

*Full Length Research Paper*

# Erythropoietin protects the retinal pigment epithelial barrier against non-lethal H<sub>2</sub>O<sub>2</sub>-induced hyperpermeability

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**Erythropoietin (EPO) is not limited to hematopoiesis; it may act as a protective cytokine. In this study, the retinal pigment epithelial (RPE) cell viability, cell monolayer integrity, RPE barrier permeability, distribution of the junction proteins ZO-1, occludin and the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were monitored to evaluate the effect of EPO on non-lethal H<sub>2</sub>O<sub>2</sub>-induced RPE barrier hyperpermeability. Results showed that, EPO increased the viability of H<sub>2</sub>O<sub>2</sub>-treated RPE cells, the disruption of junction proteins and the higher permeability caused by H<sub>2</sub>O<sub>2</sub> was partially prevented by EPO pre-treatment. EPO treatment also induced lower MDA levels and higher SOD activity in H<sub>2</sub>O<sub>2</sub> treated RPE cells. So, it is concluded that, non-lethal concentrations of H<sub>2</sub>O<sub>2</sub> could damage RPE barrier and destroy its integrity. EPO showed the protective effects on H<sub>2</sub>O<sub>2</sub>-induced hyperpermeability by stabilizing the distribution of junction proteins and reducing oxidative stress. These results indicated that, EPO may have the therapeutic potential use in the treatment of eye diseases involving RPE barrier hyperpermeability induced by oxidative stress.**

**Key words:** Erythropoietin, retinal pigment epithelial, oxidative stress, tight junction.

## INTRODUCTION

Macular edema is the final common pathway of many intraocular diseases, such as veno-occlusive disease or diabetic retinopathy. It has a complicated pathogenesis and is closely related to the blood-retinal barrier (BRB). The BRB includes the inner barrier and outer barrier. The inner barrier is formed by retinal vascular endothelial (RVE) cells and the outer barrier is formed by retinal pigment epithelial (RPE) cells, which lie between the retina and the choroid (Vinores et al., 1999; Rizzolo, 1997). Damage to RVE cells causes inner barrier damage and the release of molecules and ions from the capillaries to retinal tissue, which results in retinal edema (Antcliff and Marshall, 1999). However, not all of the retinal edema comes from retinal vessels (Antcliff and Marshall, 1999; Marmor, 1999). The formation of the RPE barrier is dependent upon the function of tight junctions, which

create a restricted diffusion barrier to non-transported solutes (Bailey et al., 2004).

Tight junctions (TJ) are important structures of RPE barrier (Hamilton et al., 2007). Destruction of TJ proteins causes outer barrier damage and liquid from the choroidal vessels accumulates in the subretinal space, which can induce macular edema, photoreceptor cell damage and eventually blindness (Marmor, 1999; Vinores, 1995; Rizzolo, 1997). Functionally, intact TJ is needed for the efficient and regulated removal of fluid from the sub-retinal space and for the barrier function of healthy RPE cells (Antcliff and Marshall, 1999; Chang et al., 1997).

TJ is the most apical components of the junctional complex and are the anatomic constituents of the oBRB, which seals the paracellular pathway between cells (Hamilton et al., 2007). TJ is predominantly made up of transmembrane proteins like occludin and claudins, as well as cytoplasmic adaptors including zonula occludens (ZO) proteins (Musch et al., 2006; Anderson and Vanitallie, 1995) and play a major role in regulating trans-epithelial resistance and paracellular permeability.

The first TJ protein to be identified was peripheral mem-

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**Abbreviations:** OBRB, Outer blood retinal barrier; EPO, erythropoietin; RPE, retinal pigment epithelial; TJ, tight junction.

brane protein ZO-1, which binds occludin (Stevenson et al., 1986) and has been shown to be the predominant component of epithelial tight junctions (Stevenson et al., 1986; Balda and Matter, 2000). A cobblestone epithelial morphology of ZO-1 around the apical aspect of the cells has been described in ARPE-19 and other RPE cultured cell lines (Kanuga et al., 2002). In addition, occludin is a transmembrane protein that has been identified in TJ (McCarthy et al., 1996) and changes in occludin expression correlate with TJ permeability (Hirase et al., 1997; Konari et al., 1995). Therefore, the continuous expression of functional molecules of TJ is required for regulating epithelial permeability. Earlier work has shown that, oxidative stress can decrease tight-junction protein expression (Bailey et al., 2004).

Oxidative stress caused by reactive oxygen intermediate species (ROIs), such as hydrogen peroxide ( $H_2O_2$ ), has been implicated in the pathogenesis of many diseases and in particular, those that are related to age (Winkler et al., 1999). Exposure of cells to increasing concentrations of  $H_2O_2$  can cause both non-lethal and lethal damage. We focused on RPE barrier damage caused by non-lethal doses of  $H_2O_2$ .

Superoxide dismutase (SOD) is a specific scavenger of oxygen free radicals and plays a vital role in the oxidation and antioxidation balance that occurs in the body. Therefore, SOD is often seen as the first line of defence against reactive oxygen species. Higher SOD activity results in a stronger antioxidant capacity in cells. Malondialdehyde (MDA) is an important free radical decomposition by-product of lipid peroxidation. MDA levels are indicative of cell peroxidation and therefore, are often used as the detection index for lipid peroxidation (Chida et al., 1999).

