Intracellular Redox Status and Cell Death Induced by H₂O₂ in a Human Retinal Epithelial Cell Line (ARPE-19)

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Abstract: Hydrogen peroxide is a normal by-product of cellular metabolism that in higher concentrations can cause oxidative stress. Reactive oxygen species impair the physiological functions of retinal pigment epithelial (RPE) cells, which are known as one major cause of ocular pathologies. Most studies investigating the influence of H₂O₂ on cells in culture but H₂O₂ concentrations are not sustained in culture medium. Continuous generation using glucose oxidase (GOx) system allows application of relevant low H₂O₂ concentrations over physiologically relevant times periods (up to 24 h). Recent findings suggest that bolus and GOx treatments can lead to different cellular response, thus warranting a quantitative comparison between the two approaches. When added as a pulse H₂O₂ is rapidly depleted. Continuous generation of H₂O₂ produces different behavior in function of GOx activities. Cytotoxicity analyses show that cells can tolerate short exposure to high H₂O₂ doses delivered as a pulse but are susceptible to lower continuous doses. Application of hydrogen peroxide causes a concentration-dependent decrease in the intracellular glutathione (GSH) content that was accompanied by a matching decrease in the glutathione peroxide activity and reducing power (FRAP).

Keywords: Hydrogen Peroxide, Glucose Oxidase, Arpe-19 Cells, Cellular Redox Status, Free Radicals

1. Introduction

Aerobic organisms must live with a multitude of free radicals and related reactive oxygen/nitrogen species. Perhaps the most ubiquitous of these species is hydrogen peroxide (H₂O₂), which is found at measurable levels in all animal tissues. H₂O₂ is most stable and can reach molecular targets distant from its site of generation. Because H₂O₂ is a small, uncharged molecule, it easily crosses cell membranes and localizes in multiple subcellular compartment [1].

Hydrogen peroxide is a physiological constituent of living cells and is continuously produced via diverse cellular pathways. The intracellular concentration of H₂O₂ is tightly controlled by various enzymatic and nonenzymatic antioxidant systems and is assumed to vary between 1 and 700 nM. Intracellular steady-state concentrations of above 1 μM are considered to cause oxidative stress inducing growth

arrest and cell death [2].

In experimental models used to investigate physiological functions and toxic effects of H₂O₂, oxidative stress responses of cells, or cytoprotection by antioxidant agents, cultured cells are often exposed to H₂O₂ added as a bolus into the culture medium. In these experimental settings substantial variations in the concentrations of H₂O₂ determined to be cytotoxic can be found, ranging from less than 10 μM to more than 1000 μM. Although H₂O₂ addition to cell cultures is a common model of stress induction, its concentration in the medium over the period of cell treatment is usually not determined or controlled. The kinetics of H₂O₂ decomposition over short time frames has been examined in cultures of several monolayer cell types including the retinal-pigmented epithelial cells [3, 4, 5].

The response of cells to either an acute (single high dose) or chronic (repeated low/moderate doses or continuous steady-state generation) exposure to oxidizing agents is quite different. Depending on the degree of the oxidizing insult, acute exposure could trigger a series of intracellular antioxidant defense mechanisms that counteract the damage caused but if these are not sufficient, cells will die by apoptosis or necrosis, again depending on the extent of the oxidative insult. In chronically exposed cells, it is anticipated that the antioxidant defense mechanism will be altered usually provokes the development of a series of adaptive responses that are distinct from those following acute exposure [6].

A continuous source of H_2O_2 by glucose oxidase (GOx) rather than an acute pulse of H_2O_2 administered reflects better the sustained and local release of this metabolite. GOx constitute an alternative to bolus treatment, which generates H_2O_2 by oxidizing glucose of the culture medium. To date it remains largely unknown how bolus and continuous low-levels H_2O_2 treatments compare to each other in terms of how the influence intracellular H_2O_2 levels over time.

Hydrogen peroxide treatment of cultured cells is a commonly used model to test oxidative stress susceptibility or antioxidant efficiency in cell types that are high risk for oxidative damage in vivo, such as cells of the retinal pigment epithelium (RPE). The RPE is a central cell type involved in age-related macular degeneration (AMD) and retinopathy-related diseases. Because of the high ambient oxygen tensions required to maintain its high metabolism that is necessary to maintain the health and function of the overlying photoreceptors and the unique, constant exposure to photo-oxidative (blue/UV light) stress, high concentrations of polyunsaturated fatty acids, as well as cigarette smoke exposure, the RPE is armed with a robust antioxidant system [4,5,7,8], making it a valuable model to study the oxidative stress.

The major focus of this investigation is to compare how the same cells under the same condition respond to different induced stress respect to the time availability, intracellular redox status and cytotoxicity of H_2O_2 delivered by two methods: as a single addition pulse or by continuous enzymatic generation using glucose/glucose oxidase. Another goal in conducting this study is to determine whether H_2O_2 could be used to model chronic oxidative stress that is sublethal for the RPE to identify therapeutically important antioxidants that protecting against cumulative injury.

2. Materials and Methods

2.1. Material

Hydrogen peroxide, catalase (from bovine liver), glucose oxidase (from *Aspergillus niger*), 3-(4,5—dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum

(FBS), penicillin, streptomycin, L-glutamine, trypsin-EDTA, iron (II) ammonium sulfate hexahydrate, xylenol orange disodium salt (3,3'-bis(N,N-di(carboxymethyl)-aminomethyl)-o-cresulfone-phatein), phosphate-buffered saline (PBS) and 5,5'-dithiobis(2-nitrobenzoic acid (DTNB), 2,4,6-tripyrindyl-s-triazine (TPTZ), sodium dodecyl sulfate, and polyacrylamide were purchased were purchased from Sigma (St. Louis, MO, USA). RIPA buffer from Thermo Scientific, caspase-3-substrate (Ac-DEVD-pNA) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), caspase-3 inhibitor I (DEVD-CHO) from Calbiochem (Merck, Millipore).

2.2. Cell Cultures

The ARPE-19 cell line is a human RPE cell and was kindly gifted from Dr. Horacio Rilo (Arizona, University, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 . The ARPE-19 cells were passaged by trypsinization (0.05% trypsin/0.53 mM EDTA) every 1-week, centrifuged, re-suspended in fresh medium and counted using trypan blue exclusion.

2.3. Trypan Blue Staining of "Dead" Rpe Cells

Viability was determined by staining cells with trypan blue. Therefore, cells were harvested, collected and re-suspended in 3 ml medium. Then the cells were stained with a final concentration of 0.04% for 5 min at room temperature. Stained and no stained cells were counted using a hemacytometer in optic microscope. No stained cell represent the viable cells, which were not permeable to trypan, blue dye.

2.4. H_2O_2 Exposure on ARPE-19 Cells

In H_2O_2 treatments experiments, the cells were plated at 5×10^4 cell/well in 96-well plates to adhere overnight before exposure to oxidant. Two treatment protocols were used: pulse delivery of a range of micro-molar and mili-molar concentrations of H_2O_2 and addition of glucose oxidase to initiate continuous enzymatic generation of the oxidant.

