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UV-generated free radicals (FR) in skin: Their prevention by sunscreens and their induction by self-tanning agents

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Abstract

In the past few years, the cellular effects of ultraviolet (UV) irradiation induced in skin have become increasingly recognized. Indeed, it is now well known that UV irradiation induces structural and cellular changes in all the compartments of skin tissue. The generation of reactive oxygen species (ROS) is the first and immediate consequence of UV exposure and therefore the quantitative determination of free radical reactions in the skin during UV radiation is of primary importance for the understanding of dermatological photodamage. The RSF method (radical sun protection factor) herein presented, based on electron spin resonance spectroscopy (ESR), enables the measurement of free radical reactions in skin biopsies directly during UV radiation. The amount of free radicals varies with UV doses and can be standardized by varying UV irradiance or exposure time. The RSF method allows the determination of the protective effect of UV filters and sunscreens as well as the radical induction capacity of self-tanning agents as dihydroxyacetone (DHA). The reaction of the reducing sugars used in self-tanning products and amino acids in the skin layer (Maillard reaction) leads to the formation of Amadori products that generate free radicals during UV irradiation. Using the RSF method three different self-tanning agents were analyzed and it was found, that in DHA-treated skin more than 180% additional radicals were generated during sun exposure with respect to untreated skin. For this reason the exposure duration in the sun must be shortened when self-tanners are used and photoaging processes are accelerated.

Keywords: Skin; UV irradiation; Free radicals; Self-tanner; Maillard reaction; ESR spectroscopy

1. Introduction

The effects of chronic sun exposure on skin are apparent when skin not typically exposed to the sun and skin regularly exposed to the sun are compared. While the sun is not the only aetiological factor in the dynamic process of skin aging, it is the primary exogenous cause among several internal and environmental elements. Thus, photoaging is a subset of extrinsic skin ageing.

Solar UVB (280–320 nm) and particularly UVA (320–400 nm) radiations have a capacity to generate reactive chemical species, including free radicals, in cells. These intermediates have been shown to be involved in various biological effects in skin (e.g. erythema, skin aging, skin wrinkling, cancer).

The molecular oxygen (O_2) present within skin cells in the mid-lower levels of the epidermis is a primary target for UV light waves that penetrate the skin.

The reactive oxygen products and other biologically important free radical species are usually very unstable in biological material due to their high reactivity. Free radicals have a characteristic half-life due to their chemical reactivity. Some radicals are stable enough to diffuse across biological membranes; others are so reactive, that they react in the chemical microenvironment at their site of formation. Very reactive free radicals (hydroxyl radicals) cause biological damage only if generated in close proximity to a potential target molecule (e.g. DNA), because they are immediately scavenged by the high concentration of organic molecules in the cell. If they are to cause cell damage directly, they need to be generated directly at the critical cellular target site [1].

Free radicals of intermediate reactivity are able to diffuse over significant distances and may then react with some specificity

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and selectivity with target molecules. They are the most likely species to lead to direct biological damage. Persistent free radicals are rather biologically unimportant with respect to direct cell damage. Persistent free radicals of physiological relevance are melanin radicals, which can be detected directly in skin and hair.

Numerous methods are used to quantify the skin damage induced by UV exposure. The most common in cosmetic industry is the SPF (sun protection factor) method that measures the MED (middle erythema dose), thus the time necessary to develop an erythema. Since skin erythema is caused mainly by the UVB radiation, due to its short wavelength and its superficial penetration depth into the epidermis, this SPF method quantifies only the biological UVB damage. Other methods quantify biological endpoints as skin pigmentation (PPD method, permanent pigment darkening) or DNA-fragmentation. All of these endpoints are very advanced steps in a biological reaction cascade in the skin, that has its beginning with the generation of free radicals. The accurate measurement of these UV-induced free radical species directly in the exposed skin is on the basis of a method able to characterize the benefits or risks of cosmetical and/or pharmaceutical dermatological products. The quantification of free radicals and free radical reactions in the skin could be a valid method for the estimation of UV damage. The quantification of free radical reactions is realized by electron spin resonance (ESR) spectroscopy. Herein we present a method for ex vivo examination of skin biopsy samples, which enables an analysis of free radical reactions as a function of UV irradiation dose, and UV intensity. From the relationship between applied UV dose and radical concentration in a skin sample a calibration curve is constructed, that allows to analyze, if a substance applied on the skin protects against UV-induced free radicals or even enhances UV damage. Sunscreen agents protect against UV-induced free radicals, if they contain UV filters that adsorb or scatter the UV radiation mainly in the UVA range (280–400 nm) and if they are sufficiently photostable to ensure a protection over longer radiation times (UV doses). Some examples of the protective effect of chemical and physical UV filters are discussed here. On the other hand there are chemicals that can enhance the free radical reactions in skin. Herein we present a study on processes of the radical induction by self-tanners as DHA (dihydroxyacetone) and erythrulose. These sugar compounds are commonly used in cosmetic self-tanning products to obtain a sunless coloring of the skin. The chemical reaction process of these sugar compounds is the Maillard reaction, where the sugars react with the proteins of the keratinocytes in the first layers of the stratum corneum and epidermis. During UV radiation free radicals and mainly superoxide anion ($O_2^{\bullet -}$) are produced, that can react with the ketoamines (Amadori products) and other intermediates of the Maillard reaction. This leads to oxidation of the sugar derivates and the consequent radical chain reactions cause a dramatic increase in the radical injury of the skin.