The retina contains an ideal environment for the generation of ROIs. Long exposure to sunlight, high blood flow through the choriocapillaris and the phagocytosis of used photoreceptor components by the RPE all result in the generation of  $H_2O_2$  (Beatty et al., 2000). Although, multiple physiologic defences exist to protect the RPE from oxidative damage, evidence suggests that cumulative oxidative exposure disrupts RPE cell junctions as well as barrier integrity and induces RPE cell blebbing, which is a sign of oxidant injury (Marin-Castano et al., 2005; Strunnikova et al., 2004). Recently, research has focused on the role of oxidative stress in RPE damage that occurs in AMD and other retinal diseases of aging (Beatty et al., 2000; Cai et al., 2000; Bailey et al., 2004). The effects of antioxidants, such as vitamin C, L-carnitine (Shamsi et al., 2007) and catalase on these cells are being considered for their potential to prevent oxidative damage (Pemp et al., 2010).

Erythropoietin (EPO) is a glycoprotein that has been shown to mediate responses to hypoxia and is recognized for its central role in erythropoiesis (Lacombe and Mayeux, 1998).

Recent studies have shown that, EPO receptors are abundantly expressed in the brain, uterus and retina.

The effects of EPO are not limited to haematopoiesis and the protein has been shown to have anti-inflammatory, anti-apoptosis and antioxidative stress effects on cells and may even act as a protective cytokine (Katavetin et al., 2007; Eid and Brines, 2002; Zhang et al., 2008a). Gawad et al. have suggested that the protective effect of EPO on RPE cells occurs as a result of the anti-apoptotic properties of the protein (Meng et al., 2008; Gawad et al., 2009). The neuroprotective and cytoprotective effects of EPO have been studied in the retina. Fu et al. and King et al. have studied the neuroprotective effects of EPO in animal models exhibiting phenotypic diseases, such as ocular hypertension and optic nerve transection (Weishaupt et al., 2004; Fu et al., 2008; King et al., 2007; Zhong et al., 2007).

The protective effects of EPO on TJ have been detected in other cells, including cells of the blood-brain barrier (BBB). Martinez-Estrada et al. (2003) found that, EPO had a protective effect against VEGF-induced hyperpermeability. Moreover, EPO was shown to prevent an increase in BBB permeability during pentylene-tetrazole-induced seizures (Uzum et al., 2006). Recently, it was demonstrated that the modulation of cellular TJ protein expression, the transport and permeation characteristics of the BBB and the oBRB, which is composed of the intercellular TJ of the RPE are surprisingly similar (Steuer et al., 2005).

Recently, Zhang et al. (2008a) found that, administration of intravitreal EPO to diabetic rats can produce therapeutic effects on BRB function; however, no assessment was made for the effects of EPO on oBRB. Wang et al. (2009) extended studies of the protective effects of EPO on RPE cells, but did not examine effects on the RPE barrier in great detail.

The purpose of this study was to investigate the oxidative injury by  $H_2O_2$  affecting the RPE barrier and whether EPO could protect the RPE barrier from  $H_2O_2$ -induced hyperpermeability. Moreover, we wished to extend the assessment performed by previous studies and understand the effects of EPO on the RPE barrier in more detail.

## MATERIALS AND METHODS

### RPE cell culture

The human RPE cell line ARPE-19, obtained from the American type culture collection (CRL-2302; ATCC, Manassas, VA, USA), was cultured in a 1:1 mixture of Dulbecco's modified eagle's medium (DMEM) and nutrient mixture F12 (Invitrogen-GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO), 50 U/ml penicillin/streptomycin (Sigma Chemical Co., St. Louis, MO, USA) and 2.5 mM glutamine (Sigma) at 37°C and 5%  $CO_2$ . For all of the experiments, cells that had been cultured between 15 and 25 passages were used.

### MTS assay

RPE cells ( $1 \times 10^4$  cells/well) were cultured in 96-well culture plates

(Corning-Costar, NY, USA). Cells were given fresh medium 24 h after plating. Once confluent, RPE cells were starved for 24 h in serum-free medium before being used in the experiments. Cells were treated with varying concentrations of H<sub>2</sub>O<sub>2</sub> or EPO for 24 h or pre-treated with EPO for 2 h and then, incubated with varying concentrations of H<sub>2</sub>O<sub>2</sub> for 24 h. Cell viability was measured using the tetrazolium compound ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) (CellTiter 96 Aqueous one solution kit, Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 20 µl MTS were added to each well. After 4 h incubation, the cell viability was determined by measuring the absorbance at 490 nm using a microplate spectrophotometer (Wallac 1420 Victor 2, Wallac Oy, Turku, Finland). Absorbance values were normalized with respect to untreated control cultures to calculate changes in cell viability.

