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Research Article

Inhibitory effect of lanosterol on cataractous lens of cynomolgus monkeys using a subconjunctival drug release system

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ABSTRACT

Background: To evaluate the effect of lanosterol on cataractous lens of cynomolgus monkeys using a subconjunctival drug release system.

Methods: Nine elder cynomolgus monkeys were used, consisting of three monkeys without cataract as controls, three monkeys with naturally occurring cortical cataract, and three monkeys with nuclear cataract as intervention groups. Nanoparticulated thermogel with lanosterol and fluorescein was administered by subconjunctival injection in the monkeys with cataract. Fluorescence changes of injected thermogel and cataract progression were observed. Lanosterol concentration in aqueous humor, solubility changes in lens proteins, and oxidative stress levels were analyzed in the lenses of the control and intervention groups.

Results: Injected thermogel showed decreased fluorescence during follow up. Lanosterol concentration in aqueous humor increased in the first 2 weeks and then gradually decreased, which was in accordance with the changes in cortical lens clarity. However, lenses with nuclear opacification showed little change. In the cortical region of lenses with cortical cataract, solubility of α-crystallin was significantly increased after administration of lanosterol, as well as the reduction of oxidative stress.

Conclusions: We demonstrated the effect of lanosterol on cataract progression based on *in vivo* models of primates. Lanosterol showed a short-term and reliable reversal effect on reducing cataract severity in cortical cataract in the early stages, possibly due to the increase in the solubility of lens proteins and changes in the oxidative stress status. Lanosterol administration using subconjunctival drug release system could be a promising nonsurgical approach for future clinical studies of cataract prevention and treatment.

Keywords: lanosterol, drug release, cataract, lens, monkeys

Introduction

Cataract is the most common cause of vision loss worldwide,[1] contributing to >90% of the total disability-adjusted life years in developing countries. As the elderly population increases globally, cataract will produce an even greater socioeconomic burden worldwide.[2] Nonsurgical treatment of cataract could greatly alleviate this situation without the burden of surgical costs.[3]

Previous studies reported that normal functions of crystallin proteins in lens fibers contribute to lens transparency and refractive properties.[4,5] Thus, recent investigations have explored promising medications to maintain the function of crystallins, for the purpose of postponing or reversing the lens opacification in cataract. Zhao *et al.*[6], in a recent landmark publication focusing on lanosterol-loaded nanoparticles for intravitreal injection and lanosterol eye drops, demonstrated that lanosterol reverses lens opacification in cataracts of dogs, suggesting a novel strategy for the prevention and treatment of cataracts.

However, intravitreal injection is neither a convenient nor a safe way to administer drugs, and may not be appropriate for long-term prevention. Using the advances in nanotechnology, we designed a drug-loading system for subconjunctival implantation in cynomolgus monkeys (*Macaca fascicularis*), which are regarded as excellent experimental models of human systems. The eye structure and ocular age-related changes of cynomolgus monkeys and humans are remarkably similar,[7] and have therefore been previously applied in cataract research of primates.[8,9] Using cynomolgus monkeys with age-related cataractous lenses to mimic the human state in terms of species and diseases, we investigated the

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relationship between the lanosterol concentration in aqueous humor and the changes of lens opacification and proteins to observe the inhibitory effect of lanosterol on age-related cataract in primates.

Methods

Ethical statement

This study was approved by the Ethics Committee of the Eye and Ear, Nose, and Throat Hospital, Fudan University, Shanghai, China. All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of lanosterol–thermogel formulations

Triblock copolymer of $PLGA_{1700}$–PEG_{1500}–$PLGA_{1700}$, obtained from Dr. Sun at the Department of Ophthalmology, Eye and ENT Hospital of Fudan University and Professor Yu's Department of Macromolecular Science of Fudan University, was synthesized and characterized as described previously.[10] Figure 1 shows the construction of the lanosterol–thermogel system. The copolymer (1.0 g) was dissolved in 3.0 g of normal saline to form 25% (w/w) thermogel solution. To make the medication sustainable for 1 month of release, lanosterol (1.6 mg) was added to 1.0 ml of the previously mentioned thermogel solution and magnetically stirred at 600 rpm and 4°C for 3 days to form a homogeneously dispersed solution to obtain the lanosterol–thermogel formulations. This thermogel with lanosterol behaves like a liquid (meaning the drug is easy to load and to be injected) below its low critical solution temperature (LCST), whereas it forms hydrogel (delivery and release of the loaded drug) when the environmental temperature reaches or exceeds the LCST. Moreover, fluorescein sodium was added as the tracer labeling for further drug visualization under slit lamp examination.

Primates are mainly used in this study, which is a further analysis based on the previous study,[6] from which the effective monthly dosage for the *in vivo* treatment is calculated. The thermogel volume for subconjunctival injection was 0.1 ml to provide enough drug release during the 1-month experiment, but not to induce significant ocular surface swelling and foreign body sensation in the experiment animals. Considering the loading capacity of thermogel and the previous data, lanosterol (1.6 mg) was added to 1.0 ml of the thermogel solution to make the medication sustainable for 1 month of release.

In vivo treatment of cataractous lenses in cynomolgus monkeys using lanosterol

Screening of cataractous monkeys

As we planned to monitor the effect of lanosterol–thermogel on natural lens aging, monkeys aged >18 years were selected for further study. Forty cynomolgus monkeys (3.0 to 6.0 kg), aged from 18 to 22 years old, were provided by Guangdong Chunsheng Biotechnology Development Co., Ltd for us to screen. We first screened these 40 aged cynomolgus monkeys with an overall anterior segment examination using a portable slit-lamp microscope (YZ3, 66 Vision Tech Co., Ltd, Suzhou, China). Next, nine female monkeys (18 eyes) were enrolled in our study. According to the cataract grading system (Lens Opacities Classification System III, or LOCSIII),[11] three monkeys with bilateral cortical (C) cataract and three monkeys with bilateral nuclear (N) cataracts were diagnosed. The remaining three monkeys without cataracts were enrolled as controls (Supplementary Table 1). During the examination, intramuscular anesthesia was applied. Those who cooperated well and showed good reaction to anesthesia were chosen in case possible violent actions occurred between selected monkeys that could have influenced the study results.

