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COMPARATIVE STUDY OF LIPID PEROXIDATION AND ACTIVITY OF ANTIOXIDANTS INCLUDED INTO LIPOSOMES PRODUCED BY EXTRUSION AND SONIFICATION

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Abstract

The properties of production and standardization of liposomes of complex composition by the methods of sonification and extrusion (through polycarbonate filters) have been studied. The study shows that both methods allow producing liposomes of different lipid composition with inclusion of various bioactive substances into lipid and intraliposomal phases. We also show that sonification causes the activation of lipid peroxidation and decreases the activity of biologically active substancesincluded into liposomes. Moreover the liposomes produced by sonification need standartization in particles size. The success of extrusion method depends on the correct choice of organic solvent, lipids and their hydration properties. It is shown that liposomes can be successfully separated from extra-liposomal substances using the method of gel-filtration.

Key words: *liposome production, liposome standartization, lipid peroxidation.*

1. INTRODUCTION

Intensive research of liposomes in recent decades has shown perspective of their application in different medicine spheres, cosmetology and food industry (Fan, Zhang, 2013; Levchenko, Hartner, Torchilin, 2012; Rani, 2013).Meanwhile there are no conventional standardization methods of the liposomal preparations. This makes difficult to compare results of different researches since they use liposomes of different size, composition, production methods which may affect their pharmacological properties (Krasnopolskiy, Stepanov, Shvets, 1999). In current practice, two methods of liposome production are generally used: ultrasound sonification and extrusion. Recent studies have shown that liposomes having the same lipid composition and obtained by extrusion and sonication, show differences in the membrane organization (Cho et. al., 2013; Lapinski et. al., 2007).

The purpose of this study is to examine the features of preparation and standardization of liposomes by extrusion and sonication.

2. MATERIALS AND METHODS

Lipid content in liposomes was determined using standard kits of Diagnostic Systems. Liposome size was estimated by measuring optical density (Sorokoumova, Selitsev, Tyurina, 2001). The content of liposomes in the medium was estimated by light scattering at the wave length of 254nm. Concentration of malondialdehyde (MDA), diene and triene conjugates, cytochrome C, reduced glutathione, superoxide dismutase (SOD) activity and catalase were determined by methods described by Vorontsova *et al*. (2005).

Statistical Analysis was carried out using the software Statistica, version 7.0. Data are presented as mean ± SEM. Statistical significances of the differences between groups were evaluated using one-way ANOVA followed Tukey's multiple comparison post hoc test. Differences were considered significant where $P<0.05$.

3. EXPERIMENTAL PROCEDURE

Production of multilamellar vesicles (MLV) is the common stage for both methods of liposome production. MLV are the particles produced as a result of mechanical dispersion of phospholipids in water. They are large multilayered particles up to 50 μm in size. In order to produce these particles, the method based on formation of lipid film on the inner surface of glass bulb followed by resuspension in aqueous media was used. In the preparation of the lipid film α-tocopherol, cholesterol and other lipophilic substances were added to its composition along with the purified lipids. The resulting lipid film was emulsified in aqueous medium containing substances intended for incorporation in the inside of the liposomes.

At the next stage small unilamellar vesicles (SUV) were produced from MLV.

When producing liposomes by sonification method, the suspension of MLV was sonicated (ultrasonic disrupter UD-20, TECHPAN, Poland). Sonification was performed in a water bath in thick-walled quartz tubes. The total sonification time was 12 minutes. After each minute of sonification, a two-minute break was made.

To produce SUV by extrusion method a laboratory extruder («Lipex», Biomembranes Inc., and Canada) was used. MLV were extruded through two layers of polycarbonate filter with 100nm or 50 nm pore diameter. Overpressure was made with compressed argon. For this method, the choice of organic solvent for lipids mixture at the stage of MLV production has turned out to be important. Traditionally used mixtures of chloroform and methanol appeared to be unsuitable for this purpose because a small part of cholesterol, after distilling off the chloroform, formed crystal particles in the lipid film which were clogging the filter pores and disturbing impeding the process of extrusion. In the preliminary experiments we found that the best results could be achieved by using a mixture of benzene-ethanol as solvent in the ratio 9:1 respectively.

