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Preparation and *in-vivo* evaluation of cytochrome-C-containing liposomes

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This study investigates the development of a method for obtaining cytochrome C-containing liposomes (LS-Cyt), and evaluates their stability and specific activity. LS-Cyt were intended for the therapy of ophthalmic diseases. LS-Cyt were prepared by high pressure homogenization technique and lyophilized to obtain freeze-dried LS-Cyt. It was proposed to use anionic phospholipid- dipalmitoylphosphatidylglycerol (DPPG-Na) and phosphatidylcholine (PC) in a nanoparticulate composition. Were investigated various concentrations of lactose and trehalose as cryoprotectants. Samples with a lactose concentration of 6% showed the best results in terms of the emulsion formation time, encapsulation and preservation of nanosize. The main technological parameters for the obtained freeze-dried LS-Cyt were encapsulation of no less than 95% of cytochrome C (Cyt C), particle size of 140-170 nm, pH of 6.85±0.1, osmolarity of 330±3 mOsmol/kg, a lysophosphatidylcholine content (LPC) of 0.65±0.05 % of the total of lipids. Stability of the freeze-dried LS-Cyt during storage was established. The freeze-dried LS-Cyt was kept for 1 year in a light protected place at the temperature of -15 °C. No changes in the composition of LS-Cyt samples were detected over the observation period. Preclinical *in-vivo* research was conducted, namely the evaluation of specific activity on the model of the penetrating corneal injury. It was established that use of LS-Cyt contributes to a more rapid process of tissue regeneration and reduction of the inflammatory response in comparison with a non-liposomal dosage form.

1. Introduction

Corneal injury is of special importance in ocular pathology. According to the World Health Organization, diseases affecting the cornea are a major cause of blindness (Whitcher et al. 2001). As a therapy for corneal damage, the possibility of using Cyt C was considered.

Cyt C is a catalyst for cellular respiration, which stimulates oxidative reactions and regeneration processes, activates metabolism in tissues, as well as reduces tissue hypoxia at various pathological conditions. It is used in ophthalmology, e.g. in preparations like "Oftan catachrom" (Santen), "Vitaphakol Eye Drops", "Cytochrome C Eye Drops" (Samson-med), "Ractovit" (Ibn Sina), "Vitafol Eye Drops" (Popular Pharmaceuticals), where it exhibits a high activity towards free radicals, binds aggressive molecules of oxidants, and protects the lens and cornea from damage. It also suppresses and prevents the development of lens opacity (cataract), and prevents degeneration of the retina.

However, water-soluble dosage forms of Cyt C have a number of significant drawbacks: Cyt C has an insignificant penetration ability through biological membranes into cells, and has a short half-life, which contributes to its extremely low bioavailability. These drawbacks of the known preparations – drops based on aqueous solutions of Cyt C – require frequent administration of the solution. Thus, it is reasonable to create LS-Cyt eye drops.

Unfortunately, some undesirable phenomena such as aggregation and hydrolysis of phospholipids can occur in aqueous solutions of LS. To solve these problems the lyophilization of LS was used.

The main objectives of this study were therefore to determine the main technological stages of LS-Cyt preparation and stability assessment, as well as to determine the specific activity of LS-Cyt on an animal model of penetrating corneal injury in comparison with a non-liposomal dosage form.

2. Investigations, results and discussion

2.1. Investigation of the technology for obtaining liposomes

Our initial task was to determine the optimal composition of LS, which ensures the maximum encapsulation of Cyt C. In earlier works (Zhang et al. 2009; Shanskaya et al. 1998), Cyt C encapsulation efficiency suggested by the authors was 38-60 %. We introduced the LS composition, which provides encapsulation of Cyt C of no less than 95%.

The ability of Cyt C to form complexes with anionic phospholipids in biological membranes is well known (Gorbenko et al 2006; Mohn et al 2014), which is the determining factor in the protein-lipid interaction of Cyt C. Considering this, it was proposed to use an anionic phospholipid-DPPG-Na in a nanoparticulate composition. As the membrane-forming lipid we used phosphatidylcholine (PC) – the main component of the membranes of eukaryotes. Having used these components in different ratios and concentrations in the experimental study, we proposed a composition that provides maximum encapsulation of Cyt C (Shobolov et al. 2015). Moreover, the degree of encapsulation of Cyt C depends on the ratio of the lipid components used and the amount of Cyt C. Under these conditions the positively charged groups on the Cyt C molecules interact with the negatively charged groups on the DPPG-Na molecule.

