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INCLUSION AND AND INTRACELLULAR DISTRIBUTION OF LIPOSOMES IN THE MYOCARDIUM

Rinat A. Mukhamadiyarov[1], Alexey V. Veremeev[1], Igor G. Khaliulin[2], Irina Yu. Zhouravleva[1]

[1] Research Institute for Complex Issues of Cardiovascular Diseases under the Siberian Branch of the Russian Academy of Medical Sciences

6 Sosnovy blvd. Kemerovo 650002 Russian Federation, rem57@rambler.ru

[2]University of Bristol, School of Clinical Science, United Kingdom, Level 7, Bristol Royal Infirmary, Bristol, BS2 8HW

Abstract

The main aim is study of the inclusion and subcellular distribution of liposomes in a myocardium. The optimal results of liposomes distribution with a ratio phosphatidylcholin/cholesterol – 2/1 were observed. The maximal inclusion of a label in a myocardium was observed using the liposomes in concentration of 10 mg/ml. This concentration of liposomes allows to avoid any negative influence on myocardial functioning after ischemia and reperfusion.

Key words: *liposomes, ischemia, reperfusion, myocardium, intracellular distribution*

1. INTRODUCTION

The most common and promising application of liposomes in biology and medicine is to create a liposomal forms of drugs. Widespread attention is paid to the question of application of liposomes for correction of ischemic organ damage, particularly infarction. However, the issue targeted delivery, including liposomes into the myocardium and their subcellular distribution remains open.

In this connection, this study investigated the inclusion and distribution of liposomal drugs in the myocardium during ischemia and subsequent reperfusion.

2. MATERIAL AND METHODS

In our study we used multilamellar vesicles prepared with phosphatidylcholine and cholesterol at different molar ratios (Table 1). Two types of liposomes were made, namely, "hard" liposomes with phosphatidylcholine-cholesterol molar ratio of 7:5, and ""soft" liposomes with phosphatidylcholine-cholesterol ratio of 2:1.

Table 1. Specifications of liposomal formulations

Types of liposomes	Concentration of liposomes, mg/ml	
"soft"	10 mg/ml	10 mg/ml
"hard"	25 mg/ml	25 mg/ml

Liposomes were prepared by ultrasonic dispersion using the UZDN-1 disintegrator. The time scoring was determined under the optical density of the solution at a wavelength of 400 nm. Sufficient value of optical density was regarded at a certain critical point L, after which a further decrease in the value of the optical density is not greater than one-hundredth (Fig. 1). The volume of the liposome suspension was 6 ml.

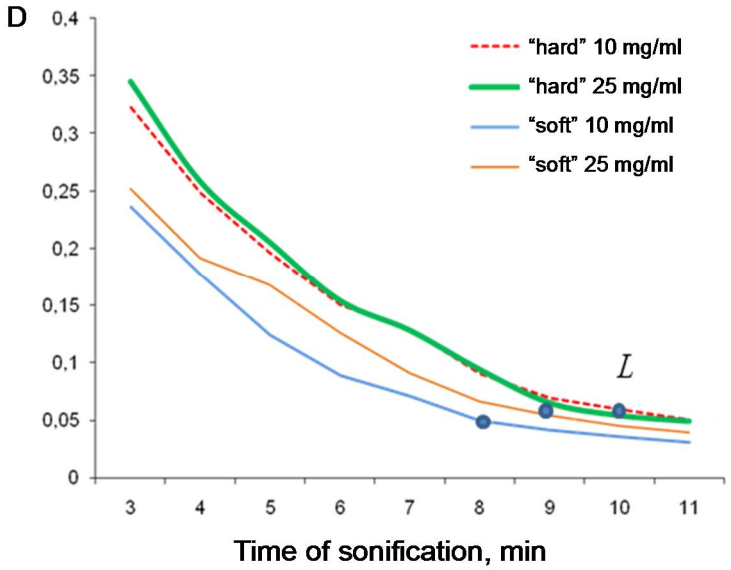


Fig. 1. The dynamics of the optical density over time for different types of liposomes

The optimal time for the scoring of "hard" and "soft" liposomes was 10 and 8 minutes at the concentration of 10 mg/ml, respectively. At the concentration of 25 mg/ml the optimal time for the scoring was 9 minutes for both types of liposomes.

Phosphatidylcholine, labeled with tritium with a specific activity of 1 MCU/mL ("Biosan", Russia) was used as a radioactive tag. Phosphatidylcholine was introduced to liposomes at the stage of the lipid film.

