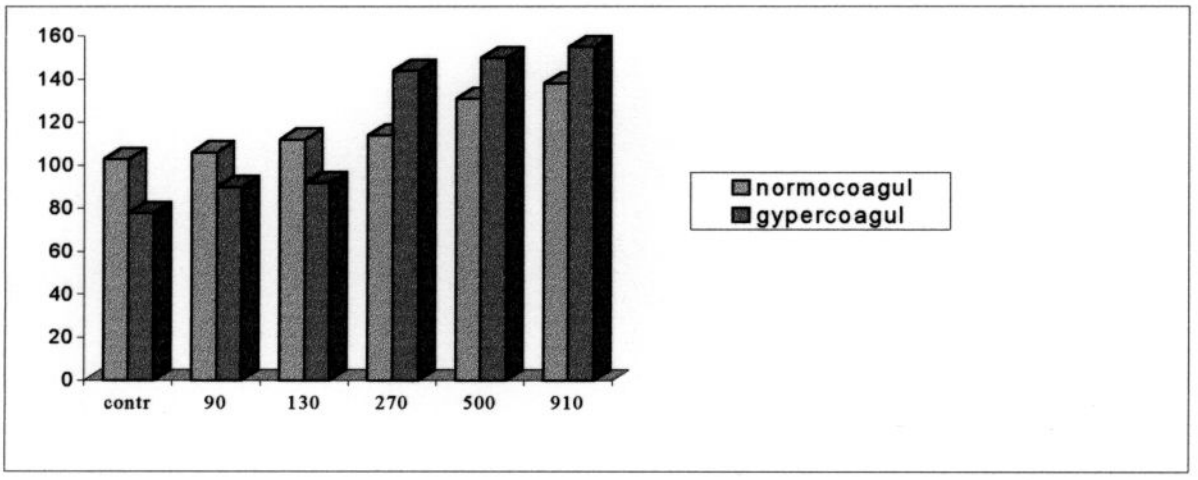

Fig.3 Changes in fibrinilytic activity in incubation of blood with normal and hypercoagulation with ozonated saline. Ozone concentrations are different.

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 erythrocyte membranes. Thrombocytes activation can be caused by hydroperoxides of polynonsaturated fatty acids generated free radical aytooxidation of phospholipids.

The results received in the fourth series of experiments to study the contents of cyclic nucleotides in the liver of tumour-inoculated rats before and after ozonation are presented in Table 2 and Table 3.

Table 2. The contents of cyclic nucleotides in the liver of tumour-inoculated rats
pM/g of tissue (M±m)

Group of animals	c-AMP	c-GMP
Интактные	754,83±19,02	9,25±0,40
2.1 – S-45 – 20	1057,83±74,04*	10,31±0,78
2.2 – S-45 – 30	845,07±35,22*	10,88±0,34*
2.1 – S-45 – 20 – ОФР	760,67±8,31**	9,86±0,55
2.2 – S-45 – 30 – ОФР	1323,93±71,50*,**	9,96±0,20

Note: * - p<0,05 compared with intact;
** - p<0,05 –compared with controls.

Table 3.The contents of cyclic nucleotides in tumour-tissue (M±m)

Group of animals	AMP, pM/g of tissue	GMP, pM/g of tissue	cAMP/cGMP
Control 2.1	837,500±34,908	24,496±1,777	39,303±7,193
Control 2.2.	1116,000±76,215	25,817±1,265	43,611±3,132
Experimental 2.1	1288,667±131,116*	10,842±0,880*	126,448±21,749*
Experimental 2.2	1061,000±47,071	21,179±1,370*	50,640±2,222

Note:* - p<0,05 –compared with controls.

The liver of experimental animals with 20 days of tumour development reveal 40% increase in cAMP contents(p>0,05) with no significant changes in cGMp level. The second control group of animals with 30 days of sarcoma-45 development showed decrease in cAMP compared with control group 2.1 but was 12% higher compared with normal range (p<0,05).

Levels of c AMP and c GMP can indirectly show the tendency and activity of cells physiological processes. In extreme conditions cAMP contents in the cells of different organs increases in response to activation of hypathalamo-hypophis-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 energygeneration.

Correction of cAMP 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.activate H^+ - ATP-ase,to provide ATP important energy-dependent processes.
2.bring K^+ - Na^+ and Ca^{2+} - ATP-ase to normal range and to regulate distribution processes in inter- and intracellular space.
3.maintain proteolysis system at the level necessary to utilize nutritional proteins and dying tissues and to keep hemostasis system at an active level.

Intraperotoneal 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 proleolytic enzymes (trypsin-like and chymothripsin-like proteinases, elastase, kallikrein, kininase and leucinaminopeptidase) can be used as a sensitive criterium to control efficacy and safety of ozonated saline.

Experiments in vitro 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 fibrinilytic activity. Concentration of 270mcg/l appeared to be a threshhold 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. Blood incubation with ozonated saline, ozone concentrations being 130-270 mcg/l cause changes in the parameters of thrombocytic homostasis by lowering the degree of throbocytes 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 LP intesity.

Ozone has its influence on secondary messengers that can be seen in cAMP and cGMP changes in the liver and tumour of sarcoma-45 inoculated animals.

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