We have introduced a scheme for obtaining LS-Cyt, which comes down to obtaining a lipid film and its emulsification with solution of Cyt C to obtain an emulsion of multilamellar vesicles; obtaining LS-Cyt by the high pressure homogenization (HPH); sterile filtration.

The advantage of obtaining LS by the HPH method is the standard and scaling, high yield of the method, minimal oxidation

and hydrolysis of the phospholipids, drug integrity, stability of LS, and control over the temperature and pressure of the technological process. The homogenization method allows to obtain LS of uniform composition, mainly represented by particles 120-170 nm in size. The optimal temperature range of homogenization was 38-44 °C with the pressure of 900 bar. All the work on obtaining liposomes was carried out under an inert (nitrogen) atmosphere. The results of the studies are presented in Table 1.

Table 1: Parameters of the LS-Cyt production technology

LS composition PC: DPPG-Na	4.0:1.0	3.6:1.0	2.4:1.0	1.20:1.0
LS average size, nm	140 nm – 95.2%, 35 nm – 4.8%	147.2 nm – 90.1%, 30.8 nm – 9.9%.	138.2 nm – 92.1%, 37.8 nm – 7.9%.	145 nm – 92.0%, 75 nm – 8%;
Encapsulation efficiency	81.2%	89.88	94.88	95.2

As a result, LS-Cyt were obtained with Cyt C encapsulation effiency of 95.2 %, and with an average particle size of 140-150 nm, pH of 6.85±0.1, osmolarity of 330±3 mOsmol/kg, a lysophosphatidylcholine content (LPC) of 0.65±0.05 % of the total of lipids. LS-Cyt were filtered through a 0.22 µm membrane filter, after which the obtained emulsion was bottled in vials and freeze-dried.

2.2. Lyophilization

Lyophilization of LS ensures stability of LS during long-term storage (Shulkin et al. 1984). The method of lyophilization influences the stability of LS, the amount of Cyt C included in them, and the particle size. With the aid of lyophilization it is possible to obtain stable LS, that are easily reconstituted with the addition of water. Lyophilization requires a careful control of the dehydration process, since the lyophilization process involves freezing the drug followed by the removal of water. This raises a problem due to the fact that at the stage of freezing and dehydration, some physical damage to the LS structure is possible.

We investigated various concentrations of lactose and trehalose as cryoprotectants, solutions of which were added during the production of LS emulsion. The following characteristics were used as evaluation criteria: appearance of the lyophilisate, encapsulation efficiency, preservation of particle nanosize, emulsion formation time, and emulsion stability (Table 2).

Table 2: Effect of cryoprotectant on particle size, encapsulation efficiency, appearance, emulsion formation time, and emulsion stability

Cryoprotectant	Lactose				Trehalose			
Cryoprotectant concentration, %	4.0	5.0	6.0	7.0	4.0	5.0	6.0	7.0
Particle size, nm	190-200	190-200	160-170	160-170	190-210	190-210	170-195	150-170
Encapsulation, %	≤ 90	90-95	≥ 95	≥ 95	≤ 90	90-93	≥95	≥ 95
Appearance	Porous mass of red color				Porous mass of red color			
Emulsion formation time, min.	4	4	3	3	4	4	4	4
Emulsion stability, min.	no less than 60 min				no less than 60 min			

As can be seen from Table 2, samples with lactose concentration of 6% showed the best results in terms of emulsion formation time, encapsulation and preservation of nanosize.

2.3. Stability

The use of LS as a medication for ophthalmic use is possible provided that the formulation remains stable during the shelf life. To monitor the stability in accordance with common recommendations (Ukrainy, D. F. 2015; US Department of Health 2015; Guideline, I. H. T. 2003), key properties controlling the pharmacological effects of LS (encapsulation efficiency, particle size, quantitative determination of concentration of the active substance, and lack of degradation products (LPC)) were tracked over 12 months.

