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Research Paper

Freeze-dried liposomes as potential carriers for ocular administration of cytochrome *c* against selenite cataract formation

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Abstract

Objectives In this study, the preparation, stability and anti-cataract effect of cationic freeze-dried liposomes containing cytochrome *c*, along with nicotinamide and adenosine, are described.

Methods Cytochrome *c*-loaded cationic liposomes (CC-L) were prepared by the thin-layer evaporation technique and lyophilized to obtain freeze-dried cytochrome *c* liposomes (CC-F). The influence of the preparation components on the liposomal encapsulation efficiency and the stability were studied. The anti-cataract effect of the CC-F was demonstrated through attenuating lens opacity development with slit lamp examination in rats with selenite-induced cataract.

Key findings Our study indicates that: (1) the liposomal encapsulation efficiency increased with increasing phosphatidylcholine content and reduced in the presence of stearylamine. Moreover, optimal encapsulation efficiency was obtained at an appropriate ratio of phosphatidylcholine to cholesterol; (2) CC-F was stable for at least 12 months at 4°C; (3) satisfactory improvements in lens opacity were shown in the cytochrome *c*-treated groups, especially for the CC-F-treated group with the decreased percentage of lens opacity at about 28% at the final examination.

Conclusions CC-F were shown to be stable superior ophthalmic carriers and were able to markedly retard the onset of cataract development.

Keywords cytochrome *c*; encapsulation efficiency; freeze-dried liposomes; lens opacity; stability

Introduction

Cataract is a progressive opacity of the lens of the eye that impairs vision and may cause blindness.[1] Nowadays, although surgical removal of the natural lens and replacement with a lens made of synthetic polymers is the major treatment for cataract, the incidence of cataracts is so high globally that surgery alone is unable to cope with the problem. Moreover, the requirement for highly trained personnel and the cost of surgery pose another economic problem. Therefore, development of pharmacological solutions for cataract is highly desirable.[2]

Numerous studies indicate that oxidative stress, mediated by reactive oxygen species (ROS) in the lens and lipid peroxides produced in the crystalline lens, is responsible for the breakdown of lens homoeostasis and describe the events leading to lens opacification.[3] Significant metabolic sources of reactive oxygen species are the respiratory chain, in which incomplete reduction of oxygen results in generation of superoxide anion, and oxidases, notably those involved in peroxisomal β-oxidation, that generate H_2O_2. The radicals converted from superoxide anion and H_2O_2 can cause various physiological dysfunctions through cellular DNA damage, protein oxidation and lipid peroxidation,[4–6] damaging the cell and whole organism.[7] Selenite cataract is a rapid and convenient model of nuclear cataract produced in young rats by an overdose of selenium, which leads to the shifting of the oxidative status to the pro-oxidant side in the developing lens. It has been employed in many experimental studies as a useful in-vivo rodent model since 1978.[8]

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The first realized function of cytochrome *c* is as an electron carrier between complex III (ubiquinol–cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase) in the respiratory chain, from which the bimetallic Cu_A site of cytochrome *c* oxidase accepts electrons.[9] Meanwhile, it was proved that cytochrome *c* plays a central role in stimulating cell apoptosis in the cytosol.[10] It was also shown that cytochrome *c* has a detoxifying function to dispose of ROS in mitochondria and keep the ROS level at the normal physiological condition. Oral administration of exogenous coenzymes of the respiratory chain, such as vitamins B1 or B2 and cytochrome *c*, and of ATP itself, has been investigated as way to compensate for dysfunctional mitochondria.[11,12] Nicotinamide is a component of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which also play an important role in the respiratory chain. It has been reported that tissue damage that is a result of over-production of superoxide can be protected against by adenosine via an elevation of the intracellular cAMP level.[13] In addition, adenosine is an endogenous nucleoside found in all cells of the body. Adenosine triphosphate (ATP) is produced by phosphorylation of adenosine. Taking the above information into account, we hoped that the combination of cytochrome *c*, nicotinamide and adenosine would facilitate the synthesis of ATP and electron transport, attenuating the oxidation process to delay or prevent the onset of cataract formation.

However, there are a number of problems: (1) cytochrome *c* does not penetrate lipid bilayers easily because of its high molecular weight and hydrophilic nature; (2) it has a low bioavailability with a comparatively short half-life; and (3) there is rapid precorneal loss and poor corneal permeability with conventional eye drops. One of the approaches to solve these problems is to incorporate the drug into cationic submicronic vectors that exploit the negative charges present at the corneal surface for increased residence time and penetration, such as liposomes, which are also capable of protecting cytochrome *c* from degradation. The cationic liposomes are attractive for creating an electrostatic interaction with the negative charges of mucin lying on the corneal surface and prolonging the retention time at the target site of the vesicles compared with conventional eye drops.[14] Unfortunately, aqueous liposome preparations are subject to a series of adverse events, such as aggregation, fusion and phospholipid hydrolysis. To address these problems, lyophilization has been employed.

