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ORIGINAL ARTICLE

Glucosamine Attenuates Hydrogen Peroxide-Induced Premature Senescence in Human Retinal Pigment Epithelial Cells *In vitro*

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Aims: The purpose of this study was to investigate the effects of glucosamine (GlcN) on hydrogen peroxide (H_2O_2)-induced premature senescence in human retinal pigment epithelial (RPE) cells *in vitro*. **Materials and Methods:** We analyzed the cell viability of H_2O_2-treated human RPE cells by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate assay. The effect of GlcN on the intracellular levels of reactive oxygen species (ROS) in H_2O_2-treated human RPE cells was examined by the fluorescent dye 2′, 7′-dichlorodihydrofluorescein diacetate. The effect of GlcN on the stress-induced premature senescence (SIPS) in H_2O_2-treated human RPE cells was evaluated by senescence-associated β-galactosidase (SA-β-Gal) staining. We quantified the effect of GlcN on the protein levels of p21 in H_2O_2-treated human RPE cells by western blotting. **Results:** H_2O_2 reduced the cell viability of human RPE cells. H_2O_2 induced the increase of intracellular ROS, whereas GlcN reduced the increase of intracellular ROS due to H_2O_2 treatment in human RPE cells. GlcN reduced the SIPS in H_2O_2-treated human RPE cells and reduced the increase of the p21 protein level in H_2O_2-treated human RPE cells. **Conclusions:** GlcN attenuates the oxidative stress caused by H_2O_2 on the increase of ROS and the induction of SIPS in human RPE cells, at least in part, by suppressing the p21 pathway. These effects may contribute to the GlcN-mediated antioxidative effects in the eye in age-related macular degeneration.

Key words: Glucosamine, hydrogen peroxide, premature senescence, retinal pigment epithelial cells

INTRODUCTION

Age-related macular degeneration (AMD) can cause severe central vision loss in patients aged 75 or older,[1] which is initially caused by an age-related, progressive degeneration in the retinal pigment epithelium in the macular area of the retina.[2,3] RPE cells, located between the neuroretina and the choriocapillaris, which comprise the outer blood–retinal barrier, participate in the selective transport of metabolites, phagocytose the outer segments that are shed from photoreceptors, and act as antigen-presenting cells.[4-6] A literature review has demonstrated that photoreceptor outer segment phagocytosis, peroxidized lipid membranes, and photo-oxidative reactive oxygen intermediates are endogenous sources of oxidative stress, which can induce damage to the RPE cells.[3]

Long-term exposure of cellular components to oxidative stress can induce progressive oxidative damage of macromolecules and is implicated in age-related diseases.[7] Likewise, RPE cells in primates incur cellular senescence during the aging process.[8] Previous studies have reported that oxidative stress can induce or accelerate the development of cellular senescence, which is termed stress-induced premature senescence (SIPS).[9,10] Hydrogen peroxide (H_2O_2), a noncytotoxic oxidative stress agent, has been found to induce SIPS in many types of human cells.[10,11] In addition, it has recently been shown that H_2O_2 promotes SIPS in human RPE cells.[12] Therefore, reduced oxidative stress in senescence-associated changes in RPE cells might be a possible preventive target for AMD.

Glucosamine (GlcN), a naturally occurring amino monosaccharide, exerts certain immunosuppressive

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effects *in vitro* and *in vivo* and is widely used as an alternative therapeutic regimen for rheumatoid arthritis and osteoarthritis.[13,14] It is a common dietary supplement, which is considered safe in humans[15] and widely used for its protective effect on osteoarthritis in healthy adults.[16] In one of our previous studies, GlcN inhibited tumor necrosis factor-alpha (TNF-α)- or interferon-gamma-induced expression of the *ICAM-1* gene in human RPE cells[17] and was found to modulate the O-linked glycosylation of the factors involved in nuclear factor-κB signaling. GlcN also reduced the N-linked glycosylation of TNF-α-induced ICAM-1 to suppress ICAM-1 expression and functions in ARPE-19 cells.[18] In addition, GlcN treatment inhibits experimental uveitis by blocking the NF-κB-dependent signaling pathway and ICAM-1 expression in a rat model of endotoxin-induced uveitis.[19] Previous studies have reported that GlcN has antioxidative stress effects in H_2O_2-treated retinal ganglion cells[20] and human chondrocytes.[21] However, the effects of GlcN on antioxidative stress in SIPS of RPE cells are not clearly understood. This prompted us to ask whether GlcN can reduce oxidative SIPS in RPE cells and be a potential preventive agent for AMD. In the present study, we investigated the effect of GlcN on the production of reactive oxygen species (ROS) and the proportion of senescence-associated β-galactosidase (SA-β-Gal)-positive cells in H_2O_2-treated RPE cells to understand the possible mechanism.

