

**NANOTECHNOLOGY AND NANOMATERIALS: USE OF VESICULAR  
NANOSYSTEMS FOR ISCHEMIC HEART INJURY REPAIR**

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**Abstract**

*In the present work studied has been the effect of preliminary systemic introduction of liposome preparations of various composition on the contractive activity of the isolated rat heart. Some antioxidants and high-energy phosphates were introduced into the liposomes. The liposomal preparations were injected via the caudal vein – 15 injections of 25 mg lipid/kg. The intermission made up 48 hours. The hearts were isolated, and total ischemia was reproduced in them. The maximal contractive force was registered; the presents and duration of the perfusate the lactate dehydrogenase activity was determined. It was shown that the liposomes with antioxidants and high-energy phosphates have a manifested protective effect by ischemia and subsequent reperfusion.*

**Key words:** liposomes, ischemia, reperfusion

**1. INTRODUCTION**

At present, there are, at least, two main mechanisms, which are thought to lead to irreversible ischemia-reperfusion cell injury.

First of all, it is the decrease in myocardial macroergic phosphates concentration less than the lower critical value (about 4  $\mu\text{mol/L}$  dry weight), which results in an irreversible cell metabolism impairment (see for example Kuzmin A.I et al., 1998, Verma D.D. et al., 2005). Secondly, it is the cell membrane damage due to phospholipases and lipid peroxidation processes activation and, consequently, the loss of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ionic gradients (see for example Allen D.G. et al., 1985, Hudeto Nagami et al., 2005, Nao-omi Omoi et al., 2006, O'Rourke Brain, 2000). Therefore, the modification of obtained liposomes was aimed at loading them with macroergic phosphates in order to restore the cell deficit or with the antioxidant defense system substances. The lipid bilayer contents were chosen based on the fact that hypoxia caused a decrease in phosphatidylcholine concentrations due to phosphatidylcholine conversion into lysophospholipids (see for example Nao-omi Omoi et al., 2006). Lipid bilayer cholesterol concentrations were chosen based on the fact that lecithin-cholesterol liposomes absorption and their distribution in the intracellular fractions depended on the 'hardness' of the lipid bilayer (see for example Caride V.J. et al., 1977, Ferrari R. et al., 1989).

It was assumed that the liposomal membrane phase could act as a donor of phosphatidylcholine and, possibly, elementary phospholipid membrane fragments for cellular organelles injured by ischemia. Macroergic phosphates could compensate for their cell deficit and antioxidants could improve cellular antioxidant defense system.

## 2. MATERIALS AND METHODS

Thus, the study used 3 types of liposomes: empty liposomes (EL), liposomal macroergic phosphates (LMP) and liposomal antioxidants (LA) (Table 1). LMP had a similar membrane to the EL, but their inner compartment contained adenosine triphosphate (ATP) ('Reanal', Hungary) and phosphocreatine (PCr) ('Reanal', Hungary) dissolved in the NaCl solution. The lipid bilayer of LAs contained  $\alpha$ -tocopherol ('Sigma-Aldrich', USA) and their inner compartment was loaded with superoxide dismutase (SOD) ('Sigma-Aldrich', USA) and reduced glutathione (GSH) ('Reanal', Hungary). Krebs-Henseleit buffer became the outer liposomal compartment.

**Table 1.** Liposomal contents in the given experiment

| Type of liposomes                     | Lipid bilayer      |             | Liposomal inner compartment    |
|---------------------------------------|--------------------|-------------|--------------------------------|
|                                       | Contents           | Molar ratio |                                |
| Empty liposomes (EL)                  | PC:Ch              | 7:5         | 0.9% NaCl                      |
| Liposomal macroergic phosphates (LMP) | PC:Ch              | 7:5         | 0.9%NaCl+2%PCr+2%ATP           |
| Liposomal antioxidants (LA)           | PC:Ch: $\alpha$ TP | 7:5:0,5     | 0.9%NaCl+2mg/mlSOD+2 mmole GSH |

PC indicates egg phosphatidylcholine; Ch, cholesterol; PCr, phosphocreatine;  $\alpha$ TP,  $\alpha$ -tocopherol; SOD, superoxide dismutase; GSH, reduced glutathione.

