

# Vitreous Humor Changes Expression of Iron-Handling Proteins in Lens Epithelial Cells

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**PURPOSE.** In humans, vitrectomy is associated with development of nuclear cataracts. Iron catalyzes free radical formation causing oxidative damage, which is implicated in cataract formation. This study was designed to determine if vitreous humor, which can initiate differentiation of lens epithelial cells, would have an effect on iron-handling proteins.

**METHODS.** Cultured canine lens epithelial cells were treated with collected canine vitreous humor. Lysates of treated and control cells were separated by SDS-PAGE. Ferritin H- and L-chains, transferrin receptor 1, and aquaporin 0 were immunodetected and quantitated with specific antibodies. Morphologic changes in treated cells were assessed.

**RESULTS.** Treatment of lens epithelial cells with a 33% (vol/vol) solution of vitreous humor changed the morphology of lens cells and induced expression of aquaporin 0, a marker of fiber cell differentiation that was undetectable in control cells. Treatment did not modify the size of iron-handling proteins but significantly increased content of ferritin from 2.9- to 8.8-fold over control and decreased levels of transferrin receptor by 37% to 59%.

**CONCLUSIONS.** Vitreous humor may significantly limit iron uptake by transferrin/transferrin receptor pathway, and by increasing ferritin levels could profoundly increase the iron-storage capacity of ferritin in lens cells. Vitreous humor may play a significant protective role against iron-catalyzed oxidative damage of lens epithelial cells and therefore in the formation of cataracts.

Keywords: iron, oxidative damage, ferritin, transferrin receptor, vitreous humor

The vitreous humor fills the space between the posterior surface of the lens and the inner retina. The viscosity and transparency of vitreous humor decrease with age.<sup>1</sup> These changes in vitreous humor are associated with pathological conditions such as posterior vitreous detachment, macular hole formation, and retinal detachment,<sup>2</sup> as well as the formation of nuclear cataracts.<sup>3</sup> The formation of age-related cataracts is not fully understood. However, it is generally agreed that oxidative damage to proteins and lipids significantly contributes to this pathology.<sup>4,5</sup> Fast-progressing lens nuclear opacification, morphologically and histologically similar to that of age-related cataract, is also observed after vitrectomy.<sup>6</sup> In humans, within 2 years after vitrectomy, most patients develop nuclear cataracts.<sup>7</sup> Recent data indicate that vitrectomy exposes the lens, which functions in a low-oxygen environment, to a higher level of oxygen.<sup>8-10</sup> This may increase the possibility of significant oxidative damage to the lens.

Iron is implicated in cataract development because of its ability to catalyze free radical formation.<sup>11</sup> Cells control iron availability by strictly regulating its uptake and safe storage in ferritin, which is the main iron-storage protein. Ferritin consists of 24 subunits of two types, heavy (H) and light (L), that are assembled in highly regulated cell type-specific ratios.<sup>12-14</sup> These stable, multi-subunit protein complexes have high binding capacity for iron (4500 iron atoms per ferritin molecule).<sup>15</sup> Iron is delivered to cells by transferrin (Tf), the plasma iron binding transport protein, via endocytosis of transferrin receptor 1 (TfR1). Translation of ferritin chains and TfR1 are regulated in an opposite manner by cytosolic levels of

iron.<sup>16</sup> Higher concentrations of cytosolic iron increase translation of ferritin chains and decrease translation of TfR. Lower concentrations of iron have the opposite effect.

Vitreous humor has a high concentration of Tf, which is synthesized in eye tissues and constitutes 30% to 40% of total vitreal proteins.<sup>17-20</sup> Vitreous humors of various species have low iron levels compared to plasma (2.4%-4.5% of plasma levels).<sup>21</sup> Under physiological conditions, low iron content and relatively high levels of unsaturated iron-binding capacity of Tf limit non-Tf-bound iron (NTBI) uptake by surrounding tissues. However, under pathological conditions such as vitreous hemorrhage, excess iron saturates vitreous Tf, creating a pool of NTBI, which could then be taken up by cells and catalyze free radical formation causing oxidative damage to the lens. Higher iron content was found in vitreous humor of humans with various vitreoretinal diseases,<sup>22</sup> as compared to normal vitreous humor.

The lens grows continuously throughout life as a result of division and subsequent differentiation of lens epithelial cells (LEC) into fiber cells. This process is initiated by fibroblast growth factor (FGF-2),<sup>23</sup> which is present in vitreous. As our previous studies documented, ferritin chains identified in human and canine fiber cells are significantly modified compared to those found in LEC<sup>24</sup> and most likely do not form functional ferritin capable of storing iron. Long-lived cells generate and accumulate, posttranslationally, modified proteins as they age. However, changes can also take place during cell differentiation. In this study we initiated differentiation of cultured LEC by treating them with vitreous humor in order to

examine if structural modification and expression of ferritin chains take place during this process and to evaluate the changes in expression of TfR1, which is responsible for the most extensively characterized route of iron uptake via the Tf-TfR1 pathway. Changes in expression of iron-handling proteins caused by vitreous humor could significantly influence iron metabolism in LEC by modifying iron uptake and storage.

## METHODS

### LEC Cultures

Eyes were obtained from mixed-breed dogs, approximately 6 months to 11 years old, that had been euthanized at the Johnston County, North Carolina Animal Shelter. The entire anterior segment of the capsule, which contained adherent epithelial cells, was removed and placed in a tissue culture dish. Lens epithelial cells that grew out from capsule were dispersed with trypsin and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Rockville, MD, USA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% antibiotic-antimycotic (Invitrogen). After reaching confluency, LEC from two or three capsules were combined and plated in 6-well plates at a density of 200,000 cells/well. Fully confluent LEC were used in the experiments.

### Collection of Aqueous and Vitreous Humors

Aqueous humor was obtained by inserting a 28-gauge needle through the cornea of enucleated dog eyes and withdrawing 200 to 400  $\mu$ L sample into a 0.5-mL syringe. Subsequently, the posterior side of the eye was cut out with scissors, exposing the eye cup filled with vitreous humor. Vitreous was carefully removed using a pipette with a large orifice tip and a pair of small sharp scissors to break gel components. Collected samples were stored at  $-20^{\circ}\text{C}$ .

