



Effects of intranasal phototherapy on nasal mucosa in patients with allergic rhinitis

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Received 25 July 2007; received in revised form 10 September 2007; accepted 23 September 2007

Abstract

Rationale: Rhinophototherapy has been shown to be effective in the treatment of allergic rhinitis. Considering that phototherapy with ultraviolet light (UV) induces DNA damage, it is of outstanding importance to evaluate the damage and repair process in human nasal mucosa.

Methods: We have investigated eight patients undergoing intranasal phototherapy using a modified Comet assay technique and by staining nasal cytology samples for cyclobutane pyrimidine dimers (CPDs), which are UV specific photoproducts.

Results: Immediately after last treatment Comet assay of nasal cytology samples showed a significant increase in DNA damage compared to baseline. Ten days after the last irradiation a significant decrease in DNA damage was observed compared to data obtained immediately after finishing the treatment protocol. Difference between baseline and 10 days after last treatment was not statistically significant. Two months after ending therapy, DNA damage detected by Comet assay in patients treated with intranasal phototherapy was similar with that of healthy individuals. None of the samples collected before starting intranasal phototherapy stained positive for CPDs. In all samples collected immediately after last treatment strong positive staining for CPDs was detected. The number of positive cells significantly decreased 10 days after last treatment, but residual positive staining was present in all the examined samples. This finding is consistent with data reported in skin samples after UV irradiation. Cytology samples examined two months after ending therapy contained no CPD positive cells.

Conclusion: Our results suggest that UV damage induced by intranasal phototherapy is efficiently repaired in nasal mucosa.

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Keywords: Phototherapy; DNA damage; Nasal mucosa

1. Introduction

Phototherapy is widely used for the treatment of several immune-mediated skin diseases like atopic dermatitis and

psoriasis [1]. In the last decade new applications have been developed and ultraviolet (UV) light has been applied with good results in the treatment of oral mucosal diseases, such as lichen planus and graft versus host disease [2,3]. Recently, intranasal phototherapy with mixed UVA–UVB–visible light (mUV/vis) has been reported to be successful for the treatment of seasonal allergic rhinitis [4].

One of the main mechanisms of action of UV light is induction of DNA damage in the irradiated cells. This

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mechanism is responsible in part for the biological effects of UV light and consequently its therapeutic use. However, DNA damage is also implicated in the mutagenic and carcinogenic potential of UV light. Knowledge of the mutagenic risk of DNA photodamage has stimulated interest to determine the wavelengths dependent distribution of different DNA photodamage types [5,6]. UV light is able to cause DNA damage by direct mechanisms (absorption of photons by the DNA) or by indirect mechanisms such as generation of reactive oxygen species [6]. UVC (100–280 nm) has been shown to induce direct DNA damage, mirrored by the preponderant production of promutagenic photoproducts, mainly cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6,4) pyrimidone photoproducts (6-4 PPs). UVB (280–320 nm) radiation is also acting mostly on DNA through direct excitation processes and results in the formation of CPDs and to a lesser extent of 6-4 PPs. It has been reported that UVC and UVB induced photodamage is more complex including also induction of strand breaks and oxidative DNA modifications [6,7]. Until recently, the formation of single-strand breaks, alkali-labile lesions and oxidative DNA modifications has been considered to be the main mechanism of UVA (320–400 nm) damage. Recent data showed that UVA induces in bacteria, cultured cells and human skin the formation of CPDs but not 6-4 PPs [8].

Cells possess repair mechanism in response to UV induced DNA damage. The primary process that removes DNA damage is the nucleotide excision repair (NER) pathway. The removal of DNA base modifications via NER requires DNA damage recognition, lesion demarcation, dual asymmetrical incisions at the 5' and 3' sites flanking the lesion, excision of nucleotides from the single-stranded loop, containing the lesion, and gap-filling by DNA synthesis and ligation [9]. Alternatively, highly damaged cells undergo cell cycle arrest, activation of the caspase cascade and finally apoptotic cell death [10].

Although, skin diseases are successfully treated with phototherapy and data from the literature support that no significant increase in skin cancer risk is present in patients treated for decades with UVB light, no data are available regarding the effect of UV light on nasal mucosa.