The first aim of this study was to evaluate the influences of a variety of components on the physical properties of CC-L (cytochrome *c*-loaded liposomes) and compare their stability with CC-F (freeze-dried cytochrome *c*-loaded liposomes). A further attempt has been made to determine the anti-cataract effect of CC-F in an experimental in-vivo setting for the first time.

Materials and Methods

Drugs and reagents

Cytochrome *c* was obtained from Qingdao Jiesheng Chemical Products Co. Ltd (Qingdao, China), nicotinamide was from Tianjin Regent Chemical Products Co. Ltd (Tianjin, China)) and adenosine from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China). Sodium selenite was purchased from Peking Chemical Works (Beijing, China). Soybean phosphatidylcholine (SPC) was from Shanghai Taiwei Pharmacy Co. Ltd (Shanghai, China). Cholesterol (CH) was from Tianjin Bodi Chemical Co. Ltd (Tianjin, China). All other chemicals used were of analytical purity and commercially available.

Animals

Eleven-day-old Sprague-Dawley rat pups were obtained from the animal centre of Shenyang Pharmaceutical University and allowed free access to a commercial diet and water. The animal room was well ventilated and a regular 12-h light–dark cycle was maintained. 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, and the protocol was approved by the local animal ethics committee.

Cytochrome *c*, nicotinamide and adenosine analysis

As for cytochrome *c*, samples with a series of concentrations were obtained by dilution with phosphate buffer solution (pH 7.3), added with 5 mg sodium hydrosulfite[15] as a reducing reagent, and determined at 553 nm using a UV-2000 spectrophotometer (Unico, Princeton, USA). The HPLC system used for nicotinamide and adenosine analysis involved a 4.6 mm × 250 mm Kromasil C_{18} column (particle size 5 μm; Phenomen (Tianjin) Technology Development Co. Ltd, Tianjin, China), an L-7100 HPLC pump and an L-7420 UV variable detector operated at a wavelength of 260 nm (Hitachi, Tokyo, Japan) using 20-μl sample volumes. The mobile phase consisted of methanol–water (3 : 7, v/v) at a flow rate of 0.8 ml/min. For these studies, the calibration curve for nicotinamide was linear over the concentration range (10.08 μg/ml to 120.96 μg/ml, $r = 0.9999$), and for adenosine the calibration curve was linear over the concentration range (1.03 μg/ml to 12.36 μg/ml, $r = 0.9999$).

Determination of cytochrome *c* activity

To confirm the effect of the preparation process, the activity of the cytochrome *c* in CC-L and CC-F was evaluated.[15] Briefly, phosphate buffer (0.2 M, pH 7.3), succinate solution (0.4 M) and sample were transferred to a Nessler cylinder fitted with a stopper, followed by the addition of cytochrome-*c*-free pig heart suspension and potassium cyanide solution (0.01 M), and finally diluted with distilled water to produce 10 ml of solution, which was mixed thoroughly. The absorbance was measured until the absorbance value ceased to increase at 553 nm, which is the absorbance due to enzymatic reduction. Sodium hydrosulfite (5 mg) was then added and the absorbance was measured until the value ceased to increase at the same wavelength. This is the

absorbance due to chemical reduction.[16] A calculation was performed using Equation 1:

$$\text{Activity of cytochrome } c\ (\%) = (\text{Absorbance due to enzymatic reduction}/\text{Absorbance due to chemical reduction}) \times 100 \quad (1)$$

Preparation of liposomes

CC-L was prepared using the thin-layer evaporation technique[17] with the components shown in Table 1.

In brief, the lipid phase consisting of a mixture of SPC, CH and stearylamine (SA) was dissolved in chloroform–methanol (1 : 1, v/v) which was then removed under reduced pressure in a rotary evaporator (Eyela N-1001; Tokyo Rikakikai Co. Ltd, Tokyo, Japan) at 50°C to make a thin film of uniform dry lipid on the wall of the bottle. A 10-ml volume of aqueous phase (0.08 % cytochrome *c* in phosphate buffer, pH 6.8, together with 2% nicotinamide and 0.2% adenosine) was added to the dried lipid film and shaken at 50°C before the mixture was sonicated using a probe type sonicator (Ningbo Scientz Biotechnology Co. Ltd, Ningbo, China) for 2 min in an ice bath under an atmosphere of nitrogen.

Lyophilization

The cryoprotectants, lactose, dextran, sucrose and mannitol (5%, w/v), were dissolved in liposomal suspension. The samples were frozen at −85°C using an MDF-U32V ultra-low temperature freezer (Sanyo Electric Biomedical Co. Ltd, Tokyo, Japan) for 12 h before being lyophilized with an Eyela FDU-1100 (Prkakikai, Tokyo, Japan). The resulting solid matrix appeared as porous cakes.