### Treatments of LEC

Medium from confluent LEC was supplemented with 33% (vol/vol) vitreous or aqueous humor, and LEC were cultured for 24 or 96 hours. Medium from 96-hour cultured LEC was replaced after 2 days with fresh vitreous containing DMEM. Cells treated just with DMEM were used as controls.

### Fractionation of Vitreous Humor

Vitreous humor was collected from both eyes of two dogs (ages 6 months and 3–5 years) and 3 mL of combined vitreous were filtered through glass wool placed inside of a 5-mL syringe. Three 900- $\mu$ L samples of filtered vitreous were processed as follows. One sample was precipitated with an equal volume of acetone for 30 minutes on ice and spun down. The precipitate was dissolved in complete DMEM. The second sample was boiled for 15 minutes. The third was filtered through Microcon 10 filter (EMD Millipore, Billerica, MA, USA), and both filtrate and retentate were collected. Separate wells of LEC were treated for 24 hours with vitreous humor that was not glass wool filtered, with glass-filtered vitreous, and with all differentially processed vitreous fractions. Each treatment was equivalent to 33% (vol/vol) of nonprocessed vitreous in complete DMEM containing 10% FBS.

### Immunodetection of Ferritin Chains and TfR1

The LEC were lysed with 10 mM Tris/HCl buffer, pH 7.4, containing 2% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA). The

protein concentrations of lysates were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples (35–50  $\mu$ g) were separated by 8% or 12% SDS-PAGE under reducing conditions, using a Tris/Tricine buffer system. Proteins were semidry transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare, Pittsburg, PA, USA) using the transfer Trans-Blot SD Cell (Bio-Rad Laboratories, Richmond, CA, USA) system. Purified, canine heart ferritin was used as a standard for ferritin chains,<sup>25</sup> and purified human placenta TfR1 (Alpha Diagnostics, San Antonio, TX, USA) was used as a standard for canine TfR1. The ferritin chains were immunodetected with canine chain-specific custom-made antibodies (Open Biosystems, Huntsville, AL, USA) produced in rabbits immunized with peptides corresponding to H- and L-chain canine ferritin-specific amino acid sequences and TrueBlot HRP (horseradish peroxidase) anti-rabbit IgG antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA) as described in an earlier publication.<sup>13</sup> Transferrin receptor 1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies (Invitrogen) and HRP-labeled goat anti-mouse IgG antibodies (Millipore, Temecula, CA, USA). All blots were reprobated with HRP anti-actin as a loading control (Santa Cruz Biotechnology, Dallas, TX, USA). Immunoreactivity was determined with an ECL Western blotting Analysis System (GE Healthcare). The images were digitized and evaluated with a ChemiDoc MP Imaging System (Bio-Rad Laboratories).

### Immunodetection of Aquaporin 0

Lens epithelial cells treated with vitreous humor for 96 hours in DMEM with 1% FBS and 1% antibiotic-antimycotic were lysed with 10 mM Tris/HCl buffer, pH 7.4, containing 2% SDS and protease inhibitor cocktail (Sigma-Aldrich Corp.). Samples of control- and vitreous-treated cell lysates containing 50  $\mu$ g proteins were separated by 12% SDS-PAGE, semidry transferred to nitrocellulose membranes (Hydrobond ECL; GE Healthcare), and probed with rabbit anti-aquaporin 0 polyclonal antibodies (Alpha Diagnostics) and subsequently with TrueBlot HRP anti-rabbit IgG antibodies (Rockland Immunochemicals). A 50- $\mu$ g protein sample of lens fiber cells sonicated in the lysis buffer was used as a positive control. Immunoreactivity was determined with an ECL Western blotting Analysis System (GE Healthcare).

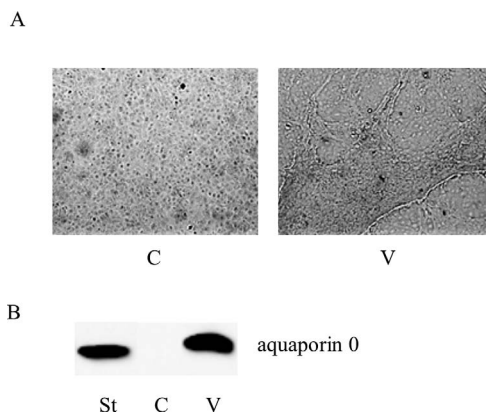
## RESULTS

### Morphologic Changes in LEC and Immunodetection of Aquaporin, a Protein Marker of Differentiation, After Vitreous Humor Treatment

Treatment of LEC for 96 hours with a 33% (vol/vol) solution of vitreous humor in DMEM containing 1% serum significantly changed LEC morphology (Fig. 1A). Some of the treated cells became elongated and accumulated in scattered areas across the culture plate while nontreated, control LEC remained in a uniform monolayer of cubical cells. Western blotting analysis of aquaporin 0 confirmed that vitreous treatment induced differentiation of LEC into cells expressing the marker of fiber cell differentiation, which was undetectable in control LEC (Fig. 1B).

### Immunodetection of Ferritin L-Chain in Control- and Vitreous-Treated LEC

We previously determined that ferritin chains in lens fiber cells are extensively altered in size and charge in comparison to that

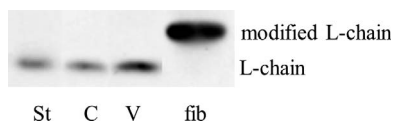


**FIGURE 1.** (A) Phase contrast image of control (C) and vitreous-treated LEC (V). LEC were treated for 96 hours with DMEM containing 1% FBS and 33% of vitreous humor. Control LEC were grown in DMEM containing 1% FBS without vitreous humor. (B) Western blot analysis of aquaporin 0 expression in treated (V) and control LEC (C). Lens lysates containing 50  $\mu$ g proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and probed with rabbit anti-aquaporin 0 polyclonal antibodies. Canine lens was dissected and the section containing lens fibers was sonicated. Fifty micrograms of protein sample of sonicated lens fiber cells was used as a positive control (St). Blot was evaluated with ChemiDoc MP Imaging System.

