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Protective effect of Coenzyme Q_{10} against oxidative damage in human lens epithelial cells by novel ocular drug carriers

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ABSTRACT

The evaluation of N-trimethyl chitosan (TMC)-coated liposomes containing Coenzyme Q_{10} as potential ophthalmic drug delivery system was carried out. Firstly, transcorneal permeation studies were conducted at 34 °C using a side-by-side diffusion apparatus. The transport process of the fluorescent marker, rhodamine B, across the corneal epithelium was visualized with confocal laser scanning microscopy. Secondly, the human lens epithelial cells (HLECs) were cultured without or with Coenzyme Q_{10} followed by addition of H_2O_2. The cell viability and apoptosis were evaluated. The permeability coefficient for rhodamine B with TMC-coated liposomes increased more than two times in comparison with the value obtained for solution as control, from $(0.42 \pm 0.018) \times 10^5$ cm s^{-1} to $(1.31 \pm 0.030) \times 10^5$ cm s^{-1}. Confocal laser scanning microscopy revealed that a TMC coating enhanced the transepithelial transport, dependent on the TMC concentration and contacting time. Coenzyme Q_{10} elevated the cell viability and reduced the oxidative damage with the decreased percentage of apoptotic cells in a positive concentration-dependent manner. The ATP content of liposome-treated cells was increased about 2-fold compared with that of H_2O_2-treated cells. Together, our findings demonstrate that with the enhanced permeation effect of the TMC coating, Coenzyme Q_{10}-loaded TMC-coated liposomes appear to be a promising ophthalmic drug delivery carrier with an efficacy in protecting HLECs against H_2O_2-induced oxidative damage.
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1. Introduction

Topical application of drugs to the eye is the common and well-accepted route of administration for the treatment of various eye disorders. Improving the poor bioavailability of ophthalmic drug is a major challenge. Such poor bioavailability, amounting to at best 10% of the applied dose, is largely due to efficient protective mechanisms of the eye. Therefore, many strategies have been adopted to develop new ocular drug delivery systems with enhanced bioavailability of drugs (Lee and Robinson, 1986). Among these new ocular systems, the use of colloidal drug delivery systems, such as liposomes, is now considered a useful strategy to enhance the ocular bioavailability of topically administrated drugs (Monem et al., 2000). Furthermore, the performance of liposomes can be improved by providing them with a chitosan coating (Borchard et al., 1996). However, chitosan is a weak base and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form. At physiological pH, most chitosan molecules will lose their charge and precipitate from solution. This inconvenience can be circumvented by synthesizing the partially quaternized derivative, N-trimethyl chitosan chloride (TMC), which is soluble over a wide pH range. To our knowledge, there are few published reports on the use of TMC in ophthalmology as a coating material.

TMC exhibits an excellent absorption-enhancing effect even at neutral pH by opening the tight junctions between adjacent cells of epithelial cell monolayers by exploiting the negatively charged sites on the cell membrane through ionic interaction (Zambito et al., 2006). It has been suggested that TMCs with a medium to high degree of quaternization are capable of significantly enhancing the permeability of ofloxacin across a stratified epithelium, for example the cornea (Di Colo et al., 2004). It was also found that TMCs could significantly change the secondary structure of keratin in stratum corneum of skin, decrease cell membrane potential and enhance cell membrane fluidity, which leading to transdermal enhancement of the drug (Hen et al., 2009). Therefore, we synthesized the poly-

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mer with a high degree of quaternization at 66.3%, as a component of the preparation. We have reported that the drug elimination of TMC-coated liposomes is significantly slower than the radiolabeled solution used as a control in gamma scintigraphy (Zhang and Wang, 2009). In this study, the capacity of TMC-coated liposomes to increase the ocular permeation of lipophilic drugs was evaluated.

Oxidative stress in the lenses is the most common damaging factor for the development of cataract. The epithelial layer of the lens is the main target of the oxidative insult (Ottonello et al., 2000). This layer is responsible for maintaining lens homeostasis and transparency, and any external insult may affect its antioxidant status (Spector, 1995). Apoptosis of lens epithelial cells, which is an important, intricate and finely orchestrated mechanism that maintains the homeostasis of viable cells (Thompson, 1995), is suggested to be a cause of cataract formation (Li et al., 1995). In the aqueous fluid and lenses of some cataract patients, the level of H_2O_2 is markedly raised (Spector and Garner, 1981) and there is clear evidence that H_2O_2 may be involved in the genesis of human nuclear cataract (Truscott and Augusteyn, 1977). Using *in vitro* organ culture studies, H_2O_2, a non-radical member of the active oxygen family, can easily penetrate lipid membranes and has been proved to be toxic to the lens (Fukui, 1976). At the molecular level, H_2O_2 generates hydroxyl radicals which are particularly damaging to DNA, resulting in mutagenesis and leading to cataract formation (Imlay and Linn, 1988), in addition to producing damage to both the cell membrane and cytosol regions as it causes a decrease in the levels of antioxidants (Spector et al., 1985; Richer and Rose, 1998). The human lens epithelial cell line, SRA 01/04 has been established by using the immortalizing gene of SV40 and is derived from a single cell with uniform characteristics. The SRA 01/04 cell line appears to be an excellent model system for investigating the cellular functions of the human lens epithelium (Ibaraki et al., 1998; Ou et al., 2008) under the oxidative damage induced by H_2O_2 in this study.

Coenzyme Q_{10} (CoQ_{10}) is known as an endogenous cellular antioxidant. It is essential for adenosine 5′-triphosphate (ATP) synthesis in mitochondria as a carrier of electrons and protons in oxidative phosphorylation and in other proton-pumping membranes, presumably by the Q-cycle (Mitchell, 1975). Its excellent ability to fulfill two established functions as an antioxidant and as electron/proton carriers in bioenergetic membranes (Tutunen et al., 2004) makes CoQ_{10} attractive as a potential anti-cataract agent.

To summarize, the two main goals of the present work were: firstly, to evaluate the effect of a TMC coating for ocular application on permeation enhancement across rabbit cornea and its transport route; secondly, to investigate the effect of CoQ_{10}-loaded liposomes coated with TMC on protecting against hydrogen peroxide-induced oxidative damage in SRA 01/04 cells with anti-apoptotic effect.

2. Materials and methods

2.1. Materials

CoQ_{10} was purchased from Taizhou Kaichuang Chemical Co., LTD. Soy phosphatidylcholine was from Shanghai Taiwei Pharmaceutical Industry Co., LTD. Cholesterol was purchased from Taizhou Hisound Chemical Co., LTD. TMC (66.3% degree of quaternization, synthesized from chitosan with an MW of 450 KDa) was synthesized in our own laboratory. Rhodamine B (RhB) was supplied by TianJin Bodi Chemical Holding Co., LTD.

Male albino New Zealand rabbits (2.5–3.0 kg, free of clinically observable abnormalities) were obtained from the animal center of Shenyang Pharmaceutical University and given a commercial diet and water *ad libitum* throughout the study. All experiments were run in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the protocols were approved by the local ethics committee for animal experimentation.

For cell culture, the human lens epithelial cell line SAR 01/04 was obtained from the Chinese Academy of Medical Sciences (Beijing, China). The reagents employed in this study were as follows: minimum essential medium (MEM; Hyclone, USA), fetal bovine serum (FBS; Gibco, USA), methylthiazol tetrazolium (MTT; Fluka, USA), Dimethyl sulfoxide (Sigma, USA). All other materials were used without further purification.

