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The contribution of DNA repair and antioxidants in determining cell type-specific resistance to oxidative stress

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Abstract
The aims of this study were; (i) to elucidate the mechanisms involved in determining cell type-specific responses to oxidative stress and (ii) to test the hypothesis that cell types which are subjected to high oxidative burdens *in vivo*, have greater oxidative stress resistance. Cultures of the retinal pigment epithelium (RPE), corneal fibroblasts, alveolar type II epithelium and skin epidermal cells were studied. Cellular sensitivity to H_2O_2 was determined by the MTT assay. Cellular antioxidant status (CuZnSOD, MnSOD, GPX, CAT) was analyzed with enzymatic assays and the susceptibility and repair capacities of nuclear and mitochondrial genomes were assessed by QPCR. Cell type-specific responses to H_2O_2 were observed. The RPE had the greatest resistance to oxidative stress ($P > 0.05$; compared to all other cell types) followed by the corneal fibroblasts ($P < 0.05$; compared to skin and lung cells). The oxidative tolerance of the RPE coincided with greater CuZnSOD, GPX and CAT enzymatic activity ($P < 0.05$; compared to other cells). The RPE and corneal fibroblasts both had up-regulated nDNA repair post-treatment ($P < 0.05$; compared to all other cells). In summary, variations in the synergistic interplay between enzymatic antioxidants and nDNA repair have important roles in influencing cell type-specific vulnerability to oxidative stress. Furthermore, cells located in highly oxidizing microenvironments appear to have more efficient oxidative defence and repair mechanisms.

Keywords: *Aging, oxidative stress, antioxidant enzymes, oxidative DNA damage, DNA repair, post-mitotic cells*

Abbreviations: *AMD, age-related macular degeneration; CAT, catalase; CuZnSOD, copper zinc superoxide dismutase; GPX, glutathione peroxidase; MnSOD, manganese superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; RPE, retinal pigment epithelium; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; QPCR, quantitative polymerase chain reaction*

Introduction

Exposure to reactive oxygen species (ROS) is ubiquitous to all cell types. ROS cause damage to the nuclear (nDNA) and mitochondrial (mtDNA) genomes, proteins and lipids [1–5]. Cells respond to toxic levels of ROS by activating a diverse array of protective responses. This includes a complex range of enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD) and non-enzymatic antioxidants such as glutathione, tocopherols and carotenoids [5,6]. Oxidative repair mechanisms also exist, which include DNA, protein and lipid repair or degradation pathways [7–12].

The exposure to endogenous and/or exogenous ROS is highly specific to a particular cells function and its unique *in vivo* micro-environment. This is particularly true in higher animals as different cell types are highly specialized to perform specific functions. For example, skin epidermal cells are subjected to several ROS-inducing stresses including light and UV radiation, and retinal pigment epithelial

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cells to light, phagocytosis of the spent tips of the photoreceptor outer segments and high oxygen fluxes [13,14]. Growing evidence suggests the diversity of physiological micro-environments may also be accompanied by an equally diverse cell type-specific sensitivity to oxidative stress [6,15–18]. We have further hypothesized that the diverse array of physiological cellular micro-environments has resulted in a cell type-specific sensitivity to oxidative stress, with cell types located in highly oxidizing environments having a greater resistance to oxidative stress.

However, to date, knowledge of the mechanisms involved in determining this cell type-sensitivity to ROS are unclear. A greater understanding of the interplay between a cells defence and repair response has the potential to provide valuable insights into aging and to shed light on novel therapeutic treatments. For instance, evidence has shown that genomic oxidative damage can be reduced by the administration of certain combinations of endogenous antioxidants [6]. In addition, during recent years it has become apparent that the oxidative stress response varies considerably between post-mitotic cells and cells that are capable of actively dividing [7,18]. Indeed, these differences may have arisen due to functional reasons, such as the fact that post-mitotic cells are expected to function for many decades. However, to date, the cellular mechanisms for the dissimilarities between the oxidative stress response of dividing and non-dividing cells remains poorly understood. It is of further note that, despite the highly damaging role of nDNA and mtDNA damage in age-related degenerative diseases, the available information regarding the roles of genomic susceptibility and repair in post-mitotic cells is still lacking.

Using a comparative *in vitro* model, we asked whether nuclear DNA (nDNA) and mitochondrial (mtDNA) repair and/or the cellular antioxidant status (CuZnSOD, MnSOD, GPX and CAT) have important roles in determining cell type-specific susceptibility to oxidative stress. Thereby, this study compared the oxidative stress response of typically post-mitotic cells (RPE and corneal fibroblasts) and cell types that are capable of actively dividing *in vivo* (skin keratinocytes and lung alveolar type II cells). Furthermore, the investigated cell types were specifically chosen because they reside in physiologically different oxidizing environments.

Materials and methods

Cell culture

The CRC-23021 (human retinal pigment epithelium), CRL-1555 (human skin epidermis) and NCI-H441 (human alveolar type II epithelium) cell lines used were obtained from the American Type Culture Collection. The human primary corneal fibroblasts were isolated as previously described [19]. The cell lines were cultured in Ham's F10 medium (Gibco-BRL, UK). All cultures were supplemented with 10% heat-inactivated foetal calf serum (LabWest, UK), 100 μl/ml streptomycin, 100 μl/ml kanamycin and 60 μl/ml penicillin (Sigma, UK). All experiments were performed on non-dividing confluent cell monolayers in 25 cm^2 flasks as previously described [20].

Oxidative treatments

All cultured cells were treated with a toxic dose of H_2O_2 (1.5 mM) for 1 h at 37°C in a humidified incubator at 5% CO_2/95% air. An important aim of this study was to highlight that RPE cells are highly resistant to damage, thus we used H_2O_2 at levels that are greater than would be found under physiological conditions, in order to show this feature of this cell type. The concentration of the acute levels of H_2O_2-induced oxidative stress used during this study was determined from various dose and time course studies (data not shown). At 1 h oxidative exposure the net oxidative damage (the total damage minus repair) was determined for protein carbonyls, nDNA and mtDNA lesions. For the repair experiments, after the 1 h oxidative stress exposure, cells were washed twice with PBS and replaced by serum-free medium. Cell cultures were allowed to recover for the indicated repair time. Control flasks were mock treated with serum-free medium. Immediately, after treatment or recovery period the cells were used for antioxidant or DNA analysis.

Cell viability assay

The cytotoxicity of the oxidative stress-inducing compounds was determined using the MTT assay, with minor modifications [21]. After experimental treatment, cells were washed with PBS and incubated with 0.5 mg/ml of MTT (Sigma, UK). The cells were incubated at 37°C and after 3 h the MTT solution was removed and acidified isopropanol containing 0.5% HCl was added to dissolve the resultant formazan crystals. Aliquots were measured on a microplate reader at an absorbance of 590 nm (Labsystems, Multiskan Ascent, UK). The cell viability was expressed as a percentage of the non-treated normalized control samples.