For pulse delivery, culture medium was first removed and cells were fed with DMEN in the presence and absence of serum (10%), containing either no H_2O_2 (control) or different ranges of concentrations of H_2O_2 , freshly prepared in medium: treatment 1-(12.5, 25, 50, 100, 200, 400, 800, 1200, 1600 µM) during 24 h with intervals up to 30 min during the first 2 h and treatment-2 (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25 mM) in 2 hours. H_2O_2 was also added to medium in culture wells lacking cells to determine H_2O_2 depletion in the absence of culture monolayers.

For continuous enzymatic generation of H_2O_2 , the cultures were first reseeded with fresh DMEM containing 10% FBS (D-10). Glucose oxidase (GOx) was then added to the medium to initiate the generation of H_2O_2 by oxidation of the glucose

contained in DMEM (4.5 mg/ml D-glucose). Stock solutions of GOx were prepared by solubilizing the enzyme in 50 mM sodium acetate buffer, pH 5.1, at a concentration of 10 KU/ml and storing aliquots at -20 °C. Just before use, stock solutions were thawed, diluted, and added to the culture medium to produce final concentrations of 3-100 mU/ml. GOx was also added to medium in culture wells lacking cells to determine H₂O₂ depletion in the absence of culture monolayers.

After addition of H₂O₂ (pulse delivery) or of GOx (to initiate continuous H₂O₂ generation), aliquots of culture medium were retrieved at intervals to determine H₂O₂ levels, and cells were harvested after 24 h (treatment-1 and GOx) or 2 h (treatment-2) to assay for cytotoxicity by the methods described below.

2.5. Hydrogen Peroxide Determination

Hydrogen peroxide concentration in culture medium was determined by a modified ferrous oxidation-xylenol orange (FOX) assay reported by Gil *et al.*, [9]. FOX reagent contained 25 mM ammonium ferrous sulfate in 3.5 M H₂SO₄ (solution A) and 0.125mM xylenol orange disodium salt (solution B). Just before use 1 volume of solution A was added to 100 volumes of solution B to produce complete FOX reagent. For the assay, an aliquot of medium retrieved from cultures (50µl DMEM or D10 in the presence and absence of ARPE-19) were mixed with 500 µl complete FOX reagent.

The oxidation of Fe²⁺ in the presence of hydroperoxides forms Fe³⁺, with reacts with xylenol orange to produce a chromosphere. Samples were incubated for 30 min at room temperature. Absorbance of the supernatant was read spectrophotometrically at 560 nm against PBS as background control. 50 µl of known concentrations of H₂O₂ (3.125 to 100 µM) were used as standard calibration curve and a best-fit line for the data was plotted using linear regression. Experimental H₂O₂ concentrations were then extrapolated substituting the unknown values into the equation derived for the standard curve.

2.6. Assay of Mitochondrial Viability (MTT Assay)

Cell viability was measured using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by Mosmann [10] method with some modification reported by Iloki *et al.*, [11]. Living cells reduced MTT to formazan, which was quantified by measuring absorbance at 570 nm and 655 nm with a microplate reader (Thermo-Scientific Multiskan Spectrum). Cells were grown to confluence in 96-well plates and incubated with H₂O₂, or GOx for 2 and 48 h. Control cells were prepared in plates containing only medium. At the end of the incubation the medium was replaced by MTT (0.5 mg/ml in medium), and the conversion of MTT into an insoluble formazan crystals for 4 h at 37 °C were solubilized in acidified isopropanol. Formazan production was expressed as a percentage of the values obtained from control cells.

2.7. Preparation of Cell Lysates from ARPE-19 Cells and Protein Quantification

One million cells were plated in 12-well plates to adhere overnight before hydrogen peroxide-stress and control cells. The medium was carefully removed and washed twice which cold PBS buffer, trypsinized and centrifuged at 1500 rpm for 5 min. For making whole cell lysates, the cells were lysed in phosphate-buffered saline (PBS; 136.75 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, 8.10 mM Na₂HPO₄, pH 7.4) by three freeze-thaw cycle (-77°C/25°C) and then disrupted by sonication. The homogenate was centrifuged for 15 min at 14,000 g. Protein concentration determination was performed by the Bradford method using bovine serum albumin as the standard at 595 nm [12].

2.8. Measurement of Apoptosis/Caspase-3 Assay

After treatment the medium was aspirated and cells were washed once in cold PBS and afterward counted using the trypan blue exclusion method. An amount of 1x10⁶ cell/ml was transferred to lysis RIPA buffer. The activity of caspase-3-like protease in the lysate was measured using colorimetric caspase-3 assay kit according to the reported by Bai *et al.*, [13]. In brief, cytosolic protein (100 µg) was mixed with caspase-3-specific substrate acetyl-Asp-Glu-Val-Asp-p-nitronilide (final concentration, 200µM) and incubated at 37°C for 90 min. The absorbance was read at 405 nm. Apoptotic cell lysates containing active caspase-3 yield a considerable compared to non-apoptotic cell lysates.

To confirm that substrate cleavage was due to caspase activity, lysates were incubated in the presence of the caspase-3-specific inhibitor acetyl-DEVD-CHO (final concentration, 20 µM) at 37 °C, before the addition of substrate. The value (in arbitrary absorbance units) of the absorbance signal of the inhibited sample was subtracted from that of the no inhibited sample.

2.9. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-Page)

SDS-PAGE was carried out in 12% (w/v) polyacrylamide gel, for the separating gel and 2% (w/v) for the stacking gel (Bio-Rad). Equal volumes (25µl) of lysates and buffer sample were mixed and then boiled for 5 min. Meanwhile, 20 µg proteins of different samples were directly loaded onto the gel. Gel electrophoresis was run at 100 V for 2 h in Tris/glycine/SDS buffer. After electrophoresis, the gel image was developed by Coomassie blue [14].

2.10. Measurement of Cellular Redox Status

2.10.1. Total Antioxidant Capacity

Total antioxidant capacity in the lysates cells and extract was assayed using the FRAP assay of Benzie and Strain

[15] with some modifications according to Iloki et al., [11]. The FRAP reagent was prepared in acetate buffer (pH 3.6), 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol HCL and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in water bath prior to use. 5 µL of samples diluted with 15 µL of PBS were added to 150 µL of FRAP reagent. The absorbance of the mixture was measured using microplate spectrophotometer reader Thermo Scientific at 595 nm after 5 min. The standard curve was prepared by iron (II) sulfate solution, and the results were expressed as µM Fe (II).

2.10.2. Cellular Thiol Quantification

Total intracellular thiol levels were quantified spectrophotometrically using 5,5'-dithiobis (2-nitrobenzoic acid (DTNB), as described previously by Gill et al., [9] with glutathione reduced (GSH) as standard. Briefly, this method is based on the reaction of the glutathione present in the lysates with DTNB to generate oxidized GSH (GSSG) and 2-nitro-5-thiobenzoic acid, a yellow compound that absorbs at 412 nm.

