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# Zebrafish (*Danio rerio*) Is an Economical and Efficient Animal Model for Screening Potential Anti-cataract Compounds

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**Purpose:** To develop a zebrafish cataract model for screening potential anti-cataract compounds.

**Methods:** Living zebrafish were anesthetized and exposed to ultraviolet-C (UV-C) irradiation at a dosage of 3250 mJ/cm$^2$/d until they developed severe cataracts. These cataracts were graded based on photographs analyzed with ImageQuant TL version 7.0. Fish with severe cataracts were used to evaluate a range of compounds for cataract treatment, including the previously demonstrated hit compound lanosterol. For the initial evaluation, fish were divided into four groups: no treatment, balanced salt solution, $\beta$-cyclodextrin ($\beta$-CD), and lanosterol dissolved in $\beta$-CD. The treatments were performed for 10 days, and the clarity of lenses was evaluated. To assess the persistence of treatment, fish were treated with $\beta$-CD and lanosterol dissolved in $\beta$-CD for seven consecutive days followed by monitoring for three days without treatment.

**Results:** The average time for zebrafish to develop severe cataracts using the present UV-C irradiation protocol was 7.8 days (range 4–15 days). Both study designs required only another 10 days to determine the effect of hit compounds. The total experimental period could be completed within one month, and the entire experiment was economical.

**Conclusions:** We could assay a large number of hit compounds at a reasonable cost and within a short time using this newly developed zebrafish cataract model. These assays may allow development of an efficient platform for screening potential anti-cataract compounds.

**Translational Relevance:** The results may facilitate the development of ani-cataract medication for humans after further experiments and investigations.

## Introduction

Cataracts that impair vision have been a significant global problem for centuries. It is estimated that the number of people with cataracts will increase by about one third over the next 20 years and that cataracts will remain the leading cause of visual impairment in all regions of the world.[1,2]

Protein aggregation is the most important factor in the development of cataracts. Protein aggregation can be induced by multiple causes, including mutations of the crystalline proteins, the major cause of congenital cataracts, and oxidative stress, the main contributor to age-related cataracts. However, the precise regulatory mechanisms by which lens proteins maintain transparency or cause opacity are not completely understood.[3–5] Furthermore, there is still no compound available in clinical practice to treat cataracts; surgical treatment remains the only effective treatment, although it may lead to complications that may result in blindness and some postoperative care issues.[6–9] Therefore discovery of a compound that effectively treats cataracts would improve patient safety.

A recent study reported that one compound, lanosterol, could reduce the severity of cataracts in dogs in vivo and increase the transparency of dissected cataractous lenses of rabbits ex vivo,[10] whereas another showed that compound 29 improved lens transparency in a mouse model of hereditary cataracts with R49C crystalline $\alpha$A and R120G crystalline $\alpha$B. Compound 29 treatment also partially restored the solubility of crystalline protein in the lenses of aged mice in vivo and in the lenses of humans ex vivo.[4] Although these experimental models provide valuable tools for testing anti-cataract compounds, they are expensive and time-consuming and have a steep learning curve for most laboratories.[11] Therefore developing a cost-effective animal model for the development of anti-cataract compounds is of clinical value.

The advantages of using zebrafish as an animal model lie in the feasibility of screening a large number of compounds and drugs for their therapeutic potential in human diseases such as Alzheimer's disease and metabolic disorders.[12] In addition, zebrafish have also been used specifically to model several ocular diseases of humans, including congenital cataract, myopia, glaucoma, diabetic retinopathy, and age-related macular degeneration.[12–18] However, there is still no documented protocol for inducing cataracts in zebrafish or for evaluating candidate compounds. Therefore we set up an economical and time-saving protocol for the development of a zebrafish cataract model to screen for anti-cataract compounds.

## Methods and Materials

### Zebrafish Cataract Model

The animal study was approved by the animal ethic Review Board of Tamkang University, Tamsui, Taiwan (Approval No. 108001). Cataracts were induced in zebrafish by ultraviolet-C (UV-C) irradiation as follows. The AB strain of zebrafish were maintained under a 14-hour light and 10-hour dark photoperiod at 28.5°C, as described previously.[19,20] At 90 days after fertilization, three-month-old healthy zebrafish weighing 360 to 440 mg were selected for experiments. Zebrafish were anesthetized with 150 ppm tricaine (Sigma-Aldrich, St. Louis, MO, USA) for induction and maintained in 50 ppm tricaine before initiating experiments.[21,22] The fish were first photographed with a special magnifier and the built-in camera of an iPhone 7, using the ProCam APP (version 11.4.7; Samer Azzam, Los Angeles, CA, USA) with fixed settings (Supplementary Figs. S1A, S1B).The right eyes of fish were irradiated in a UV crosslinker (Spectroline, Westbury, NY, USA) with 254 nm UV-C at a dosage of 3250 mJ/cm$^2$/d until a grade 3 cataract was observed (the detailed protocol is illustrated in Supplementary Fig. S2). The development of cataracts was detected by an experienced ophthalmologist (C-F. Liu) using the magnifier when daily photographs were taken. After UV-C irradiation, fish were placed in a dark water tank for 60 minutes to recover from anesthesia.

The above visual grading was only used for screening for severe cataracts in the beginning.[10] The gray scale of cataracts visually graded 3 in the photographs was further analyzed by ImageQuant TL (v 7.0; GE Healthcare, Chicago, IL, USA). The objective gray scales of ImageQuant TL ranged from 0 to 255; the higher the value, the greater number of black pixels in the unit area, which indicates greater transparency of the lens. Conversely, the lower the value, the fewer black pixels in the unit area, which indicates more severe cataract. The results of statistical analysis of the experimental lenses were used to evaluate the treatment effects of different solutions. The analysis method is similar to the Ex Vivo Porcine Lens Soaking Assay and is described in the supplementary file.

