

# DNA damage in lens epithelium of cataract patients *in vivo* and *ex vivo*

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## ABSTRACT.

**Purpose:** DNA damage has been described in the human cataractous lens epithelium, and oxidative stress generated by UV radiation and endogenous metabolic processes has been suggested to play a significant role in the pathogenesis of cataract. In this study, the aim was to explore the quality and relative quantity of DNA damage in lens epithelium of cataract patients *in vivo* and after incubation in a cell culture system.

**Methods:** Capsulotomy specimens were analysed, before and after 1 week of *ex vivo* cultivation, using the comet assay to measure DNA strand breaks, oxidized purine and pyrimidine bases and UV-induced cyclobutane pyrimidine dimers.

**Results:** DNA strand breaks were barely detectable, oxidized pyrimidines and pyrimidine dimers were present at low levels, whereas there was a relatively high level of oxidized purines, which further increased after cultivation.

**Conclusion:** The observed levels of oxidized purines in cataractous lens epithelium may support a theory consistent with light damage and oxidative stress as mediators of molecular damage to the human lens epithelium. Damage commonly associated with UV-B irradiation was relatively low. The levels of oxidized purines increased further in a commonly used culture system. This is of interest considering the importance and versatility of *ex vivo* systems in studies exploring the pathogenesis of cataract.

**Key words:** 8-oxoguanine – cataract – comet assay – DNA damage – human lens epithelium – lens – light – oxidative stress – oxidized purines

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## Introduction

Cataract is the most prevalent eye disease in the world (Pascolini et al. 2004).

It is at the top of the World Health Organization list of prioritized eye diseases. Delaying the average age of onset would significantly reduce this problem,

and preventive measures against the development of cataract are thus of potentially tremendous importance (Frick & Foster 2003).

Light, Ultraviolet radiation (UVR) and oxidative stress are considered to be important factors in the pathogenesis of age-related cataract (Soderberg 1990a,b; Spector 1995; West et al. 1998; Singh et al. 2002; Truscott 2005; Ates et al. 2010). Reported molecular targets for oxidative damage in the lens include proteins (Dische & Zil 1951), membranes (Garner et al. 1983), and deoxyribonucleic acid (DNA) (Kleiman & Spector 1993). Kleiman & Spector (1993) reported the presence of single-strand DNA breaks in human cataractous lens epithelium, and in a recent study, the DNA damage was found to be significant and arbitrary (Sorte et al. 2011). Various types of DNA damage may be associated with different types of insults (Pflaum et al. 1994a), and the relative contribution of the above-mentioned stressors to DNA damage in human cataractous lens epithelium has not previously been characterized. The purpose of this study was therefore to examine the DNA damage in freshly removed human cataractous lens epithelium to accumulate some initial information related to the damage profile.

*In vitro* systems are valuable tools in experiments focusing on the

pathogenesis of lens damage and cataract (Ibaraki et al. 1998; Wormstone et al. 2006). However, alterations in molecular damage profile induced by transfer of the lens epithelium from the eye to such a system have not previously been explored. Such changes may to some extent influence the sensitivity to subsequent stressors in an experimental setting. We therefore examined to what extent a transfer of the cataractous lens epithelium to such a commonly used system influenced the DNA damage profile.

## Materials and Methods

### Sampling

The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. The regional committee for medical research ethics approved the research. Consecutive capsulotomy specimens obtained from 18 age-related cataract patients who underwent phacoemulsification procedures were analysed.

A clear corneal 2.75-mm incision was made, viscoelastic material (Healon GV) introduced and the anterior capsule in all cases extracted by an experienced surgeon. The tissue samples were with no further handling or irrigation immediately put into Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) with 15% fetal bovine serum (FBS) and 100 U/l Penicillin/Streptomycin at 0–4°C. Eleven samples were analysed at once for DNA damage with the comet assay, while 11 others were incubated in the same medium at 37°C with 5% CO<sub>2</sub> for 1 week before carrying out the same assay. (In four cases, capsulotomy specimens were divided in two, one half for immediate analysis and the other half for incubation. For the purpose of this study, they are regarded as independent samples.)

