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# Lens aquaporins function as peroxiporins to facilitate membrane transport of hydrogen peroxide

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## Abstract

High levels of reactive oxygen species such as hydrogen peroxide ($H_2O_2$) cause oxidative stress in the lens and lead to cataractogenesis. The present investigation was undertaken to find out whether the mammalian lens aquaporins (AQPs) 0, 1, and 5 perform $H_2O_2$ transport across the plasma membrane to reduce oxidative stress. Our *in vitro* cell culture and *ex vivo* lens experiments demonstrated that in addition to the established water transport role, mouse AQP0, AQP1 and AQP5 facilitate transmembrane $H_2O_2$ transport and function as peroxiporins. Human lens epithelial cells expressing AQP1, AQP5 and AQP8, when treated with 50μM $HgCl_2$ water channel inhibitor showed a significant reduction in $H_2O_2$ transport. Data obtained from the experiments involving $H_2O_2$-degrading enzyme glutathione peroxidase 1 (GPX1) knockout lenses showed $H_2O_2$ accumulation suggesting $H_2O_2$ transport level by AQPs in the lens is regulated by GPX1. Under hyperglycemic conditions, there was an increased loss of transparency, and enhanced production and retention of $H_2O_2$ in AQP5$^{-/-}$ lenses compared to similarly-treated WT lenses. Overall, the results show that lens AQPs function as peroxiporins and cooperate with GPX1 to maintain lens $H_2O_2$ homeostasis to prevent oxidative stress, highlighting AQPs and GPX1 as promising therapeutic drug targets to delay/treat/prevent age-related lens cataracts.

### Keywords

Lens AQP0; AQP1; AQP5; AQP8; Peroxiporin; $H_2O_2$

## 1. Introduction

Aquaporins (AQPs) are transmembrane proteins that are assembled in a homotetrameric manner in the plasma membrane. Each monomer functions as a channel that facilitates the influx and efflux of water. In mammals, thirteen AQPs (AQP0-AQP12) are expressed in various tissues. The ocular lens, which has a monolayer of anterior epithelial cells and

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Declaration of competing interests

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multiple layers of fiber cells, expresses three AQPs, namely, AQP0, AQP1 and AQP5. These AQPs are classified as classical AQPs, permeable selectively to water. AQP1 and AQP5 are expressed in the epithelial cells, and AQP0 and AQP5 are expressed in the fiber cells. The avascular lens generates a circulating flux of ions that cause water to flow through AQPs. This microcirculation [1,2] carries anti-oxidants, glucose and other nutrients into the lens and removes metabolic byproducts. Thus, AQPs play a major role in the lens microcirculation, transparency and homeostasis [3–14].

In the lens, AQPs play diverse roles such as volume regulation [1–3], cell-to-cell adhesion [6,15], and establishment of biomechanics (16) and refractive index gradient (7). Recent studies report that some mammalian AQPs, such as AQP3, AQP8, and AQP9 transport hydrogen peroxide ($H_2O_2$) across the membrane and function as peroxiporins [17–21]. $H_2O_2$ is a major reactive oxygen species (ROS) that are produced in various mammalian systems due to environmental radiation, aerobic metabolism and endogenous biochemical processes. Accumulation of $H_2O_2$ oxidant causes oxidative stress and leads to cataractogenesis [22–24]. Even though the underlying mechanism by which AQPs permeate $H_2O_2$ remains unclear, structural analyses favor the notion that the classical AQPs should be able to permeate $H_2O_2$ [19,20,24] owing to the structural similarities of $H_2O$ and $H_2O_2$ molecules.

The present study was undertaken to determine whether the lens AQPs, AQP0, AQP1, and AQP5 permeate $H_2O_2$ bidirectionally across the plasma membrane. We used MDCK cells stably expressing mouse AQP0, AQP1, AQP5 and human lens epithelial cells to determine the role of the specific AQPs in $H_2O_2$ transport. Lenses of WT and AQP knockout mouse models were also tested *ex vivo* for $H_2O_2$ transport and hyperglycemia-induced $H_2O_2$ production and transport.