MATERIALS AND METHODS

Isolation and culture of human RPE cells

Choroidal membrane samples were obtained during the standard procedures of evisceration. The procedures were done with the approval of the Institutional Review Board of Tri-Service General Hospital (TSGHIRB No: 2-105-05-114). The tenets of the Declaration of Helsinki were followed, and informed consent was obtained from all participants. Human RPE cells were harvested from the samples, as described previously.[22] Briefly, the choroid membrane was digested in 0.25% trypsin for 30 min at 37°C until the RPE detached from the stroma. Then, the RPE cells were centrifuged and resuspended in Dulbecco's Modified Eagle's medium (DMEM)-F-12 (Invitrogen-Gibco, Grand Island, NY, USA) and supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS; Invitrogen-Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C in the presence of 5% CO_2. The culture medium was replaced every 3 days until confluency. The successful growth of RPE cells was confirmed by immunocytochemical staining for cytokeratin expression.[22,23]

Cell viability assay

The cell viability was measured using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1; Roche Diagnostics, Indianapolis, IN, USA) assay, according to the manufacturer's recommended protocol. In brief, confluent monolayer cells were incubated with indicated concentrations (62.5, 125, 250, and 500 μM) of H_2O_2 (Sigma-Aldrich) in serum-free medium for 24 h, and 10 μL of the WST-1 reagent was added to the medium in each well. The cells in the multi-titer plate were incubated in a humidified atmosphere at 37°C in 5% CO_2/95% air for 1 h, shaken for 1 min, and then, the absorbance was read at 450 nm. The background absorbance was measured in wells containing only the dye solution and culture medium. The cell viability data were obtained from at least three experiments with at least six wells at each concentration in separate 96-well plates. The mean optical density values, corresponding to the untreated controls, were defined as 100%. The results were expressed as the percentage of the optical density of treated cells, relative to that of untreated controls.

Measurement of intracellular reactive oxygen species

The intracellular ROS were detected using 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich), according to the manufacturer's protocol. In brief, cells were rinsed twice with DMEM (Invitrogen-Gibco) before 10 μM H_2DCFDA in DMEM was added and incubated for 30 min at 37°C, followed by two washes with DMEM. After treatment with H_2O_2 in the presence or absence of GlcN (Sigma-Aldrich), cells were washed twice with phosphate-buffered saline (PBS), and then, the intracellular ROS were measured with a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) with excitation at 485 nm and emission at 535 nm.

Induction and analysis of oxidative stress-induced premature senescence

Premature senescence was induced by oxidative stress in H_2O_2-treated human RPE cells, as previously described with minor modifications.[12] Briefly, confluent human RPE cells were incubated daily in the presence of 125 μM H_2O_2 for 2 h for 3 days. After each stress, the cells were washed with PBS and allowed to recover with complete culture medium for 22 h. For the control cells, the culture medium was replaced with the complete culture medium daily during the 3-day period according to the same time schedule. In the cotreatment group, cells were cotreated with H_2O_2 and GlcN (2.5 or 5 mM) or N-acetylcysteine (NAC) (1 mM), a ROS scavenger,[24] for 2 h, followed by the treatment with fresh GlcN or NAC in

complete culture medium for another 22 h. After the last H_2O_2 treatment, the cells were allowed to recover for 3 days before further experiments were carried out.

Cellular senescence was analyzed with the SA-β-Gal staining kit (Sigma), according to the manufacturer's recommendations. In brief, treated RPE cells were washed twice with PBS, fixed with 1x Fixation Buffer for 7 min, washed twice with PBS, and then incubated under light protection with the staining mixture (containing X-gal) at 37°C for 24 h. The positive cells were examined and photographed at low magnification (×200) using a light microscope. The percentage of positive cells was counted in three fields of view per sample.