### Measurement of TER

Collagen-coated filter inserts (Corning Transwell-COL hydrophilic polytetrafluoroethylene (PTFE) membranes, diameter 6.5 mm, growth surface 0.33 cm<sup>2</sup>, pore size 0.4 µm) were used. RPE cells were seeded at a density of 10<sup>4</sup> cells/filter in 24-well culture plates. The volumes of the upper and lower chambers were 0.1 and 0.6 ml, respectively and the fluid pressure was equal in both chambers. The cultures were incubated in a humidified atmosphere (37°C, 5% CO<sub>2</sub>). The medium was changed in the following day and every second day thereafter, for the duration of the experiment. Seven days after seeding, transepithelial resistance (TER) was measured every other day using a Millicell-ERS electrical resistance system (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Resistance was measured across two filters at several time points after plating and the net TER measurements were calculated by subtracting the values obtained from a blank set of membranes without RPE cells.

### Permeability assay

All permeability experiments were conducted as described in Bailey et al. (2004) using tracer leakage. Briefly, cells were seeded onto 0.4 µm pore size membrane filters. Once tight junctions had formed, as determined by the TER measurements, the cells were starved for 24 h in serum-free medium and then treated with 0.4 mM H<sub>2</sub>O<sub>2</sub> or pre-treated with 1 U/ml EPO for 2 h followed by 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h or were left untreated as control group. Medium containing 1 mg/ml rhodamine isothiocyanate-dextran (RD) was added to the upper chamber, while the lower chamber was continually incubated with serum-free medium without tracer. At each time point, 50 µl of medium was removed from the lower chamber of the filter, diluted in PBS and measured in a fluorometer at an excitation wavelength of 570 nm and emission wavelength of 595 nm (Safire2, Tecan, Mannedorf, Switzerland).

### Immunofluorescence microscopy

RPE cells were grown on glass cover slips placed inside 24-well culture plates and given fresh medium 24 h after plating. Once confluent, RPE cells were starved for 24 h in serum-free medium before being used in the experiments. Cells were treated with 0.4 mM H<sub>2</sub>O<sub>2</sub>, or pre-treated with 1 U/ml EPO for 2 h followed by 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h. Cells were washed three times with phosphate-buffered saline (PBS), fixed for 10 min with a methanol:acetone (1:1) mixture and then washed again three times with PBS. After treatment with 0.5% Triton X-100, the fixed cultures were blocked with 5% bovine serum albumin (BSA) in PBS for 30 min at

room temperature. Cultures were then incubated overnight with a rabbit anti-ZO-1 antibody (2.5 µg/ml, Catalog No.40-2200, Invitrogen, Carlsbad, CA, USA) or rabbit anti-occludin antibody (10µg/ml, Catalog No.71-1500, Invitrogen, Carlsbad, CA, USA), rinsed three times with PBS and incubated for 1 h with a fluorescein-conjugated goat anti-rabbit secondary antibody (1:300, Invitrogen, Carlsbad, CA, USA). The slides were then, washed three times with PBS, sealed under 10% glycerol and analyzed by fluorescence microscopy (Olympus, Shinjuku, Japan).

### Determination of MDA levels and SOD activity

RPE cells were grown in 6-well culture plates and given fresh medium 24 h after plating. Once confluent, RPE cells were starved for 24 h in serum-free medium before being used in the experiments. Cells were treated with 0.4 mM H<sub>2</sub>O<sub>2</sub> or 1 U/ml EPO for 24 h or pre-treated with 1 U/ml EPO for 2 h and then, incubated with 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h. RPE cell cultures were then washed three times with PBS and lysed with RIPA buffer for 30 min. The homogenate was centrifuged and the supernatant was collected. Assays for measuring SOD activity and MDA levels were performed according to the manufacturer's instructions using detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A Coomassie blue assay was used to determine protein levels.

### Statistical analysis

All experiments were repeated at least three times and all results were expressed as means ± standard deviations (SD). Statistical analyses were performed using one-way ANOVA and t-tests in SPSS 16.0 software for windows (SPSS Inc., Chicago, IL, USA). P < 0.05 was considered significant.