## 2. Materials and methods

### 2.1. Mice

The wild type (WT), and knockout mouse models used in this investigation were in the C57BL/6J strain. AQP0 original knockout (in a mixed strain) was from Dr. Shiels (Washington University, MO) and we strain-transferred to C57BL/6J [8]. $AQP1^{-/-}$ was originally developed by Dr. Verkman Lab [25]. AQP5 knockout was from Dr. Menon Lab. (University of Cincinnati, OH;[26]). Glutathione peroxidase 1 (GPX1) knockout was developed by Dr. Ho Lab. (Wayne State University, MI; [27–28]). For animal procedures, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health's (NIH; Bethesda, MD, USA) "Guide for the Care and Use of Laboratory Animals" and protocols approved by Stony Brook University Animal Care and Use Committee, were followed.

### 2.2. Western blotting and immunofluorescence analyses

Previously established MDCK cells that stably-express mouse WT AQP0, AQP1 or AQP5 [6,29,30] were used for both analyses. HLE lysates from ScienCell Research Laboratories (Carlsbad, CA) were also used for Western blotting. Details are given in the Supplementary Section as Appendix I).

### 2.3. In vitro analysis of $H_2O_2$ transport by lens AQPs

**2.3.1. Using a cell culture expression model—**MDCK cells stably-expressing mouse WT AQP0, AQP1 or AQP5 were cultured in 24-well plates in serum-free medium 199 (Gibco). Quantitative $H_2O_2$ assay was done using the $H_2O_2$-specific AbGreen indicator ($H_2O_2$ Assay Kit; Abcam, Cambridge, MA), according to the manufacturer's instructions. The cell-permeable AbGreen reacts with $H_2O_2$ and produces a green fluorescence. The AbGreen probe was added to MDCK control (parental) cells, and AQP0, AQP1 or AQP5 stably-expressing cells, incubated for 30 min and washed three times 5 min each in 1XPBS. AbGreen-loaded cells were exposed to 10 μM $H_2O_2$ and imaged simultaneously for 15 min using a Zeiss confocal epifluorescent microscope with a FITC filter. Fluorescence intensity was quantified using the SigmaScan Pro, Version 5 (Systat Software, Inc., San Jose, CA). $H_2O_2$ transported through each AQP was calculated by subtracting the background fluorescence value for MDCK control cells from the fluorescence intensity value of AQP0, AQP1 or AQP5-expressing cells.

**2.3.2. Using human lens epithelial (HLE) cells—**Two groups of HLE cells (HLEpiC, ScienCell Research Laboratories, Carlsbad, CA) were cultured in 8-well plates in triplicates using ScienCell epithelial medium. At ~70% confluence, experimental cells were treated with 50 μM mercuric chloride ($HgCl_2$) for 30 min to inhibit $H_2O_2$ uptake by AQPs. $H_2O_2$ assay was done as described above (section 2.3.1). HLE cells not treated with $HgCl_2$ served as control. Cells were imaged and AbGreen fluorescence due to $H_2O_2$ uptake was quantified as above.

### 2.4. Ex vivo investigation of $H_2O_2$ transport by mouse lens AQPs

**2.4.1 Fluorometric Assay using WT and AQP0$^{+/-}$ or WT and AQP5$^{-/-}$ mouse lenses, and AbGreen probe—***Ex vivo* quantitative measurement of $H_2O_2$ in 1-month-old lenses of WT and AQP0$^{+/-}$, and 4-month old lenses of WT and AQP5$^{-/-}$ mice was done using the $H_2O_2$ Assay Kit (Abcam). WT, AQP0$^{+/-}$ and AQP5$^{-/-}$ lenses were dissected out gently, placed on a BSA-coated culture dish containing lens culture medium (in mM: NaCl 150, KCl 4.7, MgCl2 1, glucose 5, HEPES 5, pH 7.4), incubated at 37°C for 24 hours and exposed to AbGreen probe for 1 hour. The lenses were washed three times 5 min each using lens culture medium to remove the probe in the extracellular spaces of the lens. Exposure of the lenses to 50μM $H_2O_2$ and fluorescence intensity imaging were done simultaneously as described in section 2.3.1; fluorescence intensity was quantified using the SigmaScan Pro software. $H_2O_2$ uptake was calculated using the pixel brightness intensity, which is proportional to $H_2O_2$-specific AbGreen uptake.