Antioxidant enzyme assays and protein determination

Following oxidative stress treatment or a recovery period, the cell cultures were washed with PBS and incubated with 0.2% triton X-100 in distilled water for 5 min at 4°C. The supernatant was used immediately for protein determination and antioxidant assays.

Catalase (CAT) activity

CAT activity was measured using a previously described method [22]. Briefly, the cell lysate was incubated for 5 min with 6 mM H_2O_2 and the reaction quenched with the addition of 0.75 N H_2O_2 and 50 mM $FeSO_4$. Potassium thiocyanate was added and the absorbance of the ferrithiocyanate product was measured at 460 nm using a microplate reader (Labsystems, Multiskan Ascent, UK). The cell cultures were analyzed kinetically at 2 time points and the results were expressed as units per mg of protein using the following equation.

$$\text{Catalase units} = \frac{k}{\text{protein}} = \frac{\left[\frac{\text{In}\left(\frac{A_1}{A_2}\right)}{t}\right]}{\text{protein}}$$

where In is the natural log, A_1 and A_2 are the observed mean absorbance at the two selected time points and t is the time difference between the two points.

Glutathione peroxidase (GPX) activity

GPX activity was assessed using a commercially available kit following the manufacturer's protocol (Cayman Chemical Company, USA). Briefly, assay buffer (50 mM Tris–HCl, pH 7.6, 5 mM EDTA) was added to co-substrate (containing NADPH, glutathione and glutathione reductase) and the experimental sample. The reaction was initiated by the addition of cumen hydroperoxide. The absorbance at 340 nm using a microplate reader was recorded as a function of time, to obtain the linear portion of the reaction. From this slope, two time points were used to determine the change in absorbance using a calculation provided by the manufacturer.

Copper zinc (CuZnSOD) and manganese (MnSOD) superoxide dismutase activity

The dismutation activities were determined using a method previously described [23]. Briefly, the cell lysate was incubated with 50 mM phosphate buffer containing 0.1 mM EDTA, 62 μM nitroblue tetrazolium (NBT) and 98 μM NADH. The reaction was initiated by the addition of 3.3 μM PMS. The absorbance at 560 nm was measured using a microplate reader after a 5 min incubation period and used as an index of NBT. The activities of CuZnSOD and MnSOD were separately distinguished by adding 15 mM potassium cyanide to the sample to inhibit CuZnSOD activity. SOD activity was calculated from the standard curve of known activity of SOD.

Protein carbonyl measurement

Cells were scraped into lysis buffer (10 mM Tris, pH 7.2, 0.5% Triton X-100, 50 mM NaCl, 1 mM EDTA). One mg/ml each of aprotinin, leupeptin, pepstatin, 0.3 mM phenylmethylsulfonyl fluoride, and 0.01% butylated hydroxytoluene was also added to prevent oxidation of proteins during preparation. Cells were vortexed and freeze-thawed three times, centrifuged at 16,000*g* for 10 min at 4°C, and supernatants were collected and protein concentrations were determined using the BCA protein assay kit (BioRad, UK). The protein carbonyl content was determined in cell lysates by the ELISA method of Buss et al. [24].

Protein determination

Protein levels were measured using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's protocol using a BSA standard curve.

DNA damage and repair experiments

After the 1 h H_2O_2 exposure DNA was extracted at this point. For DNA repair assessment, serum-free medium was added and the cultures maintained at 37°C and allowed to recover for 6 and 12 h.

Genomic DNA was extracted using a commercially available High Pure PCR Template Preparation Kit (Roche, UK). The QPCR assay measures the average oxidative lesion frequency. The lesion frequency (λ) was calculated as the amount of amplification of damaged DNA samples (A_d) relative to the amplification of control (A_0) (no damage) as previously described [20,25]. Briefly, this PCR assay is an indirect indicator of DNA damage and has previously been reported to detect a wide array of oxidative lesions from an extensive source of oxidative stressors, including paraquat, UV radiation, photoreceptor outer segments, blue light and hydrogen peroxide [8,20,25,26]. Furthermore, as this study used acute levels of oxidative stress the amount of non-oxidative lesions would be negligible. The PCR conditions used in this study were based on published data using previously reported sequences for nDNA and mitochondrial DNA (mtDNA) primers, with minor modifications [20,25]. QPCR was carried out on a DNA Engine thermal cycler (DYAD, UK) with all reactions being a total volume of 100 μl containing 15 ng of total genomic DNA, 1unit of XL rTth polymerase, 3.3 XL PCR buffer II (containing potassium acetate, glycerol and DMSO) and final concentrations of 200 μM dNTP's, 1.2 mM $Mg(AOC)_2$ and 0.1 μM primers.

The gene fragments were amplified using the following thermo-cycling profile: The PCR was initiated with the addition of 1 unit of XL rTth

polymerase when samples had reached a temperature of 75°C. This was followed by an initial denaturation for 1 min at 94°C, cycles of denaturation at 94°C for 30 s and primer extension at 66°C for 12 min. After the PCR cycles had been completed a final extension at 72°C for 10 min was performed. The nuclear and mitochondrial gene products underwent 32 cycles and 26 cycles of thermo-cycling, respectively. After the completion of the QPCR, the gene products were resolved on a 1% agarose gel and digitally photographed on a UV transilluminator (UVi Tec, UK). The intensity of the PCR product bands was quantified with Scion Image analysis software (Scion Corporation, Version Beta 4.0.2).

Statistical analysis

Data represents the mean ± SEM of three separate experiments. Statistical analysis was performed using PRISM (GraphPad Software), by a one-way analysis of variance adjusted with a Bonferroni's correction. Values of significance were taken as $\star P < 0.05$.

Results

Cell type-specific sensitivity to oxidative stress

To determine whether there was a cell type-specific susceptibility to oxidative stress in our study, we utilized two indices of cell viability. Firstly, we measured the percentage cell viability during a 1 h H_2O_2 treatment and at 6 and 12 h post-treatment (Figure 1a,b). Secondly, we treated the cell cultures with a wide range of lethal H_2O_2 concentrations for up to 24 h and determined the lethal dose 50 values over a 24 h exposure period (LD_{50}; concentration of H_2O_2 to kill 50% of the cell population) (Figure 1c). Furthermore, in order to determine whether cell viability was due to cell type-specific behaviour and not to differences between the cell densities, cell counts were taken at all time points. We observed no significant difference in cell numbers between the investigated cell types throughout the study.