Subconjunctival injection of lanosterol

All procedures were performed after an intramuscular injection of ketamine HCl (50 mg/kg) and xylazine (10 mg/kg)/Zoletil 50 (Virbac Animal Health, France) by the same surgeon (K.K.Z.). The six monkeys with cataract then received a subconjunctival injection of thermogel with lanosterol. The eye was briefly cleaned with 0.5% povidone iodine, and 0.1 ml of thermogel with lanosterol was injected into the subconjunctival space in the superior temporal region of each eye (Fig. 1). Topical Cravit Eye Drops (levofloxacin; Santen Pharmaceutical Co. Ltd, Osaka, Japan) were administered to the eye that was operated on daily for 1 week.

Post-injection inspections

Signs of conjunctival irritation, inflammation or infection at the site of injection were searched for. The monkeys were also monitored for any significant changes such as eye discharge, squinting, or abnormal behavior suggesting pain or severe discomfort. The Modified Hackett–McDonald Scoring Scale was used to grade conjunctival injection, conjunctival swelling, discharge, corneal clarity, and aqueous flare. We compared the Modified Hackett–Macdonald Scores of the implantation site by calculating the percentage increase in score from pre-implantation to the end of the study in each monkey enrolled in the study.

Evaluation of thermogel degradation and cataract progression

To monitor the process of thermogel degradation, changes in fluorescence at the injection sites were recorded using slit lamp examination with cobalt-blue light illumination. To assess the progression of cataract, anterior segment photography (SL-D7, Topcon Medical Systems Inc., Oakland, NJ, USA) with fully dilated pupils was performed prior to the injections and weekly thereafter (7, 14, and 21 days). After capturing images under standardized and unified conditions, the use of retro-illumination images was proposed in the further analysis of cortical cataract and the use of slit-lamp images was proposed in nuclear cataract. Severity of cataract was manually assessed by two blind-test examiners according to the LOCSIII grading system. The reduction rate of lens opacity was calculated as (original lens grading − lens grading after injection)/original lens grading × 100%.

Estimation of lanosterol concentration in aqueous humor using the liquid chromatography–mass spectrometry (LC–MS) method

Aqueous humor acquisition

Aqueous humor samples for the control and cataract groups were obtained prior to the thermogel injections as baseline (0 day) and then weekly after routine slit-lamp examination (7, 14, and 21 days). Immediately after the paracentesis was performed, aqueous humor samples (50 to 100 μl) were aspirated with a 1-ml syringe with a 30-gauge needle, aliquoted into microtubes, and centrifuged at 400*g* for 5 minutes to remove any particulates or debris. The cell-free supernatant was immediately stored at −80°C until further use.

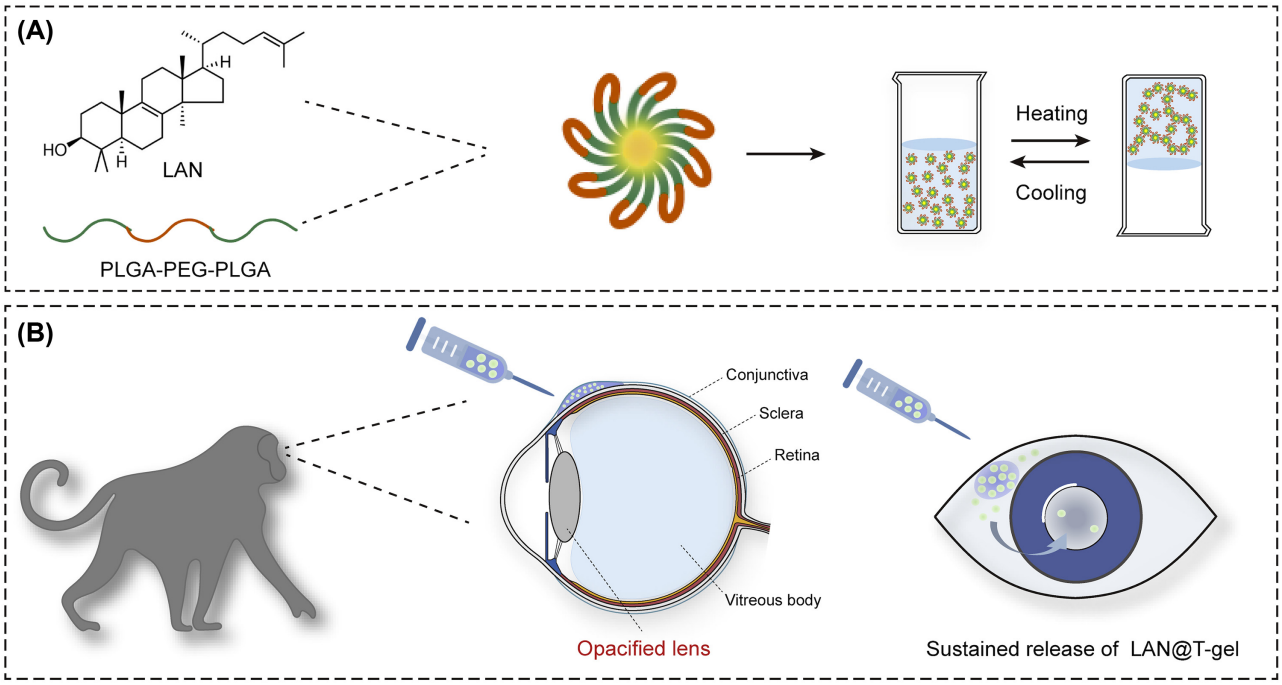

Figure 1. Construction of the lanosterol–thermogel system and the *in vivo* subconjunctival injection. (**A**) Preparation of lanosterol–thermogel formulations. (**B**) Schematic diagram showing the subconjunctival delivery of lanosterol using the thermogel mixture.