Determination of encapsulation efficiency

Liposome encapsulation efficiency (EE%) was determined using the ultrafiltration technique for separating the non-entrapped drug from liposomes.[18,19] Five millilitres of drug-loaded liposomal dispersion was placed in a stirred cell (Millipore 8010; Millipore Corporation, Bedford, USA) which was fitted with a filter membrane (molecular weight cut off: 50 000 MW) under nitrogen. The ultrafiltrate was collected, and the drug contents in the ultrafiltrate (C_{free}) and the total drug content (C_{total}) after chloroform extraction were both assayed as mentioned above. The percentage of the encapsulation efficiency was calculated according to Equation 2:

$$EE\% = [(C_{total} - C_{free})/C_{total}] \times 100 \quad (2)$$

Table 1 Effect of soybean phosphatidylcholine content (A), the ratio of phosphatidylcholine to cholesterol (B) and stearylamine content (C) on the encapsulation efficiency of cytochrome *c*

A (%)	1	2	3	5
EE%	24.4	32.9	36.8	39.2
B	3 : 1	5 : 1	8 : 1	12 : 1
EE%	31.6	36.8	38.2	34.4
C (%)	0	0.1	0.2	0.4
EE%	41.6	36.8	35.4	33.0

EE%, encapsulation efficiency (n = 3).

Characterization of liposomes

The liposomal particle size and surface charge were determined by dynamic light scattering and electrophoretic light scattering, respectively, using an ELS 800 apparatus (Otsuka, Japan) at 25°C. The freeze-dried liposomes were reconstituted in de-ionized water. Equation 3 was used for converting the electrophoretic mobility to the zeta-potential:[20]

$$\zeta = \eta\mu/\varepsilon \quad (3)$$

where ζ is the zeta-potential, μ is the electrophoretic mobility, ε is the dielectric constant of the solvent and η is the viscosity.

The pH of the liposome suspension was measured with a pH meter (PB-10; Sartorius Science Instrument Ltd, Beijing, China), while the osmotic pressure was obtained with a Vapor Pressure Osmometer (Vapro 5520; Wescor, Logan, USA) at 25°C. Each measurement was repeated in triplicate.

Stability studies

The physical stability of the freeze-dried products protected from light at 4°C was assessed by evaluation of the liposomes, which were reconstituted in de-ionized water at predetermined time points.

Animal treatment protocol

The litters were allocated to four groups. The blank control group (n = 8) received no injection and was treated with normal saline (5 μl/20 g body weight) on the eyes. The model control group (n = 8) received a subcutaneous sodium selenite injection (0.38 μmol/20 g) and was treated with normal saline (5 μl/20 g) on the eyes. Group 1 and Group 2 (cytochrome *c*-treated groups, n = 8 each) received the same amount of sodium selenite injection and were treated with ophthalmic administration of cytochrome *c* solution and CC-F, respectively (5 μl/20 g). Cytochrome *c* solution was prepared according to the marketed Vitaphakol Eye Drops (composition: cytochrome *c* 0.05%, nicotinamide 2%, adenosine 0.2%). The instillations were repeated three times per day for eight days. Cataract development was assessed every two days by slit lamp examination. The pupils were dilated with tropicamide 0.5% and phenylephrine hydrochloride 2.5% before taking slit images with a photo slit-lamp microscope and an anterior eye segment analysis system, EAS-1000 (Nidek, Aichi, Japan), equipped with a CCD camera, as described previously.[21] The observer did not know the identity of the rats beforehand.

The area of lens opacity, expressed as pixels, was analysed by computer with image analysis software connected to EAS-1000 and calculated by Equation 4:

$$\text{Pixels within opacity} = \text{pixels within outline} - \text{pixels within transparent area} \quad (4)$$

The percentage of lens opacity of each group was identified by Equation 5:

$$\text{Opacity}\,(\%) = (\text{Area}_{\text{opacity in the treated group}}/\ \text{Area}_{\text{opacity in the model group at the 8th day}}) \times 100 \quad (5)$$

where the treated group received a subcutaneous sodium selenite injection and were treated with CC-L or CC-F and the model group received a subcutaneous sodium selenite injection alone.

Statistical analysis

The mean value of each parameter in each individual group of rats was calculated from eight individual values. Statistical analysis was performed using a one-way analysis of variance with SPSS software. $P < 0.05$ was considered significant.

Results

Factors influencing encapsulation efficiency

Effect of phosphatidylcholine content

On the basis of preliminary experiments, the amount of lipids was the major factor affecting the EE% of liposomes. Table 1 shows the encapsulation efficiency of formulations with different SPC contents (1.0, 2.0, 3.0 and 5.0%, w/v). The EE% increased markedly to 39.2% with 5.0% lipids. When the SPC concentration was above 5.0%, the vesicles might be prone to aggregate and precipitate due to lipid fragments.

Effect of the ratio of phosphatidylcholine to cholesterol

The encapsulation efficiency of cytochrome *c* in formulations with different ratios of SPC to cholesterol (3 : 1, 5 : 1, 8 : 1 and 12 : 1, w/w) is shown in Table 1. With the increasing ratio of lipids, the loading efficiency increased with the optimal encapsulation efficiency reaching 38.2% at the ratio of 8 : 1, and this decreased when the ratio was above 8 : 1.

Effect of stearylamine content

The encapsulation efficiency of CC-L decreased with increasing concentrations of stearylamine (0, 0.1, 0.2 and 0.4%, w/v) (Table 1).