2.2. Preparation and characterization of liposomes

Small unilamellar liposomes composed of soy phosphatidylcholine, cholesterol (83:17, w/w) and α-tocopherol were prepared by ethanol injection method followed by sonication. Briefly, the lipid fraction (1.0%, w/v) and CoQ_{10} (0.1%, w/v) were dissolved in 2 ml of ethanol, and the solution was then injected into 10 ml of phosphate buffer (pH 6.8) at 50 °C. The obtained suspension was sonicated.

For preparation of polymer-coated liposomes, TMC was dissolved in phosphate buffer (pH 6.8) to give a series of concentrations (0.1, 0.2, 0.5, and 1.0%, w/v). These solutions were mixed with the same volume of small unilamellar liposomes under magnetic stirring, and then incubated at 10 °C for 60 min.

The size and zeta potentials of the liposomes were measured by dynamic light scattering and electrophoretic light scattering, respectively, both using an ELS 800 apparatus (Otsuka, Japan) at 25 °C. For size measurements, the liposomal dispersions were diluted about 100 times in the same buffer used for their preparation. Dust particles were eliminated by filtration (0.45 μm) from the vesicle preparation as well as from the buffer solution. The dispersion medium for the evaluation of zeta potential of liposomes was Millipore-filtered water (pH = 7.0) and ions were eliminated to avoid influence on the surface electric charge of liposomes. The equations (Nicoli et al., 1997) used for converting the electrophoretic mobility to the zeta potential are:

$$\zeta = \frac{\eta\mu}{\varepsilon}$$

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

Liposome entrapment efficiency (EE%) was determined using the ultrafiltration technique for separating the non-entrapped drug from liposomes (Zhang and Wang, 2009; Lei et al., 2006). The ultrafiltrate was collected by using a stirred cell (Millipore 8010, Millipore Corporation, Bedford, USA), and the drug content in the ultrafiltrate (c_{free}) was determined by HPLC on a C_{18} Kromasil column (Phenomenex, USA) (150 mm × 4.6 mm) with a particle size of 5 μm at 275 nm. The mobile phase consisted of methanol–ethanol (1:1, v/v) with a flow-rate of 1.0 ml/min at 25 °C. Then liposomal suspension was diluted with mobile phase to determine the total CoQ_{10} (c_{total}) by HPLC. The EE% was calculated by:

$$EE\% = \frac{c_{total} - c_{free}}{c_{total}} \times 100$$

2.3. Ocular irritation study

Ocular irritation was evaluated in four rabbits and the treatment was performed according to the modified Draize technique (Draize et al., 1944). Either 0.1 ml of saline or TMC-coated liposomes containing CoQ_{10} with the concentration of TMC at 0.5% (w/v) was instilled randomly into four eyes, in the way that no rabbit received the same type of drops and each preparation was instilled to two right and two left eyes. The instillation was performed four times a day for seven consecutive days. Any signs of irritation (redness,

inflammation, chemosis or increased tear production) were monitored periodically after instillation.

At the end of the seventh day, following the final examination, the rabbits were sacrificed using an overdose of pentothal. The eyes were enucleated, and fixed in formalin solution 8% (w/w) for 24 h, and then examined blind under a microscope (Olympus, Japan) by hematoxylin and eosin staining in co-operation with a professional oculist.

2.4. Transcorneal permeation studies of the colloidal carriers

The liposomes were prepared as described above but adding RhB, instead of CoQ_{10}, as a fluorescence marker, to the liposomes for *in vitro* transcorneal measurements (Suhonen et al., 1998). Briefly, rabbits were sacrificed by a marginal ear-vein injection of an overdose of sodium pentobarbital. The whole eyes were enucleated within 20 min after death and the cornea, together with a 2 mm ring of sclera, was excised. The cornea was mounted between two half-cells of a side-by-side perfusion apparatus which maintained the corneal curvature and was placed in a thermostated bath at 34 °C, facing a blank of glutathione-bicarbonate Ringer's solution (GBR, preadjusted to pH 6.8 at 34 °C) on both sides. After an equilibration period of 30 min, the buffer was replaced with TMC-coated liposomes containing RhB on the donor (epithelial) side, and with GBR buffer on the acceptor (endothelial) side. Liposomes incorporating RhB and RhB solution were used as control. Carbogen (a mixture of 95% O_2 and 5% CO_2) was bubbled through both chambers to maintain tissue viability and to ensure mixing.

Sample of 100 μl was withdrawn from the acceptor side at suitable time intervals over 180 min, and the same volumes were replaced with freshly prepared acceptor medium. The samples were analyzed by HPLC with a Kromasil C_{18} (5 μm, 150 mm × 4.6 mm) analytical column (Phenomenex, USA). An injection volume of 20 μl was used and RhB could be detected at 545 nm with the mobile phase consisting of methanol–water (3:1, v/v) at a flow rate of 1.0 ml/min at 25 °C. The experiment was done in triplicate.

The apparent permeability coefficient (P_{app}) in units of centimeters per second, was calculated by

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{60AC_0}$$

where the term $\Delta Q/\Delta t$ was the steady state slopes of linear plots of the amount of drug in the receptor cell (Q) vs. time (t), A the exposed corneal surface area (0.78 cm^2), C_0 the initial permeant.

The steady state flux (J_{ss}, μg cm^{-2} s^{-1}) was calculated by

$$J_{ss} = C_0 P_{app}$$

The percent corneal hydration level (%) was calculated by

$$\text{Hydration level\%} = \frac{W_a - W_b}{W_a} \times 100$$

where W_a was the wet cornea weight, and W_b the corresponding dry cornea weight after a desiccation of 12 h at 70 °C.

2.5. Transcorneal visualization studies of the colloidal carriers

Confocal laser scanning microscopy (CLSM) was used to visualize the interaction of the colloidal particles with the corneal samples (Calvo et al., 1996). The contact of encapsulated RhB with the corneas was performed as aforementioned in transcorneal permeation experiment with the concentration of RhB at 1 μg ml^{-1}. With different TMC concentrations and scheduled time intervals, the donor and acceptor compartments were washed with fresh buffer to remove the excess fluorescent permeant. The corneal specimens were directly mounted on glass slides and examined by CLSM without additional tissue processing. The CLSM system (TCS SP2/AOBS, Leica, Germany) linked to an inverted microscope with the HCX PL APO 100× /1.40–0.70 oil CS objective (DM IRE2, Leica, Germany) was used. RhB was excited with the 543 nm line of the HeNe laser. Optical sections in the vertical plane were made by scanning in the x and y direction, and sections in the horizontal plane were made by scanning at different depths in the tissue (x and z direction). LEICA Confocal Software was used for image acquisition and analysis. To ascertain reproducibility, each experiment was conducted three times.

2.6. Assessment of protective effect of CoQ_{10}-loaded liposomes against lens oxidative damage

2.6.1. Cell culture and treatments

Human lens epithelial cell line, SAR 01/04, was cultured in MEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in an atmosphere of 5% CO_2. The SRA 01/04 cells were passaged by trypsinization every two days. Cells were seeded in multi-well plates or dishes in MEM–10% FBS medium. After 12–24 h, the culture medium was changed as follows to provide model, control and test groups. In the model group, exposure to oxidative stress was performed in a serum-free medium containing 100 μM H_2O_2 for 60 min. Normal cells were treated exactly as the model cells except that H_2O_2 was not included in the medium. In the test groups, after a 24-h pretreatment in the presence of TMC-coated liposomes with CoQ_{10} concentration at 38 μM and 76 μM, medium containing 100 μM H_2O_2 was added to these cultures for 60 min at 37 °C. Afterwards, the cells were cultured in liposomes for another 24 h and then the epithelial cells were collected by trypsinization.