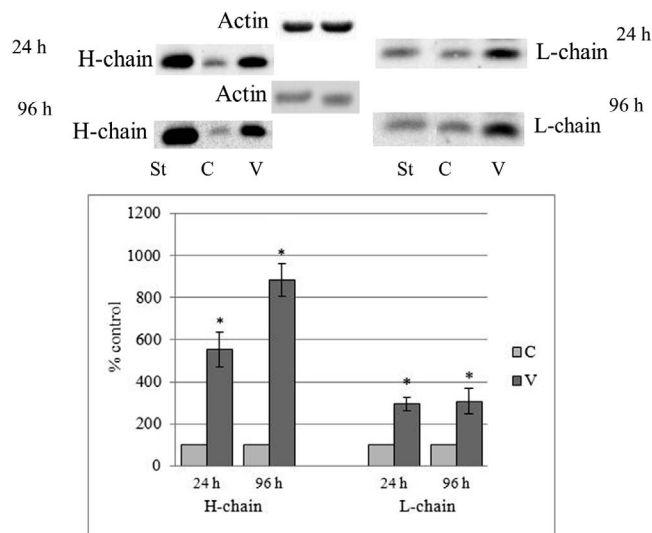
of undifferentiated LEC.<sup>24</sup> In this study, we immunoprecipitated ferritin L-chains from lysates of control- and vitreous-treated LEC expressing aquaporin 0 in order to determine whether these changes take place during initiation of differentiation of LEC into fibers. Ferritin L-chains from lysates of control- and vitreous-treated LEC had the same gel mobility (C, V), which was higher than that of ferritin L-chains from samples of sonicated canine lens fiber cells (fib) (Fig. 2). This suggests that modification of ferritin L-chain is not associated with initiation of differentiation of LEC into lens fibers.

### Quantitation of Ferritin H- and L-Chains in Control- and Vitreous-Treated LEC

Treatment of LEC with vitreous humor did not change the mobility characteristics of L-chains (Fig. 2), but it visibly elevated L-chain content in treated LEC. We quantitated both ferritin L- and H-chains in lysates of control- and vitreous-treated LEC by Western blotting using chain-specific antibodies. Vitreous humor treatment increased the content of ferritin H-chains from 5.5-fold after 24 hours to 8.8-fold after 96 hours (Fig. 3). Increased levels of ferritin L-chains were much less pronounced, 2.9-fold after 24 hours, and did not progress over time (Fig. 3).



**FIGURE 2.** Western blot analysis of ferritin L-chain in the lysates of control LEC (C), vitreous-treated LEC (V), and sonicated lens fiber cells (Fib). Protein samples, 35 to 50  $\mu$ g, were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system. Proteins were transferred to nitrocellulose membranes and probed with antibodies specific to ferritin L-chain. Purified canine heart ferritin was used as standard (St).



**FIGURE 3.** Western blot analysis of ferritin H- and L-chains in the lysates of control (C) and vitreous-treated LEC (V). Confluent LEC were cultured in DMEM with 10% FBS with (V) or without (C) 33% (vol/vol) of vitreous humor for 24 or 96 hours. LEC lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified canine heart ferritin was used as standard (St) for ferritin chains. Blot was reprobbed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The significance of differences was determined by paired *t*-test. Data represent the mean  $\pm$  SEM; *n* = 11; statistically different from corresponding C, \**P* < 0.05.

### Quantitation of TfR1 in Control- and Vitreous-Treated LEC

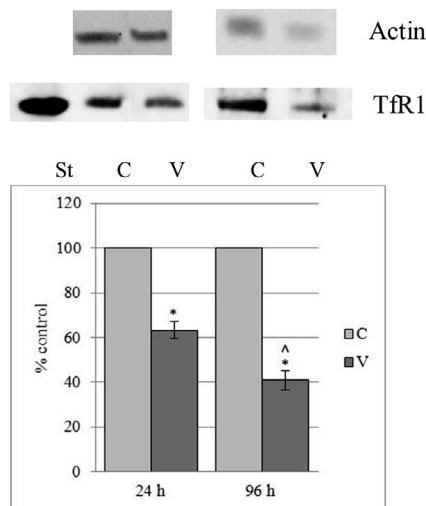
Expression of iron-handling proteins such as ferritin chains and TfR1 is regulated translationally by intracellular iron. Elevated cytosolic iron activates expression of ferritin H- and L-chains; it had an opposite effect on TfR1 expression. We quantitated TfR1 in the lysates of control- and vitreous-treated LEC to determine if vitreous treatment would show the same regulatory pattern. Western blotting analysis revealed a significant decrease of TfR1 after 24 and 96 hours of treatment with vitreous humor, characteristic of the decrease produced when cytosolic iron is increased (Fig. 4).

### Effects of Iron Depletion on Ferritin Chain and TfR1 Levels in Control- and Vitreous-Treated LEC

Depletion of iron by the iron chelator deferoxamine (DFO) abolished expression of both ferritin chain types in control- and vitreous-treated LEC (Fig. 5A). Iron chelation increased TfR1 levels in control LEC and had an opposite effect to that of vitreous treatment. Transferrin receptor 1 levels in vitreous- and DFO-treated LEC were higher than TfR1 levels in LEC treated only with vitreous (Fig. 5B).

### Effects of Cycloheximide on Expression of Ferritin Chains and TfR1 in Control- and Vitreous-Treated LEC

Iron is a key regulator of the translation of ferritin chains and TfR1. As our data indicated, vitreous humor required iron to exert its effects on expression of these proteins. We blocked translation with cycloheximide and analyzed, by Western blotting, changes in levels of ferritin chains and TfR1 in



**FIGURE 4.** Western blot analysis of TfR1 in control (C)- and vitreous-treated LEC (V). Confluent LEC were cultured in DMEM with 10% FBS with or without 33% (vol/vol) of vitreous humor for 24 or 96 hours. LEC lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified human placenta TfR1 was used as a standard (St) for canine TfR1. TfR1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies. Blot was reprobed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The significance of differences was determined by paired *t*-test. Data represent the mean  $\pm$  SEM; *n* = 9; statistically different from corresponding C, \**P* < 0.05; statistically different from corresponding V 24 hours, <sup>^</sup>*P* < 0.05.

control- and vitreous-treated LEC (Figs. 6A, 6B). Cycloheximide reduced ferritin chain levels by half in control LEC and completely obliterated stimulation of ferritin chain expression by vitreous treatment. Cycloheximide treatment did not have a statistically significant effect on TfR1 expression in control- and vitreous-treated LEC.