After the preliminary experiment, the optimal composition of the cytochrome *c*-loaded liposomes was obtained by orthogonal experimental design as follows: SPC 3%, SPC/CH 8 : 1 (w/w), SA 0.1%, cytochrome *c* 0.08%, nicotinamide 2%, adenosine 0.2%.

Cryoprotectant investigation

Liposomal lyophilization is one of the most promising procedures to keep the liposomes stable during long-term storage.[22] Therefore, the cryoprotectants, lactose, dextran, sucrose and mannitol (5%, w/v), were evaluated as candidates after preliminary experiments according to the appearance, morphology and stability of the lyophilized liposomes after rehydration.

As to the protective effect of lyoprotectants (lactose, sucrose and dextran), the size and encapsulation efficiency of freeze-dried liposomes were similar to these of cytochrome *c* liposomes. The dried product obtained with sucrose was satisfactory in appearance and easy to reconstitute with unchanged and uniform size distribution. As far as mannitol was concerned, the lyophilized product was difficult to rehydrate but possessed smooth and bright appearance. Considering mannitol as the protein stabilizer, when it was combined with sucrose (1 : 1, w/w) to a final sugar concentration of 5%, the lyoprotective effect was similar to that of sucrose and, furthermore, the freeze-dried liposomes possessed excellent loose features and could be reconstituted in de-ionized water easily and rapidly.

Characteristics and stability of liposomes

Characteristics of liposomes and freeze-dried liposomes after rehydration

The encapsulation efficiency, median particle size, zeta-potential (ζ), pH and osmotic pressure of CC-L and CC-F are given in Table 2. The composition of freeze-dried liposomes was as follows: SPC 3%, SPC/CH 8 : 1 (w/w), SA 0.1%, cytochrome *c* 0.08%, nicotinamide 2%, adenosine 0.2%, cryoprotectants (mannitol–sucrose 1 : 1, w/w) 5%. CC-F were reconstituted in de-ionized water and it was seen that the encapsulation efficiency, ζ, pH and osmotic pressure of liposomes changed little after lyophilization except that the median particle size was larger, increasing from 136.4 ± 17.2 nm to 346.6 ± 44.9 nm. Notwithstanding, the properties of the lyophilized liposomes were still within the physiologically compatible range.

Stability of liposomes

No significant change in vesicle size, pH, drug content or cytochrome *c* activity was observed for liposomes formed by rehydration of CC-F during the long-term stability study, and they had a smooth, full and compact appearance and could be rapidly reconstituted. For the liposome suspension in the stability study, the activity of cytochrome *c* decreased 15%, the content of adenosine decreased 9% and the encapsulation

Table 2 The characteristics of liposomes and freeze-dried liposomes after rehydration

Preparation	EE%	Median particle size diameter (nm)	ζ (mV)	pH value	Osmotic pressure (mmol/kg)
Liposomes	38.4 ± 1.5	136.4 ± 17.2	21.06 ± 0.26	6.82 ± 0.05	285 ± 2.3
Freeze-dried liposomes	35.2 ± 1.7	346.6 ± 44.9	17.25 ± 0.35	6.85 ± 0.09	283 ± 3.1

Data are means ± SD, $n = 3$. Composition of freeze-dried liposomes: SPC 3%, SPC/CH 8 : 1 (w/w), SA 0.1%, cytochrome *c* 0.08%, nicotinamide 2%, adenosine 0.2%, cryoprotectants (mannitol–sucrose 1 : 1, w/w) 5%. The dispersion medium was de-ionized water (pH 7.0) at 25°C and ions were eliminated to avoid influence on the surface electric charge of liposomes. CH, cholesterol; EE%, encapsulation efficiency of cytochrome *c*; SA, stearylamine; SPC, soybean phosphatidylcholine; ζ, zeta-potential.

Table 3 Storage stability of cytochrome-*c*-loaded liposomes (A), freeze-dried liposomes (B) and reconstituted liposomes (C) with SPC concentration of 3% at 4°C

Time (months)	Cytochrome *c* content (%)		Cytochrome *c* activity (%)		Nicotinamide content (%)		Adenosine content (%)		EE%		Median particle size diameter (nm)		pH value	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	100.3 ± 1.6	99.8 ± 0.8	102.1	101.5	102.5	99.2	99.6	100.2	38.4 ± 1.3	35.2 ± 1.7	136.4 ± 17.2	346.6 ± 44.9	6.82 ± 0.06	6.85 ± 0.08
1	101.5 ± 2.0	98.9 ± 1.8	97.3	100.3	100.5	99.5	100.2	100.7	37.3 ± 2.1	36.4 ± 1.2	138.6 ± 57.6	351.6 ± 11.3	6.80 ± 0.05	6.88 ± 0.08
3	98.6 ± 1.4	100.3 ± 1.5	96.3	99.5	98.2	98.3	98.9	99.4	35.2 ± 1.4	35.2 ± 0.9	142.1 ± 22.8	349.2 ± 35.2	6.86 ± 0.05	6.95 ± 0.06
6	99.5 ± 1.7	99.3 ± 1.5	97.0	98.7	96.3	99.3	98.1	99.0	31.8 ± 2.3	34.9 ± 1.5	159.0 ± 34.9	361.0 ± 7.9	6.92 ± 0.08	6.78 ± 0.05
12	93.3 ± 1.3	98.9 ± 2.1	87.2	97.6	97.1	98.5	90.7	98.6	25.0 ± 1.6	33.8 ± 1.1	205.4 ± 118.0	358.0 ± 39.6	6.88 ± 0.07	6.83 ± 0.06
	C		C		C		C		C		C		C	
0	99.5 ± 1.9		102.2		100.9		101.4		35.32 ± 0.8		340.4 ± 19.4		6.79 ± 0.03	
1/3	100.1 ± 1.7		101.2		98.8		99.5		35.01 ± 1.6		354.6 ± 33.6		6.83 ± 0.06	
2/3	99.7 ± 1.1		99.8		99.4		100.9		34.82 ± 1.1		343.1 ± 41.7		6.92 ± 0.07	
1	99.2 ± 1.5		97.8		98.0		99.4		32.10 ± 2.1		359.3 ± 37.0		6.85 ± 0.04	