The experiment was carried out on male rats of Wistar weighing 270 ± 20g. General anesthesia was performed using the thiopental. Thereafter, the heart was dissected at the level of the aortic arch and placed in a Krebs-Henseleit solution cooled to 4^0C (see for example Bell R.M. et al., 2011). After stopping, the heart was fixed on the aortic perfusion cannula system. At the same time, the retrograde perfusion was carried out using the oxygenized Krebs-Henseleit solution, according to Langendorff. After 15 minutes, we switched to the antegrade perfusion through a cannula introduced into the left atrium, according to Neely (see for example Qu Y. et al., 2013).

At the 10th minute of the antegrade perfusion, we recorded the value of cardiac output (CO). At the 15th minute of the antegrade perfusion, we simulated ischemia by clamping all perfusate-carrying arteries. Thereafter, we put the heart into the chamber filled with Krebs-Henseleit solution at 40°C, 14°C or 37°C. The value of CO was used as a criterion of the adequacy of the myocardium. Only those hearts that showed the CO value at least 45 ml/min were used for further manipulations

Thereafter, the aortic cannula was connected to a peristaltic pump for injection of liposomes at a rate of 0.1 ml/min for 1 hour at 4°C and 14°C, or at a rate of 0.2 ml/min for 30 minutes at 37°C.

In our study we used 109 hearts which were divided into groups depending on the temperature and the composition and concentration of liposomes (Table 2).

After the end of ischemic exposure, we measured the volume of liquid in each chamber and took 3 ml of Krebs-Henseleit solution to determine the radioactivity (sample V_1). Thereafter, the miocardium was reperfused for 15 minutes, according to Langendorff. The total volume of perfusate was measured and 3 ml was taken to determine the radioactivity (sample V_2).

Finally, the heart was removed from the perfusion system. We cut off about one-third of the tissue at the base, and the remainder part was quickly weighed and frozen in liquid nitrogen.

Table 2. Conditions of ischemia, the lipid composition of liposomes and cardiac output in studied groups

Group	Quantity	Conditions of ischemia		Liposomes		OC (ml/min)
		Time (minutes)	Temperature, C^0	Concentration (mg/ml)	Composition (ratio)	
1	13	30	37	25	7:5	52±2
2	13	30	37	25	2:1	50±2
3	13	30	37	10	7:5	52±2
4	13	30	37	10	2:1	49±3
5	10	60	14	25	7:5	60±3
6	10	60	14	25	2:1	56±3
7	10	60	14	10	7:5	56±2
8	10	60	14	10	2:1	55±2
9	10	60	4	10	7:5	59±2
10	10	60	4	10	2:1	53±3

2.1. Method for determination of radioactivity

0.1 ml of V_1 and V_2 were collected from the perfusate of each sample. Hearts were homogenized in a glass homogenizer with a glass piston at 40^0C in a 0.9% solution of NaCl (4 ml per 1 g of tissue mass of the heart). Thereafter, sequential centrifugation at 3000 g at 80^0C was performed. Homogenate (1 ml) was divided into subcellular fractions, namely, crude fraction of cell membrane, mitochondrial and microsomal fractions.

Precipitation after each centrifugation was resuspended in 1 ml of 0.9% solution of NaCl.

Thereafter, 0.2 ml of 0.9% NaCl solution and 0.4 ml of chloroform were added to an aliquot of 0.2 ml suspension of sediments and the supernatant of the last centrifugation. After the separation of chloroform and aqueous phase by centrifugation at 10^4g for 1 min, 0.1 ml of chloroform was collected to determine the radioactivity.

The activity of aliquots was measured using the liquid-scintillation counter RackBeta-II (LKB, Sweden) in vials with 5 ml of standard toluene scintillator. To estimate the extinction scintillation

solution in each vial, we added 10 ml of ^{3}H-PC in chloroform with the known activity of tritium (about 10^5cpm) and again measured the activity of the samples.

The activity of tritium was measured as follows:

$I=I_1I_0/(I_2-I_1)$,

where I_1 and I_2 – count rate of the first and second measuring the activity of the sample, cpm, respectively;

I_0 – count rate of the added ^{3}H-PC, cpm.