2.10.3. Activity of Antioxidant Enzymes

Catalase (CAT) activity was measured by using a protocol adapted from Gill et al., [9] 2003. Catalase activity was assayed by adding 50 µL of sample to 450 µL of 50 mM phosphate buffer, pH 7.0, and 250 µL of 50 mM H₂O₂ in a final volume of 750 µL. Absorbance decrease was measured at 240 nm in a Cary spectrophotometer for 1 min and calculations were performed using an extinction coefficient of 0.043 mM⁻¹cm⁻¹. Catalase activity is expressed as µmol of H₂O₂ /min*ml. All experiments were carried out by using three biological replicates and three technical replicates for each sample.

2.10.4. Assay of GSH-Peroxidase Activity

GSH (200 µM) and H₂O₂ (200 µM) were incubated at 37 °C for 1 h in PBS containing various lysates of ARPE-19 cells. The volume of the enzymatic reaction was 120 µL. Residual peroxide was measured spectrophotometrically at 560 nm by a modified ferrous oxidation-xylenol orange (FOX) assay [9].

2.11. Microscopic Imaging

Samples from ARPE-19 cells treated or not with were evaluated using an inverted microscope (Faga-Lab-Labomed, TCM 400) to detect structural changes. A series of structural images were collected with a bottom-mount digital camera for the analysis of treatments.

2.12. Statistical Analyses

Data were expressed as the mean ± standard deviation (SD). Significance between experimental groups was determined by GLM-ANOVA followed by post hoc Tuckey using NCSS software. Values of p ≤ 0.05 were considered

statistically significant. Figures were performed using Origin 8.0 software and the error bars represent the SD.

3. Results

3.1. Pulse Delivery of H₂O₂

Numerous studies have analyzed the response of cultured cells to H₂O₂ when added to the culture medium as a single or repeated bolus. Usually, the purpose of these experiments is to investigate the cellular response to "oxidative stress", as it may occur naturally in retinal pigment epithelium that are high risk for oxidative damage *in vivo*. Most of these studies have been conducted by adding diluted H₂O₂ to the cell culture medium (H₂O₂ bolus treatment) assuming that this at least partially mimics conditions and elicits responses that may also occur *in vivo*. H₂O₂ was utilized to generate oxidative stress because it is a common intermediate produced by multiple oxidative pathways and is involved in cellular redox signaling.

Preliminary experiments were conducted to confirm that the presence of serum did not affect H₂O₂ measurement. As shown (Fig. 1), hydrogen peroxide determinations did not differ for a range of concentrations of H₂O₂ (10-200 µM) added to DMEM either lacking serum or containing 10% FBS. However H₂O₂ concentrations in PBS is nearly an order of magnitude higher than in medium.

Exogenously added of H₂O₂ has a short half-life due to its rapid degradation in culture medium. For monitor the decay of the H₂O₂ bolus in our experiments and to know how long and to what extent H₂O₂ levels remain elevated after a bolus treatment, the kinetics of H₂O₂ degradation was determined in the presence and absence of cells. In the absence of cells initial concentration is not sustained in the medium (Fig. 2) and therefore the concentration of the agent and the time of exposure is not well controlled. As illustrated for an initial nominal concentration of 250 µM, the depletion rate is almost similar over 2 h at 37 °C in the absence and presence of serum. Although in the presence of serum (D10) H₂O₂ concentration undergoes exponentially more rapid, the half time of depletion is 30 min with 10% FBS while in DMEM (without serum) is 45 min. The rapid degradation of H₂O₂ in medium with serum is due to own serum antioxidant joined to its amino acids present in the medium. H₂O₂ concentration in PBS declines slowly with the time.

Because oxidation of ferrous to ferric ions in biological samples could also result from the interaction of ferrous ions with organic hydroperoxides, H₂O₂ specificity was confirmed by measuring concentrations in 500 µM H₂O₂ in D10 without and with catalase at a final concentration of 200 U/ml in the assay mix (Fig. 2). Catalase (CAT) dismutase H₂O₂ into H₂O and O₂. Catalase is likely to be particular relevance when cells are exposed to high H₂O₂ concentrations, because of its essentially nonsaturable first-order kinetics.

The relationship between H₂O₂ concentration and incubation time shows an exponential decline (Fig. 3). Exponential decay was more rapid in medium with serum in presence of ARPE-19 ($k_d = -0.065 \text{ min}^{-1}$), similar decay

rate constants (k_d) were found in medium with 10% of serum and DMEM without serum in presence of ARPE-19 (-0.041 and -0.037 min^{-1} respectively).

Fig. 1. Calibration curves for H₂O₂ levels in the range 10–200 μM using ferrous-xylenol orange with 10 % FBS (D10; linear regression $y = 0.0022x + 0.0149$, $R^2 = 0.98616$ and without FBS (DMEM linear regression: $y = 0.0025x + 0.0157$, $R^2 = 0.98643$). Therefore H₂O₂ determination was measured in PBS.

Fig. 2. Degradation of 250 μM H₂O₂ added as single pulse to medium without serum (DMEM) and with 10% FBS (D10) in the absence of ARPE-19. Exponential regression of 250 μM H₂O₂ in D10 $y = 245.54e^{-0.029x}$, $R^2 = 0.96447$. Addition of catalase 200 U/ml to the 500 μM H₂O₂ with serum confirms the specificity of the determinations for H₂O₂.

The near perfect exponential decay (shown for 400 μM bolus in Fig. 3 demonstrates that H_2O_2 degradation closely follows first-order kinetics with dependence on substrate concentration. Fitting of the measured concentrations to the function.

$$[\text{H}_2\text{O}_2] = [\text{H}_2\text{O}_2]_{\text{ini}} \times e^{-kt}$$

Where $[\text{H}_2\text{O}_2]_{\text{ini}}$ is the initial concentration and k (min^{-1}) the first order rate constant. The rate constant was used to calculate elimination half-life ($t_{1/2}$) and the time of 99% H_2O_2 disappears from the supernatant (t).

$t_{1/2} = \ln 2/k$, $t_{1/2} = \ln 2/0.065$, $t_{1/2} = 0.693/0.065$ $t_{1/2} = 10.67$ min

$t = \ln(0.01)/-k$; $t = \ln(0.01)/-0.065$; $t = 70.85$ min

For ARPE-19 cells grown in DMEM with 10% FBS using

the specific elimination rate constant, $k_d = -0.065 \text{ min}^{-1}$ derived from the relationship between peroxide concentration and the time (Fig. 3), it can be estimated that the half-life elimination ($t_{1/2}$) was 10.67 min and the elimination total time was 70.85 min. By this time the concentration of H_2O_2 in culture medium had diminished more than 10-fold.

ARPE-19 cells were exposed to various H_2O_2 nominal concentrations by delivering oxidant to culture as a single addition (pulse) with serum-free culture medium (Fig 4A) and with 10% of serum (4B). The concentration-effect relationship shows that the response of cultured cells is probably determined by both the concentration of the agent and the time of exposure (Fig. 4).