Composition of freeze-dried liposomes: SPC 3%, SPC/CH 8 : 1 (w/w), SA 0.1%, cytochrome *c* 0.08%, nicotinamide 2%, adenosine 0.2%, cryoprotectants (mannitol–sucrose 1 : 1, w/w) 5%. The dispersion medium was de-ionized water (pH 7.0) and ions were eliminated to avoid influence on the surface electric charge of liposomes at 25°C. Drug contents (cytochrome *c*, nicotinamide or adenosine) are a percentage of the initial concentrations as measured at time zero. CH, cholesterol; EE%, encapsulation efficiency of cytochrome *c*; SA, stearylamine; SPC, soybean phosphatidylcholine.

efficiency of cytochrome *c* was only 65% of the original level at the 12th month. Ophthalmic liposomes will be used for a relatively long period after rehydration, unlike those used for parenteral administration. Hence, the stability of CC-F after rehydration storage at 4°C was also investigated. There was no appreciable change in the physical properties over a month (Table 3), and it could be assumed that the liposomal eye drops were stable.

Assessment of opacity changes in lenses

High concentrations of selenium have been reported to produce nuclear cataract when injected subcutaneously to young rat pups.[8] The slit images of four groups are shown in Figure 1. Our experiments involving the treatment of suckling rats with selenite alone resulted in formation of nuclear cataract within four days in the model group and the cataracts developed rapidly over six days to a mature dense opacity involving the entire lens. In the control group, lenses remained clear throughout the experiment. As far as Group 1 was concerned, the onset of cataract was later than in the model group, the percent of opacity was lower and the size was smaller. There was a significant reduction in opacification of the lenses during the same period in Group 2. Although the transparency of the crystalline lenses was impaired over the six days and opacification occurred on the 8th day, the onset of cataract was markedly delayed compared with groups treated with normal saline or liposome-free solution.

Figure 2 shows the percentage of opacity after the pups were treated with cytochrome *c* solution (Group 1) and CC-F (Group 2). Compared with the blank control group, the opacity of the model control group reached nearly 100% at the end of the examination, while that of the treated groups was 51% and 28% for Group 1 and Group 2, respectively. Following treatment with cytochrome *c* solution, the increase in opacity began from the 6th day, when there was barely any change in transparency in the CC-F-treated group. The differences among groups were statistically significant ($P < 0.01$).

Discussion

Optimization of cytochrome-*c*-loaded liposome formulation

Numerous studies have focused on the preparation of liposomes. In general, the choice of phospholipids is often limited to the family of the phosphatidylcholines because of toxicological considerations and their ready availability as pure compounds. The phase transition temperature (Tc) is critical to the physical states of the phospholipids. When the surrounding temperature is below the Tc, lipids are in a well-ordered gel-like state. When the surrounding temperature is above the Tc, lipids are in a disordered fluid-like or liquid-crystalline state and the bilayer is less stable and more permeable to solute, especially in the case of hydrophilic solute.[23] After the incorporation of cholesterol, the fluidity of the lipid bilayer can be improved, with decreased rotational freedom of hydrophobic chains of phospholipids