Material preparation

Primary and deuterated lanosterol standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents were high-performance liquid chromatography-grade or better all chemicals were ACS grade (American Chemical Society grade) or better. The aqueous humors were aspirated into a syringe and transferred into amber-colored LC–MS vials with insets. The lanosterol amount in the aqueous humor was estimated by the LC–MS method, as described next.

Instrumental analysis and quantification

The simple protein precipitation with methanol was applied for handling. The 50 μl of aqueous fluid and 150 μl of methanol were thoroughly mixed by vortexing for 30 s. The mixture was then centrifuged at 9000*g* for 20 minutes after standing in −20°C for ∼30 minutes. The 200 μl of supernatant were taken and dried at room temperature.

Shimadzu Nexera X2 LC-30AD was used. Buffer A consisted of 5% acetonitrile and 20 mM ammonium acetate, pH 9.45, while buffer B consisted of 100% acetonitrile. The sample was redissolved in 200 μl of 95% acetonitrile, and then 5 μl was loaded onto Waters UPLC BEH Amide column (1.7 μm × 2.1 mm × 100 mm column) in buffer A and separated with a linear gradient of buffer B at a flow rate of 400 nl/min controlled: 90% buffer B for 0.1 min, 55% to 90% buffer B for 5 min, 40% to 55% buffer B for 1 min, 40% buffer B for 1.7 min, 40% to 90% buffer B for 1 min, and 95% buffer B for 2.2 min. A quality control (QC) sample was set for the stability and repeatability of the system.

Mass spectrometry was performed using the 5500 QTRAP mass spectrometer (SCIEX, Framingham, MA, USA) in positive ion mode. The parameters of the atmosphere pressure chemical ionization are as follows: Source Temperature 550, Ion Source Gas1: 40, Ion Source Gas2: 50, Curtain Gas: 25, Nebulizer current: 3.0. The energy mass spectrometric detection was optimized in the positive ion ion transition for lanosterol (*m*/z) 409.5–109.1 detection mode by multiple reaction monitoring.

Integration of areas under elution curves for LC–MS was performed using Chemstation software. Quantitative values were calculated using isotope dilution and single-point calibration through a relative response factor calculation. The relative response factor standard was run at the beginning, middle, and end of each sample set.

Measurement of solubility changes in lens proteins

Intact lens tissues were obtained from all the monkeys by intracapsular cataract extraction at Day 21 and separated into two fractions, with one fraction containing the outer cortex and the other containing the nucleus. The cortical and nuclear lens samples were further homogenized in 50 μl of buffer A (10 mM phosphate, pH 7.0 containing 0.1 mM ethylene glycol tetra-acetic acid, and 1:1000 protease inhibitor). The homogenate was centrifuged at 100 000*g* for 30 min at 4°C, and the supernatant was collected and the pellet was homogenized with buffer A for an additional three times to yield a total volume of ∼200 μl containing the water-soluble protein. The insoluble pellet was then suspended in 50 μl of buffer B (10 mM Tris, pH 8.0 containing 8 M urea), homogenized and centrifuged at 20 000*g* for 30 min at 4°C. The pellet was re-extracted with 50 μl of buffer B and the supernatants were combined to yield the water-insoluble protein fraction. Protein content in the supernatant and total homogenate was measured using the Micro BCA assay (Pierce, Rockford, IL, USA) in triplicate, to determine the protein content of the water-soluble and -insoluble fractions. The ratio of soluble vs. total protein was then calculated to determine the solubility changes in the cortex and nucleus of lenses.

Each component of various peaks from lens extracts, including α-, β-, and γ-crystallin from the cortical and nuclear region of lenses, was further isolated by gel filtration

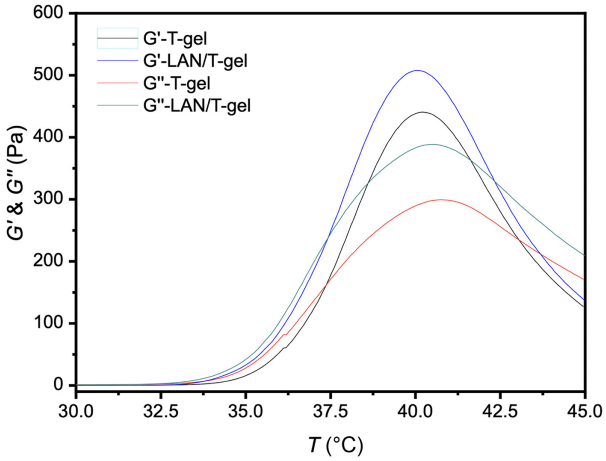

Figure 2. LCST of the thermogel.

chromatography (using Sephadex G-200 Column, Sigma-Aldrich, Darmstadt, Germany). The ratios of soluble α-, β-, and γ-crystallin vs. total protein were also calculated.

Changes of oxidative stress levels in lens

The total antioxidant capacity (TAC) of lenses was measured on sample lens supernatant by a 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation decolorization assay, according to the manufacturer's instructions. The $ABTS^{+}$ radical was generated by chemical reaction with potassium persulfate. For these purposes, 25 ml of ABTS (7 mM) was spiked with 440 μl of potassium persulfate (140 mM) and allowed to stand in darkness at room temperature for 12 to 16 h (time required for the formation of the radical). Trolox was used as standard, and the TAC of samples was defined as the concentration of Trolox having equivalent activity as μmol/g eye wet weight. Total glutathione (GSH + GSSG) and GSSG were measured according the GSSG and GSH Assay Kit (Beyotime Biotechnology, China) using kinetic determination methods under a 405-nm wavelength (Bio-Rad; model 680), and the GSSG/GSH ratio (oxidized/reduced glutathione) was calculated.