Fig. 3. Dissipation of H_2O_2 (400 μM nominal concentration) from the culture medium in presence or absence of ARPE (50,000 cell/well) with or without serum. Decay rate constants were $k_d = -0.065, -0.041, -0.037, -0.026 \text{ min}^{-1}$ for D10+ARPE, D10, DMEM+ARPE and DMEM respectively.

A **B**

Fig. 4. Concentration of H_2O_2 versus time curves containing different nominal concentrations 400, 600, 800 and 1200 μM with serum-free medium (A) and with 10% FBS (B) in presence of ARPE. Data represent means of triplicate measurements of 3 independent experiments. 1200 μM of H_2O_2 in absence of ARPE was determined in parallel culture.

A rapid exponential depletion of H_2O_2 from serum-free medium exposed to RPE culture was obtained (Fig. 4A), although elimination of H_2O_2 in ARPE-19 cell undergoes a much more rapid with incubation time in the presence of serum (4B). The concentration of H_2O_2 in the medium of ARPE-19 cell cultures with serum starts to decrease immediately after administration of the peroxide. At least for the first 10-15 min of incubation H_2O_2 disappears from the culture. By 2 h the concentration of H_2O_2 in culture medium

had diminished totally for all concentrations.

An initial exponential decline of the H_2O_2 concentration in the culture medium was observed with H_2O_2 concentrations between 100 and 1600 μM . In general, concentrations of $H_2O_2 \leq 100 \mu M$ were completely eliminated during the first 30 min after administration. At initial H_2O_2 concentrations of $\geq 200 \mu M$ after the initial rapid phase the elimination became slower and the concentration of H_2O_2 leveled off. Regardless of the

kinetics, from the pragmatic point of view, the significant observation is that H_2O_2 is not sustained at the added concentration when the agent is delivered as a pulse.

ARPE-19 cultures with different cell number ($2, 4, 8 \times 10^5$ cells/well) were exposed to various nominal H_2O_2 concentrations (400, 800, 1600 μM) in 1ml /well to evaluate the elimination of H_2O_2 from culture medium and the cytotoxic action. Figure 5 shows that increasing the cell

concentration accelerate the peroxide depletion. Raising the cell concentration by increasing the cell number resulted in a shift of the nominal concentration-effect curves for the elimination of H_2O_2 toward higher concentrations (Fig. 5 A, B). The elimination of H_2O_2 changes with variation of cell number, although this effect is almost independent of the cell number to low cell dose of H_2O_2 (Fig. 5 C).

A

B

C

Fig. 5. Influence of cells number on the elimination of hydrogen peroxide in ARPE cells. Cells were incubated with various concentrations of H_2O_2 1600 μM (A), 800 μM (B) and 400 μM (C) for the indicated times. Data are means of three independent experiments.

It is well established that H_2O_2 added to the medium of ARPE-19 cells as a single pulse produces an oxidant-dependent cytotoxicity that can be detected by MTT assay 24 h after treatment (Fig. 5). Because H_2O_2 is largely

depleted from culture medium within 2 h (Fig. 4), one would expect similar cytotoxicity whether the medium was replaced with fresh medium at 4 h after oxidant addition or remained unchanged until the time of assay.

Fig. 6. Cytotoxic action of hydrogen peroxide in ARPE-19 cells. A range of concentrations of H₂O₂ from 12.5 to 1600 μM was added to serum-containing DMEM. Cytotoxicity was estimated 24 h after addition by the MTT assay in cultures in which the medium was not replaced (solid bars) or was replaced at 4 h with fresh H₂O₂-free DMEM (hatched bars) or was measured at 4h (dotted bars). * IC₅₀ in 24 h ** IC₅₀ 4 h with replace (4 h + 20 h). Data expressed as a percentage of the untreated control, are from three independent experiments and are the means ±SD.

The figure reveals that H₂O₂ concentrations ≤100 μM are not cytotoxic at 24 h when H₂O₂ is delivered as a pulse due to the oxidant is depleted fairly rapidly in these concentrations in the culture medium. Additionally ARPE-19 cell is not very sensitive to oxidative stress. In the case of 200 μM had little effect, but cytotoxicity was substantial at higher concentrations. The minimal concentration necessary to elicit 50% of the cytotoxic effect (IC₅₀) was calculated to be 916 μM at 24 h similar to 896 μM whether the medium was replaced with fresh medium at 4 h after oxidant addition. As, shown, similar outcomes were in fact obtained (Fig. 6).

In the case of H₂O₂, incubation time is not equivalent to exposure time when oxidant is delivery as single addition. The clearance measurements reveal that H₂O₂ is rapidly eliminated obviously the cell death is not immediate and the window between initial exposure and cell death is relevant because is in this step that antioxidant could function to increase the fraction of surviving cells.

The manifestation of the incipient cytotoxicity of H₂O₂ at 4 h means that cytotoxicity of H₂O₂ takes several hours longer than the exposure to H₂O₂.

It has to be noted that different sensitivities of the cells to H₂O₂ due to cell type. ARPE-19 cells are not sensitive to low concentrations of H₂O₂ (i.e. 12.5 to 200 μM), presumably because their antioxidant defense mechanisms

are sufficient to counteract the damaging effects of the low H₂O₂ concentrations applied. However at high H₂O₂ concentrations (i.e. 400 -1600 μM), the antioxidant defense mechanism in the ARPE-19 cell line appear to be insufficient to cope with such high oxidant insult, resulting in a high percentage of cell death.

Additionally the ARPE-19 cells were exposed to a short exposure time (30, 60 and 120 min) with H₂O₂ without or with serum to find the cytotoxicity effect (Fig. 7). The amounts of H₂O₂ that have to be added to ARPE-19 cell cultures to produce 50% cytotoxicity increased about 10-fold by shorting the incubation time. Exposure to H₂O₂ concentrations ≤ 0.5 mM showed little effect on both cultures cells, however, 5mM H₂O₂ exposure by 60 and 120 min resulted in a significant loss of cell viability, with a 50 % reduction in free-serum culture compared to 10 mM for the ARPE-19 cells containing serum in the same short periods. Increasing exposure to 25 mM resulted in an 80% loss of viability in ARPE-19 cells in both cultures types at 120 min. Short-term exposure to high H₂O₂ levels induced marked toxicity, suggesting that under these experimental conditions necrosis was the predominant mode of cell death. It is unclear whether necrotic-like cell death was the result of primary, secondary, or programmed necrosis. The duration of incubation with H₂O₂ is important for the determination of H₂O₂ concentration required to induce

death cell.

The IC_{50} value revealed that the cytotoxic potency in serum-free culture medium was increased nearly twice fold over the ARPE-19 culture grown in 10% FBS (Fig. 7). This

high sensitivity to oxidation was produced to serum starvation-induced apoptosis owing to the lack of serum antioxidants.

A

B

Fig. 7. Influence of incubation time on the concentration dependency of the cytotoxic action of hydrogen peroxide in ARPE-19 cells. Cells were incubated for the indicated time with various concentrations of the peroxide in free serum culture medium (A) and with serum (B). Data are means of at least three independent experiments.