The mean age of the donors of the capsulotomy specimens was 71.36 years (53–88 years) in the noncultivated group and 70.27 years (50–87 years) in the cultivated group (Table 1). There were six women and five men donors in both groups. In both groups, two patients had underwent intraocular surgery. Two persons in the noncultivated group had used

**Table 1.** Clinical data.

	Age	Gender	Type of cataract	Additional information
<b>Fresh</b>				
If*	66	F	N2, C5, P5	Acute Lymphatic Leukemia, bone marrow transplant, immunosuppressiva
2f	88	M	N3	Diabetes mellitus type 2
3f	53	M	C5	High energy trauma
4f	75	F	C3–4, N2,P5	Choroidal malignant melanoma, rutenium irradiation
5f	83	M	N4, C2, P1	
6f	82	F	C1,N4	
7f	65	F	N4	
8f <sup>†</sup>	80	F	N3	
9f <sup>†</sup>	61	M	C3, N2	Corneatranplantation
10f <sup>†</sup>	71	F	N2, P4	Rheumatoid Arthritis, corticosteroids
11f <sup>†</sup>	67	M	N2, P3	
<b>Cultured</b>				
1c*	66	F	N2, C4, P4	Acute Lymphatic Leukemia, bone marrow transplant, immunosuppressiva
2c	87	F	C4, N3	Polymyalgia rheumatica, prednisolone
3c	50	M	N1, P1	Myopia
4c	68	F	N4	Macular hole, vitrectomy
5c	70	M	Mature	Uveitis
6c	66	M	N3	
7c	83	F	C3, N3	
8c <sup>†</sup>	80	F	N3	
9c <sup>†</sup>	61	M	C3, N2	Corneatranplantation
10c <sup>†</sup>	71	F	N2, P4	Rheumatoid arthritis, corticosteroids
11c <sup>†</sup>	67	M	N2, P3	

f = fresh, that is, capsulotomy specimens analysed immediately after retrieval, c = cultured, that is, capsulotomy specimens analysed after 1 week in culture. Cataracts were classified according to Lens Opacities Classification System III (LOCS III). <sup>†</sup> Same patient, that is, 1f is the whole capsulotomy specimen from the first operated eye, 1c from the same person's second eye, subsequently operated. \* Same capsulotomy specimens, that is, capsulotomies 8–11 were divided, one half analysed fresh (8f–11f), the other after 1 week of cultivation (8c–11c).

systemic and three local steroids, whereas three had used systemic and two local steroids in the cultivated group. There were six patients with mixed, four with nuclear and one with cortical cataract in the noncultivated group. In the cultivated group, seven had mixed, three nuclear cataract, and one patient had an almost clear lens (N1, P1) (Table 1) according to the Lens Opacities Classification System III (Chylack LT Jr, et al. 1993). All patients were Caucasians.

### Comet assay

The comet assay, or single-cell gel electrophoresis (Collins 2004), provides a sensitive method to measure DNA breaks, and in combination with lesion-specific endonucleases also oxidized or alkylated bases as well as UVC-induced pyrimidine dimers. Comet-like images are produced on electrophoresis of nucleoids (DNA from lysed cells) embedded in agarose; the DNA break frequency is indicated

by the '% DNA in tail' because breaks relax supercoiling and allow DNA loops to extend towards the anode.

A suspension of single cells was easily obtained after pipetting the lens epithelium several times. Cell suspensions were then centrifuged at 200 g for 5 min at 4°C; the supernatant was discarded and cells were resuspended in 90 µl of phosphate-buffered saline (PBS). Thirty microlitres of the cell suspension was mixed with 140 µl of 1% low melting point agarose at 37°C and ten 5 µl drops were placed onto a glass slide (precoated with agarose and dried) as two rows of five (without coverslips). Gels were allowed to set at 4°C and lysed in 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10 and 1% Triton X-100 (pH 10) overnight at 4°C. Slides were then rinsed three times, 5 min each, in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0 with KOH) at 4°C. Using a silicone gasket and a plastic chamber (Shaposhnikov et al.

