Molecular Biology Reports (2024) 51:637
https://doi.org/10.1007/s11033-024-09595-2

ORIGINAL ARTICLE

Vitamin C alleviates hyperglycemic stress in retinal pigment epithelial cells

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Received: 2 February 2024 / Accepted: 29 April 2024

Abstract

Background Retinal pigment epithelial cells (RPECs) are a type of retinal cells that structurally and physiologically support photoreceptors. However, hyperglycemia has been shown to play a critical role in the progression of diabetic retinopathy (DR), which is one of the leading causes of vision impairment. In the diabetic eye, the high glucose environment damages RPECs via the induction of oxidative stress, leading to the release of excess reactive oxygen species (ROS) and triggering apoptosis. In this study, we aim to investigate the antioxidant mechanism of Vitamin C in reducing hyperglycemia-induced stress and whether this mechanism can preserve the function of RPECs.

Methods and results ARPE-19 cells were treated with high glucose in the presence or absence of Vitamin C. Cell viability was measured by MTT assay. Cleaved poly ADP-ribose polymerase (PARP) was used to identify apoptosis in the cells. ROS were detected by the DCFH-DA reaction. The accumulation of sorbitol in the aldose reductase (AR) polyol pathway was determined using the sorbitol detection assay. Primary mouse RPECs were isolated from adult mice and identified by *Rpe65* expression. The mitochondrial damage was measured by mitochondrial membrane depolarization. Our results showed that high glucose conditions reduce cell viability in RPECs while Vitamin C can restore cell viability, compared to the vehicle treatment. We also demonstrated that Vitamin C reduces hyperglycemia-induced ROS production and prevents cell apoptosis in RPECs in an AR-independent pathway.

Conclusions These results suggest that Vitamin C is not only a nutritional necessity but also an adjuvant that can be combined with AR inhibitors for alleviating hyperglycemic stress in RPECs.

Keywords Hyperglycemia · Retinal pigment epithelial cell · ROS · Apoptosis · Vitamin C

Abbreviations

AGEs	Advanced Glycation End products
AR	Aldose Reductase
ARPE	Adult Retinal Pigment Epithelium
DM	Diabetes Mellitus
DR	Diabetic Retinopathy
FOXO3	Forkhead box O3
PARP	Poly (ADP-ribose) Polymerase
PKC	Protein Kinase C
ROS	Reactive Oxygen Species
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
HG	High Glucose
LG	Low Glucose
RPEC	Retinal Pigment Epithelial Cell

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Published online: 10 May 2024

Introduction

The pathophysiology of diabetes mellitus (DM) gradually progresses to affect various body organs and tissues [1]. DM is characterized by hyperglycemia, which arises from metabolic dysfunctions in either insulin synthesis, insulin activity, or both [2]. Diabetic retinopathy (DR) is a severe microvascular complication of diabetes that targets the blood vessels of the retina and can progressively lead to other vision complications including vitreous hemorrhage, retinal detachment, and glaucoma [3, 4]. Globally, the number of people with DR will grow from 103 million in 2020 to 160 million in 2045 and the number with vision-threatening diabetic retinopathy will increase from 28 million to 45 million during the same time [5]. Among the molecular mechanisms that underlie the pathogenesis of DR are increased polyol pathway flux, heightened levels of reactive oxygen species (ROS), and dysregulated inflammatory response [6]. All these pathways directly or indirectly induce oxidative stress, leading to several cellular changes including the upregulation of vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF) that eventually contribute to the pathogenic mechanism of DR [7–9]. Therefore, oxidative stress and inflammation play a crucial role in the progressive loss of neurons and pericytes observed in DR [10, 11].

The polyol pathway, a two-step pathway of glucose metabolism, is implicated as a plausible contributor to the underlying mechanism of diabetic retinopathy as it leads to the overproduction of ROS and increased proinflammatory response during hyperglycemic conditions [6]. The activity of aldose reductase (AR), the first and rate-limiting enzyme of the polyol pathway, is associated with the hyperglycemia-induced apoptosis of retinal pericytes and endothelial cells [12]. AR catalyzes the reduction of glucose to sorbitol in the presence of nicotinamide adenine dinucleotide (NAD) and phosphate (NADPH), which serves as a cofactor in the reaction [13]. Due to elevated glucose concentrations (over 125 mg/dL before a meal or greater than 180 mg/dL 2 h postprandial) [14], the acceleration of AR polyol pathway causes the accumulation of sorbitol in both the lens and retina [15]. Since sorbitol does not passively diffuse through cell membranes, it accumulates at excessive levels, triggering osmotic stress within the eye [16]. Additionally, the increased activity of AR enhances the depletion of NADPH, which is essential for the production of GSH (antioxidant coenzyme) [17]. As the intracellular levels of GSH diminish, the cellular antioxidant capacity decreases, leading to an increased susceptibility of retinal cells to oxidative stress [18]. Retinal pigment epithelial cells (RPECs) not only function to absorb light [19], but also to maintain photoreceptor excitability [20]. In a hyperglycemic environment, a buildup of sorbitol in the AR polyol pathway leads to ROS elevation and apoptosis in RPECs [21]. Therefore, the reduction of the up-regulated activity of AR in RPECs under hyperglycemic conditions serves as a promising therapeutic strategy to alleviate or prevent the complications of diabetic retinopathy [22].