**2.4.2. Colorimetric Assay using WT, AQP0$^{+/-}$, AQP1$^{-/-}$, AQP5$^{-/-}$ and GPX1$^{-/-}$ mouse lenses, and OxiRed probe—**Three-month-old transparent lenses of WT, AQP0$^{+/-}$, AQP1$^{-/-}$ AQP5$^{-/-}$ and GPX1$^{-/-}$ were used for experiments using the OxiRed probe as given in the Supplementary section, Appendix I.

### 2.5. Effect of Hyperglycemia in AQP5$^{-/-}$ mouse lens transparency and $H_2O_2$ transport

The hyperglycemic effect on WT and AQP5$^{-/-}$ mouse lenses was tested as described previously with modifications [31]. $H_2O_2$ was quantified by means of the Fluorometric

Assay using the AbGreen probe as described on section 2.3.1. Details are provided in the Supplementary section as Appendix I.

### 2.6. Statistical analysis

Statistical analysis was performed using the two-tailed Student's t-test.

## 3. Results and Discussion

We sought to find out whether lens AQP0, AQP1 and AQP5 permeate $H_2O_2$ in the cultured cells *in vitro*. As a first step, MDCK cells stably-expressing AQP0, AQP1 or AQP5 [6,29–31] were immunostained with the corresponding antibody to verify the expression of the respective protein; Figure 1 shows the expression AQP0 (A), AQP1 (B) and AQP5 (C). To investigate $H_2O_2$ transport by these AQPs, the MDCK cells expressing AQP0, AQP1 or AQP5 were exposed to AbGreen for 30 minutes. The AbGreen-loaded cells were exposed to 10 μM $H_2O_2$ and instantaneously imaged for 15 minutes to record the progression in the green fluorescence due to $H_2O_2$ uptake (Fig. 1D). Fluorescence intensity was quantified and expressed in pixel brightness intensity (Fig. 1E) which is proportional to the $H_2O_2$ transport into the cells. Compared to the MDCK control cells, the fluorescence intensity values for AQP0, AQP1 or AQP5-expressing cells were higher indicating the presence of significantly ($P<0.01$) more intracellular $H_2O_2$ (Fig. 1E). This experiment demonstrates that each of these AQPs can transport $H_2O_2$ *in vitro*; the transport efficiency can be represented as AQP5>AQP1>AQP0.

Next, we tested *in vitro* $H_2O_2$ transport by the AQPs expressed in the human lens epithelial (HLE) cells. The expression of the different AQPs in these cells was explored by Western blotting using the appropriate antibodies. We found that in addition to the already reported AQP1 and AQP5, HLE cells also express AQP8 (Fig. 2A, lane 3). $H_2O_2$ transport by AQPs in HLE cells was verified by conducting an inhibition experiment. Experimental HLE cells were treated with 50μM $HgCl_2$ for 30 minutes, to inhibit $H_2O_2$ transport; Figure 2B shows inhibition of $H_2O_2$ in $HgCl_2$-treated cells (right panel) compared to untreated control cells (left panel). Quantification of the fluorescence intensity data (Fig. 2C) showed that the reduction in $H_2O_2$ transport was statistically significant ($P< 0.01$).

*Ex vivo* experiments for $H_2O_2$ transport were conducted in one-month-old WT and $AQP0^{+/-}$, and 4-month-old WT and $AQP5^{-/-}$ mouse lenses using the AbGreen probe. Figure 3A shows higher levels of intracellular $H_2O_2$ in the WT than in the other lenses. Quantification of $H_2O_2$ uptake showed that loss of 50% AQP0 or 100% AQP5 significantly ($P< 0.001$) reduced $H_2O_2$ transport into the lens by ~44 and ~50% respectively (Fig. 3B). *Ex vivo* quantification studies were pursued using another probe OxiRed combined with a Colorimetric method. The experiments were conducted in 3-month-old $AQP0^{+/-}$, $AQP1^{-/-}$, $AQP5^{-/-}$ and $GPX1^{-/-}$ mouse lenses. GPX1 is an $H_2O_2$ scavenging enzyme; lenses lacking GPX1 were tested to find out the role of GPX1 in lenticular $H_2O_2$ homeostasis. Monitoring and quantifying the $H_2O_2$ transport for 30 minutes showed that compared to the WT lenses, there was significant ($P<0.01$) reduction in the transport in the lenses of the mouse models (Fig. 3C). The WT lenses, with AQP0, AQP1, AQP5 and GPX1, transported $H_2O_2$ at a rate of 90 ± 5 nM/s. Loss of 50% AQP0 reduced the rate to ~73 ± 5 nM/s. $AQP0^{-/-}$ lenses were

