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BASIC SCIENCE

Triamcinolone acetonide prevents oxidative stress-induced tight junction disruption of retinal pigment epithelial cells

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Abstract
Purpose Oxidative stress is known to disrupt the integrity of retinal pigment epithelium (RPE) tight junctions. The goal of this study is to evaluate the effect of triamcinolone acetonide (TA) on the junctional integrity of RPE under oxidative stress and to identify the underlying mechanisms.
Methods Second passage porcine RPE cells were cultured on 6-well membrane inserts until 4 weeks after reaching confluence. Cells were incubated with TA (10^{-5} M) for 30 min. FITC-containing medium was added to the upper chamber (cell's apical side). The cells were then challenged with 1 mM Hydrogen Peroxide (H_2O_2). After 5 h, the fluorescence intensity of the medium from lower chamber (cell's basolateral side) was measured using a fluorescence spectrofluorophotometer. This transepithelial flux of FITC-dextran was measured until the 21st day. The immunolocalization of occludin and F-actin was examined with fluorescence microscope. Reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio was determined by a colorimetric assay kit.
Results Non-lethal oxidative stress by H_2O_2 increased transepithelial flux of FITC-dextran significantly. TA inhibited this increase and preserved the lower flux through the whole experimental period. This permeability change by H_2O_2 was reversible and recovered to the normal level within 3 weeks. In immunohistological study, H_2O_2 reduced linear occludin staining at the cell border and increased actin stress fibers. TA prevented H_2O_2-induced disruption of junctional assembly of occludin and F-actin. Glutathione assay demonstrated that intracellular GSH/GSSG ratio decreased significantly with H_2O_2, while TA preserved this ratio by up-regulating GSH synthesis.
Conclusions TA has a protective effect against oxidative stress-induced disruption of RPE tight junction by preserving cellular redox state.

Keywords Retinal pigment epithelium · Oxidative stress · Triamcinolone acetonide · Tight junction · Glutathione

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Introduction

In pathogenesis of chorioretinal disorders, such as age-related macular degeneration (AMD) and diabetic retinopathy, oxidative stress is considered to be one of the crucial factors [1, 2]. In retinal pigment epithelial (RPE) cells, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are generated during the phagocytosis of the oxidized photoreceptor outer segment in the physiological process of photoreceptor renewal [3]. The nondegradable end products of this phagocytosis lead to the accumulation of cholesterol ester and oxidized lipids, which are considered to be related to the early pathogenesis of AMD [4]. In diabetic patients, high glucose level is implicated in ROS production [1] and thus, the level of oxidative stress elevates [5, 6].

Oxidative stress has been shown to affect the expression of heat shock proteins [7–9], catalase and metallothionein gene expression [5], fibroblast growth factor (FGF) 2 [10], FGF receptors [11], RPE-65 and cellular retinaldehyde-binding protein [11] in RPE cell. Kinetic microarray study by Strunnikova et al. demonstrated that the nonlethal oxidative stress by H_2O_2 upregulates the gene expression of the protective proteins from oxidative stress, chaperon

proteins, anti-apoptotic factors, and DNA-reparing factors, and down regulates pro-apoptotic genes. They also showed the recovery with the normalization of gene expression to the baseline levels [12].

H_2O_2 has been utilized to induce oxidative stress in a number of in vitro studies. The cellular events following H_2O_2 treatment include actin reorganization, [13] membrane blebbing, [12] apoptosis, [14] redistribution of paracellular junctional proteins [9] and the change of paracellular permeability [9, 15]. Tight junction of RPE cells, which is the most apical component of junctional complex, function as the outer blood retinal barrier (BRB), which contributes to a restricted diffusion barrier between the retinal and the choroidal perfusion. The integrity of tight junction is preserved by a number of interaction of proteins, including occludin, ZO-1, −2, and −3. The disruption of tight junction barrier function causes the increase of outer BRB permeability, which leads to the impairment of efficient removal of subretinal fluid, and as result, may cause the prolonged visual disturbance and functional loss of outer retina.