3.2. Continuous Enzymatic Generation of H_2O_2

Because H_2O_2 is labile in culture medium, sustained exposure to a given concentration is difficult to achieve when the agent is delivered in a single pulse. An alternative method for sustained treatment of cultures is to continuously

generate the product from medium glucose using GOx. Glucose oxidase catalyzes the direct two-electron reduction of oxygen to H_2O_2 , using reducing equivalents from the oxidation of glucose. This approach has been used for short-term treatment of cultured cells including, ARPE-19 cells.

The addition of GOx to culture medium (DMEM)

produces a linear rate of accumulation of H₂O₂ as a function of enzyme concentration during the first hour of incubation in both the absence and the presence of ARPE-19 cells (Fig. 8), although the rate of accumulation in the absence of cells

is approximately twice as great as when cells are present. The generation of H₂O₂ by GOx can be assuming pseudo-zero order kinetics (i.e., assuming an unlimited supply of Glc and O₂).

Fig. 8. H₂O₂ concentration in culture medium generated from D-glucose after 1 h post addition of 0-10 mU/ml glucose oxidase (GOx). GOx was added to the medium of culture well without or with confluent cultures of ARPE-19 cells to illustrate the initial linear accumulation of H₂O₂ as a function of enzyme concentration.

In the absence of cells, H₂O₂ concentration continues to increase over 48 h at a higher rate than during the first hours, perhaps because of partial H₂O₂ depletion by medium was overcome by hydrogen peroxide accumulation for glucose oxidase (Fig. 9A). In the presence of cells the concentration of in the culture medium over time after GOx addition is strikingly different from in the absence of cells, and the pattern differs with enzyme concentration (Fig. 9B).

With lower amount of GOx (5 mU/ml) H₂O₂ concentration in the medium is nearly stationary in the presence of cells during 48 h. The rates of H₂O₂ production and decomposition are approximately equal. At middle GOx amounts (8 or 10 mU/ml), H₂O₂ concentration exhibits a complex dynamic. The concentration continues to rise after the first hour of enzyme addition, peaks at approximately 6 h, and then decreases thereafter. Using 10 mU/ml GOx delivery to the ARPE-19 cultures a maximum concentration of 60 µM at 6 h and then declined to 0 µM by 48 h. With higher concentration (25 mU/ml) the rate of H₂O₂ production continues to increase over 48 h reached maximum value of 280 µM much lower than in the absence of cells (Fig. 9B). These changes in medium content of H₂O₂ are released to cytotoxic response of ARPE-19 cells.

As for delivery of H₂O₂ in a single pulse (Fig. 6), GOx addition to culture medium also produces a dose-dependent cytotoxic response in confluent ARPE-19 cultures quantified by the MTT assay at different time (Fig. 10). Low amounts of added enzyme (3 or 5 mU/ml) had little effect, but cytotoxicity was substantial at higher concentrations. This observation is noteworthy because the peak concentrations of H₂O₂, achieved at 6 h after addition of middle concentrations enzyme (Fig. 9B), were relatively low compared to peak concentrations that produced cytotoxicity when H₂O₂ was added in a single pulse (Fig. 6).

Using 10 mU/ml GOx, the peak concentration of 60 µM produced by this amount of enzyme reduced MTT in 50% at 48 h, while 25 mU/ml achieved 280 µM of H₂O₂ with almost 50% of inhibition at 24 h (Fig. 11), yet 800-900 µM H₂O₂ was required to produce comparable MTT reductions when the oxidant was delivered as a pulse (Fig. 6). Clearly, however, the dynamics of oxidant exposure to cells differ under the two conditions. When delivered as a pulse, H₂O₂ is depleted fairly rapidly, whereas the GOx-treated cells were exposed to more sustained moderate concentrations over the full 48-h incubation time.

Fig. 9. H_2O_2 concentration in culture medium generated from D-glucose after addition of 5, 8, 10 and 25-mU/ml glucose oxidase (GOx) during 48 h. GOx was added to the medium of culture wells (A) without or (B) with confluent cultures of ARPE-19 cells. Because higher medium concentrations are achieved in the absence of cells, different y-axis scales are used. Data are from three independent experiments and are the means of three culture wells per group in each experiment.

Fig. 10. Influence of glucose oxidase concentrations on the cytotoxic action of hydrogen peroxide in confluent cultures of ARPE-19 cells. The viability was determined as a percentage of the untreated controls using the MTT assay for the indicated times. IC₅₀ 32.5 mU/ml and 14.5 mU/ml at 24 h y 48 h respectively.

Cell lysates from the experimental cultures were tested for functional caspase activity using the caspase-3-specific substrate Ac-DEVD, this peptide-PNA conjugate is nonchromophore until PNA is cleaved from the peptide by an active caspase, and consequently, increased absorbance is indicative of caspase activity.

Treatment with H₂O₂ increased the activity of caspase-3 in both addition and enzymatic generation to a significantly greater extent than ARPE-19 cells control (Fig. 11). The cells control showed a very minor caspase-3 activity (≈ 1); however, once the cells were treated with GOx 25 mU/ml or

800-1600 μ M H₂O₂, the caspase-3 activity was increased 1.5 and 2 fold.

Although H₂O₂ addition system caused an increase in the activation of caspase-3 compared to enzymatic method. Indeed, severe oxidative stress conditions resulted a drastic drop in the apoptosis (i.e. Gox 50 mU/ml and 1600 μ M), consistent with the notion that pro-oxidant conditions can inactivate caspases by oxidative modification of key cysteine residues, thereby impairing the apoptotic program and promoting a switch to necrosis [21].

Fig. 11. Caspase-3 activity induced by H₂O₂ addition (800 or 1600 μ M) and GOx (25 or 50 mU/ml) in ARPE-19 cells for 24 h. Control cells (untreated), positive control cells and cells GOx50 mU/ml+ CAT (200 U/ml) were treated with H₂O₂, C⁺ (the specific caspase-3 control). Values are presented as means $\pm$ SD of at least 3 independent experiments. * $p < 0.05$ significantly different from value of control vs. 1600 μ M addition.

Exposure to high H₂O₂ concentrations (1600 μ M or 50 mU/ml) exerted extensive damage leads to massive cell death. Necrotic-like cell death was observed, rather than apoptosis. At a moderate H₂O₂ cytotoxic dose (800 μ M or 25 mU/ml) was a significant increase in caspase-3 activity. Our current data further confirmed that caspase-3 might be a partially target involved in H₂O₂-induced apoptosis in the retinal pigmented epithelium. On the other hand, the low correlation between caspase-3 activity and the level of cell death indicates that concentrations of H₂O₂ influence the mode of cell death, i.e., apoptosis vs. necrosis. The higher H₂O₂ concentrations induced severe toxicity, with over 90% cell death, suggesting that under these experimental conditions necrosis was the predominant mode of cell death.

Oxidative stress induced by H₂O₂ is capable of inducing cell death by both apoptosis and necrosis; mild oxidative stress causes apoptosis whereas severe oxidative stress triggers necrosis [29]. The extent of oxidative stress determines the level of lysosomal membrane damage. H₂O₂ interacts with intralysosomal iron to generate highly reactive hydroxyl radicals that initiate lipid peroxidation of lysosomal membranes and subsequent lysosomal membrane permeabilization.