Vitamin C, also known as L-ascorbic acid, is a natural vitamin that can be obtained from many fruits and vegetables. Vitamin C is known as a vital physiological antioxidant that plays a critical role in scavenging oxygen-derived free radicals [23]. There are two major forms of Vitamin C: ascorbic acid and dehydroascorbate (DHA) [24, 25]. The oxidized form of Vitamin C, DHA, passes through the plasma membrane mainly via glucose transporter protein type 1 (GLUT1) and is intracellularly converted to ascorbic acid via the activity of dehydroascorbate reductase (DHAR) [26, 27]. Ascorbic acid, which is the reduced form of Vitamin C, diffuses across the plasma membrane via Sodium-Dependent Vitamin C Transporters (SVCTs) [28]. SVCT2, specifically, exhibits high expression in the brain, and thus appears to be the main source of ascorbic acid uptake in the retina [29]. Studies have shown that the inhibition or depletion of SVCT2 from the plasma membrane reduces the intracellular uptake of AA [30], and induces microglia activation [31] and oxidative stress [32] in the ocular cell system. Studies have shown that the concentrations of Vitamin C in diabetic patients with retinopathy were lower compared to those without [33–35], suggesting that the antioxidant capacity of Vitamin C is reduced under hyperglycemic conditions [36]. Vitamin C has low toxic effects and has been demonstrated to be effective in reducing oxidative damage through the removal of intracellular ROS [37]. However, the effect of Vitamin C on alleviating oxidative-induced stress in adult retinal pigment epithelial (ARPE-19) cells, which is central to retinal health and homeostasis as well as is involved in the pathophysiology of DR [38, 39], is still unclear. In this study, we aimed to study whether the antioxidant mechanism of Vitamin C can prevent oxidative-induced death in RPECs. We examined the efficacy of Vitamin C in protecting cell viability, reducing of sorbitol accumulation and ROS formation, and alleviating apoptosis-induced cell death in RPECs exposed to high glucose (HG) conditions. Our results suggest that the antioxidant mechanism of Vitamin C is potent in reducing programmed cell death under hyperglycemic conditions. However, Vitamin C was not able to reduce sorbitol accumulation, which might suggest that Vitamin C protects RPECs in an AR-independent manner. Overall, the data derived from cell culture experiments indicate that Vitamin C may mitigate oxidative stress and cellular demise induced by hyperglycemia in RPECs. However, further investigations are warranted to elucidate its potential therapeutic efficacy in clinical settings.

Methods

Materials and cell culture

Adult retinal pigment epithelium (ARPE-19) cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Corning Cellgro, Manassas, VA, USA) formulated with low glucose (1 g/L) and supplemented with 4 mM l-glutamine, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cultures of ARPE-19 cells were allowed to grow at 37 °C in a 5% CO_2 humidified atmosphere. Vitamin C was purchased from (PHR1008-25; Sigma-Aldrich). Cell viability was measured by MTT, as described previously [21]. Primary RPE cells were collected from ~8–16 weeks C57BL/6 mouse eyes and purified as described previously [40].

Polymerase chain reaction

To confirm the primary tissues from the mouse are RPE, we collected the mouse lens (negative control) and primary PRE culture for PCR, examined by the RPE-specific primer. Total RNA was isolated (RNeasy Microarray Tissue Mini Kit; Qiagen) according to the manufacturer's protocol. Using the iScript cDNA Synthesis Kit (Bio-Rad), RNA (5 µg) was reverse-transcribed according to the manufacturer's protocols. Rpe65 was used as a primer (Forward: AA GGCTCCTCAGCCTGAAGTCA; Reverse: GAGAACCT C-AGGTTCCAGCCAT) in PCR to determine the presence of RPE cells, and GAPDH (Forward: CATCACTGCCAC CCAGAAGACTG; Reverse: ATGCCAGTGAGCTTCCC GT-TCAG) was used as the loading control for lens tissues and RPE cells. The PCR process was conducted according to the protocol. Twenty-five cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) were used for PCR reactions with *Vio-Taq* DNA polymerase. After amplification, the same volume of each PCR product was loaded onto 1.5% agarose gel containing SYBR Safe DNA Gel Stain (ThemorFisher Scientific). The DNA gel was imaged with GelDoc Go Imaging system (Bio-rad).

