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Effects of ultraviolet B on epidermal morphology, shedding, lipid peroxide, and antioxidant enzymes in Cope's rat snake (*Elaphe taeniura*)

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Abstract

Cope's rat snakes (*Elaphe taeniura*) favor to expose under sunlight in order to increase their body temperature simultaneously increasing the risk of skin damage by ultraviolet B (UVB) irradiation. We have investigated the effects of UVB irradiation on their skin. Results show that the UVB transmission of the keratinous layer was only $5.1 \pm 0.36\%$. The peak of epidermal damage and malondialdehyde (MDA) content, a product of lipid peroxidation, simultaneously occurred 72–96, 48 or 24 h after exposure to 300, 500 and 800 mJ/cm^2 of UVB radiation, respectively. Superoxide dismutase (SOD) activity was inhibited by UVB and the lowest activity occurred 24, 48, 12 and 12 h after exposure to 110, 300, 500 and 800 mJ/cm^2 of UVB, respectively. SOD activity recovered later to some extent but mostly remained below control level. After exposure to different doses of UVB radiation, catalase (CAT) activity was inhibited immediately, and then gradually recovered and even increased to peak levels above control level. The highest CAT levels accompanied the most serious damage of skin morphology. Later on, CAT activity decreased and recovered again close to or below control level, which was accompanied by shedding off the damaged epidermal complex. This indicated that the epidermal damage induced by UVB is closely related to lipid peroxidation, where CAT acts as a primary antioxidant enzyme. Moreover, the keratinous layer protects the viable cell layer against UVB damage as well.

Keywords: Ultraviolet B; Snake; *Elaphe taeniura*; Epidermis; Morphology; Malondialdehyde; Lipid peroxide; Antioxidant enzymes

1. Introduction

UVB radiation is known to affect many biological processes, and is largely detrimental to individual organisms [1–3]. It has been reported that UVB radiation induces an increase in lipid peroxide (LPO) [4,5], changes of antioxidant enzyme activities [6–8], apoptosis [9,10] and sunburn cell formation in various animal skin or cultured cells [11–16]. UVB radiation generates reactive oxygen species (ROS) and by this also induces cellular oxidative stress. Superoxide dismutase (SOD) and catalase (CAT) are among the most active scavengers of ROS, providing defence against cellular oxidative stress [6–8].

The Squamata species are ectotherm organisms. They usually like to expose under sunlight to increase their body temperature in order to raise their activity [17–19]; however, the body surface of Squamata species is covered with keratinous scales rather than protective feather or hair. So their skin might easily be damaged by UVB radiation. Nevertheless, one can safely assume that during evolution, snakes will have developed a special, perfect mechanism to protect their skin from UVB damage. It has been reported that green anoles (*Anolis carolinensis*) are resistant to UV radiation induced immunosuppression [16]. However, the resistance of green anoles to UV radiation is not yet completely understood. In general, research and knowledge about UV tolerance in reptiles is quite limited so far.

In the present study we investigate the effects of different doses of UVB irradiation on morphology, lipid

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peroxidation and activities of antioxidant enzymes in the skin of Cope's rat snake (*Elaphe taeniura*).

2. Materials and methods

2.1. Animals

The adult snakes (body length: 1456 ± 53 mm, $n = 75$) were purchased from the market and reared in the laboratory. Two days post-shedding, the experimental snakes were exposed to UVB radiation and natural light and the control snakes were only exposed to natural light at room temperature.

2.2. Irradiation and experimental procedures

UVB radiation was provided by three B-297 lamps (Nanjing Ultraviolet Source Institute Products, Nanjing, China) emitting a UVB spectrum with the emission peak at 297 nm and the spectral output in 290–310 nm. Irradiance (E_e) of UVB was measured with a UVB-297 spectroradiometer. The different irradiance (E_e) of UVB was obtained by controlling the distance between B-297 lamps and animals. In the present experiments, exposure time was always 2 h. Radiation exposure dose was expressed as mJ/cm^2 calculated from $E_e \times t$ (t is the exposure time) [11]. Animals were exposed to four different doses of UVB radiation. Skin pieces of animals irradiated by UVB were collected at different time periods after exposure (Table 1).