The significant change in cellular morphology and increase in H₂O₂ levels in ARPE-19 cells were associated with a dose-dependent decrease in protein concentrations (Fig. 12 A). The loss of protein from the cell layer compared to the untreated control cultures was related with cytotoxic

action of H_2O_2 . Because the decrease in protein concentrations could represent a cytostatic and cytotoxic effect; MTT assay were performed in control and H_2O_2 treated cells. Whereas (100 μM of H_2O_2 or 5 mU/ml GOx)

treatment was nontoxic at 24 h, the surviving fraction decreased approximately 50% in 800 μM of H_2O_2 addition or 32.5 mU/ml GOx and 80% in 1600 μM or 50 mU/ml GOx treated cells (Fig. 12 B).

Fig. 12. Effects of H_2O_2 -induced oxidative damage on cell protein concentration in ARPE-19. H_2O_2 generated by GOx (50, 25, 12.5 mU/ml) or in addition (1600, 1000 or 800 μM) (A). Relationship between values for cell proliferation and the protein concentration (B). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cell morphology is also presented (original magnification 100 x) (C). Values are presented as means $\pm$ SD of at least 3 independent experiments.

In Fig. 12 B it is clearly visible that hydrogen peroxide-treated cells are losing their dividing ability in a concentration-dependent manner. The proliferating ability had not recovered after 24 h after the start of oxidative challenge. This permanent inhibition in proliferation might be attributed to the persistent presence of protein aggregates in ARPE-19 cells.

The oxidative attack leads first to a slight protein oxidation, causing misfolding in the native protein state; if the stress persists, further oxidation aims at the hydrophobic residues that are normally buried inside proteins and these become heavily oxidized, which favors the formation of protein aggregates. Accumulation of oxidized proteins is

normally degraded by the proteasome, however this process may be impaired when the rate of oxidized protein formation exceeds proteasomal capacity or when the proteasome itself has decreased activity. The formed aggregates interact with the proteasome, inhibit its activity, and lead to a loss of functionality, e.g. proliferation.

The morphologic images confirmed the toxic H_2O_2 -induced oxidative damage (Fig. 12 C). ARPE-19 control cells have elongated spindle shape characteristic of a retinal epithelial morphology. By exposure these cells to peroxide treatments these take a very different morphology that is accentuated with the severity of oxidative stress. Observed that the cell loses fusiform globular appearance and is

difficult perception of cell nuclei. The cells mixed with H₂O₂ and 200 U/ml catalase showed no cytotoxicity. From these results, we can conclude that H₂O₂ induced ARPE-19 cell death and prevented by catalase.

3.3. Intracellular Redox Status

To investigate the possible changes in cellular redox homeostasis after response of cells to H₂O₂-induced cell injury, the level of oxidants and antioxidant capacity were simultaneously measured. The antioxidant power in the control cells and cells exposed to H₂O₂ were determined to assess oxidative stress. It is well established that the detoxification of H₂O₂ can be achieved through non-enzymatic (glutathione) and enzymatic ways; two different

enzymatic systems, namely, catalase (CAT) and glutathione peroxidase (GPx). In this study glutathione, the CAT and GPx were measured.

3.3.1. Evaluation of the Intracellular Glutathione Levels

To determine whether the reduced intracellular levels of glutathione dependent of exposure to oxidant agents (single high dose) or (enzymatic generation), the glutathione levels were monitored. GSH, a thiol-containing tripeptide, is the major intracellular antioxidant but it can also be the major substrate of peroxidases reducing hydroperoxides. The sulfhydryl group (SH) serves as an electron donor and reacts with ROS (H₂O₂, OH[•], O₂^{•-}) maintaining a reducing cellular environment.

Fig. 13. Effects of GOx and H₂O₂ in addition on glutathione reduced (GSH) at 24 h (A). ARPE-19 treated with 12.5, 25 and 50 mU/ml of GOx and pulse of H₂O₂ 800, 1000, 1600 μ M and control cells or control catalase (50 mU/ml GOx+200 U/ml CAT) (B) The levels of H₂O₂ in GOx at 24 h, the medium was analyzed using FOX reagent. Values were expressed in means $\pm$ SD in μ M (n=3 independent experiments).

The results revealed that cells treated with GOx had the lowest amount of GSH, i.e., 40 to 80 μ g/ml of SH content while the cells exposed to H₂O₂ addition significantly increased the level (60-100 μ g /ml) compared to GOx cells

(Fig. 13 A). As can be seen, following H₂O₂ treatment, the glutathione levels decreased in a dose-dependent manner in ARPE-19 cells, reflecting the antioxidant protection by GSH against H₂O₂-induced oxidative stress. The results showed

that moderate but sustained H_2O_2 concentrations (GOx) and pulse (1600 μM H_2O_2) deplete the intracellular glutathione content almost half values. However low concentrations of addition (800 and 1000 μM) decreased less the glutathione content.

The lower amount of endogenous reduced glutathione indicates the increased consumption of antioxidant to counteract the elevated level of H_2O_2 (Fig. 13 B). This behavior results mainly by the accumulation of H_2O_2 as a function of enzymatic concentration, whereas the delivery of H_2O_2 in a single pulse is not sustained. The elevation in

glutathione in cells treated with H_2O_2 in pulse can be related to more required concentration to produce comparable MTT reductions when the oxidant was delivered as a pulse (Fig. 6) indicating the possible involvement of GSH in the cytoprotection. The GSH redox cycle represents the most important H_2O_2 elimination pathway in the RPE cells. In our study, we found that GOx (12.5-50 mU/ml) or addition of 1600 μM H_2O_2 caused a dose-dependent decrease in glutathione of up to 60%, exceeding the 40% depletion level that render cells vulnerable to oxidative

Fig. 14. Diagram of the glutathione redox system. Glutathione is a tripeptide of cysteine, glutamate, and glycine, which undergoes oxidation and forms a homodimer GSSG. GSSG can participate in posttranslational modification of proteins by S-thiylation. GSSG can also be recycled back to reduce glutathione by GSH reductase in a reaction that utilizes NADPH.

GSH serves as an antioxidant by scavenging ROS, which oxidize the cysteine moiety. Oxidation of GSH drives the formation of glutathione disulfide (GSSG); which can then be directly recycled to GSH through the enzyme glutathione disulfide reductase, a reaction requiring NADPH. The ratio of GSH to GSSG is often used as an indicator of intracellular redox status; more highly oxidized redox state is associated with differentiation and apoptosis. GSH can also act as a cofactor for GSH-utilizing antioxidant enzymes, such as GSH peroxidase, glutaredoxin and glutathione S-transferases (Fig. 14).

