



White Light-mediated DNA Strand Breaks in Lens Epithelial Cells

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It was hypothesized that white light can induce DNA single-strand breaks/alkali labile sites in lens epithelial cells during the dissection process when lenses are being collected for study. Dissection of lenses in white light significantly increased 'Integrated Intensity' ($P = 0.0216$), an index of DNA single strand breaks/alkali labile sites, using the alkaline microgel electrophoresis assay. In addition, we investigated the DNA damaging effect of visible light in a human lymphocyte model in which cells were exposed to white light, red light or minimal indirect light for 5 min. Results of these experiments confirm our conclusion that visible light induces DNA damage in cells. These findings may be relevant in a genotoxicity study on cells *ex vivo*. © 2002 Elsevier Science Ltd.

Key words: visible light; lens epithelial cells; alkaline microgel electrophoresis; DNA single strand breaks.

1. Introduction

Exposure to light in mammalian cells can lead to DNA damage, the severity of which is dependent on the wavelength and the internal and external cellular environment (Epe et al., 1989; Balasubramanian, 2000; Yusifov et al., 2000; Pavilack and Brod, 2001). Lens epithelial cells provide a good model for relatively early age-related increase in oxidative stress (Wolf and Penn, 2001). Stress has been shown to be associated with DNA damage (Harman, 1998). DNA damage due to oxidative stress may be significantly lower in the *in vivo* environment of lens when compared with lens *ex vivo*, as the lens *ex vivo* is exposed to 20 % oxygen during dissection while the *in vivo* lens is exposed to only 3 % oxygen. Compensatory antioxidant defenses, such as glutathione, catalase and superoxide dismutase (Giblin, 2000; Yusifov et al., 2000) are released in response to light-induced oxidative stress that would result in macromolecular damage (DNA, crystallin). Also, it is likely that several DNA repair enzymes are induced by exposure to light, in order to maintain the long-term functionality of the lens epithelial cells. Compromised anti oxidant defenses (due to various factors including a longer duration dissection/removal process) can lead to excessive DNA damage in lens epithelial cells as has been shown in retinal cells (Organisciak et al., 2000). The most abundant lesion produced is DNA single strand breakage, which can be sensitively detected by the alkaline microgel electrophoresis (COMET) assay (Singh et al., 1995; Singh, 2000). Additionally, the COMET assay has been

previously used to detect DNA single strand breaks in lens epithelial cells (Kleiman and Spector, 1993; Bucolo et al., 1994; Sasaki et al., 1998). However, results of DNA damage estimation are influenced by several factors. One of the most important is light (Pflaum et al., 1994, 1998; Kielbassa et al., 1997) during the dissection process.

In this report we have investigated the effect of white light during dissection of the lens on DNA single strand breaks/alkali labile sites using the sensitive technique of COMET. The technique involves the mixing of 1000–10 000 single cells with agarose, making a microgel on microscopic slides, lysing these embedded cells in agarose by detergents and high salt, electrophoresing nuclear DNA in microgels on slides to allow broken pieces of negatively charged DNA to move towards the positive electrode in a size dependent manner, fixing the DNA in agarose by dehydrating the microgels, and finally staining the DNA from individual cells with a sensitive dye, YOYO-1, and quantifying DNA damage using an image analysis program.

2. Materials and Methods

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) unless mentioned otherwise. Brown Norway male rats, approximately 8 weeks of age, were purchased from Harlan Labs (Indianapolis, IN, U.S.A.). They were housed in a temperature-controlled (22°C) vivarium maintained at a 12 hr light–dark cycle.

Rats were killed by sealing the top of the individual plastic cages with a Styrofoam lid, and pumping CO₂ gas, for approximately 4 min through a hose

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perforating the lid. The whole eye was removed from the rat and placed corneal-side down on sterile gauze, and held in place by forceps. Two incisions, at right angles to each other, were made across the surface where the optic nerve enters the eye, and the sclera was pulled back to expose the lens. The iris and ciliary muscles, which are attached on the equatorial plane of the lens, were gently teased away with forceps as the lens was removed from the eye. Orientation of the lens was maintained using the remnants of the pigmented equatorial plane where the iris was attached to the lens. The lens epithelial cells are located on the hemispherical surface oriented toward the cornea.