not included in the study because they develop cataracts even at embryonic stages. Knockout of epithelial cell AQP1, reduced the $H_2O_2$ transport to a rate of 76 ± 5 nM/s; loss of AQP5, which is present both in the lens epithelial and fiber cells and accounts for ~0.5% of the membrane protein in the lens, reduced the $H_2O_2$ transport to 67 ± 5 nM/s. Based on the data obtained from our experiments, the degree of *ex vivo* $H_2O_2$ transport can be represented as AQP5>AQP0>AQP1. However, based on the quantity of each type of AQP protein expressed in the lens, the extent of $H_2O_2$ transport could be AQP0>AQP5>AQP1. AQP0 contributes about 45% of the total membrane protein in the lens, justifying the possibility of being the most significant $H_2O_2$ transporter in the lens, a noncanonical role for a classical water pore. By functioning as peroxiporins, AQPs could reduce $H_2O_2$-induced oxidative damage to the lens. The $H_2O_2$ transported into the cells by AQPs are scavenged as necessary by GPX1 to maintain homeostasis. The loss of GPX1 reduced the $H_2O_2$ transport to 77 ± 4 nM/s (Fig. 3C). Absence of GPX1 causes accumulation of $H_2O_2$ in the lens, which eventually reaches an equilibrium with the extracellular $H_2O_2$ and prevents the further influx of $H_2O_2$ through AQPs. During aging, a reduction in GPX1 protein level or activity could cause $H_2O_2$ accumulation in the lens and lead to oxidative stress-induced cataractogenesis [28,32,33].

Hyperglycemia induces increased production of $H_2O_2$ in the mitochondria and leads to oxidative stress [34,35]. We tested hyperglycemia-induced intracellular $H_2O_2$ levels in the lenses of WT and AQP5$^{-/-}$ mouse lenses, which were cultured in 3 mM (normal, control) or 30 mM (hyperglycemic) glucose for 48 hours. At the end of the experiments, transparency was reduced in the lenses cultured under hyperglycemic conditions (Fig. 4A, right column). Lenses of AQP5$^{-/-}$ showed a significant decrease in transparency ($P< 0.01$) at high glucose compared to those of WT (Fig. 4B); in the Figure, the pixel brightness intensity is inversely proportional to transparency. WT and AQP5$^{-/-}$ lenses were exposed to AbGreen for one hour and imaged for the green fluorescence due to the level of $H_2O_2$ present. The intracellular $H_2O_2$ levels were quantified. Figure 4 panels C and D show that the high glucose in the culture medium causes an increase in the intracellular $H_2O_2$ levels; in panel D, the pixel brightness intensity is proportional to the $H_2O_2$ level. When the levels of $H_2O_2$ were compared, AQP5$^{-/-}$ lenses subjected to hyperglycemia showed significantly ($P<0.01$) higher $H_2O_2$ level than the WT control, the WT lenses exposed to the same hyperglycemic conditions or the untreated AQP5$^{-/-}$ lenses. In the WT, when there is a higher level of intracellula$H_2O_2$, AQP5-facilitated-efflux from the cells helps to maintain $H_2O_2$ balance and homeostasis, as there is no significant build-up of $H_2O_2$ (Fig. 4D). In AQP5$^{-/-}$ lenses exposed to high glucose, the AQP5-facilitated efflux route is not present and there is a significant build-up of $H_2O_2$ causing lens opacity, possibly due to oxidative damage.

$H_2O_2$ plays a paradoxical dual role. At low concentrations, $H_2O_2$ functions as a signaling molecule [36,37], while at high concentrations it leads to oxidative damage that alters cellular functions and causes apoptosis [38,39]. High concentrations of $H_2O_2$ are cytotoxic to a wide range of animal, plant and bacterial species depending upon the physiological state and duration of exposure of the tissue [22, 40–42]. In the lens, $H_2O_2$ is mainly produced in the mitochondria and endoplasmic reticulum of epithelial and peripheral fiber cells. Acute $H_2O_2$-induced oxidative stress in human lens epithelial cells showed significant up- and down-regulation involving 1171 genes [43]. High production of ROS during aging or a

significant decrease in the ROS scavenging ability of the lens, causes oxidative stress and leads to cataracts [44–48]. $H_2O_2$ concentration in the aqueous humor is ~30–70 μM; higher concentrations (>600 μM) are seen in certain cataract patients [47–49].