The epidermal biopsies were detached from the snake body, incubated at 80 °C in water for 3 s and carefully scraped off the stratum germinativum. The excised epidermis, consisting of Oberhäutchen, α-layer, mesos layer and β-layer, defined here as the keratinous layer, was covered over the detector, and the transmission (E'_e) of UVB was measured. Percent penetration was calculated by $E'_e/E_e \times 100\%$, where E_e is the emissive irradiance [11]. Five biopsies of skin from the back of each snake exposed to UVB radiation were fixed in Bouin's fluid, dehydrated in ethanol, and embedded in paraffin. Paraffin sections stained with hematoxylin–eosin were used to study the morphology of the skin.

2.3. Methods

Malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured to estimate the extent of lipid peroxidation. MDA concentration in skin and epidermis was determined using the thiobarbituric acid (TBA) method [20,21] with little modification. Briefly, 200 mg excised skin or epidermal pieces were washed twice with bi-distilled water, added to 2 ml bi-distilled water and homogenized on ice. 0.2 ml of 8.1% sodium dodecyl sulfate (SDS) and 1.5 ml of 20% acetic acid buffer (pH 3.5) were added to 0.2 ml homogenate. The mixture was centrifuged at 5000g. Supernatant removed from the tube was mixed with 1.5 ml of 1% thiobarbituric acid reagent (TBA) and 1 ml bi-distilled water, then incubated at 90 °C for 60 min, and cooled subsequently. 1.5 ml bi-distilled water substituted for TBA in the control solution. The absorbance of the solution was read at 532 nm. The MDA concentration was expressed as nmol per gram of protein (nmol/g pr).

Superoxide dismutase (SOD) activity was assayed by monitoring the self-oxidation rate of pyrogallol at 325 nm in a reaction medium consisting of 4.5 ml of 100 mM Tris–HCl buffer (pH 8.2) and 4.2 ml bi-distilled water or 3.9 ml bi-distilled water and 0.3 ml sample homogenate incubated at 25 °C for 10 min, immediately supplemented with 0.3 ml of 3 mM pyrogallol in 10 mM HCl or 0.3 ml of 10 mM HCl (control) at 25 °C [22,23]. One unit of superoxide dismutase activity was defined as the amount of enzyme that inhibited pyrogallol self-oxidation by 50% under assay conditions. The results were calculated as unit per minute per milligram of protein (U/mg pr · min).

Catalase (CAT) activity was assayed by following the rate of H_2O_2 consumption at 240 nm in a reaction medium containing 10 mM H_2O_2 (control soloution) or 10 mM H_2O_2 and 10–30 μl sample solution (the amount of sample solution was added to limit the change rate of 240 nm absorbance value between 0.02 and 0.07 per minute) in phosphate buffer (pH 7.0) at 25 °C [24]. Catalase activity was expressed as nmol per minute per milligram of protein (nmol/mg pr · min).

Protein content was determined by the method described by Bradford [25].

3. Results

3.1. Morphology of epidermis exposed to UVB

The epidermis of Cope's rat snake consists of the "dead" cell layers termed as the keratinous layer,

Table 1
Different doses of UVB and time points of skin sampling

Doses (mJ/cm^2)	Sampling time (h)								
110	0	12	24	48	72	–	–	–	–
300	0	12	24	48	72	96	120	144	168
500	0	12	24	48	72	96	–	–	–
800	0	12	24	48	72	–	–	–	–

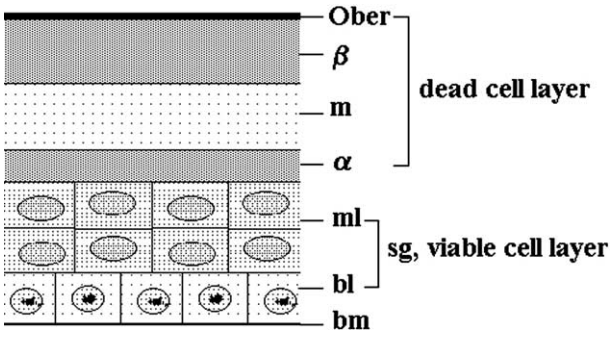

Fig. 1. Schema of epidermal morphology in the resting phase of *Elaphe taeniura* (modified from Landmann, 1979 [27]). Ober, Oberhäutchen; β, β-layer; m, mesos layer; α, α-layer; ml, middle layer; bl, basal layer; sg, the viable cells of stratum germinativum; and bm, epidermal basement membrane.

including Oberhäutchen (Ober), α-layer (α), mesos layer (m) and β-layer (β), and a viable cell layer belonging to stratum germinativum (sg) which is divided into a middle layer (ml) under β-layer and a basal layer (bl) connecting with epidermal basement membrane (bm) in the resting phase (Figs. 1, and 2a) [26,27].