Statistical analysis

Statistical analysis included descriptive statistics, where the mean and standard deviation (SD) were calculated for the continuous variables, while frequency distribution and percentages were used for categorical variables. Comparisons between categorical variables were conducted with a Fisher exact test, whereas a one-way repeated measures analysis of variance was used for means. A P value <0.05 was considered statistically significant.

Results

Low critical solution temperature of the lanosterol–thermogel

The LCST data are provided in Fig. 2. The prepared formulation (LAN/T-gel) and PLGA-PEG-PLGA (T-gel) present a sol-gel change when the temperature increases. The energy storage module (G') and the loss module (G'') change with the temperature. G' was lower than G'' at a relatively lower temperature, showing a free flowing sol state and good injectability of the LAN/T-gel and T-gel. The lower critical solution temperature and the sol-gel transition were observed at ~37.5°C at which G' was equal to G'', and G' and G reach peak values of almost 40°C in the gel state.

Modified Hackett–Macdonald scores evaluation

Slit-lamp photography confirmed subconjunctival location of thermogel with lanosterol in the superior temporal aspect of a monkey's eye immediately after implantation, as shown in Fig. 3A. Subsequent slit-lamp examination revealed no abnormal local tissue scarring or scleral thinning at the implantation sites (Fig. 3B–D). Evaluation of the Modified Hackett–Macdonald Scores showed that was no significant differences in discharge, corneal clarity, or aqueous flare before and after implantation. Only mild conjunctival swelling was evident, which subsided within 1 week (Supplementary Table 2).

Fluorescence changing to monitor the *in vivo* lanosterol–thermogel degradation

In our study, the degradation process *in vivo* was also recorded after subconjunctival injection (Fig. 3). To monitor the process of thermogel degradation, changes in fluorescence at the injection

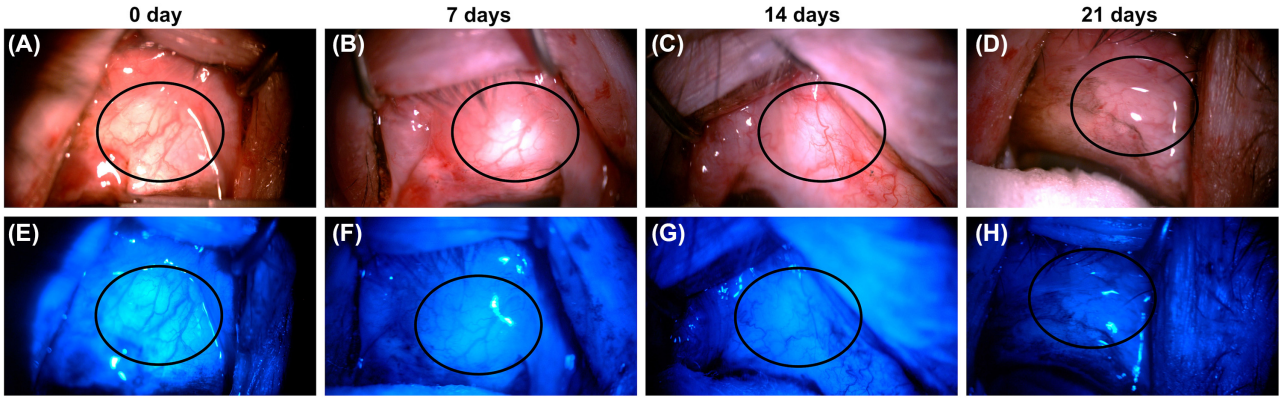

Figure 3. Slit lamp photography of the operated monkey eye. Subconjunctival location of drug delivery in the superior temporal region of the monkey eye was observed immediately after injection (**A**), 7 days after injection (**B**), 14 days after injection (**C**), and 21 days after injection (**D**). Fluorescence in the injection site was also observed immediately after lanosterol administration (**E**). The subsequent slit lamp examination performed in the follow up revealed a gradually decreasing fluorescence in 7 days after injection (**F**), 14 days after injection (**G**), and 21 days after injection (**H**). Black circles show the injection site.