Oxidative stress has been reported to affect the distribution of RPE junctional proteins [9, 15, 16]. The distributions of occludin, ZO-1 in tight junctions and cadherin in adherens junction are disrupted by the oxidative stress [16]. This oxygen-mediated junctional disruption was prevented by pigment epithelium-derived factor (PEDF) [16] and keratinocyte growth factor (KGF) [15].

Triamcinolone acetonide (TA) is a corticosteroid suspension that has been administrated periocularly for the treatment of ocular inflammatory diseases. In recent years, TA is used with intravitreal or trans-tenon's retrobulber infusion also for the treatment of macular edema [17–21], AMD with [22–24] or without [25] photodynamic therapy, serous macular detachment in central retinal vein occlusion, [26] in a combination with vitrectomy [27, 28], or with anti-vascular endothelial growth factor treatment for AMD [29]. The efficacy of TA has been well proven from the enormous clinical data, though its side effects [30–32] and the reports about its toxicity [33–36] are to be argued. However, the effects of TA during the healing process and its role in treatment remain to be elucidated.

With ECV304 cell line, TA decreased phorbol 12-myristate 13-acetate-induced paracellular permeability [37]. The protective effect of TA against oxidative stress in hair cells has been reported [38]. In ophthalmic field, TA is known to reduce VEGF expression from RPE cells [39], and thus the permeability of retinal vascular endothelium (inner BRB) is reduced, which may be followed by the reduction of edema. TA inhibits the increase of the albumin permeability in the retina of diabetic rat model [40]. It has been also shown that TA has a protective effect from streptozotocin-induced acute inflammation and early vascular leakage in rat retina [41]. For the healing of macular edema or AMD, the stabilization of both inner and outer BRB is necessary. However, the effect of TA on RPE cell junctional properties (outer BRB) has not yet been reported.

The aim of the present study is to elucidate the role of TA in the treatment of oxidative stress-related ocular diseases. Therefore, we investigated the effect of TA on cultured RPE paracellular permeability and junctional molecule distribution under oxidative stress. In order to understand if TA has an antioxidative effect, intracellular glutathione levels (total/oxidized/reduced) and the ratio of reduced/oxidized glutathione (GSH/GSSG ratio) were also investigated.

Materials and methods

Cell culture

Porcine eyes were obtained from a local slaughterhouse, and RPE cells were isolated as previously described [42]. Cells were cultivated with Dulbecco's modified Eagle's medium (DMEM; PAA, Cölbe, Germany) supplemented with penicillin/streptomycin, L-glutamine, sodium pyruvate and 10% porcine serum (PAA, Cölbe, Germany). The cells were incubated at 37°C under 5% CO_2. The medium was changed every 2 days, and the cells were subcultured by trypsin-ethlendiaminetetracetic acid (EDTA) digestion when they reached confluence as assessed by phase contrast microscopy. The second passage cells were used in all experiments.

Permeability assay

The transepithelial permeability was evaluated as previously described [9]. In brief, second passage RPE cells were cultured on top of transwell-clear polyester membrane chamber for six-well plates (0.4 micrometer pore size) (Sigma, St. Louis, MI) and used in experiments 4 to 5 weeks after reaching confluence. The cells were treated with TA (10^{-5} M) (Sigma, St. Louis, MI) for 30 min. A 1000 fold TA stock was used and methanol control was included. The concentration of TA (10^{-5} M) was decided due to the previous studies [37, 39], in which the protective effect of TA was obvious in the range of 10^{-6} M-10^{-4} M. Medium in the upper chamber (cell's apical side) was then replaced to 4 kDa Fluorescein isothiocyanate (FITC)-dextran (4FD; Sigma, St. Louis, MI)-containing medium and cells were challenged with 1 mM hydrogen peroxide (H_2O_2). Five hours later, the medium from lower chamber (cell's basolateral side) was collected and the amount of fluorescence was measured using a fluorescence spectrofluorophotometer (FP-550, Jasco, Japan) with an excitation

wavelength of 488 nm and an emission wave length of 530 nm. Fluorescence intensity of the normal medium was measured as a blank, which was subtracted from the intensity of the samples.