Western blotting

ARPE-19 cells (2.5×10^6 cells) were coated on 60 mm dishes overnight at 37 °C in a humidified incubator containing 5% CO_2. Cells were pretreated with Vitamin C and low glucose or low glucose only and incubated for 1 h at 37 °C, 5% CO_2. Cells in each dish were then treated with one of four treatments: low glucose (LG, 5.5 mM), high glucose (HG, 25 mM), low glucose and Vitamin C, or high glucose and Vitamin C. Treated cells were incubated for 72 h at 37 °C, 5% CO_2. Following a DPBS wash, cells were harvested, suspended in RIPA buffer (Thermo FisherScientific), and sonicated. Cell lysates were then heated to 95 °C for 10 min, and protein quality was monitored by SDS-PAGE (4,561,096; Bio-Rad). Protein samples were transferred to PVDF membranes (L002045A; Bio-Rad). For immunodetection, the following primary antibodies were used: PARP rabbit antibodies (1:1000) and GAPDH rabbit antibodies (1:4000) (from Cell Signaling Technology, Inc.). To detect chemiluminescence, secondary goat anti-rabbit antibodies (1:5000, IRDye® 800CW; LI-COR) and LI-COR Odyssey® Imaging System were used. Three independent samples from different times were collected for this experiment.

Sorbitol colorimetric assay

ARPE-19 cells (2.5×10^6 cells) were incubated in 60 mm dishes overnight at 37 °C. After pretreatment with Vitamin C and low glucose or low glucose only, cells were incubated for 1 h at 37 °C. Cells were then treated with LG, HG, LG and Vitamin C, or HG and Vitamin C and incubated for 48 h at 37 °C, 5% CO_2. After washing with DPBS twice, cells were scraped from the dishes and suspended in nuclease-free water (AM9937; Thermo Fisher Scientific). Sorbitol was measured in the protein samples using a D-Sorbitol Colorimetric Assay Kit and protocol (MKA010-1KT; Sigma-Aldrich) as well as SpectraMax M3 Plate Reader. Three independent samples from different times were conducted for this experiment.

Measurement of ROS levels

ROS measurement was conducted as previously described [21]. ARPE-19 cells (3.6×10^3 cells) were coated on a 96-well plate. Cells were first treated with LG, HG, LG and Vitamin C, or HG and Vitamin C for 1 d followed by incubation with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for another 30 min. Cells were then washed with DPBS before measurement. The fluorescence was measured by SpectraMax M3 Plate Reader at excitation of 488 nm and emission of 525 nm. Three independent samples from different times were conducted for this experiment.

Measurement of mitochondrial membrane potential

Primary RPE cells were coated on a clear bottom black 96-well plate. After pretreatment with Vitamin C for 1 h, cells were incubated with low glucose or high glucose treatment for 5 h. 50 µl of JC-10 dye loading solution (Abcam, ab112134) was added into each well (containing 100 µl medium) for 1 h at 37 °C. Then, 50 µl assay buffer B was directly added to each well. JC-10 is capable of selectively

entering mitochondria, and reversibly changes its color from green to orange as membrane potentials increase. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates. However, in apoptotic and necrotic cells, JC-10 exists in monomeric form and stains cells green. The fluorescence intensities were monitored at Ex/Em = 490/525 nm (cut off at 515 nm) and 540/590 nm (cut off at 570 nm) for ratio analysis. The change of mitochondrial membrane potential was measured as the ratio between aggregate (Em = 590 nm) and monomeric forms (Em = 515 nm) of JC-10. Increasing ratios (515/590) indicate mitochondrial membrane depolarization. Three independent samples from different times were conducted for this experiment.

Statistical analysis

Data were denoted as the means ± SEM of at least three independent experiments. Student's t-test was used to analyze experimental results. The criterion for significance was $P < 0.05$.