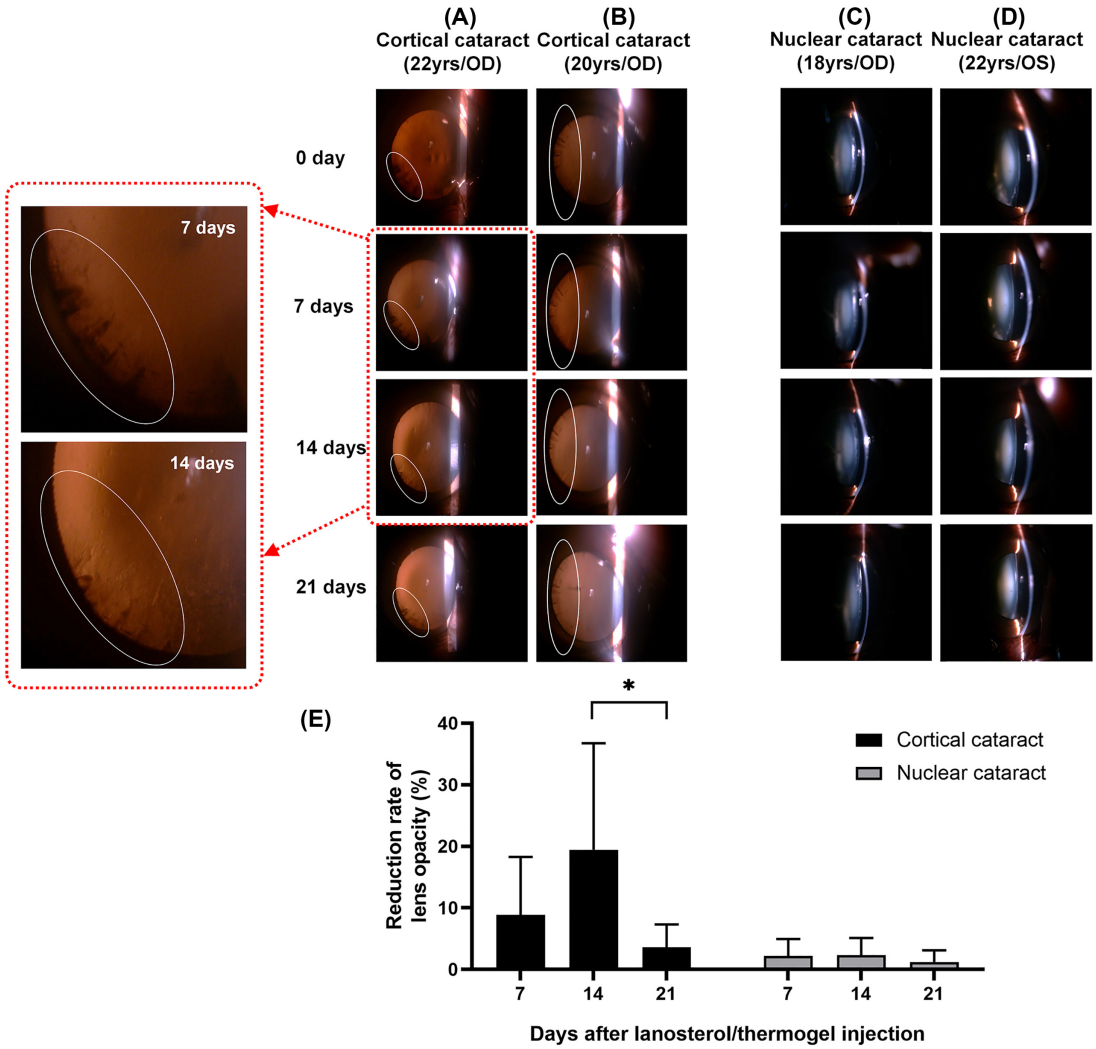

Figure 4. (**A, B**) Changes in cataract severity in the eyes with cortical cataract during follow-up. We observed a trend of reduction in cataract severity in the eyes with cortical cataract, as demonstrated by an increase in lens clarity, marked with a wedge-shaped opacification reduction in peripheral cortex. However, 21 days after lanosterol administration, the lens opacification aggravated again. White circles showed the region with opacification changing. (**C, D**) Changes in cataract severity in the eyes with nuclear cataract during follow-up. The monkey with the nuclear cataract showed almost no change in cataract severity after lanosterol administration. (**E**) Reduction rate of lens opacity in the eyes with cortical and nuclear cataract. *$P < 0.05$.

sites were recorded using slit lamp examination with cobalt-blue light illumination. Slit-lamp photography was able to confirm the change in fluorescence using cobalt-blue light around the subconjunctival location of each monkey eye after injection. The subsequent slit-lamp examination performed during the follow-up revealed a gradually decreasing tendency of fluorescence density (Fig. 3E–H). The fluorescence signal was seldom detected after 21 days.

Cataract progression after lanosterol administration

Cortical cataract is characterized as the wedge-shaped opacification located in the periphery of the lens. In the lens of cortical cataract, the peripheral opacification decreased with time, especially during the first 2 weeks after lanosterol–thermogel injection (Fig. 4A and B). However, eyes with nuclear cataract showed almost no change in cataract severity after drug injection (Fig. 4C and D). The changes of lens grading were shown in the Supplementary Table 1.

In cortical cataractous eyes, the reduction rate of lens opacity in 14 days post-injection were the highest during the follow-up period, which was significantly higher than those at 21 days ($P < 0.05$) (Fig. 4E). After reaching the highest reduction rate of lens opacity at 14 days, the opacification recovered at 21 days. In nuclear cataractous eyes, the reduction rate of lens opacity showed no significant changes during the 21-day follow-up (Fig. 4E).

Lanosterol concentration in aqueous humor with time

We identified the peak of lanosterol using the LC–MS technique (Fig. 5A). The release of lanosterol from the drug films to the anterior chamber was expressed in terms of cumulative drug release concentration over time, as shown in Fig. 5B. A slow and sustained release of the drug was achieved at the peak of the drug loading volume being released at 14 days after implantation, and then gradually retreated to a relatively lower concentration at 21 days. Changes in lanosterol concentration during the 21-day follow-up were statistically significant (Supplementary Table 3). At Day