After collecting the lower medium at the first day (day 1), the medium was completely replaced by the new one and the transepithelial flux of FITC-dextran for 5 h was investigated as described above at day 2, 4, 6, 8, 10, 14, 21.

Immunohistochemistry

The second passage porcine RPE cells were cultured on type I collagen-coated cover glass until 4 to 5 weeks after reaching confluence. The cells were treated or untreated with TA and then challenged with 1 mM H_2O_2. Five hours after H_2O_2 stimulation, the cells were washed with PBS, fixed in 3% paraformaldehyde in PBS for 15 min on ice and again washed three times with PBS at room temperature. Then the cells were permeabilized with 0.1% triton X-100 in PBS for 15 min. Cells were then incubated in PBS containing 1% bovine serum albumin (BSA) (blocking buffer) for 20 min at room temperature and incubated with primary antibody (mouse anti-occludin antibody, diluted 1:100; Sanko Junyaku, Tokyo, Japan) overnight at 4°C. After three times rinsing with blocking buffer, cells were incubated with secondary antibody (TRITC-conjugated anti rat antibody, diluted 1:100; Santa Cruz Biothec. Santa Cruz, CA) for 1 h at room temperature. F-actin was costained with FITC-conjugated phalloidin (Sigma, St. Louis, MI). Coverslips were washed and mounted with antifade regent on slides and examined under a fluorescence microscope (Carl Zeiss, Jena, Germany).

Glutathione assay

The second passage RPE cells were cultured on 35 mm cell culture dish (Nunc, Roskilde, Denmark) and were stimulated by H_2O_2 (1 mM) with or without pretreatment of TA (10^{-5} M) for 30 min. Three hours later, the cells were collected by trypsinization, and cellular total glutathione, oxidized glutathione (GSSG) and reduced glutathione (GSH) were measured by using a commercial kit according to the manufacture's protocol (Trevigen Inc., Gaithersburg, MD). This assay utilizes a kinetic enzymatic recycling reactions of glutathione. Once GSSG reacts with NADPH2, glutathione reductase reduces GSSG to GSH, which reacts with 5, 5′-dithiobis-2-nitrobenzonic acid (DTNB) to produce a yellow-colored 5-thio-2-nitrobenzonic acid (TNB) that absorbs at 405 nm. Another product of this reaction, GSTNB (GSH and TNB), is reduced by glutathione reductase to produce GSH and as a result, produces more TNB. The rate of TNB production, which was monitored over 10-min period, is directly proportional to the concentration of total glutathione in the sample. With the addition of 4-Vinylpyridine at the beginning of the assay, every free thiol reaction can be blocked, thus any contribution to the cycling reaction caused by GSH is eliminated. Therefore, only GSSG concentration can be measured. Reduced glutathione concentration can be obtained by subtracting GSSH from total glutathione.

Statistical analysis

Each experiment was conducted triplicates and repeated three times.

Statistical significance was determined by paired Student's *t*-test. A P-value less than 0.05 was considered to be significant.

Results

The time after confluence affects the resistance of RPE cells against oxidative stress

The resistance of cultured RPE cells against H_2O_2 stimulation was apparently different dependent on the time after confluence. This was preliminary confirmed (data not shown) and the data were consistent with the previous report by Bailey et al, in which the cultured RPE cells 5 weeks after confluence was more resistant to H_2O_2 stimulation both in viability and paracellular permeability compared to the cells of 1 week after confluence [9]. Concerning the concentration of H_2O_2, according to the preliminary experiments using the RPE cells 5 weeks after confluence, 1 mM was the best concentration just to induce non-lethal oxidative junctional damage. 2 mM H_2O_2 was always lethal for these cells, and 0.5 mM sometimes did not have any effect on juctional integrity. Therefore, in this study, the cells 5 weeks after confluence were used and the concentration of H_2O_2 was determined 1 mM to induce non-lethal junctional damage.