Liposomes were prepared by forcing suspensions of lipids through polycarbonate filters with the LIPEX extruder (Biomembranes Inc. Canada). Before the extrusion a rotary evaporator was used in order to obtain a film of lipids with the necessary contents using benzol as a solvent. The film of lipids was then hydrated with an aqueous vehicle and shaken to obtain multilamellar vesicles. The vesicles were then subjected to a freeze-thaw procedure repeated 10 times to improve hydration. The obtained suspension was forced through polycarbonate filters (Costar, Japan) with a pore size of 100nm. The purification of liposomes was done by gel-filtration using a sterile column filled with Sepharosa 2B (Sigma). Liposome-containing fraction was diluted in NaCl until the lipid concentration reached 25 mg/ml.

The liposomes were administered either intracoronary into an ischemized part of the myocardium or into the tail vein of the experimental animals (see for exemple Caride V.J. et al., 1977, Verma D.D. et al., 2005, Zhuravleva I.Y. et al., 1995).

The experiments were conducted on 40 *mature Wistar male rats weighing 250-300g. The rats were divided into 4 groups (Table 2). The first (control) group was administered with 0.9% NaCl (1ml/kg); the second group, with EL; the third group, with liposomal macroergic phosphates and the fourth group, with liposomal antioxidants (Table 2). The liposomal drugs were injected into the tail vein (15 injections at 48h intervals) in an amount of 25 mg of lipids/kg.*

**Table 2.** Experimental grouping

| Groups                          | Controls (without liposomes) | Empty liposomes | Liposomal macroergic phosphates | Liposomal antioxidants |
|---------------------------------|------------------------------|-----------------|---------------------------------|------------------------|
| Number of rats in the group (n) | 10                           | 10              | 10                              | 10                     |

24 hours after the last injection the rats had their chest opened under general anaesthesia with tiopental, their heart was quickly taken, arrested in a cooled Krebs-Henseleit buffer and Langendorf-perfused in the same buffer, which had been previously gassed with O<sub>2</sub> (95%) and CO<sub>2</sub> (5%).

After 20 minutes of perfusion the perfusate-containing line was completely blocked, so that 30-min total normothermic ischemia could be reproduced. Then perfusion was resumed and after 10 minutes of reperfusion 1 ml of Krebs-Henseleit buffer, containing 10<sup>-8</sup>M adrenaline, was injected through the aortic cannula.

An ECG machine with silverchloride electrodes for isolated heart study (TOO 'Topaz', Russia) was used to record the ECG and the contractile force of the heart (with a photoelectric isometric force transducer).

A force curve showed the tension force of the heart on the aortic cannula, maximum contractile force (F<sub>max</sub>), maximum contractile force increase rate (MCFIR), and maximum relaxation rate (MRR) of the heart on the aortic cannulae. After 15 minutes of perfusion the tension force was increased from 0 to 2 g and was maintained at that level until the start of prolonged ischemia, then at the 10<sup>th</sup> minute of the reperfusion period it was reset at 2g.

An electrocardiogram was used to assess the duration of arrhythmia during reperfusion.

The outflowing perfusate was biochemically measured for lactate dehydrogenase (LDG) activity.

SPSS Statistics 6.0 software was used for statistical processing of the data. In order to describe the variables distributed other than normal a median and an interquartile range (25<sup>th</sup> and 75<sup>th</sup> percentiles) were indicated. The analysis of variance (ANOVA) was used for independent samples. The differences were considered to be significant when p value was less than 0.05.

### 3. RESULTS AND DISCUSSION

All the liposome types in this experiment had an impact on the myocardial contractility, the duration of reperfusion arrhythmias and LDG release into the perfusate after an ischemia-reperfusion cycle. 30-min normothermic ischemia and subsequent reperfusion resulted in a significant myocardial function impairment in the control group. Indeed, this group of rats showed the most significant (p<0.05) decrease in the contractile force by 60% (Figure 1), the longest arrhythmias (Figure 2) and a 1.5-2 times LDG activity increase in the outflowing perfusate compared with the other groups (Figure 3). This was mainly due to the activation of free radical oxidation processes, in particular lipid peroxidation, with a decreased activity of antioxidant systems. These processes cause cell membrane damage, calcium hemostasis and enzymatic systems damage, which, in turn, results in a significant myocardial contractility reduction and arrhythmias.