Fish with gray scale <150 in ImageQuant TL analysis were selected for the next compound-treatment experiment. In the first stage of experiments, we evaluated the effect of compounds for cataract treatment by dividing zebrafish into four groups: no drug (ND), balanced salt solution (BSS), $\beta$-cyclodextrin ($\beta$-CD) dissolved in BSS, and lanosterol dissolved in $\beta$-CD in BSS. The right eye of the fish was covered by placing a compound-infiltrated sponge on the cornea for five minutes twice a day for 10 consecutive days (the detailed protocol is described in the legend for Fig. 1B). The results are summarized in Figures 1C, 1D and 1E. In the second stage of experiments, to evaluate the persistence of the effects of treatment with hit compounds, fish cataracts with gray

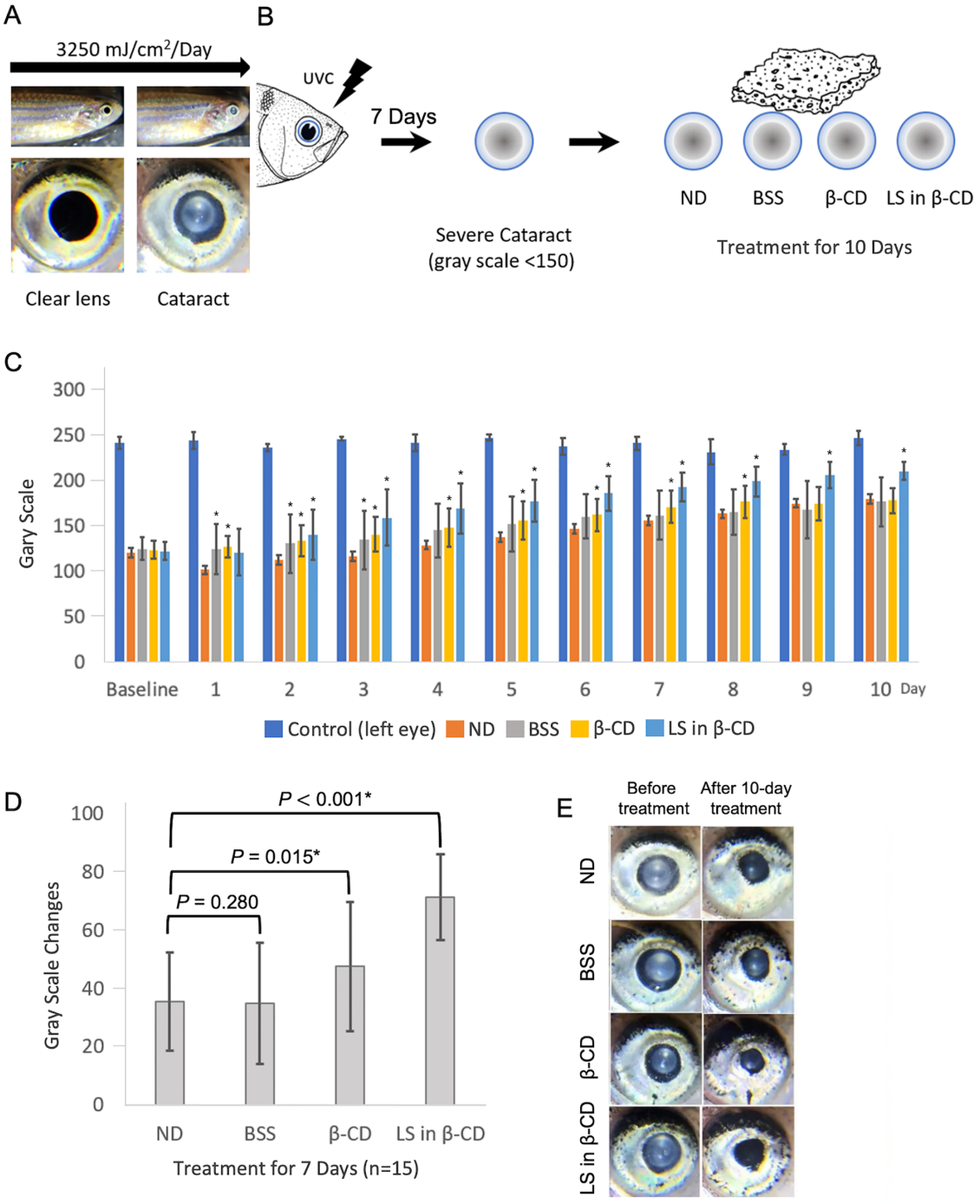



**Figure 1.** Constitutive lanosterol treatment reversed cataract in zebrafish. (A, B) Schematic of experimental procedure using zebrafish. (C, D and E) Schematic of experimental results. (A) The lenses of zebrafish were irradiated by 254 nm UV-C at a dosage of 3250 mJ/cm$^2$/d for 7 days to induce cataract. (B) After cataract induction, the fish with a severe cataract phenotype were divided into four treatment groups as follows: no drug, BSS, $\beta$-CD, and lanosterol (LS) in $\beta$-CD. Lenses were covered with a compound-infiltrated sponge for five minutes twice a day for 10 days. The zebrafish were anesthetized and photographed using the same method used for cataract induction. After a 5-$\mu$L instillation of the intended compound, a 5 $\times$ 5 mm sponge infiltrated with the intended compound was applied on the right eye. After five minutes, the zebrafish was placed in a dark water tank for 60 minutes to recover from the anesthesia. (C) Gray-scale quantification of lenses of fish in the experiment. The results are expressed as means $\pm$ standard deviation. Gray scale 255 indicates totally clear, whereas 0 means totally opaque. The average gray scale of the untreated left eye was 241 at the beginning of the experiments, and the value was stable throughout the course of the experiments. *Asterisks* indicate significant differences ($P < 0.05$, independent *t*-test) between ND and other groups. (D) Changes in gray scales after seven days treatment with indicated compounds. Detailed gray scales of lens and sample numbers are summarized in Supplementary Tables S1 to S4. *$P < 0.05$, independent *t*-test. (E) Images of day 10 zebrafish lenses treated with no drug, BSS, $\beta$-CD, or LS in $\beta$-CD.