2010), each gel in the slide was isolated and incubated with 30 µl of the following buffers and enzymes: lysis buffer (without Triton X-100), enzyme buffer, formamidopyrimidine DNA glycosylase (FPG), endonuclease III (endoIII) and T4 endonuclease V (T4endoV). Two gels were incubated with each of the solutions for 30 min at 37°C in a moist chamber. Subsequently, the DNA was allowed to unwind in electrophoresis solution (0.3 M NaOH and 1 mM Na<sub>2</sub> EDTA, pH 13) for 20 min at 4°C before electrophoresis at

1.4 V/cm, 300 mA for 30 min at 4°C. Slides were neutralized in PBS and rinsed in ultrapure water for 10 min each at 4°C. Then, slides were fixed first in 70% ethanol and then in absolute ethanol for 30 min each and dried on the bench overnight. DNA was stained by immersing the slides in SYBR Gold in TE buffer (10 mM Tris base, 1 mM EDTA and pH 8.0), at the concentration recommended by the manufacturer, for 40 min at 4°C, followed by washing in water, drying, placing a drop of water on each gel and covering with a cover slip. A Nikon Eclipse TS-100 fluorescence microscope was used to evaluate the nuclei visually or using a semi-automated image analysis system (Comet Assay IV; Perceptive Instruments Ltd, Bury St Edmunds, UK). Twenty-five comets were analysed per gel. As there were duplicate gels, 50 comets were analysed per treatment.

using PASW 18 (Aspire Software International, Ashburn, VA, USA).

## Results

DNA damage was estimated in freshly isolated and cultured lens epithelium from cataract patients, using the comet assay. We found very low levels of strand breaks with a mean value of DNA in the tail of 0.2% and 0.6%, respectively, before and after cultivation (Table 2, Fig. 1). Figure 2A shows a typical image from lens cells tested with the standard comet assay; the nucleoid shows no detectable damage, with all the DNA in the 'head' of the comet and no visible 'tail'.

**Table 2.** Experimental data. The numbers show % of DNA in the tail in each sample analysed.

	Lysis	FPG	EndoIII	T4endoV
<b>Fresh</b>				
1f*	0.12	22.69	0.81	5.31
2f	0.35	20.44	-0.83	-7.14
3f	0.22	30.30	1.82	9.29
4f	0.19	17.51	4.47	4.01
5f	1.20	25.18	0.01	-0.67
6f	0.05	22.42	7.14	8.54
7f	0.06	28.15	5.77	2.51
8f†	0.02	24.46	3.50	4.66
9f†	0.02	16.33	2.25	0.30
10f†	0.00	11.74	2.72	0.71
11f†	0.02	10.47	-0.75	-0.54
Mean	0.21	20.88	2.45	2.45
SD	0.35	6.35	2.61	4.67
<b>Cultured</b>				
1c*	0.34	26.09	2.10	4.13
2c	3.28	42.49	4.74	1.47
3c	1.04	33.53	3.48	4.28
4c	0.23	31.94	4.52	1.33
5c	0.57	29.78	2.82	0.69
6c	0.02	38.49	0.84	-0.77
7c	0.52	45.44	7.52	1.76
8c†	0.47	40.61	2.30	0.72
9c†	0.10	35.23	8.71	2.44
10c†	0.06	36.35	4.21	1.46
11c†	0.05	25.89	2.30	-0.05
Mean	0.61	35.08	3.96	1.59
SD	0.94	6.42	2.38	1.56

Net sites are shown, which means that the comet score from the buffer has been subtracted from the other actual scores, which again explains why some numbers are negative. Lysis shows DNA strand breaks, formamidopyrimidine DNA glycosylase (FPG) shows damage in purines (8-oxoguanine), Endo III shows damages in pyrimidines and T4 endoV shows damage caused by UV radiation.

\* Same capsulotomy specimens, that is, capsulotomies 8-11 were divided, one half analysed fresh (8f-11f), the other after 1 week of cultivation (8c-11c).

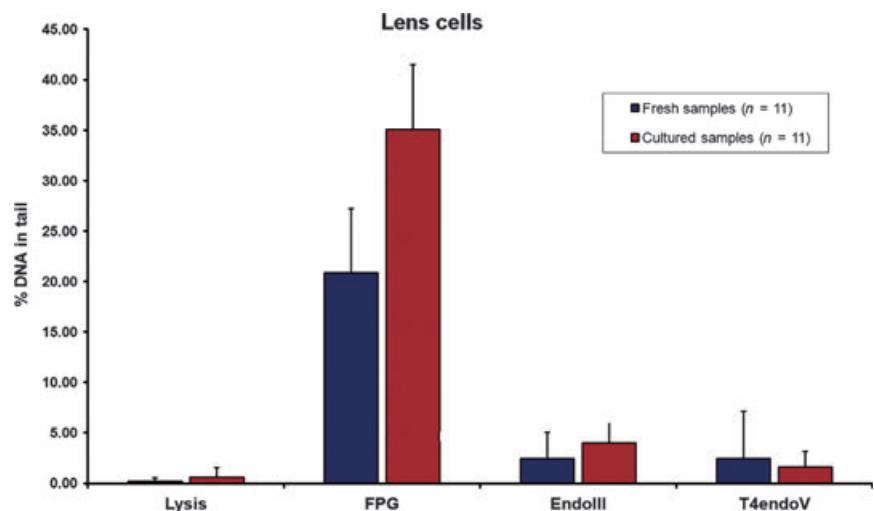
† Same patient, that is, 1f is the whole capsulotomy specimen from the first operated eye, 1c from the same person's second eye, subsequently operated.