Free radical oxidation in normal individuals is known to be neutralized by antioxidant systems. For example, SOD can catalyze the dismutation of

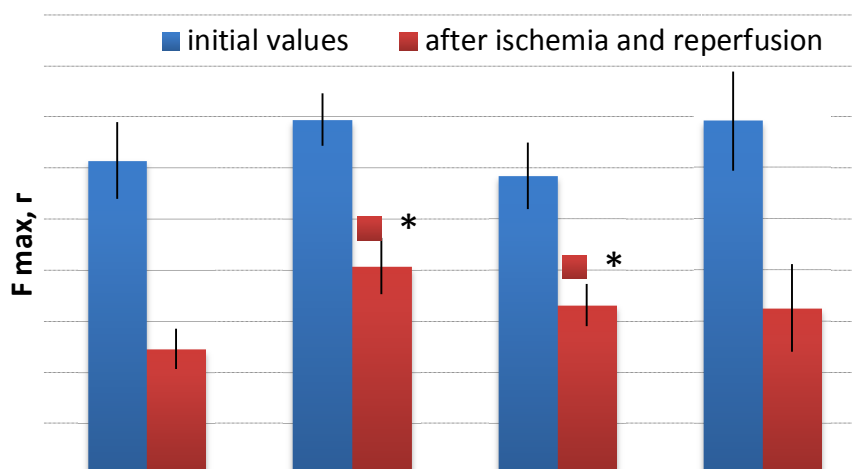


**Fig. 1.** Maximum heart contractile force (F max) after the pretreatment with systemic liposomal drug injection, 30-min ischemia and subsequent 10-min reperfusion.

\*  $p < 0.05$  compared with the controls.

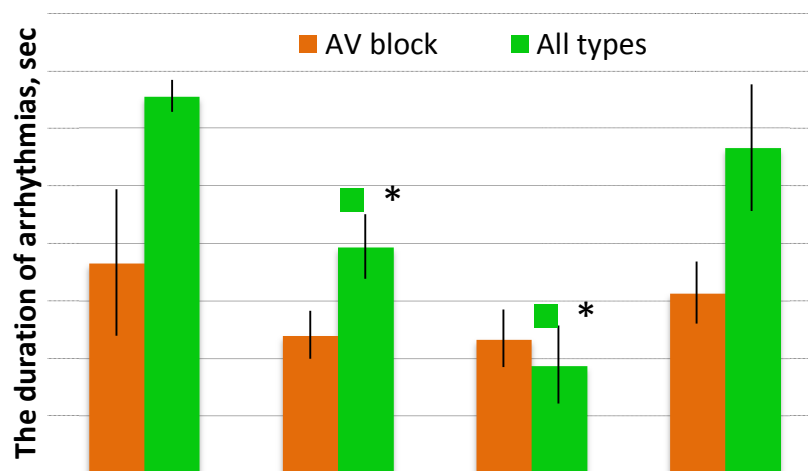


**Fig. 2.** The duration of reperfusion arrhythmias and conduction impairment after the pretreatment with systemic liposomal drug injection, 30-min ischemia and subsequent 10-min reperfusion. AV block – atrio-ventricular block.

\*  $p < 0.05$  compared with the controls.

superoxide to hydrogen peroxide. Hydrogen peroxide is further reduced by either catalase or glutathione peroxidase (GP). The latter can be used as a cofactor for glutathione peroxidase.  $\alpha$ -tocopherol is also known to have high antioxidant activity (see for example Ferrari R. et al., 1989). This activity neutralizes both oxygen radicals and cytotoxic fatty acid radicals and forms a

tocopheroxile radical, which reacts with another tocopheroxile radical either producing a dimer or being neutralized by ascorbic acid.

