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Detection of reactive oxygen species in the skin of live mice and rats exposed to UVA light: a research review on chemiluminescence and trials for UVA protection†

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Received 12th November 2004, Accepted 24th March 2005
First published as an Advance Article on the web 12th April 2005

The harmful effects of ultraviolet (UV) exposure on the skin are associated with the generation of reactive oxygen species (ROS) such as superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) as well as with lipid peroxides and their radicals (LOOH and LOO$^{\bullet}$). To give direct proof that such ROS are generated in UV-exposed skin, we proposed the *in vivo* detection and imaging method in which both a sensitive and specific chemiluminescence (CL) probe, such as CLA, and an ultralow-light imaging apparatus with a CCD camera were used. With this method we found that $^{\bullet}O_2^-$ is formed

† Presented at the 14th International Congress on Photobiology, at Jungmoon, Jeju Island, South Korea, 10th–15th June 2004.

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DOI: 10.1039/b417319h

intrinsically and that $^1O_2$ and $^{\bullet}O_2^-$ are generated in the UVA-exposed skin of mice. In addition, we indicated that antioxidative ability against ROS in the skin of hairless rats decreased as age increased. Using these findings, we demonstrated the protective abilities of sodium ascorbate, caffeic acid, essential aroma oils, and zinc(II) ion and its complexes, which we administered to mice both topically and orally. We present a review for the current state of our research proposing the sensitive CL method as a useful *in vivo* tool in photobiological research for the detection of oxidative stress as well as for the evaluation of antioxidative agents to the skin.

Introduction

When skin exposed to air is irradiated by ultraviolet (UV) light consisting of UVA (320–400 nm) and UVB (290–320 nm) on the Earth, reactive oxygen species (ROS) including superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), lipid peroxides (LOOH), and their radicals (LOO$^{\bullet}$) are formed. These, in turn, induce skin aging, phototoxicity, inflammation, and malignant tumors.[1–3] Such results were derived from separated skin as epidermis or from homogenated tissues by using electron paramagnetic resonance (EPR) spectroscopy, although disputable observations were reported[4–6] due to the lack of *in vivo* results. To examine the oxidative stress of the skin *in vivo*, several evaluation methods, such as chemiluminescence, photoemission, and fluorescence[7–11] as well as EPR spectroscopy using spin probes,[12–14] have been applied. In 2000, we proposed an *in vivo* real-time detection and two-dimensional imaging method of endogenously generated ROS in the skin of live animals after UVA light irradiation, by using a chemiluminescence probe (*cypridina hilgendorfii luciferin analog*: CLA; 2-methy-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one) combined with an ultralow-light imaging apparatus equipped with a CCD camera (NightOWL).[15] After many trials, this method has been concluded to be very useful not only for characterizing the ROS generated in the UV light-exposed skin but also for searching protective agents or materials against UV light-induced skin injury or disorder.[16–19]

In this research review, we describe not only the usefulness of the imaging and detection methods of ROS generated in the skin of hair-depilated and hairless mice and rats under UVA exposure but also the evaluation of protective compounds such as sodium ascorbate, caffeic acid, essential aroma oils, and zinc(II) ion and its complexes, which were topically applied to the skin surface or administered orally to mice, in suppressing ROS generation in the skin under UVA exposure.

Detection of ROS in the skin of live mice under UVA exposure by the chemiluminescence imaging method

The sensitivity and spatial resolution of Peltier air-cooled CCD cameras provided improved sensitivity, dynamic range, and excellent image quality and robustness,[15,20–23] which led to the development of new applications in the life sciences.[15–19] These instruments have enabled studies based on the quantitative localization of low levels of chemiluminescence (CL) signals with adequate spatial resolution on a targeted surface of tissue.