### Statistics

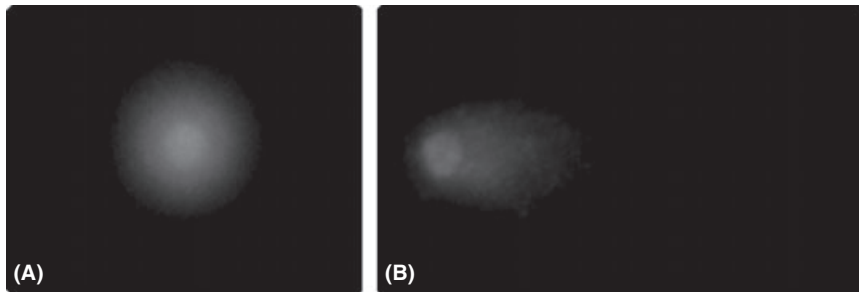
The median of the 50 comets scored per treatment was calculated. To calculate the net enzyme-sensitive sites for FPG, endoIII and T4endoV, the value obtained after the buffer treatment was subtracted from the value after the enzyme treatment. The mean and the standard deviation (SD) of the values from different tissues (fresh and after a week in culture) were calculated. Student's *t*-test was used to compare the amounts of different DNA lesions before and after culture

Specific types of DNA damage were detected using lesion-specific enzymes. The levels of FPG-sensitive sites, expressing the presence of oxidized purines (8-oxoguanine lesions and ring-opened FaPy bases), were high in freshly isolated cells, with a mean value of 20.9% of DNA in the tail (Table 2, Fig. 1). After 1 week in culture, the mean value increased to 35.1% of DNA in the tail, indicating a significant increase ( $p < 0.001$ ) in oxidative damage to purines (Table 2, Fig. 1). Figure 2B shows a typical image from lens cells incubated with FPG; a high proportion of the DNA is in the tail indicating the presence of a substantial number of oxidized bases.

Endonuclease III-sensitive sites, reflecting the presence of oxidized



**Fig. 1.** DNA damage of fresh (blue bars) and 1-week cultured epithelial lens cells (red bars). The mean (SD) of 11 samples are shown. Lysis shows DNA strand breaks, formamidopyrimidine DNA glycosylase (FPG) shows damage in purines (8-oxoguanine), Endo III shows damage in pyrimidines and T4 endoV shows damage caused by UV radiation.



**Fig. 2.** (A) Nucleoids from lens cells tested with the standard comet assay for strand breaks. They show no detectable damage, the entire DNA is in the 'head' of the comet and there is no visible 'tail'. (B) Nucleoids from lens cells incubated with formamidopyrimidine DNA glycosylase (FPG) to detect oxidized bases. A high proportion of the DNA is in the tail indicating the presence of a substantial number of oxidized bases.

pyrimidines, were also measured. The scores were low with mean values of 2.4% and 4.0% of DNA in the tail before and after incubation, respectively (Table 2, Fig. 1).

The levels of T4 endo V-sensitive sites, recognizing cis-syn-cyclobutane pyrimidine dimers caused by UV irradiation, were also low, with mean values of 2.5 and 1.6% of DNA in the tail before and after incubation (Table 2, Fig. 1).

In the uncultivated group, the youngest patient (53 years) with a pure cortical cataract (C5) (Table 1), possibly induced by trauma, had the highest level of 8-oxoguanine lesions (sample 3f; 30.30% of DNA in the tail) (Table 2). The three patients with predominantly posterior subcapsular cataract (sample 4f, 10f and 11f) (Table 1) had three of the four lowest scores in oxidative purine lesions (respectively 17.51%, 11.74% and 10.47% of DNA in the tail) (Table 2).