Maintaining a balance of $H_2O_2$ is critical for lens transparency since $H_2O_2$ in excess causes oxidative damage to lens proteins, lipids, and DNA that results in opacity and cataract [50]. The current investigation demonstrates that lens AQPs, AQP0, AQP1 and AQP5 can transport extracellular and intracellular $H_2O_2$ into and out of cells, respectively, with varying degrees of efficiency. *In vitro* expression of AQP0, AQP1 or AQP5 into MDCK cells increased the uptake of exogenous $H_2O_2$. Treatment with AQP-inhibitor mercury significantly reduced the uptake of $H_2O_2$ by HLE cells. Absence of AQP0 (partial), AQP1, AQP5 or GPX1 significantly inhibited the entry of $H_2O_2$ into the lens. Reduction in $H_2O_2$ transport by the reduction or loss of lens AQPs or GPX1 further suggests that lens AQPs and GPX1 coordinate in maintaining optimum levels of $H_2O_2$ to prevent oxidative damage to the long-lasting proteins in the lens; age-related loss or reduction of these proteins could lead to lens cataract. Hyperglycemic condition induced increased $H_2O_2$, and loss of AQP5 increased $H_2O_2$ accumulation. Our results support the involvement of lens AQPs in $H_2O_2$ transport in both mouse and human lens cells.

Among the three AQPs tested, the AQP5 channel appears to be the most efficient $H_2O_2$ transporter in the lens. AQP5 is expressed in both epithelial and fiber cells. Previous studies have shown that after the synthesis, AQP5 is stored in the cytoplasmic vesicles and upon demand, traffics to the plasma membrane. It is a regulatory AQP in the lens [30,51]. Therefore, AQP5 could be acting as the predominant regulator of $H_2O_2$ to prevent oxidative stress in the lens. Under normal conditions, AQP0 in the fiber cells and AQP1 in the epithelial cells function as $H_2O_2$ transporters for housekeeping; however, under stressful conditions, AQP5 could be trafficking to the membrane to reduce $H_2O_2$ accumulation.

In conclusion, our *in vitro* and *ex vivo* investigations demonstrate the noncanonical function of lens AQPs as $H_2O_2$ transporters/ peroxiporins. In coordination with GPX1, AQPs play a significant role in maintaining $H_2O_2$ homeostasis in the lens, thereby reducing $H_2O_2$-induced oxidative damage. Further studies involving lens AQPs and $H_2O_2$ detoxifying enzymes will help to understand their coordinated roles in modulating $H_2O_2$ transport and managing oxidative stress to prevent lens age-related cataracts.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- Mammalian lens aquaporins transport hydrogen peroxide
- AQP0, AQP1 and AQP5 function as peroxiporins
- Normal human lens epithelial cells express AQP1, AQP5 and AQP8
- Hyperglycemia enhances $H_2O_2$ production and accumulation in the lens
- Knockout of AQP5 causes significant increase in $H_2O_2$ accumulation in the lens