Firstly, as shown in Figure 1a, cell type-specific differences in cell viability after a 1 h H_2O_2 exposure was observed. The RPE displayed the greatest cell survival compared to all other cell types (~75%; $P < 0.05$). No significant differences in cellular sensitivity occurred between the other cell cultures. As shown in Figure 1b, at 6 h post-treatment, no significant change in percentage cell viability was measured compared to the respective 1 h cell viability levels. At 12 h post-treatment, only the RPE and corneal fibroblasts showed levels of cell viability that were greater than the respective 1 h cell viability levels ($P < 0.05$). Secondly, as shown in Figure 1c, utilizing LD_{50} values, the RPE demonstrated the greatest levels of oxidative resistance compared to all other cell types

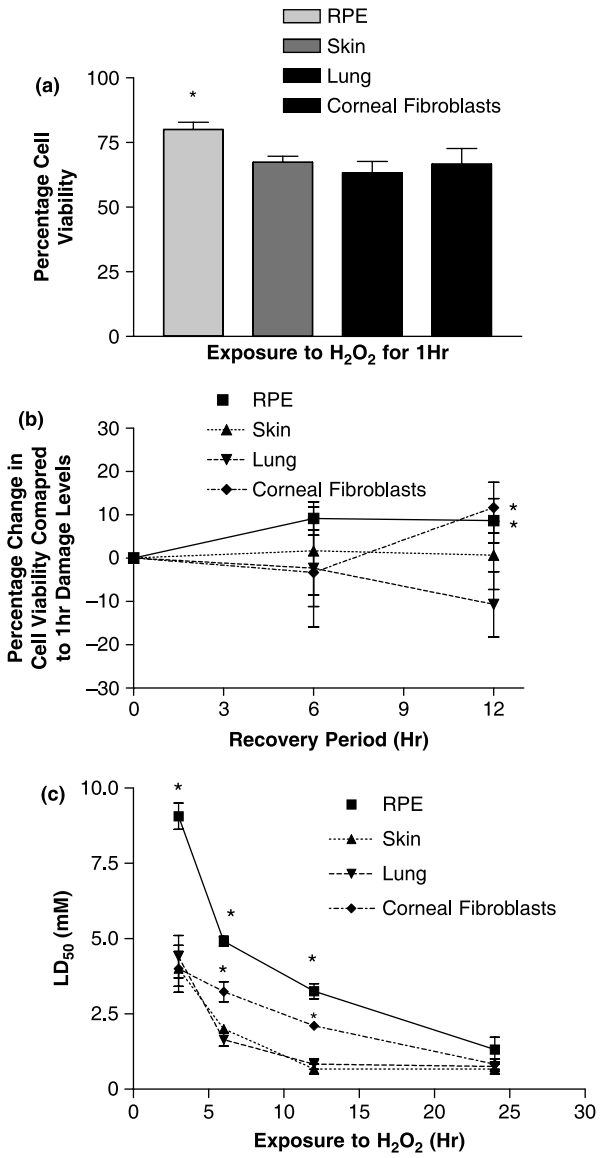

Figure 1. The effect of H_2O_2-induced oxidative stress on cell viability: (a) confluent cultures were exposed to H_2O_2 for 1 h and the resistance to oxidative stress was determined; (b) all cultures were allowed to recover up to 12 h post-treatment; (c) confluent cultures were exposed to various H_2O_2 concentrations for up to 24 h. The LD_{50} values were determined using PrismGraph Pad software. The data are expressed as the mean ± SEM from three experiments. Cellular viability was determined by the MTT assay and expressed as percentage cell viability of untreated control cultures. Furthermore, during the recovery period, the results are expressed as the percentage increase in cell survival compared to the respective 1 h cell viability levels. Cultures that displayed significantly different levels of cellular resistance or LD_{50} values compared to all other cell types were shown as $\star P < 0.05$.

at 3, 6 and 12 h H_2O_2 exposure (9, 4.8 and 3.2 mM, respectively; $P < 0.05$). The corneal fibroblasts demonstrated LD_{50} values that were significantly greater than the skin and lung cells at 6 and 12 h H_2O_2 exposure (3.2 and 2.1 mM, respectively; $P < 0.05$). Whereas, no significant differences between the LD_{50} values of the skin and lung cells was evident through out the oxidative exposure.