Effect of H_2O_2 on RPE junctional integrity and the protective effect of TA

In permeability assay, the flux of FITC-dextran through the non-treated RPE cell culture (5 weeks after confluence on the membrane) was detected as very low (close to zero) fluorescence intensity, and this level was determined as a control level (100%) (Fig. 1). H_2O_2-treated RPE cells showed a significantly higher transepithelial flux of FITC-dextran (283% of control, $P<0.01$). TA reduced this increase significantly (TA+H_2O_2-treated cells; 203% of control, 71% of H_2O_2-treated cells, $P<0.01$). TA-treated cells did not show a significant difference from control

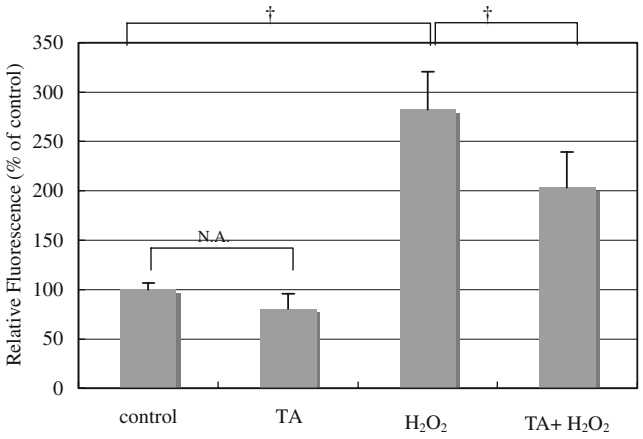

Fig. 1 Transepithelial flux of FITC-dextran for 5 h through the confluent RPE cell culture under the condition with no treatment (control), 30-min pretreatment of 10^{-5} M triamcinolone acetonide (TA), 1 mM hydrogen peroxide (H_2O_2), and 30-minute pretreatemnt of 10^{-5} M TA+1 mM H_2O_2 (TA+H_2O_2): H_2O_2 incrased transepithelial flux and TA inhibited this increase significantly. †P<0.01. Error bar shows standard deviation (SD)

cells. There were no morphological changes or defects of the cells in all cultures under the light microscopic examination.

Time-course change of the paracellular permeability after H_2O_2 stimulation and the protective effect of TA

We also investigated the time-course changes of the transepithelial flux of FITC-dextran until the 21st day (Fig. 2). The H_2O_2-treated RPE cells showed the highest flux at the first day (283% of control). After the removal of

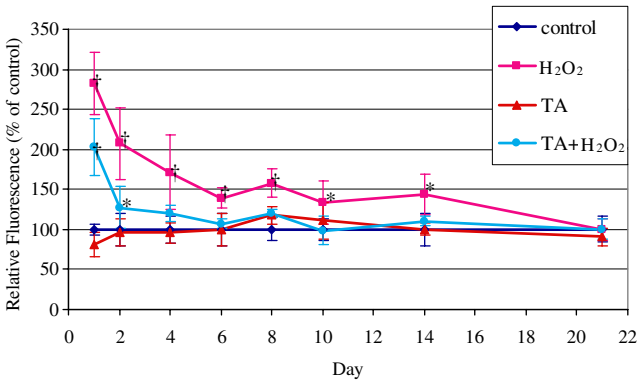

Fig. 2 Time-course change of transepithelial flux of FITC-dextran. After the test on day 1 (result shown in Fig. 1), the medium was completely changed to fresh one (no treatment). On day 2, 4, 6, 8, 10, 14, 21, the transepithelial flux of FITC-dextran for 5 h was measured in the same way as on day 1: H_2O_2 –treated and TA+H_2O_2 –treated cells showed the recovery to the normal level within 21 days. TA+H_2O_2 –treated cells maintained the lower flux level than H_2O_2 –treated cells during the experimental period and recovered to the normal level earlier. †P<0.01, * P<0.05 compared to the non-treated control cells. Error bar shows standard deviation (SD)

oxidant stimulation, the flux reduced and recovered to the normal level in 21 days. Transepithelial flux through TA+H_2O_2-treated RPE cells reduced dramatically at day 2 (from 203% to 127%) and maintained the lower flux level during the experimental period. TA-treated RPE cells did not show any significant difference from control cells.