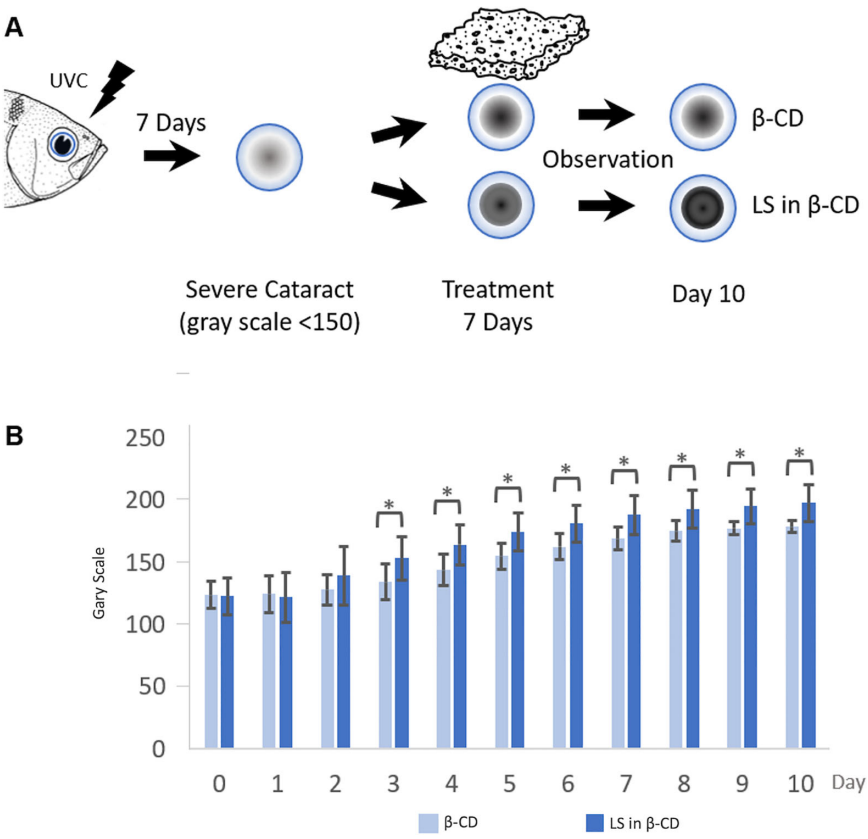



**Figure 2.** The treatment effect is sustained for three days after cessation of lanosterol treatment. (A) Schematic of cataract induction and lanosterol treatment. Zebrafish with severe cataract were treated with $\beta$-CD and lanosterol (LS) for 7 days followed by observation without treatment for three days. (B) Gray-scale values of zebrafish lenses. The results are expressed as means $\pm$ standard deviation. Details of gray-scale values of lens and sample numbers are summarized in Supplementary Tables S5 and S6. *$P < 0.05$, independent *t*-test.

scale <150 were treated with the indicated compounds twice a day for seven consecutive days, and then the changes in gray scale were evaluated for three days without any treatments. The results are summarized In Figure 2B. To fulfill the requirements for ethical animal care and protection, we developed the methods to test the compound in vitro and ex vivo in advance, and the detailed methods of in vitro turbidity clearance assay and ex vivo porcine lens soaking assay are both described in the supplementary file.

## Results

### Lanosterol Treatment Produced Cost-Effective Reversal of Cataract in the Zebrafish Model

To determine the anti-cataract effect of lanosterol in vivo, we developed an animal cataract model using zebrafish, which is economical, time-saving, and reproducible. As shown in Figures 1A and 1B, severe cataracts, gray scale <150, can be induced in zebrafish by exposing their right eyes to UV-C irradiation at 3250 mJ/cm$^3$/d for seven consecutive days. The left eyes of zebrafishes were left untreated to maintain the vision of the fish and used as a control for the eye with cataract. This induction of cataract caused some damage to the fish, because the mortality rate in the end of our experiments was around 20% (Supplementary Fig. S3). To evaluate this zebrafish cataract model for screening anti-cataract compounds, we performed the following two sequences of assays using lanosterol.

#### Stage 1: Examining the Anti-Cataract Effect of Lanosterol Using the Zebrafish Cataract Model

To examine whether zebrafish cataracts can be reversed by anti-cataract compounds, we used zebrafish with severe cataracts (gray scale <150) and lanosterol (Fig. 1B). The fish were divided into four treatment groups: lanosterol in $\beta$-CD and BSS, $\beta$-CD in BSS, BSS, and ND groups (Fig. 1B). Each group contained 15 cataract-bearing fish that were treated for 10 consecutive days. Table 1 summarizes the demographic features, none of which showed any significant between-group differences. Supplementary Tables S1 to S4 list the gray-scale values of cataracts during the period of treatment. As shown

**Table 1.** The Demographic and Ocular Data for Zebrafish Before the First-Stage Experiment

| Treat 10 Days | Lanosterol | $\beta$-Cyclodextrin | BSS | No Drug | *P* Value[d] |
|---|---|---|---|---|---|
| Sex | | | | | 0.798 |
| Male | 8 | 5 | 7 | 7 | |
| Female | 7 | 10 | 8 | 8 | |
| Weight (mg) | 412 ± 28 | 413 ± 405 | 408 ± 271 | 402 ± 213 | 0.712 |
| Age (mos.) | 3 | 3 | 3 | 3 | |
| Length (mm) | 29.5 ± 2.8 | 28.9 ± 1.2 | 28.5 ± 1.0 | 28.5 ± 0.9 | 0.350 |
| Eyeball width (mm) | 2.33 ± 0.49 | 2.20 ± 0.41 | 2.13 ± 0.35 | 2.26 ± 0.46 | 0.619 |
| Pupil width (mm)[a] | 1.0 | 1.0 | 1.0 | 1.0 | |
| Lens width (mm)[b] | 1.07 ± 0.09 | 1.08 ± 0.12 | 1.09 ± 0.10 | 1.11 ± 0.10 | 0.818 |
| Baseline gray scale[c] | 122.5 ± 12.8 | 123.4 ± 10.0 | 125.2 ± 8.7 | 120.4 ± 9.7 | 0.650 |