Results

Vitamin C alleviates hyperglycemia-induced cell viability reduction

Under hyperglycemic conditions, the reduction of glucose to sorbitol via the activity of AR leads to excessive accumulation of ROS in various tissues of DM [41]. In particular, previous studies have shown that increased levels of ROS production under HG conditions trigger apoptosis in retinal cells [42]. As a result, we aimed to investigate the protective role of Vitamin C in the reduction of ROS production and prevention of oxidative stress-cell death. We first tested the cytotoxicity of different concentrations of Vitamin C on ARPE-19 cells. Our data showed that a low concentration (5 µM) and high concentration (100 µM) of Vitamin C have similar effects on ARPE-19 cell viability **(**Fig. 1A**)**. However, multiple studies have reported that the antioxidant activity of Vitamin C is enhanced at a concentration of 100 µM [43]. In this study, therefore, we used 100 µM Vitamin C to investigate the effect of the antioxidant on ARPE-19 exposed to oxidative stress. Compared to ARPE-19 cells grown under LG conditions (5.5 mM), the growth of cells in HG conditions (25 mM) decreased by 22% and 30% after 24–48 h of incubation, respectively **(**Fig. 1B**)**. A 48-h incubation of ARPE-19 cells with 25 mM D-glucose in the presence of Vitamin C enhanced cell viability by 20%, compared to the control treatment group **(**Fig. 1C**)**.

Vitamin C treatment attenuates HG-induced apoptosis

Poly(ADP-ribose) polymerase (PARP) is a ubiquitous nuclear enzyme that is involved in various cellular processes including DNA repair, regulation of transcription, and programmed cell death [44]. PARP is cleaved in fragments of 89 and 24 kDa by caspase enzymes during apoptosis,

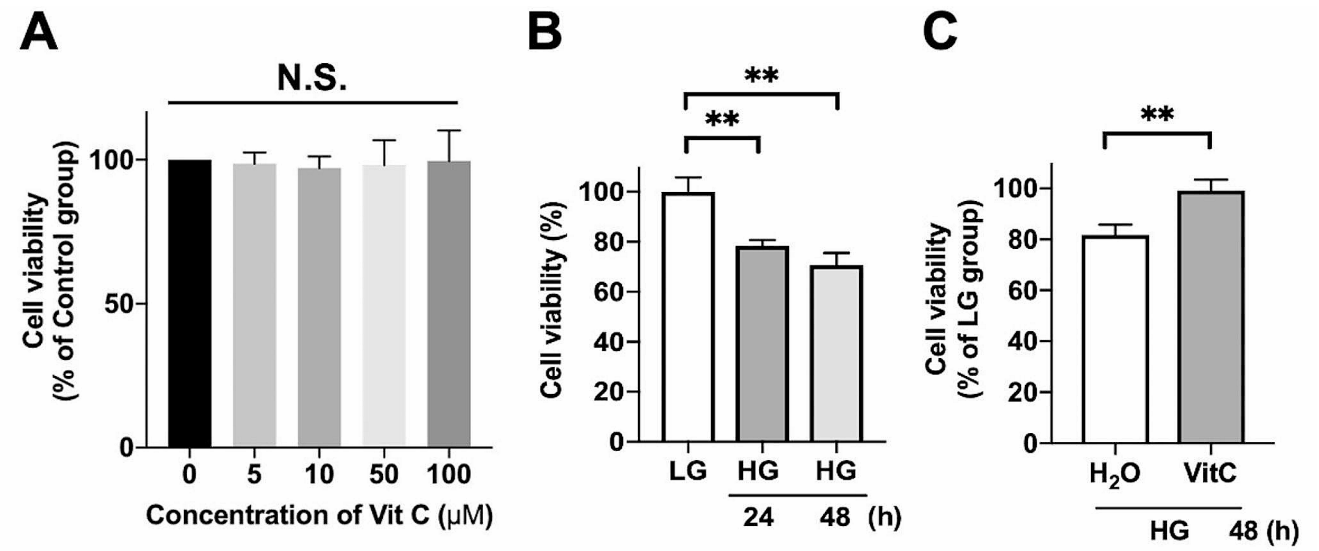

Fig. 1 Cell viability of ARPE-19 cells is improved after Vitamin C treatment hyperglycemic levels. **(A)** ARPE-19 cells were cultured with various concentrations of Vitamin C for 48 h. No significant difference in cell viability was observed within groups. **(B)** ARPE-19 cells were cultured in low glucose (LG, 5.5 mM) or high glucose (HG, 25 mM) for 24–48 h. Hyperglycemia reduces cell viability in ARPE-19 cells. **(C)** ARPE-19 cells were co-culture with Vitamin C (100 µM) and HG for 48 h. Cell viability of ARPE-19 cells was improved by Vitamin C treatment. Data shown are means ± SEM ($N = 3$). $^{**}P < 0.01$., N.S. = Not Significant