The results were recalculated according to the weight of the heart and the total volume of solution from a chamber (V_1) and reperfusion solution (V_2). Results are presented in relative terms. In each experiment, 100% radioactivity consisted of:

$R_0 = R_1 + R_2 + R_C$,

where $R_1 + R_2$ – total radioactivity in perfusion solutions V_1 and V_2 (10^6cpm);

R_C – total radioactivity of subcellular fractions, isolated from the mass of the myocardium (10^6cpm).

The share of ^{3}H-PC was calculated as follows:

$R_{Comn} = R_C / 100\ R_0$.

3. RESULTS AND DISCUSSION

The results suggest that the inclusion and distribution of liposomes in ischemic myocardium depends on their concentration and lipid composition, as well as on the temperature of exposure.

A significant share of liposomes (17-20%) were included in the heart at a temperature of 4°C (Figure 2). However, approximately half of them were found in the cytoplasmic membranes; only one third was detected in the mitochondrial membranes, and only 3-6% liposomes were observed in microsomal membranes (Fig. 3).

Observed effect may be possibly due to the fact that hypothermia may dramatically change the properties of the cell and the liposomal membrane, and this may lead to a violation of the interaction between liposomes and the cell (see for example Zabelinskiy S.A. et al., 2011, Burattini S. et al., 2010). It could be suggested that the inclusion of ^{3}H-PC may be due to so-called flip-flop effect (see for example Gummadi S.N. et al., 2005). Thus, the penetration of liposomes to the cell structure is complicated, and liposomes are not effective agents for intracellular transport of drugs.

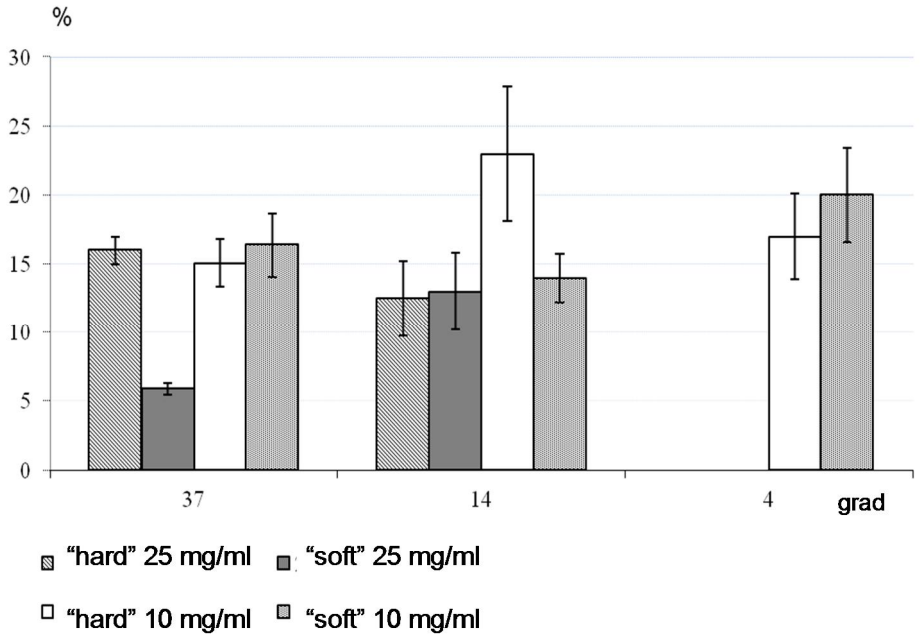


Fig. 2. Inclusion of tritium-labeled liposomes with different concentrations (25 or 10 mg/ml), and different ratios of lipids (7:5 or 2:1) in subcellular fractions at different temperatures.

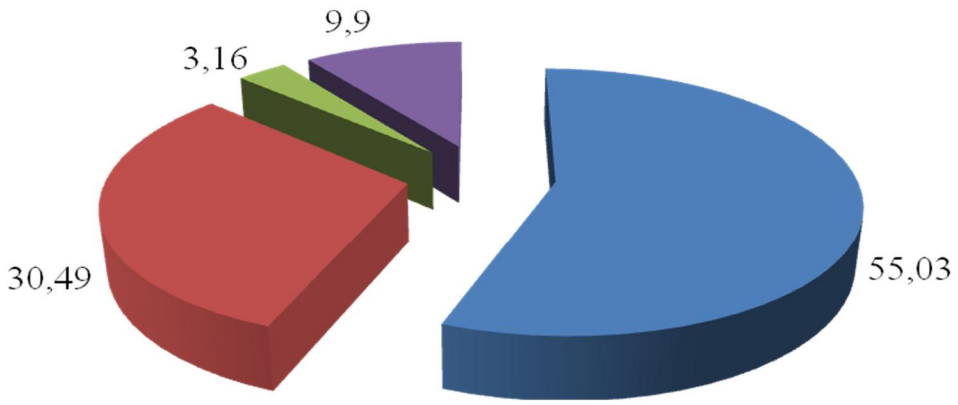
In addition, deep ischemia (4-6^{0}C) generally damages cell membranes in a large extent [50]; therefore, further investigations in the field should either avoid the usage of low temperature in the protection of ischemic myocardium, or to focus on the modification of the liposomal membrane. The latter approach may be done by changing the composition of the lipid components and by introducing compounds with cryoprotective properties.