The results are presented as the mean with standard deviation (mean ± SD).
[a]The pupil width was measured using a built-in ruler in the dissecting microscope (MZ9; Leica, Wetzlar, Germany).
[b]The lens width was measured using a built-in ruler of the light microscope (XL-1000; Leica).
[c]Baseline gray scale was measured after cataract induction.
[d]Sex was analyzed by Fisher's exact test, others by one-way analysis of variance.

**Table 2.** The Demographic and Ocular Data for Zebrafish Before the Second-Stage Experiment

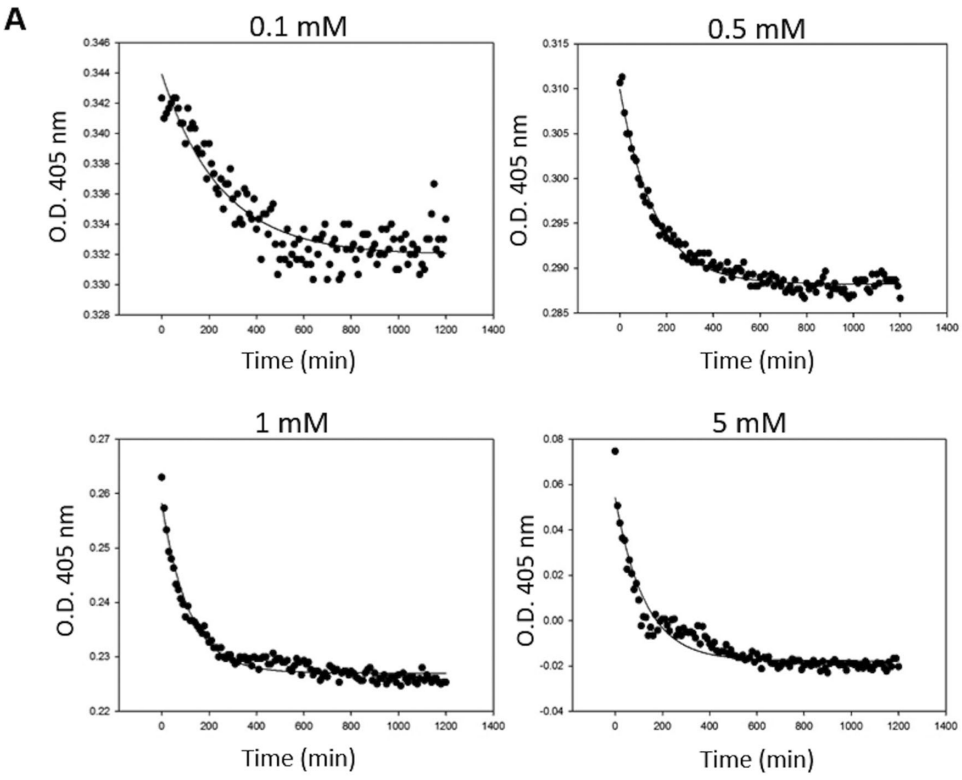
| | Lanosterol | $\beta$-Cyclodextrin | *P* Value[b] |
|---|---|---|---|
| Sex | | | 0.710 |
| Male | 5 | 7 | |
| Female | 10 | 8 | |
| Weight (mg) | 404 ± 25 | 404 ± 16 | 0.959 |
| Age (mos.) | 3 | 3 | |
| Length (mm) | 28.2 ± 0.9 | 29.0 ± 1.4 | 0.065 |
| Eyeball width (mm) | 2.10 ± 0.26 | 2.00 ± 0.00 | 0.326 |
| Pupil width (mm) | 1.0 | 1.0 | |
| Lens width (mm) | 1.17 ± 0.11 | 1.14 ± 0.09 | 0.463 |
| Baseline gray scale[a] | 122.9 ± 15.2 | 124.1 ± 11.3 | 0.811 |

The results are presented as the mean with standard deviation (mean ± SD).
[a]Baseline gray scale was measured after cataract induction.
[b]Sex was analyzed by Fisher's exact test, others by independent *t*-test.

in Figure 1C, all gray-scale values increased gradually in a time-dependent manner that was probably related to the self-recovery of fish. Interestingly, the lenses treated with lanosterol displayed a significantly greater increase in gray scale at the end of the experiment, indicating an enhanced anti-cataract effect. To compare the amplitude of increases induced by self-recovery and lanosterol treatment, we calculated the differences in gray scale between day 0 and day 7. As shown in Figure 1D, the gray scale increased by 69.6 in lanosterol-treated lenses, but by $< 50$ in the ND, BSS, and $\beta$-CD groups. Although lanosterol cannot completely reverse the development of zebrafish cataracts to the clear state, most lanosterol-treated lenses displayed a gray scale $>200$ at the end of the experiment (Figs. 1C and 1E).

### Stage 2: An Extended Anti-Cataract Effect of Lanosterol in Zebrafish Cataract Model

Based on the above 10-day experiment, lanosterol treatment has superior anti-cataract activity compared with other treatments in vivo. To evaluate the duration of this effect further, we performed an experiment in which monitoring of the gray scale of lenses was prolonged without further compound treatment after 7 days (Fig. 2A). In this experiment, the baseline demographic and ocular phenotype of zebrafish lenses displayed no significant between-group differences for any measurements (Table 2). The results are summarized in Figure 2B. Consistent with the above results, the anti-cataract effect of lanosterol was significantly higher than that of vehicle control from day 3 to day 7 (In the presence of treatments, Supplementary Fig.