In the present study we have evaluated the *in vivo* effect of intranasal phototherapy, by assessing DNA damage and repair in nasal mucosa.

2. Materials and methods

2.1. Intranasal phototherapy of ragweed allergic patients

The examinations were performed during the 2005 ragweed season in Szeged, Hungary in eight ragweed allergic patients undergoing intranasal phototherapy. Positive skin prick tests and/or an elevated level of ragweed-specific IgE antibody confirmed the diagnosis. We excluded potential subjects from the study if they had any significant nasal structural abnormalities, or had had asthma, perennial rhini-

tis or upper or lower respiratory infection within 4 weeks prior to the beginning of the study, or had used any of the following drugs: systemic corticosteroids within 4 weeks, topical corticosteroids within 2 weeks, membrane stabilizers within 2 weeks, antihistamines within one week, nasal decongestants within 3 days or immunotherapy 5 years prior to the beginning of the study. The Human Investigation Review Board of the University of Szeged had approved the protocol. All patients gave their written informed consent. All subjects were symptomatic at the time of enrollment. Each intranasal cavity was irradiated 3 times a week for 2 weeks, using gradually increasing doses of mUV/vis. The irradiations were performed with a broad band light source (Rhino-light, Hungary, 180 mW, spectral composition 5% UVB, 25% UVA and 70% visible light). Nasal cytology samples were collected with a disposable plastic curette (Rhino-probe, ASI, Arlington, Texas) before starting therapy, immediately after last treatment, 10 days and 2 months after last treatment.

2.2. DNA damage detection with COMET assay

DNA strand breaks and alkali-labile sites were quantified by Comet Assay (single cell gel electrophoresis). As the enzyme, ultraviolet DNA endonuclease (UVDE) from *Schizosaccharomyces pombe* participates in an alternative excision repair pathway in which DNA is cut immediately 5' of CPDs or (6-4) photoproducts, this endonuclease can be used for identification and measurement of UV induced damage by these specific lesions in Comet Assay. The protocol was a modification of the previously described alkaline Comet assay [11]. Seventy micro liter of 0.5% normal agarose (Sigma-Aldrich, St. Louis, Mo, USA) in PBS was spread on each fully frosted microscope slide, covered with a coverslip, and kept at 4 °C to solidify until subsequent use. Centrifuged cells were suspended in 70 µl of 0.5% low melting agarose (Sigma-Aldrich, St. Louis, Mo, USA) in PBS kept at 37 °C, and transferred onto the first agarose layer from which the coverslip had been previously removed. The slides were covered with coverslips again and left at 4 °C for 5 min. The coverslips were removed and the slides were then incubated in cold, freshly prepared lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, and 1% Triton X-100 added fresh) for 1 h to remove cellular proteins. This treatment leaves residual nuclei embedded in the gel and makes the DNA susceptible to lesion-specific endonuclease treatment.

After the lysis, the slides were washed three times for 5 min with 300 µl UVDE buffer (20 mM HEPES, 10 mM MgCl₂, 1 mM MnCl₂, 100 mM NaCl, pH 6.5). The UVDE enzyme (Trevigen, Gaithersburg, MD, USA) was freshly diluted (2500×) before use and 50 µl was used for each slide; the untreated slides were covered with 50 µl buffer. The slides were incubated for 1 h at 30 °C in a humidified chamber.

After the incubation the coverslips were removed, and the slides were washed three times for 5 min with neutral-

ization buffer (0.4 M Tris-HCl, pH 7.5). After the neutralization the slides were placed in a horizontal electrophoresis tank filled with fresh 4 °C electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13). The DNA was left to unwind for 20 min before electrophoresis. Electrophoresis was conducted for 20 min at 25 V and 300 mA in an ice-cooled tank. After electrophoresis the slides were drained and flooded with three changes of neutralization buffer at 4 °C. After neutralization 50 μ l of 500 μ g/ml ethidium-bromide in PBS was dropped to the slides. The slides under coverslips were visualized in a fluorescence microscope.