Generally, stratum germinativum showed morphological damage in response to UVB radiation. The UVB-induced epidermal damage was graded into five categories according to the epidermal morphology: Grade 0, no damage, both the middle and basal layers consist of squamous cells (Fig. 2a). Grade 1, little damage, only proliferation of stratum germinativum was induced (Fig. 2b). Grade 2, low level damage, some sunburn cells were induced and dispersed among the cells of stratum germinativum (Fig. 2c). Grade 3,

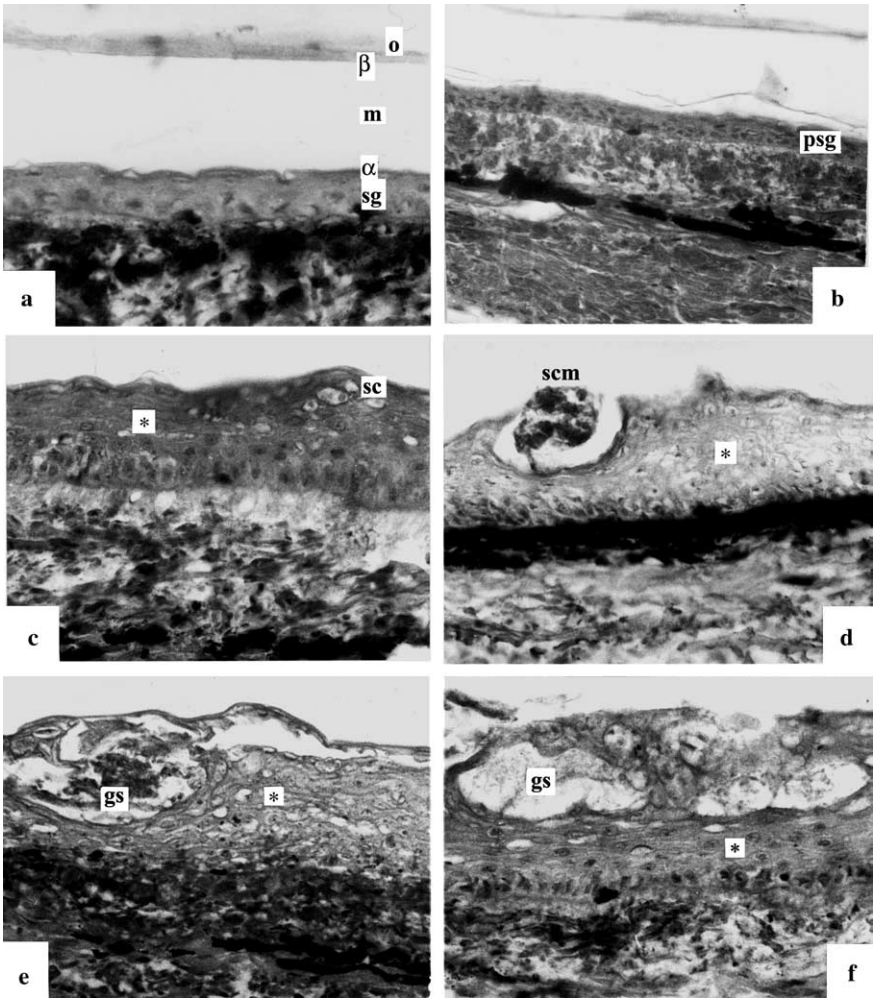

Fig. 2. Representation of five grades of epidermal damage induced by UVB. *Proliferation of stratum germinativum was markedly induced by UVB. (a) Grade 0: Control epidermis consists of Oberhäutchen (O), α-layer (α), mesos layer (m), β-layer (β), and the viable cells of stratum germinativum (sg). The same morphology is seen when collected at 0, 144 and 168 h irradiated by 300 mJ/cm^2, 96 h (500 mJ/cm^2), and 72 hr (800 mJ/cm^2). (b) Grade 1: Proliferation of stratum germinativum (psg) was induced at 12–72 h (110 mJ/cm^2), 12 and 120 h (300 mJ/cm^2), 0 and 72 h (500 mJ/cm^2) and 48 h (800 mJ/cm^2). (c) Grade 2: Sunburn cells (sc) were induced at 24 h (300 mJ/cm^2), 12 h (500 mJ/cm^2) and 0 h (800 mJ/cm^2). (d) Grade 3: Large sunburn cell masses (scm) were induced at 48 h (300 mJ/cm^2), 24 h (500 mJ/cm^2) and 12 hr (800 mJ/cm^2). (e, f) Grade 4: Giant sunburn cell masses (gs) collapsed at 72 and 96 h (300 mJ/cm^2), 48 h (500 mJ/cm^2) and 24 h (800 mJ/cm^2).