In brief, the luminograph used (NightOWL Molecular Light Imager, luminograph LB 981, EG & G Berthold, Germany) was a high-performance low-light imaging system which is able to detect any type of luminescence emission (400–600 nm) over a wide range of intensities. The analytical performance of the NightOWL was sufficiently evaluated for application to the quantitative detection of ultralow-light CL in terms of background, sample size, geometry, sensitivity, resolution, accuracy, and precision. The NightOWL slow-scan CCD camera was a Peltier-air-cooled camera (−73 °C) for ultralow-light imaging with a high sensitivity, the resolution being defined by 385 × 578 pixels. The luminescent signals on a photocathode in the image intensifier were detected as photons. The system was controlled by DOS/V personal computer (OS: Microsoft Windows 95) provided with software for quantitative image analysis. The whole instrument was installed in an airconditioned room (24 °C and 35% relative humidity).

Animals were anaesthetized by intraperitoneal injection of pentobarbital (50 mg kg^{-1} body weight), fixed on a black polystyrene 96 well microplate, covered with black cloth on which two circles with the same area (78.5 mm^2) were cut, and placed in light-tight box to prevent interference by external light. The measured areas were divided into two parts (right and left sides of the back skin) for comparative investigation. For the detection of intrinsic and UVA-induced ROS in each animal, one area in the skin was treated with UVA irradiation and the other was left without treatment. The skin areas of the live animals were exposed to UVA irradiation (320–400 nm) for 3 min through a UVA filter and a flexible fiber at a dose of 100 mW cm^{-2} generated with a Supercure-203S (San-Ei Electric MFG, Japan). During UVA exposure, the anaesthetized animals remained under the detector. 5 μl of 400 μM CLA solution dissolved in H_2O were applied to the two circles on the animals' back skin immediately after UVA irradiation. Measurement of the light emission due to the *in vivo* CL began following the application for CLA to the skin. Identification of ROS and the ability of topical applications containing superoxide dismutase (SOD) (62.5 μM in H_2O), catalase (CAT) (20 μM in H_2O), β-carotene (10 mM in 50% ethanol), deferoxamine (DFO) (2 mM in H_2O) and other antioxidative compounds were examined, in which each portion of 5 μl of the compounds and vehicle was applied to the treated and untreated skin before UVA irradiation, respectively. No effects of any vehicle on the measurements were confirmed in advance. The light emitted from the sample was accumulated for 1 min and integrated for 10 s on the camera tube's target at 0.5 min intervals for 40 min, which was sufficient to gather acceptable light emission date. The light emission output was then recorded on a memory device. The image was processed with a nonlinear gray scale to modify the contrast. A pseudo-color function converted the different gray shades to the colors. The quantification was expressed as the measurement of light from a single given area (78.5 mm^2). The background was calculated for the black cloth over the animal with a signal/noise ratio of ≥3 considered as the detection limit. The results were expressed as photons s^{-1} pixel^{-1} as well as AUC (area under the curve due to the time-dependent CL intensity change) values for the CL measurement time. Animal numbers are expressed in each figure or table.

We have tried to detect and identify ROS generated in the skin of live mice exposed to UVA, in which sensitive and specific CL probes (Fig. 1) and the CCD camera were used. When CLA was used, UVA-induced (18 J cm^{-2}) and intrinsic CL signals were detectable in the skin of a live hair-depilated mouse in a time-dependent manner. Clearly increased CL intensities in the skin were observed following UVA exposure (Fig. 2).[15] The results indicated that CL imaging can be used to estimate the spatial distribution of CL signals produced by ROS that were generated intrinsically or induced by UVA in the skin of mice.

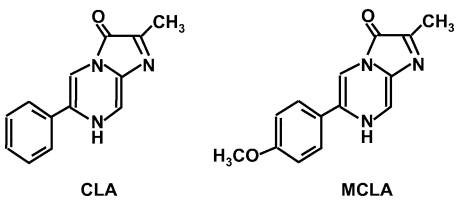

Fig. 1 Structures of CL probes, CLA and MCLA.