Observations were made at a final magnification of 400 $\times$ using a fluorescence microscope (Zeiss, Axioscope, Germany) equipped with Axiocam Digital Camera using Komet 5.0 video image analysis software (Kinetic Imaging Ltd., Liverpool, UK). This software is designed to differentiate comet head (nuclei) from tail (damaged DNA). The percentage of DNA in the Comet tail is linearly related to DNA break frequency, therefore, this parameter of 100 cells/slides was measured and the mean was calculated. The mean of 100 cell percentage tail DNA was found to be a valuable characteristic for a given slide. Results are shown as mean $\pm$ std dev.

2.3. Nasal epithelial cell culture

Positive control experiments were conducted on second passage nasal epithelial cell cultures, isolated from patients undergoing mucosal resection of the concha nasi inferior. Specimens were first washed in an isotonic solution supplemented with antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, Mo, USA). The tissue was incubated in 0.25% Trypsin-EDTA (Sigma-Aldrich, St. Louis, Mo, USA) for 20 min at 37 °C. After incubation 10% fetal bovine serum (Sigma-Aldrich, St. Louis, Mo, USA) was added to neutralize protease activity, and epithelial cells were detached by gentle agitation. The cell suspension was filtered through a 100 μ m nylon mesh (BD, Falcon) and centrifuged at 1000 rpm for 10 min at 4 °C. Determination of cell concentration and percentage of viable cells was carried out with trypan blue staining using a haemocytometer. The cells were seeded on 25 cm² tissue culture plastic flasks in RPMI 1640 culture medium supplemented with 10% fetal bovine serum.

Nasal epithelial cells in second passage were harvested by trypsinization and washed in PBS. Cell suspensions were put in 500 μ l PBS and irradiated with mUV/vis light with the following doses: 225 mJ/cm² and 450 mJ/cm². After irradiation cell suspensions were collected into Eppendorf tubes and centrifuged at 200g for 4 min and processed using both the conventional and the UVDE modified Comet assay protocols.

2.4. CPD detection in nasal mucosa

Nasal cytology samples were stained with monoclonal antibodies against CPDs (anti-thymine dimer, clone

KTM53, Kamiya, Seattle, WA, USA). Control samples were included in each experiment using negative control reagents that are routinely applied to histologic sections. Immunostaining was performed using an immunoperoxidase kit (mouse IgG Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). 3,3'-diaminobenzidine (DAB) substrate, which results in a brown-colored precipitate, was used as chromogen. The samples were counterstained with haematoxylin.

2.5. Statistical analysis

The data between different time-points were analyzed for statistical significance using the Dunnett test. To compare the conventional Comet assay with the UVDE modified technique at the same time points paired *t*-test was used. Value of *p* < 0.05 was considered to be statistically significant for comparison between data sets.

3. Results

3.1. Kinetics of DNA damage detected by Comet assay in patients undergoing intranasal phototherapy

Comet assay was performed on nasal cytology samples of eight allergic rhinitis patients before starting the treatment protocol, immediately after last irradiation and 10 days after last treatment. Four of the eight patients returned for the 2 month follow-up visit when nasal cytology samples were collected.

DNA damage was significantly higher in nasal cytology samples collected immediately after completing the 2 weeks treatment regimen than before starting therapy (*p* = 0.02 in conventional Comet assay and *p* = 0.002 in UVDE modified assay) (Fig. 1). DNA damage assessed by the UVDE modified method ($49.42 \pm 7.97\%$) was compared with that obtained by the conventional Comet assay ($48.48 \pm 8.08\%$) in samples collected immediately after last treatment. A

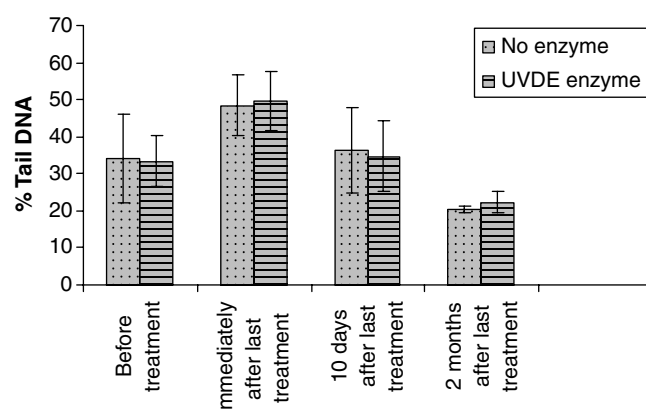


Fig. 1. DNA damage detected by conventional and UVDE modified Comet assay in nasal cytology samples before starting intranasal phototherapy, immediately after last treatment, 10 days and 2 months after last treatment.

