Repair of Sparfloxacin-Induced Photochemical DNA Damage *In Vivo*

Melanie Struwe[1,2], Karl-Otto Greulich[2], Elias Perentes[3], Hans-Jörg Martus[1], Willi Suter[1] and Ulla Plappert-Helbig[1]

The induction and subsequent repair of photochemically induced DNA damage by sparfloxacin was assessed in different tissues of juvenile Wistar rats. The animals were treated once orally with 500 mg kg^{-1} of sparfloxacin and irradiated 3 hours later with 7 J cm^{-2} UVA. Induction and repair of DNA damage was studied in the skin, retina and cornea using the alkaline comet assay. After a tissue-specific increase in the initial DNA damage (higher in the cornea than in skin and retina), an exponential decrease was found in the skin and retina, whereas in cornea a further increase of the DNA damage after 1 hour followed by an exponential decrease was observed. The half-lives for DNA repair were approximately 3 hours for skin and retina and 1 hour for cornea. After a recovery time of 6 hours, the majority of the induced DNA damage detectable with the comet assay had been removed. In conclusion, the data indicate that (1) photochemically induced DNA damage by sparfloxacin is efficiently removed in skin, retina and cornea, (2) repair of these DNA lesions follows an exponential decrease, (3) the induction and repair of sparfloxacin-mediated photochemical DNA damage might be tissue specific.

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INTRODUCTION

Sparfloxacin belongs to the class of fluoroquinolone antibiotics, which are used to treat various bacterial infections by inhibiting bacterial DNA synthesis (Hooper, 1999). An undesirable side effect of many fluoroquinolones is their photocytotoxic, -allergic and -genotoxic effects, which has been demonstrated in different *in vitro* (Chetelat *et al*., 1996; Zhang *et al*., 2003; Neumann *et al*., 2005; Struwe *et al*., 2007) and *in vivo* models (Makinen *et al*., 1997; Itoh *et al*., 2002; Zhang *et al*., 2003, 2004; Neumann *et al*., 2005; Yabe *et al*., 2005) as well as in clinical studies (Ferguson, 1995; Arata *et al*., 1998; Tokura, 1998; Pierfitte *et al*., 2000; Dawe *et al*., 2003). Fluoroquinolone administration in combination with the exposure to UVA light was furthermore shown to cause morphological changes in the retina (Shimoda *et al*., 1993, 2001; Thompson, 2007). In contrast to the aforementioned studies on the induction of photochemical DNA damage, very little is known about DNA repair *in vivo*, particularly in ocular tissues such as cornea and retina. In a previous study, (Struwe *et al*., 2008), we demonstrated the photogenotoxic effect of sparfloxacin in the skin and eye (retina and cornea) of rats. Furthermore, fluoroquinolones are known to enhance the incidence and shorten the latent period of UVA-induced skin tumors (Johnson *et al*., 1997; Klecak *et al*., 1997; Makinen *et al*., 1997). Thus, the efficient removal of DNA lesions is one important mechanism to prevent tumorigenesis in light-exposed tissues. In general, photochemical DNA modifications are formed in parallel with UV induced DNA lesions, due to the UV-irradiation needed to excite or activate a photosensitizing compound. The induction and repair of UV-induced DNA damage *in vitro* has been extensively studied (Cadet *et al*., 1997; Griffiths *et al*., 1998; Ravanat *et al*., 2001).

The phototoxic potential of fluoroquinolones has been mainly associated with the generation of reactive oxygen species, such as hydroxyl radicals, singlet oxygen, and superoxide that have been directly detected upon irradiation (Umezawa *et al*., 1997; Araki and Kitaoka, 1998). The phototoxic effect of fluoroquinolones is strongly dependent on the C-8 substituent at the quinolone ring. Although a fluoro-substituent confers a strong phototoxicity, hydrogen- and methoxy substituent have little effect (Yabe *et al*., 2005). Sparfloxacin has a fluoro-substituent that is eliminated upon exposure to UV light (Engler *et al*., 1998). In consequence, a highly reactive carbene at the C-8-position is formed that can cause direct DNA single-strand breaks. Furthermore, hydrogen peroxide may also be generated that is converted to hydroxyl radicals by the Fenton reaction (Nakatani *et al*., 1995; Martinez *et al*., 1997, 1998). Besides oxidatively generated DNA damage (such as oxidized bases, abasic sites, single-strand breaks), fluoroquinolones have also been demonstrated to photosensitize pyrimidine dimer formation