The effect of TA on H_2O_2-induced disruption of occludin and F-actin distribution

In control cells, occludin displays linear staining at the cell border (Fig. 3a). F-actin filaments are also distributed at the cell border and show the linear staining (actomyosin ring) (Fig. 3b). In H_2O_2-treated cells, the linear occludin staining at the cell border is less clear (Fig. 3c, arrowhead), and more occludin staining can been seen in cytoplasm (Fig. 3c, asterisk). H_2O_2 increased the actin stress fibers and the disruption of actomyosin ring (Fig. 3d, arrowhead). TA pretreatment reduced H_2O_2-induced these changes in occludin and F-actin. Occludin linear staining is more apparent, although some disturbance by H_2O_2 can still be seen (Fig. 3e, f). These findings are consistent to the results of permeability assay, in which TA partially inhibited oxidant-induced paracellular permeability increase at day 1 (Fig. 1, 2). Occludin and F-actin localization of TA-treated cells were comparable to the control (data not shown).

The effect of TA on glutathione balance

From the results above, it was suggested that TA has a protective effect against oxidative stress on RPE cells. Thus, we investigated the effect of TA on the cellular glutathione level and redox balance in RPE cells. Table 1 shows the amount of total, reduced (GSH) and oxidized (GSSG) glutathiones, and the GSH/GSSG ratio of non-treated control cells and the differently treated cells. TA increased the cellular glutathione level (128.7 pmol/10^6 cells, P<0.05) by increasing GSH level (127.0 pmol/10^6 cells, P<0.05), and GSH/GSSG ratio (73.2) was slightly higher than the control cells (statistically not significant). H_2O_2 increased cellular GSSG level (2.40 pmol/10^6 cells, P<0.05). GSH level also increased in H_2O_2-treated cells (122.7 pmol/10^6 cells, P<0.05), but it was not enough to preserve the GSH/GSSG ratio, which decreased significantly compared to control cells (52.6, P<0.05). In TA+H_2O_2-treated cells, GSSG was higher than control, but significantly lower than H_2O_2-treated cells (2.17 pmol/10^6 cells, P<0.05). They showed high GSH level (138.4 pmol/10^6 cells, p<0.01), and thus the GSH/GSSG ratio (65.6) was preserved to be comparable to the control cells. There was no significant differences among GSH/GSSG ratios of the control, TA-treated, and TA+H_2O_2-treated cells.