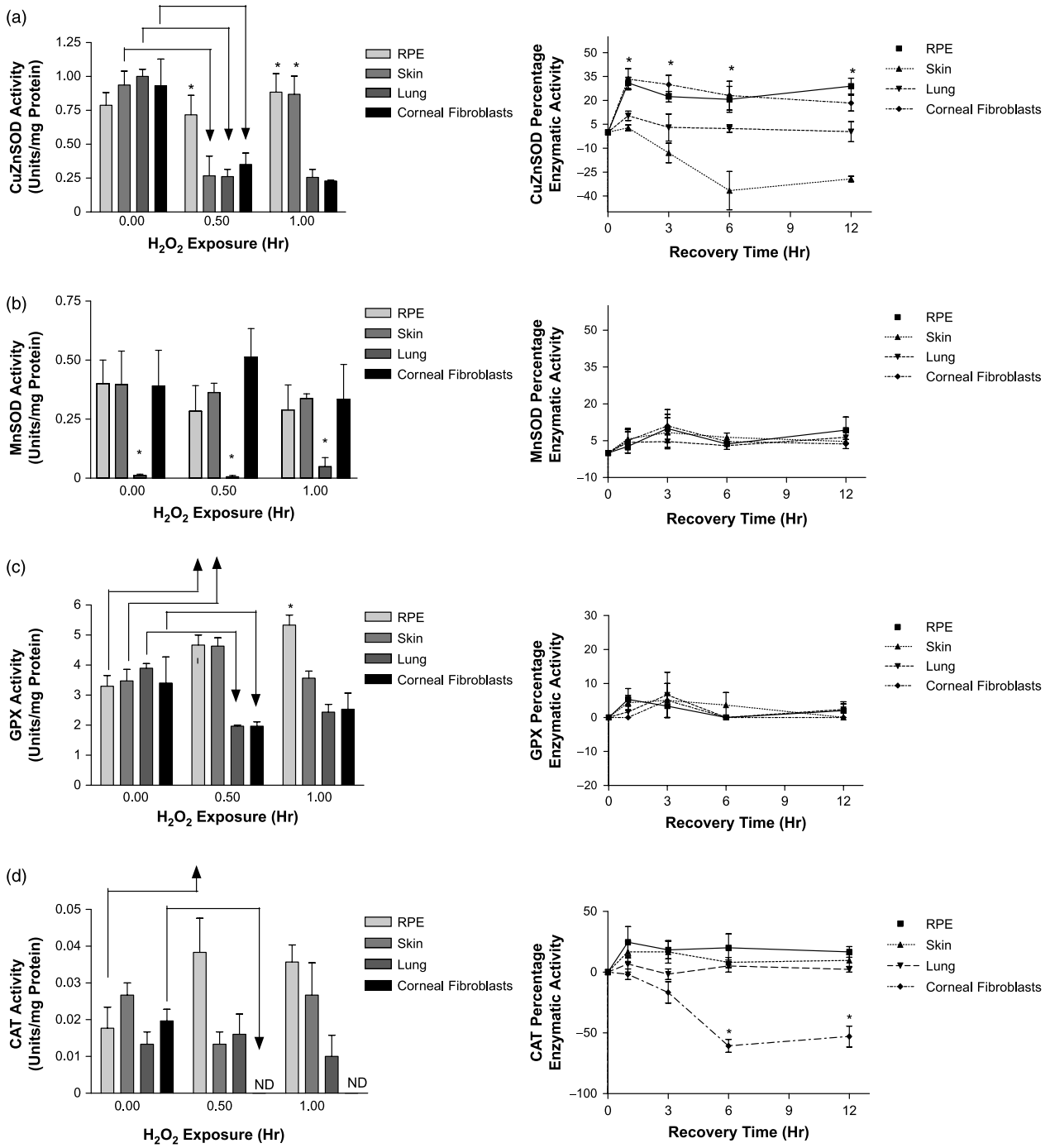

Figure 2. Cell type-specific antioxidant behaviour during and post- oxidative treatment. The enzymatic activities were analyzed, immediately before the toxic challenge, 15, 30, 60 min and 3, 6 and 12 h following the H_2O_2 challenge: (a) CuZnSOD activity, (b) MnSOD activity, (c) GPX activity, and (d) CAT activity. The data are expressed as the mean ± SEM from three experiments. Cultures that displayed significantly different enzymatic levels compared to the other cell types were shown as $\star P < 0.05$. In addition, significant modulations ($P < 0.05$) in antioxidant activity during the time course are represented by either an upward arrow (increase in activity) or a downward arrow (decrease in activity) during the time course.

Antioxidant activity

The enzymatic activity of major endogenous antioxidants was measured during and after oxidative exposure (Figure 2). A difference in antioxidant activity between cell types and in response to oxidative stress was observed. However, there was no correlation with susceptibility to oxidative stress.

CuZnSOD activity

As shown in Figure 2a, during control conditions, no significant difference in enzyme activity between cell types was evident. After 30 min H_2O_2 treatment, a decrease in activity occurred in the skin (3.6-fold; $P < 0.05$), lung (3.8-fold; $P < 0.05$) and corneal fibroblasts (3-fold; $P < 0.05$) (as shown as downward

arrows). In contrast, the RPE did not demonstrate any significant change in CuZnSOD activity which was greater than all other cell types ($P < 0.05$). At 1 h H_2O_2 treatment, the CuZnSOD activity in skin cells returned to control levels. All other cell types did not demonstrate any significant modulation in activity compared to respective 30 min levels. During the recovery period, significant differences in SOD activity was observe between cell types. The RPE and corneal fibroblast cells demonstrated the greatest CuZnSOD activity up to 12 h post-treatment ($P < 0.05$, respectively).

MnSOD activity

As shown in Figure 2b, during control conditions and during H_2O_2 treatment, significant differences in MnSOD enzymatic activities were observed, with the lung showing the lowest dismutase activity ($P < 0.05$; compared to all other cell types). During the recovery period, no significant modulation in MnSOD activity for all cell types was observed.

GPX activity

As shown in Figure 2c, during control conditions no significant differences in GPX activity between cell types was observed. After a 30 min H_2O_2 treatment, enzyme activity for the RPE and skin cells increased (1.2-fold; $P < 0.05$ compared to the respective control levels) (as shown as upward arrows). By contrast, enzyme activity for the corneal fibroblasts and lung cells decreased (2-fold; $P < 0.05$ compared to the respective control levels) (as shown as downward arrows). At 1 h H_2O_2 treatment, the GPX activity of the RPE was significantly greater than the other cell types and its activity at control conditions ($P < 0.05$). At 1 h post-treatment, all cell types displayed enzymatic activity that had returned to the respective control conditions and did not change throughout the recovery period.

CAT activity

As shown in Figure 2d, during the control conditions distinct cell type variations in CAT activity were observed. After a 30 min H_2O_2 treatment, only the RPE showed an increase in enzymatic activity (2-fold; $P < 0.05$) (as shown by an upward arrow). The corneal fibroblasts activity decreased to a point that it could not be detected. At 1 h H_2O_2 treatment no cell type demonstrated any change in activity compared to respective 30 min levels. During post-treatment, no significant difference in activity compared to their respective 1 h levels was observed for RPE, skin and lung. Furthermore, CAT activity could still not be detected in the corneal fibroblasts.

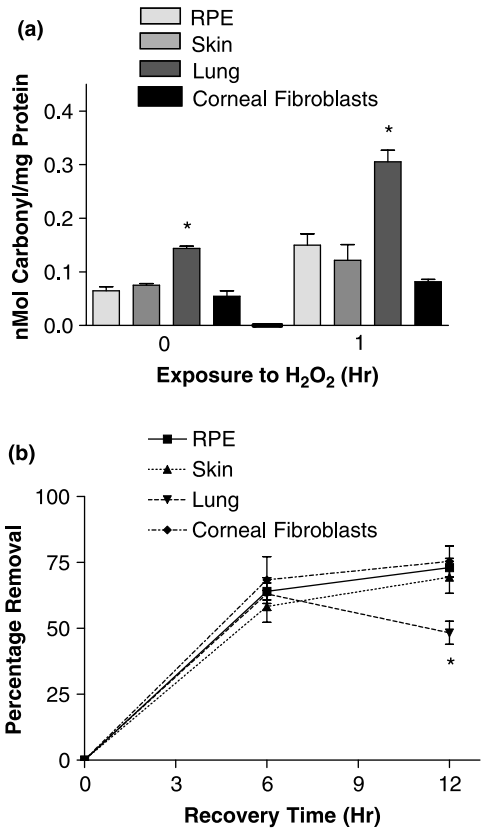

Figure 3. Protein carbonyl content and its subsequent removal after a H_2O_2 exposure. Confluent cultures were exposed to H_2O_2 for 1 h and the level of protein oxidation was measured using an ELISA method: (a) shows the levels of carbonyl groups immediately before and at 1 h H_2O_2 treatment; (b) shows levels of carbonyl groups at 6 and 12 h post-treatment. The data are expressed as the mean ± SEM from three experiments. Cultures that displayed significantly different levels of carbonyl groups from the other cell types were shown as $*P < 0.05$.