2.6.2. Quantification of cell viability

The cell-protective activity of the liposomes was assessed by MTT assay with another two experimental groups established. Normal cells were treated exactly as the test groups except that TMC-coated liposomes containing CoQ_{10} were replaced by empty TMC-coated liposomes and free CoQ_{10} (concentration at 38 μM), respectively. In all the incubations with H_2O_2, H_2O_2 was detoxified by adding 100 U/ml catalase (Sigma Chem. Co., St. Louis, MO) at the end of the 60-min incubation period to terminate all activity of extracellular H_2O_2. After the cell treatments described in the previous section, 10 μl 5 mg/ml MTT was added to each well of a 96-well plate, and incubation proceeded at 37 °C for 4 h. The formazan granules obtained were then dissolved in dimethyl sulfoxide, and the absorbance at 570 nm was detected with a Model 550 microplate reader (Bio-Rad, USA). The absorbance of HLECs in the normal group was taken as the 100% cell survival.

2.6.3. Acridine orange/ethidium bromide staining

Morphological demonstration of apoptosis was obtained by means of acridine orange/ethidium bromide staining. Cells were prepared under control and experimental conditions. After removal of the incubation medium, cells were harvested with trypsin solution (0.25% trypsin; Sigma–Aldrich, USA) and treated with dye mixture (Normal/Apoptotic/Necrotic Cell Detection Kit, Nanjing KeyGen Biotech. Co. Ltd., China). Double-staining by these two agents provides the percentage of live, apoptotic and necrotic cells. The cells were observed immediately using an Olympus B×61 fluorescence microscope (Tokyo, Japan) and the appropriate fluorescein filter. Acridine orange exhibits green fluorescence and ethidium bromide exhibits red fluorescence when bound to DNA. It is possible to distinguish between viable cells (green fluorescence with intact nucleus), early apoptotic (green fluorescence with chromatin condensation), late apoptotic (orange fluorescence with chromatin condensation) and necrotic

Table 1
The particle size, zeta potentials and EE% of liposomes coated with or without TMC (data represent the mean ± S.D., $n = 3$).

Preparations	TMC concentration (%)	Particle size		Zeta potential (mV)	EE%
		D1 (nm)	D2 (nm)		
Uncoated liposomes	0	149.0 ± 8.2		−18.36 ± 6.6	97.6 ± 1.3
TMC-coated liposomes	0.1	63.0 ± 12.6	156.1 ± 10.8	8.83 ± 0.9	96.9 ± 2.5
	0.2	77.9 ± 7.8	243.1 ± 3.6	10.60 ± 2.3	98.1 ± 1.7
	0.5	78.0 ± 11.7	317.0 ± 9.5	18.43 ± 2.7	97.9 ± 2.2
	1.0	78.0 ± 21.5	334.2 ± 13.4	24.50 ± 1.1	98.0 ± 1.0

cells (orange fluorescence with intact nucleus) (Bradbury et al., 2000).

2.6.4. Annexin V/propidium iodide double staining

Untreated and CoQ_{10}-loaded liposomes-treated adherent cells were detached by trypsin, and washed twice. Apoptotic cells were identified by the binding of fluorescein-labeled annexin V to exposed phosphatidylserine on the cell surface and necrosis by nuclear staining with propidium iodide. Cell clusters were identified as described in the annexin V-FITC staining kit (Nanjing KeyGen Biotech. Co. Ltd., China) according to the manufacturer's recommendations with flow-cytometric analysis (BD FACSCalibur, USA), and a dot plot of FL1 (annexin V) and FL2 (propidium iodide) was constructed to show the gated events. Quadrant markers were applied to identify the four cell populations (annexin V−/propidium iodide+, annexin V+/propidium iodide+, annexin V−/propidium iodide−, annexin V+/propidium iodide−). The results were expressed as a percentage of the gated events.

2.6.5. ATP measurements

ATP was measured in the cell extracts using a firefly luciferase-based ATP Assay Kit (Beyotime, China) in a 1450 LSC & Luminescence Counter (PerkinElmer, USA). Following CoQ_{10} treatment at 24 h after oxidant assault, cells were lysed using the provided cell lysis buffer and assayed according to the manufacturer's instructions. ATP concentrations were calculated by generating a standard curve based on known amounts of ATP and presented as nmol/mg protein. The protein content in the cells was

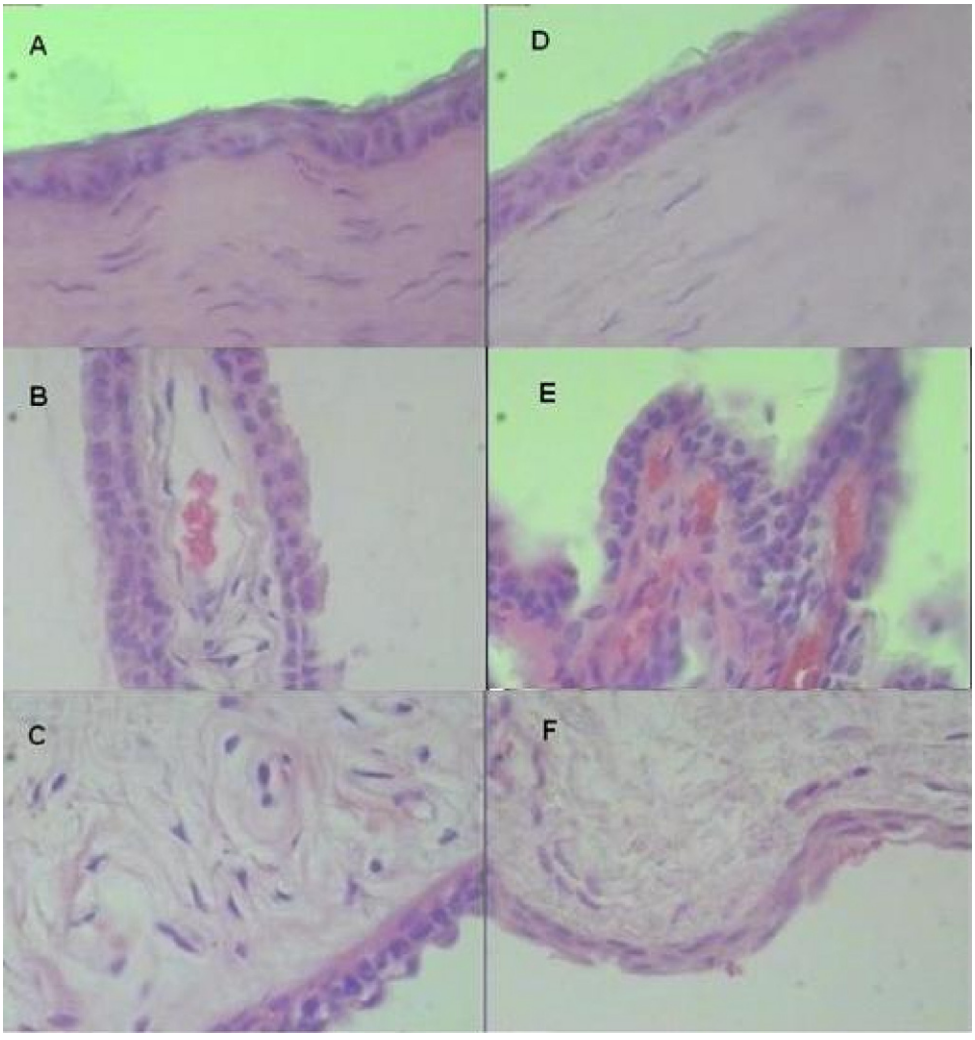

Fig. 1. Light micrographs of ocular tissue: saline-treated group (A: cornea, B: iris, C: bulbar conjunctiva); TMC-coated liposomes-treated group (D: cornea, E: iris, F: bulbar conjunctiva).

calculated by a commercially available Enhanced BCA Protein Assay Kit (Beyotime, China).