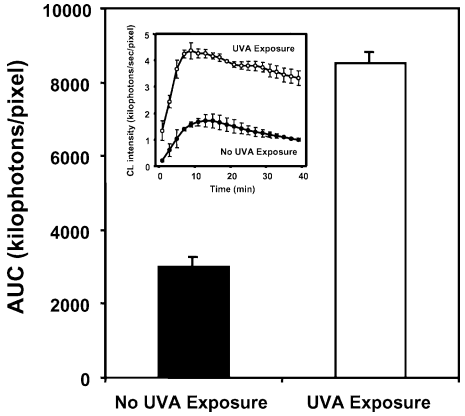

Fig. 2 The calculated AUC values for 0–40 min after the CL measurement and (insert) time-dependent profiles of the quantified CL intensities measured on the skin of mice treated with or without UVA light. Quantified CL intensities were expressed as photons s^{-1} $pixel^{-1}$. The CL intensities in the skin of live mice treated with UVA exposure (open circle in the insert) were found to be significantly higher than those in the intact skin without UVA (closed circle in the insert). From the data, the AUC values were calculated. Data are expressed as means ± standard deviations ($n = 4$).

Identification of ROS in the skin of mice under UVA exposure

The significant difference in the intensity between intrinsic and UVA-induced CL in the skin of mice suggested a difference in the chemical species of ROS in the skin. The chemical forms of ROS were then examined by topical application of typical ROS scavengers and quenchers as well as a chelating agent.

The application of SOD ($^{\bullet}O_2^-$ scavenger) and β-carotene ($^1O_2$ quencher) greatly reduced UVA-induced CL intensity in the skin (Fig. 3).[15] SOD also highly reduced intrinsic CL in the skin, whereas β-carotene barely did so. It is interesting to note that applications of denaturated SOD by heating, CAT (H_2O_2 scavenger), and DFO (a metal chelator) exhibited no ability to reduce either intrinsic or UVA-induced CL in the skin (data not shown). SOD has been known to have dual abilities to scavenge $^{\bullet}O_2^-$ (second-order rate constant 2.0×10^9 M^{-1} s^{-1})[24] and quench $^1O_2$ (quenching rate constant 2.6×10^9 M^{-1} s^{-1}),[25] and β-carotene quenches $^1O_2$ at the rate constant of $3–30 \times 10^9$ M^{-1} s^{-1}.[25] These data indicated that the intrinsic CL in the skin of mice was attributable mostly to $^{\bullet}O_2^-$, while UVA-induced CL was attributable predominantly to $^1O_2$ and $^{\bullet}O_2^-$.

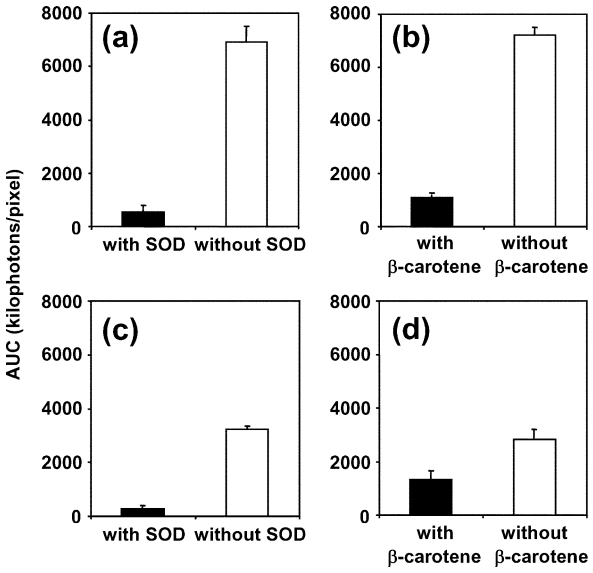

Fig. 3 Suppressive effects of SOD and β-carotene on chemiluminescence due to UVA-induced and intrinsic ROS generation in the UVA-exposed and intact skin of live mice in terms of AUC for 0–40 min of CL measurement. Because 5 μl of 62.5 μM SOD (a) and 10 mM β-carotene (b), which were topically applied to the skin before UVA exposure, suppressed the UVA-induced ROS generation, $^{\bullet}O_2^-$ and $^1O_2$ were indicated to have been produced in the skin of live mice treated with UVA exposure. While 5 μl of 62.5 μM SOD topically applied (c) suppressed intrinsic ROS generation and 10 mM β-carotene (d) did not, indicating that $^{\bullet}O_2^-$ was predominantly produced in the intact skin of live mice without UVA exposure.