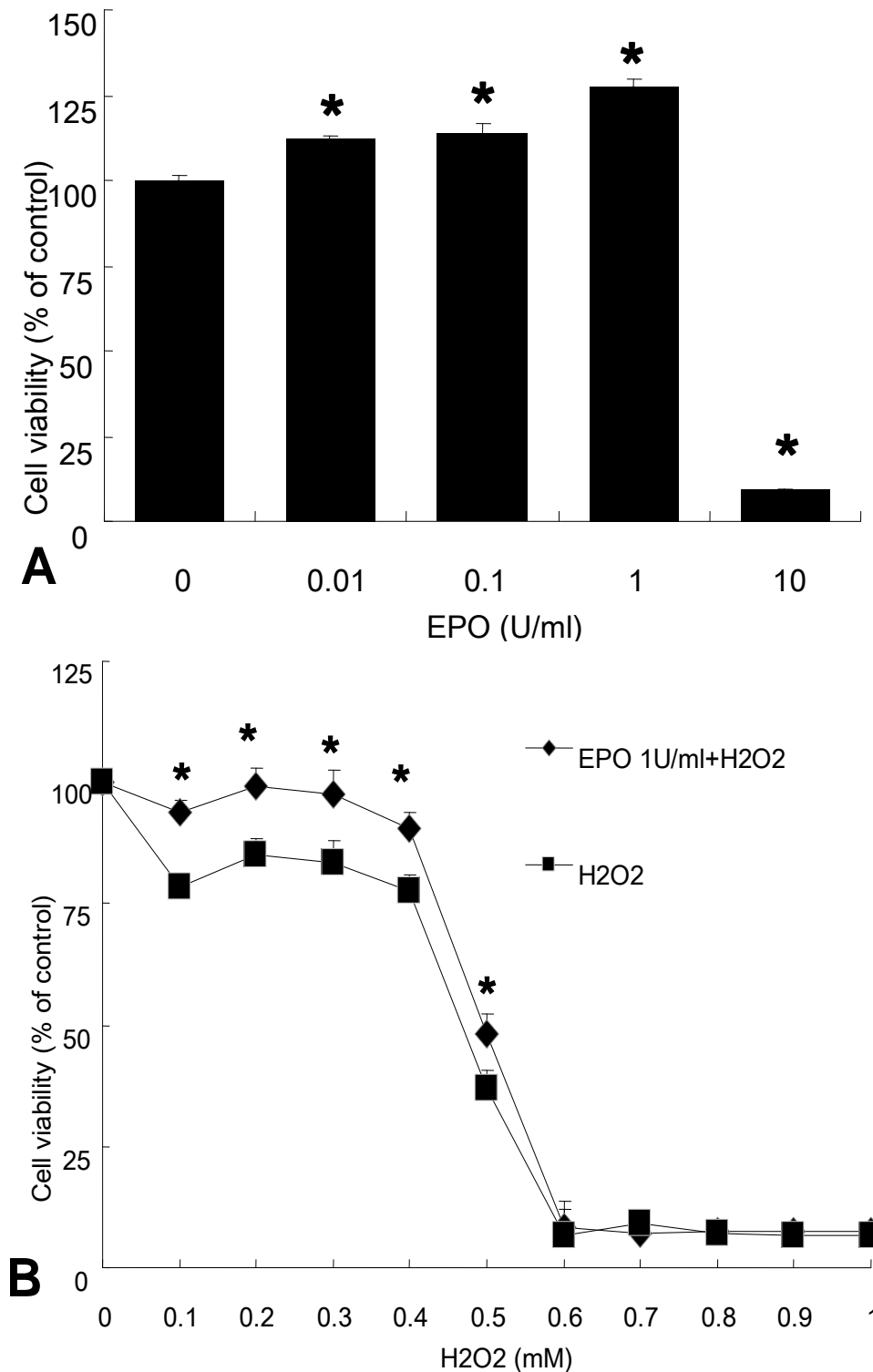
## RESULTS

### The negative effects of H<sub>2</sub>O<sub>2</sub> on RPE cell viability are antagonized by EPO

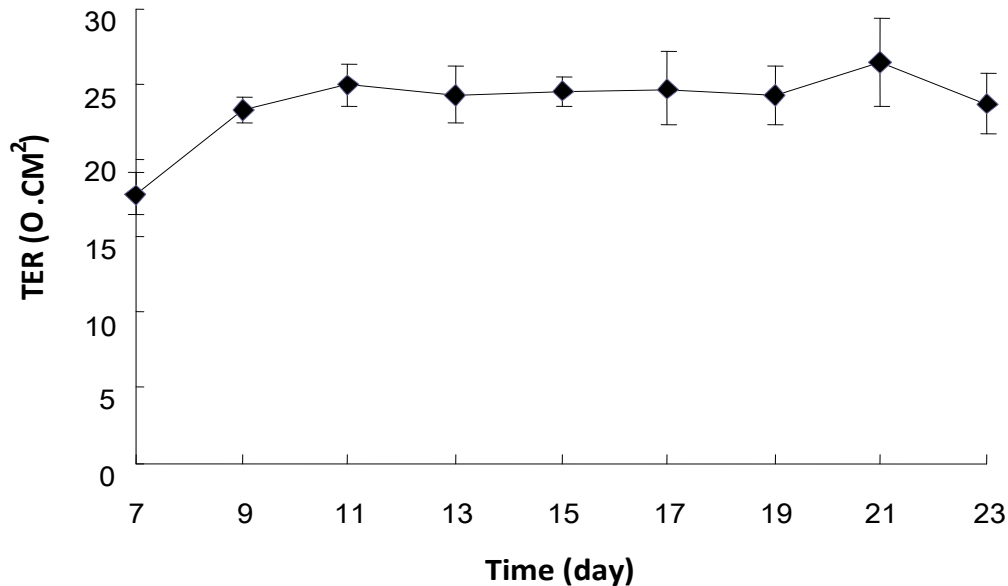
Cell viability was reduced by H<sub>2</sub>O<sub>2</sub> in a concentration dependent manner. Cell viability remained at 81% when the cells were treated with 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h, but decreased to approximately 36% at 0.5 mM H<sub>2</sub>O<sub>2</sub> (Figure 1a). The cell viability between the 0.4 mM H<sub>2</sub>O<sub>2</sub> treatment and control groups had no significant difference (P > 0.05). Cell viability significantly increased when cells were treated with 0.01 to 1 U/ml EPO, but decreased to 9.2% when the cells were exposed to 10 U/ml EPO (P < 0.05) (Figure 1b). Pre-treatment with 1 U/ml EPO partially protected the cell viability from the effects of H<sub>2</sub>O<sub>2</sub> (P < 0.05). However, EPO had no significant protective effect on cells exposed to H<sub>2</sub>O<sub>2</sub> concentrations that were greater than 0.6 mM (Figure 1a).