moderate level damage, sunburn cells were abundantly induced and partly form large sunburn cell masses (Fig. 2d). Grade 4, high level damage, clumped sunburn cells were abundantly induced and form "giant", collapsed sunburn cell masses (Figs. 2e and f). Finally, the damaged epidermis was shed off and the epidermis recovered to the normal morphology, Grade 0 or Grade 1 (Figs. 2a and b).

The results (Figs. 2 and 3) show clearly that the peak of damage events occurred the faster, the higher the applied UVB dose had been. The peak of damage occurred at 72–96, 48 and 24 h after exposure to 300, 500 and 800 mJ/cm^2 of UVB radiation, respectively. The epidermis was scarcely damaged after exposure to 110 mJ/cm^2 of UVB radiation.

3.2. Penetration of UVB radiation in the keratinous layer

The UVB transmission of the keratinous layer was only $5.1 \pm 0.36\%$ and showed no change with the increased irradiance of UVB radiation ($r = 0.996 > r_{0.01} = 0.661$) (Fig. 4).

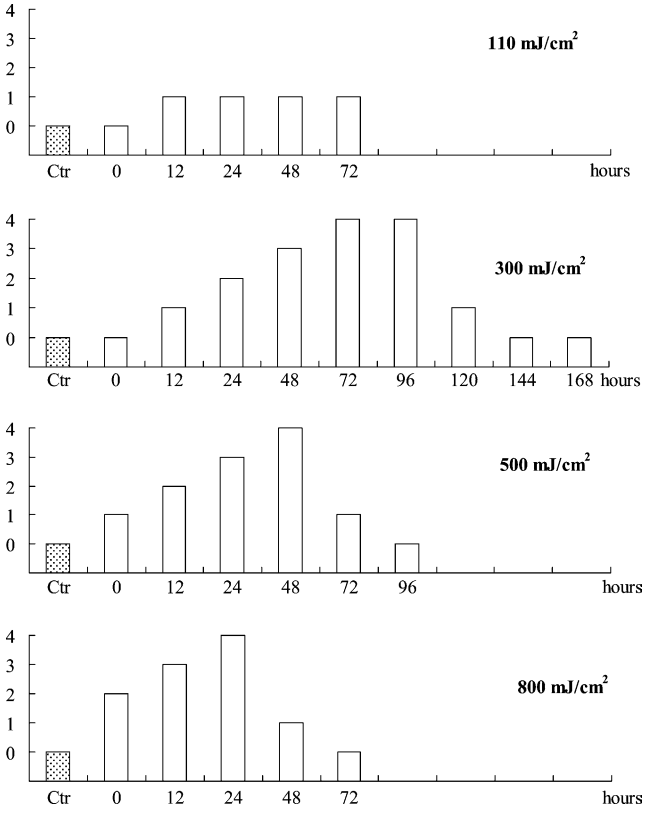

Fig. 3. Morphological parameters show the maximum epidermal damage manifested faster with increasing UVB dose (for grading see text). Ctr, control group; ordinate, damage grade; abscissa, hours after exposure.