H_2O_2-induced carbonyl damage

As shown in Figure 3a, during control conditions, significant differences in the carbonyl levels between cell types was evident. The lung cells showed the greatest levels of carbonyl modifications during control conditions ($P < 0.05$). After a 1 h exposure to H_2O_2, all cell types sustained significant increased levels of protein oxidation ($P < 0.05$; compared to the respective control conditions). However, the lung cells continued to demonstrate the greatest extent of carbonyl modifications compared to the other cell types ($P < 0.05$). In contrast, no significant difference in the levels of carbonyl groups was observed between all other cell types.

Removal of H_2O_2-induced carbonyl damage

The ability of the cell types to remove the protein carbonyl groups was determined by measuring the levels of protein oxidation at 6 and 12 h post-treatment. As shown in Figure 3b, significant

differences in the removal capability of damaged protein were observed. The skin cells demonstrated an impaired ability to remove damaged protein with approximately 50% of the carbonyl modifications still remaining. In contrast, all other cell types demonstrated relatively efficient removal rates with no significant differences between the RPE, lung and corneal fibroblasts (~80% removal at 12 h post-treatment).

H_2O_2-induced nDNA damage

In order to understand the effect of oxidative genomic damage to both the nuclear and mitochondrial genomes, we utilized an assay to measure DNA lesions. As shown in Figure 4a,b, after a 1 h exposure to H_2O_2, all cell types sustained significant levels of DNA damage. However, the corneal fibroblasts demonstrated greater levels of nDNA damage compared to the other cell types ($P < 0.05$). In contrast, no significant difference in the levels of DNA damage was observed between all other cell types.

Repair of H_2O_2-induced nDNA damage

To verify the DNA repair capability of the different cell types we measured the extent of DNA lesions at 6 and 12 h post-treatment. As shown in Figure 4c, significant differences in the percentage repair of damaged nDNA were observed. The RPE showed the greatest percentage removal of DNA damage at 6 and 12 h post-treatment ($P < 0.05$; compared to all cell types).

H_2O_2-induced mtDNA damage

As shown in Figure 5a,b, after a 1 h H_2O_2 exposure, all cell types sustained significant levels of mtDNA damage. The lung cells demonstrated the greatest levels of DNA damage compared to the other cell types ($P < 0.05$). Furthermore, in all cell types, the mtDNA suffered significantly more lesions compared to the respective cellular nDNA ($P < 0.05$).

Repair of H_2O_2-induced mtDNA damage

A comparison of the repair capabilities of mtDNA damage revealed that all four cell types demonstrated a limited DNA repair activity towards H_2O_2-induced injury. As shown in Figure 5c, no significant change in the extent of mtDNA damage in the RPE, lung and corneal fibroblasts occurred compared to their respective 1 h H_2O_2 levels up to 12 h post-treatment. However, the skin demonstrated a significant increase in DNA damage at 12 h post-treatment compared to its 1 h damage levels ($P < 0.05$).

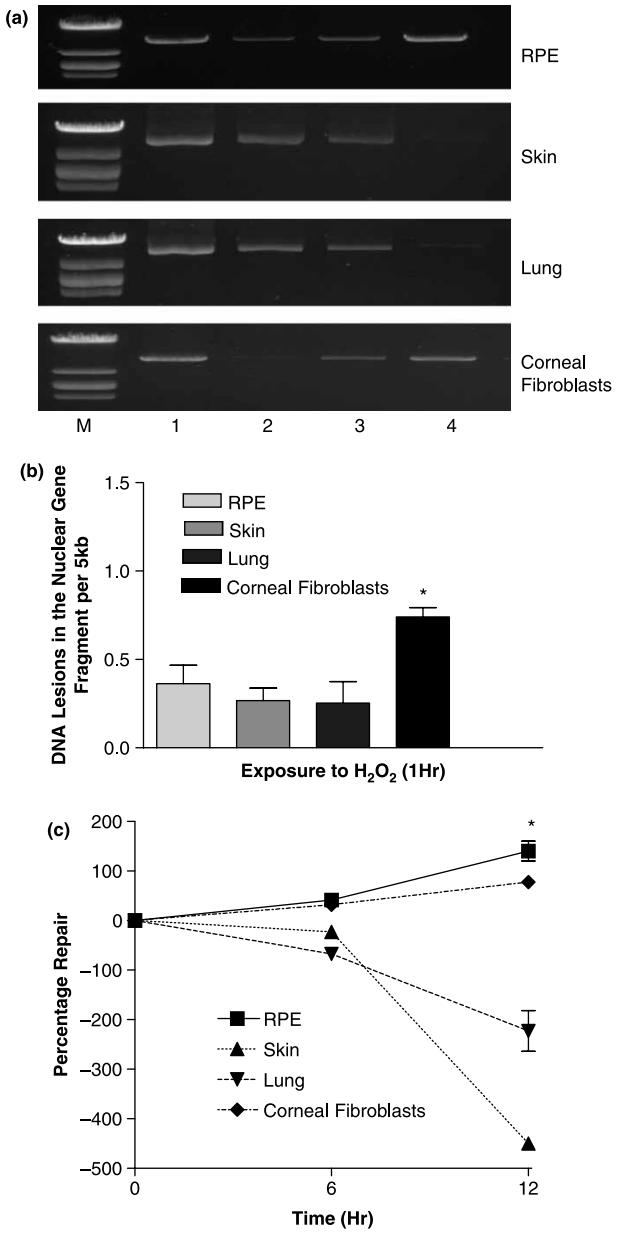

Figure 4. nDNA oxidative damage and its subsequent repair after a H_2O_2 exposure: (a) shows representive agarose gels. The lanes correspond to (1) control cells (untreated); (2) 1 h H_2O_2 damage; (3) 6 h repair; (4) 12 h repair; and (5) a 21 kb molecular DNA ladder; (b) shows the mean number of H_2O_2-induced nDNA lesions per 5 kb during and after oxidative exposure; (c) shows the percentage repair of the H_2O_2-induced nDNA damage at 6 and 12 h post-treatment. QPCR was carried out on freshly isolated DNA and the levels of DNA damage were determined from the relative intensities of the 10.4 kb *hprt* gene fragment, using the poisson equation. The untreated control cells were considered zero class damage. The data are expressed as the mean ± SEM from three experiments. Cultures that displayed significantly different levels of DNA lesions from the other cell types were shown as $*P < 0.05$.