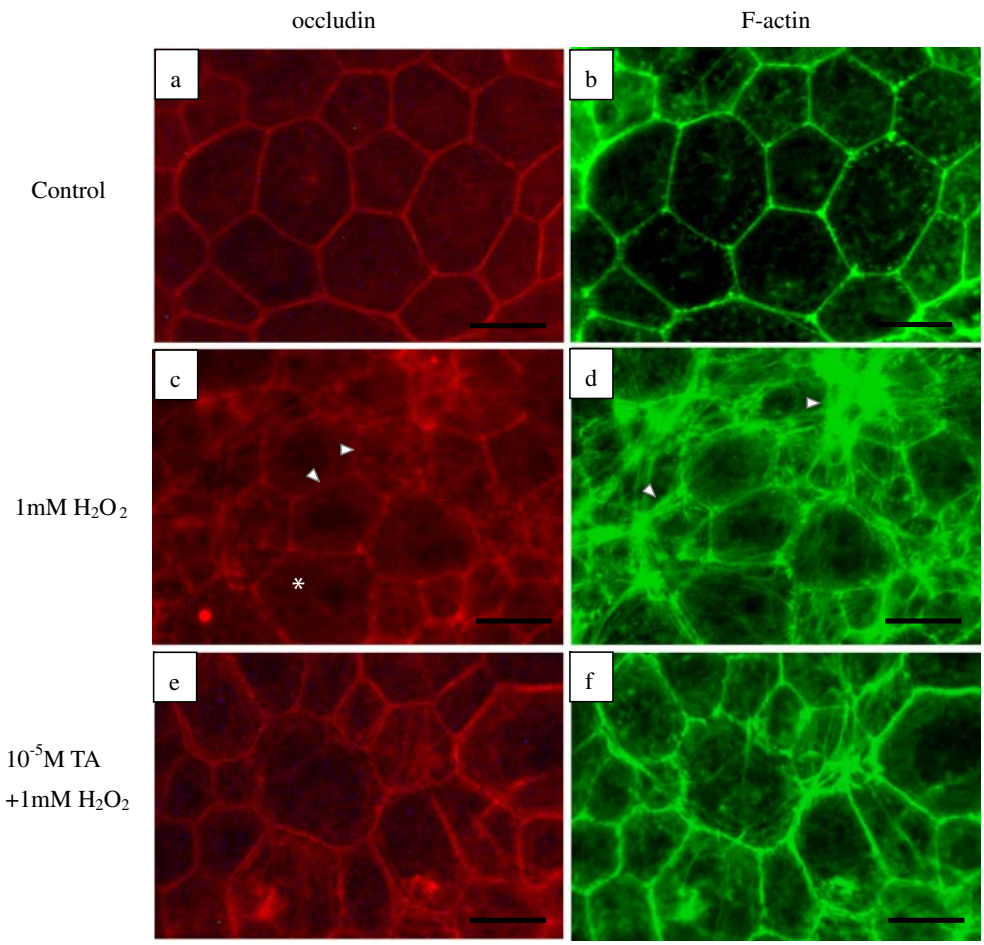

Fig. 3 Immunofluorescein staining of occludin (**a, c, e**) and F-actin (**b, d, f**) of the RPE cells 5 weeks after confluence. Occludin was stained with anti-occludin antibody and TRITC-conjugated second antibody. F-actin filament was co-stained with FITC-conjugated phalloidin. **a, b** control cells, **c, d** H_2O_2 (1 mM)-treated cells, **e, f** TA (10^{-5} M)+H_2O_2 (1 mM)-treated cells: H_2O_2 –treated cells showed less occuldin linear staining at the cell border (**c**, arrow head) and more in cytoplasm (**c**, asterisk), and more actin stress fibers (**d**, arrowhead) compared to the control cells. TA reduced H_2O_2-induced these changes of occluding and actin fibers (**e, f**). Scale bar=10 μm

Discussion

Oxidative stress can damage the cells in many degrees, from nonlethal to lethal. It attacks a variety of cellular component, mainly DNA [43, 44] and cellular cytoskeleton [13, 45], and if the cells are not repaired properly, the accumulation of this damage can lead to cell death [46]. In this study, we focused on the oxidant-induced junctional damage with nonlethal oxidative stress.

Tight junctions create a strong barrier to the movement of water, solutes and immune cells, and its dysfunction leads to the increased paracellular permeability [47]. Tight junctions anchor physically into the apical actin cytoskeleton circumscribing the cell [48] which is known as the perijunctional actomyosin ring. The retention of the perijunctional actin ring results in the stable barrier function [49]. In our study, oxidative stress by H_2O_2 resulted in disruption and internalization of occludin protein along with reorganization of perijunctional actin rings and the increase of actin stress fiber formation. Some previous studies demonstrated the mechanisms of oxidative stress-induced actin reorganization in vascular endothelial cells [13, 50–52]. Regarding RPE cells, however, information about the mechanisms of this actin reorganization is not available yet and further investigation is needed.

Table 1 The effects of triamcinolone acetonide (TA) and hydrogen peroxide (H_2O_2) on glutathione balance in cultured RPE cells

	control	TA	H_2O_2	TA+H_2O_2
Total glutathione	119.0±7.7	128.7±9.6*	125.1±10.1*	140.5±7.7†
Oxidized (GSSG)	1.79±0.38	1.79±0.38	2.40±0.56*	2.17±0.45*
Reduced (GSH)	117.2±7.7	127.0±9.7*	122.7±9.9*	138.4±7.7†
GSH/GSSG ratio	67.6±14.9	73.2±16.8	52.6±10.7*	65.6±14,5

(pmol/10^6 cells)
†$P<0.01$, *$P<0.05$ compared to the non-treated control cells

To our knowledge, time-course follow-up of RPE paracellular permeability after H_2O_2 stimulation has not been investigated before.