#### Effects of Actinomycin D on the Expression of Ferritin Chains and TfR1 in Control- and Vitreous-Treated LEC

As we have shown, the effects of vitreous humor on expression of iron-handling proteins were facilitated through an iron-regulated translational pathway. To examine the possibility of vitreous involvement in transcriptional regulation, we treated LEC with an inhibitor of transcription, actinomycin D, and measured the levels of ferritin chains and TfR1 in control- and vitreous-treated LEC by Western blotting (Figs. 7A, 7B). Surprisingly, actinomycin D stimulated expression of ferritin H- and L-chains in control LEC. The combination of actinomycin D and vitreous treatment had an additive stimulatory effect on expression of ferritin chains (Fig. 7A). Actinomycin D did not modify expression of TfR1 in control LEC, but completely obliterated the suppression of TfR1 caused by vitreous treatment of LEC (Fig. 7B).

#### Effects of Vitreous Humor Fractions on Levels of Ferritin Chains in LEC

To determine which fraction of vitreous humor had a stimulatory effect on expression of iron-handling proteins, we first removed aggregates of collagen fibrils and large molecules of hyaluronic acid by filtering through glass wool.

After testing the effects of the resulting filtrate (Vf) on iron-handling proteins, we determined that the effects of Vf did not differ from those of the nonfiltered vitreous sample (V) as examined by Western blot analysis of ferritin H- and L-chains (Fig. 8). Subsequently, we aliquoted filtered vitreous (Vf) into three samples of equal volume and processed each sample differently. The activity of each sample was tested by Western blot analysis of ferritin H- and L-chain levels. Filtering the Vf sample through a Microcon 10 filter yielded a filtrate (Vmf) that was significantly more active in elevating ferritin chains than the retentate (Vmr). The Vf sample acetone precipitate, dissolved subsequently in complete DMEM, had no effect on ferritin chain levels (Va). Boiling the Vf sample for 10 minutes (Vb) eliminated its stimulatory effect.

#### Effects of Vitreous Humor From Dogs of Different Age on the Expression of Ferritin Chains and TfR1

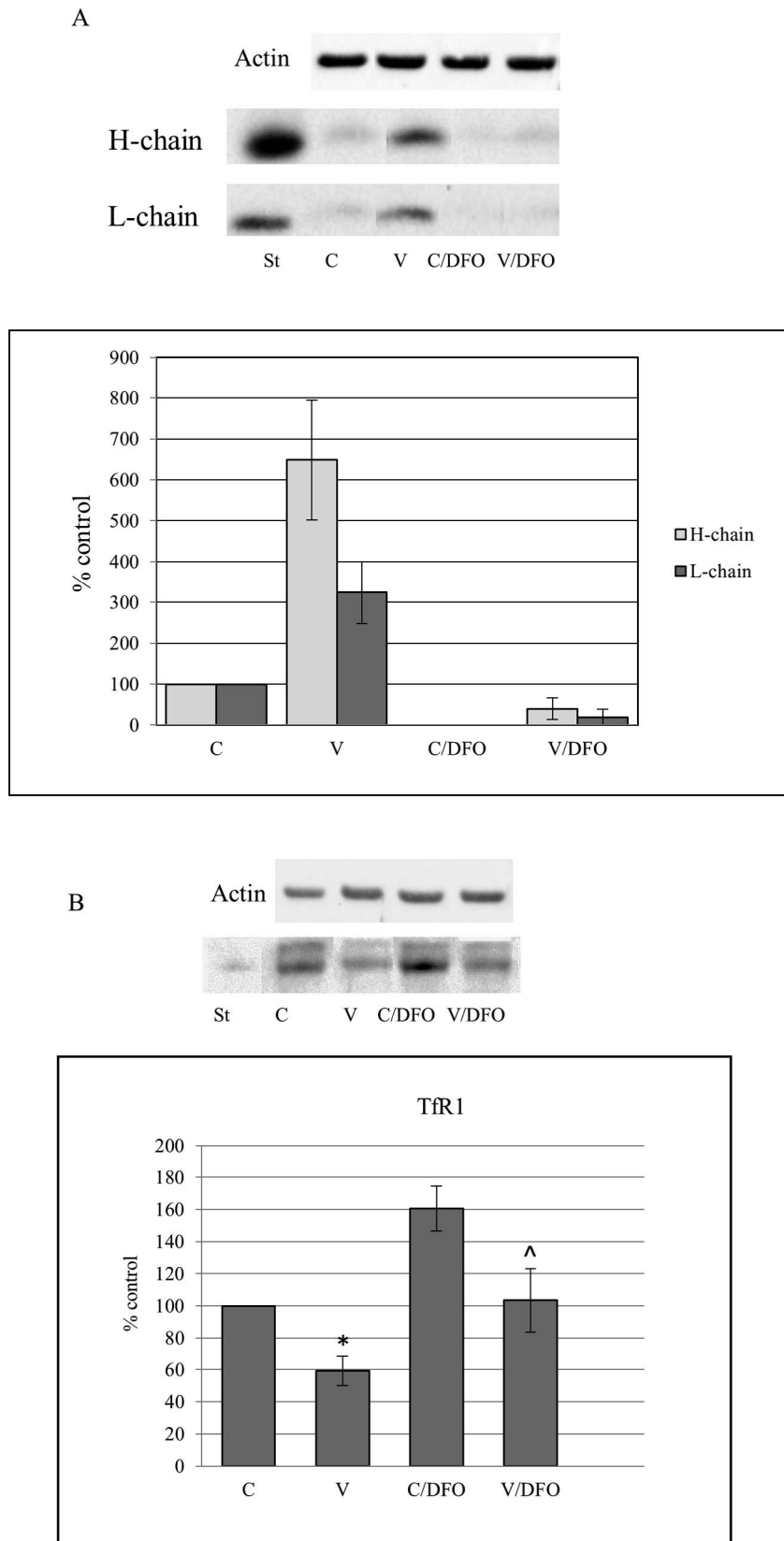
We examined the effects of vitreous humor from dogs aged 1 to 3 years and compared them to those produced by vitreous humor from dogs 7 to 10 years old. There were no differences between these two groups in terms of the stimulatory effect on expression of ferritin chains and the decrease in TfR1 expression as measured by Western blot analysis (data not shown). However, vitreous humor collected from dogs with age-related cataracts produced significantly higher stimulation of ferritin L-chain expression as compared to that produced by vitreous humor from dogs of similar age but without cataracts (Fig. 9).