[1]Novartis Pharma AG, Preclinical Safety, Genetic Toxicology and Safety Pharmacology, Basel, Switzerland; [2]Leibniz Institute for Age Research—Fritz Lipmann Institute, Single cell and single molecule techniques, Jena, Germany and [3]Novartis Pharma AG, Preclinical Safety, Toxicologic Pathology, Basel, Switzerland

Correspondence: Dr Ulla Plappert-Helbig, Novartis Pharma AG, Preclinical Safety, Genetic Toxicology and Safety Pharmacology, MUT-2881.5.14, CH-4002 Basel, Switzerland. E-mail: ulla.plappert_helbig@novartis.com

Abbreviations: CMC, carboxymethylcellulose; $t_{1/2}$, repair half-life

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(Traynor and Gibbs, 1999; Sauvaigo *et al*., 2001; Lhiaubet-Vallet *et al*., 2007). As the alkaline comet assay detects a variety of these DNA modifications, either directly or indirectly due to DNA repair processes with high sensitivity, it was considered to be the appropriate system for the present study (Bock *et al*., 1998; Collins, 2007).

The aim of this study was to investigate DNA repair in skin and two ocular tissues, that is retina and cornea, of Wistar rats after a single oral dose of sparfloxacin and subsequent irradiation with UVA light.

RESULTS

Clinical symptoms and histopathology

During the experiment, animals were monitored for clinical signs of toxicity. Clinically, irradiation (7 J cm^{-2} UVA) of the animals treated with 500 mg kg^{-1} sparfloxacin caused erythema of the skin, ears, and partly the tail, possibly due to a hyperthermic reaction (vasodilatation).

As cytotoxicity may cause DNA fragmentation, for example, due to the activation or release of endonucleases in necrotic or apoptotic cells (Henderson *et al*., 1998; Quintana *et al*., 2000), samples from all tissues were examined by histopathology, as recommended in the literature (Hartmann *et al*., 2003; Burlinson *et al*., 2007). In the skin of the irradiated vehicle group, the epidermal cells presented a minimal degeneration after 6 hours. In the sparfloxacin-treated groups, increased incidence and/or severity of epidermal cell degeneration/necrosis/apoptosis and koilocytosis were observed in sparfloxacin-treated animals after 3 hours. These findings were more pronounced after 6 hours. In the eyes, no alterations within 6 hours following irradiation were observed. The main histopathological findings are summarized in Table 1.

Previously, we demonstrated that 1% carboxymethylcellulose (CMC) and a higher dose of sparfloxacin (1,000 mg kg^{-1}) without or with irradiation induced no histopathological alterations directly after the exposure to 7 J cm^{-2} UVA (that means 0 hour of recovery time; Struwe *et al*., 2008). Thus, in this study, histopathological analysis was only done 3 and 6 hours after irradiation. As the identified alterations were neither observed in all animals nor in all parts of the tissues and with only very few or slight/few grading a cytotoxicity impact on the comet assay was excluded.

Table 1. Histopathology analysis of the skin following irradiation after 3 and 6 h recovery time

	1 % CMC		500 mg kg^{-1}	
	3 h	6 h	3 h	6 h
Koilocytosis	—	—	2 (1–2)	2 (1–2)
Degeneration (epidermis)	—	1 (1)	2 (1)	3 (2)
Inflammation	—	—	—	3 (1)
Atrophy	—	—	—	1 (1)

CMC, carboxymethylcellulose.
Numbers given represent number of animals showing the respective finding; numbers in brackets indicate the respective histopathological grading: (1) minimal/very few; (2) slight/few; n=3 animals for all other groups.

DNA damage and repair

The irradiation of Wistar rats treated with 500 mg kg^{-1} sparfloxacin induced a significant increase in tail moment in the skin, retina, and cornea as compared to the irradiated vehicle group (0 mg kg^{-1}, that is 1% CMC). The absolute tail moment values as well as the fold-increase between the vehicle-treated and the sparfloxacin-treated irradiated groups were highly tissue specific (Figure 1). In comparison to the vehicle treatment, sparfloxacin induced an increase of the tail moment in the cornea by a factor of 68, followed by the skin (factor 25) and the retina (factor 9).