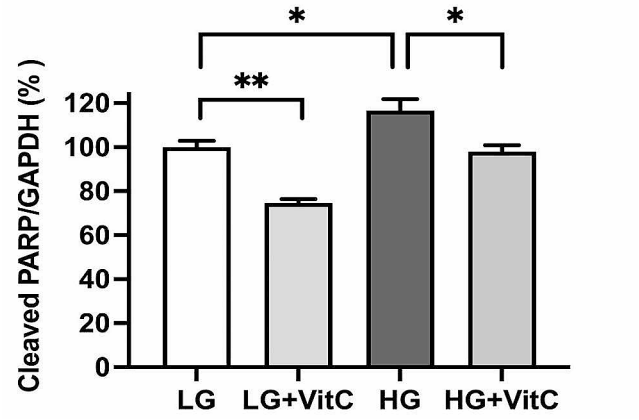

Fig. 2 Hyperglycemia-induced apoptosis is attenuated by Vitamin C treatment in ARPE-19 cells. ARPE-19 cells were treated with the following conditions: low glucose (LG, 5.5 mM), low glucose + 100 µM Vitamin C (LG + VitC), high glucose (HG, 25mM), or high glucose + 100µM Vitamin C (HG + VitC) for 3 d. The apoptosis was confirmed by Western blot using a cleaved PARP antibody. Data shown are means ± SEM ($N = 3$). *$P < 0.05$, **$P < 0.01$

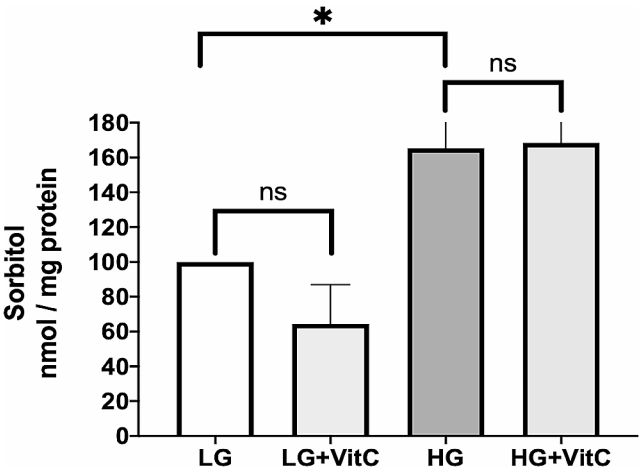

Fig. 3 Vitamin C does not inhibit the aldose reductase polyol pathway. ARPE-19 cells were treated with either of the following conditions: low glucose (LG, 5.5 mM), low glucose + 100 µM Vitamin C (LG + VitC), high glucose (HG, 25 mM), or high glucose + 100µM Vitamin C (HG + VitC) for 48 h. The sorbitol level in cell lysates was measured by using a sorbitol colorimetric assay. Data shown are means ± SEM ($N = 3$).*$P < 0.05$, ns = Not Significant

serving as an important hallmark of oxidative stress-induced death in ARPE-19 cells [45]. Therefore, we studied whether Vitamin C can potentially mitigate cell death that results from the cleavage of PARP. Our results suggested that HG conditions increased apoptotic cell death by 16% compared to LG conditions **(**Fig. 2**)**. Under physiological glucose concentration (5.5 mM), ARPE-19 cells that were treated with Vitamin C showed a significant reduction of apoptosis (74%) in comparison to cells that did not receive the treatment **(**Fig. 2**)**. Our data also demonstrated that Vitamin C was able to reduce the induction of apoptosis in cells treated with high glucose concentration (18% less apoptosis in cells treated with Vitamin C under hyperglycemic condition) **(**Fig. 2**)**. Taken together, these results suggest that Vitamin C plays a protective role in ARPE-19 cells under hyperglycemic conditions.

Vitamin C does not influence sorbitol accumulation

AR catalyzes the reduction of glucose to sorbitol, leading to the formation of nicotinamide adenosine dinucleotide phosphate (NADP+) as a byproduct [46]. Under hyperglycemic conditions, the depletion of GSH contributes to excess ROS production while the accumulation of sorbitol in the polyol pathway further promotes ROS formation as well as pro-inflammatory responses [47]. Our study investigated the potential inhibitory effect of Vitamin C on the mechanism of AR. The results of our study showed that ARPE-19 cells treated with Vitamin C (100 µM) had similar levels of sorbitol contents under LG (5.5 mM) and HG (25 mM) conditions **(**Fig. 3**)**. Our results may indicate that the Vitamin C does not interfere with the catalytic activity of AR in the polyol pathway for preventing hyperglycemia-induced cell death.

Vitamin C attenuates hyperglycemia-induced ROS production

In ARPE-19 cells subjected to high glucose concentrations, studies report elevated levels of ROS, leading to mitochondrial dysfunction and reduction in the cellular antioxidant capacities [48]. Sustained exposure to ROS, such as that observed in DR, triggers the modification of regulatory genes and further increases ROS overproduction. In this study, we examined how the antioxidant activity of ascorbic acid can impact the increased production of ROS in ARPE-19 cells treated with high glucose concentration. Our data demonstrated that ARPE-19 cells that received Vitamin C (100 µM) as a treatment under high glucose culture conditions (25 mM) had a 16% reduction in ROS levels, compared to cells that received only the high glucose treatment **(**Fig. 4A**)**. These results manifested that the mechanism of Vitamin C is critical in protecting ARPE-19 cells against oxidative stress provoked by increased ROS levels.