3.3. Effects of UVB on the MDA level and SOD and CAT activities

3.3.1. Effects of UVB on the MDA level of skin

MDA, a terminal product of lipid peroxidation, was used to estimate the extent of lipid peroxidation. After exposure to the different doses of UVB radiation, levels of MDA gradually increased to peak levels and gradually decreased later but remained above control level

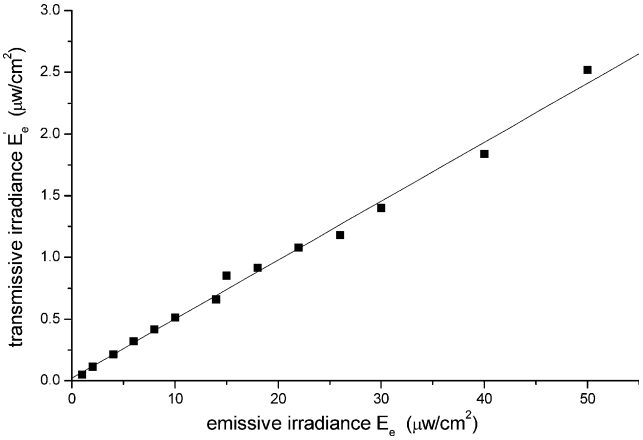

Fig. 4. The UVB transmission of the keratinous layer.

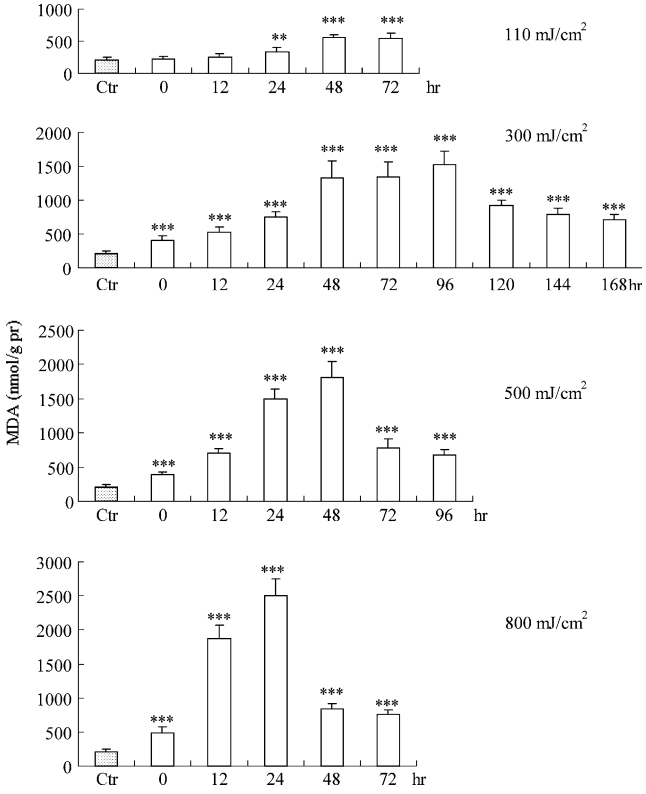

Fig. 5. The dynamic process of the MDA level of skin depended on hours (h) after exposure and doses of UVB radiation. ***$P < 0.001$ and **$P < 0.01$ vs control group (Ctr), respectively.

except for the 110 mJ/cm^2 group. The higher the UVB dose applied, the faster the MDA peak occurred. The MDA peak occurred at 96, 48 and 24 h after exposure to 300, 500 and 800 mJ/cm^2 of UVB radiation, respectively, which coincided with the maximum morphological damage of epidermis. Compared to the same UVB dose, similar dynamic patterns were found for the biochemical and morphological parameters under investigation. (Figs. 3 and 5).

3.3.2. Effects of UVB on SOD activity

The SOD activity was inhibited by UVB and the lowest activity was observed at 24, 48, 12 and 12 h after exposure to 110, 300, 500 and 800 mJ/cm^2 of UVB, respectively. Although SOD activity recovered later to some extent it mostly remained below control level (Fig. 6).

3.3.3. Effects of UVB on CAT activity

CAT activity was inhibited almost immediately by UVB, the lowest activity occurred at 48, 48, 0 and 0 h after exposure to 110, 300, 500 and 800 mJ/cm^2 UVB, respectively. Later, CAT activity gradually increased to even higher levels than control, reaching peak level, followed by a decrease again close to or below control level (Fig. 7).