2.7. Statistical analysis

Data are presented as means ± S.D. Statistical comparisons were made using a one-way ANOVA with SPSS software (version 17.0). A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Characterization of polymer-coated liposomes

CoQ_{10} is a water-insoluble drug and ethanol injection is considered to be an appropriate method to prepare it in liposome form. Table 1 shows that the physicochemical properties of the particles were greatly affected by the coating. More specifically, as expected, the coating with TMC increases the particle size and led to an inversion (from negative to positive) of the surface charge. Compared with uncoated liposomes, the increased particles size of the TMC-coated liposomes was ascribed to the positively charged TMC chains locating mainly on the outer leaflet of the layer membrane. This would increase the curvature of the liposome particles, indicating an increase in the thickness of the coating layer. Table 1 also shows that EE% of CoQ_{10} was very high, irrespective of the formulation.

3.2. Ocular irritation study

According to the observations on the rabbits' eyes, excellent ocular tolerance was noted as average scores of irritation for each formulation were zero. Histological images revealed that tissues (cornea, iris and bulbar conjunctiva) in the drug-treated group were not different from that of physiological saline group (Fig. 1).

3.3. Transcorneal permeation studies

The permeation profiles of the three preparations are given in Fig. 2 and transcorneal permeation parameters and absorption enhancement ratios ($Er = P_{app}(sample)/P_{app}(control)$) are listed in Table 2. Under the conditions described, a relatively low permeability was found in the solution group, and the accumulative amount of RhB transported up to 3 h was (3.04 ± 0.14)%. The permeability increased in the liposomes groups and the accumulated content for uncoated liposomes and TMC-coated liposomes, transported up to 3 h, were (7.14 ± 0.36)% and (9.74 ± 0.22)%, respectively. This suggests that a TMC coating resulted in an increase in transport and P_{app}. With all three preparations, P_{app} for the polymerized formulation was significantly increased in the presence of TMC, being 0.32–2.11-fold superior to those of uncoated liposomes and solution, respectively. In concentration curve of polymerized liposomes the significantly increased J_{ss} ($13.10 \pm 0.30\ \mu g\,cm^{-2}\,s^{-1}$) is observed. The TMC-coated liposomes produced the most pronounced permeation effect.

At the end of each experiment the percent corneal hydration level (%) was calculated to assess possible corneal damages.

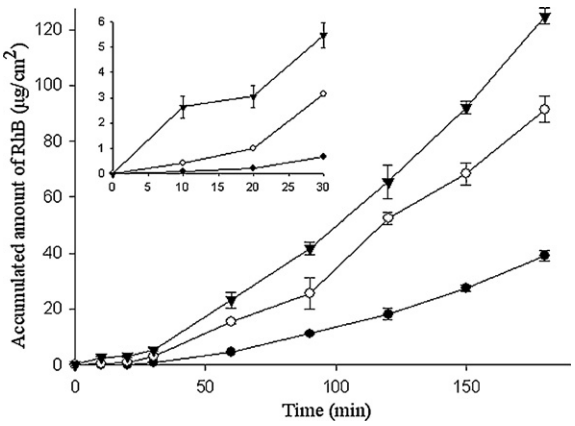

Fig. 2. *In vitro* transcorneal permeation profiles of RhB in different preparations (data represent the mean ± S.D., $n = 3$). (●) RhB solution, control; (○) uncoated liposomes labeled by RhB; (▼) TMC-coated liposomes labeled by RhB.

Table 3
Cornea hydration level (%) for different preparations labeled by RhB after *in vitro* transcorneal permeation experiment (data represent the mean ± S.D., $n = 3$).

Preparations	Hydration level (%)
Untreated	78.4 ± 0.8
Solution	80.1 ± 1.6
Uncoated liposomes	81.0 ± 1.7
TMC-coated liposomes	82.2 ± 1.5

This parameter is a sensitive indicator of corneal alteration, and 83–92% hydration level denotes damages of the epithelium and/or endothelium (Schoenwald and Huang, 1983). The hydration level (%) values of corneas exposed to different preparations ranged between (78.4 ± 0.8)% and (82.2 ± 1.5)% (Table 3). No significant difference in the hydration level (%) was observed among the corneas of test groups.

3.4. Transcorneal visualization studies

In order to obtain a true image of the fluorescence distribution covering the entire thickness of the epithelium by confocal laser scanning microscopy, total encapsulation of the marker and no release during the experiment were necessary (Calvo et al., 1996). RhB was chosen as the fluorescent marker and there was no leakage from the liposomes for RhB over 180 min in GBR solution at 34 °C (data not shown). The corneal RhB disposition was investigated by examining the samples of rabbit cornea by CLSM for, respectively, RhB solution, uncoated liposomes labeled by RhB and TMC-coated liposomes labeled by RhB.

Fig. 3 shows the images of corneal epithelia exposed *in vitro* to the colloidal systems with different TMC concentrations at 120 min post-permeation. In the presence of TMC, the amount of RhB distributed in the epithelium increased enormously, and the gain was adjusted to a lower value (Hoogstraate et al., 1996). Differences were observed in the depth of permeation of RhB. With the increasing concentration of TMC, the fluorescence signals were found deeper in the epithelium. The TMC-coated liposomes (TMC %: 1.0%)

Table 2
Transcorneal permeation parameters for different preparations labeled by RhB (data represent the mean ± S.D., $n = 3$).

Preparations	Linear	r	J_{ss} ($\times 10^3\ \mu g\,cm^{-2}\,s^{-1}$)	P_{app} ($\times 10^5\ cm\,s^{-1}$)	Er
Solution	$Q = 0.1986t - 7.6951$	0.9861	4.24 ± 0.18	0.42 ± 0.018	1.0
Uncoated liposomes	$Q = 0.4659t - 15.5486$	0.9904	9.93 ± 0.67*	0.99 ± 0.067*	2.4
TMC-coated liposomes	$Q = 0.6146t - 18.6472$	0.9929	13.10 ± 0.30*,#	1.31 ± 0.030*,#	3.1

* $p < 0.01$ vs. solution group.
\# $p < 0.01$ vs. uncoated liposome group.