High glucose induces mitochondrial depolarization in both ARPE-19 and primary mouse RPE

RPE65 is a protein expressed in abundance in RPE and its production is critical for the function of the visual cycle [49]. The lack of functional RPE65 in mice has been shown to induce apoptosis in retinal cells [50] and is associated with vision impairment [51]. To confirm the finding that

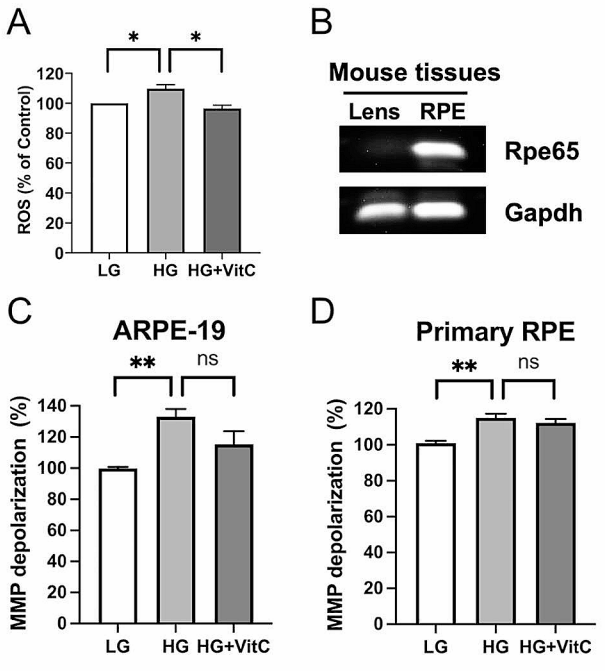

Fig. 4 High glucose induces ROS production and MMP depolarization. **(A)** ARPE-19 cells were treated with low glucose (LG, 5.5 mM), high glucose (HG, 25mM) or high glucose + 100μM Vitamin C (HG + VitC) for 1 d. Vitamin C attenuates ROS production in the HG condition. **(B)** The RNA from the mouse lens and RPE tissues were collected for PCR. The result confirmed the primary RPE culture by probing *Rpe65*. ARPE-19 cells **(C)** and primary mouse RPECs **(D)** were treated with LG, HG, and HG + VitC for 5 h. The HG condition increased mitochondrial depolarization in both ARPE-19 cells and primary mouse RPECs. The Data shown are means ± SEM ($N = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$. ns = Not Significant

observed in the RPE cell line is also validated in primary RPE cells, we isolated primary RPE cells from mouse retinas, identified by RPE marker *Rpe65*, for additional study **(**Fig. 4B**)**. We then examined the mitochondrial membrane potential (MMP) of primary RPE cells and ARPE-19 cells under a high glucose environment. Our data demonstrated that 5 h of high glucose treatment disrupted the MMP of both ARPE-19 cells **(**Fig. 4C**)** and primary mouse RPE **(**Fig. 4D**)**, suggesting that high glucose induces mitochondrial depolarization in ARPE-19 and primary RPE. However, Vitamin C did not attenuate mitochondrial depolarization suggesting that Vitamin C may play a role in chelating free radicals released from mitochondrial but does not protect mitochondria from damage.

Discussion

RPECs play a vital role in the hemostasis and development of photoreceptors residing in the retina [19, 20]. The pathophysiology of diseases that target the retinal vasculature, including glaucoma and retinal detachment, is associated in part with the dysfunction of RPECs. Oxidative-induced stress triggered under hyperglycemic conditions is implicated as one of the critical contributors to the mechanism of DR [52]. An imbalanced production of ROS is generated as a result of the accumulation of sorbitol in the polyol pathway, signaling the progression of the deregulated inflammatory response as well as endothelial dysfunction [53]. Therefore, the reduction of excess production of ROS can be a potential and promising approach for the prevention or alleviation of diabetes-related diseases. The reaction mechanism of AR has been suggested as a plausible target for the development of effective interventions for DR and its different complications [6].

In this study, we studied the protective role of Vitamin C in reducing oxidative-induced stress triggered in ARPE-19 cells under high glucose concentrations. Previous studies have shown that Vitamin C functions as a scavenger of intracellular free radicals, potentially preventing cell damage and apoptosis [54]. By treating ARPE-19 with Vitamin C, we observed that Vitamin C does not affect cell viability but improves high glucose-induced ROS and apoptosis. A previous study showed that vitamin C was able to inhibit AR in erythrocyte [55]. Therefore, we wanted to know whether this is also the case in RPECs. Interestingly, our data showed that vitamin C does not inhibit AR activity in RPECs, suggesting that vitamin C may lead to different physiological influences in different cell types due to the different regulatory mechanisms.