A different picture was observed with normothermic ischemia. The inclusion of "soft" liposomes at a higher concentration (25 mg/ml) was 2.5-3 times lower ($p<0.001$) in comparison to other 3 types of liposomes. The maximal inclusion was observed for "soft" liposomes at a lower concentration (10 mg/ml). Regarding the ^{3}H-PC label, 61±6%, 4±1%, and 27±3% were detected in the mitochondrial fraction, microsomal fraction, and in crude membranes, respectively (Fig. 4)

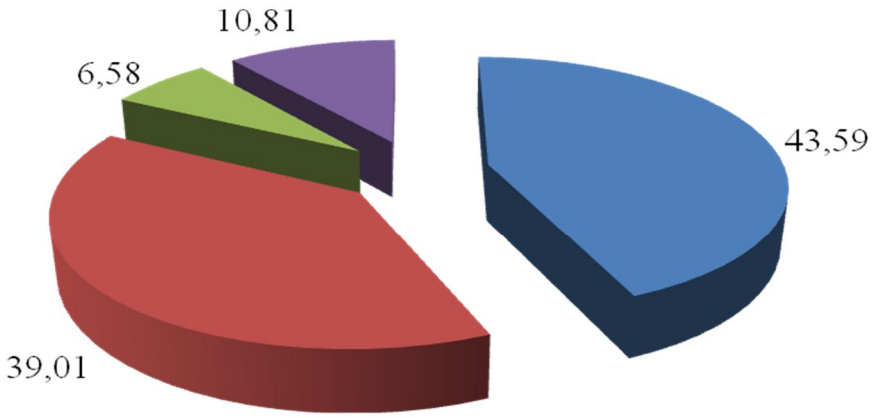
Interestingly, 60% of the ^{3}H-PC label was included in mitochondria, and total radioactivity was 55-65% in all series of experiments (Fig. 4, 5)

It should be noted that higher concentration of lipids (25 mg/ml) causes inhibition of myocardial contractility in the reperfusion period. The contractile activity of hearts restored only in 30% of cases in both groups higher concentration of lipids.

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a



b

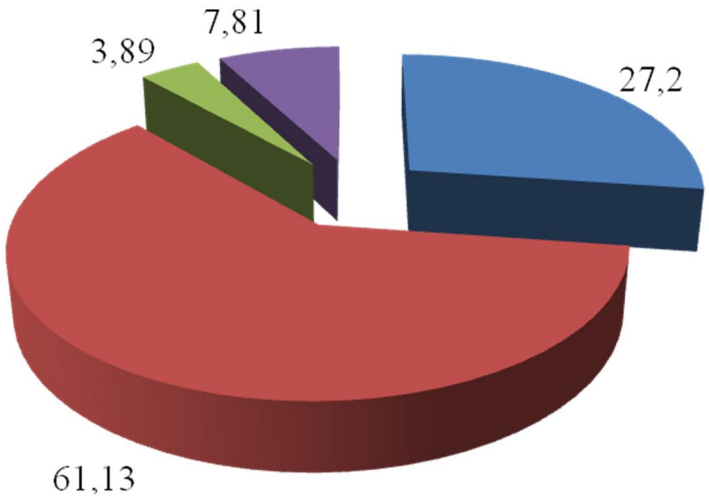


a – phosphatidylcholine-cholesterol molar ratio – 2:1 («soft» liposomes)