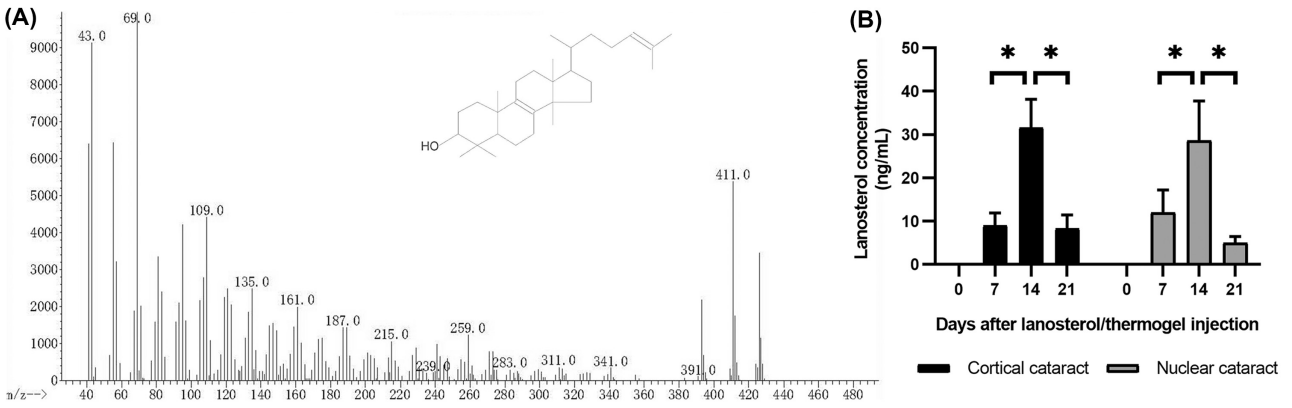

Figure 5. Identification of the peak of lanosterol using liquid chromatography–mass spectrometry (LC–MS) technique. (**A**) Molecular structure and product ions of lanosterol. (**B**) Tendency of lanosterol concentration changes during follow up. The release of lanosterol from the thermogel to the anterior chamber was evaluated using the LC–MS technique and expressed in terms of cumulative drug release concentration over time. A slow and sustained release of the drug was achieved at the peak of the drug loading volume being released at 14 days after implantation and retreated to the lowest concentration at 21 days. *$P < 0.05$.

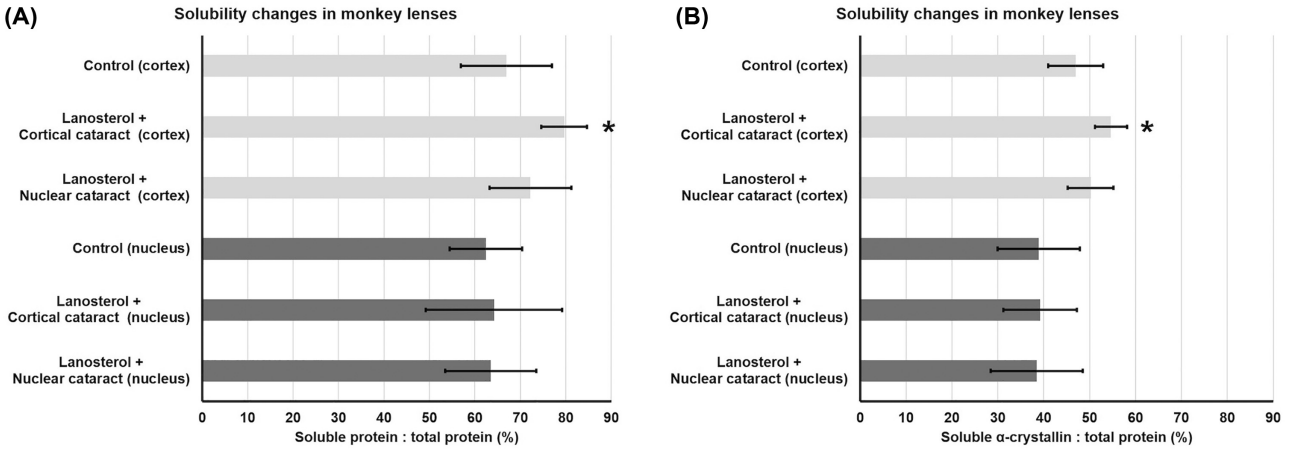

Figure 6. Changes in the protein solubility in the lens cortex and nucleus. (**A**) Changes in the ratio of soluble protein and total protein. (**B**) Changes in the ratio of soluble α-crystallin and total protein. *$P < 0.05$.

14, the lanosterol in aqueous humor of the cortical and nuclear cataract groups significantly increased to 31.61 and 28.58 ng/ml compared to the 7 and 21 days ($P = 0.002$ and 0.005, respectively). This finding was in accordance with the changing tendency of lens opacification in eyes with cortical cataracts.

Protein solubility changes in lens after lanosterol administration

We measured the total amount of soluble and insoluble protein in a subset of the lenses, as shown in Fig. 6A. In the lens cortex from the lanosterol-treated cortical cataractous monkeys, the average ratio of soluble protein/total protein was higher than that of the controls (77.6% vs. 66.9%, $P = 0.012$, Fig. 6A). In the lens cortex from the lanosterol-treated cortical cataractous monkeys, the average ratio of soluble α-crystallin/total protein was higher than that of the control (54.6% vs. 46.9%, $P = 0.039$, Fig. 6B). No significant differences were observed in the solubility of β- and γ-crystallins between the cortex or nucleus from lanosterol-treated cortical or nuclear cataractous monkeys and those of controls (data not shown).

Changes of oxidative stress levels in lens after lanosterol administration

The TAC in the lens cortex and nucleus significantly increased in all the intervention groups (Fig. 7A). The GSSG/GSH ratio decreased in the lens cortex of the lanosterol-administered cortical cataract, while it increased in the lens nucleus of the lanosterol-administered cortical and nuclear cataract (Fig. 7B). Therefore, lanosterol exposure mainly enhanced TAC and reduced GSSG/GSH ratio in the lens cortex. These results showed the antioxidative properties of lanosterol in the lens, and demonstrated that cortical cataract could be reduced by lanosterol through a reduction in oxidative stress.