### Tight-junction formation in RPE cells stabilizes between days 11 and 23 in culture

The TER initially increased between days 7 and 11 in cultured RPE cells (17.8 ± 1.4 Ω·cm<sup>2</sup> and 24.9 ± 1.4 Ω·cm<sup>2</sup> on days 7 and 11, respectively). The TER values



**Figure 1.** The negative effects of H<sub>2</sub>O<sub>2</sub> on RPE cell viability are antagonized by EPO. (A) Cultures were treated with varying doses of EPO for 24 h. Treatment with 0.01-1 U/ml EPO increased cell viability, while treatment with 10 U/ml EPO decreased cell viability. \*P < 0.05 versus control group. The control group was RPE cells that were not treated; (B) Cells were pre-treated with 1 U/ml EPO for 2 h or not treated, and then treated for 24 h with increasing concentrations of H<sub>2</sub>O<sub>2</sub>. Cell viability was reduced by H<sub>2</sub>O<sub>2</sub> in a concentration-dependent manner. 1 U/ml EPO partially protected the cell viability from the effects of H<sub>2</sub>O<sub>2</sub> concentrations that were less than 0.6 mM. \*P < 0.05 versus H<sub>2</sub>O<sub>2</sub> group.



**Figure 2.** Time course of the development of transepithelial resistance (TER) in RPE cells. The density of RPE cells was  $10^4$  cells/filter. TER was measured every other day using a Millicell-ERS electrical resistance system ( $n = 3$ ). TER values generally increased between 7 and 11, peaking at day 11 and remaining stable until day 23. The values shown represent the means  $\pm$  SD.

stabilized after day 11 ( $24.2 \pm 1.8$ ,  $24.7 \pm 0.9$  and  $24.0 \pm 2.0 \Omega \cdot \text{cm}^2$  on days 13, 15 and 23, respectively) (Figure 2).

#### Disrupted distribution of tight junction proteins caused by $\text{H}_2\text{O}_2$ can be partially prevented by EPO treatment

Immunofluorescence of control cell cultures showed that ZO-1 localized to the cell borders and gave the appearance of paving stones (Figure 3a). In  $\text{H}_2\text{O}_2$ -treated cells, the ZO-1 staining at the cell borders was disrupted and disorganized (Figure 3b). Pre-treatment with EPO reduced the  $\text{H}_2\text{O}_2$ -mediated changes that were observed. Under these conditions, the ZO-1 staining pattern was restored to that of the control cells, although, some  $\text{H}_2\text{O}_2$ -mediated disruption was still observed, such as ellipsis (Figure 3c).

Immunofluorescence of occludin showed a similar sub-cellular localization as ZO-1 and was predominately localized to the contours of the cell borders (Figure 3d). Moreover, treatment with  $\text{H}_2\text{O}_2$  disrupted the localization pattern of occludin (Figure 3e). Similar to ZO-1, pre-treatment of cells with EPO prevented the  $\text{H}_2\text{O}_2$ -mediated disruption of occludin staining (Figure 3f).