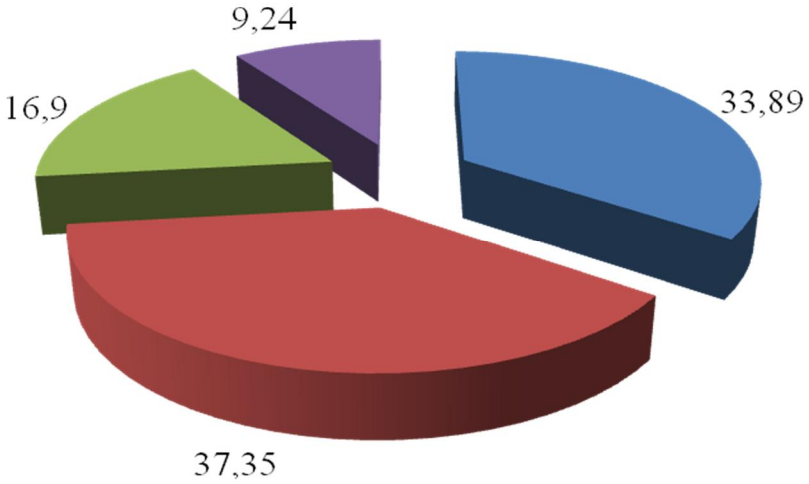
b – phosphatidylcholine-cholesterol molar ratio – 7:5 («hard» liposomes)

Fig. 3 – Distribution of ^{3}H-PC label at 4^{0}C and 10 mg/ml.

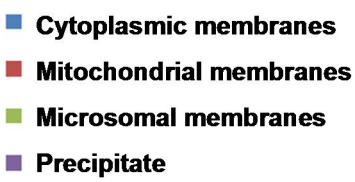
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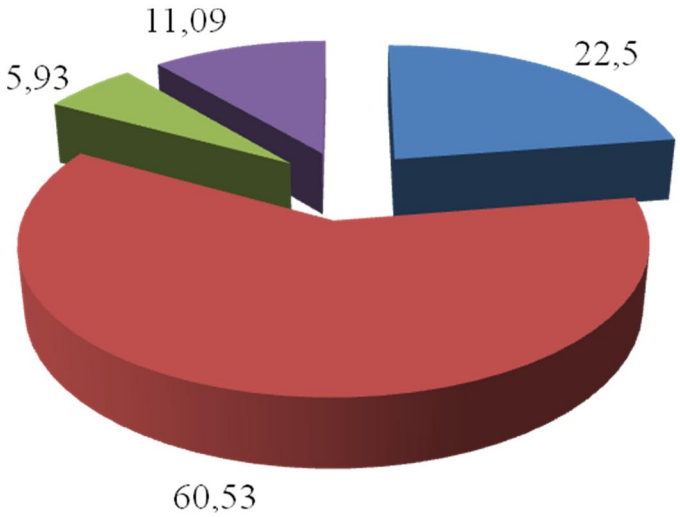
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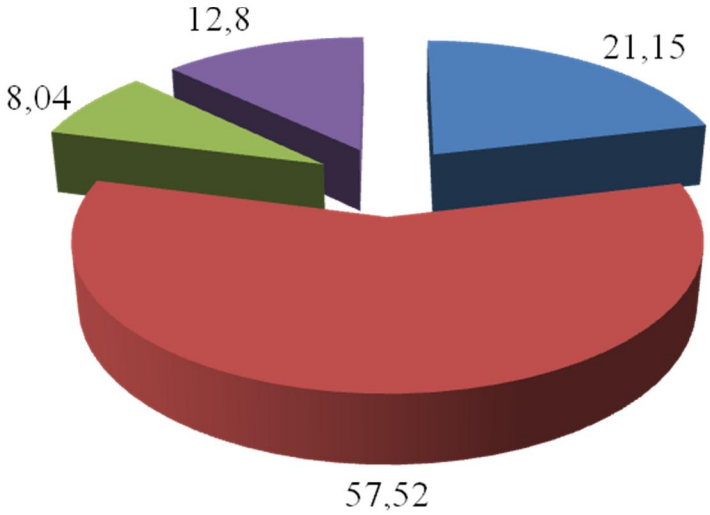
a – phosphatidylcholine-cholesterol molar ratio – 2:1 («soft» liposomes)

b – phosphatidylcholine-cholesterol molar ratio – 7:5 («hard» liposomes)

Fig. 4 – Distribution of ^{3}H-PC label at 37^{0}C and 10 mg/ml.