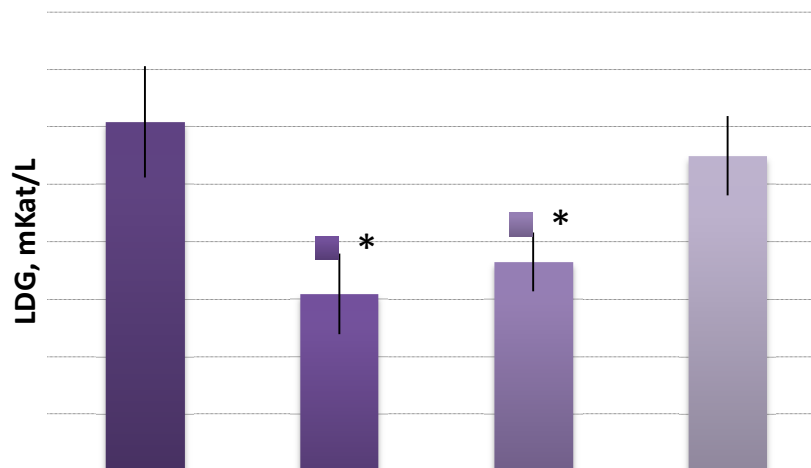


**Fig. 3.** LDG activity in the perfusate after the pretreatment with systemic liposomal drug injection, 30-min ischemia and subsequent 10-min reperfusion.

\*  $p < 0.05$  compared with the controls.

However, during ischemia-reperfusion the antioxidant system activity is significantly reduced due to the loss of energy, free radical-induced enzyme damage and the decrease in kinetic properties of the membrane-linked fractions of the antioxidant enzymes. Therefore, preliminary administration of additional liposomal SOD, GT, and  $\alpha$ TP markedly prevented ischemia-reperfusion injury to an isolated heart. This is shown by a significant ( $p < 0.05$ ) 1.5-times increase in myocardial contractility compared with the controls (Fig. 1), a significantly less duration of arrhythmias (Fig. 2) and a significant ( $p < 0.05$ ) two-fold reduction in LDG activity in the outflowing perfusate compared with the controls (Fig. 3). Of note is that all the groups, except the one with liposomal antioxidants administration, developed a post-ischemic myocardial contracture of various severity.

Lipid peroxidation development during ischemia-reperfusion causes ADP phosphorylation, ATP transport and utilization impairment. Additionally, a myocardial contractility gets decreased because contraction and relaxation processes greatly depend on ADP and ATP metabolism. In turn, ATP synthesis impairment leads to the build-up of cell hydrolysis products, which have prooxidant action. Energy deficit leads to reduced antioxidant systems activity. This pathological cycle worsens myocardial injury and further reduces myocardial viability.

The pretreatment with liposomal macroergic phosphates was observed to reduce arrhythmias, increase the contractile force by 20% and decrease LDG activity 1.5 times. However, the obtained data demonstrate that liposomal ATP and PCr were less effective in preventing reperfusion-induced contractile function impairment in an isolated heart than liposomal antioxidants.

Additionally, liposomes per se have a slight cardioprotective effect. Indeed, the empty liposomes group showed a tendency towards an increased contractile force, less durable reperfusion-induced arrhythmias and less active LDG unlike the same factors in the controls. The impact of empty liposomes can be explained by a liposomal ability to absorb toxic metabolites and lipid peroxidation products. It can be also assumed that liposomal phospholipids compete with membranes in free radical

reactions of lipid peroxidation, thus, protecting biological membrane structure, or can serve as a 'construction material', which also promotes better preservation of cell structures in ischemia-reperfusion.

At present, it is uncontroversial that a significant increase in myocardial catecholamine concentrations is one of the most important alternative pathogenetic factor of ischemia-reperfusion cell damage, especially if the endocrine and autonomic nervous system continue regulate the activity of the heart. Adrenaline is known not only to stimulate myocardial contractility but also to activate lipid peroxidation. In turn, the activation of lipid peroxidation processes can decrease a stimulating impact of adrenaline on myocardial contractility. Antioxidants-containing liposomes help decrease this negative effect of adrenaline. Indeed, after adrenaline administration at the 10<sup>th</sup> min. of reperfusion myocardial contractility parameters in group 4 were much higher than those in groups 1 and 2 (Fig 4). Pretreatment with liposomal antioxidants is likely to protect not only an isolated heart from reperfusion injury but also the heart inside the body where its activity is regulated by the other systems.