The induced DNA damage decreased in all tissues within 6 hours after the irradiation (Figure 2). Although in the skin and retina the maximum tail moment was observed directly after the irradiation (0 hour), for the cornea an increase of the tail moment within 1 hour after the irradiation was detected. However, after 6 hours the tail moment for the cornea was at the same level as for the irradiated vehicle-treated animals. For the skin and retina, the tail moments observed were slightly higher than in the respective irradiated vehicle control group. Although the tail moments in the irradiated vehicle control groups remained stable between 0 and 6 hours of recovery time, it was concluded that a dose of 7 J cm^{-2} UVA light alone induced no remarkable DNA damage in the analyzed tissues. Mathematical analysis, that means regression analysis showed an exponential decline of the maximum-induced DNA damage in the comet assay in all three tissues. The DNA repair half-lives were similar in the skin and retina (skin: 187 ± 9 minutes, retina: 187 ± 21 minutes, $n = 3$ animals per group), but clearly lower in the cornea. As in the cornea an increase of the tail moment was observed within 1 hour after irradiation, the regression analysis was done from 0 to 6 hours recovery time and from 1 to 6 hours. The repair half-lives observed were 68 ± 9 min-

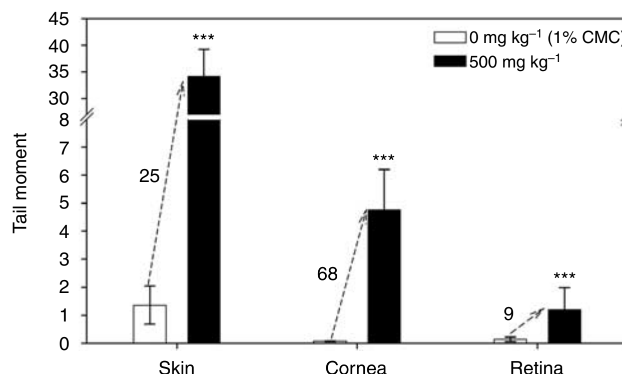

Figure 1. DNA damage in skin, retina and cornea following the irradiation with 7 J cm^{-2} UVA from animals treated with the vehicle (1% CMC, white bars) or with sparfloxacin (500 mg kg^{-1}, black bars). The arrow indicates the factor by which the tail moment increased from the vehicle to the sparfloxacin-treated group (mean ± SD, $n = 3$), ***$P < 0.001$ *vs* 0 mg kg^{-1} (one-way ANOVA).