Discussion

Over recent years much evidence has implicated a major role for ROS in cellular aging [8,9,12,25–28]. Interestingly, due to the diversity of the physiological micro-environments and cellular functions found in

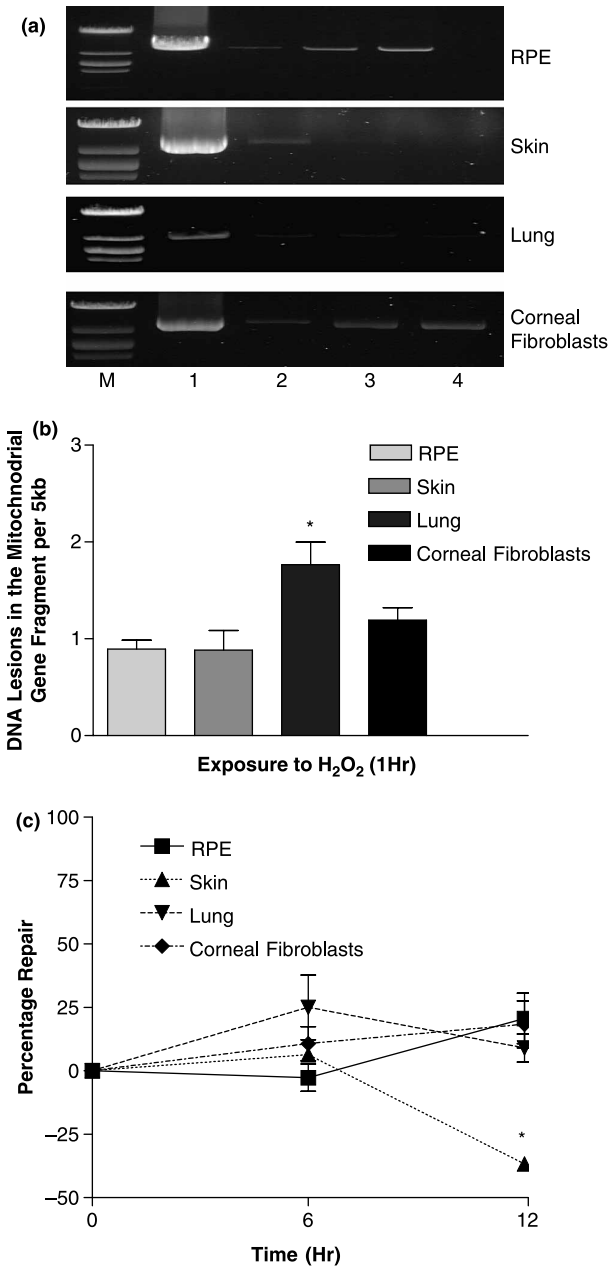

Figure 5. mtDNA oxidative damage and its subsequent repair after a H_2O_2 exposure: (a) shows representive agarose gels. The lanes correspond to (1) control cells (untreated); (2) 1 h H_2O_2 damage; (3) 6 h repair; (4) 12 h repair; and (5) a 21 kb molecular DNA ladder; (b) shows the mean number of H_2O_2-induced mtDNA lesions per 5 kb during and after oxidative exposure; (c) shows the percentage repair of the H_2O_2-induced mtDNA damage at 6 and 12 h post-treatment. QPCR was carried out on freshly isolated DNA and the levels of DNA damage were determined from the relative intensities of the 8.9 kb mitochondrial gene fragment, using the poisson equation. The untreated control cells were considered zero class damage. The data are expressed as the mean ± SEM from three experiments. Cultures that displayed significantly different levels of DNA lesions from the other cell types were shown as $\star P < 0.05$.

higher organisms, exposure to endogenous and/or exogenous ROS is highly specific to a particular cell type. Thus not surprisingly, cell and tissue-specific responses to various types of stress have been previously reported [15,29–34]. However, to date, the mechanisms involved in determining cell type-specific resistance to oxidative stress has not been fully elucidated. We asked whether nDNA and mtDNA repair and/or the cellular antioxidant status have important roles in determining cell type-specific susceptibility to oxidative stress and oxidative tolerance. In addition, this investigation allowed us to study our hypothesis, that cell types which are routinely exposed to highly oxidizing micro-environments *in vivo*, would have a greater resistance to oxidative stress compared to other cells even *in vitro*.

This report has provided novel findings, which have firstly, shown that cell type-specific oxidative sensitivities involved both variations in the cellular endogenous antioxidant potential and nDNA repair capacity. However, the extent of protein oxidation and the subsequent removal did not seem to have a measurable role in determining cell type-specific susceptibility to oxidative stress. Secondly, this study demonstrated post-mitotic cell types appear to have a more efficient nDNA repair capacity compared to cell types that are capable of cell division *in vivo*. Thirdly, our study suggests that cell types which are physiologically challenged by chronic high levels of oxidative stress, such as the RPE, appear to have more efficient oxidative defence and repair mechanism(s) compared to the other cell types.

This study compared the intrinsic cellular susceptibilities of four human cell types to oxidative stress; retinal pigment epithelium (RPE), corneal fibroblasts, alveolar type II lung cells and skin keratinocytes. The cell types used in this study were specifically chosen as they represent cells that have been well documented to be exposed to high levels of oxidative stress, under normal physiological conditions [14,19,34–36]. However, it should be pointed out the immortalisation of cell lines may result in ambiguities in comparison to an *in vivo* situation. Thus caution must always be used when interpreting *in vitro* data. However, the studied cell lines were chosen due to the fact that they have been reported to exhibit morphological and functional characteristics that are similar to their respective primary cultures [14,34–36].

This present study, confirmed that cellular antioxidant behaviour responded in a highly cell type-specific manner towards H_2O_2-induced oxidative stress. However no general trend in antioxidant activity and oxidative stress resistance either during or post-oxidative treatment was observed. The exact reason for this lack of correlation is unclear, however, multiple factors, are likely to be involved in determining a cell type's genomic susceptibility to oxidative stress for instance various enzymatic and non-enzymatic antioxidants, proteasome activity and heat shock proteins [3,29,31]. A generalized cellular antioxidant response observed during this study was that all cell types displayed MnSOD activity greater

than the respective control conditions post-treatment. This may be an adaptive mechanism which is required to prevent cumulative mtDNA damage which has grave consequences for cellular survival [18,37].

Cell type-specific patterns of protein oxidation were observed during oxidative exposure. In fact, even during conditions that were near absent of oxidative stress, cell type-specific variations in carbonyl levels existed. This damage presumably results from ROS that are produced during routine cellular metabolism [1]. We observed that the skin cells suffered the greatest levels of protein oxidation and interestingly, also had the weakest ability to remove (degrade) protein carbonyls. The 20S proteasome is largely responsible for the degradation of oxidized proteins. However, excessive protein oxidation renders the damaged protein resistant to proteolytic breakdown by the proteasome [38,39]. It is possible, that the inefficient repair of carbonyl groups demonstrated by the skin cells is due to the fact that this cell type sustained a relatively large extent of protein damage during oxidative stress exposure.