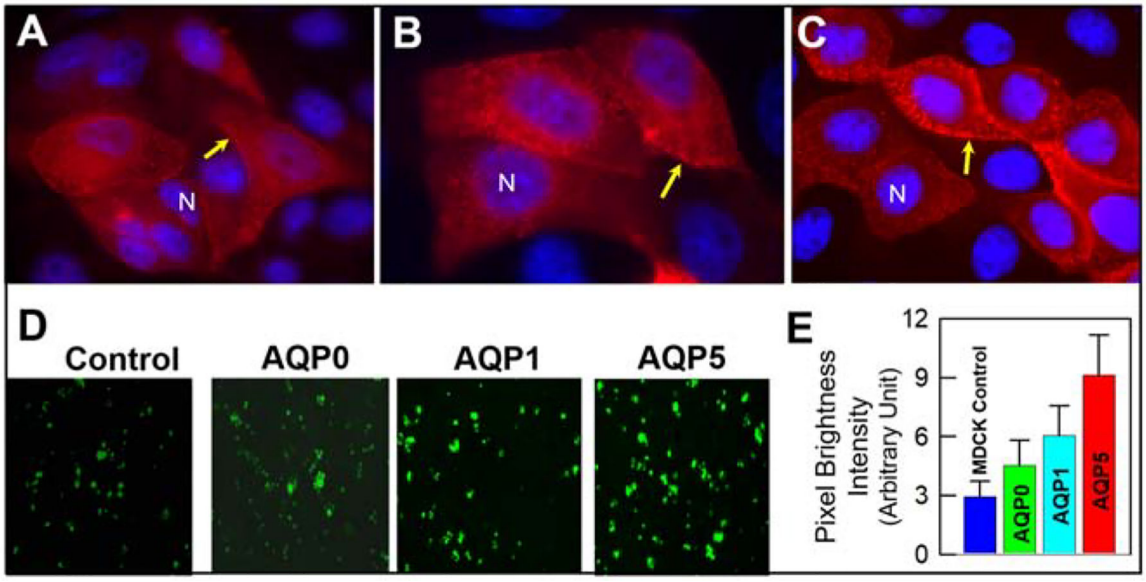


**Fig.1.**

Expression of lens aquaporins and $H_2O_2$ transport. MDCK cells expressing (**A**) AQP0, (**B**) AQP1 or (**C**) AQP5 immunostained with anti-AQP0, anti-AQP1 and anti-AQP5. Arrows - Texas-Red tagged secondary antibody binding to the respective antibody. N- Nucleus. **D**. AbGreen fluorescence due to $H_2O_2$ transport by control MDCK cells or MDCK cells expressing transfected AQP0, AQP1 or AQP5. **E**. Quantification of $H_2O_2$ levels. AQP-expressing cells transported $H_2O_2$ significantly more compared to the Control cells, $P< 0.05$.

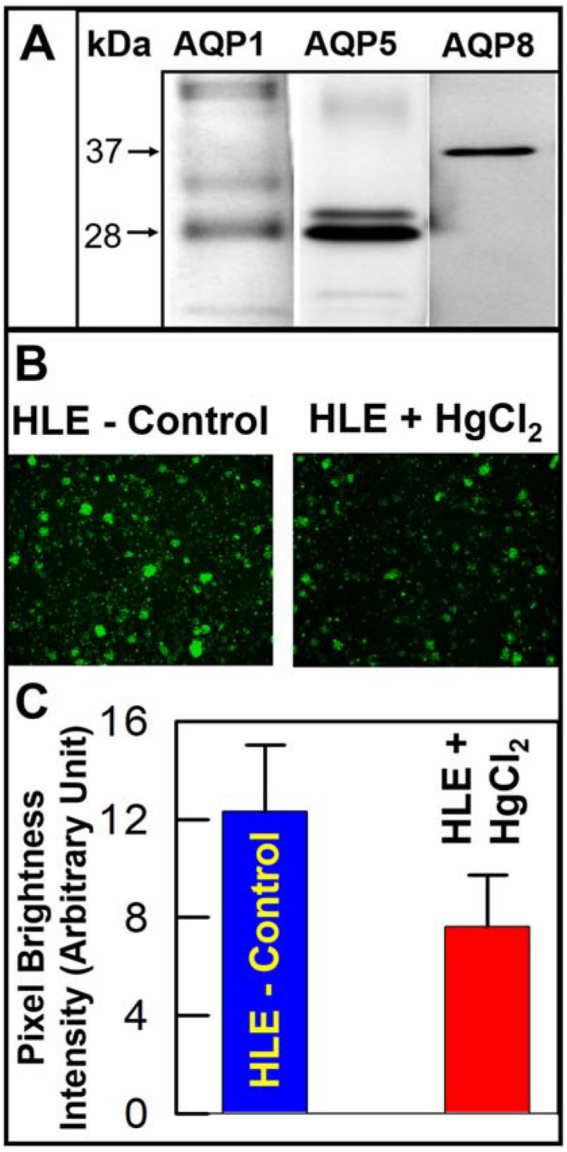


**Fig. 2.**

Expression of AQPs in human lens epithelial (HLE) cells and $H_2O_2$ transport. **A**. Western blotting showing AQP1, AQP5 and AQP8 expression in human HLE cells, using AQP-specific antibodies. AQP1 and AQP5 antibodies each bound to a ~28 kDa band. Antibody to AQP5 also bound to a slightly larger molecular size band, most likely a product of posttranslational modification. AQP8 antibody immunoreacted with a ~37 kDa protein. **B**. AbGreen fluorescence in HLE cells due to $H_2O_2$ transport by the AQPs expressed (left panel) and that in HLE cells treated with $HgCl_2$ to inhibit $H_2O_2$ transport (right panel). **C**. Quantification of $H_2O_2$ transport level in control HLE cells and those treated with $HgCl_2$.