In addition, we examined the possibility of detecting lipid peroxyl radicals (LOO$^{\bullet}$) in the skin of mice. *t*-Butylperoxyl radical (*t*-BuOO$^{\bullet}$) as a model for LOO$^{\bullet}$ was generated in a chemical system consisting of *t*-BuOOH and methemoglobin,[26] and CL intensity was clearly detectable when MCLA (2-methyl-6-(4-methoxypheryl)-3,7-dihydroimidazo-[1,2*a*]pyrazin-3-one) (Fig. 1) was used as a CL probe. Although LOO$^{\bullet}$ was not detectable in UVA- or UVB-exposed mouse skin, CL was definitely observed in UVB-exposed skin on which *t*-BuOOH was topically applied.[27] These results suggest a possibility that LOOH-accumulated skin forms LOO$^{\bullet}$ when the skin is exposed to UVB.

We were thus able to demonstrate direct *in vivo* evidence for the intrinsic generation of $^{\bullet}O_2^-$, as well as the UVA-induced generation of $^1O_2$ and $^{\bullet}O_2^-$. In addition, we suggested a possible formation of LOO$^{\bullet}$ in the lipid-rich skin under UVB exposure.

Based on these results, the proposed method constitutes a unique *in vivo* tool for photobiological study involving skin research, particularly for detecting oxidative stress processes and evaluating antioxidative agents to the skin. The obtained information is very important for understanding the mechanism and biological function of endogenously generated ROS, especially regarding the skin's response to UV light stress.

Age-dependent generation of ROS in the skin of live hairless rats exposed to UVA

Aging proceeds by complicated biochemical processes, in which ROS containing free radical species have been implicated. For many years, the relationship between UV-induced photoaging and ROS generation has been proposed.[28] However, it has been difficult to show direct proof that ROS are generated in the aged skin under UV exposure.

Using our proposed CL method, we studied the age-dependent changes in ROS generation in the skin of female HWY/slc hairless rats at the ages of 8–80 weeks old under UVA exposure.[16] CL levels attributable to ROS in the skin of untreated and UVA-exposed rats decreased as age increased, and the CL intensities in old rats were significantly lower than those in young rats (Fig. 4).[16] Next, we examined the age-dependent enhancing effect of UVA exposure, which was fixed

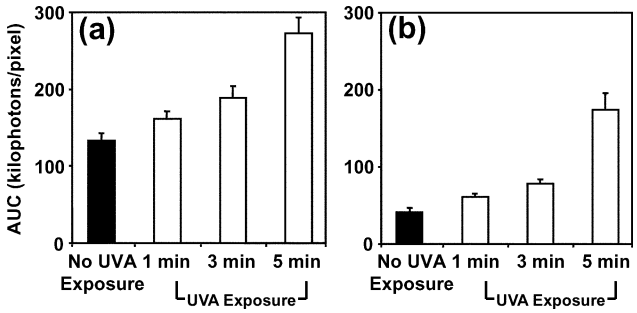

Fig. 4 UVA induced enhancement of the CL intensities in the skin of female hairless rats aged (a) 8 and (b) 80 weeks in terms of AUC for 0–30 min of CL measurement. UVA intensity due to ROS generation was measured in the back skin of hairless rats aged (a) 8 and (b) 80 weeks by setting the UVA exposure time for 1, 3, and 5 min, at an intensity of 20 mW cm^{-2}. From the time-dependent change of the CL intensity, the AUC values were calculated for 0–30 min of CL measurement. Data are expressed as means ± standard deviations ($n = 6$).

at 20 mW cm^{-2} × 5 min, on inducible ROS generation in the skin of the untreated and UVA-exposed rats.[16] The ratios of CL intensities in the UVA-exposed skin to those in the untreated skin were significantly enhanced, and this enhancement increased as age increased (Fig. 5).[16] These results suggest that antioxidative ability against ROS generation in the skin, which is maintained by several endogenous antioxidative enzymes involving SOD, CAT and glutathione peroxidase as well as low molecular weight antioxidative compounds such as ascorbic acid, α-tocophenol, and glutathione, decreases as age increases. This may, in turn, cause the photoaging of the skin.