B

f=y0+a*exp(-b*x)

| | 0.1 mM | 0.5 mM | 1 mM | 5 mM |
|---|---|---|---|---|
| y0 | 0.3320 | 0.2882 | 0.2270 | -0.0177 |
| a | 0.0119 | 0.0217 | 0.0312 | 0.0719 |
| b | 0.0040 | 0.0067 | 0.0087 | 0.0080 |

**Figure 3.** Lanosterol enhances the speed of clearing of extracted porcine lens protein in vitro. (A) The turbidity clearance assay of the effect of the indicated concentration of lanosterol on extracted porcine lens protein. The turbidity was monitored every 20 minutes for a total of 20 hours. The exponential regression curves (*solid lines*) are shown. O.D., optical density. (B) The equation and parameters for the exponential regression curves in (A). (y0 = remaining amount, a = clearable area, b = clearing speed, exp = exponent, x = time).

S4), and this difference extended from day 8 to day 10 (in the absence of treatment, Supplementary Fig. S4). Detailed measurements of lenses are shown in Supplementary Tables S5 and S6 for lanosterol and $\beta$-CD, respectively.

## Lanosterol Restored the Clarity of Solution Containing Extracted Porcine Lens Protein In Vitro

To test the anti-cataract effects of lanosterol, in vitro turbidity clearance assays were carried out using sodium selenite and lens protein solution. The detail methods are described in the supplementary file (In Vitro Turbidity Clearance Assay). The average absorbance of sodium selenite-treated samples reached 0.528 (measured at 405 nm), indicating successful cataract simulation. Notably, the turbidity of the lens solution was decreased in a time-dependent manner by lanosterol treatment (Fig. 3A). In addition, regression analysis showed that the rate of lanosterol-induced clearance was dose-dependent and saturated at a concentration of 1 mM lanosterol (Fig. 3B). As expected, our results validated the anti-cataract function of lanosterol in vitro and encouraged us to test its function further using physiological samples.

## Lanosterol Reversed the Transparency of Isolated Porcine Lens Ex Vivo

To test the anti-cataract effect of lanosterol in a more physiological context, we used fresh porcine lens in which cataract was induced by incubating the lens in Jao-Liu (JL) solution (0.05 g [ethylenediaminete-

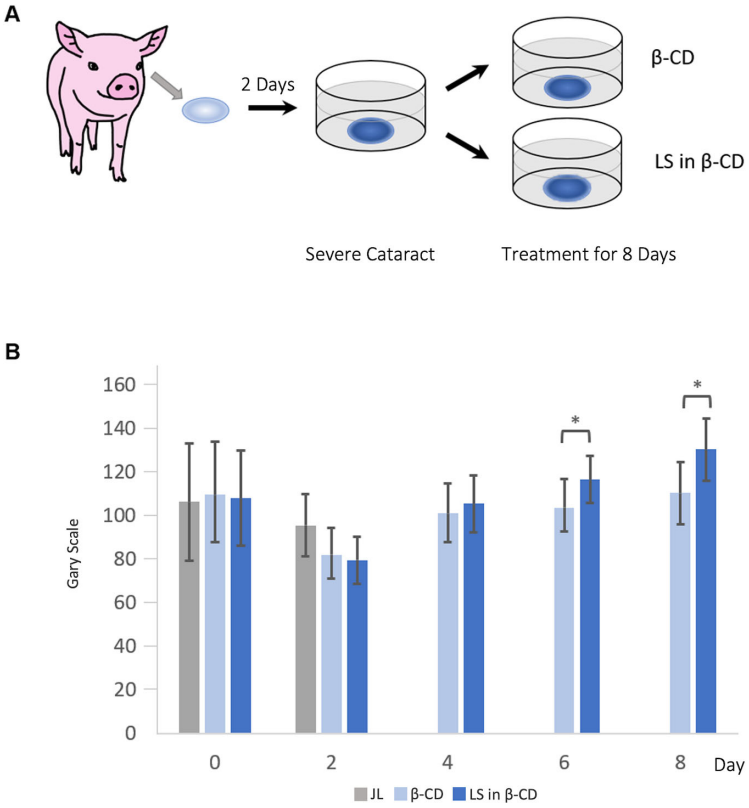



**Figure 4.** Lanosterol reverses the loss of transparency of isolated porcine lens ex vivo. (A) Schematic of soaking assays. The isolated porcine lenses were treated with Jao-Liu (JL) solution for two days followed by incubation with vehicle $\beta$-CD and lanosterol (LS) in $\beta$-CD solution for eight days. The clarity of lenses was determined every two days. The detail baseline and treatment data are summarized in Supplementary Table S7. (B.) Gray-scale quantification of porcine lenses. The results are expressed as means $\pm$ standard deviation. Detailed gray scales and sample number are summarized in Supplementary Tables S8 and S9. *$P < 0.05$, independent *t*-test.

traacetic acid]2Na + balanced salt solution to 50 mL) followed by treatment with lanosterol-containing or lanosterol-free $\beta$-CD (Fig. 4A). The detail methods are described in the supplementary file (Ex Vivo Porcine Lens Soaking Assay). The gray scale of these porcine lenses in the photographs was analyzed by ImageQuant TL software and used to determine the severity of cataract: high values for gray scale denote higher transparency and vice versa. Notably, we observed that incubation in JL solution bloated the lenses and that most lenses in the JL solution group were fractured after 2 days because of an imbalance in osmolarity. However, the lack of gray-scale analysis of these lenses in JL does not affect the comparison between the other groups. In addition, although shrinkage of porcine lenses may have affected the severity of cataracts because the gray scale significantly decreased at treatment day 2 in all groups (Fig. 4B), there was no difference in the width of porcine lenses treated with different compounds (Supplementary Table S7).