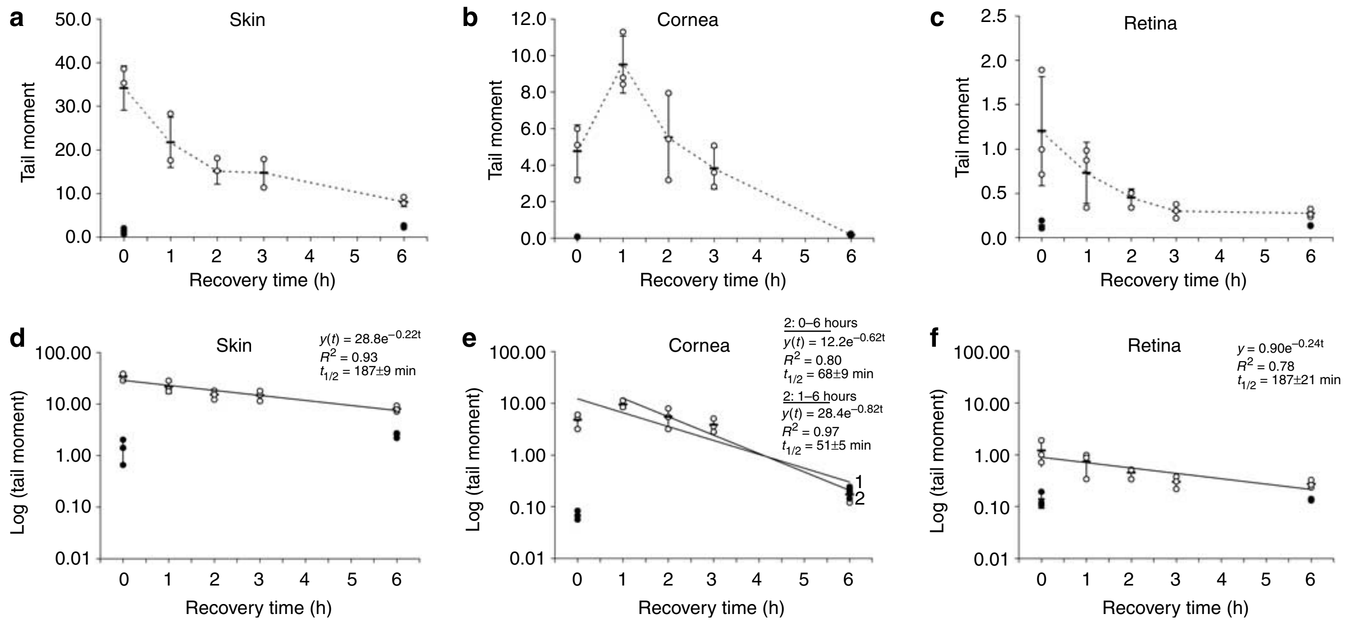

Figure 2. DNA repair of sparfloxacin-induced photochemical DNA damage. Linear (**a–c**) and logarithmic (**d–f**) representation of DNA repair *in vivo* in skin (**a**, **d**), cornea (**b**, **e**), and retina (**c**, **f**), following the administration of 1% CMC (●) or 500 mg kg^{-1} sparfloxacin (○) and irradiation. Following the irradiation, the DNA damage was assessed over a recovery time of 6 hours. Each data point (●, ○) represents one animal. Additionally the mean ± SD (–) of the groups consisting of three animals each is shown. (**a–c**) The raw data of the measured tail moment values and (**d–f**) the mathematical analysis. The tail moment over 6 hours recovery time of all tissues followed an exponential decay.

utes (0–6 hours) and 51 ± 5 minutes (1–6 hours), $n = 3$ animals per group. The raw data of the comet assay results are shown in Table S1 in the Supplementary Information, including tail DNA and tail moment.

DISCUSSION

As little is known about tissue-specific DNA repair of photochemically induced DNA damage *in vivo*, the aim of this study was to investigate the induction and repair of these DNA modifications in different rat tissues with the alkaline comet assay. Besides directly induced DNA-strand breaks, the comet assay also detects transient DNA-strand breaks that occur during DNA repair processes of base modifications such as 8-oxo-7,8-dihydro-2′-deoxyguanosine or cyclobutane pyrimidine dimers (Speit and Hartmann, 1995) that are known to be generated by sparfloxacin upon irradiation (Rosen, 1997; Traynor and Gibbs, 1999; Lhiaubet-Vallet *et al.*, 2007). These base modifications are removed by excision repair due to incision and followed by rejoining of the DNA ends. In this *in vivo* study, the DNA repair process was assessed to investigate the global DNA repair of photochemically induced DNA modifications in different rat organs, without differentiating between various types of DNA damage.

The results showed that the sparfloxacin-induced DNA damage is tissue specific, whereby the highest increase in the tail moment was observed in the cornea, followed by the skin and retina. Although the skin and cornea as the outermost layer of the eye are exposed to the complete solar UV-visible spectrum, a substantial amount of UV light is absorbed by the lens before reaching the retina (Sliney, 2002). Thus, it is reasonable that the lowest increase in the DNA damage was observed in cells of the retina.

DNA damage induced by sparfloxacin-mediated photosensitization was efficiently repaired *in vivo*. The data obtained indicates that the DNA repair can be described by an exponential decrease in the tail moment with time. In cells derived from rat skin and retina, the calculated DNA repair half-life was almost identical at ~3 hours (skin: $R^2 = 0.93$; $t_{1/2} = 187$ minutes; retina: $R^2 = 0.78$; $t_{1/2} = 187$ minutes) and most of the strand breaks were rejoined after 6 hours. The results obtained for the cornea were less straightforward. Until 1 hour after treatment, an increase in DNA damage was seen in this tissue. From this point on the DNA damage decreased, similar to skin and retina. The mechanism leading to an increase in the tail moment within the first hour following the irradiation in the cornea is not known. Therefore, for regression analysis the time course was split into different segments, from 0 to 6 hours and 1 to 6 hours. Mathematical analysis of the data indicated that an exponential removal starting at the highest expressed tail moment (at 1 hour recovery time) returned the highest regression coefficient indicating that this approach reflects the DNA repair most accurately ($R^2 = 0.97$; $t_{1/2} = 51$ minutes). A regression analysis of the phase from 0 to 6 hours returned similar results (with $t_{1/2} = 68$ minutes), but with a lower regression coefficient ($R^2 = 0.80$). In conclusion, the regression analysis over 0–6 and 1–6 hours suggests a DNA repair half-life for the cornea of around 1 hour, which was faster than in skin or retina. Although the number of animals per group was relatively small ($n = 3$), we exclude technical reasons for the observed differences between cornea and the