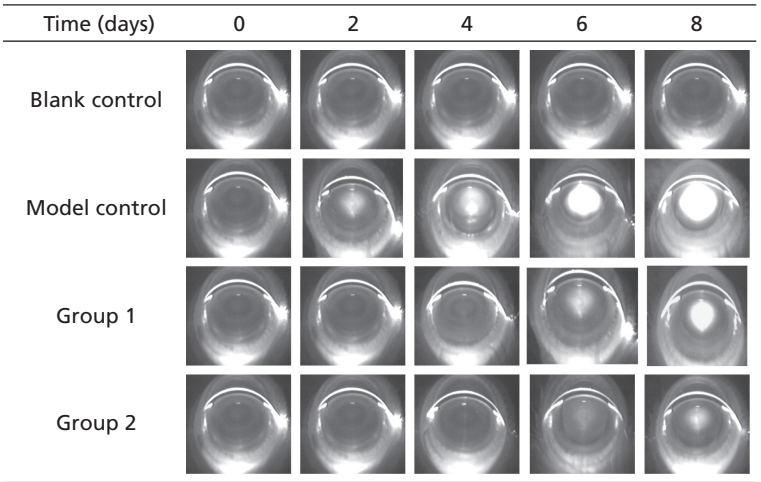

Figure 1 Slit images of eyes from selenite cataract rats, untreated or treated with cytochrome *c*. The blank control group received no injection and was treated with normal saline; the model control group received a subcutaneous sodium selenite injection and was treated with normal saline; Group 1 was treated with cytochrome *c* solution (composition: cytochrome *c* 0.05%, nicotinamide 2%, adenosine 0.2%); Group 2 was treated with lyophilized cytochrome-*c*-loaded liposomes (composition: SPC 3%, SPC/CH 8 : 1 (w/w), SA 0.1%, cytochrome *c* 0.08%, nicotinamide 2%, adenosine 0.2%, cryoprotectants (mannitol–sucrose, 1 : 1) 5%) and reconstituted with de-ionized water. CH, cholesterol; SA, stearylamine; SPC, soybean phosphatidylcholine.

and elimination of phase transition,[24] leading to less drug leakage.

According to the results obtained by analysis of liposomal components, optimal encapsulation efficiency was obtained using 3% phospholipids. Elevation of EE% did not accompany the increment of the lipid amount, which may be due to aggregation or fusion as a result of the presence of lipid fragments or a larger vesicle size.[25] Along with the influence of phosphatidylcholines, the amount of cholesterol added could augment the EE% to some extent and the highest cytochrome *c* entrapment efficiency was observed at the ratio 8 : 1 (SPC : CH). This could be explained by the fact that cholesterol acts like a 'buffer' to accommodate the fluidity of the membrane. The more cholesterol that is incorporated into the bilayer, the larger the curvature of the membrane and the more stable it is. As a result, the EE% should increase because of less drug leakage from the liposomes. When stearylamine was incorporated, the amount of encapsulated drug decreased, and this can be explained by the interaction of cytochrome *c* with lipid membranes depending on the electrostatic attraction. Cytochrome *c* (pI = 10.2–10.8) possesses a positive charge in phosphate buffer (pH 6.8) and phosphatidylcholine carries a negative charge, leading to a higher encapsulation efficiency. On the other hand, stearylamine is a positive-charge-inducing agent. Since cytochrome *c* and stearylamine both bear positive charges, there was a competition between them for the affinity to phospholipid, leading to the reduced cytochrome *c* encapsulation efficiency.

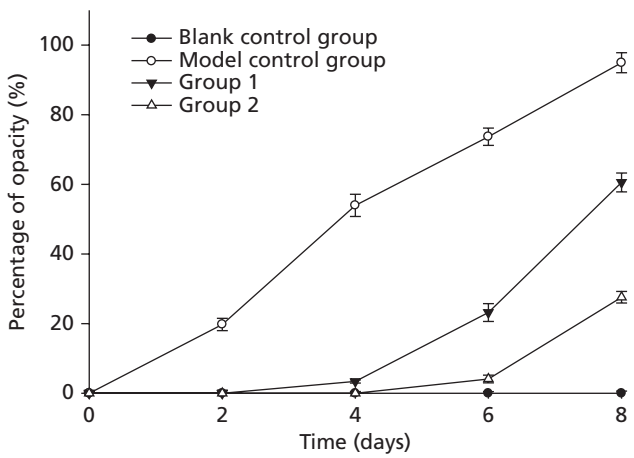

Figure 2 Effect of cytochrome *c* on the opacification of selenite cataract eye lenses in rats. Data are means ± SD, $n = 8$. The blank control group received no injection and was treated with normal saline; the model control group received a subcutaneous sodium selenite injection and was treated with normal saline; Group 1 was treated with cytochrome *c* solution (composition: cytochrome *c* 0.05%, nicotinamide 2%, adenosine 0.2%); Group 2 was treated with lyophilized cytochrome-*c*-loaded liposomes (composition: SPC 3%, SPC/CH 8 : 1 (w/w), SA 0.1%, cytochrome *c* 0.08%, nicotinamide 2%, adenosine 0.2%, cryoprotectants (mannitol–sucrose, 1 : 1) 5%) and reconstituted with de-ionized water. CH, cholesterol; SA, stearylamine; SPC, soybean phosphatidylcholine.

Effect of lyoprotectors on physical properties of freeze-dried cytochrome *c* liposomes

The gel-to-lipid crystalline phase transition temperatures (Tm) of liposomes based on unsaturated lipids can increase during freeze-drying.[26] Therefore freeze-dried liposomes undergo a gel-to-liquid phase transition. The changes in Tm affect the permeability of the bilayer, which is transiently leaky and prone to fusion during rehydration.