#### Non-lethal $\text{H}_2\text{O}_2$ concentrations increased barrier permeability and EPO can mitigate this effect

Permeability assays were used to detect the flux of RD

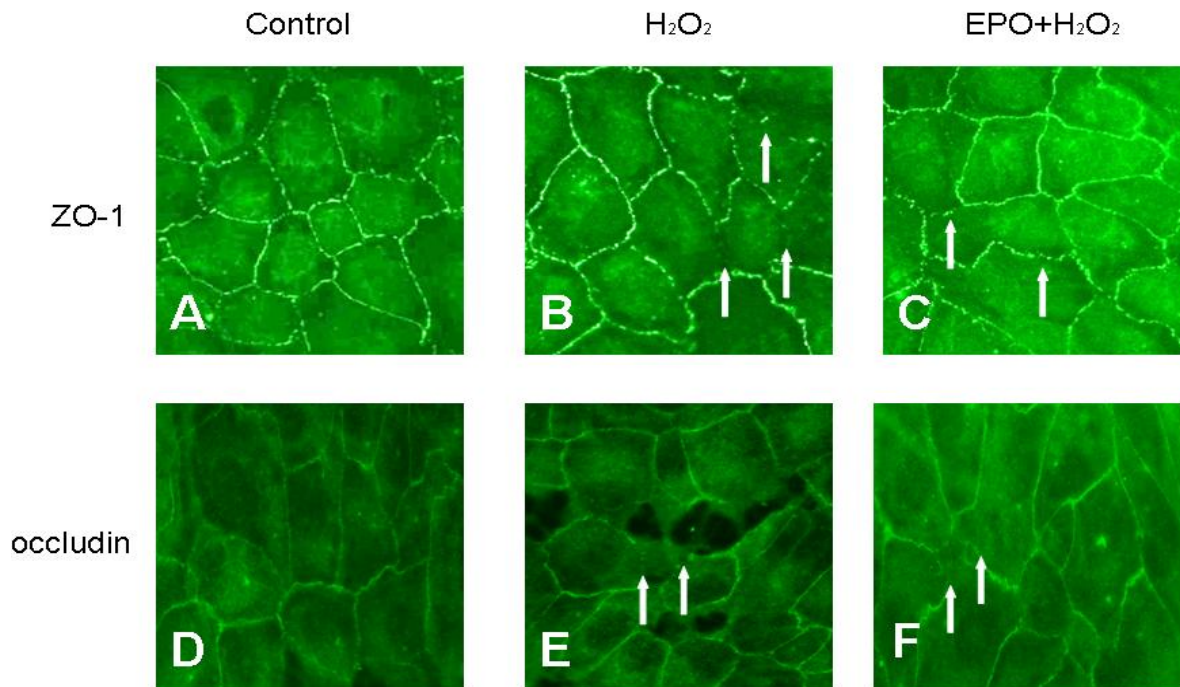
across RPE cells that had stable tight junctions. In the control group, the transepithelial flux was very low. This level was established as the control level (100%). In cultures treated with  $\text{H}_2\text{O}_2$ , the transepithelial flux was significantly higher. (329%, of control,  $P < 0.05$ ) (Figure 4).

Pre-treatment with EPO significantly decreased the transepithelial flux caused by  $\text{H}_2\text{O}_2$  (EPO +  $\text{H}_2\text{O}_2$ -treated cells; 175% of control, 53% of  $\text{H}_2\text{O}_2$ -treated cells,  $P < 0.05$ ) (Figure 4).

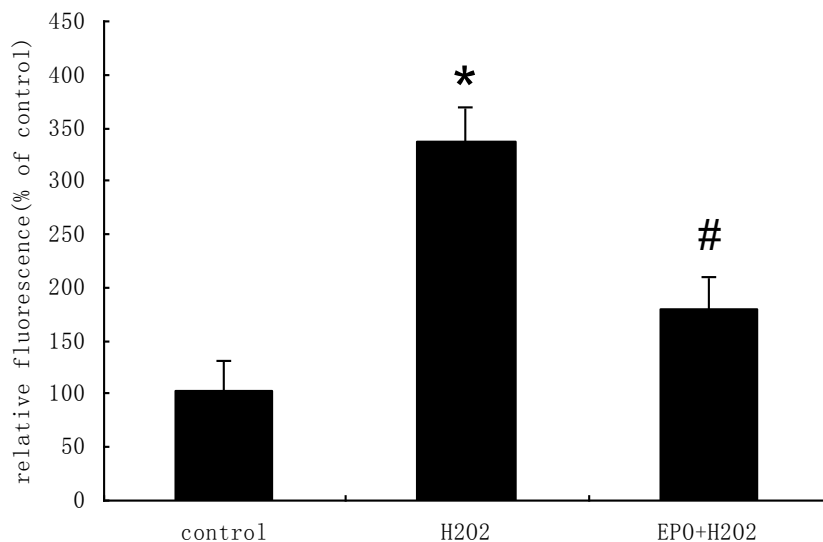
#### EPO reduces MDA production and enhances SOD activity in RPE cells exposed to $\text{H}_2\text{O}_2$

To estimate the anti-oxidative effect of EPO, the MDA level and SOD activity of RPE cells were measured. SOD is a pivotal enzyme that scavenges ROS *in vivo*. SOD activity in  $\text{H}_2\text{O}_2$ -treated cultures was significantly reduced compared with the control ( $14.3 \pm 0.7$  versus  $43.7 \pm 2.8$  U/mg protein, respectively,  $P < 0.05$ ). EPO treatment significantly prevented the decrease in SOD activity seen with  $\text{H}_2\text{O}_2$  treatment ( $32.9 \pm 4.1$  versus  $14.3 \pm 0.7$  U/mg protein, respectively,  $P < 0.05$ ). The SOD activity between the EPO treatment group and the control group is statistically insignificant ( $56.9 \pm 7.4$  versus  $43.7 \pm 2.8$  U/mg protein, respectively,  $P > 0.05$ ; Table 1).

MDA is produced during lipid peroxidation. We found that the MDA levels in  $\text{H}_2\text{O}_2$ -treated RPE cells were significantly higher than the control group ( $2.8 \pm 0.3$  versus  $1.1 \pm 0.6$  nmol/ml, respectively,  $P < 0.05$ ). EPO pre-



**Figure 3.** Sub-cellular distribution of tight junction proteins ZO-1(A, B and C) and occludin (D, E and F). Cells were treated without (A, D) or with (B, E) 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h or pre-treated with EPO for 2 h and then incubated with 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h (C, F). Immunofluorescence staining of ZO-1 (A, B, C) and occludin (D, E, F) are shown. RPE cells that were treated with H<sub>2</sub>O<sub>2</sub> showed less ZO-1(B) and occludin (E) linear staining at the cell border compared with the control group. EPO reduced the H<sub>2</sub>O<sub>2</sub>-induced changes in ZO-1 (C) and occludin (F) distribution. Arrow marks: Cell borders that have been disrupted.