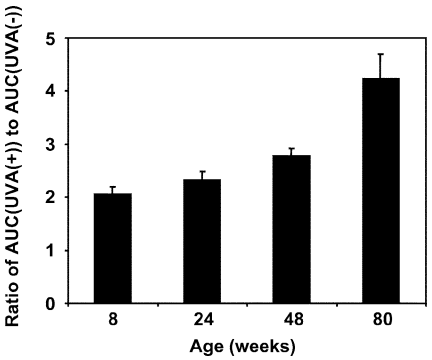

Fig. 5 Age-dependent enhancement of the ratio of CL intensities attributable to ROS generation in UVA-exposed skin to those in the untreated skin of hairless rats aged 8, 24, 48, and 80 weeks. CL intensities due to ROS generation in the skin of the untreated and UVA-exposed (20 mW cm^{-2} × 5 min) rats at 8, 24, 48, and 80 weeks old were measured, and AUC values for the CL levels were calculated from the time-dependent curves, and then the ratio of AUC [UVA (+)) to AUC (UVA (−)] was calculated in the rats at the corresponding age. Data are expressed as means ± standard deviations ($n = 6$).

Evaluation of protective ability of natural antioxidative compounds and essential oils against skin damage by reducing ROS generation in live mice

To find compounds that would effectively reduce damage to the skin caused by ROS generation in live hair-depilated or hairless HR-1 mice under UVA exposure, we examined the effect of topically applied sodium ascorbate (Asc). Asc was applied for 30 min to the surface of the skin at doses of 1 and 10 mM without UVA exposure. After 30 min, CLA was applied. The CL intensity attributable to ROS was not reduced at 1 mM Asc but was significantly reduced at 10 mM Asc. When Asc was applied at the same doses for 30 min and then removed before UVA irradiation (18 J cm^{-2}), Asc reduced the CL intensity dose-dependently (Fig. 6). Similarly, the effect of caffeic acid (CA), a kind of catechol compound contained in green and roasted coffees, was examined. Like Asc, CA reduced the CL intensity in the skin of hairless mice exposed to UVA in a dose-dependent manner in the dosage range of 1–10 mM.[29]

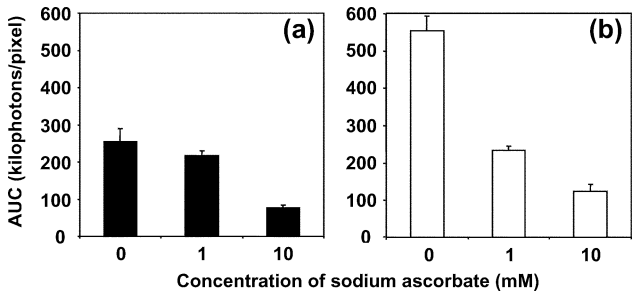

Fig. 6 Suppressive effects of sodium ascorbate (Asc) treatment on CL intensity due to ROS generation in both the intact and UVA-exposed skin of live mice in terms of AUC values for 0–30 min of CL measurement. Asc suppressed dose-dependently both intrinsic (a) and UVA-induced (b) ROS generation. The suppressive effects were more remarkable in the skin of UVA-treated live mice. Data are expressed as means ± standard deviations ($n = 3$).

For many years, lavender oil has been known to exert anti-inflammatory and sedative effects, and it is widely used as a substitute for pharmaceutical therapeutics. Using the EPR spin trap method, we therefore examined whether or not three lavender oils made in different countries—England, France, and Japan—as well as their chemically common components, such as linalool and linalyl acetate, have $^1O_2$ quenching activity in the skin of hairless mice exposed to UVA. Using the CL method, we also examined whether or not these oils and components would reduce UVA-induced ROS generation in the same mice.