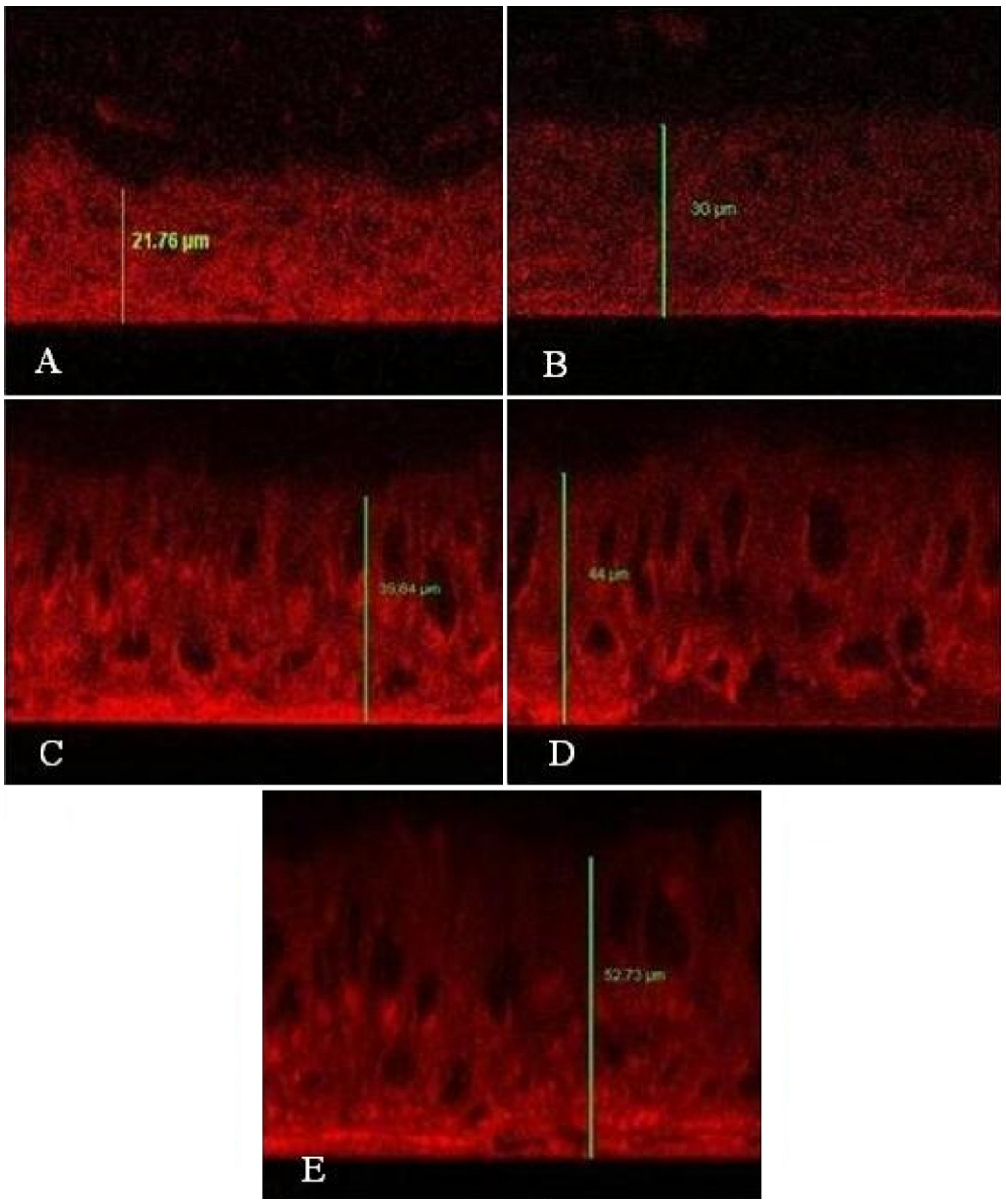

Fig. 3. Vertical sections of permeation for RhB in different preparations in rabbit corneal epithelium at 120 min post-permeation. (A) The cornea treated with RhB solution; (B) the cornea treated with uncoated liposomes labeled by RhB; (C–E) the cornea treated with TMC-coated liposomes labeled by RhB (TMC%: 0.2%, 0.5%, 1.0%, w/v).

were found at a depth of 52-μm in the corneal epithelium, and those without the coating were mainly located at a depth of 30-μm. The appearance was very different from that of the cornea exposed to RhB solution. In the latter case, fluorescence of RhB was found predominantly at about 20 μm from the surface. Fig. 4 shows the images of corneal epithelia exposed *in vitro* to RhB-labeled liposomes with TMC concentration at 1.0% (w/v) for 30 min (A), 60 min (B) and 120 min (C). With the increasing contacting time of TMC-coated liposomes, the fluorescence signals were observed deeper in the epithelium. Fig. 5 shows a stack of horizontal sections of corneal epithelium after 120 min diffusion of TMC-coated liposomes containing RhB, starting from the surface and moving inwards into the epithelium with steps of 10–50 μm below the surface. The fluorescence could be detected down to 50 μm below the surface (Fig. 5F). From these observations it was concluded that the application of TMC resulted in a deeper distribution of fluorescence signals to 50 μm below the surface.

3.5. Protective effects of liposomes against oxidative damage induced by H_2O_2 in SRA 01/04 culture

Our evaluation of cell viability revealed that 100 μM H_2O_2 treatment for 60 min reduced cell viability by approximately (70.5 ± 0.73)% of the control. However, when treated with 38 μM and 76 μM CoQ_{10}, the cell viabilities of test groups were increased significantly by (20.1 ± 6.47)% and (60.6 ± 18.4)% of model group, respectively. Fig. 6 shows that the cell death induced by H_2O_2 was significantly inhibited in test groups, especially for TMC-coated liposomes with higher CoQ_{10} concentration. The cell viability of empty TMC-coated liposomes-treated group at (31.1 ± 3.67)% was not different from that of model group significantly ($p > 0.05$), indicating that the protective effect was caused by CoQ_{10} but not by other components of the liposomes. The cell viability of TMC-coated liposomes containing CoQ_{10} was increased significantly in comparison with that of free CoQ_{10} with CoQ_{10} concentration at 38 μM

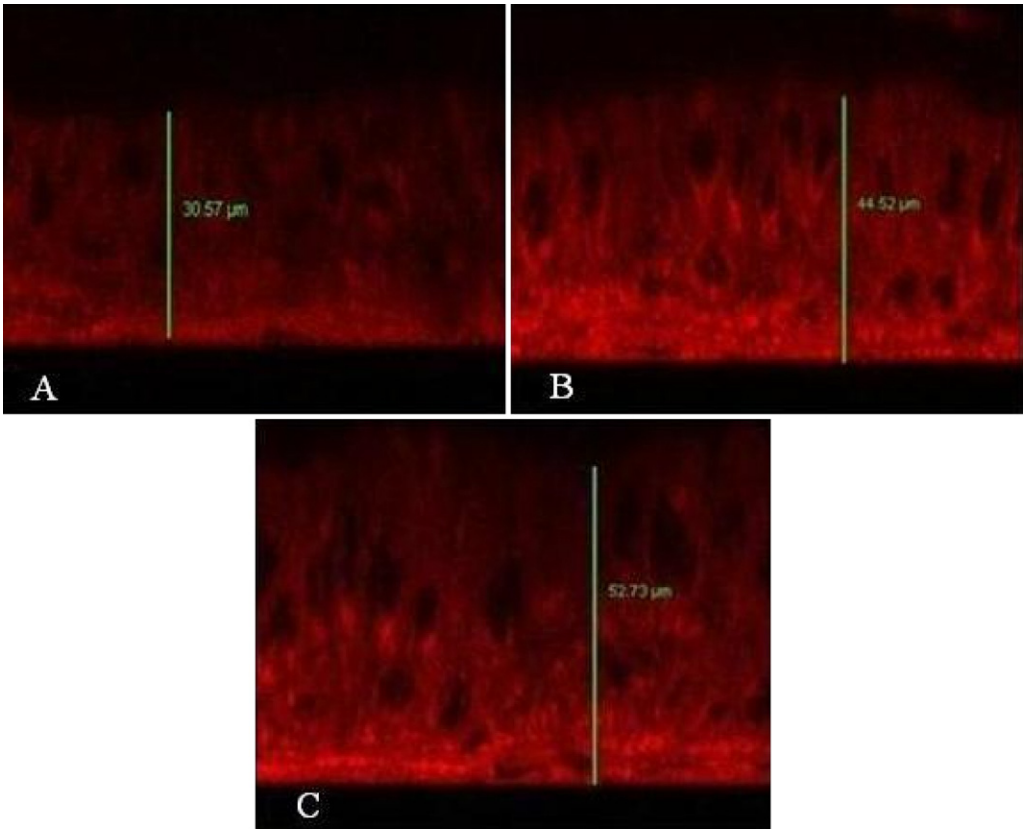

Fig. 4. Vertical sections of permeation for TMC-coated liposomes labeled by RhB (TMC%: 1.0%, w/v) in rabbit corneal epithelium at different time intervals (A) 30 min; (B) 60 min; (C) 120 min.