Soaking the lenses in lanosterol-containing buffer for six days significantly restored the transparency of cataracts compared with lenses in vehicle control $\beta$-CD. The gray scale of lanosterol-treated lenses was significantly improved up to the end of the experiment (Fig. 4B), whereas the gray scale of vehicle-treated lenses increased slightly during treatment day 2 to day 4 (Fig. 4B).

Although we were unable to cure the cataracts in isolated porcine lenses completely, our results strongly suggested that lanosterol treatment does have an effect on facilitating the recovery of transparency in porcine lens with induced cataracts ex vivo ($P = 0.026$, day 8, Supplementary Table S8).

## The New Zebrafish Cataract Model May Serve as an Economical and Efficient Screening Platform for Anti-Cataract Compounds

The zebrafish cataract model established here has an efficient cataract-inducing protocol and allows rapid verification of anti-cataract compounds. The prices of the equipment used in the zebrafish cataract model, turbidity clearance assays and lens-soaking experiments are listed in Table 3. The cost of the animals

**Table 3.** The Costs of the Equipment and Waste in the Present Study

| | Price (USD) |
|---|---|
| Equipment | |
| Optical microscope (XL-1000; Leica) | $26,000 |
| Dissecting microscope (MZ9; Leica) | $13,000 |
| ELISA reader (MQX200 uQuant; Bio Tek, Winooski, VT, USA) | $10,000 |
| UV Crosslinker (Spectroline, Westbury, NY, USA) | $3000 |
| Camera (Canon 50D; Canon Inc., Tokyo, Japan) | $500 |
| Magnifier for zebrafish (Eschenbach Optik, Nuremberg, Germany) | $130 |
| Infrared thermometer | $33 |
| Tweezers (Vetus Tools, Shanghai, China) | $28 |
| Magnifier for porcine lens | $26 |
| Thermometer | $16 |
| Sponge punching device | $6 |
| Fish tank (Taikong Corporation, New Taipei City, Taiwan) | $5 |
| Waste | |
| Lanosterol (Carbosynth, Staad, Switzerland) | $500/25 g |
| Beta-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA) | $293/100 g |
| Tricaine (Tokyo Chemical Industry, Tokyo, Japan) | $83/25 g |
| EDTA-2Na (Tokyo Chemical Industry) | $56/50 g |
| Balanced salt solution (Alcon, Geneva, Switzerland) | $30/500 mL |
| Scale slides | $15/sheet |
| 24-well culture plate | $8/plate |
| Zebrafish (Taikong Corporation) | $3/fish |
| Porcine lens (from traditional market) | $0.3/lens |
| 100-mm cell culture dish | $0.3/dish |
| Glass slides for porcine lens (Muto Pure Chemicals, Tokyo, Japan) | $0.16–$0.33/sheet |
| Glass slides for zebrafish (Matsunami, Osaka, Japan) | $0.16–$0.33/sheet |
| Sponge (3M, St. Paul, MN, USA) | $0.06/fish |
| Dark color paper | $0.03/piece |

All costs are counted in New Taiwan dollars (NTD), and converted into U.S. dollars (USD) with an exchange rate of $1 USD to 30 NTD.

EDTA, ethylenediaminetetraacetic acid.

and chemicals used in this newly-developed zebrafish cataract model for anti-cataract compound testing is very low (approximately $120 USD, including a cohort of 30 zebrafish [half for testing a new compound, and the others for control], three culture dishes, 0.15 g tricaine, and 1 g lanosterol). Furthermore, the proposed assays require a relatively short time so that a complete experimental run can be achieved within one month.

## Discussion

In this study, we used lanosterol to validate a newly established zebrafish animal model for anti-cataract assays. To fulfill the requirements for ethical animal care and protection, we examined the role of lanosterol for first-line anti-cataract compound screening using both in vitro and ex vivo methods. The in vivo experiment included two stages. In stage 1, zebrafish cataracts were treated successfully with 10 consecutive days of lanosterol (Fig. 1). In stage 2, the therapeutic effect was sustained after the end of treatment (Fig. 2). The turbidity clearance assays in our study also demonstrated a time- and dose-dependent cataract-clearing effect of lanosterol (Fig. 3). In addition, the results showed that lanosterol significantly reversed the decrease in transparency of porcine lens in a soaking experiment (Fig. 4). This series of experiments provided a cost-effective way of verifying anti-cataract compounds within one month using lanosterol as a positive control.

By comparison, other animal models used for drug screening require higher cost and more time. The genetically engineered mouse model may take more than a

year for gene editing, while the subsequent induction of cataract and compound testing may take a further 6 months. For example, mice with R120G cryAB knock-in showed lens opacity at around 20 weeks of age and then required another two weeks for compound testing.[4,23] By contrast, the total experimental time required for our newly developed zebrafish cataract model is less than one month, and it is also possible to evaluate a large number of samples in a short time. Zhao et al.[10] used dissected rabbit lenses for in vitro and dogs for in vivo experiments. Compound testing in these animals is very expensive and time-consuming and has ethical issues. The assays we developed using porcine lenses and the zebrafish cataract model have relatively lower cost and greater efficiency with a flatter learning curve. Moreover, zebrafish, a nonmammalian species, is a suitable animal model for preclinical evaluation of drug safety and therapeutic assessments without many animal ethics concerns. These features make zebrafish a useful drug-screening platform that can also be applied to hit compound refinement.[12]