In regard to genomic damage, our investigation showed that, all cells were vulnerable to H_2O_2-induced nDNA damage, and obvious cell type-specific differences in the extent of nDNA protection were observed. The corneal fibroblasts displayed the greatest susceptibility to H_2O_2-induced nDNA damage, when no significant differences between the other cell types were apparent. Previous studies have also shown a wide range of genomic susceptibilities to oxidative stress [40–43]. However, to date, the reasons behind the variations in genomic susceptibility are not fully understood [8,25,30]. It is tempting to speculate that a major contributing factor behind the cell type-specific variations in genomic susceptibility may result from the differing antioxidant capabilities between cell types. Our study supports this observation, as it seems that the antioxidant capacity generally correlates with DNA damage levels in the investigated cell types. For instance, the corneal fibroblasts suffered the greatest extent of DNA damage as well as demonstrating the lowest levels of CAT activity during H_2O_2 exposure.

In regard to the nuclear genomic repair capacity, it appears that nDNA repair plays a crucial role in providing cellular resistance to oxidative stress. The RPE and corneal fibroblasts were the only cell types to demonstrate nDNA repair during the recovery period, which coincided with increased cell viability post-treatment. In contrast, the skin and lung cells exhibited a somewhat inefficient DNA repair process. In fact, increased levels of DNA damage were observed in these cell types. This is likely due to the fact that, even after the initial oxidative exposure was removed secondary oxidative reactions are resulting in continued genomic damage [29,37]. The observed divergent cellular repair capacities may result from different sensitivities of DNA repair enzymes to become inactivated by ROS, thus reducing the effectiveness of the repair pathways [44]. Thereby, by taking the DNA susceptibility and repair data together, we can postulate that the initial quantity of oxidative damage to the nuclear genome does not have a major role in determining the oxidative sensitivity of a cell type. However, the amount of damage that persists (unrepaired) seems to play a greater significance in conferring a cell's sensitivity to oxidative stress. This is supported by emerging data showing the ability of a cell to repair/remove damaged macromolecules generally correlates to oxidative resistance [18,30].

It appears that the nDNA oxidative repair efficiency may be an important survival factor for post-mitotic cell types such as the RPE and corneal fibroblasts. Of further note, the RPE cell line was the only cell type that seemed to possess an nDNA repair compensatory response to H_2O_2-induced damage, which resulted in lower levels of DNA lesions compared to the initial damage. This indicates that repair is occurring of not only induced injury but also pre-existing endogenous damage. A possible reason to explain the presence of efficient nDNA repair, in such cell types, could be ascribed to the fact that these two cell types are post-mitotic *in vivo* and are expected to function efficiently (in a highly oxidatively-damaging environments) throughout an individual's life time. Thus, in contrast to cells with a short half life such as the alveolar type II epithelial cells or skin keratinocytes, these cells may rely on apoptosis when confronted with DNA damage. This is not an option for the long-lived post-mitotic cells, thus a competent DNA repair could be a critical factor in slowing the aging process and cellular dysfunction.

In regard to the mitochondrial genome, the mtDNA, had a higher sensitivity to oxidative damage and showed inefficient repair compared to the nDNA in all cell types. This data confirms earlier studies demonstrating that the mitochondrial genome is highly vulnerable in a wide array of cell types [8,18,20,25,30]. In addition, our investigation showed that the susceptibility of mtDNA to H_2O_2-induced oxidative stress significantly differed between cell types. By contrast, the repair capability did not vary between cell types. To date, the factors implicated in the divergent oxidative mtDNA sensitivities are largely unknown. However, one likely explanation may involve the major mitochondrial enzymatic antioxidant (MnSOD). Our study showed that cell types which had a lower MnSOD activity also suffered higher levels of mtDNA damage. In further support of the critical role that MnSOD provides against oxidative stress, knockout animal models have clearly demonstrated increased susceptibility to oxidative damage and oxidative stress related pathologies [45].

In summary, based on this present study, it is reasonable to assume that a synergistic interaction between antioxidants and nDNA repair plays an important role in determining cell type-specific sensitivity to oxidative damage. This observation is further strengthened by several recent reports that have shown efficient resistance to oxidative stress is not observed with either elevated antioxidants or DNA repair alone [46,47]. Furthermore, we have provided evidence that the *in vivo* physiological micro-environment of a cell may influence its intrinsic oxidative stress susceptibility *in vitro*. A particular interestingly finding was that the RPE demonstrated the greatest tolerance to a lethal oxidative exposure. Therefore, this study has supported our hypothesis that cell types which are subjected to high oxidative burdens *in vivo* have developed a greater intrinsic resistance to oxidative stress than other cell types.