All lavender oils exhibited dose-dependent quenching activity against UVA-induced $^1O_2$, which was generated in hematoporphyrin solution under UVA irradiation at pH 7.[19] The results showed that the lavender oils from England and France have better chemical quenching activity with respect to the $1/IC_{50}$ value than that from Japan (Fig. 7). Corresponding well with their chemically evaluated $^1O_2$ quenching activity, these oils reduced dose-dependently the CL intensity in the skin of untreated mice and that of mice exposed to UVA (Fig. 8). Both linalool and linalyl acetate also reduced CL intensity, indicating the partial contribution of such components to the antioxidative effects of lavender oils.[19]

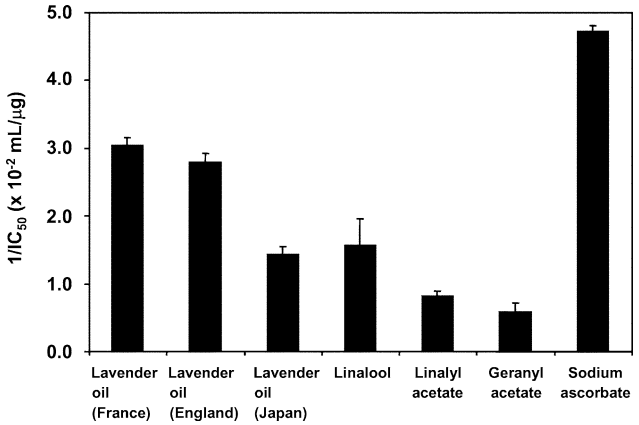

Fig. 7 Singlet oxygen ($^1O_2$) quenching activity in terms of $1/IC_{50}$ value of lavender oils and their major components, compared with sodium ascorbate IC_{50} value was defined as 50% inhibitory concentration of the materials for the generated $^1O_2$ in the hematoporphyrin–UVA system.

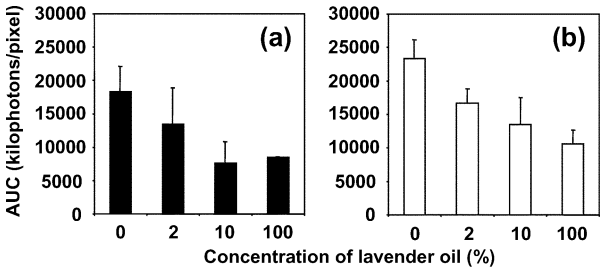

Fig. 8 Suppressive effect of a lavender oil made in France on CL intensity attributable to ROS generation in both the intact and UVA-exposed skin of live mice in terms of AUC values for 0–30 min of CL measurement. Lavender oil suppressed dose-dependently at the concentrations of 2, 10 and 100% both intrinsic (a) and UVA-induced (b) ROS generation. Data are expressed as means ± standard deviations ($n = 3$–5).

These results suggest that the antioxidative abilities of Asc, CA, and lavender oils against ROS generation protect the skin against UV-induced dermal injury and consequently suppress skin aging.

Table 1 AUC due to ROS in the skin of hairless mice treated with zinc compounds and UVA exposure[a]

Compound	Day after treatment	AUC/kilophotons pixel^{-1}	
		Intrinsic	UVA-exposed
Control	1	9200 ± 400	18200 ± 3400[c]
	3	14300 ± 600	21400 ± 2600[c]
$ZnCl_2$	1	7400 ± 2900	10100 ± 600[e]
	3	9700 ± 3500	14300 ± 1500[e]
$Zn(pic)_2$	1	10300 ± 1600	15000 ± 800[b,f]
	3	8800 ± 2400[d]	14600 ± 2500[b,e]

[a] Male hairless mice were orally given $ZnCl_2$ and $Zn(pic)_2$ at a daily dose of 10 mg Zn kg^{-1} for 7 consecutive days. The CL_{max} was the maximum of chemiluminescent intensity measured from 0 to 27 min after application of CLA. [b] $p < 0.05$. [c] $p < 0.01$ *vs.* intrinsic (no UVA) levels on the same day. [d] $p < 0.05$. [e] $p < 0.01$ *vs.* control on the same day. [f] $p < 0.05$ *vs.* $ZnCl_2$ on the same day.