The route of drug administration is also an issue for cataract treatment. In a previous study to assess the effect of lanosterol treatment on cataracts in vivo, dogs were given intravitreous injections of lanosterol.[10] However, such treatment is too inconvenient to improve the quality of life of patients with cataracts, and it also has some potential risks,[24] which could restrict the use of lanosterol on humans; the use of topical drops would be a better solution. Lanosterol is a tetracyclic triterpenoid compound that is an intermediate product of the cholesterol synthesis pathway. Although we did not determine the corneal penetration of lanosterol, we expect that this was similar to the corneal penetration of prednisolone acetate, which is also a lipophilic sterol-derived compound with a corneal permeability of 3.3E-5 cm/s and can easily penetrate the corneal epithelium, stroma, and endothelium.[25,26] We also applied $\beta$-CD to increase the penetration of lanosterol.[27–29] This compound is used in pharmaceutical applications because of its drug solubility-enhancing properties, improved bioavailability, and improved formulation stability, which make it capable of masking a drug's irritating effects. This compound is generally regarded as safe.[28] We further applied a compound-infiltrated sponge to the fish cornea (Figs. 1B and 2A). The results showed that lens opacity was reversed by lanosterol treatment (Figs. 1C and 2B). Topical usage of lanosterol may be more likely to receive US Food and Drug Administration approval for cataract treatment in humans. Furthermore, if related or other candidates are identified, our platform can also be applied to rapid and cost-effective verification of these drugs.

A previous study demonstrated that lanosterol was an effective treatment for cataracts.[10] However, that study did not investigate how long the pharmacological effects lasted after cessation of treatment. Because the mortality in the zebrafish experiment increased after day 7 (Supplementary Fig. S3), we established a treatment time of seven days in the second-stage experiment to allow better sample-size preservation. In addition, in the stage one experiment, we observed that the ImageQuant TL size of the pupils shrank to less than 750 units after day 10, which may cause difficulty in measurement and analysis. Therefore the experiment was terminated on day 10. We also observed that the cataract-clearing effect of topical treatment disappeared immediately after treatment cessation after seven days (smaller gray-scale increase in the observation period than during the treatment period). However, the difference between the two groups persisted. This may imply that the effect on previously treated cataracts may last for some time. The most appropriate treatment duration and the issue of recurrence require further investigation.

In the first-stage experiment using the zebrafish cataract model, the gray scale in all groups fluctuated after day 7; this may have been caused by some measurement bias because of pupil shrinkage. The pupil shrinkage may result from the lens sinking posteriorly because of UV-C-induced damage to the suspensory ligament. In addition, the continuous experiment put a life-threatening burden on the fish, and there was a decline in the number of surviving fish after seven days. To improve the survival rate, we made every effort to avoid other injuries, including shortening the time for photography (less than one minute) to reduce the burden on fish, but to no effect. Further investigation may identify means to improve the survival rate.

Interestingly, the zebrafish cataracts did improve without treatment. In the first stage of the zebrafish experiment, the gray scale of the ND group increased by an average of 35.7 from day 0 to day 7. In the second-stage experiment, the gray scale increased about 10 units during the day 7 to day 10 observation period in both the lanosterol and $\beta$-CD groups. We presumed that this cataract-clearance effect may be related to some of the zebrafish recovery genes: several studies have demonstrated outstanding self-healing ability from diseases in zebrafish. Zebrafish can even regenerate functional nervous tissue such as retina after major injury.[30,31] Considering the self-healing ability of living zebrafishes, we may have overestimated the effects of lanosterol and $\beta$-CD on cataract formation. To avoid this, we have included the data for an untreated group for comparison. The observed differences between the treated and untreated groups may have minimized the effect of the self-healing ability of

zebrafish, and they provide information about the true effects of lanosterol and $\beta$-CD (Fig. 1C). Therefore, for the purpose of screening, this method may be able to identify rapidly the effects of hit compounds and may be useful in further explorations. The mechanism for this cataract-clearing ability should also be investigated further as a possible treatment for cataracts.

The porcine and zebrafish cataracts were both induced to grade 3, but we used stricter criteria to define the zebrafish cataracts. The severity of the porcine lens cataracts was determined only by a visual grading method[10] because this ex-vivo experiment was used only for initial screening for effective compounds. By contrast, because the new zebrafish cataract model was the main screening model for drug assessment, only zebrafish lenses with grade 3 cataracts that were confirmed by ImageQuant TL software (gray scale $<$ 150) were included in further treatment experiments. This may ensure the consistency and reliability of further experiments.

After cataract induction, most cataracts in the in vivo (zebrafish) and ex vivo (porcine) models became more severe on day 1 and day 2 after treatment, respectively. The explanation for this may be that the treatment compound required time to achieve its therapeutic effects while destruction of the lens structure by UV-C continued. In addition, we found that the porcine lenses became clearer as the temperature rose during the ex vivo experiment, possibly because of the increasing solubility of aggregated lens protein. We noticed that temperature has a strong effect on the solubility of lens protein; therefore it should be carefully monitored for the entire experiment. This is also the reason that we conducted the entire porcine lens experiment on an ice tank to keep the temperature consistent.

The present study has several limitations, including its small sample size and lack of mechanism identification. However, in current medical practice, there is still no effective medicine for cataract treatment. Lanosterol shows great potential for clinical application in humans. There are several factors involved in cataractogenesis, including an imbalance of calcium homeostasis, genetic mutations, oxidative stress, and exposure to UV light. These factors can cause misfolding of the lens proteins and aggregation, resulting in cataracts.[3,4] Lanosterol is presumed to dissolve the aggregations and hence restore the transparency of the lens. However, the precise mechanism by which lanosterol restores the transparency of the lens is not fully understood, and the hypothesis needs confirmation using more molecular-based studies.

In conclusion, this new zebrafish cataract model is very economical and can analyze a large number of samples in a short time. It was able to demonstrate the effectiveness of topical lanosterol application for cataract treatment. Before these in vivo experiments, lanosterol had been demonstrated as a hit compound using in vitro turbidity clearance assays and ex vivo porcine lens experiments. The order of experiments in this model suggests that future verification of anti-cataract compounds will be feasible without major ethical issues regarding the use of animals. This newly developed zebrafish cataract model is cost-effective and will contribute to faster screening of anti-cataract compounds in the future.