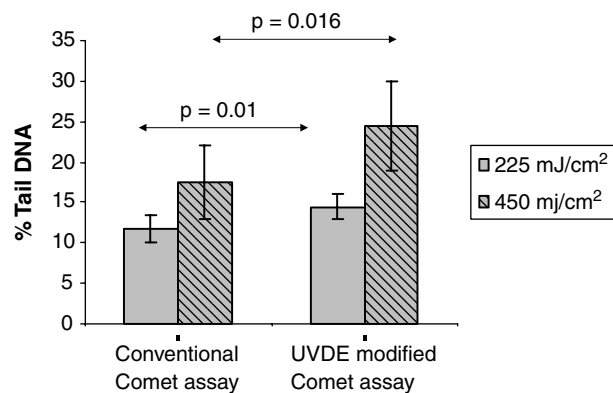


Fig. 2. DNA damage detected by conventional and UVDE modified Comet assay in cultured nasal epithelial cells exposed to UV light.

slight, but not significant increase was detected in all patients with the UVDE modified technique compared to the conventional Comet assay ($p = 0.7$). In positive control experiments in which nasal epithelial cell cultures were used, a significant increase ($p < 0.05$) of UVDE detected DNA damage was observed compared to data obtained by conventional Comet assay (Fig. 2). DNA damage of control, non-irradiated nasal epithelial cells was low and no difference was detected between conventional and UVDE modified Comet assay ($5.26 \pm 1.82\%$ and $6.07 \pm 1.43\%$, respectively).

Ten days after the last intranasal treatment a significant decrease in DNA damage was observed compared to data obtained immediately after finishing the treatment protocol with the UVDE modified Comet assay method ($p = 0.003$) (Fig. 1) and a slight, statistically not significant ($p = 0.057$) decrease was observed using the conventional Comet assay technique (Fig. 1). No significant difference in DNA damage detected by UVDE modified and conventional Comet assay was observed ($p = 0.52$) at this time-point. The difference between baseline (before treatment) and 10 days after last treatment was not statistically significant ($p > 0.05$ for both Comet techniques).

Four of these patients were also examined off-season, at the 2 month follow-up. All patients were symptom-free when the samples were collected. The DNA damage detected by both Comet techniques was significantly decreased compared to that detected 10 days after last treatment ($p = 0.04$ in conventional Comet assay and

$p = 0.04$ in UVDE modified assay) and was similar to previously reported data from healthy individuals (Fig. 1).

3.2. Kinetics of CPD detection in nasal mucosa of patients undergoing intranasal phototherapy

Nasal cytology samples of the 8 patients undergoing rhinophototherapy were stained for CPDs before starting therapy, immediately after last irradiation and 10 days after the last treatment. None of the samples collected before starting intranasal phototherapy stained positive for CPDs. In all samples collected immediately after last treatment strong positive staining for CPDs was detected. The number of positive cells decreased significantly 10 days after last treatment, but scattered residual positive cells were present in all examined samples. Cytology samples of 4 patients treated during the season with rhinophototherapy which were examined off-season (two months follow-up) showed no positive CPD staining (Fig. 3).

4. Discussion

Although, UV light has been previously successfully applied for the treatment of diseases of the oral and nasal mucosa, no data exist regarding DNA damage and repair of oral and/or nasal mucosal epithelial cells. Fornace et al have reported that bronchial fibroblasts and epithelial cells show similar DNA damage and repair as human skin fibroblasts, suggesting that DNA repair mechanism are equally efficient in all cell types [12]. The study conducted in allergic rhinitis patients undergoing intranasal phototherapy is the first assessing UV induced DNA damage in nasal epithelium.