Protective effect of zinc(II) supplementation against skin damage by reducing ROS generation in live mice

Zinc (Zn), with an atomic number of 30 and an atomic weight of 65.37, is one of the most important essential trace elements found in all living systems. This metal is necessary as a catalytic component for more than 200 enzymes and is also a structural component of many proteins, hormones, neuropeptides, and hormone receptors.[30] Among Zn(II)'s many pharmacological and nutritional roles, its anti-inflammatory activity has been well known for a long time.[31–33] In fact, zinc oxide (ZnO) is clinically used in many countries as an anti-inflammatory agent for the treatment of wounds. Recently we revealed that Zn(II) applied topically to the skin of live hairless mice prevented skin damage under UVA exposure.[18] Based on those results, we examined whether or not oral Zn(II) and its complex supplementations would prevent ROS generation in UVA-exposed skin.

When the hairless mice received oral $ZnCl_2$ supplementation at a daily dose of 10 mg Zn kg^{-1} of body weight for 7 consecutive days, the AUC due to ROS in their skin under UVA exposure was significantly suppressed after one day of the last $ZnCl_2$ administration (Table 1). However, after 3 days of the last administration, this suppressive effect was reduced. No such Zn effects were observed on skin not exposed to UVA.[34] Then, hairless mice were given an oral $Zn(pic)_2$ (bis(picolinato)Zn(II) complex) supplementation on the same experimental schedule and at the same dose as for the $ZnCl_2$ administration.

The AUC in the skin of the UVA-exposed mice was suppressed after one day of the last complex administration, but without significance at $p < 0.05$ (Table 1). However, after 3 days of the end of complex administration, the suppressive effect was further enhanced (Table 1), indicating that $Zn(pic)_2$, with a partition coefficient of 0.018 in an octanol–saline system at 37 °C,[35] had a long-term protective effect against UVA-induced ROS generation, unlike $ZnCl_2$. Interestingly, the oral $Zn(pic)_2$ complex suppressed the AUC in the skin of mice not exposed to UVA, indicating that the complex pretreatment also reduced the intrinsic generation of ROS (Table 1).

The reason why oral Zn(II) and its complex supplementations exhibited antioxidative activity in UVA-exposed skin is difficult to explain, since many factors contribute to this activity. In a preliminary study, we examined the induction of metallothionein (MT), which is proposed to have antioxidative activity, in the skin and other several different organs of animals and HaCaT cells treated with $ZnCl_2$, and found that MT levels were higher in the treated cells than in the nontreated cells.[36]

Future perspective

In this research review, we described the *in vivo* non-invasive and real-time CL-probe-dependent method for determining ROS in the skin of UVA-exposed live animals. This method will be useful not only for the study of the mechanism underlying photoaging in relation to ROS generation, but also for the development of antiphotoaging agents.

The most important investigation that needs to be conducted is the determination of the localization or distribution of ROS in skin with or without UV exposure, because localization of some antioxidative enzymes involving catalase and SOD in the stratum corneum has been revealed.[37,38] Thus, the relationships among the localization or distribution of ROS, the chemical forms of ROS, and antioxidative enzymes will be a key area of research in which the proposed method will be indispensable.

When the localization of ROS is revealed, the most suitable antioxidative agents will be proposed. Toward this end, it will be essential also to determine the physico-chemical features of antioxidative agents, such as their lipophilicity, redox potential, acid dissociation constant, and ROS scavenging or quenching activity. In this research review, we proposed several antioxidative compounds including natural antioxidants as well as Zn(II) and its complexes.

On the basis of the results, we conclude that the development of Zn(II) complexes, which are less toxic and might induce some antioxidative enzymes, will be proposed for the treatment of photoaging.

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