We developed the following methods: determination of encapsulation efficiency of Cyt C (Katsai et al. 2016), assay of Cyt C, and assay of phospholipids (PC, DPPG-Na and LPC as their main related substance in LS-Cyt) (Katsai and Ruban 2017).

Table 3: Study of stability of lyophilized LS-Cyt during storage

Month	0	1	3	6	12
Encapsulation,%	95.3	95.0	95.5	95.0	95.0
Median particle size, nm	165	160	166	164	168
pH	6.90	6.81	6.80	6.77	6.65
LPC, %	0.65	0.66	0.65	0.67	0.70
Cyt C, %	100.2	99.8	99.8	99.8	99.6
PC, %	99.5	99.5	99.7	99.4	99.4
DPPG-Na, %	100.3	100.2	100.2	100.2	100.1

As shown in Table 3, no changes in the composition of LS-Cyt samples were detected over the observation period. Also, during the entire storage term, the lyophilisates retained their appearance, formation time, and emulsion stability.

The content of the main degradation product (LPC) remained stable during the study period, and is at the level of LPC content during the preliminary study (0 month), which indicates the preservation of LS-Cyt during 1 year of observations under the selected storage conditions.

2.4. Determination of specific activity

Study of specific activity of LS-Cyt was carried out on an animal model of penetrating corneal injury in comparison with the non-liposomal form of Cyt C. The corneal injury was done by injuring the optical zone of the rabbit's right eye (Kanyukov et al. 2014). The operation was performed under local anesthesia.

Effusion of fibrin in the anterior chamber is a frequent complication after scarring the cornea and leads to a slow recovery of visual functions and ophthalmic hypertension (Boyko et al. 1997; Siatiri et al. 2005). In addition, fibrin clots in the process of inflammation often attach to the anterior capsule of the lens (Fig. 1), which can cause a damage to it and, as a result, edema and opacity of the lens. Therefore, the ability of the medication to influence the frequency and intensity of the onset of a fibrin effusion symptom is preferable when choosing a drug for treatment of the corneal injury.

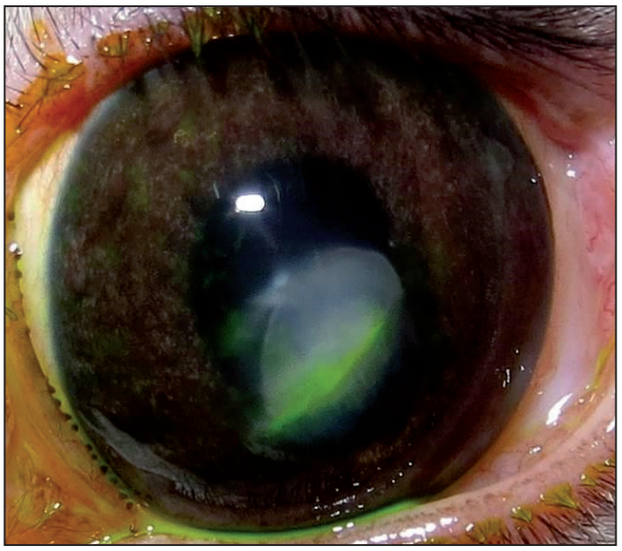

Fig. 1: Infiltration and effusion of fibrin in a test-animal from the control group on the 2nd day after corneal injury.

On the first day, the lowest fibrin effusion intensity into the anterior chamber of the eye was observed in the group of animals receiving LS-Cyt at the highest dose (1.2 mg/test-animal). When applying the LS-Cyt at a dose of 1.2 mg/test-animal immediately after the corneal injury, fibrin effusion into the anterior chamber of the eye occurred in only 20% of the animals; in the control group, fibrin effusion was observed in 70% of the animals on the first day after the induction. The formation of fibrin in the group receiving LS-Cyt at a dose of 1.2 mg/test-animal occurred only 2-4 h after injury, whilst in the control group a small amount of fibrin was noticed just an hour after the injury.
Development and enhancement of local inflammatory response was observed in all the experimental animals, which was manifested by an increase in the amount and density of fibrin in the anterior chamber of the eye and development of edema and corneal infiltration. The greatest effect from the application of the LS-Cyt was noted on the second day after the induction of pathology (Table 4).