Western blot analysis

To detect the protein expression levels of p21, western blot analyses were performed. Actin was used to normalize the protein expression. RPE cells after senescence induction were washed twice with a PBS solution and detached by scraping. Cells were pelleted at 1000 g, resuspended, and sonicated in cold lysis buffer (50 mM Tris-HCl (pH 7.5), 2% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride). The insoluble debris was removed by centrifugation at 12,000 g at 4°C for 15 min. The protein concentration was determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. Lysates (20 μg) were separated using one-dimensional SDS – polyacrylamide gel electrophoresis. The separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA) and blocked with 5% (w/v) milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with antibodies directed against p21 (diluted 1:1,000 in TBST; Cell Signaling Technology, Beverly, MA, USA) and actin (diluted 1:5,000 in TBST; Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were washed and incubated with horseradish-peroxidase-conjugated secondary antibodies (1:25,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature, and the protein was visualized using an enhanced chemiluminescence (ECL) procedure (ECL reagent; Millipore, Billerica, MA, USA). Images of western blots were acquired using a UVP BioSpectrum 500 and analyzed using VisionWorks LS software (UVP, Upland, CA, USA).

Statistical analysis

The data were compared using a one-way analysis of variance. Differences between group means were determined to be statistically significant when a $P < 0.05$. When there were significant differences between the group means, multiple comparisons of the means were made using Tukey's *post hoc* test. Data were presented as mean ± standard deviation (SD) and obtained from at least three independent experiments. Statistical analyses were performed using the GraphPad Prism version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

Effect of hydrogen peroxide on the cell viability of human RPE cells

To evaluate cell viability in the presence of H_2O_2, human RPE cells were incubated with the indicated concentrations of H_2O_2 for 24 h, and the cell viability was determined by the WST-1 assay [Figure 1]. The cell viability of human RPE cells after treatment with 62.5, 125, 250, and 500 μM of H_2O_2 was 95.44% ±8.74%, 79.52% ± 12.87%, 71.19% ± 8.63%, and 31.94% ± 6.04%, respectively. The concentrations of 125 and 250 μM H_2O_2 led to a reduction in cell viability

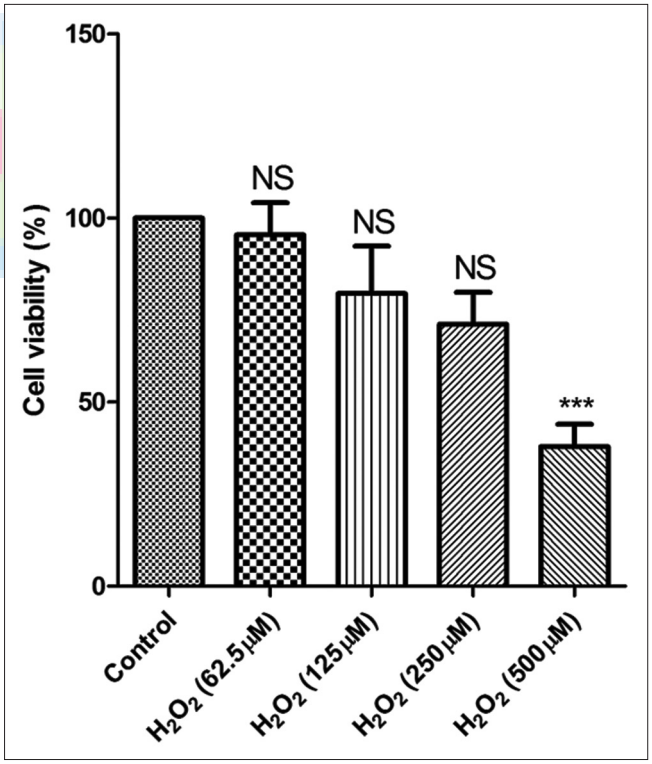

Figure 1: Effects of H_2O_2 on the cell viability of human RPE cells. The WST-1 assay was used to determine the cell viability in H_2O_2-treated human RPE cells. Confluent cells were incubated with the indicated concentrations of H_2O_2 (0, 62.5, 125, 250, and 500 μM) for 24 h. The results are the mean values ± SD of three independent experiments. ***$P < 0.001$ versus the control group. NS = Not significant versus the control group; SD = Standard deviation; H_2O_2 = Hydrogen peroxide; WST-1 = 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

compared with the control group, but the reduction was not significant. In addition, 500 μM H_2O_2 significantly reduced cell viability ($P < 0.001$) and led to the approximately half-maximal cytotoxicity of H_2O_2 on human RPE cells. Given that previous studies have reported that subtoxic oxidative stress, likely due to H_2O_2, can induce SIPS in various types of human cells,[10-12] we thus selected 125 μM of H_2O_2 for subsequent experiments.