Cells that present DNA damage after UV irradiation may follow different paths, depending on the extent of the damage and the efficiency of repair mechanism: (1) they repair the damage and assume normal function; (2) they succumb to apoptosis and necrosis and are eliminated from the tissue; or (3) they survive despite UV-induced photodamage is not repaired and may represent potential sources for formation of mutations during further divisions. DNA repair has a decisive influence on UV carcinogenesis, since the amount of unrepaired lesions will depend on a competition between repair rate and the time available for DNA repair within the cell cycle. The time needed for repair has

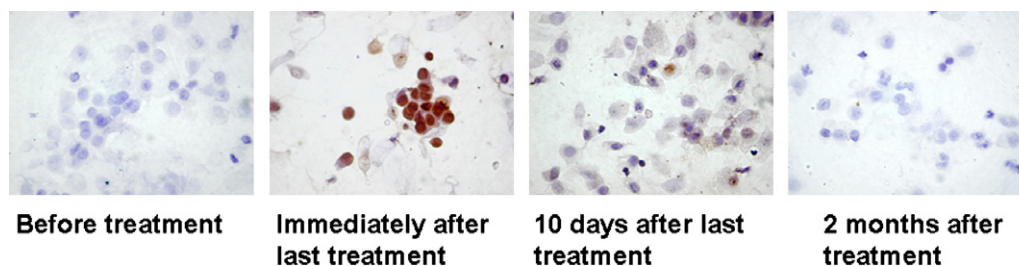


Fig. 3. Detection of CPDs in nasal cytology samples before starting intranasal phototherapy, immediately after last treatment, 10 days and 2 months after last treatment.

been shown to be dependent on the irradiation dose and consequently on the initial amount of damage [13,14]. Beside NER, apoptosis is considered to be a higher repair mechanism at the cellular level as it removes unrepaired DNA damage completely, by cell death. Although, the amount of photospecific damage is crucial in determining whether the cells undergo apoptosis or survive following UV irradiation, other mechanism may also play a role. Recent studies have suggested that different wavelengths show different capacities of inducing apoptosis which is not fully related to the amount of DNA damage. Takasawa et al. have shown that UVB irradiation of HaCaT keratinocytes activates death receptors on the cell surface which is followed by direct activation of caspase 8 leading to apoptosis. This alternative mechanism of apoptosis induction was not activated after UVC irradiation, suggesting that this mechanism may be responsible for the observed higher apoptosis rate induced by UVB light compared to UVC, at doses which are equivalent in production of specific photoproducts [15]. Induction of apoptotic cell death, especially of immune cells, is also one of the main mechanisms by which phototherapy exert its therapeutic effects. One of the pathways that results in cell cycle arrest and apoptosis is the activation of p53, which functions as “the guardian of the genome”. Activation of p53 is more intense after UVB irradiation compared to UVA. Therefore, it has been suggested that UVA induced DNA damage, even at much lower level than after UVB irradiation, have a higher chance to escape protective mechanisms and to result in mutation. Kappes et al have suggested that combination of UVA and UVB light (as it is in mUV/vis) might have a protective role, since simultaneous induction of DNA damage results either in activation of repair mechanisms and survival of a healthy cell or in additional activation of p53 and cellular death [16].

The DNA damage detected by Comet assay in the present study can be attributed in part to strand breaks as the direct effect of UV on DNA, but may represent also detection of apoptosis and necrosis not related to UV light. A portion of the cells collected are undergoing apoptosis or necrosis, which reflects in part physiologic apoptosis occurring in human tissues related maintenance of normal homeostasis and also cell death due to the procedure of collecting cells and most important due to inflammation. DNA repair mechanisms also induce new strand breaks which are detected by Comet assay [17,18]. We have observed that oxidative stress associated with the inflammatory process in the nasal mucosa of symptomatic allergic rhinitis patients results in increased DNA damage detected by Comet assay in nasal epithelial cells (Koreck, unpublished data). Considering that at baseline and immediately after last treatment most of the patients were still symptomatic, Comet assay results reflect also DNA damage caused by the disease itself. Comet assay is a technique which allows the detection of DNA lesions with a complex etiology and therefore is not detecting UV specific damage. The addition of the UVDE enzyme increased the specificity