Samples of aqueous humor collected from eyes of dogs of different ages did not have an effect on the expression of iron-handling proteins (data not shown).

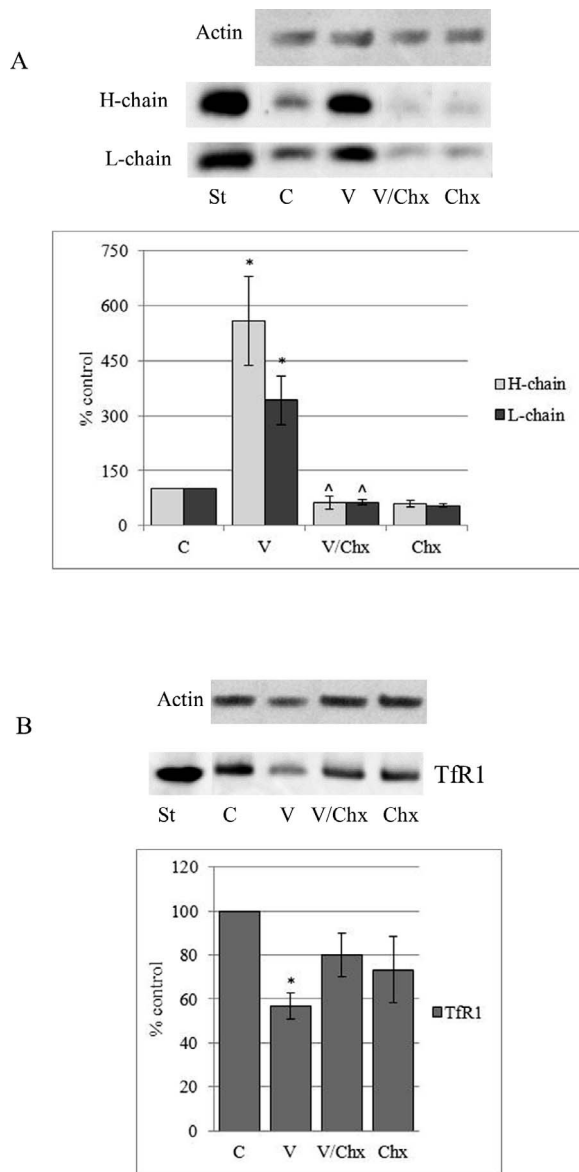
#### DISCUSSION

After 96-hour exposure to vitreous humor, LEC underwent morphologic changes characteristic of fibers and expressed aquaporin 0, a marker of differentiation that was undetectable in control LEC. However, Western blot analysis of ferritin L-chains from lysates of cells expressing aquaporin 0 did not detect the modified L-chain detected in homogenates of control canine lens fibers. This suggests that modification of ferritin chains does not take place during lens epithelial-to-fiber cell differentiation but most likely is a consequence of posttranslational modification of these proteins during the lens aging process.

Expression of ferritin H-chain in vitreous-treated LEC was increased significantly more than that of ferritin L-chain. That was a surprising result since ferritin chains assemble in a tissue-specific ratio of H:L therefore a proportional increase of each chain should be expected. Assembled ferritin of LEC contains more H- than L-chain; therefore an overall higher level of H-chain in vitreous-treated cells could be expected. However, in our previous investigation of overexpression of each or both chains together,<sup>13,14</sup> LEC controlled the levels of ferritin H-chain more tightly than L-chain. When overexpressed, H-chain is released into the culture media<sup>13</sup> or preferentially degraded<sup>14</sup> unless there is enough L-chain present to assemble into ferritin of the proper H:L ratio. Strict control of ferritin H-chain is important, since, in addition to facilitating incorporation of iron into ferritin, H-chain has other important biological functions.<sup>26,27</sup> H-chain has ferroxidase activity and when overexpressed it very efficiently chelates intracellular iron, creating an iron-deficient phenotype.<sup>28</sup> In the present study, 96-hour treatment of LEC with vitreous humor elevated H-chain by an additional 3.3-fold over 24 hours but had no further effect on the level of ferritin L-chain after 24 hours. It is difficult