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## References

1. Pascolini D, Mariotti SP, Pokharel GP, et al. 2002 global update of available data on visual impairment: a compilation of population-based prevalence studies. *Ophthalmic Epidemiol*. 2004;11:67–115.
2. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. *Bull World Health Organ*. 2004;82:844–851.
3. Moreau KL, King JA. Protein misfolding and aggregation in cataract disease and prospects for prevention. *Trends Mol Med*. 2012;18:273–282.
4. Makley LN, McMenimen KA, DeVree BT, et al. Pharmacological chaperone for alpha-crystallin partially restores transparency in cataract models. *Science*. 2015;350:674–677.

5. Tsao YT, Wu WC, Chen KJ, et al. An assessment of cataract severity based on antioxidant status and ascorbic acid levels in aqueous humor. *Antioxidants (Basel)*. 2022;11:397.
6. Lartey S, Armah P, Ampong A. A sudden total loss of vision after routine cataract surgery. *Ghana Med J*. 2013;47:96–99.
7. Chen HC, Lee CY, Liu CF, et al. Corneal endothelial changes following early capsulotomy using neodymium:yttrium-aluminum-garnet laser. *Diagnostics (Basel)*. 2022;12:150.
8. Lee CY, Lu TT, Meir YJ, et al. Refractive changes following premature posterior capsulotomy using neodymium:yttrium-aluminum-garnet laser. *J Pers Med*. 2022;12:272.
9. Chen HC, Huang CW, Yeh LK, et al. Accelerated corneal endothelial cell loss after phacoemulsification in patients with mildly low endothelial cell density. *J Clin Med*. 2021;10:2270.
10. Zhao L, Chen XJ, Zhu J, et al. Lanosterol reverses protein aggregation in cataracts. *Nature*. 2015;523:607–611.
11. Shah M, Cabrera-Ghayouri S, Christie LA, Held KS, Viswanath V. Translational preclinical pharmacologic disease models for ophthalmic drug development. *Pharm Res*. 2019;36:58.
12. Chhetri J, Jacobson G, Gueven N. Zebrafish—on the move towards ophthalmological research. *Eye (Lond)*. 2014;28:367–380.
13. Goishi K, Shimizu A, Najarro G, et al. AlphaA-crystallin expression prevents gamma-crystallin insolubility and cataract formation in the zebrafish cloche mutant lens. *Development*. 2006;133:2585–2593.
14. Li XQ, Cai HC, Zhou SY, et al. A novel mutation impairing the tertiary structure and stability of gammaC-crystallin (CRYGC) leads to cataract formation in humans and zebrafish lens. *Hum Mutat*. 2012;33:391–401.
15. Chen CC, Yeh LK, Liu CY, et al. Morphological differences between the trabecular meshworks of zebrafish and mammals. *Curr Eye Res*. 2008;33:59–72.
16. Yeh LK, Liu CY, Kao WW, et al. Knockdown of zebrafish lumican gene (zlum) causes scleral thinning and increased size of scleral coats. *J Biol Chem*. 2010;285:28141–28155.
17. Kuo YK, Chen YT, Chen HM, et al. Efficacy of myopia control and distribution of corneal epithelial thickness in children treated with orthokeratology assessed using optical coherence tomography. *J Pers Med*. 2022;12:278.
18. Liu CF, Chen SC, Chen KJ, et al. Higher HbA1c may reduce axial length elongation in myopic children: a comparison cohort study. *Acta Diabetol*. 2021;58:779–786.
19. Chen YH, Wang YH, Chang MY, et al. Multiple upstream modules regulate zebrafish myf5 expression. *BMC Dev Biol*. 2007;7:1.
20. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995;203:253–310.
21. Collymore C, Tolwani A, Lieggi C, Rasmussen S. Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). *J Am Assoc Lab Anim Sci*. 2014;53:198–203.
22. Nordgreen J, Tahamtani FM, Janczak AM, Horsberg TE. Behavioural effects of the commonly used fish anaesthetic tricaine methanesulfonate (MS-222) on zebrafish (Danio rerio) and its relevance for the acetic acid pain test. *PloS One*. 2014;9:e92116.
23. Andley UP, Hamilton PD, Ravi N, Weihl CC. A knock-in mouse model for the R120G mutation of alphaB-crystallin recapitulates human hereditary myopathy and cataracts. *PLoS One*. 2011;6:e17671.
24. Falavarjani KG, Nguyen QD. Adverse events and complications associated with intravitreal injection of anti-VEGF agents: A review of literature. *Eye (Lond)*. 2013;27(7):787–794, doi:10.1038/eye.2013.107.
25. Miao L, Nielsen M, Thewalt J, et al. From lanosterol to cholesterol: structural evolution and differential effects on lipid bilayers. *Biophys J*. 2002;82:1429–1444.
26. Prausnitz MR, Noonan JS. Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye. *J Pharm Sci*. 1998;87:1479–1488.
27. Cal K, Centkowska K. Use of cyclodextrins in topical formulations: practical aspects. *Eur J Pharm Biopharm*. 2008;68:467–478.
28. Moiseev RV, Morrison PWJ, Steele F, Khutoryanskiy VV. Penetration enhancers in ocular drug delivery. *Pharmaceutics*. 2019;11:321.
29. Loftsson T, Stefansson E. Cyclodextrins in eye drop formulations: enhanced topical delivery of corticosteroids to the eye. *Acta Ophthalmol Scand*. 2002;80:144–150.
30. Mokalled MH, Patra C, Dickson AL, Endo T, Stainier DY, Poss KD. Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. *Science*. 2016;354:630–634.
31. Rao MB, Didiano D, Patton JG. Neurotransmitter-regulated regeneration in the zebrafish retina. *Stem Cell Reports*. 2017;8:831–842.