Table 4: Intensity of clinical signs of penetrating corneal injury on the 2nd day after the induction of pathology

№ gr	Description	Dose, mg/animal	Infiltration	Fibrin effusion
			Points, median±QR	
1	Intact	0	- -	-
2	Control	0	2.5 ± 2.0	2.0 ± 2.0
3	LS-Cyt	1.2	**1.0** ± 1.0	**1.0** ± 1.0
4		0.8	1.5 ± 1.0	1.5 ± 1.0
5		0.4	2.0 ± 1.0	2.0 ± 1.0
6	Cyt C (non-liposomal form)	1.2	1.5 ± 1.0	1.5 ± 1.0
7		0.8	1.5 ± 1.0	1.5 ± 1.0
8		0.4	2.0 ± 2.0	2.0 ± 1.0

Groups receiving Cyt C (non-liposomal) and LS-Cyt at the highest doses demonstrated, lesser extent of both fibrin effusion and corneal infiltration than the control group of animals. At the same time, it should be noted that LS-Cyt had the greatest impact on these symptoms (Fig. 2).

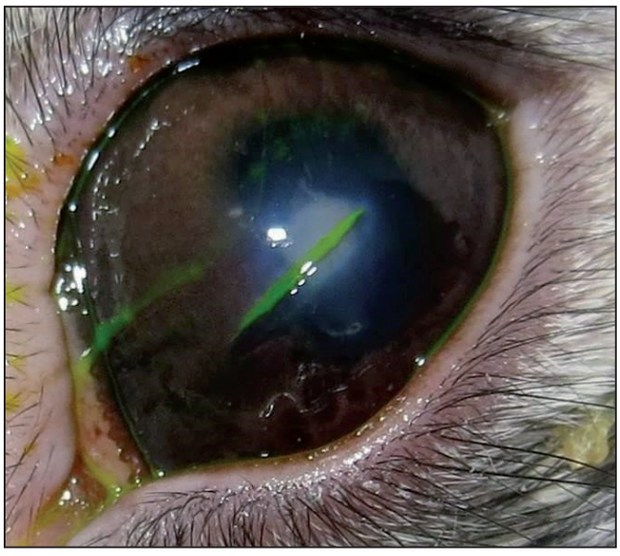

Fig. 2: Infiltration and effusion of fibrin in an animal receiving LS-Cyt at a dose of 1.2 mg on the 2nd day after injury.

On the second day, in the test animals receiving LS-Cyt and Cyt C in the highest dose, beginning of the epithelialization process was also observed. In the control group, epithelization of the injury did not occur on the second day.
Use of LS-Cyt in all the studied doses led to a decrease of the infiltration, density and size of the fibrin clot by a factor of 1.5 - 2 compared with the control group. When using the non-liposomal form of Cyt C, greatest effects were observed at a dose of 1.2 mg/animal.
On the third day after the injury, edema and infiltration of the cornea weakened in all the experimental groups. All groups also exhibited a decrease of the density and size of the fibrin clot in the anterior chamber of the eye.
In the groups receiving LS-Cyt, decrease in the manifestations of the symptoms described above was more pronounced than in the control group of animals and the group receiving Cyt C (non-liposomal form).
From the 4th to the 9th day of the study, the infiltration and epithelialization processes and the amount of fibrin in the anterior chamber of the eye changed gradually, and by the 10th day, the beginning of scarring was observed in the control group of animals. At the same time the process of infiltration and epithelization was also pronounced (Table 4). The size and density of the fibrin clot in the anterior chamber of the eye of the control animals did not appreciably change as compared to the 3rd day of the study (Fig. 3).