Saccharides, such as lactose, sucrose and dextran, are known to protect membranes from damage, replacing the water molecules in associating with the polar head of phospholipid to produce less aggregation and reducing surface tension during lyophilization. At the same time, the

sugar molecules may also maintain the phospholipid in a fluid-like state to avoid passage through the gel to the liquid-crystalline phase, thereby preventing the phospholipid bilayer disruption,[27,28] which is one possible reason for the effect of lyoprotectants on the encapsulation efficiency of freeze-dried liposomes, similar to that occurring with cytochrome *c* liposomes. The increased median particle size of lyophilized liposomes may be explained by an increased hydrodynamic diameter of the liposomes due to coating of the vesicle surface with the saccharides through hydrogen bonding with lipids. Mannitol has also been tentatively used as a cryoprotectant. Since there is no interaction between mannitol located in the aqueous part and the lipid head groups,[29] a weaker protective effect is expected.

Stability of cytochrome-*c*-loaded liposomes and freeze-dried liposomes

Stability is always a limiting factor for the application of liposomes. Cytochrome *c* is chemically unstable with a short half-life. It was found that CC-L requires lyoprotectants since without stabilizer they would aggregate completely during freeze drying and, apparently, lyophilization is a more stressful process for long-term storage at 4°C compared with CC-L (Table 3). No significant changes were observed in vesicle size, pH, drug content or cytochrome *c* activity during 12 months.

The inhibitory activity of cytochrome *c* against rat lens opacity

In lens cataracts, cell opacity results when the index of refraction fluctuation is large enough to scatter light.[30] The oxy-radical-induced oxidative stress on the lens is assumed to play a critical role in the aetiology of the disease. Much of the data from selenite cataract show that the lens epithelium may be the first cell layer exposed to selenite. Some critical changes in metabolism have been documented in lens epithelium during formation of selenite cataract, while accelerated apoptosis (programmed cell death) might be a fairly early event in selenite cataract.[31] Cytochrome *c* would eliminate superoxide anion and H_2O_2 when ROS were over-generated. It is rational that the less cytochrome *c* transfers electrons, the more electrons leak out and the more ROS are generated. The ROS burst could be the earlier event to drive cells to apoptosis.[32] It has been reported that the oxygen can be reduced to toxic superoxide anion by the leaked electrons from complex I and complex III and then the superoxide anion is dismutated to H_2O_2. The ferricytochrome *c* scavenges the superoxide anion and the ferrocytochrome *c* scavenges H_2O_2, which make cytochrome *c* play a role in keeping superoxide anions and H_2O_2 at the normal physiological level.[33]

With this in mind, we conducted studies with cytochrome *c*, a protein with ironic porphyrin as an active centre, located on mitochondria. The reversible oxidation–reduction reactions occurred between the different oxidation states of cytochrome *c*, which participate in the electron transport in the respiratory chain. Meanwhile, nicotinamide and adenosine are necessary for ATP synthesis and energy metabolism. Therefore, these studies were conducted with a combination of these three drugs.

Various experimental models have been reported to delineate the mechanism of cataractogenesis and to identify crucial targets in the process. The selenite cataract is a rapidly induced and convenient model of cataractogenesis, and is a useful in-vivo rodent model for primary drug evaluation. The selenite-induced cataract may be related to the oxidative damage to the lens epithelium caused by selenite, leading to a progressive decline in the transport of cations and the activity of some critical enzymes with a loss of lens transparency.[34]

With regard to the toxicity of selenium and the preparations, we examined the tissues, including the cornea, iris and sclera, and found no toxicity or irritation in the model control group or the cytochrome-*c*-treated groups (data not shown).

The slit images indicated that even though cytochrome *c* cannot prevent the development of cataract completely, it can be effective in attenuating the severity of lens opacity, through facilitating the synthesis of ATP and improving the energy metabolism. It appears that cytochrome *c* solution prevented the onset of cataract to a lesser extent than CC-F, which may be due to the liposomal protection of cytochrome *c* and the prolonged retention time on the eye surface obtained by incorporating a positively charged inducing agent in the formulation.

Conclusions

In this study the preparation, stability and anti-cataract effect of cationic freeze-dried liposomes containing cytochrome *c*, along with nicotinamide and adenosine, are described. With optimal formulation, the encapsulation efficiency of cationic freeze-dried liposomes after rehydration reached 35% with unimodal size distribution. The lyophilized liposomes were more stable than the CC-L and able to protect the drug for at least 12 months.