3.3.2. Evaluation of Catalase (Cat) and Glutathione Peroxidase (Gpx) Activities

It is well established that most of the H_2O_2 in cells is eliminated by the intracellular antioxidant enzymes, catalase and GPx. The effect of different treatments on the activity of catalase and glutathione peroxidase is shown in Fig. 15. To investigate whether kind of induction system was related to their catalase activity, the basal level of catalase activity was determined spectrophotometrically by following the rate of H_2O_2 consumption in both models systems. The CAT activity was slightly lower in the H_2O_2 cells than in the control cells (Fig. 15 A). The result showed no significant difference in the level of catalase activity between addition and enzymatic generation of H_2O_2 . Catalase is likely to be of particular relevance when cells are exposed to high H_2O_2 concentrations, because of its essentially non-saturable first-order kinetics. Catalase exerts a “filter” function that very

efficiently prevents accumulation of cytosolic H_2O_2 levels by dismutation into H_2O and O_2 .

In addition to catalase, the GPx activity was measured in both addition and enzymatic generation-induced systems in ARPE-19 cells. GSH-peroxidase activity was evidenced by measuring the residual H_2O_2 during the incubation of 200 μM H_2O_2 with different treatments or in the presence of 500 μM GSH. We evaluated whether ARPE-19 cultures with H_2O_2 or GOx had different depleting effect. The results (Fig. 15 B) showed that GPx was significantly different between the addition and enzymatic H_2O_2 . In enzymatic generation the residual peroxide was diminished concentration-dependent. At the highest concentration tested (50 mU/ml), there was a decrease in the GSH-peroxidase activity as indicated by the higher H_2O_2 residual. In contrast, 1600 μM of H_2O_2 added to the medium of ARPE-19 cells as a single pulse produces the maximum activity may to counteract the severe damage and improve oxidative stress tolerance.

However catalase activity is quite similar between the ARPE-19 cells induced to H_2O_2 by addition or enzymatically, suggesting that catalase has a minor role in detoxifying H_2O_2 . One explanation for the relative importance of GPx and not catalase in detoxifying H_2O_2 in ARPE-19 cells relates to the intracellular location of these enzymes. Catalase enzyme is located in peroxisomes and its access to cytosolic is limited. However GPx, unlike catalase, is mainly present in the cytosol and requires reduced glutathione to complete the catalytic cycle. So it is likely that cells in dependence of severity of injury adopted the

more convenient cytosolic GPx/reduced glutathione cycle for efficient removal of H₂O₂ because this more accessible than the compartmentalized catalase.

Antioxidant capacity by measuring the ferric reducing antioxidant power decreased after treatment with H₂O₂ addition or with H₂O₂-generating GOx compared to culture control. Treatment with H₂O₂ or GOx for 24 h significantly

decreased around threefold of antioxidant power in cells in absence of extract compared to control cells (Fig. 16). The significant correlation of Pearson showed that the lower reducing power in ARPE-19 cells correlated with low values for GSH and H₂O₂ residual (GPx) mainly in continuous enzymatic generation ($r=0.957$ and -0.996 respectively).

A

B

Fig. 15. The effect of hydrogen peroxide by enzymatic generation (GOx 50, 25 and 12.5 mU/ml) and pulse (1600, 1000, 800 µM) on catalase activity (A) and GSH-peroxidase activity assay (B). H₂O₂ (initially, 200 µM in PBS) was incubated for 1h, at 37 °C with different lysates, control GOx 50 mU/ml+ catalase 200 U/ml or GSH (500 µM). Results are expressed as the means ± SD of three independent studies (each analyzed in triplicate).

Fig. 16. Effect of glucose oxidase (GOx) or H_2O_2 addition treatment on antioxidant capacity measured by FRAP assay. Significant change in the antioxidant index in indicated (* $p < 0.05$).

4. Discussion

4.1. Pulse Delivery of H_2O_2

This study demonstrated that a nominal concentration of H_2O_2 that exerts cytotoxicity and modulating in status redox in ARPE-19 cells cultures is largely dependent of H_2O_2 -induced systems. Nominal concentrations of H_2O_2 in cell cultures in fact are initial concentrations that are eliminated rapidly from the culture medium [2, 4, 18-21]. Treatments of bolus and GOx can lead to different cellular response. The concentration of H_2O_2 in the medium of ARPE-19 cell cultures with serum decreases immediately after administration of the peroxide (Figures 2, 3 and 4). This finding is consistent with a rapid exponential depletion of H_2O_2 is consistent reported for other cell types [2, 17] and for RPE [4]. Exogenously added H_2O_2 has a short half-life due to its rapid degradation by cellular catalase and peroxidases. It is not clear which component (s) of DMEM is responsible for the slow but measurable decomposition of H_2O_2 in medium. Perhaps amino acids present in DMEM, such as methionine, cysteine and tryptophan, interact with H_2O_2 leading to its consumption [4, 16]. Therefore different supplementation of culture medium with antioxidants and precursors, as well as different contents of peroxidase-consuming additive in the culture medium such as pyruvate and serum albumin is a known H_2O_2 scavenger [2, 22, 23].

The minimal incubation time necessary to determine the incipient IC_{50} value for the cytotoxic action of H_2O_2 was approximately 24 h (see Fig. 6). A similar results was obtained in ARPE-19 cells by Kaczara et al. [4]. They reported that ARPE-19 cells needed 24 h to develop half

cytotoxicity effect at 300 μM nominal concentration but the cells were grown in MEM. Different supplementation of culture medium exerts variability in the cytotoxic potency of peroxides [5]. Gulden et al., [2] revealed that the cytotoxic potency of H_2O_2 in C6 astrogloma cell increased about 17 fold by prolonging the incubation time. Antunes and Cadenas, [19] observed that Jurkat cells needed 12 h after a 1-h exposure to a steady-state concentration 25 μM H_2O_2 to develop maximum cytotoxicity.

ARPE-19 cells exposed to a short exposure time with H_2O_2 without or with serum increased the cytotoxicity effect (Fig. 7) about 10-fold by shorting the incubation time. The duration of incubation with H_2O_2 is important for the determination of H_2O_2 concentration required to induce death cell. This high sensitivity to oxidation was even more obvious when the cells are exposed to serum starvation-induced apoptosis owing to the lack of serum antioxidants. Neuronal cells cultured in serum-free medium for 48 h experienced about 40% cell death. The cell population displayed decrease in S phase and an emergence of sub- G_0/G_1 phase compared with those cultured in normal serum. Serum starvation successfully blocked the progression of cells from G_0/G_1 phase into S phase and induced cells toward apoptosis [25].

The retinal pigmented epithelium is armed with a robust antioxidant system because of the high ambient oxygen tensions required to maintain its high metabolism that is necessary to maintain the health and function of the overlying photoreceptors, and the unique, constant exposure to photo-oxidative stress [7]. Despite a potent antioxidant system, the RPE cell become progressively dysfunctional and finally dies by apoptosis. Part of this deterioration has

been attributed to inadequately neutralized oxidative stress. Oxidative damaged biomolecules have been identified in the RPE from AMD samples [7, 24].

When H₂O₂ is delivered to or generated outside cells, a gradient is established across the cell membrane that depends not only on the extracellular concentration of the oxidant, but also on plasma membrane permeability to H₂O₂ and on the amount and availability of enzymes that decompose H₂O₂ [2, 19-21]. H₂O₂ was found to be distributed in various subcellular compartments, i.e., the cytosol, the mitochondria, and the nucleus [21].