Effect of glucosamine on hydrogen peroxide-induced reactive oxygen species in human RPE cells

Previous studies have reported that exogenous H_2O_2 can induce oxidative stress in RPE cells[12,25] and that GlcN can reduce H_2O_2-induced intracellular ROS in retinal ganglion cells and chondrocytes.[20,21] Therefore, we investigated whether GlcN can attenuate oxidative stress in H_2O_2-treated human RPE cells by measuring intracellular ROS. As shown in Figure 2, compared to the control group, treatment with GlcN did not influence the production of intracellular ROS in RPE cells. In contrast, H_2O_2 treatment stimulated the production of intracellular ROS in RPE cells compared to the control group. In addition, compared to the group treated with H_2O_2 alone, cotreatment with the indicated concentration of GlcN (2.5 or 5 mM) reduced the production of intracellular ROS in H_2O_2-treated RPE cells in a dose-dependent manner ($P < 0.001$ for each). Overall, this result demonstrated that GlcN attenuated H_2O_2-induced oxidative stress in human RPE cells.

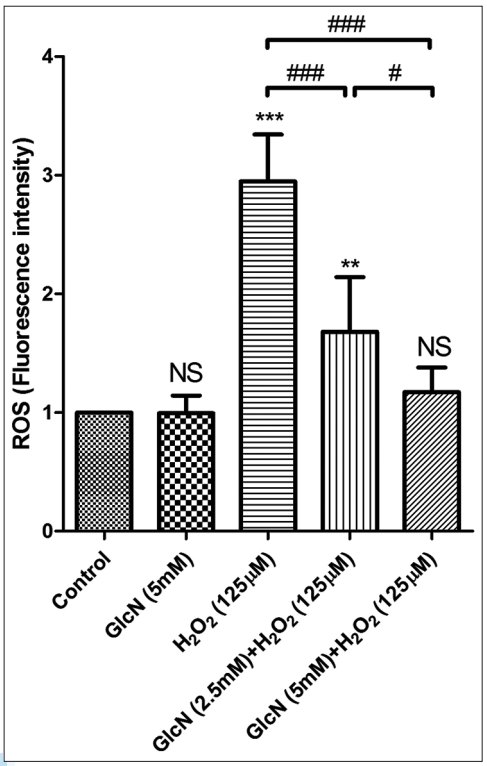

Figure 2: GlcN reduced the production of intracellular ROS in H_2O_2-treated human RPE cells. Cells were treated with or without H_2O_2 for 24 h in the absence or presence of 2.5 or 5 mM GlcN, respectively, and then the DCF signal was measured. Data are expressed as the mean ± SD of results of three experiments. $^{**}P < 0.01$ versus the control group; $^{***}P < 0.001$ versus the control group; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$. NS = Not significant; SD = Standard deviation; H_2O_2 = Hydrogen peroxide; GlcN = Glucosamine; DCF = Fluorescent 2′,7′-dichlorofluorescein

Effect of glucosamine on premature senescence in hydrogen peroxide-treated human RPE cells

Previous studies have reported that the activity of SA-β-Gal is a marker for identifying senescent RPE cells and that subtoxic oxidative stress-induced SA-β-Gal activity confirmed the presence of senescent RPE cells.[8,12] In addition, ROS are a potent inducer of SIPS.[26] Another previous study reported that NAC treatment decreases senescence markers in H_2O_2-treated ARPE-19 cells.[24] Our above result showed that GlcN attenuated the production of intracellular ROS in H_2O_2-treated human RPE cells [Figure 2]. Therefore, we further investigated the effects of GlcN on H_2O_2-induced premature senescence in human RPE cells by SA-β-Gal staining. NAC, an antioxidant agent, was used as a positive control.[24] As shown in Figure 3, after senescence induction by H_2O_2, the proportion of cells positive for SA-β-Gal activity was 21.75% ± 3.43%, 17.27% ± 7.87%, 16.71% ± 6.69%, 74.03% ± 4.76%, 52.65% ± 5.82%, and 52.93% ± 5.69%, in the control, GlcN alone, NAC alone, H_2O_2 alone, cotreatment with GlcN and H_2O_2, and cotreatment with NAC and H_2O_2 groups, respectively. Compared to the control group, there was a significant increase in SA-β-Gal-positive cells in the H_2O_2-treated group ($P < 0.001$). Cotreatment with GlcN or NAC reversed this effect in the H_2O_2-treated RPE cells ($P < 0.001$ for each). This result demonstrated that GlcN attenuates the increase of SA-β-Gal-positive cells in H_2O_2-treated RPE cells. Taken together, these results suggested that GlcN attenuated the H_2O_2-induced premature senescence, at least in part, by reducing the production of intracellular ROS.