of the modified Comet assay in detecting the UV specific damage attributed to photoproducts (CPDs and 6-4 PP). This was mirrored by the significantly increased damage detected by the UVDE modified Comet assay technique in cultured epithelial cells exposed to mUV/vis light. Cultured cells exhibited a significantly lower baseline DNA damage level of non-irradiated cells compared to DNA damage of cells collected from allergic subjects with active disease state. Although immediately after last treatment a slight increase in DNA damage was observed in all subjects with the UVDE technique compared to the classical Comet assay, the difference was not significant. These results suggest a limited contribution of UV specific DNA damage to the overall cell damage of nasal mucosa in symptomatic allergic rhinitis patients detected at this time-point with the Comet assay technique. Ten days after last treatment the DNA damage detected by the UVDE modified technique showed a significant decrease while the DNA damage detected by the conventional method proved to be not significant. However, when comparing DNA damage results detected by the UVDE modified technique with those obtained with the classic Comet assay, no significant difference was detected. Although, these results suggest that 10 days after last treatment repair of UV specific photoproducts occurred they also highlight the limitations of Comet assay (both techniques) in analyzing the superimposed DNA damage (specific to UV light) in an inflamed nasal mucosa which is characterized by high baseline DNA damage values characteristic to the disease itself. Therefore, direct detection of UV specific photoproducts by techniques such as immunostaining of tissue specimens is of outstanding importance for evaluating DNA damage and repair of nasal mucosa.

For an accurate evaluation of the UV specific DNA damage, CPD staining was performed. Absorption of UV radiation by adjacent pyrimidine bases in DNA induces primary CPDs and 6-4 PPs [19]. Several studies performed in mouse, fish, monkey and human skin showed that 6-4 PPs are repaired more rapidly than CPDs [20–23]. Studies performed in human skin showed that 7 days after irradiation, CPDs are still detected in the epidermis and dermis, meanwhile 6-4 PPs are almost completely removed from the tissue 6 h after irradiation, with no positive staining at day 7 [24]. Moreover, studies performed in mice and humans suggested that after chronic UVB irradiation of the skin, CPD retaining cells persist for longer time, and they can be detected even after 60 days from the last irradiation [25]. Based on the long half life of CPDs and their persistence in the tissue they are considered the most important photoproducts for mutagenesis. Our results showed that CPDs can be detected in nasal mucosa samples immediately after irradiation and residual staining was present 10 days after last irradiation. These results are in concordance with previously published data in the skin. Samples taken 2 months after the last irradiation showed no residual damage in nasal mucosal samples, suggesting that after intranasal phototherapy no long-term

residual CPDs remain in nasal cells. Collection of nasal epithelial cells using Rhinoprobes is a very convenient and easy technique and have been previously used to assess the effect of air pollution on DNA damage of nasal mucosa [26,27]. However, for a complete evaluation of the safety of phototherapy in airway diseases the results reported here should be replicated in future studies by staining for CPDs in biopsy specimens of the nasal mucosa exposed to UV light with special emphasis on DNA damage and repair of the basal cell layer. UV light-induced carcinogen effect is linked to the cumulative doses of UV light (usually requiring many years). Development of skin cancers has always been a concern when applying long-time phototherapy in dermatological patients. Therefore, several prospective and retrospective studies regarding association of phototherapy with increased risk for skin cancer have been conducted in psoriasis, atopic dermatitis and vitiligo patients. Recently, Lee et al. performed a complex study based on extensive literature search (MEDLINE between 1966 and 2002) to assess the risk of skin cancer associated with UVB phototherapy [28]. No significant increase in the risk of developing skin cancer was found. They stated that evidence suggests that UVB phototherapy remains a very safe treatment modality. Since skin and mucosa are characterized by differences in structure and cell turn-over rate we have compared in a separate study the ability of these tissues to repair UV specific photoproducts (CPDs and 6-4 PPs). We found that skin and airway mucosa exhibit similar kinetics in repairing UV induced DNA damage (manuscript in preparation). Cumulative doses in rhinophototherapy are much lower compared to that used in skin phototherapy which suggests that the safety profile of mucosal phototherapy is similar to that of skin.

In this pilot study we have shown for the first time that nasal mucosa exposed to UV light possess the capacity to repair DNA damage which suggests that the multistep process of carcinogenesis has not been triggered. However more studies are needed in the future to characterize UV specific DNA damage and repair of the nasal mucosa.

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