4.2. Continuous Enzymatic Generation of H₂O₂

Because H₂O₂ is labile in culture medium, sustained exposure to a given concentration is difficult to achieve when the agent is delivered in a single pulse. An alternative method for sustained H₂O₂ treatment of cultures is to continuously generate the product from medium glucose using GOx. This approach has been used for short-term treatment of cultured cells [4, 26]. There are several reasons why GOx is a reliable H₂O₂ source for cell culture applications: (a) in contrast to other oxidases such as xanthine oxidase, GOx exclusively converts oxygen to H₂O₂ in a stoichiometrically simple 1:1 relationship, (b) GOx continuously generates low levels of (an importantly no other ROS) from its substrate, glucose, (c) its product (D-gluconolactonate) is metabolically inert, (d) GOx works well at physiological pH of 7.4, (e) is highly substrate specific, (f) is stable and remains fully active over 24 h at 37 °C, and (g) GOx does not interfere with endogenous enzyme expression because it does not exist in mammalian cells.

GOx addition to culture medium also produces a dose-dependent cytotoxic response in confluent ARPE-19 cultures (Fig. 10). Clearly, reductions when the oxidant was delivered as a pulse differ under enzymatic generation of H₂O₂. When delivered as a pulse, H₂O₂ is depleted fairly rapidly, whereas the GOx-treated cells were exposed to more sustained moderate concentrations over the full 48-h incubation time. Cytotoxicity analyses show that cells can tolerate short exposure to high H₂O₂ doses delivered as a pulse but are susceptible to lower continuous doses.

In a study by Kaczara *et al.* [4] the slight differences between concentrations of H₂O₂ generated by GOx compared to our results might be the different supplementation of culture medium with antioxidants and precursors, as well as different contents of peroxide-consuming additives in the culture medium such as pyruvate and serum albumin is a known H₂O₂ scavenger.

As shown in Fig. 11 the apoptotic marker protein caspase-3 exhibits elevated caspase-3 activity H₂O₂-induced apoptosis. Exposure to sustained continuous generations invoke extensive cell apoptosis as evident from the activation of the classical apoptotic pathway in which elevated the cleavage of procaspase-3 into caspase-3, and loss of plasma membrane integrity all occurred in these cells (Fig. 11 and 12). Yu *et al.*, [27] found that 200 µM H₂O₂-induced in human lens epithelial B3 (HLE B3) cell injury is

mediated primarily by caspase-associated apoptosis. HLE B3 cells were grown in serum deprivation MEM before exposure to a bolus of H₂O₂.

Changes in the cell cycle caused by H₂O₂ eventually led to apoptosis due to increased sub-G1 phase as the arrest of the G2/M phase increased. When HT-22 cells were treated with 100 µM H₂O₂ for 30 h, a decrease in the G1 phase and an increase in the G2/M phase were observed. The amount of Bax (antiapoptotic) gradually decreased, whereas Bcl-2 (proapoptotic) gradually increased [28]. Progression of death stimuli to apoptosis and necrosis depended on the mitochondrial-mediated damage and on ATP levels. The presence of ATP is essential for the activation of apoptosis protease and subsequent activation of caspases that induce apoptosis. It is well known that caspase-3 has a primordial role in triggering the cascade of events that lead to apoptosis [5,13,27].

The significant change in cellular morphology and increase in H₂O₂ levels in ARPE-19 cells were associated with a dose-dependent decrease in protein concentrations. Castro *et al.*, [31] in Jurkat cells found that actin aggregation diminished functional cell, namely proliferation. The rupture of lysosome membranes releases hydrolytic enzymes (nucleases, proteases, phospholipases, lipases, phosphatases, sulfatases, and glycosidases) normally reside inside lysosomes into cytosol, which may in turn trigger apoptotic signaling by hydrolyzing their substrates. However, severe lysosomal membrane damage leads to a massive exodus of lysosomal enzymes into the cytosol, indiscriminately digesting cellular components, causing deleterious cytoplasmic acidification, and ultimately inducing necrosis instead of apoptosis [28, 29, 30].

4.3. Intracellular Redox Status

GSH had an essential role in preserving nuclear function from deleterious oxidative modifications. A minimal GSH concentration is essential to protect the nucleus against oxidative damage and the maintaining nuclear function during mild H₂O₂ treatment is the key parameter of cell survival [32]. ARPE-19 cells possess significantly higher capacity in the retention of diminution of intracellular level glutathione than Jurkat J16. In this cell line H₂O₂ concentrations higher than 0.1 mM deplete the total intracellular glutathione content of the J16 cell line almost entirely. However in the polyclonal HJ16 cell line, H₂O₂ treatment only decreased the GSH content by half [6]. In the yeast *Saccharomyces cerevisiae* when cells were GSH depleted for 8G, mutation frequency increased significantly, showing that nuclear DNA stability is strongly affected by H₂O₂ treatment under very low GSH concentrations [32].

GSH had an essential role in preserving nuclear function from deleterious oxidative modifications. A minimal GSH concentration is essential to protect the nucleus against oxidative damage and the maintaining nuclear function during mild H₂O₂ treatment is the key parameter of cell survival. When GSH concentration is high (> 1mM), all cell

functions are preserved from oxidative inhibition. As a result, enzymes of the detoxification machinery are induced and intracellular H_2O_2 is rapidly degraded. At a lower concentration (≈ 0.1 mM), GSH loses its cytosolic protective effect but still efficient for the protection of the nucleus against oxidative damage. Despite the transcriptional induction of stress-responsive genes, the detoxification machinery remains at a low level, because of the inhibition of translation. Therefore, is a slowly degraded and oxidized protein accumulating in the cytosol. But upon stress release, the functional nucleus can support growth resumption. When GSH concentration drops under 0.03 mM, both nuclear and cytosolic components become highly sensitive to oxidative damage, leading to cell death [32].

In the RPE, oxidative stress is a powerful risk factor for age-related macular degeneration (AMD). The susceptibility of RPE cells to oxidative stress progressively increases with age, and cumulative oxidative damage contributes to RPE dysfunction and apoptosis [33] (Wang et al, 2014). Thurman et al, [34] found that H_2O_2 depleted nuclear factor erythroid-derived 2-related factor 2 (Nrf2). Nrf2 regulates the inducible expression of antioxidant and cytoprotective enzymes via the cis-acting antioxidant response element. In human, RPE accumulates oxidative damage with age and individuals with high oxidative burden, such as smokers, are at increased risk for AMD. We speculate therefore that long-term increase in antioxidant intake will probably reduce RPE oxidative damage in the human eye and may delay onset of age-related visual impairment.

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Author Contributions

L.L.L.M and I.A.S.B proposed the ideas, planning the experimental methods, analyzed the data and wrote the manuscript; F.A.D. collected the samples and carried out the chemical analyses; G.S.A.A, L.E.C.L and R.P.J.L statistic analyzes of data, reviewing and editing the manuscript. All authors read and approved the final manuscript.

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