Glucosamine attenuated the increase of p21 protein expression in hydrogen peroxide-treated human RPE cells

H_2O_2-induced premature senescence is due to the overexpression of the cyclin-dependent kinase inhibitor, p21 protein, in RPE cells.[12,27] Hence, we used western blot analysis to evaluate the effect of GlcN on the protein expression of p21 in RPE cells after senescence induction. As shown in Figure 4, compared to the control group, treatment with GlcN alone did not influence the expression of the p21 protein in RPE cells. As expected, the p21 protein was highly expressed in H_2O_2-induced prematurely senescent RPE cells compared to

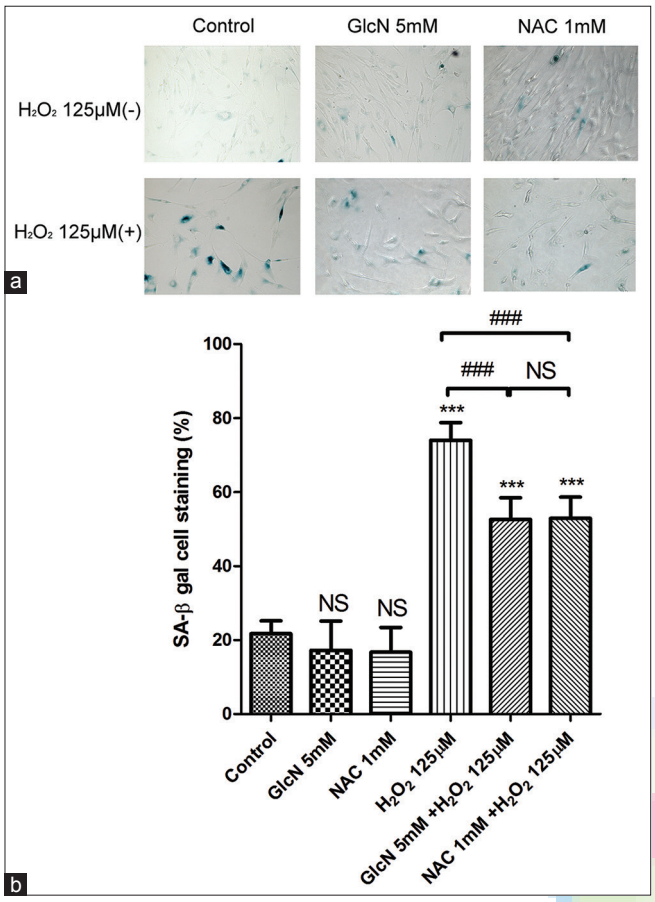

Figure 3: GlcN attenuates H_2O_2-induced premature senescence in human RPE cells. (a) Cells were treated daily with H_2O_2 only (125 μM) for 2 h, before treatment with fresh, complete medium for later 22 h for 3 days. Others were treated with or without GlcN (5 mM) or NAC (1 mM) only for 24 h, or cotreated with H_2O_2 (125 μM) and GlcN (5 mM), or NAC (1 mM) for 2 h, followed by treatment with fresh GlcN or NAC for latter 22 h for 3 days. H_2O_2 induced the increase of SA-β-Gal-positive cells in RPE cells. Cotreatment with GlcN or NAC reduced the H_2O_2-induced increase of SA-β-Gal-positive cells. SA-β-Gal staining was observed by light microscopy (×200). (b) The percentage of SA-β-Gal-positive cells was scored by counting at least 3 fields in light microscopy photomicrographs of representative fields. The data are shown as mean ± SD. ***$P < 0.001$ versus control group; ###$P < 0.001$. NS = Not significant; SD = Standard deviation; GlcN = Glucosamine; SA-β-Gal = Senescence-associated β-galactosidase; NAC = N-acetylcysteine

the control group ($P < 0.001$). However, cotreatment with the indicated concentration of GlcN attenuated the increase of p21 protein expression in H_2O_2-induced prematurely senescent RPE cells in a dose-dependent manner ($P < 0.05$ and $P < 0.001$). Overall, this result demonstrated that GlcN attenuated the H_2O_2-induced premature senescence, at least in part, through the p21 pathway.