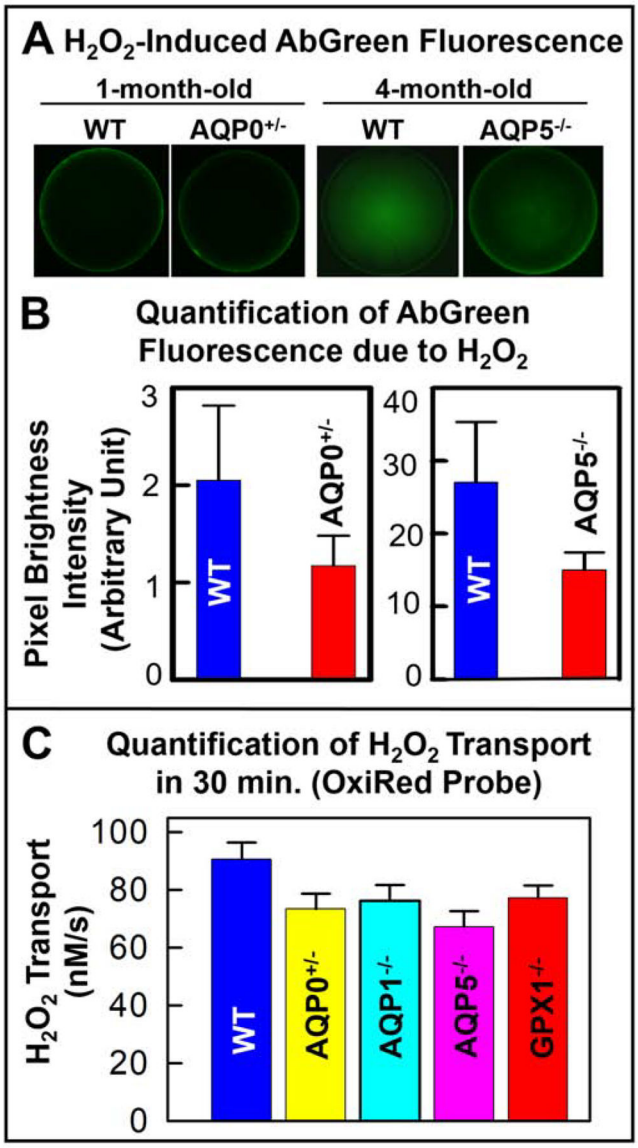


**Fig. 3.**

$H_2O_2$ transport in the lenses of mouse models. **A**. $H_2O_2$ uptake by 1-month-old lenses of WT and AQP0$^{+/-}$ or 4-month-old lenses of WT and AQP5$^{-/-}$ exposed to 50μM $H_2O_2$ for 30 min., using a Fluorometric assay; **B**. Quantification of the $H_2O_2$ levels. **C**. Quantification of $H_2O_2$ transport (in 30 min.) in 4-month-old lenses of WT, AQP0$^{+/-}$, AQP1$^{-/-}$, AQP5$^{-/-}$ and GPX1$^{-/-}$, using OxiRed probe and a Colorimetric Assay.