other organs. First, the initial increase in tail moment, within the first hour, was seen in each single animal, which speaks against an artifact. This is particularly true when considering that in general an animal organism is a very controlled system compared to, for example, cell culture models, and physiological disturbances leading to artifactual results are unlikely. Second, cells of all organs, in particular the retina and cornea were isolated in parallel under comparable conditions. Therefore, for the time being, the reason for the difference in the DNA repair kinetics between skin, retina, and cornea remains speculative. These results may be correlated to the observation that, in contrast to the skin, primary cancers of corneal endothelial cells are extremely rare, despite the fact that this tissue is, such as the skin, constantly exposed to solar UV light. It can be hypothesized that various very efficient mechanisms that prevent damage to the DNA have evolved in the cornea (Uma *et al.*, 1996; Cai *et al.*, 1998; Kolozsvari *et al.*, 2002; Pappa *et al.*, 2003). It has been reported that the nuclear factor damaged DNA-binding protein (DDB) 2 is higher expressed in human corneal cells than in the skin (Inoki *et al.*, 2004). DDB2 together with DDB1 forms a heterodimer that is involved in the recognition of several types of DNA modifications, preferentially cyclobutane pyrimidine dimers and (6–4) photoproducts, as part of the nucleotide excision repair pathway. Thus, in the cornea, an overexpression of damaged DNA-binding protein 2 DDB2 has been demonstrated to enhance the repair process of this DNA damage (Inoki *et al.*, 2004). However, sparfloxacin photosensitizes predominantly the generation of oxidatively generated DNA modifications that are mainly repaired by base excision repair (Memisoglu and Samson, 2000; Barzilai and Yamamoto, 2004; Evans *et al.*, 2004; David *et al.*, 2007) and partly by nucleotide excision repair (Brooks *et al.*, 2000; Kuraoka *et al.*, 2000; Rybanska and Pirsel, 2003). In conclusion, a higher DNA repair activity due to the nucleotide excision repair could explain the shorter DNA repair half-life observed in the cornea. The increased tail moment within the first hour of recovery seems to be reasonable, as this is often seen for the DNA repair by the nucleotide excision repair in the comet assay or alkaline elution assay (Lehmann *et al.*, 1998).

Another aspect is the difference in the distribution of sparfloxacin. Although sparfloxacin is distributed to the skin and retina directly by the blood stream, the cornea is exposed by aqueous humor and lachrymal fluid. Furthermore, the corneal stroma can act as an ocular deposit (Koneru *et al.*, 1986). In consequence, the tissue concentrations of sparfloxacin in the cornea might differ from that in the skin and retina, which may have an impact on the induction and subsequent repair of DNA lesions during irradiation.

A single treatment with sparfloxacin plus UVA irradiation induced significant DNA damage in the skin, cornea, and retina, which is efficiently repaired. With regard to the clinical use for humans it might be concluded that a single oral dose of the antibiotic sparfloxacin causes DNA damage, which is efficiently repaired after a few hours. However, the comet assay only detects primary DNA lesions and, thus, the induction of mutations cannot be assessed, but this was also not the focus of this work. As it is known that fluoroquinolones are photomutagenic *in vitro* (Chetelat *et al.*, 1996), sparfloxacin served as a model compound to determine the repair of photochemically induced DNA damage with the comet assay in different organs.

In conclusion, our data indicate that the level of photochemically induced DNA damage and rate of repair *in vivo* was tissue specific, whereby the velocity of the induction and kinetics of the DNA repair was similar between skin and retina differs remarkably in the cornea.

MATERIALS AND METHODS

Irradiation conditions

The animals were exposed to 7 J cm^{-2} UVA light using a Mercury vapor lamp Psorisan 900 with H1 filter (Dr Hönle Medizintechnik, Kaufering/Germany) with an emission spectrum from 320–700 nm. An UVA/UVB meter (Gigahertz-Optik, Opto.Cal GmbH, Movelier, Switzerland) was used to control the irradiation dose and to calculate the irradiation time. To irradiate the animals with 7 J cm^{-2} UVA light, the animals were irradiated for 25 minutes. (Struwe *et al.*, 2008). The emission spectrum of the lamp is shown in Figure 3.