DISCUSSION

In the present study, we investigated the effects of GlcN on H_2O_2-induced premature senescence and the mechanisms underlying its effects in human RPE cells. We demonstrated that GlcN treatment attenuated the increase of intracellular ROS in H_2O_2-treated RPE cells. GlcN reduced the proportion of SA-β-Gal-positive cells, a senescence marker, in H_2O_2-induced prematurely senescent RPE cells. In addition, we found that GlcN treatment reduced the expression of the p21 protein in RPE cells after senescence induction. Based on these data, our results provide evidence for the use of GlcN as a potential supplement to attenuate oxidative stress-induced senescence, at least in part, through the p21 pathway *in vitro*.

The pathogenesis of AMD is related to cumulative oxidative stress caused by ROS, and Retinal pigment epithelium is the prime target of oxidatively damaged tissues.[28,29] RPE cells continually undergo oxidative stress throughout life, which comes from photoreceptor outer segment phagocytosis, peroxidized lipid membranes, and photo-oxidative reactive oxygen intermediates.[25] Prolonged oxidative exposure disrupts multiple functions of RPE cells and leads to AMD.[30,31] H_2O_2, which can cross the plasma membrane, is one of the exogenous sources of oxidative stress that increases the production of intracellular ROS in RPE cells.[12,25] In addition, treatment with GlcN can reduce the oxidative stress-induced increase of intracellular ROS *in vitro*.[20,21] Consistent with previous studies,[12,20,21,25] the present study demonstrated that treatment with H_2O_2 induced the increase of intracellular ROS in RPE cells, whereas GlcN attenuated the effect of H_2O_2 in terms of the increase of intracellular ROS.

The senescence of Retinal pigment epithelium is one of the potential contributors to the complex pathogenesis of AMD.[32] Sublethal doses of H_2O_2 promote the SIPS of RPE cells,[12] whereas a ROS scavenger, NAC, decreases the SIPS in H_2O_2-treated RPE cells.[24] SIPS is a mechanism of accumulation of senescent-like phenotypic cells *in vivo*, and these cells display several features of cellular senescence, including the increase in the proportion of SA-β-Gal-positive cells.[10] SA-β-Gal activity is a biomarker for the identification of senescent cells,[33] and this feature is displayed in senescent RPE cells of human eyes.[8] In this study, our results showed that treatment with GlcN, like NAC treatment, reduced the increase in the proportion of SA-β-Gal-positive cells after induction of SIPS. Consistent with previous studies,[12,24] our study indicates that GlcN attenuated the SIPS in H_2O_2-treated RPE cells through reducing the increase of intracellular ROS.

Previous studies have reported that SIPS is primarily mediated through two signal transduction pathways: p21-Rb and p16-Rb pathways[11,34,35] and that the upregulation of p21 or p16 or both pathways leads to the subsequent induction of senescence in the various kinds of stressors and cell types.[10,11,35] Exposure of human fibroblasts and melanocytes to oxidative stress, as well as the SIPS of cells, upregulates both