($p < 0.05$), indicating that CoQ_{10}-loaded liposomes had stronger ability to block apoptosis of HLECs than free CoQ_{10}.

To investigate the protective effects of CoQ_{10} against oxidative damage, the cells were stained with acridine orange/ethidium bromide, which combine the differential uptake of fluorescent DNA binding dyes acridine orange and ethidium bromide, allowing one to distinguish viable, apoptotic and necrotic cells. Fig. 7 shows late apoptotic cells (yellow stain) and necrotic cells (red stain) when exposed to 100 μM H_2O_2 for 60 min. When treated with CoQ_{10}, the number of apoptotic and necrotic cells was significantly decreased, in parallel with the drug concentration.

To confirm these morphological findings, flow cytometric analysis with annexin V and propidium iodine staining was performed to detect apoptosis by targeting the loss of phospholipid asymmetry of the plasma membrane (van Engeland et al., 1998). Cells were analyzed by flow cytometry after staining of phosphatidylserine translocation with FITC-annexin V in combination with propidium iodine. Phosphatidylserine is a lipid found on the inner surface of the cell membrane. When undergoing apoptosis, cells externalize phosphatidylserine and can then be labeled with fluorochrome-conjugated annexin V. All SRA 01/04 were divided into 4 groups, namely, mechanically injured cells (left upper part, annexin V−/propidium iodine+), healthy living cells (left lower part, annexin V−/propidium iodine−), cells at the stage of early apoptosis (right lower part, annexin V+/propidium iodine−) and necrotic cells or cells exhibiting late stage apoptosis (right upper part, annexin V+/propidium iodine+). The cells within each cluster were then counted and represented as a percentage of the gated cells. Cells of both the right upper and right lower parts were considered as apoptotic. There was a significant difference among the experimental groups. Fig. 8 indicates that the percentage of early apoptotic cells in the control group was 2.6%, while that of the model group was 60.4%. When cells were treated with 38 and 76 μM CoQ_{10}, the percentage of early apoptotic cells decreased to 22.3% and 16.9%, respectively, showing a significant reduction in apoptosis.

With regard to cell bioenergy, although its role in apoptosis has not been fully evaluated, ATP is a key molecule of cellular regulation and maintenance of ion homeostasis. The decrease in ATP level generates a strong apoptotic signal (Garland and Halestrap, 1997). Fig. 9 shows that the loss of ATP was (77.3 ± 4.1)% by 100 μM H_2O_2 after 60 min of incubation compared with that of normal cells, but this loss was significantly reduced to (17.8 ± 10.3)% in the presence of a higher concentration of CoQ_{10}.

4. Discussion

Enhancement of corneal penetration has been advocated as one of the possible strategies for overcoming the poor topical bioavailability of ophthalmic drugs. Basically, corneal drug penetration can be improved by: (1) increasing the drug lipophilicity through ion-pair formation or prodrug derivatization (Lee and Li, 1989); (2) transiently altering the corneal permeability with penetration enhancers (Lee and Robinson, 1986; Liaw and Robinson, 1993). Therefore, TMC-coated liposomes were developed and studied as a potential ocular drug delivery system for CoQ_{10} for the first time. Our hypotheses here were (i) that a TMC coating could increase the versatility of the resulting system, in terms of the ability to achieve drug permeation enhancement, and (ii) the excellent ability of anti-apoptosis makes CoQ_{10} attractive as a novel potential anti-cataract agent.

In order to evaluate the capacity of the developed system to enhance the corneal permeation of lipophilic drugs *in vitro* studies were performed, including transcorneal permeation study and

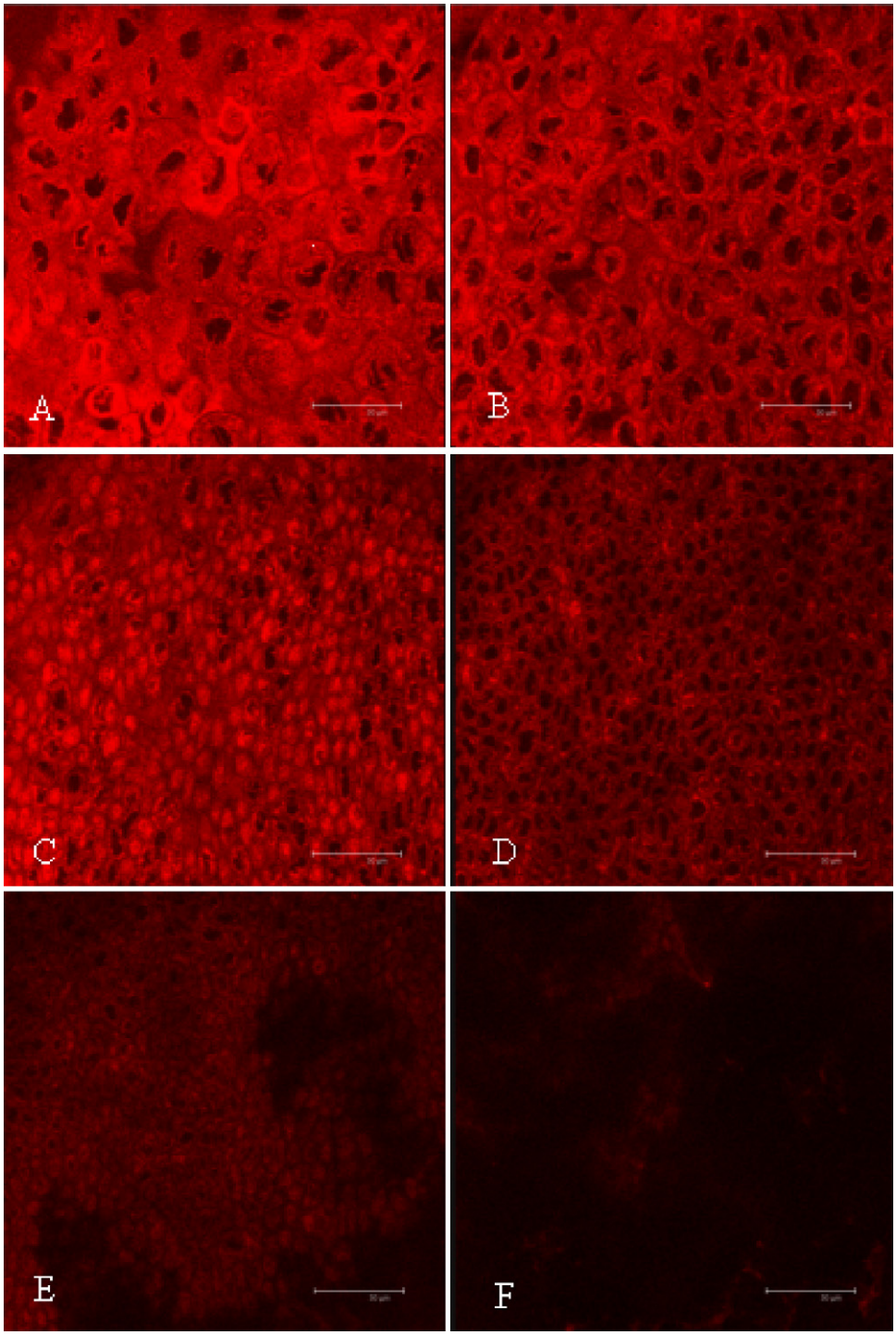

Fig. 5. Optical sections of permeation for TMC-coated liposomes labeled by RhB in rabbit corneal epithelium at 120 min post-permeation, starting from the surface layer (A) with steps of 10 μm (B–E) to 50 μm (F) below the surface of the epithelium. Bar equals 30 μm.