We also demonstrated for the first time that cytochrome-*c*-loaded freeze-dried liposomes exhibited a more significant effect than the conventional cytochrome *c* solution on retarding the onset and progression of cataract in rats. This effect was associated with delayed lens opacity, which was nearly 28% at the end of eight days. Unfortunately, the exact molecular mechanisms of its functions have not been elucidated so far. Although our preliminary results are encouraging, further studies are desirable at biochemical and pharmacological levels.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Geraldine P *et al*. Prevention of selenite-induced cataractogenesis by acetyl-L-carnitine: an experimental study. *Exp Eye Res* 2006; 83: 1340–1349.
2. Varma SD, Hegde KR. Effect of alpha-ketoglutarate against selenite cataract formation. *Exp Eye Res* 2004; 79: 913–918.
3. Yu XL *et al*. Antioxidant activities of compounds isolated from *Dalbergia odorifera T. Chen* and their inhibition effects on the decrease of glutathione level of rat lens induced by UV irradiation. *Food Chem* 2007; 104: 715–720.
4. Minard KI, McAlister-Henn L. Antioxidant function of cytosolic sources of NADPH in yeast. *Free Radic Biol Med* 2001; 31: 832–843.
5. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 1997; 272: 20313–20316.
6. Sohal RS, Windruch R. Oxidative stress, caloric restriction, and aging. *Science* 1996; 273: 59–63.
7. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* 2000; 29: 222–230.
8. Oštádalová I *et al*. Cataract induced by administration of a single dose of sodium selenite to suckling rats. *Experientia* 1978; 34: 222–223.
9. Malatesta F *et al*. Electron entry in a Cu_A mutant of cytochrome *c* oxidase from *Paracoccus denitrificans.* Conlusive evidence on the initial electron entry metal center. *FEBS Lett* 1998; 434: 322–324.
10. Tudor G *et al*. Cytotoxicity and apoptosis of benzoquinones: redox cycling, cytochrome *c* release, and BAD protein expression. *Biochem Pharmacol* 2003; 65: 1061–1075.
11. Yamada Y, Harashima H. Mitochondrial drug delivery systems for macromolecule and their therapeutic application to mitochondrial diseases. *Adv Drug Deliv Rev* 2008; 60: 1439–1462.
12. Tanaka J *et al*. Treatment of mitochondrial encephalomyopathy with a combination of cytochrome *c* and vitamins B1 and B2. *Brain Dev* 1997; 19: 262–267.
13. Si QS *et al*. Adenosine inhibits superoxide production in rat peritoneal macrophages via elevation of cAMP level. *Immunopharmacology* 1997; 36: 1–7.
14. Cortesi R *et al*. Cationic liposomes as potential carriers for ocular administration of peptides with anti-herpetic activity. *Int J Pharm* 2006; 317: 90–100.
15. State Pharmacopeia Commission of China. *Pharmacopeia of the People's Republic of China (Second Part)*. Beijing: Chemical Industry Press, 2005: Appendix 102.
16. Zhang YL, Wang YS. Discussion of method for activity assay of cytochrome *c*. *Chinese J Biochem Pharm* 1994; 15: 59–61.
17. Bangham AD *et al*. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol* 1965; 13: 238–252.
18. Lei GF *et al*. Determination of entrapment efficiency for liposomal formulation of breviscapine using ultrafiltration-HPLC method. *J Shenyang Pharmaceutical University* 2006; 23: 237–243.
19. Li BQ *et al*. Determination of entrapment efficiency of topotecan liposomes by ultrafiltration-HPLC. *Chinese J New Drugs* 2007; 16: 58–61.
20. Nicoli DF *et al*. Zeta potential and particle size analysis of colloids using ELS and DLS. *Am Lab* 1997; 29: 12.
21. Ito Y *et al*. Correlation between prevention of cataract development by disulfiram and fates of selenium in selenite-treated rats. *Curr Eye Res* 1999; 18: 292–299.
22. Shulkin PM *et al*. Lyophilized liposomes: a new method for long-term vesicular storage. *J Microencapsul* 1984; 1: 73–80.
23. Siler-Marinkovic S *et al*. Liposomes as carriers of antimicrobial drugs. *Drug Dev Ind Pharm* 1997; 23: 483–488.
24. Sharma A, Sharma US. Liposome in drug delivery: progress and limitations. *Int J Pharm* 1997; 154: 123–140.
25. Wu PC *et al*. The characterization and biodistribution of cefoxitin-loaded liposomes. *Int J Pharm* 2004; 271: 31–39.
26. Christensen D *et al*. Trehalose preserves DDA/TDB liposomes and their adjuvant effect during freeze-drying. *Biochim Biophys Acta* 2007; 1768: 2120–2129.
27. Crowe JH *et al*. Interactions of sugars with membranes. *Biochim Biophys Acta* 1988; 947: 367–384.
28. Wolkersa WF *et al*. Preservation of dried liposomes in the presence of sugar and phosphate. *Biochim Biophys Acta* 2004; 1661: 125–134.
29. El Maghraby GMM *et al*. Drug interaction and location in liposomes: correlation with polar surface areas. *Int J Pharm* 2005; 292: 179–185.
30. Benedek GB. Theory of transparency of the eye. *Appl Opt* 1971; 10: 459–473.
31. Tamada Y *et al*. Evidence for apoptosis in the selenite rat model of cataract. *Biochem Biophys Res Commun* 2000; 275: 300–306.
32. Lee M, Xu JX. Detoxifying function of cytochrome *c* against oxygen toxicity. *Mitochondrion* 2007; 7: 13–16.
33. Xu JX. The role of electron leakage of mitochondrial respiratory chain in cell apoptosis. *Pro Biochem Biophys* 2003; 30: 655–657.
34. Shearer TR *et al*. Selenite cataract: a review. *Curr Eye Res* 1987; 6: 289–300.