In the cultivated group, the three highest scores in 8-oxoguanine lesions (42.49%, 45.44% and 40.61% of DNA in the tail, respectively) (Table 2) belonged to the three oldest patients (sample 2c; 87 years, sample 7c; 83 years and sample 8c; 80 years, respectively) (Table 1). However, the numbers of capsulotomy specimens were too small to significantly predict any correlations between age, type and amount of cataract, gender, medication and the levels of oxidative damage to purines.

## Discussion

The purpose of our study was to examine the DNA damage profile in the

human cataractous lens epithelium. Such a mapping of specific lesions could shed some further light onto the relative contribution of recognized molecular stressors in the pathogenesis of lens epithelial cell damage. Our main finding was a relatively high level of oxidized bases (8-oxoguanine) in freshly removed epithelium.

Patients with cataract have previously been shown to have a decreased systemic level of antioxidant capacity (Virgolici et al. 2009), and this is accompanied by an increased level of 8-hydroxy 2-deoxyguanosine (8-OHdG) in the DNA of blood lymphocytes (Ates et al. 2010).

Here, examining freshly removed human cataractous lens epithelium, we found that FPG-sensitive sites (8-oxoguanine and ring-opened FaPy bases) were present to the extent of 20.9% of DNA in the tail, which is somewhat higher than is generally found in normal human lymphocytes (Moller 2006).

When cells are exposed to UVA or visible light, FPG-sensitive sites have been shown to predominate over endoIII-sensitive sites (Pflaum et al. 1994b). In our samples, Endo III-sensitive sites were less abundant than seen in lymphocytes (Moller 2006). The spectrum of damage in the epithelium resembles that induced by light/UV-A in the presence of photosensitizers and/or oxidative stress (Pflaum et al. 1994b; Kielbassa et al. 1997).

Culture for 1 week leads to a highly significant increase in FPG-sensitive sites; endoIII-sensitive sites show a nonsignificant increase. This finding is of relevance considering the importance and versatility of *ex vivo* systems

in experimental trials exploring the pathogenesis of cataract. A multitude of changes in the microenvironment surrounding the lens epithelial cells *ex vivo*, including an increased oxygen tension, reduced levels of antioxidants and altered metabolism and proliferative status might explain such an increase in DNA base oxidation. On removal from the eye, the cells are exposed to higher levels of oxygen, from a reported level in rabbits of 17.2 mm Hg *in vivo* (Shui et al. 2006) to atmospheric conditions of 159 mm Hg. This effect will be exacerbated by the decline in antioxidant levels from the reported 1.42 mm of total ascorbate (Taylor et al. 1991) and 1.9  $\mu\text{mol}$  of reduced glutathione (Riley et al. 1980) in human aqueous humour to insignificant levels in culture medium.

The incidence of lens opacities is known to increase with increasing UV-B exposure (West et al. 1998). The levels of T4endoV sensitive sites, a type of DNA damage considered to be specific for DNA damage caused by UV-B or UV-C, were low in our samples with mean values of 2.5% and 1.6% of DNA in the tail before and after cultivation. It should be noted that T4endoV (as well as FPG and endoIII) detects apurinic/apyrimidinic (AP)-sites in addition to cyclobutane pyrimidine dimers (Friedberg et al. 1995) and so the level of specific UV-B induced damage is probably even lower.

The low levels of strand breaks seen with and without incubation also indicate the excellent quality and ease of handling of these cells. In a report of DNA damage in the lens epithelium in cataractous lenses, strand breaks were detected and measured with the comet assay (Kleiman & Spector 1993). A precise comparison is impossible, because of critical differences in the methods of scoring comets, but it is clear from an examination of their data that the level of damage was generally very low. The frequency of strand breaks does not increase significantly after culture of the cells for a week.

In conclusion, the pathogenesis of age-related cataract is multifactorial and includes a continuous molecular stress induced by light, UV irradiation and oxidative reactions. We here examined human cataractous lens epithelium and present the first report



on the relative magnitude of DNA lesions linked to these different stressors.

The highest levels were represented by oxidized purines or 8-oxoguanine lesions commonly associated with oxidative stress and visible light/UV-A in the presence of an endogeneous photosensitizer, and the level of DNA damage induced by UV-B was relatively low.

The number of samples in our study is low, and further investigations may allow for examining possible correlations between the level and profile of DNA damage and clinical as well as experimental parameters.

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