To produce the maximum incorporation of active substances into the liposomes by both methods, a supplementary stage of lipids hydration was added, according to the method described earlier (Hope et. al., 1985). This method is based on freeze/thawing which provides the equilibrium distribution of solutes between lamellas and adequate hydration of lipids. The original technique includes 5-fold freezing of a sample in cryotube by liquid nitrogen and thawing in warm water (40-60^{0}C). However, our studies have shown that this procedure subsequently worsens the extrusion process during the production of liposomes of complex lipid composition with high cholesterol and lipophilic substances. This is related to the fact that the formed structures quickly clog the filter pores and the extrusion process becomes impeded.

A hydration procedure by freezing the sample in a freezer at -18^{0}C for 24 hours followed by slow thawing in the air just prior to extrusion turned to be more acceptable. In this case, the extrusion process doesn't cause special difficulties.

Liposomes produced by sonification were centrifuged at 10000g (4ºC) in order to separate the particles of ultrasonic disrupter and the remaining MLV.

SUV produced by both methods need further washout from non-incorporated substances. The liposomes produced by sonification also need standardization in size. Washout from the incubation medium and liposome sizing were performed by gel-filtration using Toyopearl 45 (Japan). The size of liposomal particles and total lipid content were determined in the fractions (Fig.1).

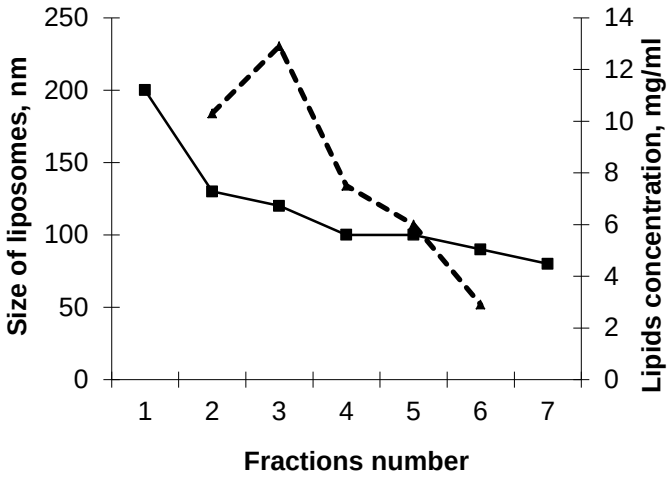

Fig.1. Lipids concentration (---) and diameter of liposomes (—), produced by sonification in the fractions of SUV after gel-filtration using Toyopearl 45. The column volume was 50 ml, the flow rate was 4 ml/min. Initial lipid concentration was 50 mg/ml.

During the gel-filtration, the efflux of liposomes was followed by the efflux of non-incorporated particles. An example of the separation of liposomes containing SOD from the extraliposomal SOD in the column is shown in Fig. 2.

Method of liposome production may affect the activity of the incorporated bioactive substances. We have studied the influence of extrusion and sonification on the activity of SOD, catalase, reduced glutathione and cytochrome C. After a standard sonification procedure or extrusion of the suspensions of multilamellar vesicles, we determined the activity or concentration of the above mentioned substances relatively to the initial values. If necessary, for release of the substances incorporated into the liposomes, the membranes were destroyed by adding the detergent triton X100.