**Figure 4.** Permeability changes of rhodamine isothiocyanate-dextran (RD) for 5 h through the RPE monolayer. Cells were either treated with 0.4 mM H<sub>2</sub>O<sub>2</sub>, pre-treated with 1 U/ml EPO for 2 h followed by 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h (EPO + H<sub>2</sub>O<sub>2</sub>) or were untreated (control group). Membranes containing RPE cell monolayer (control group) blocked RD flux. RD flux in the H<sub>2</sub>O<sub>2</sub> group was significantly higher than that in control group (\*P < 0.05 versus control group). Pre-treatment with 1 U/ml EPO before treatment with H<sub>2</sub>O<sub>2</sub> significantly reduced RD flux (# P < 0.05 versus H<sub>2</sub>O<sub>2</sub> group).

**Table 1.** EPO reduces MDA production and enhances SOD activity in RPE cells exposed to H<sub>2</sub>O<sub>2</sub>.

Parameter	Control	EPO	H <sub>2</sub> O <sub>2</sub>	EPO + H <sub>2</sub> O <sub>2</sub>
SOD (U/mg protein)	43.7 ± 2.8	56.9 ± 7.4 <sup>c</sup>	14.3 ± 0.7 <sup>a</sup>	32.9 ± 4.1 <sup>b</sup>
MDA (nmol/ml)	1.1 ± 0.6	1.0 ± 0.3 <sup>c</sup>	2.8 ± 0.3 <sup>a</sup>	1.4 ± 0.5 <sup>b</sup>

Changes in SOD activity and MDA levels. Cells were either treated with 0.4 mM H<sub>2</sub>O<sub>2</sub> or 1 U/ml EPO for 24 h or pre-treated with 1 U/ml EPO for 2 h and then treated with 0.4 mM H<sub>2</sub>O<sub>2</sub> for 24 h (n = 3). The SOD activity in the H<sub>2</sub>O<sub>2</sub> group was significantly lower than that in control group (P < 0.05). SOD activity in the EPO treatment group was higher than that in control group (P > 0.05). Pre-treatment with 1 U/ml EPO increased SOD activity compared with the H<sub>2</sub>O<sub>2</sub> group (P < 0.05). The MDA levels in the H<sub>2</sub>O<sub>2</sub> treatment group were significantly higher than those in the control group (P < 0.05). The MDA levels in the EPO treatment group were lower than those in the control group (P > 0.05). Pretreatment with 1 U/ml EPO significantly decreased the MDA levels compared with those in the H<sub>2</sub>O<sub>2</sub> treatment group (P < 0.05). (<sup>a</sup>P < 0.05 versus control group; <sup>b</sup>P < 0.05 versus H<sub>2</sub>O<sub>2</sub> treatment group; <sup>c</sup>P > 0.05 versus control group).

treatment significantly inhibited H<sub>2</sub>O<sub>2</sub>-mediated MDA production (1.4 ± 0.5 versus 2.8 ± 0.3 nmol/ml, respectively, P < 0.05). The MDA level between the EPO treatment group and the control group is statistically insignificant. (1.0 ± 0.3 versus 1.1 ± 0.6 nmol/ml, respectively, P > 0.05; Table 1)

## DISCUSSION

In this study, we evaluated the effect of EPO on RPE barrier damage caused by non-lethal H<sub>2</sub>O<sub>2</sub> exposure. We found that, EPO improved the oxidative parameters of RPE in an *in vitro* model and prevented RPE barrier hyperpermeability caused by H<sub>2</sub>O<sub>2</sub> exposure, indicating that EPO has protective effects on the RPE barrier during oxidative stress.

This study focused on RPE barrier damage caused by non-lethal doses of H<sub>2</sub>O<sub>2</sub>. In order to exclude possible RPE monolayer defects caused by cell death, we used a concentration of H<sub>2</sub>O<sub>2</sub> (0.4 mM) that did not show significant toxicity to RPE cells in the MTS assays or in a previous study by Bailey et al. (2004).

Although, RPE cells have a greater capacity to survive exposure to H<sub>2</sub>O<sub>2</sub> than to other oxidative stressors (Lu et al., 2006), they are unable to survive repeated, non-lethal challenges. Injured cells that undergo apoptosis are generally too severely damaged to function properly, as is seen in the geographic atrophy of AMD (Katavetin et al., 2007). Antagonizing apoptotic pathways is unlikely to be an effective protective strategy, since damaged cells that survive may not function appropriately or may eventually undergo necrotic cell death (Katavetin et al., 2007). However, preventing the cellular effects of ROIs in the early stages of injury may be effective. The study also showed that, EPO had no significant protective effect on cells exposed to H<sub>2</sub>O<sub>2</sub> concentrations that were greater than 0.6 mM, which means that, EPO's protective effect on RPE was limited. We should use EPO earlier before lethal damage has happened.