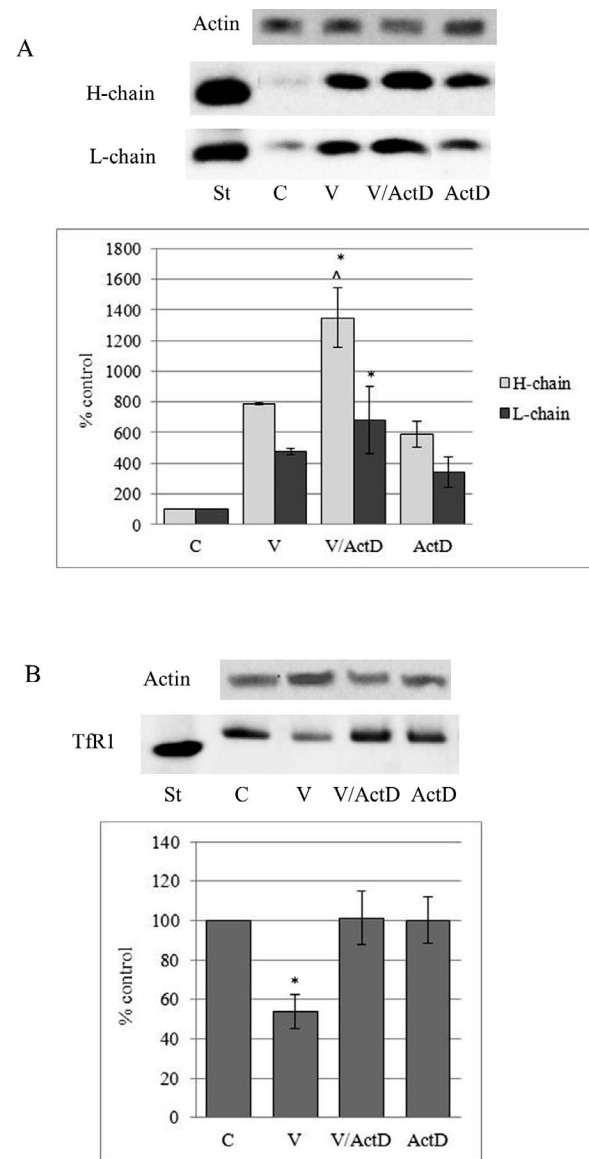


**FIGURE 5.** Effects of DFO on expression of ferritin chains (A) and TfR1 (B) in LEC treated with vitreous humor (V) and control LEC (C). LEC were treated with 100  $\mu$ M DFO (C/DFO;V/DFO) for 24 hours in serum-free DMEM without (C) or with 33% vitreous humor (V). Cell lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified canine heart ferritin was used as standard (St) for ferritin chains. Purified human placenta TfR1 was used as a standard (St) for canine TfR1. After Western transfer to a nitrocellulose membrane, ferritin chains were immunodetected with canine chain-specific custom-made antibodies. TfR1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies. Blots were reprobbed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The significance of differences was determined by paired *t*-test. Data represent the mean  $\pm$  SEM; *n* = 4; statistically different from C, \**P* < 0.05; statistically different from C/DFO, ^*P* < 0.05.



**FIGURE 6.** Effects of cycloheximide on expression of ferritin chains (A) and TfR1 (B) in LEC treated with vitreous humor (V) and control LEC (C). LEC were treated with 10  $\mu$ M cycloheximide for 24 hours in DMEM containing 10% FBS without (Chx) or with 33% vitreous humor (V/Chx). Cell lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified canine heart ferritin was used as standard (St) for ferritin chains. Purified human placenta TfR1 was used as a standard (St) for canine TfR1. After Western transfer to a nitrocellulose membrane, ferritin chains were immunodetected with canine chain-specific custom-made antibodies. TfR1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies. Blots were reprobed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The significance of differences was determined by using Tukey's HSD test. Data represent the mean  $\pm$  SEM;  $n = 3$ ; statistically different from corresponding C, \* $P < 0.05$ ; statistically different from corresponding V,  $\wedge P < 0.05$ .

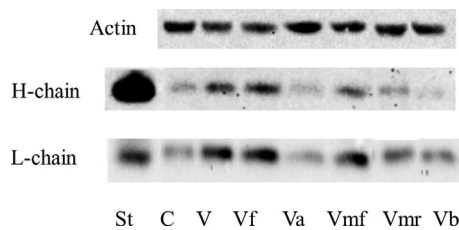
to hypothesize why the limited availability of L-chain did not prevent the accumulation of H-chain in differentiating LEC. However, accumulation of ferritin H-chain could increase iron storage, as ferritin having high H:L ratio more efficiently stores iron.<sup>28</sup> This could benefit metabolically inactive fiber cells by



**FIGURE 7.** Effects of actinomycin D on expression of ferritin chains (A) and TfR1 (B) in LEC treated with vitreous humor (V) and control LEC (C). LEC were treated with 1  $\mu$ g/1 mL actinomycin D for 24 hours in DMEM containing 10% FBS without (ActD) or with 33% vitreous humor (V/ActD). Cell lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified canine heart ferritin was used as standard (St) for ferritin chains. Purified human placenta TfR1 was used as a standard (St) for canine TfR1. After Western transfer to a nitrocellulose membrane, ferritin chains were immunodetected with canine chain-specific custom-made antibodies. TfR1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies. Blots were reprobed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The significance of differences was determined by using Tukey's HSD test. Data represent the mean  $\pm$  SEM;  $n = 3$ ; statistically different from corresponding C, \* $P < 0.05$ ; statistically different from corresponding V,  $\wedge P < 0.05$ .

limiting iron-catalyzed free radical formation, which is known to increase during hypoxia.

Vitreous treatment progressively decreased TfR1 levels from 63% at 24 hours to 41% at 96 hours. This decline in TfR1 could significantly reduce the uptake of Tf iron by vitreous-treated LEC. Decreased expression of TfR1 and concomitant elevation

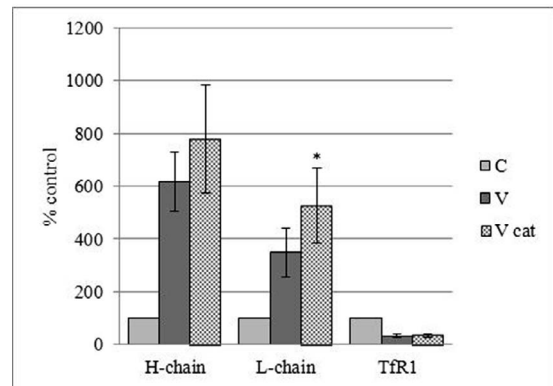
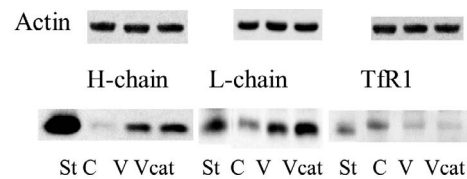


**FIGURE 8.** Western blot analysis of ferritin H- and L-chains in the lysates of LEC treated with differentially processed fractions of vitreous humor. LEC were treated for 24 hours without (C) or with 33% of vitreous humor (V) and with equivalent amount of differentially processed vitreous fractions, in DMEM containing 10% FBS. Vf, vitreous humor filtered through glass wool; Va, acetone precipitate of Vf dissolved in complete DMEM; Vmf, Microcon 10 filtrate of Vf; Vmr, Microcon 10 retentate of Vf; Vb, Vf boiled for 10 minutes. Cell lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified canine heart ferritin was used as standard (St) for ferritin chains. After Western transfer to a nitrocellulose membrane, ferritin chains were immunodetected with canine chain-specific custom-made antibodies. Blots were reprobbed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The blot shown is representative of two experiments.

of ferritin chains are characteristic of iron's translational regulation of iron-handling proteins.<sup>16</sup> Increased levels of iron stimulate ferritin synthesis and reduce TfR1. This promotes safe iron storage and reduces its uptake. To assess the possibility of intracellular iron involvement in vitreous humor regulation of iron-handling protein expression, we tested levels of ferritin chains and TfR1 after treatment with iron chelator DFO. We found that DFO abolished expression of both ferritin chains in control- and vitreous-treated LEC. This indicated that iron is essential for vitreous humor stimulation of ferritin chain expression. Transferrin receptor 1 expression in an iron-deficient environment was increased in vitreous-treated LEC; however, it was lower than expression of TfR1 in control LEC treated with DFO. We concluded that there was an additional, non-iron-mediated mechanism by which vitreous humor lowered expression of TfR1.