a



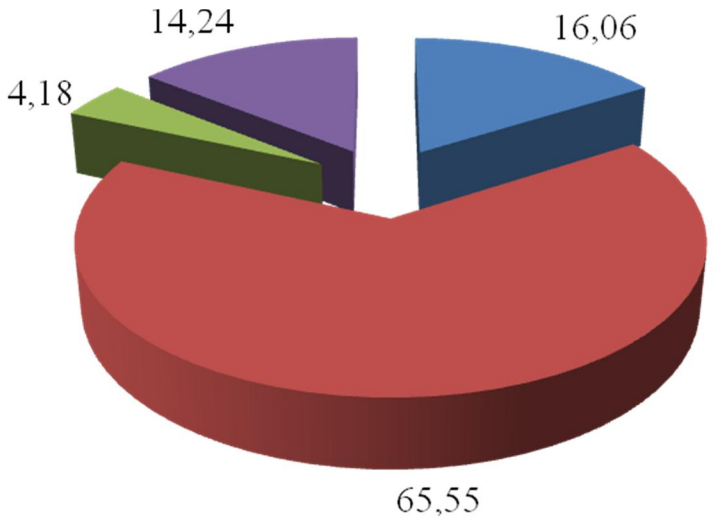
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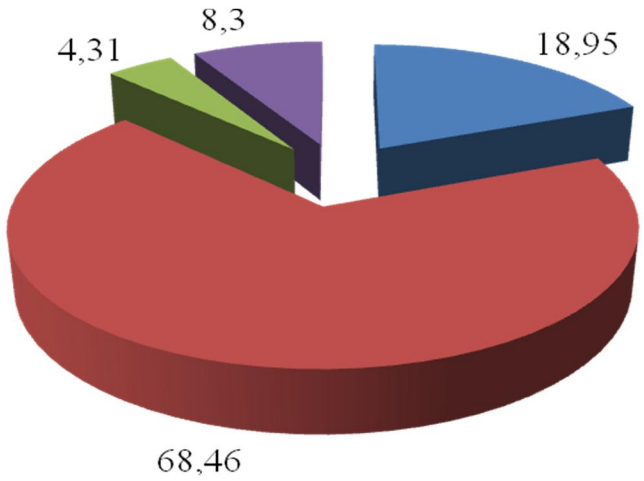
a – phosphatidylcholine-cholesterol molar ratio – 2:1 («soft» liposomes)

b – phosphatidylcholine-cholesterol molar ratio – 7:5 («hard» liposomes)

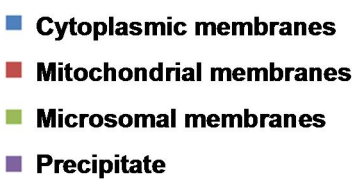
Fig. 5. – Distribution of ^{3}H-PC label at 37^{0}C and 25 mg/ml.



a



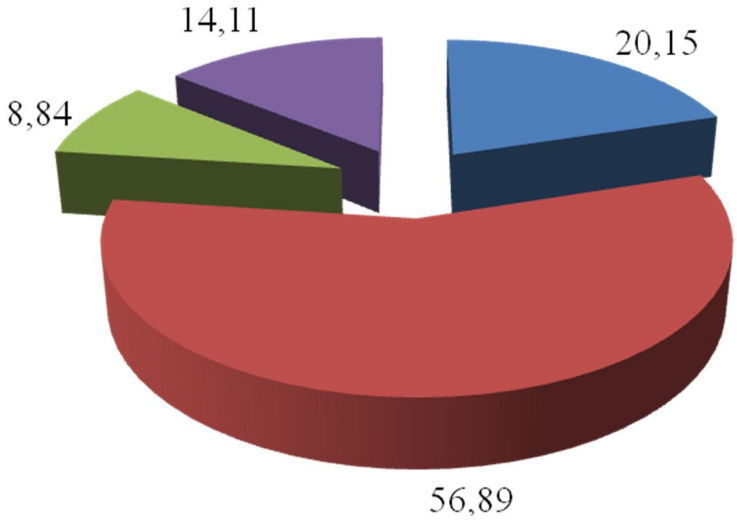
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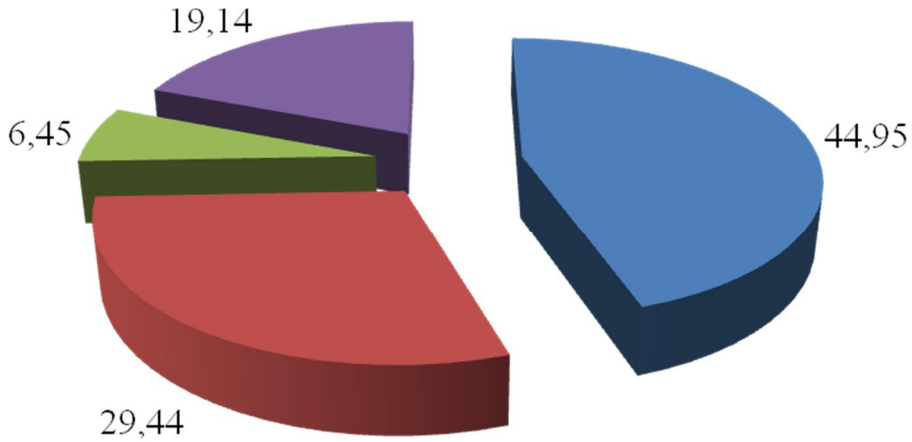
a – phosphatidylcholine-cholesterol molar ratio – 2:1 («soft» liposomes)