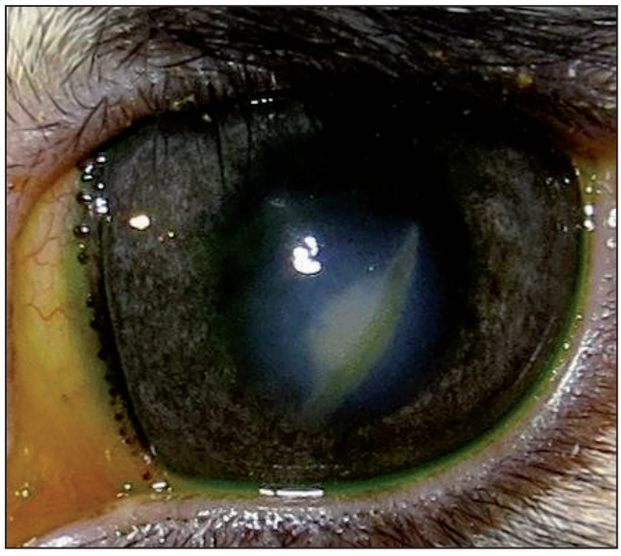

Fig. 3: Clinical condition of the eye of a control group animal 10 days after the corneal injury

In the groups of animals receiving LS-Cyt, the clinical picture looked different (Fig. 4).

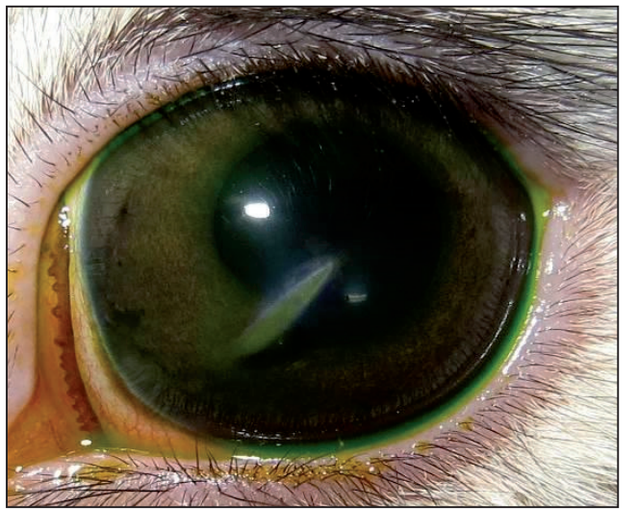

Fig. 4: Clinical condition of the eye of an animal receiving LS-Cyt at a dose of 1.2 mg 10 days after the corneal injury

Epithelialization of the damaged tissue was also more pronounced when LS-Cyt was used. The greatest wound healing was noted in the groups receiving LS-Cyt at the maximum dose.
Quality and rate of reconstruction of scar tissue were evaluated by the number of cases of a compact scar formation in each group by the end of the observation period. Formation of a soft compact scar occurred in most animals receiving LS-Cyt. In the group of animals receiving LS-Cyt at the highest dose, the formation of a compact scar was noted in all the test animals.
In the research done, effectiveness of Cyt C preparations for treatment of penetrating corneal trauma was demonstrated. Effectiveness of the drugs manifested in the decrease of the clinical intensity of the tissue inflammatory reaction, intensification of the regeneration processes expressed in the activation of the injury epithelization and in formation of a compact scar, as well as in the reduction of the inflammatory infiltration of the fibrin clot in the anterior chamber of the eye and in the absence of the scar germination by newly formed vessels. Some dose dependence was observed for Cyt C preparations in both liposomal and non-liposomal forms.
The current research also demonstrates that use of LS-Cyt eye drops is more effective than the use of Cyt C related to the inflammation and regeneration processes according to the results of clinical and histological studies.

2.4. Conclusion

A technological platform for obtaining LS-Cyt was developed. The optimal composition of the liposome membrane was established; this composition ensures a maximum encapsulation of cytochrome C in nanoparticles – no less than 95%, with a particle size of 140-170 nm. Optimal parameters of lyophilization of the preparation were determined, and storage stability of the preparation was established. The technology demonstrated in this study determines the following: encapsulation efficiency of Cyt C, particle size, the amount of LPC, storage stability, and also affects the pharmacological properties.
The following methods for assessing the quality of the developed LS-Cyt were also developed: the determination of encapsulation in LS, the determination of the lipid composition of LS-Cyt and its stability by the HPLC method.
It was observed that use of LS-Cyt contributes to a faster tissue regeneration process and decreased inflammatory response, as compared to non-liposomal Cyt C. It is therefore important to continue to study the specific pharmacological activity of LS-Cyt for other eye pathologies, such as retinopathy, cataract, and glaucoma, as well as engage in further clinical trials at the most effective doses.