Red light (600–700 nm) or white light (broadband) was used during dissection. A light bulb (21 V, 150 W) was fitted into an 18 inch fiber-optic light guide DPL operated by an illuminator (Model T-Q/FOI-1 from Techni Quip Corp, Hollywood, CA, U.S.A.). The intensity at the site of dissection (a distance of approximately 3.5 inches from the source of light) was adjusted to approximately 100 fc for both lights (as measured by a Sylvania Digital Light Meter, Model DS-2000, OSRAM, E.I.L. Instruments, Clarence, NY, U.S.A.). No UVA, UVB or UVC light was detected during 5 min of exposure to white light source when measured using UV detectors (Filters: UVA#1285, UVB#16022, UVC#5924, International Light, Newburyport, MA, U.S.A.) fitted in a UV light meter (IL 1400 Research Radiometer, for UVA and UVB and IL 1700 Research Radiometer for UVC, International Light, Newburyport, MA, U.S.A.). Approximately 5 min of dissection time was used for each lens using a dissecting microscope (Unitron, ZST, Japan) at a magnification of $8\times$ (eye-piece: $10\times$ and objective: $0.8\times$).

All steps after this time were conducted in dim and indirect light. Immediately after dissection, the lens was immersed in 5% sucrose in cold phosphate buffered saline (PBS) with freshly added 200 μmol of *N*-t-butyl-alpha-phenyl nitron (PBN), a potent spin trap to minimize DNA damage by free radicals. The lens was placed on a wire grid of a cold Mini Tissue Press (Biospec Products Inc, Bartlesville, OK, U.S.A.). The Mini Tissue Press is composed of a steel piston, which can move up and down in a steel barrel in a rotational movement. At the lower end of the barrel, a stainless steel wire screen (hole size $250 \times 250 \mu\text{m}$) is held with a supporting plate. After assembling this unit, the whole lens was dispersed into 1 mm cubes by forcing it through the wire grid into a microfuge tube having cold PBS with PBN. The tissue pieces thus obtained were pelleted and the supernatant was discarded, leaving a volume of approximately 20 μl on top of the pellet. The tissue pieces were dispersed into a single cell suspension by repeated pipeting. Ten μl of the single cell suspension from the whole lens was mixed with 50 μl of freshly made 0.5% agarose (High resolution agarose 3:1 from Amresco, Solon, OH, U.S.A.) in PBS. Fifty μl of this mixture was quickly

placed onto a clear window frosted slide (MGE slides, Erie Scientific Co, New Haven, CT, U.S.A.) already coated with the dry and wet first layer of microgel (Singh, 2000). A cover glass was placed on top of the cell-agarose mixture to make a second layer of microgel. The slide was placed on an ice-cold plate for 1 min and then the cover glass was removed and a third layer of microgel was made using 200 μl of agarose. The slides were then immersed in lysing solution (prewarmed to 37°C) having 1.25 M NaCl, 0.01% sodium salt of *N*-lauroyl sarcosine, 50 mM tetra sodium salt of EDTA, 100 mM, tris pH10, with freshly added 0.5 mg/ml proteinase K (Amresco, Solon, OH, U.S.A.) and 2 mg/ml Glutathione (Free Acid, Reduced Form) and incubated for 1 hr at 37°C . The slides were placed in a special electrophoretic unit (Ellard Instrumentation, Monroe, WA, U.S.A.) with 1 l of 300 mM NaOH, 1 mM EDTA, 0.2% DMSO and 0.01% 8 hydroxyquinoline (pH > 13.5). The DNA from the lysed cells in the microgels was electrophoresed at 12 V (0.4 V cm^{-1}), 250 mA for 20 min at room temperature in indirect, incandescent low light. During electrophoresis, the solution was recirculated at a rate of 100 ml min^{-1} . The slides were immersed in 50% ethanol having 1 mg ml^{-1} of spermine and 20 mM of Tris-HCl (pH 7.4) for 1 hr at room temperature. The slides were dried in air. Fifty μl of the pre-staining solution (5% sucrose in 10 mM NaH_2PO_4) was placed on each slide and covered with a coverglass. The coverglass was removed and 50 μl of 0.25 μM YOYO in 5% DMSO and 5% sucrose was added to each slide.

In another experiment, lymphocytes were isolated from freshly drawn human blood as described previously (Singh et al., 1995). Microgels were made as described earlier. The cells in the microgels (placed on ice) were exposed to white light or red light at an intensity of 100 fc (1076 lx) for 5 min at a distance of 3.5 inches. A third sample of lymphocytes was similarly kept on ice for 5 min in low indirect, incandescent light (a reading of less than 1 fc on a light meter scale). The cells in microgels were lysed immediately and processed for estimation of DNA single strand breaks as described earlier.