b – phosphatidylcholine-cholesterol molar ratio – 7:5 («hard» liposomes)

Fig. 6 – Distribution of ^{3}H-PC label at 14^{0}C and 10 mg/ml.



a



b



a – phosphatidylcholine-cholesterol molar ratio – 2:1 («soft» liposomes)

b – phosphatidylcholine-cholesterol molar ratio – 7:5 («hard» liposomes)

Fig. 7 – Distribution of ^{3}H-PC label at 14^{0}C and 25 mg/ml.

The observed effect can be associated with both vasoconstrictor effect of liposomes, and with the mechanical occlusion of small coronary vessels by liposomal aggregates (see for example Heusch G. 2008, Maslov L.N. et al., 2012, Wiclund L. 1994). In addition, the diffusion of lipid vesicles in the vascular wall is restricted even with an increase in the porosity of the endothelium under the influence of ischemia [8]. According to our findings, it is necessary to search for the optimal concentration of liposomes administered intracoronary. Optimal diameter of liposomes as well as their composition should be found in further investigations on this topic (see for example Tang C.S. et al., 1993, Caride V.J. et al., 1977, Verma D.D. et al., 2005).

Certain patterns of a change in the concentration and composition of lipids were identified in conditions of "gentle" hypothermia (14°C) (Fig. 6, 7). Maximal inclusion of ^{3}H-PC label was observed in groups 5-8 using the lower concentration of lipids (10 mg/ml). The inclusion was significantly higher for "hard" liposomes (23±3%, $p<0.01$). As for the subcellular distribution of labels, it was roughly the same in these two groups and in the group of "soft" liposomes with the lipid concentrations of 25 mg/ml.

It is important to note that all the liposomes at all temperatures effectively incorporated and distributed within the cells. In conditions of normothermia, the maximum of inclusion was observed for "soft" liposomes in a concentration of 10 mg/ml. In conditions of "gentle" hypothermia, the maximum of inclusion was observed for "hard" liposomes in a concentration of 10 mg/ml; moreover, it was the best value of inclusion in the whole experiment. In conditions of deep hypothermia, no significant differences between "soft" and "hard" liposomes were observed. Therefore, it could be concluded that "soft" liposomes should be used for the prior administration and administration in the conditions of normothermia. "Hard" liposomes should be used for intracoronary administration and for the administration of intraoperative hypothermia of various kinds.

We also found that the "soft" liposomes in a concentration of 10 mg/ml are embedded in the mitochondrial membrane; moreover, the total inclusion of liposomes in subcellular structures reaches up to 70-80%, which gives the prerequisites for the use of liposomes as a transport container for energy substrates.

4. CONCLUSIONS

Thus, liposomal transport of bioactive substances into the cell is possible at all temperatures; however, a subtle balance between lipid composition and concentration of lipids should be maintained for better efficacy. On the one hand, the structural "rigidity" of the liposomal membrane plays a great role in the interaction between cell and liposomes , the highest inclusion into the myocardium was demonstrated for "hard" liposomes with a concentration of 10 mg/ml. On the other hand, a 2.5 fold increase in lipid concentration leads to worse inclusion, and subcellular distribution of the lipids. We suggest that the concentration of 25 mg/ml is too much; therefore and the use of liposomes at this concentration of lipids may negatively affect the myocardial reperfusion period.

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