Increased uptake of extracellular iron could be a possible mechanism by which vitreous humor initiated changes in expression of iron-handling proteins. Elevated content of cytosolic iron would increase its deposition into ferritin, and via a negative feedback mechanism, would limit iron uptake by Tf/TfR1. However, vitreous humor itself has a low iron content,<sup>21</sup> and its effects were the same when LEC were treated in media supplemented with 10% serum or without serum supplementation. We hypothesized that vitreous humor changes intracellular trafficking of iron, making it more available for the iron responsive protein/iron responsive element (IRP/IRE) regulatory system rather than increasing its uptake.

To confirm that vitreous humor regulation is at the translational level, LEC were treated with the protein translation inhibitor cycloheximide. Cycloheximide completely abolished the stimulatory effect of vitreous treatment on expression of ferritin H- and L-chains. Such a profound effect suggested that vitreous treatment effects were translational and were not due to decreased degradation of ferritin chains. Cycloheximide effects on expression of TfR1 in both control- and vitreous-treated LEC were not significant. This confirmed our previous finding that synthesis of TfR1 is not regulated exclusively by iron on a translational level. It should be pointed out that expression of TfR1 is also regulated by cytokines and growth factors on a transcriptional level.<sup>29</sup>



**FIGURE 9.** Effects of treatment of LEC with vitreous humor collected from dogs 7 to 10 years old with (Vcat) or without (V) age-related cataract on expression of ferritin chains and TfR1. LEC were treated with 33% vitreous humor in DMEM containing 10% FBS for 96 hours. Cells nontreated with vitreous humors were used as controls (C). Cell lysates containing 35 to 50  $\mu$ g protein were separated by 12% SDS-PAGE, under reducing conditions, using a Tris/Tricine buffer system and transferred to nitrocellulose membranes. Purified canine heart ferritin was used as standard (St) for ferritin chains, and purified human placenta TfR1 was used as a standard (St) for canine TfR1. After Western transfer to a nitrocellulose membrane, ferritin chains were immunodetected with canine chain-specific custom-made antibodies. TfR1 was immunodetected with monoclonal mouse anti-human TfR1 antibodies. Blots were reprobbed with HRP-goat anti-human  $\beta$ -actin as a loading control and evaluated with ChemiDoc MP Imaging System. The significance of differences was determined by paired *t*-test. Data represent the mean  $\pm$  SEM; *n* = 7; statistically different from corresponding V, \**P* < 0.05.

Actinomycin D significantly increased levels of ferritin H- and L-chain in both control- and vitreous-treated LEC. We observed this paradoxical action of actinomycin D in our previous studies concerning the effects of ascorbic acid on ferritin synthesis in LEC.<sup>30</sup> In that study, actinomycin D treatment increased the total level of ferritin measured by ELISA and de novo synthesis of ferritin, but decreased the stimulatory effect of ascorbic acid on ferritin expression. This paradoxical effect of actinomycin D is difficult to explain. It is possible that actinomycin D may repress transcription of factors that limit expression of ferritin chains.

Actinomycin D did not alter TfR1 levels in control LEC, but completely obliterated the inhibitory effect of vitreous treatment, bringing TfR1 levels up to that found for the control LEC (Fig. 7B). Seiser et al.,<sup>31</sup> in their study on iron-dependent degradation of TfR1 mRNA, reported that actinomycin D treatment effectively suppressed degradation of TfR1 mRNA in mouse fibroblasts. Based on this finding, we hypothesized that vitreous humor treatment could inhibit expression of TfR1 by increasing the degradation rate of TfR1 mRNA through the iron-dependent IRP/IRE regulatory system. However, this may not be the only mechanism by which vitreous humor decreased TfR1 level, since the iron chelator DFO did not fully reverse the decline of TfR1 levels in vitreous-treated LEC.

In order to determine which fraction of vitreous humor is responsible for changing expression of iron-handling proteins, we removed the collagen fibrils and fibril-associated macro-

molecules by filtration. After determining that activity was associated with the filtrate we differentially processed this fraction. Differential fractionation revealed that the active compound is thermolabile and most likely of low molecular weight. Ascorbic acid, which has been known to greatly stimulate ferritin synthesis in LEC<sup>30</sup> and is present in high concentrations in vitreous humor of diurnal animals,<sup>32</sup> is consistent with these properties. However, ascorbic acid degrades rapidly and irreversibly under culture conditions.<sup>33</sup> Thus it is unlikely that it would have such a strong effect on cells cultured from 24 to 96 hours. The nature of the low molecular weight compound of vitreous humor that regulates expression of iron-handling proteins in LEC requires further investigation. Aqueous humor collected in parallel from the same eyes did not alter expression of iron-handling proteins (data not shown).

During the aging process, vitreous humor undergoes significant changes, such as aggregation of fibrils, liquefaction, and posterior detachment.<sup>2</sup> In our study there was no difference in the effects of vitreous humor collected from young dogs and visibly liquefied vitreous humor of old dogs on the expression of iron-handling proteins (data not shown). Surprisingly, vitreous humor collected from old dogs with age-related cataract increased the expression of ferritin L-chain significantly more than vitreous humor collected from old dogs without visible cataract.