Oxidative stress is involved in the development and progression of RPECs degeneration and dysfunction [56]. Increased levels of ROS contribute to the reduced expression of the Sirtuin 1 (SIRT1) protein, which plays a critical role in the regulation of apoptosis, inflammation and oxidative stress [57]. SIRT1 targets the functions of various cellular proteins including p53, which controls cell division and death, and Forkhead box O3 (FOXO3) expression, which is involved in the regulation of autophagy and cell survival [58]. Wei, et al. reported that Vitamin C enhances cell survival and protects against oxidative stress by increasing the expression of SIRT1 [59]. In this work, we studied the protective effect of Vitamin C on the viability of ARPE-19 cells under high glucose concentrations. Excessive or chronic ROS production can also alter the morphology and function of the mitochondrial membrane, thus disrupting MMP [60, 61]. Trudeau, Molina et al. reported that the mitochondrial morphology of rat PRECs recovered following a brief exposure to HG. Under sustained hyperglycemia, however, the researchers showed that RPECs exhibited a significant increase in fragmented mitochondria and underwent permanent changes in mitochondrial morphology [62]. To further validate our findings in primary RPECs, we then conducted

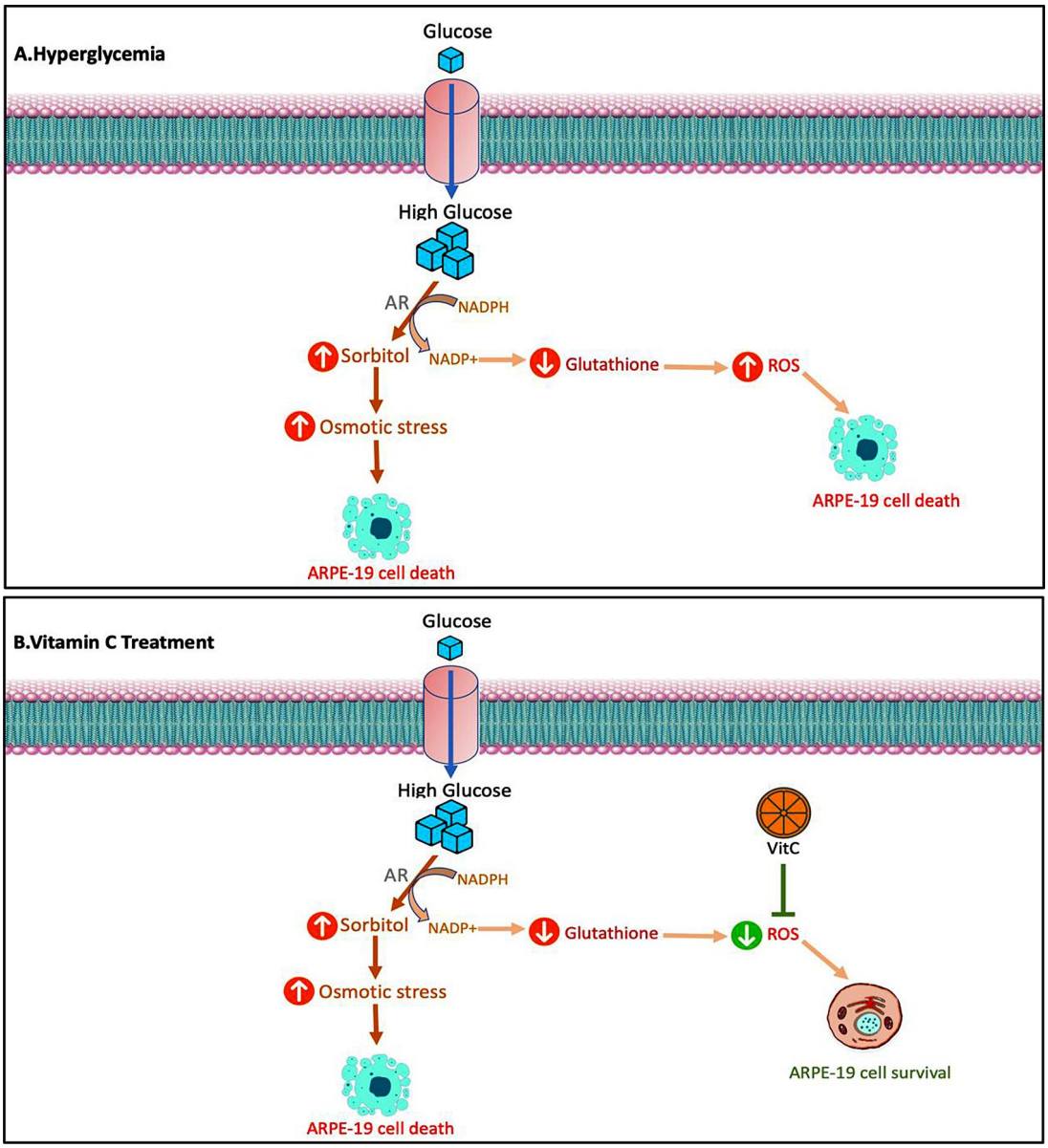

Fig. 5 Schematic diagram of the biochemical pathway involved in the induction of apoptosis, and the mechanism through which Vitamin C is predicted to attenuate oxidative stress. **(A)** As high levels of glucose enter the cell through the plasma membrane, the glucose can be converted into sorbitol via the activity of aldose reductase (AR) in the polyol pathway. Since sorbitol poorly diffuses through the cell membrane, the molecule accumulates inside the cell, causing water to move across cell membranes and trigger osmotic stress leading to cell death. The buildup of the reducing agent, NADP+, contributes to the reduction of glutathione and increased formation of ROS and eventually causes cell death. **(B)** Vitamin C passes through the plasma membrane via SVCT2 and inhibits ROS formation, increasing cellular stress resistance and the survival rate of ARPE-19 cells

a mitochondrial experiment in both ARPE-19 and primary mouse RPECs. The data showed that HG similarly increases MMP depolarization in both cell types, which warrants our experiments using ARPE-19 for the RPE study. However, vitamin C treatment slightly but not significantly reduces MMP depolarization, suggesting that vitamin C treatment induces other pathways for RPEC protection in the HG condition.

Under hyperglycemic conditions, ROS can be generated by a variety of cellular pathways including polyol pathway, hexosamine pathway, enediol pathway, protein kinase C (PKC) activation pathway, advanced glycation end products (AGEs) pathway in addition to the mitochondrial electron transport chain [63]. Excess levels of ROS trigger the release of cytochrome c from the mitochondrial intermembrane to the cytoplasm, leading to the activation of caspase-3, which initiates cell-programmed death through