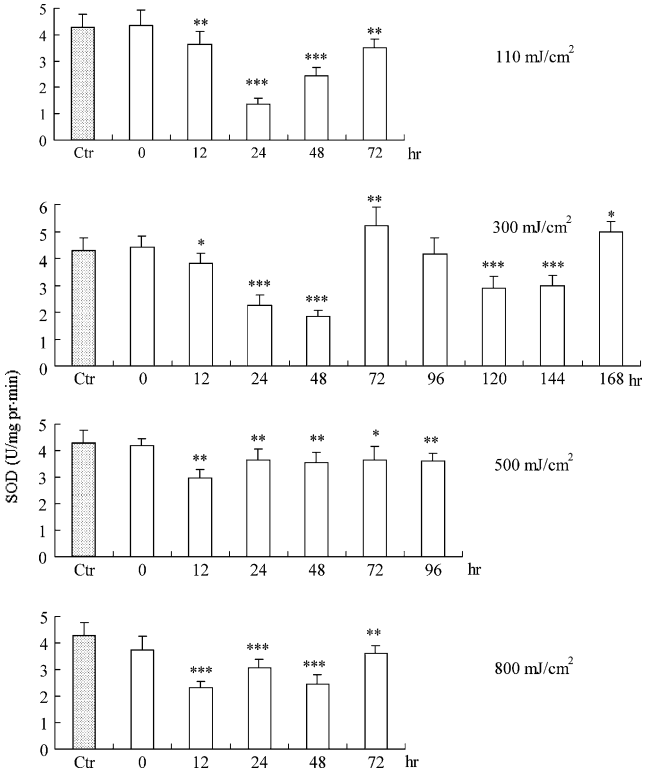

Fig. 6. Effect of UVB on SOD activity. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ vs control group (Ctr), respectively.

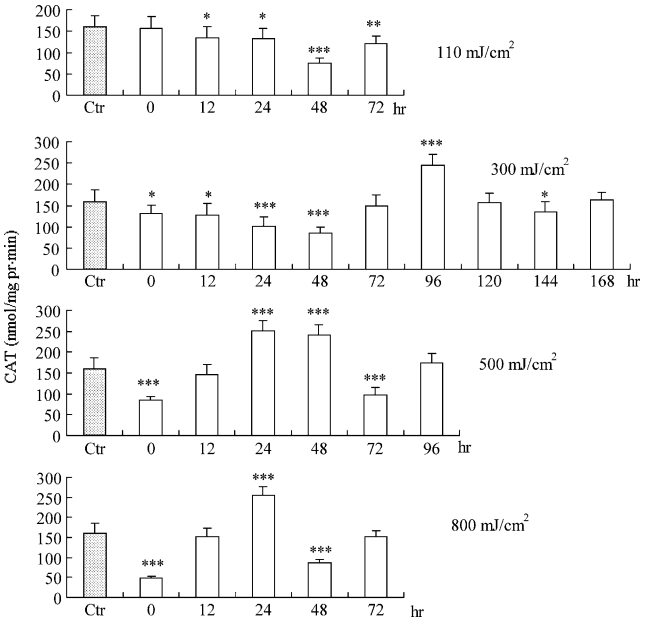

Fig. 7. Effect of UVB on CAT activity. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ vs control group (Ctr), respectively.

4. Discussion

The Squamata species are ectotherm vertebrates without isolating epidermal derivatives like feather or hair. For any meaningful activity they need to increase their body temperature from external sources, usually they favor to expose themselves to sunlight [17–19] subjecting their skin to maximum doses of the full, unshaded solar spectrum. In the present study, we observed that in Cope's rat snake only 5.1% of UVB radiation transmits through keratinous layers to the epidermal stratum germinativum, in contrast to about 30% of UVB through Caucasian epidermis without the basal cell layer and about 10% in Negro epidermis [28]. Proteins are major targets for photo-oxidation within cells due to their high abundance, the presence of endogenous chromophores within the protein structure, their ability to bind exogenous chromophoric materials, and their rapid rates of reaction with other excited state species. Photo-oxidation of protein involves not only direct oxidation of the protein arising from the absorption of UV radiation by the protein structure or bound chromophores, but also indirect oxidation of the protein via the formation and subsequent reaction of singlet oxygen [3,29–31]. Oberhäutchen, α-keratin and β-layer of Squamata species contain α- and/or β-, α-, and β-keratin, respectively [27]. Generally, sunburn cells are largely produced in the viable cell layer of epidermis of human and other animals after exposure to UVB radiation. For example, 12–24 h after exposure to 40 mJ/cm^2 UVB radiation, human epidermis produces many sunburn cells, but Cope's rat snakes required comparatively