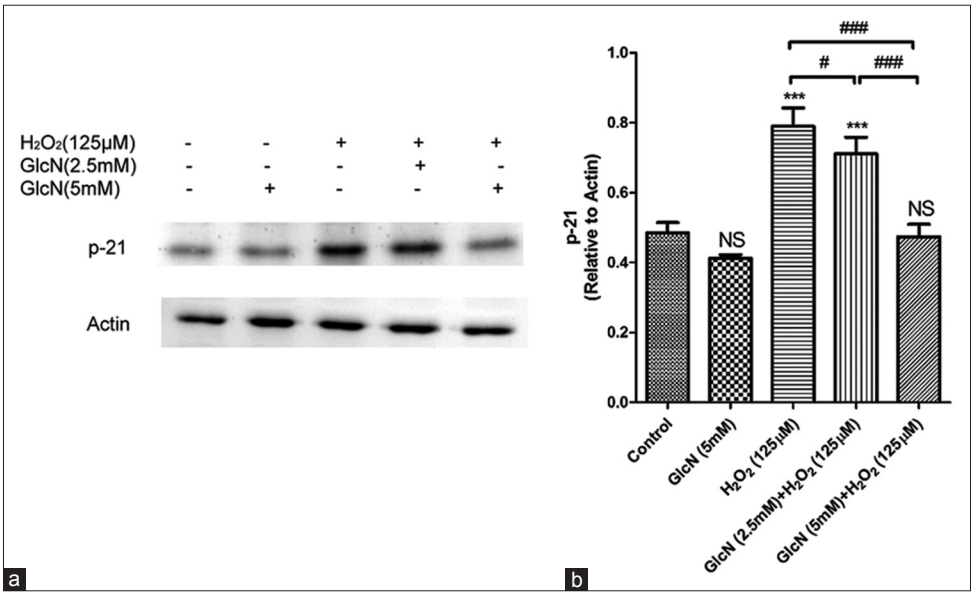

Figure 4: Effect of GlcN on the expression of the p21 protein in H_2O_2-induced prematurely senescent RPE cells. (a) Cells were treated with H_2O_2 only (125 μM) for 2 h, followed by treatment with fresh, complete medium for later 22 h for 3 days. Others were treated with GlcN (5 mM) only for 24 h or cotreated with H_2O_2 (125 μM) and the indicated concentration of GlcN (2.5 or 5 mM) for 2 h, followed by treatment with fresh GlcN for latter 22 h for 3 days. Cell lysates were immunoblotted with anti-p21 antibodies. Protein loading was normalized using actin as the reference protein. The data are representative of at least three independent experiments. (b) Quantitative data for p21 expression are presented as mean ± SD. ***$P < 0.001$ versus the control group; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$. NS = Not significant versus the control group; SD = Standard deviation; GlcN = Glucosamine

the p16 and p21 signaling pathways.[10] In endothelial cells, the SIPS of cells increased the expression of the p16 protein.[11] In human RPE cells, oxidative stress increases the expression of p21 and involves the p21 senescence-related pathway in the aging process.[12] Similar to a previous study,[12] the current study found that H_2O_2 induced the increase of p21 protein expression in human RPE cells. Moreover, our results suggest that GlcN reduced the increase in p21 protein expression after senescence induction, indicating that GlcN attenuated the SIPS of RPE cells, at least in part, through the p21 pathway.

This preliminary study was designed to give an overview of the possible applications of GlcN to prevent the pathogenesis of AMD, such as the SIPS of RPE cells. Hence, additional studies are needed to investigate the details of the mechanisms by which GlcN reduces H_2O_2-induced premature senescence and the processes involved in AMD. It should be noted that cells in SIPS can increase the expression levels of senescence-associated genes, such as apolipoprotein J, connective tissue growth factor, fibronectin, and transgelin.[10,12] Therefore, it is important to investigate the effects of GlcN on the expression of senescence-associated genes in H_2O_2-treated RPE cells. Furthermore, a senescent cell develops the senescence-associated secretory phenotype (SASP), which produces and releases pro-inflammatory cytokines, chemokines, proteases, growth factors, and other peptides.[36,37] SIPS in senescent RPE cells can upregulate the proinflammatory cytokines IL-6 and IL-8 and vascular endothelial growth factor, which are involved in AMD pathogenesis.[24] It will also be interesting to investigate the effects of GlcN on the expression of the SASP in H_2O_2-treated RPE cells. Finally, as we known, p21-Rb and p16-Rb pathways are two major signal pathways, which participate SIPS in many cell types.[11,34,35] In the present study, we found that treatment of H_2O_2 increased the p21 protein expression in RPE cells and treatment with GlcN decreased the increase of p21 protein expression in H_2O_2-treated RPE cells. However, the expressions of p16 and Rb proteins are also very important in SIPS in RPE cells. Therefore, the relationship between the expressions of p21-Rb and p16-Rb proteins in SIPS in RPE cells needs to be investigated in the future study.

CONCLUSION

This study showed that GlcN attenuated the increase of intracellular ROS and SIPS in H_2O_2-treated RPE cells, at least in part, through the p21 pathway. On the basis of our findings, we propose a possible role underlying the purported antioxidative effects of GlcN in SIPS in AMD.

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Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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