DNA damage was primarily assessed using the parameter 'Integrated Intensity', as an index of DNA single strand breaks, with the help of VisComet image analysis software (Impulse Bildanalyse GmbH, Gilching, Germany) and a CCD camera CV252 (JAI Corporation, Kanagawa, Japan) attached to a Reichert fluorescent microscope with excitation at 490, dichroic at 500, and emission at 515 nm. The parameter 'Integrated Intensity' was used to measure three-dimensional changes in length, breadth and intensity of nuclear DNA due to damage. Fifty cells were analysed from each slide. The unpaired one-tailed Student's *t*-test was used to compare mean of means of DNA damage levels from 50 lens epithelial cells exposed to red light with those exposed to white light.

Similarly, *t*-test was used to compare means of mean from 50 lymphocytes exposed to white light or red light or indirect, incandescent low light (a reading of less than one on light meter scale). The Mann-Whitney U-test was used to compare the sums of means of DNA damage levels from 50 lens epithelial cells (from one lens) exposed to red light with those exposed to white light.

3. Results

Examples of DNA migration in cells from lenses dissected in red and white light are shown in Figs. 1 and 2, respectively. DNA damage, as indicated by increased DNA migration, is significantly lower in the majority of the cells exposed to red light (Fig. 1) compared to those exposed to white light during lens dissection (Fig. 2). The means and standard deviations of integrated intensity from 8 lenses from 4 animals (50 cells per lens) are plotted as a bar graph in Fig. 3. Exposure to white light during dissection of the lens induced significantly higher levels of DNA single strand breaks/alkali labile sites (one-tailed *t*-test, $P = 0.021$), than those dissected in red light. Other commonly used parameters as indices of DNA single strand breaks/alkali labile sites, such as tail moment ($P = 0.045$), comet extent ($P = 0.046$), tail extent ($P = 0.048$) and tail breaks ($P = 0.048$) were also increased (data not shown). However, not all the cells were uniformly damaged. Fig. 4 shows the variation in DNA damage levels from lens to lens. The Mann-Whitney U-test indicates the significant effects of white light on DNA damage levels ($P = 0.041$) as predicted. Higher DNA damage ($P > 0.0001$, two-tailed, unpaired *t*-test) was found in human lymphocytes exposed to white light compared to cells exposed to red light. There was no significant difference between cells exposed to red light and cells exposed to minimal indirect light ($P = 0.324$, unpaired, two-tailed *t*-test), as seen in Fig. 5.

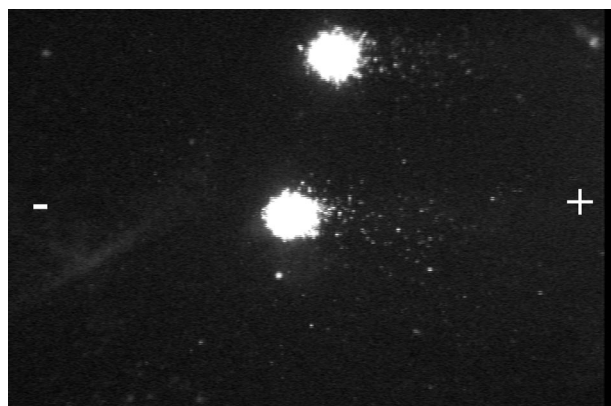


FIG. 1. DNA migration pattern from lens epithelial cells exposed to red light (magnification: $400\times$, dye: YOYO 1). The symbols (+ and -) indicate the cathode and anode, respectively, during electrophoresis of negatively charged DNA.

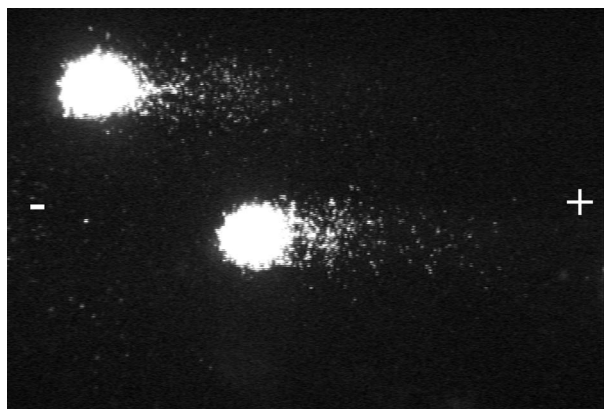


FIG. 2. DNA migration pattern from lens epithelial cells exposed to white light (magnification: $400\times$, dye: YOYO-1). The symbols (+ and -) indicate the cathode and anode, respectively, during electrophoresis of negatively charged DNA.