CLSM visualization study. As shown in Fig. 2, the RhB transport across the cornea was significantly increased by the physical presence of TMC-coated liposomes ($p < 0.01$), thus suggesting that these carriers have permeability enhancing properties. The permeation study of CLSM images also showed that TMC-coated liposomes exhibit a higher permeation into deeper corneal layers, especially for the liposomes with higher TMC concentration and longer contacting time.

TMC is a derivative of chitosan with superior solubility, compared with other chitosan salts, and positively charged on the quaternary amino group on the C-2 position. TMC has been evaluated as a permeation-enhancer in intestinal and nasal epithelia (Kotzé et al., 1998a,1998b). As previously reported, TMC solutions (degree of quaternization around 10%) were able to enhance the transport of peptide drugs across intestinal epithelial cells *in vitro*, and TMC most likely acts in the same manner with chitosan to open tight junctions (Kotzé et al., 1997). The mechanism of the opening of the tight junctions under the influence of chitosan and its derivatives has not yet been elucidated. Actually, most cationic macromolecules such as chitosan, can interact with anionic com-

ponents of glycoproteins on the surface of the cells. Moreover, the interior of the tight junctions (pores) is highly hydrated and contains fixed negative charges. A change in the relative concentration of specific ion species in the pores would cause alterations in tight junction resistance, leading to loosening or opening of the pores (Thanou et al., 1999; Madara, 1989). It has been shown that chitosan glutamate was able to induce changes in the F-actin distribution, increasing the paracellular permeability (Artursson et al., 1994; Meza et al., 1982). In fact, the corneal surface and the conjunctiva were covered by mucin. The mucin layer is secreted by goblet cells of the conjunctiva, and it consists of a protein or polypeptide core with carbohydrate side chains branching off the core (Qi et al., 2007). Accordingly, at physiological pH, the corneal surface is negatively charged. Therefore, the TMC coating investigated in this work most likely has a similar action on the junctional complex that could favor the permeation of the nanosystems across the cornea.

Using TMC-coated liposomes as drug carriers, we examined the effects of CoQ_{10} against H_2O_2-induced oxidative cell damage in cultures of SRA 01/04 *in vitro*.

CoQ_{10} is a lipid redox agent that is endogenously synthesized in mammals. Our results demonstrated that the apoptosis caused by the exposure of HLECs *in vitro* to H_2O_2 could be attenuated by the application of CoQ_{10}, in a concentration-dependent manner for the first time.

In many models of apoptosis, cells are induced to die as a result of changes in environmental stimuli. In the SRA 01/04 culture, H_2O_2 was used to induce oxidative stress. H_2O_2 is not a free radical and has a rather low toxicity; however, because of its relatively long half-life (in seconds) and its ability to pass through cell membranes, it is able to distribute the damage induced by free radical-generating processes over progressively larger areas (Boveris and Cadenas, 1997). In the presence of metal ions, H_2O_2 forms a very toxic hydroxyl radical (Fenton's reaction). H_2O_2 also has the potential of generating hydroxyl radical in a reaction with the superoxide anion, catalyzed by transitional metal ions such as Fe^{2+} and Cu^{2+} (The Haber–Weiss reaction) (Petersen et al., 2008). It is estimated that of the total molecular damage sustained as a consequence of free radicals, over 50% is attributable to the hydroxyl radical (Hassan, 1997). The attack by the hydroxyl radical is totally random. This indiscriminate plundering leads to the molecular disfiguration of lipids, DNA, proteins, and carbohydrates (Reiter et al., 2000), resulting in mutagenesis and leading to cataract formation (Imlay and Linn, 1988). Our results show

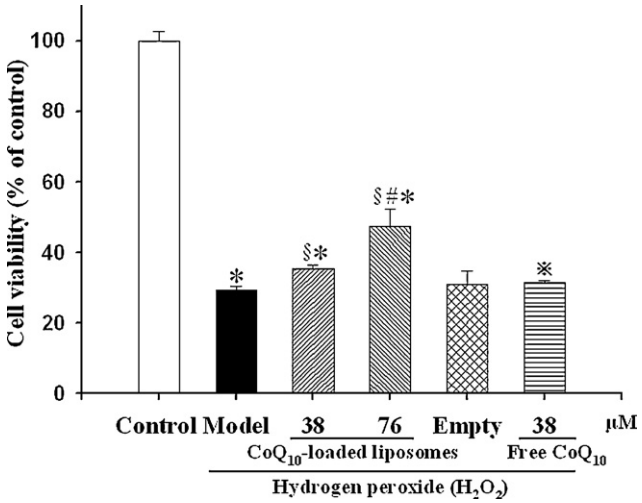

Fig. 6. Cell viability of SRA 01/04 in different groups measured by MTT method (data represent the mean ± S.D., $n = 3$). $^{*}p < 0.01$ vs. control group; $^{§}p < 0.05$ vs. model group; $^{\#}p < 0.01$ vs. 38 μM CoQ_{10}-loaded liposomes group; ※ $p < 0.05$ vs. 38 μM CoQ_{10}-loaded liposomes group.

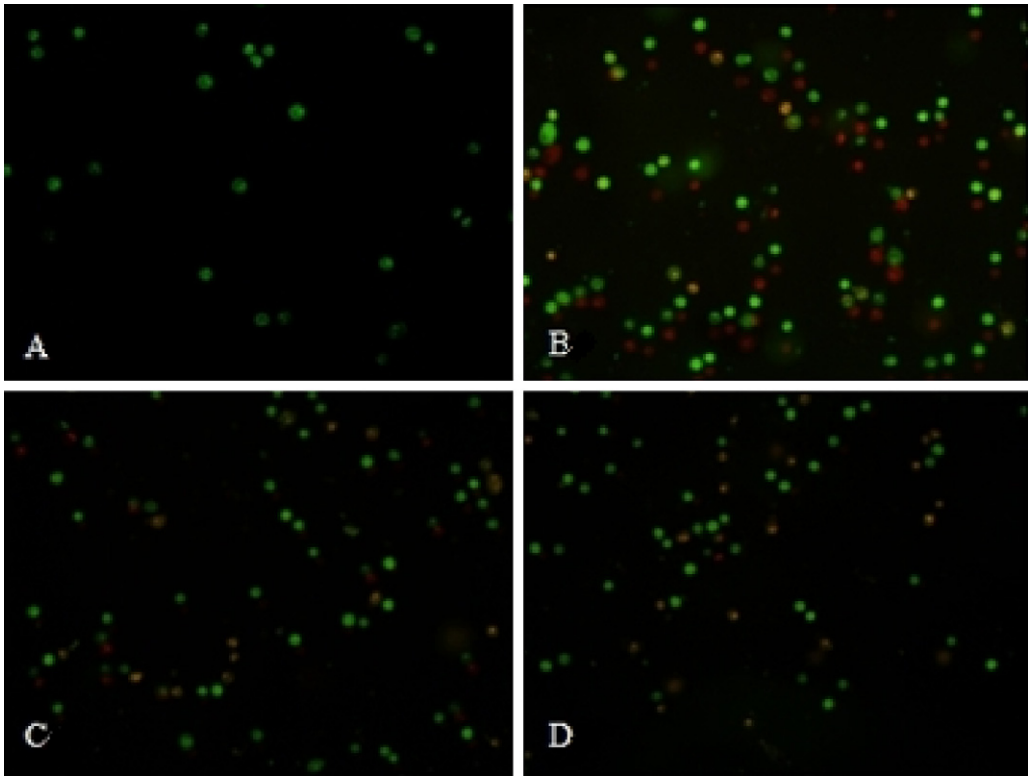

Fig. 7. Fluorescence microscope detection of SRA 01/04 in different groups by acridine orange/ethidium bromide double staining. (A) Normal cells; (B) cells treated with 100 μM H_2O_2; (C) cells treated with 38 μM CoQ_{10}; (D) cells treated with 76 μM CoQ_{10}.