3. Experimental

3.1. Materials

3.1.1. Chemicals

Cytochrome C (Hebei Lead Bio-Chemicals Co., Ltd, China), Cytochrome C Eye Drops (a non-liposome form) (LLC "Samson-Med", Russia) – a reference drug, phosphatidylcholine (EPC), Lipoid E 100 (Lipoid GmbH, Germany) dipalmitoylphosphatidylglycerol (DPPG-Na) (Lipoid GmbH, Germany), phosphatidylcholine standard Lipoid E PC RS (Lipoid GmbH, Germany), dipalmitoylphosphatedylcholine standard DPPG-Na RS (Lipoid GmbH, Germany), lysophosphatidylcholine (Lipoid GmbH, Germany), lactose (Fluka), trehalose (Fluka), chloroform (Sigma Aldrich), methanol (Sigma Aldrich), potassium dihydrogen phosphate (Fluka), sodium dihydrogen phosphate (Sigma Aldrich), disodium hydrogen phosphate (Sigma Aldrich).

3.1.2. Animals

In the *study of specific activity*, rabbit females of the Soviet chinchilla line were used as a test system. Selection of these animals as a test system was suggested by literature data on similar studies of the specific pharmacological activity (Samoylov and Petrova 2005). The choice of the animal gender (females) was justified by the more convenient animal care and stable behavior of females of this species. The number of animals used in the study is sufficient for a complete statistically significant recording of the effects studied and is the least rational from the point of view of ethical principles.
The animals were kept under standard conditions in accordance with regulations of the National Research Council (2010).
All procedures adhered to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and Guiding Principles in the Care and Use of Animals; ARRIVE guidelines; the EU Directive 2010/63/EU for animal experiments (Kilkenny et al 2010; Directive, E. U. 2010) and the protocol was approved by the local animal ethics committee.

3.2. Preparation of LS-Cyt

3.2.1. Preparation of liposomes

Liposomes were prepared according to a reported procedure (Shobolov et al 2015): DPPG-Na and EPC were dissolved in a mixture of chloroform:ethanol (4:1). The resulting mixture was evaporated in a rotary evaporator BUCHI Rotavapor R215 (Switzerland) until a lipid film formed. The lipid film was hydrated with the Cyt C solution at a ratio of Cytochrome C: lipids 1:16-35 respectively in the IKA Werke orbital shaker (Germany) until a homogeneous emulsion of multi-lamellar vesicles was formed.
The emulsion of multi-lamellar vesicles was homogenized in a high-pressure homogenizer M110P Microfluidizer (USA) under various pressure conditions (from 600 to 1200 bar) until unilamellar LS with an average particle size in the range of 100 to 200 nm were obtained. In cases where the inclusion of cytochrome C was incomplete, free Cyt C remains in the aqueous medium of the nanoemulsion and can be separated by ultrafiltration. The resulting liposomal emulsion was exposed to the sterilizing filtration through 0.22 µm membrane filters Pall (USA).

3.2.2. Lyophilization

Lactose and trehalose were tested as a cryoprotectant. Before lyophilization the samples of liposomes with cytochrome C were frozen beforehand to a temperature of - 60 ^{0}C in a low-temperature chamber Thermo Scientific Forma 8600 during 12 hours. The lyophilization was carried out in a freeze-dryer Quarco MD (China).

3.3. Characterization of liposomes

3.3.1. Preparation of the LS emulsion for the research.

Freeze-dried LS-Cyt were dissolved in water for injection to a Cyt C concentration of 0.675 mg/ml.

3.3.2. Assay of Cyt C

The total concentration of Cyt C in the freeze-dried LS-Cyt (C_{total}) was quantified spectrophotometrically on a Shimadzu UV 1800 (Japan) spectrophotometer using the UV absorption spectrum of a diluted emulsion of liposomes with cytochrome C in the range of 400-560 nm.