4. Discussion

Light has both beneficial and detrimental effects on cellular macromolecules (Ryter and Tyrell, 1998). In mammalian cells light mediates synthesis of vitamin D (Holick et al., 1980). In plants light is responsible for photosynthesis and mediates many other cellular effects via generation of hydrogen peroxide (Ishida et al., 1998; Karpinski et al., 1999). In bacteria white light induces repair of UV-induced DNA damage by photoreactivation. DNA damaging effects of visible light on DNA single strand breaks/alkali labile sites have been demonstrated in a variety of cell types (Erickson et al., 1980; Sideris et al., 1981; Christensen et al., 1990; Churchill et al., 1991; Pflaum et al., 1998; Specht et al., 2000) using the alkaline elution assay. Visible light has been shown to be mutagenic and carcinogenic due to its capability to damage DNA, directly or indirectly, via cellular chromophores such as porphyrins and riboflavin (Yamamoto et al., 1992; Kielbassa et al., 1997; Pflaum et al., 1998). DNA lesions (DNA single and double strand breaks) in retinal cells in vivo, induced by visible light, were observed even when the retina was no longer exposed to the light (Specht et al., 2000).

Visible light-induced damage is not a special case confined to lens epithelial cells, as was shown by our experiment with human lymphocytes, a cell type of different embryonic origin, function and species. In addition, there exists the possibility that DNA damage due to broadband white light was secondary to other macromolecular damage (Nitzan and Ashkenazi, 2001), such as damage to membranes and activation of endonucleases (Zaidi et al., 1993).

Our results, in rat lens epithelial cells and human lymphocytes exposed to visible light, support earlier reports on the induction of DNA single strand breaks/alkali labile sites by light (Erickson et al., 1980; Sideris et al., 1981; Christensen et al., 1990; Churchill et al., 1991; Pflaum et al., 1998). Erickson et al. (1980) did not find these lesions to be mutagenic in V 79 cells.

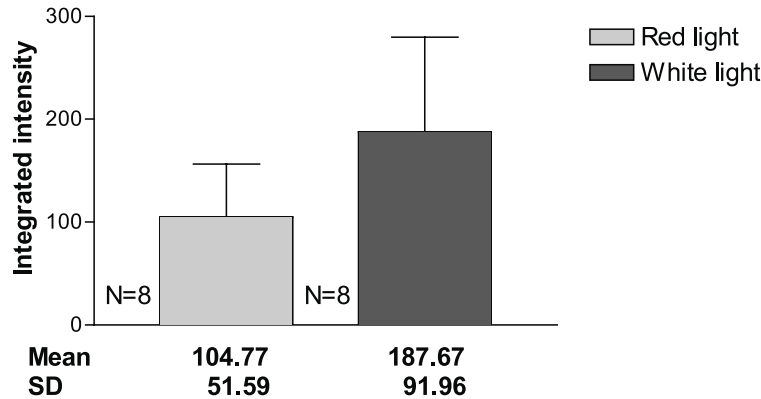


FIG. 3. Fifty lens epithelial cells per animal were analysed (a total of four animals, with two lenses from each animal, N = number of lenses). Use of white light during dissection of lens induced significantly more DNA damage than red light (one-tailed t -test, $P = 0.021$). Analysis of DNA damage was also conducted using parameters other than integrated intensity and similar results were found: tail moment ($P = 0.045$), comet extent ($P = 0.046$), tail extent ($P = 0.048$) and tail breaks ($P = 0.048$).

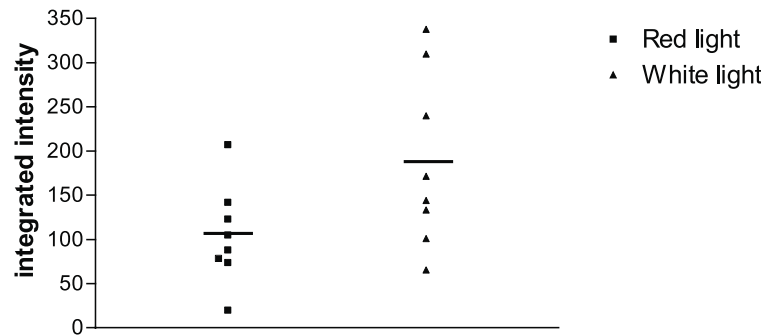


FIG. 4. Means of 50 cells from each of eight lenses, exposed to red and white light during dissection were plotted. The horizontal line represents mean of means from eight lenses (Mann-Whitney U-test, one tail, $P = 0.041$).