In vivo experiments with Wistar rats (HanRcc:Wist rat strain)

Juvenile female Wistar rats (aged 42–49 days and weighing between 120 and 150 g) purchased from RCC Ltd. (Ittingen, Switzerland) were acclimatized for at least 5 days. The study was performed in conformity with the Swiss Animal Welfare Law (Tierschutzgesetz, 1978, 1981) and in accordance with the in-house Standard Operating Procedures and guidelines for care and use of laboratory animals. Commercial pelleted standard rodent diet and tap water from the domestic supply were available *ad libitum*.

Three animals per group were treated with 500 mg kg^{-1} sparfloxacin or the vehicle CMC (1% aqueous carboxymethylcellulose solution (Sigma-Aldrich, Buchs, Switzerland)) once by gavage with 10 ml kg^{-1} body weight. After 3 hours of treatment, the animals were exposed to 7 J cm^{-2} UVA light and killed 0, 1, 2, 3, and 6 hours following the irradiation. As it was demonstrated previously that a dose of 1,000 mg kg^{-1} sparfloxacin does not increase DNA fragmentation without irradiation (Struwe *et al.*, 2008), no unirradiated sparfloxacin-treated group was included in this study.

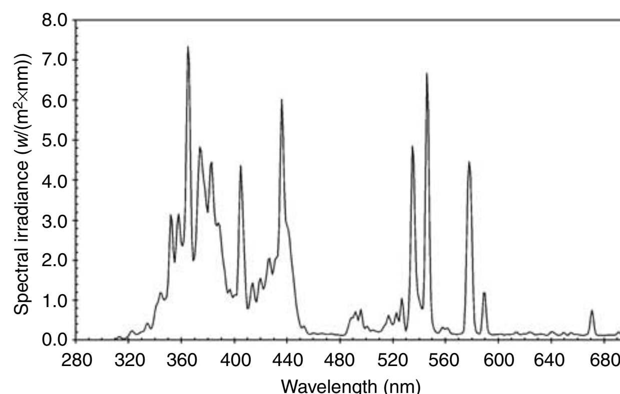

Figure 3. Spectrum of the Psorisan 900 lamp with a maximum emission in the UVA (320–400 nm). The lamp was used to irradiate the animals to cause a photogenotoxic effect of sparfloxacin.

The animals were killed by CO_2 inhalation. Immediately after death, the animals were dissected and skin samples of the dorsal area and the eyes were removed. The cornea and retina were dissected from the eyes and kept separately in precooled Hank's balanced salt solution buffer (Sigma-Aldrich), containing 25 mM EDTA/10% dimethyl sulfoxide (pH 7.0–7.5) at 4 °C. For the comet assay the skin was washed and kept in precooled PBS (4 °C). The cells of the epidermis, cornea, and retina were isolated according to Struwe *et al*. (2008). For histopathological evaluation skin samples were fixed in phosphate-buffered 4% formalin (JT Baker, Basel, Switzerland), and the eyes were fixed in Davidson's solution (96% ethanol, 2% formaldehyde, glacial acetic acid, methylene blue). Subsequently, the tissues were embedded in Paraplast, sectioned at nominally 4 μm and stained with hematoxylin and eosin.

Comet assay

The alkaline comet assay, which allows the detection of DNA-strand breaks of single cells due to the different migration of intact and damaged DNA in an electric field, was performed as previously described (Singh *et al*., 1988; Collins and Horvathova, 2001; Mohanty *et al*., 2002). In this test, cells are embedded in an agarose matrix and lysed. This is followed by an electrophoresis, whereby unraveled DNA loops caused by DNA-strand breaks migrate faster in the electric field than undamaged DNA.

To detect photochemically induced DNA damage *in vivo*, the photo comet assay was used as described previously (Struwe *et al*., 2008). Cells isolated from the retina and cornea were examined with a fully automatic image analysis system, developed in-house (Frieauff *et al*., 2001). The parameter used to determine DNA damage was the Olive tail moment as it was shown to be one of the parameters which reflect DNA fragmentation most accurately (Kumaravel and Jha, 2006; Olive and Banath, 2006). The mean value of three slides was used, calculated from the median of the Olive tail moment from 100 cells per slide. Slides from the skin were blinded and analyzed using the comet III software (Perceptive Instruments, Haverhill, UK), whereby the median tail moment of 50 cells per slide was determined and the mean of two slides was calculated.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Table S1. DNA damage expressed as tail moment and tail DNA in skin, retina and cornea.

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