Discussion

Cataract is the leading cause of blindness worldwide. Currently, the only way to treat cataracts is to surgically remove the opacified lens, which is an expensive procedure that increases the socioeconomic burden of the disease. The recently new finding of lanosterol as a promising agent for nonsurgical intervention to treat

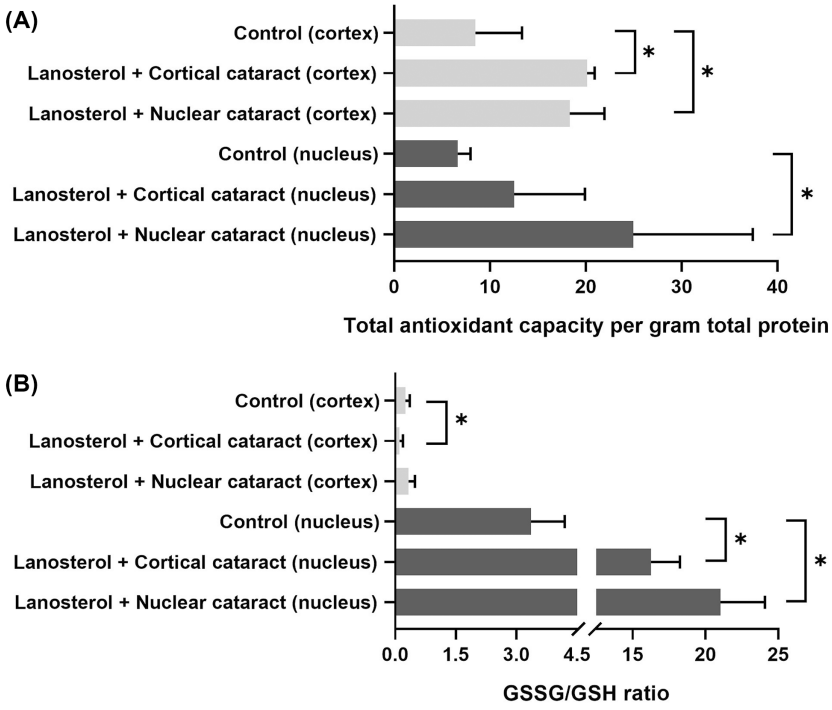

Figure 7. Changes of oxidative stress levels in lens after lanosterol administration. (**A**) Changes of the TAC in the lens cortex and nucleus. (**B**) Changes of the GSSG/GSH ratio in the lens cortex and nucleus. *$P < 0.05$.

cataract has brought about some new insights. However, there are still some problems that need to be addressed before further application, including the administrative method, effects in primates, and influences on lens proteins. In our study, the subconjunctival lanosterol–thermogel treatment showed a short-term but reversible effect on reducing cataract severity and increasing lens clarity in the cynomolgus monkey with cortical cataract, which was also in accordance with the trend of lanosterol concentration detected in the aqueous humor. Further changes in lens protein solubility and aggregation were also observed in our study. This is the first report on the inhibitory effect of lanosterol on the cataract progression based on *in vivo* models of primates.

Lanosterol treatment has also showed impressive effects on reducing cataract severity by increasing lens clarity in dogs.[6] In our study, we further confirmed the *in vivo* effect of lanosterol in the cynomolgus monkeys with naturally occurring adult-onset cataracts, especially in those with cortical cataract. Due to their relatively easy upkeep in captivity, wide availability, and closeness to humans anatomically and physiologically, cynomolgus monkeys have been used extensively in medical and biological research on human and animal health-related topics.[12,13] Therefore, the breed is regarded as an excellent animal model for studies of human systems, compared to other *in vivo* animal models using nonprimates such as rabbits, dogs, and rats. Our observation of naturally occurring cataract in primates is important to the study on the effect of lanosterol in the field of animal experimentation.

Regarding the drug administration method, we induced the lanosterol-loaded thermogel and applied a subconjunctival route for drug administration. We did not observe any clinical complications such as conjunctival infection or anterior chamber inflammation on slit-lamp examination. As there is almost no drug loss and no organic solvents used in the whole preparation process, we speculate that the drug loading efficiency is almost 100%, which was also consistent with previous studies.[14–16] In previous pharmacokinetic study of the lanosterol-loaded thermogel, *in vitro* drug release experiment showed that the drug concentration can remain above 50 ng/ml for up to 3 weeks after administration of a single dose of 400 μg/g formulation which indicated a sustained release profile.[17] The *in vivo* degradation of the PLGA–PEG–PLGA thermogels lasted 4 to 6 weeks after subcutaneous injection as the previous study described.[15] In the previous study of Zhao *et al.*,[6] lanosterol was loaded into a lipidpolymer hybrid nanoparticle through an adapted nanoprecipitation method before being injected into the vitreous cavity in a dog's eye using a 28-gauge needle. They also applied a topical eye drop with lanosterol to enhance the effect. Compared to the intravitreous route, our method requires only a simple external operation on the eyes of cynomolgus monkeys, which is much safer and reduces the risk of serious vision-threatening complications, including the bleeding, infection, and retinal detachment brought about by intravitreous injection. Moreover, compared to the topical route, the subconjunctival injection of the nanoparticulated and drug-loaded thermogel provides a quantitatively controlled and stable release for lanosterol during follow-up. Finally, there would be no need for the daily administration of eye drops, which could reduce the influence on the ocular surface.

After lanosterol treatment, we observed a trend of reduction in cataract severity in the eyes with cortical cataract as indicated by the decrease of wedge-shaped opacification in the periphery of the lens. Moreover, the decrease in cortical lens opacification was also in accordance with the trend of lanosterol concentration in the aqueous humor. However, little change was observed in eyes with nuclear cataract after lanosterol administration. It is possible that the reversible inhibitory effect of lanosterol may be more representative in the early stage of cataract, while in nuclear cataract, where protein denaturation and entanglement is a proven fact, it has hardly any effect on lens clarity. On the one

hand, the denatured proteins formed by aggregation and entanglement of crystallins in those severely opacified lenses[18,19] could possibly reduce the drug penetration and concentration within lenses. On the other hand, the density of the nuclear cataract is directly proportional to the aggregated proteins within the crystalline lens. It still could be possible that the concentration is not enough or the observation time is not long enough to observe significant effect on nuclear cataract. According to previous study of Song *et al.*, in a dose-dependent fashion, lanosterol treatment (from 0.25 to 2.5 μM) led to a decrease of antireductase reactivity, which could possibly achieve a higher effective concentration with a better solubility when delivered directly to ER membranes *in vitro* and thus exhibit an enhanced potency.[20] It has been shown that the efficiency of lanosterol is dependent on cataract severity by an *ex vivo* study of human cataractous lens samples.[21] It is suggested that the time duration for which the nucleus needs to be immersed in the lanosterol solution to achieve cataract reversal may be different for different nuclear densities.[6] Moreover, considering the inner barrier between the cortical and nucleus region, the transportation of lanosterol from the outer cortex to the inner nucleus could be the main obstacle for the potential target medication to treat lens opacity,[22,23] which could take longer time for lanosterol transportation to nucleus. As our drug-loading system did not show significant effectiveness in the lens of nuclear cataract, it may be necessary to further explore the drug-loading concentration and release time in the future.