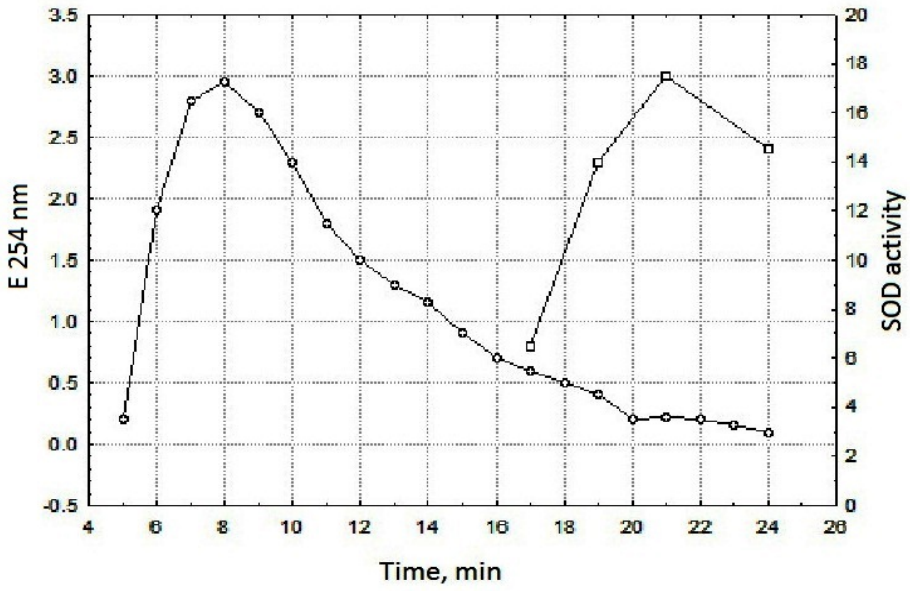

Fig.2. Separation of liposomes (D254) and extraliposomal superoxide dismutase by gel-filtration using Toyopearl 45. The column volume was 50 ml, the flow rate was 4ml/min.

The results (Table 1) show that extrusion insignificantly reduced activity of superoxide dismutase and α-tocopherol and concentration of glutathione and cytochrome C. Meanwhile sonification decreased activity of catalase approximately 20-fold and cytochrome C concentration was also significantly reduced.

Table 1. Influence of 10 min sonification and extrusion on the activity of substances, incorporated into liposomes.

Markers	Content ratio or activity, %	
	Extrusion	Sonification
Superoxide dismutase	97,3±2,4	94,6±3,2
Catalase	96,8±2,2	5,5±4,1
Reduced glutathione	98,4±3,1	92,3±6,7
α- tocopherol	99,2±0,6	91,5±4,2
Cytochrome C	97,4±1,8	88,7±2,4

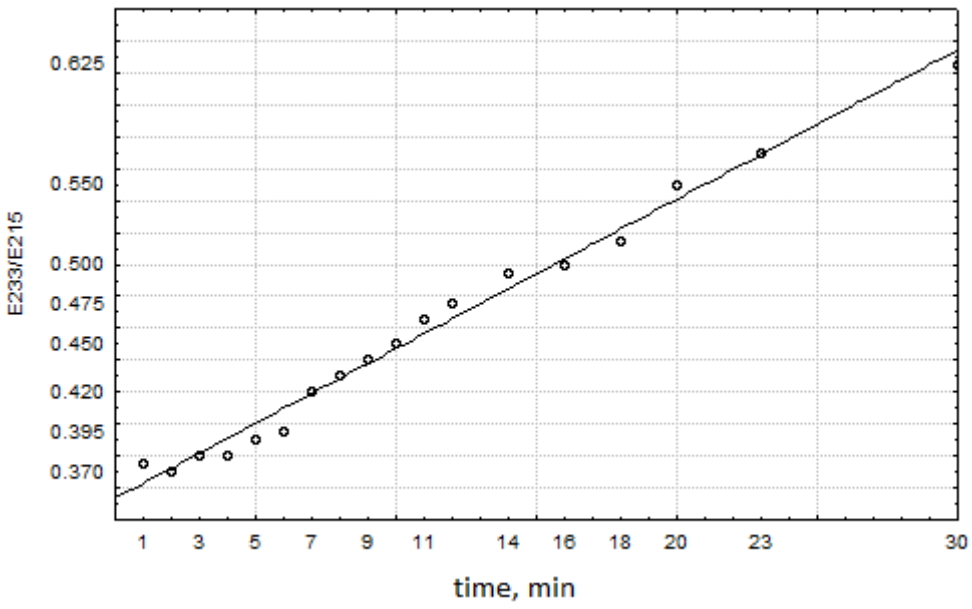

Fig.3. Influence of sonification time of MLV consisting of lecithin and cholesterol (7:5) on the formation of diene conjugates (E233/E215).

A comparative study of the influence of liposome production method on the formation of lipid peroxidation products in the liposomes was carried out. We found that 12-minute sonification resulted in the increase of MDA concentration from 7.02±0.43 mmol/mg to 20.7±0.81 mmol/mg. Increase in the sonification time brought about almost linear increase in concentration of lipid peroxidation of the primary products, diene conjugates (Fig. 3). The extrusion process was not accompanied by increase of lipid peroxidation products in liposomes.

4. CONCLUSIONS

Thus, production of liposomes by extrusion has several advantages over sonification method: the possibility to produce liposomes of strictly calibrated size, considerably reduced probability of lipid peroxidation and insignificant influence on the activity and content of bioactive substances. In our view, monitoring of the liposome size, incorporation of active substances into extraliposomal and inner phases, content of lipids and lipid peroxidation products allows to produce liposomal products of standard composition and consequently with the predictable therapeutic effect.

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