Additionally, a significant increase in contractile force in response to adrenaline suggests that cAMP-dependent mechanisms causing contractile force increase are sustainable and potent in the hearts treated with liposomal antioxidants compared with those not treated with liposomal drugs (Fig.4). The increase in this parameter can also suggest better sustainability of cellular metabolic processes because, at least partially, the effect of cAMP on the myocardium is due to the cellular energy metabolism activation.

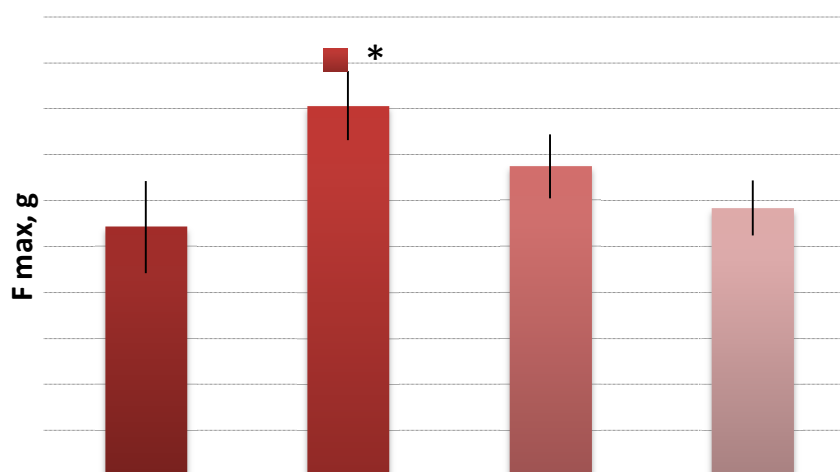


**Fig 4.** Maximum contractile force after adrenaline administration in the hearts pretreated with systemic liposomal drugs injection, after 30-min ischemia and subsequent reperfusion.

\* -  $p < 0.05$  compared with the controls.

As for maximum contractile force increase rate, all the groups showed significantly different MCFIR after ischemia-reperfusion cycle compared with the baseline ( $p < 0.05$ ) (Fig. 5). For example, post-ischemic MCFIR in the controls made up 63.1% of the baseline value ( $p < 0.05$ ) and 72.6% ( $p < 0.05$ ) and 60.1% ( $p < 0.05$ ) in the EL and LPM groups, respectively. In the LA group, MCFIR made up 75.5% of the baseline value ( $p < 0.05$ ), which was 33.1% ( $p < 0.05$ ) higher than in the controls.

Similar dynamics was observed in maximum myocardial relaxation rate characteristics (Fig.6). In the controls, MRR after ischemia-reperfusion made up 43.6% of the baseline ( $p<0.05$ ). MRR in the EL and LMP groups made up 57.5% ( $p<0.05$ ) and 53.1% ( $p<0.05$ ), respectively. In the LA group MRR after ischemia-reperfusion cycle did not differ significantly from the baseline and made up 76.3%, which is 44.5% greater than the same value in the controls ( $p<0.05$ ).

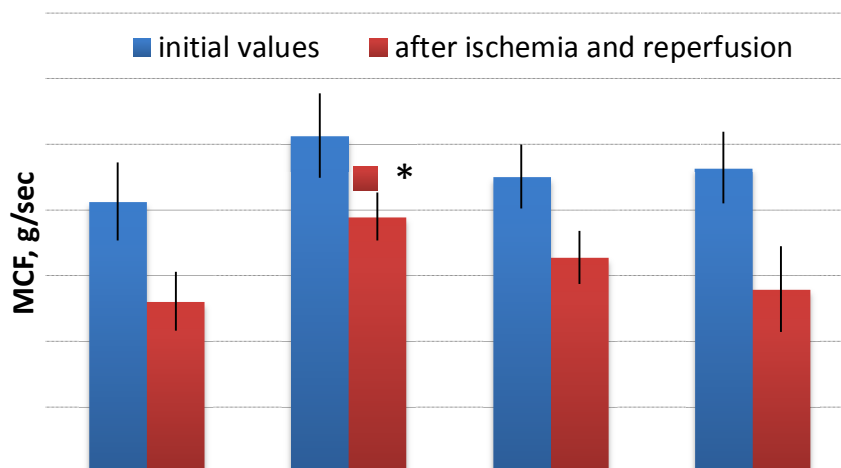


**Fig 5.** Maximum contractile force increase rate in the hearts after the systemic pretreatment with liposomal drug injection, 30-min ischemia and subsequent reperfusion.