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References

[1] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993;90:7915–7922.
[2] Sawyer DE, Van Houten B. Repair of DNA damage in mitochondria. Mutat Res 1999;434:161–176.
[3] Stadtman ER. Protein oxidation in aging and age-related diseases. Ann N Y Acad Sci 2001;928:22–28.
[4] Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 2003;25:207–218.
[5] Choudhary S, Xiao T, Srivastava S, Zhang W, Chan LL, Vergara LA, Van Kuijk FJ, Ansari NH. Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells. Toxicol Appl Pharmacol 2005;204:122–134.
[6] Dusinska M, Kazsimirova A, Barancokova M, Beno M, Smolkova B, Horska A, Raslova K, Wsolova L, Collins AR. Nutritional supplementation with antioxidants decreases chromosomal damage in humans. Mutagenesis 2003;18: 371–376.
[7] Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: Induction, repair and significance. Mutat Res 2004;567:1–61.
[8] Ballinger SW, Van Houten B, Jin GF, Conklin CA, Godley BF. Hydrogen peroxide causes significant mitochondrial DNA damage in human RPE cells. Exp Eye Res 1999;68:765–772.
[9] Bohr VA. Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic Biol Med 2002a;32:804–812.
[10] Chevanne M, Caldini R, Tombaccini D, Mocali A, Gori G, Paoletti F. Comparative levels of DNA breaks and sensitivity to oxidative stress in aged senescent human fibroblasts: A distinctive pattern for centenarians. Biogerontology 2003;4:97–104.
[11] Sitte N, Merker K, Grune T. Proteasome-dependent degradation of oxidized proteins in MRC-5 fibroblasts. FEBS Lett 1998;440:399–402.
[12] Stadtman ER. Cyclic oxidation and reduction of methionine residues of proteins in antioxidant defense and cellular regulation. Arch Biochem Biophys 2004;423:2–5.
[13] Scharffetter-Kochanek K, Brenneisen P, Wenk J, Herrmann G, Ma W, Kuhr L, Meewes C, Wlaschek M. Photoaging of the skin from phenotype to mechanisms. Exp Gerontol 2000; 35:307–316.
[14] Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. Mol Vis 1999;5:32.
[15] de Waard H, de Wit J, Gorgels TG, van den Aardweg G, Andressoo JO, Vermeij M, van Steeg H, Hoeijmakers JH, van der Horst GT. Cell type-specific hypersensitivity to oxidative damage in CSB and XPA mice. DNA Repair 2003;2:13–25.
[16] Dreher I, Schmutzler C, Jakob F, Kohrle J. Expression of selenoproteins in various rat and human tissues and cell lines. J Trace Elem Med Biol 1997;11:83–89.
[17] Schul W, Jans J, Rijksen YM, Klemann KH, Eker AP, de Wit J, Nikaido O, Nakajima S, Yasui A, Hoeijmakers JH, van der Horst GT. Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. EMBO J 2002;21:4719–4729.
[18] Grishoko VI, Rachek LI, Spitz DR, Wilson GL, LeDoux SP. Contribution of mitochondrial DNA repair to cell resistance from oxidative stress. J Biol Chem 2005;280:8901–8905.
[19] Ha NT, Nakayasu K, Murakami A, Ishidoh K, Kanai A. Microarray analysis identified differentially expressed genes in keratocytes from keratoconus patients. Curr Eye Res 2004;28:373–379.
[20] Jarrett SG, Boulton M. Antioxidant up-regulation and increased nuclear DNA protection play a key role in adaptation to oxidative stress in epithelial cells. Free Radic Biol Med 2005;38:1382–1391.
[21] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunonol Methods 1983;65:55–63.
[22] Cohen G, Kim M, Ogwu V. A modified catalase assay suitable for a plate reader and for the analysis of brain cell cultures. J Neurosci Methods 1996;67:53–56.
[23] Ewing JF, Janero DR. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anals Biochem 1995;232:243–248.
[24] Buss H, Chan TP, Shuish B, Domigan NM, Winterbourn CC. Protein carbonyl measurement by sensitive ELISA method. Free Radic Biol Med 1997;23:361–366.
[25] Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci USA 1997;94:514–519.
[26] Ayala-Torres S, Chen Y, Svoboda T, Rosenblatt J, Van Houten B. Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction. Methods 2000;22: 135–147.
[27] Ma W, Kleiman NJ, Sun F, Li D, Spector A. Peroxide toxicity in conditioned lens epithelial cells—evaluation of multi-defense systems. Exp Eye Res 2003;77:711–720.
[28] Beckman KB, Ames BN. Endogenous oxidative damage of mtDNA. Mutat Res 1999;424:51–58.
[29] Burczynski JM, Hayes JR, Longhurst PA, Colby HD. Species differences in adrenal lipid peroxidation: Role of α-tocopherol. Free Radic Biol Med 1999;26:987–991.
[30] Hollensworth SB, Shen C, Sim JE, Spitz DR, Wilson GL, LeDoux SP. Glial cell type-specific responses to menadione-induced oxidative stress. Free Radic Biol Med 2002;28: 161–1174.

[31] Hurst JS, Saini MK, Jin G, Awasthi YC, Van Kuijk FJGM. Toxicity of oxidized β-carotene to cultured human cells. Exp Eye Res 2005;81:239–243.
[32] Meehan WJ, Spencer JP, Rannels DE, Welch DR, Knobbe ET, Ostrander GK. Hydrogen peroxide induces oxidative DNA damage in rat type II pulmonary epithelial cells. Environ Mol Mutagen 1999;33:273–278.
[33] Wang Y, Chaung R, Tung L. Comparison of the cytotoxicity induced by different exposure to sodium arsenite in two fish cell lines. Aquat Toxicol 2004;69:67–79.
[34] Weidauer E, Lehmann T, Ramisch A, Rohrdanz E, Foth H. Response of rat alveolar type II cells and human lung tumor cells towards oxidative stress induced by hydrogen peroxide and paraquat. Toxicol Lett 2004;151:69–78.
[35] Manevich Y, Sweitzer T, Pak JH, Feinstein SI, Muzykantov V, Fisher AB. I-cys peroxiredoxin overexpression protects cells against phospholipid peroxidation-mediated membrane damage. Proc Natl Acad Sci USA 2002;99:11599–11604.
[36] Masella R, Straface E, Giovannini C, Di Benedetto R, Scazzocchio B, Viora M, Cantafora A, Malorni W. Subcellular alterations induced by UV-oxidized low density lipoproteins in epithelial cells can be counteracted by alpha-tocopherol. Photochem Photobiol 2000;71:97–102.
[37] Jin GF, Hurst JS, Godley BF. Rod outer segments mediate mitochondrial DNA damage and apoptosis in human retinal pigment epithelium. Curr Eye Res 2001;23:11–19.
[38] Davies KJ. Degradation of oxidized proteins by the 20S proteasome. Biochimie 2001;83:301–310.
[39] He K, Nukada H, McMorran PD, Murphy MP. Protein carbonyl formation and tyrosine nitration as markers of oxidative damage during ischaemia-reperfusion injury to rat sciatic nerve. Neuroscience 1999;94:909–916.
[40] Bishop A, Marquis JC, Cashman NR, Demple B, Kagawa K. Adaptive resistance to nitric oxide in motor neurons. Free Radic Biol Med 1999;26:978–986.
[41] Roos WP, Binder A, Bohm L. Determination of the initial DNA damage and residual DNA damage remaining after 12 h of repair in eleven cell lines at low doses of irradiation. Int J Radiat Biol 2002;76:1493–1500.
[42] Suzuki T, Spitz DR, Gandhi P, Lin HY, Crawford DR. Mammalian resistance to oxidative stress: A comparative analysis. Gene Expr 2002;10:179–191.
[43] Arrigo A, Firdaus WJJ, Mellier G, Moulin M, Paul C, Diaz-latoud C, Kretz-remy C. Cytotoxic effects induced by oxidative stress in cultured mammalian cells and protection provided by Hsp27 expression. Methods 2005;35:126–138.
[44] Laval F, Wink DA, Laval J. An discussion of mechanisms of NO genotoxicity: Implication of inhibition of DNA repair proteins. Rev Physiol Biochem Pharmacol 1997;131: 175–191.
[45] Liang L, Patel M. Mitochondrial oxidative stress and increased seizure susceptibility in Sod2$^{-/+}$ mice. Free Radic Biol Med 2004;36:542–554.
[46] Kisby GE, Lesselroth H, Olivas A, Samson L, Gold B, Tanaka K, Turker MS. Role of nucleotide- and base excision repair in genotoxin-induced neuronal cell death. DNA Repair 2004;3:617–627.
[47] Dargusch R, Schubert D. Specificity of resistance to oxidative stress. J Neurochem 2002;81:1394–1400.