The results of this study also showed that cell viability significantly increased after treatment with 0.01 to 1 U/ml

EPO, but decreased to 9.2% in cultures exposed to 10 U/ml EPO. The optimal concentration of EPO was found to be 1 U/ml, which is similar to the peak plasma concentration of EPO (1 to 2 U/ml) achieved in patients (1200 U /kg) (Ramakrishnan et al., 2004). Recently, a study has shown that, 5 to 10 U EPO had a neuroprotective effect, while 25 U EPO had a neuroregeneration effect (King et al., 2007). Tsai et al. (2005) found that, 200 ng EPO protected retinal ganglion cell (RGC) viability. Studies have also found that, 2 U EPO can rescue RGCs after ocular hypertension injury (Fu et al., 2008; Weishaupt et al., 2004). Zhang et al. (2008b) found that, administration of 0.05 to 200 ng EPO protected the BRB.

However, all of the studies to date have not addressed RPE cells. The results of this study have shown that the administration of high doses of EPO was toxic to RPE cells and that an optimal concentration of EPO was required. These results are consistent with the results shown by Gawad et al. (2009) and Wang et al. (2009, 2010).

Recently, a study has found that the vitreous EPO concentration was markedly higher in patients with diabetic macular edema than in the control subjects (Garci-Arumi et al., 2009). We hypothesize that the increase in EPO in macular edema is a pathological phenomenon, since our data showed that high doses of EPO had toxic effects on RPE cells. This toxicity may lead to the destruction of RPE transport function and the eventual development of macular edema.

To choose an optimal time point for the permeability assay, TER was used to test tight-junction formation. Stable TER is required for this assay or it would be difficult to determine whether the permeability increase was caused by H<sub>2</sub>O<sub>2</sub> or defective tight junctions.

Earlier studies have reported TER values for ARPE-19 cells cultured in DMEM/F12 of approximately 30 Ω·cm<sup>2</sup> and our results were consistent with the values previously reported for this cell line (Dunn et al., 1996; Luo et al., 2006). Results from electron microscopy also supported the correlation of these TER values with the assembly of tight junctions (data not shown).

In our study, the RPE barrier was damaged by non-lethal H<sub>2</sub>O<sub>2</sub>, which can be detected as ZO-1 and occludin delocalization and a concurrent increase in permeability. Pre-treatment with EPO for 2 h prevented the damage.

Therefore, data from both the paracellular permeability experiments and the immunofluorescence detection of tight junction proteins suggest that, non-lethal concentrations of H<sub>2</sub>O<sub>2</sub> can destroy the RPE barrier, while pre-treatment with EPO can partially protect tight junction integrity. It is important to note that EPO pre-treatment did not restore the H<sub>2</sub>O<sub>2</sub>-mediated changes of RPE barrier permeability to basal levels (control), as shown by immunofluorescence staining and the permeability assay, which suggests that the protective effects of EPO are limited. Other treatment, such as triamcinolone acetonide (TA) (Miura and Roider, 2009) and anti-VEGF drugs (Iturralde et al., 2006); laser photocoagulation may need to be used in combination with EPO for the treatment of macular edema.

The redox status of cells influences cellular anti-oxidant defences. Our study demonstrated that, H<sub>2</sub>O<sub>2</sub> decreased SOD activity and increased MDA levels, whereas EPO was able to partially reverse these effects. Our results were consistent with values previously reported for this cell line (Zhao-Yang, 2009). These data indicated that the EPO-mediated protection of the RPE barrier was induced by the anti-oxidative effects of the protein. Gwada et al. (2009) hypothesized that, EPO-mediated protection of RPE cells against the effects of oxidative stress occurred through the anti-apoptotic properties of the protein and not the antioxidative properties. However, we have observed that the antioxidant effects are beneficial to AMD patients, though some criticisms have been raised about the required dose needed and the side effects that can occur. The SOD activity and MDA levels support our hypothesis that, EPO protects the RPE cell barrier against the effects of oxidative stress through the induced antioxidative properties of the protein.

The results from this study have shown that, EPO has protective effects against the RPE barrier during oxidative stress, which has important implications on the pathophysiology of certain diseases. The application of an optimal concentration of EPO can have a partial protective effect on the RPE barrier. These results provide a theoretical basis for the molecular mechanism underlying macular edema and diseases related to the RPE barrier. In fact, EPO has recently been used in clinical practice for the treatment of macular edema. Li et al. (2010) injected EPO intravitreally into the eyes of 5 patients with macular edema and found that the disease was reduced to some degree. These clinical results suggest that, EPO-mediated stabilization of the outer BRB may contribute to the healing process of macular edema and the stabilization of visual acuity.

In this study, we have concluded that, non-lethal concentrations of H<sub>2</sub>O<sub>2</sub> destroyed oBRB, while EPO was

shown to have a protective effect. However, high doses of EPO were toxic to the RPE and EPO's protective effect is limited. EPO was also shown to protect against H<sub>2</sub>O<sub>2</sub>-induced hyperpermeability by stabilizing the distribution of junction proteins and reducing oxidative stress. Therefore, EPO is a potential drug that could be used to treat retinal disease associated with this type of stress damage.

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