\* -  $p<0.05$  compared with the controls.

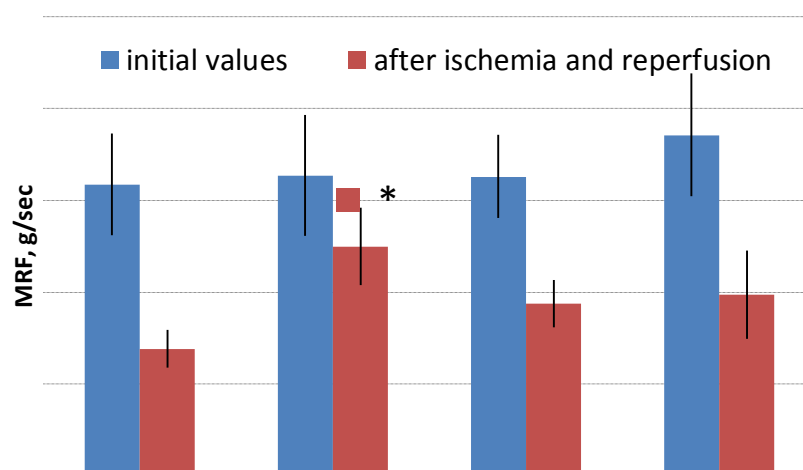


**Fig 6.** Maximum relaxation force rate in the hearts after the systemic pretreatment with liposomal drug injection, 30-min ischemia and subsequent reperfusion.

\* -  $p<0.05$  compared with the controls.

Thus, MCFIR and MRR in the LA group, first of all, showed that the ion transport mechanism, in particular,  $\text{Ca}^{2+}$  ions, through cardiomyocyte and sarcoplasmic reticulum membrane was preserved. And, finally, this explains a better sustainability of physical and mechanical properties of the hearts, less durable reperfusion arrhythmias and a myocardial contracture absence in this group.

The analysis of the obtained results show that the pretreatment with systemic liposomal antioxidants and macroergic phosphates injection can be useful to reduce ischemic and reperfusion injury. At present, there are studies devoted to myocardial protection with SOD, GT,  $\alpha\text{TP}$  and ATP without liposomes. Though these substances reduced myocardial injury, they still caused a range of side effects. This can be related to the issue of the drug delivery to target-organs in a body.

In the above-mentioned studies of an isolated heart the drugs were either added to the perfusate or to a cardioplegic solution. However, it is known that SOD interacts with substrates and other proteins in the blood flow. If SOD molecules still reach the target region, an issue of their transport into a cardiomyocyte cytoplasm emerges, because high molecular weight proteins are known to enter the molecule only through endocytosis. ATP and PCr are also metabolized in the blood flow and have a difficulty entering cardiomyocytes (see for example Gordon J.L. 1986). The most  $\alpha\text{TPs}$  are accumulated in the liver and endocrine glands (see for example Ferrari R. et al., 1989).

However, the issue of drug delivery to the affected organs can be successfully addressed by incorporating those drugs into a lipid layer or an internal liposomal phase. The most important liposomal property allowing them to transport medications is their ability to enter the cytoplasm by endocytosis or mix with a cell membrane and so liposomal drugs, including high molecular weight compounds, can get inside the cell.

Although all the studied liposomal drugs reduce ischemia-caused impairment of a contractile function of an isolated heart to a greater or lesser extent, intravenous pretreatment with liposomes, containing antioxidants, caused less reduction in the contractile force of the heart after total ischemia and reperfusion, compared with the controls, and led to a significant increase in the contractile force in response to adrenaline and less durable reperfusion arrhythmias. Additionally, pretreatment with liposomal antioxidants resulted in LDG activity decrease in the outflowing perfusate.

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