The present study demonstrates that the H_2O_2-induced increase of paracellular permeability of RPE is reversible. This finding is supported by a previous study, in which the survival of RPE cell after prolonged oxidative stress was investigated [12]. In our study, only 30-min pretreatment of TA prevented the initial damage by H_2O_2, which can be detected as occludin and actin dislocalization accompanied by permeability increase, and resulted in an earlier recovery of RPE junctional function than H_2O_2-treated cells. In the pathogenesis of AMD or diabetic retinopathy, lethal damage is the last stage of degeneration, as seen in geographic atrophy of dry AMD. In most cases, nonlethal damage might be repeated and affect cell function gradually, which decreases the cellular recovery potential in the long run. Marin-Castaño reported that nonlethal oxidative stress to human RPE cells causes cells membrane blebbing [53] and repetitive damage decreased extracellular matrix turnover and induced sub-RPE deposits [54]. We suspect that repetitive oxidative stress affects not only the turnover of extracellular matrix, but also many other functions of RPE cells, which have to be elucidated in future investigations. Therefore, cellular damage from oxidative stress should be minimized even if nonlethal and recoverable, and the protective effect of TA shown in this study has a significant implication.

The redox status of the cells is of significance to determine the cellular anti-oxidant defense. It is largely determined by reduced glutathione (GSH), which is a major nonprotein cellular thiol in mammalian cells, and accounts for more than about 98% of intracellular total glutathione in healthy cells [55–57]. GSH and its precursors have been reported to protect cultured RPE cells from oxidant-induced apoptosis [58–60]. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) (GSH/GSSG ratio) has been widely used as an indicator of cellular redox status [61]. The preservation of cellular GSH/GSSG ratio means less oxidative damage in the cell. Thus, for the pathogenesis and treatment of AMD and diabetic retinopathy, this redox status is one of the important factors which may play crucial roles.

H_2O_2 stimulation decreased cellular GSH/GSSG ratio significantly, with the increase of GSSG. Decreased GSH/GSSG ratio indicates cellular oxidative stress and less antioxidant defense. Redox imbalance can induce the oxidative damage of intracellular proteins and nucleotides, thus the functional damages, which can be lead to cell death, either apoptosis or necrosis [61]. Our result suggests that TA increases GHS synthesis to preserves the redox balance, thus maintains adequate cellular antioxidant defenses. Recently, the protection of RPE cells from oxidative stress is of significant interest among researchers [62–66]. The protective effect of TA, however, has not yet been reported, even though TA is being widely used in clinical practice.

The modulation of cellular tight junction protein expression and paracellular permeability by corticosteroid has been demonstrated in some cells, such as brain endothelial cells [67], mouse mammary epithelial cells [68] and retinal vascular endothelial cells [69]. In cultured RPE cells in this study, however, TA alone showed no significant effect on paracellular permeability. Thus we assume that, in RPE cells under oxidative stress, TA itself has no, or very little, direct effect on junction proteins of RPE cells, but has the effect mainly to protect them from oxidative injury, and in consequence, leading to the preservation of junctional integrity.

From previous studies, it has been shown that the mechanism of reducing macular edema by TA is considered to be via its anti-inflammatory/angiogenic effects on vascular endothelial cells [39] and down-regulation of VEGF expression [37]. Also in the treatment of AMD, it is thought that TA reduces the formation of choroidal neovasucularization (CNV) by downregulating of VEGF [37, 70]. TA promoted RPE cell proliferation to enclose the areas of CNV [39, 71]. Our study shows an additional effect and suggests that stabilized outer BRB by TA might contribute to the healing process and the stabilization of visual acuity.

In this study, the effectiveness of TA in protecting RPE cellular junctional integrity under oxidative stress was proven. The results provide further important information in understanding the therapeutic effect of TA. Further investigations are needed to elucidate more details, such as intracellular signaling pathways.

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