The potential rebound effect is probably due to the estimated 1-month subconjunctival persistence of the thermogel. Adequate dosing, in combination with the sustained effect of a subconjunctival depot and the decreased clearance effect from encapsulation of the free drug, allows the therapeutic effect to be maintained for an intended period of 1 month. However, in the last 2 weeks since Day 14, the thermogel could hardly be detected in the previous injection site using the cobalt-blue light of the slit-lamp microscopy. There could be a turn-on threshold to trigger the anti-aggregation reaction of lanosterol in lens. Therefore, the reverse process of lens opacity could not be sustained longer as the lanosterol in anterior chamber was only just detectable but not enough to maintain the function in the first 14 days. We will keep on conducting multi-round drug administration with longer observation time for further investigation.

The amphipathic nature of lanosterol allows it to intercalate into and coat hydrophobic core areas of large protein aggregates, effectively allowing these aggregations to gradually become water soluble again.[6] *In vitro* studies showed the coordinated actions of disaggregation by lanosterol and refolding by heat shock proteins might facilitate the cells to recycle proteins from aggregates.[24,25] It has been suggested that high molecular weight protein aggregate is an intermediate in the formation of the water-insoluble protein, which has been reported to have a composition consisting mainly of α-crystallin.[26] Since protein conformation plays a role in light scattering,[27] it is important to study the aggregation-induced conformational change. The addition of lanosterol reversed the negative effects of the G91del mutation under external stress including proteolysis, solubility, and structural stability.[28] Moreover, the effect of lanosterol in binding α-crystallins and reversing their aggregation *in vitro* has been confirmed previously,[29] indicating that protein aggregation is not an endpoint and could be reversed with specific small-molecule drugs.[30] However, a recent study of *in vitro* rat lens along with a study of human lens protein solubilization showed that lanosterol failed in binding aggregated lens protein to dissolve cataracts.[31] Therefore, it is necessary to obtain more experimental evidence based on *in vivo* study. Increased protein solubility, decreased protein aggregation, and different tertiary structures in the lenses of cynomolgus monkeys with the lanosterol administration were confirmed in our study. It is possible that lanosterol influences the conformational change process from LMW α-crystallin to high molecular weight α-crystallin, and then reduces the light scattering of lens. This explains why the solubility and aggregation of proteins that we observed are mainly due to the change in α-crystallin.

Over the course of its life, the lens is exposed to biochemical, physiological, and functional changes, as a result of the natural process of aging. Protein-damaging stress, deficiency of glutathione, and oxidative damage are several specific mechanisms responsible for senile cataract.[32] The lens has substantial supplies of antioxidant reserves, antioxidant enzymes, and secondary defenses to prevent cataract formation. The major antioxidants in the lens are GSH and ascorbic acid.[33–36] Depletion of antioxidants renders the lens susceptible to oxidative damage, which results in accumulation of oxidized residues in long-lived lens proteins and enzymes. The effect is a loss of normal metabolic function and disorganization of the normal intracellular protein necessary for transparency. A common feature of nuclear cataract is the low concentration of GSH in the center of the lens.[35] GSH is an obvious compound for defending the lens against oxidative insult, by directly involving in reducing disulfides and acting as a pivotal cofactor in the detoxication of H2O2 and also a free-radical quencher.[37] Such data can be interpreted as suggesting that the intracellular environment of the lens is primarily a reducing environment. The deleterious nature of the presence of GSSG is apparent from the reports of protein GSH-mixed disulfides formed as a result of the presence of GSSG. Such mixed disulfides can lead to protein disulfides and further modification. Superoxide and hydroxyl radicals cause damage to cell membrane lipids and proteins, which are deposited on the surface of the lens, causing opacities. In our study, lanosterol seemed to reduce the production of radical species. The role that lanosterol plays in helping an endogenous antioxidant system neutralize oxidative stress requires further study with a larger sample size, which may provide evidence for possible medical intervention, as well as offer a way to diminish the risk of cataract.

Conclusion

In summary, based on the previous landmark findings that lanosterol plays a role in reducing cataract formation, we performed *in vivo* study on the inhibitory effect of sustainably released lanosterol by subconjunctival injection in the primates, suggesting a promising strategy for the prevention and treatment of cataracts, particularly in the early stage of the cortical type.

Supplementary data

Supplementary data is available at *Precision Clincal Medicine* online.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Design of the study (X.J.Z.); conduct of the study (K.K.Z., W.W.H., Y.D.); sample collection (K.K.Z., W.W.H., Y.G.Z., X.K.W., J.Z.); collection and management of the data (K.K.Z., W.W.H.); analysis and

interpretation of the data (K.K.Z., W.W.H.); and preparation, review, and approval of the manuscript (X.J.Z., K.Z., Y.L.).

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Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Eye and Ear, Nose, and Throat Hospital, Fudan University, Shanghai, China. All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. In addition, as an Editorial Board Member of *Precision Clinical Medicine*, the corresponding author Kang Zhang was blinded from reviewing or making decisions on this manuscript.

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