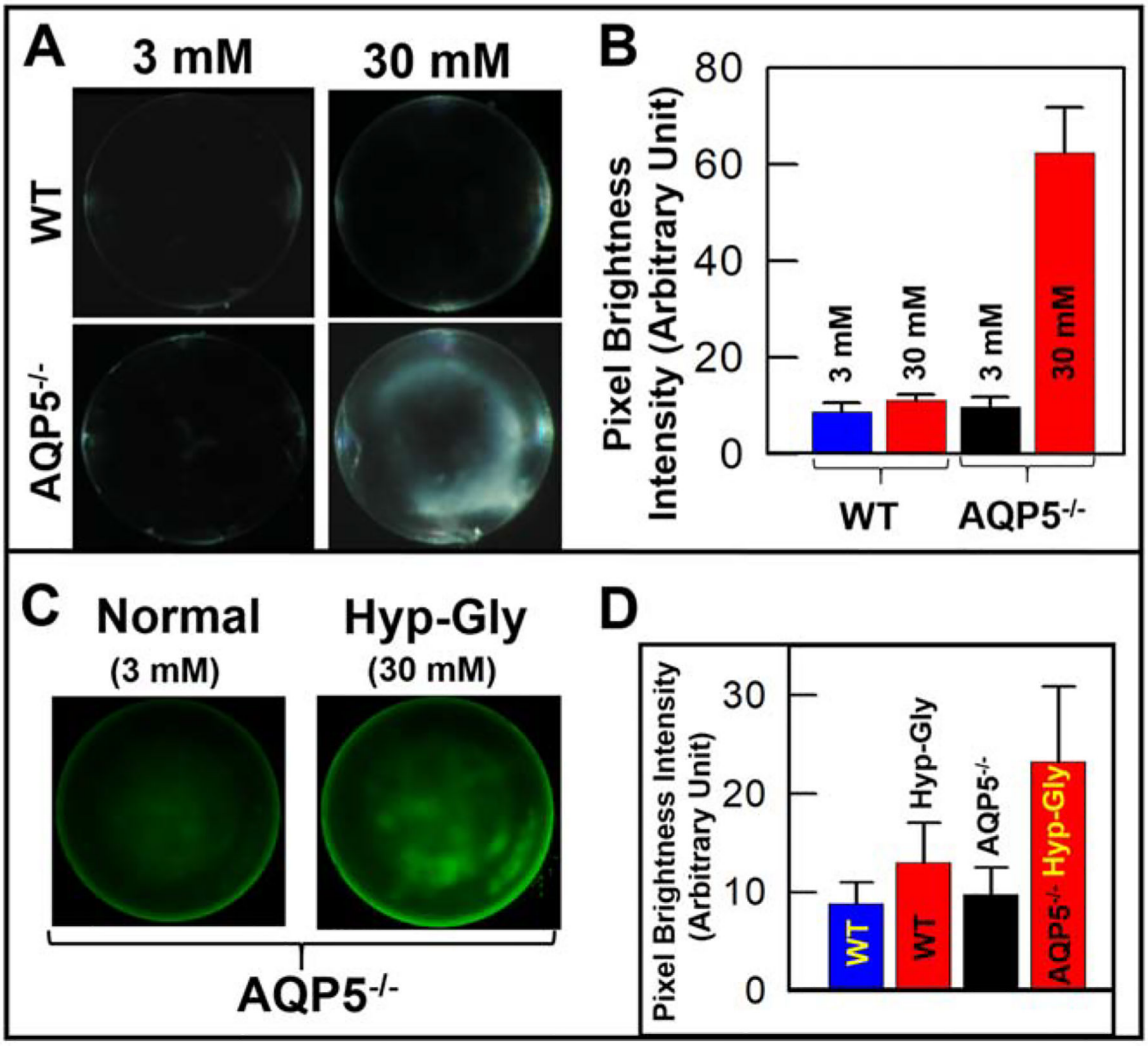


**Fig. 4.**

Effect of hyperglycemia on lens transparency. **A**. Lens transparency in 4-month- old WT and $AQP5^{-/-}$ mouse lenses incubated in normal (3mM glucose) or hyperglycemic (30mM glucose) culture medium for 48 hrs. **B**. Quantification of the lens transparency due to glucose treatment. Pixel brightness intensity is inversely proportional to lens transparency. **C**. AbGreen fluorescence due to $H_2O_2$ transport in 4-month-old $AQP5^{-/-}$ lenses incubated in normal glucose (3 mM) and hyperglycemic glucose (Hyp-Gly; 30 mM) conditions. WT lenses not shown. **D**. Quantification of $H_2O_2$ level in 4-month-old lenses of WT and $AQP5^{-/-}$ subjected to normal glucose (blue and black bars) and hyperglycemic (red bars; Hyp-Gly) conditions. Pixel brightness intensity is directly proportional to the $H_2O_2$ level.