the cleavage of PARP [64]. By promoting the proteolysis of PARP, caspase-3 inhibits critical cellular processes such as DNA repair and chromatin structure modulation [65]. In this study, we tested whether the antioxidant mechanism of Vitamin C can interfere with the processes of ROS production, potentially preventing the cleavage of PARP, and inhibiting or delaying cellular damage. ARPE-19 cells that were exposed to hyperglycemic conditions and treated with Vitamin C demonstrated reduced apoptotic activity and ROS formation.

Sorbitol accumulation in the polyol pathway during hyperglycemia initiates the depletion of glutathione (GSH) [66], promoting ROS production [67] and leading to the augmentation of oxidative-induced stress. As sorbitol has poor cell diffusion, the molecule accumulates excessively, triggering osmotic stress within the cell **(**Fig. 5A**)**. In the current study, we aimed to investigate whether the antioxidant activity of Vitamin C has an inhibitory effect on the catalytic mechanism of aldose reductase (AR). However, our study demonstrated that ARPE-19 cells that received either the control or Vitamin C treatments exhibited similar levels of sorbitol accumulation, suggesting that Vitamin C may not directly inhibit the catalytic conversion of glucose to sorbitol. Overall, our results indicate that while Vitamin C does not exert an inhibitory effect on the AR polyol pathway, however, its antioxidant properties can play a protective role against oxidative stress in ARPE-19 cells exposed to high glucose concentration **(**Fig. 5B**)**.

Conclusions

In summary, the finding in this study suggests that daily consumption of Vitamin C can effectively reduce ROS production in the eye, subsequently ameliorating RPEC damage. In a clinical setting, Vitamin C could be a promising adjuvant in parallel with AR pathway inhibition treatment in managing and alleviating the symptoms of hyperglycemia-induced retinal damage.

Acknowledgements We thank Chandler Meadows, Hui-Chun Cheng, Soureesh Moturi, and Shining Wang for their technical assistance.

Author contributions HA, CCL, ER, VYL conducted experiments and analyzed data; HA, PR and KCC wrote the manuscript; KCC supervised the whole project.

Funding The work was supported by the NIH Core Grant P30-EY008098, the Eye and Ear Foundation of Pittsburgh, an unrestricted grant from Research to Prevent Blindness, New York, NY, the Shaffer grant from Glaucoma Research Foundation, and the Biomedical Masters Program at University of Pittsburgh. VYL was supported by Hillman Academy.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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