Vitreotomy in humans is done to address such pathological conditions as tears in the retina, retinal detachment, vitreous hemorrhage, or proliferative retinopathy. This procedure exposes the lens to potentially toxic oxygen levels,<sup>9,10</sup> eliminates antioxidants present in vitreous humor,<sup>10,32</sup> and potentially reduces protective effects of vitreous humor as seen in the present study. Together these results indicate that removal of the vitreous humor could result in an increase of iron-catalyzed free radical formation. There are no previous data on vitreous humor involvement in the regulation of iron uptake and storage by LEC. Considering how significantly vitreous humor limits the Tf/TfR1 iron uptake pathway and how it profoundly increases the iron-storing capacity of ferritin, it is reasonable to speculate that vitreous humor may play a significant protective role against iron-catalyzed oxidative damage of LEC and in the formation of cataracts.

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### References

- Sebag J. Ageing of the vitreous. *Eye (Lond)*. 1987;1(pt 2):254-262.
- Le Goff MM, Bishop PN. Adult vitreous structure and postnatal changes. *Eye (Lond)*. 2008;22:1214-1222.
- Harocopos GJ, Shui YB, McKinnon M, et al. Importance of vitreous liquefaction in age-related cataract. *Invest Ophthalmol Vis Sci*. 2004;45:77-85.
- Spector A. Oxidative stress-induced cataract: mechanism of action. *FASEB J*. 1995;9:1173-1182.
- Truscott RJ, Augusteyn RC. Oxidative changes in human lens proteins during senile nuclear cataract formation. *Biochim Biophys Acta*. 1977;492:43-52.
- Hsuan JD, Brown NA, Bron AJ, et al. Posterior subcapsular and nuclear cataract after vitrectomy. *J Cataract Refract Surg*. 2001;27:437-444.
- Melberg NS, Thomas MA. Nuclear sclerotic cataract after vitrectomy in patients younger than 50 years of age. *Ophthalmology*. 1995;102:1466-1471.
- Holekamp NM, Shui YB, Beebe DC. Vitrectomy surgery increases oxygen exposure to the lens: a possible mechanism for nuclear cataract formation. *Am J Ophthalmol*. 2005;139:302-310.
- Beebe DC, Holekamp NM, Siegfried C, et al. Vitreoretinal influences on lens function and cataract. *Philos Trans R Soc Lond B Biol Sci*. 2011;366:1293-1300.
- Li Q, Yan H, Ding TB, et al. Oxidative responses induced by pharmacologic vitreolysis and/or long-term hyperoxia treatment in rat lenses. *Curr Eye Res*. 2013;38:639-648.
- Loh A, Hadziahmetovic M, Dunaief JL. Iron homeostasis and eye disease. *Biochim Biophys Acta*. 2009;1790:637-649.
- Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta*. 1996;1275:161-203.
- Goralska M, Holley BL, McGahan MC. Identification of a mechanism by which lens epithelial cells limit accumulation of overexpressed ferritin H-chain. *J Biol Chem*. 2003;278:42920-42926.
- Goralska M, Nagar S, Fleisher LN, et al. Differential degradation of ferritin H- and L-chains: accumulation of L-chain-rich ferritin in lens epithelial cells. *Invest Ophthalmol Vis Sci*. 2005;46:3521-3529.
- Theil EC. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem*. 1987;56:289-315.
- Hentze MW, Caughman SW, Rouault TA, et al. Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science*. 1987;238:1570-1573.
- Dernouchamps JP, Vaerman JP, Michiels J, et al. Transferrins in rabbit ocular fluids [in French]. *Ophthalmologica*. 1975;170:72-83.
- Van Bockxmeer FM, Martin CE, Constable IJ. Iron-binding proteins in vitreous humour. *Biochim Biophys Acta*. 1983;758:17-23.
- Laicine EM, Haddad A. Transferrin, one of the major vitreous proteins, is produced within the eye. *Exp Eye Res*. 1994;59:441-445.
- McGahan MC, Harned J, Goralska M, et al. Transferrin secretion by lens epithelial cells in culture. *Exp Eye Res*. 1995;60:667-673.
- McGahan MC, Fleisher LN. A micromethod for the determination of iron and total iron-binding capacity in intraocular fluids and plasma using electrothermal atomic absorption spectroscopy. *Anal Biochem*. 1986;156:397-402.
- Konerirajapuram NS, Coral K, Punitham R, et al. Trace elements iron, copper and zinc in vitreous of patients with various vitreoretinal diseases. *Indian J Ophthalmol*. 2004;52:145-148.
- Lovicu FJ, McAvoy JW. Growth factor regulation of lens development. *Dev Biol*. 2005;280:1-14.
- Goralska M, Fleisher LN, McGahan MC. Ferritin H- and L-chains in fiber cell canine and human lenses of different ages. *Invest Ophthalmol Vis Sci*. 2007;48:3968-3975.
- Cham BE, Roeser HP, Nikles A, et al. A procedure for the purification of ferritin from human liver by heating a methanol-treated homogenate. *Anal Biochem*. 1985;151:561-565.
- Guo JH, Juan SH, Aust SD. Suppression of cell growth by heavy chain ferritin. *Biochem Biophys Res Commun*. 1998;242:39-45.
- Cozzi A, Levi S, Corsi B, et al. Role of iron and ferritin in TNFalpha-induced apoptosis in HeLa cells. *FEBS Lett*. 2003;537:187-192.



28. Cozzi A, Corsi B, Levi S, et al. Overexpression of wild type and mutated human ferritin H-chain in HeLa cells: in vivo role of ferritin ferroxidase activity. *J Biol Chem.* 2000;275:25122-25129.
29. Tacchini L, Gammella E, De Ponti C, et al. Role of HIF-1 and NF-kappaB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals. *J Biol Chem.* 2008;283:20674-20686.
30. Goralska M, Harned J, Grimes AM, et al. Mechanisms by which ascorbic acid increases ferritin levels in cultured lens epithelial cells. *Exp Eye Res.* 1997;64:413-421.
31. Seiser C, Posch M, Thompson N, et al. Effect of transcription inhibitors on the iron-dependent degradation of transferrin receptor mRNA. *J Biol Chem.* 1995;270:29400-29406.
32. Rose RC, Gogia R, Richer SP. Properties of electrochemically active components in mammalian vitreous humor. *Exp Eye Res.* 1997;64:807-812.
33. Frikke-Schmidt H, Lykkesfeldt J. Keeping the intracellular vitamin C at a physiologically relevant level in endothelial cell culture. *Anal Biochem.* 2010;397:135-137.