far high doses for producing the same effects in their epidermis [11]. We observed that the epidermis of Cope's rat snake initially formed sunburn cells 24, 12, 0 h after exposure to 300, 500 and 800 mJ/cm^2 UVB radiation, respectively. Massive formation of sunburn cells occurred 48, 24 and 12 h after exposure to 300, 500 and 800 mJ/cm^2 UVB radiation, respectively. These results indicate that the skin of Cope's rat snake is more resistant to UVB irradiation than human skin partly due to a higher abundance of keratin.

Generally, the shedding of Squamata species is a normal and regulative physiological regeneration process of their epidermis which is characterized by proliferation and differentiation of the stratum germinativum [26,27,32]. The frequency of their physiological shedding is influenced by ambient temperature and their regeneration is also caused by mechanical damage of the epidermis [26,33]. Many reports show that cell proliferation is induced by UVB radiation [1]. Our results show that in Cope's rat snake cell proliferation is also induced, sunburn cells are generated and the damaged epidermis is finally shed off. This process is UVB dose dependent, higher doses of UVB accelerating the process and increasing the intensity of the response. That means, shedding of skin serves not only the physiological needs of body growth in Squamata species [26], but is also an important mechanism assuring normal skin function by inducing shedding of damaged epidermis outside of the normal physiological schedule.

It is known that lipid peroxides (LPO) are induced by UVB irradiation [4,5]. The dynamics of LPO levels in Cope's rat snake skin after UVB exposure show the typical pattern of initial increase to peak level, followed by decrease and recovery to normal levels. The time course was closely related to the overall epidermal response. A clear relationship to the UVB dose applied was observed. The considerable levels of LPO detected are probably produced directly by absorption of UVB irradiation in the mesos layer due to its high content of lipids [26,27]. However, in this case the free radicals induced by UVB irradiation are rather trapped at their point of generation, because the lipid-rich mesos layer is sitting between the α-layer and β-layer, both low on water, which limits free radical diffusion [34,35]. In this way the viable cells of stratum germinativum are protected by the upper layers of skin. Reactive oxygen species (ROS) can be produced by UVB exposure and cause oxidative damage in lipids, DNA and proteins [2–4]. It is known that antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) play important defensive roles by scavenging $O^{\cdot -}$ or H_2O_2 [6,36]. It was reported that UVB irradiation induced hyperproliferation of keratinocytes in various species accompanied by a decrease in SOD activity. During the initial hypoproliferative phase following UVB irradiation no alteration in SOD activity was noted [38–41]. The present result also indicates that SOD acts not as a primary defence against oxidative stress induced by UVB irradiation. For CAT activity a transient decrease effected by UVB irradiation has also been observed, with a subsequent recovery or increase [37,38,41,42]. In the present study, we also observed after UVB exposure a transient decrease of CAT activity, gradual recovery reaching the peak levels, followed by another decrease and recovery. The peak level in CAT activity coincided with the most serious damage of skin morphology, while the second recovery marked the shedding of the damaged epidermis.

The direct redox reaction of superoxide with itself (dismutation) and/or other reactions catalyzed by enzymes or induced by UVB radiation will give rise to H_2O_2. Fenton-type reduction of H_2O_2 would produce hydroxyl radicals. No enzymes are known to eliminate hydroxyl radicals, instead they are mainly trapped and neutralized by scavengers. Accumulation of H_2O_2 could produce a wide variety of oxidative damage. However, hydrogen peroxide can be removed by Catalase, changing H_2O_2 to H_2O [6,36,43]. Thus catalase in Cope's rat snake probably acts as an important defence against oxidative stress induced by UVB irradiation.

Our results show that the defence mechanism protecting the skin of Cope's rat snake from UVB damage might involve three major routes. The first route is the physical defence. Due to the high optical density of the upper-keratinous-dead cell layer in the UVB range the viable cells of the epidermal stratum germinativum are protected. The second route involves the biochemical defence, antioxidative enzymes, as shown for CAT activity in the present study. The third route involves the cellular defence, including generation of sunburn cells, limitation of diffusion of UVB induced free radicals by the α- and β-layers, and finally the shedding of the damaged epidermis.

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