Conversely, [Sideris et al. \(1981\)](#) showed these lesions to be lethal to some Chinese hamster cells in culture. [Wolf and Penn \(2001\)](#) did not find any effect from long-term fluorescent light exposure on the incidence of cataract in mice, while [Delcourt et al. \(2000\)](#) found a higher incidence of cortical and mixed

cataract in human subjects who are exposed to excessive sunlight.

We observed wide variations in DNA damage levels from lens to lens in both groups (dissected in red and white light). There appears to be no obvious reason for such variations. It is possible that such a wide range of

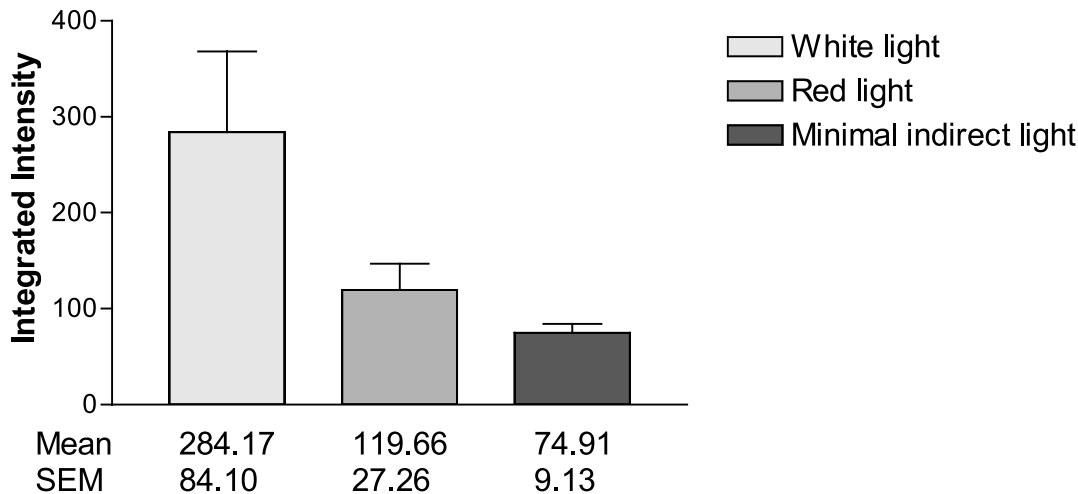


FIG. 5. Each data point is an average of means from 3 different experiments. In each experiment 50 lymphocyte cells were analyzed for each data point. Error bars represent standard deviations of means. Significantly higher levels of DNA damage ($P = 0.016$, one-tailed, unpaired t -test) were induced by white light as compared to red light. Note that, proportionately, white light induced more DNA damage in lymphocytes than in lens epithelial cells.

DNA damage is due to variations in dissecting and handling of lenses and assay procedures. Wide animal to animal variation in DNA damage levels has also been observed in our past *in vivo* studies (Singh et al., 2001).

It is possible that very low levels of UV light were also present in the light source that was used, though UVA, UVB and UVC were unable to be detected using a photometer. However, UV induced damage is not expressed to a detectable level within 15 min post-irradiation and requires incubation of cells at 37°C (Beisker and Hittelman, 1988; Mullaart et al., 1989; Sidjanin et al., 1993). The DNA damage induced by UV requires incision at sites by recognition enzymes. The lens epithelium was never exposed to 37°C during dissection or post-dissection processing except when cells were mixed in liquid agarose for less than a minute, thus it is unlikely that the damage we have observed is due to UV light.

Our results underscore the importance of minimizing the use of bright white light during dissection or handling of tissues and during the various steps for alkaline microgel electrophoresis. This study shows that white light (at an intensity of 1076 lx) produces detectable DNA damage in lens epithelial cells within 5 min *ex vivo*. To put this information in perspective, a Philips 60 W incandescent bulb operated at 120 V provides 1076 lx at a distance of 1 ft. Our findings also suggest the possible use of the alkaline microgel electrophoresis assay to evaluate the role of antioxidants in preventing light-induced macromolecular damage in lens.

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