3.3.3. Determination of the free Cyt C (C_{free})

Free Cyt C was determined by the gel chromatography method. A Shimadzu "Nexera" chromatograph was used, a chromatography column Tricorn (GE Healthcare) of 5/200 size filled with a "superose 12" sorbent; mobile phase: 4.515 g/L KH_2PO_4 pH to 6.0, with 2 M NaOH; flow rate 0.5 ml/min; detection at a wavelength of 409 nm; column temperature 25 °C. The solutions of the cytochrome C substance and liposomes with cytochrome C were chromatographed alternately.
The encapsulation efficiency was calculated by the formula:
$EE\ \% = [(C_{total} - C_{free})/ C_{total}] \times 10$

3.3.4. Assay of phospholipids and their related substances (lysophosphatidylcholine)

The content of phospholipids in liposomes was determined by HPLC using a Shimadzu (Japan) chromatograph under the following conditions: PerfectChrom 100 Diol 0.125 m x 4 mm, 5 µm column, column temperature 55 °C, detector ELSD Sedere SEDEX 85 (France), sample volume 20 µl. Mobile phase: A) 1341.6 g of n-hexane, 334.1 g of 2-propanol, 39.4 g of acetic acid, and 1.45 g of triethylamine (or 2.0 mL of triethylamine). B) 663.5 g of 2-propanol, 140.0 g of water, 15.8 g of acetic acid, and 0.58 g of triethylamine.
Gradient according to Table 5.

Table 5: Gradient of mobile phase composition

Time (min)	Flow rate (ml/min)	Phase A (% vol.)	Phase B (% vol.)
0	1.0	95	5
5.0	1.0	80	20
8.5	1.0	60	40
15.0	1.0	0	100
17.5	1.0	0	100
17.6	1.0	95	5
21.0	1.0	95	5
22.0	2.0	95	5
27.0	2.0	95	5
29.0	1.0	95	5

The phospholipids included in liposomes and phospholipid standards were dissolved in chloroform:methanol:water (74:23:3). The content of phospholipids was calculated using the calibration curves of PC, DPPG-Na and LPC.

3.3.5. Physicochemical characterization of liposomes

The particle size was determined by the dynamic light scattering technique using a Malvern Zetasizer Nano S (UK). The pH of the liposome emulsion with Cyt C was measured using a pH meter by SCHOTT Instruments Lab 860 (Germany). The osmolarity was determined with the help of the osmometer Gonotec OSMOMAT 3000 (Germany).

3.4. Stability studies

The lyophilized LS was divided into 3 sample sets and subjected to the stability studies, in triplicate, as per ICH guidelines. The freeze-dried LS-Cyt was kept for 1 year in a light protected place at the temperature of minus 15 °C.
The studies were carried out for a period of 0, 1, 3, 6 and 12 months on the particle size, encapsulation, content of the main components (DPPG-Na, PC, Cyt C), content of the main LPC degradation product, appearance, emulsion formation time, emulsion stability.

3.5. Investigation of the LS-Cyt specific activity

The corneal injury was done by injuring the optical zone of the rabbit's right eye with a scalpel blade. After the pathology simulation and selection of animals, the test groups were formed according to the scheme presented in Table 6.

Table 6: Characteristics of the groups

№ group	Number of animals, gender	Specimen	Daily dose of active substance, mg/rabbit	Daily volume of the solution, ml	Drug administration
1	10, female	Intact Reference solution	0	0.6	10 days
2	10, female	Control Reference solution*	0	0.6	
3	10, female	LS-Cyt	1.2	0.6	
4	10, female		0.8	0.4	
5	10, female		0.4	0.2	
6	10, female	Cyt C (non-liposomal form)	1.2	0.6	
7	10, female		0.8	0.4	
8	10, female		0.4	0.2	

* - a solution containing all the auxiliary substances, but not containing Cyt C.

Freeze-dried LS-Cyt were dissolved in water for injection to a Cyt C concentration of 2.0 mg/ml.
The investigation of the eye surface structures condition was carried out by examining the anterior part of the eye with a slit lamp. The evaluation of the eye surface structures condition was carried out before the damage was caused and then daily for 10 days.
For all the data, descriptive statistics were applied: the data were checked for the distribution normality. Most of the data were presented in points and analyzed using nonparametric methods of descriptive statistics. The median and the interquartile range were determined. The intergroup differences were analyzed by a nonparametric method – the Mann-Whitney U test was used for the dependent and independent variables for multiple comparisons. The differences were determined at a 0.05 significance level. The statistical analysis was performed using Statistica 6.0 software (StatSoft, USA).

Conflicts of interest: None declared.

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