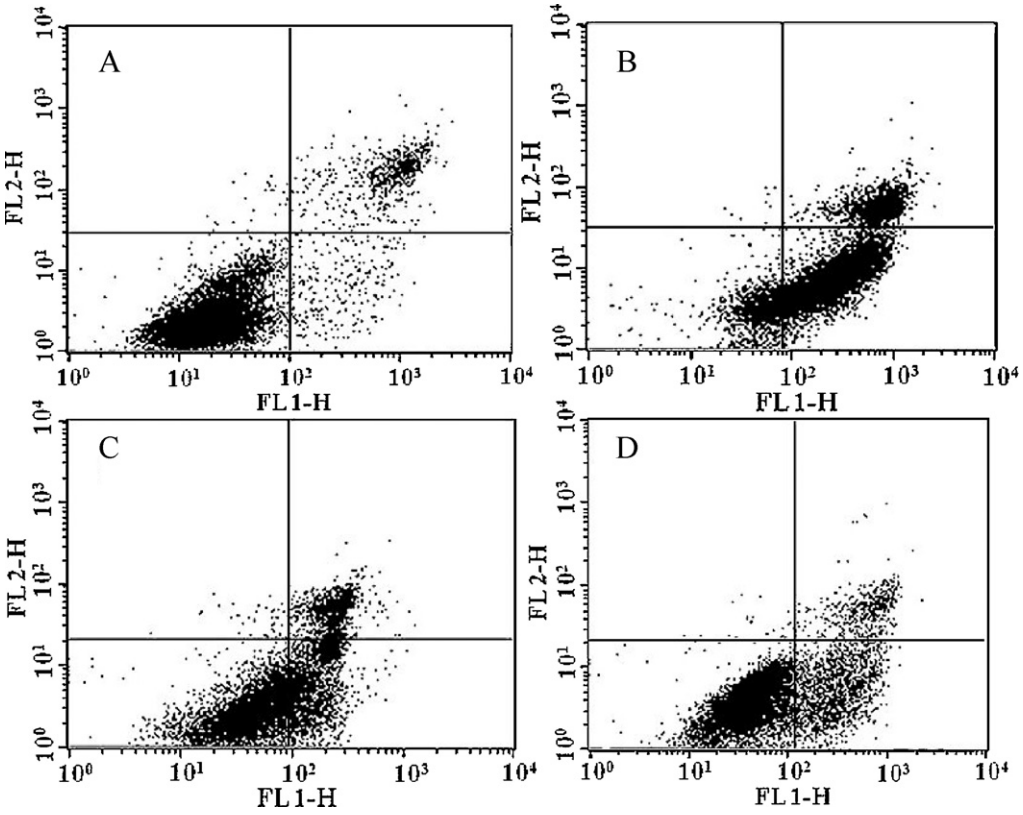

Fig. 8. Dual parameter flow cytometry analysis of SRA 01/04 cells with annexin V/propidium iodide staining. (A) Normal cells; (B) cells treated with 100 μM hydrogen peroxide; (C) cells treated with 38 μM CoQ_{10}; (D) cells treated with 76 μM CoQ_{10}.

that 100 μM H_2O_2 can significantly induce apoptosis in HLECs, as shown by acridine orange/ethidium bromide double-staining and annexin V/propidium iodine double-staining assays. Typical apoptosis features in H_2O_2-treated cells were observed with acridine orange/ethidium bromide staining under fluorescence microscopy. CoQ_{10} has the clear ability to block apoptosis of HLECs. A critical mechanism for the recognition of apoptotic cells takes advantage of the changes in phospholipid asymmetry that are seen in old or dying cells. Annexin V has a high affinity for phosphatidylserine and binds to cells with exposed phosphatidylserine (Ou et al., 2008).

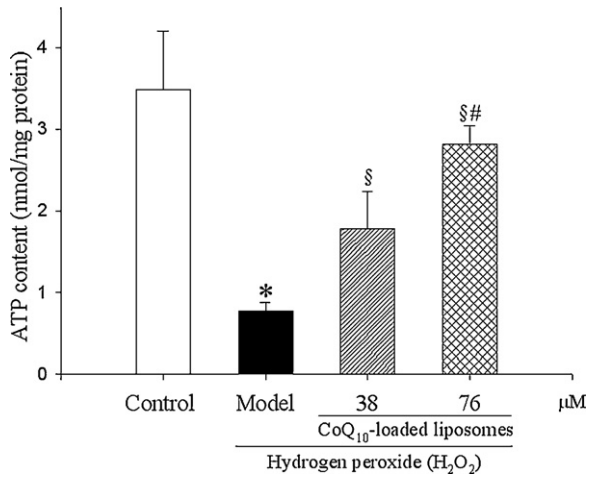

Fig. 9. Effects of CoQ_{10} on cellular ATP content in SRA 01/04 cells in different groups. $^{*}p < 0.05$ vs. control group; $^{\S}p < 0.05$ vs. model group; $^{\#}p < 0.05$ vs. 38 μM CoQ_{10} group.

Furthermore, apoptosis is an active energy-dependent process requiring functioning mitochondria. In apoptotic cells the mitochondrial membrane integrity has been reported to be disrupted due to opening of the permeability transition pores, leading to a reduction in the mitochondrial transmembrane potential (Kroemer et al., 1997). One of the consequences of this is a reduction in ATP, as well as the release of apoptogenic factors into the cytosol. The ATP results show that a loss of ATP plays an important role in the effect of H_2O_2 and that CoQ_{10} counteracts this effect by providing the cells with ATP. Indeed, the mitochondria are responsible for the production of 80–90% of the ATP needed for cell respiration and survival and play an important role in the pro- and anti-apoptotic stimuli (Dias and Bailly, 2005). ATP depletion has been demonstrated as one of the major mechanisms of apoptosis (Nakamura and Wada, 2000). CoQ_{10} functions as an electron carrier in the respiratory chain which produces a proton-motive force for synthesizing ATP. Administration of CoQ_{10} has also been demonstrated to increase H^+ transport (Echtay et al., 2001). Meanwhile, CoQ_{10} might influence the superoxide anion production by stabilizing the electron transfer chain and reducing the uncoupling of reactions during electron transport that could otherwise result in superoxide anion production (Ishii et al., 2004). Our data show that CoQ_{10} is a positive regulator of the death pathway induced by H_2O_2 in human lens epithelial cells.

5. Conclusion

The results of this work have shown that nanosystems consisting of TMC penetrate deeper into the corneal epithelium, in comparison with non-coated liposomes. Furthermore, CoQ_{10} can inhibit the early stage of H_2O_2-induced HLECs apoptosis *in vitro*. In summary, although further experiments are warranted, all these data support the belief that TMC-coated liposomes are a useful

nanosystem for the anti-cataract effect of ophthalmic delivery of CoQ_{10}.

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