Both the protective effect of sunscreens as well as the radical induction effects of self-tanners can be quantitatively determined by means of the same RSF (radical sun protection factor) method [2].

2. Experimental

2.1. Materials

2.1.1. Skin biopsy samples

Skin biopsy samples from pig were used in all experiments. Pig skin has the greatest similarity to human skin and has the main advantage of a high structural and functional homogeneity. The ears of 6-months-old pigs from local slaughter were washed, the cartilage and the subdermal fat was removed, the skin was cut into 1 cm × 1 cm pieces and stored in PBS buffer until used at 4 °C for maximum 6 h.

2.1.2. Marker for ESR analysis

PCA probe—2,2,5,5-tetramethyl piperidine-*N*-oxyl (Sigma–Aldrich, Germany) at 1 mM concentration in water was used to detect free radical reactions.

2.1.3. UV filters

Commercially available sunscreen products containing different concentrations of four chemical UV filters (B1, A, B2, AB) were used.

2.1.4. Self-tanning agents

DHA (dihydroxyacetone) was purchased from Sigma–Aldrich, Germany, erythrulose from Kraeber, Germany and liposomal encapsulated DHA from ROVI Cosmetics International, Germany. Five percent, 10%, and 20% (w/w) concentrations of all tanning agents were prepared in distilled water and used for the experiments within 12 h.

2.2. Methods

Direct evidence of free radical formation in skin tissues following exposure to UV radiation can be obtained by ESR spectroscopy at low temperature (77 K). However, these signals are very broad and usually give only limited information about the chemical identity of the free radical structure. For quantitative determination of radical reactions as a function of UV dose neither this direct detection nor spin trapping agents are suitable. There are multiple requirements to an ESR probe able to monitor radical reactions in skin at room temperature: the probe must penetrate into the dermal and epidermal layer of the skin without signal intensity loss to the enzymatic and non-enzymatic biological reaction. Further, it must be photostable and non-degraded by UV radiation. It must react with the ROS and free radicals (FR) generated inside the skin during UV radiation. The nitroxyl spin probe PCA fulfils all these requirements and is a suitable probe for the detection of radical reactions by ESR spectroscopy [3–5].

2.2.1. UV irradiation

A sun simulator SOL F2 (Hönle AG, Germany) with UVA 25 mW/cm^2 and UVB 2.6 mW/cm^2 was used for all experiments.

2.2.2. ESR instrumentation

A X-band ESR spectrometer Miniscope MS200 Magnettech, Germany was used for all experiments. The following spectra acquisition parameters were used: 0.1 mT modulation amplitude, 20 dB attenuation and 0.14 s time constant, gain 200.

A special tissue cell was used.

2.3. Experimental design

1 cm × 1 cm skin samples were placed for 5 min on a paper imbued with a 1 mM solution of PCA. After labelling, the skin was dried on paper to remove the excess of PCA; the test products were applied on the epidermal side of the skin and allowed to penetrate for different times. Afterwards, skin samples of 4 mm diameter were taken with a biopsy punch, the skin was fixed on the ESR tissue cell and the first ESR spectrum was recorded. The skin in the tissue cell was UV irradiated with different UV doses (corresponding to different irradiation times) and after each dose an ESR spectrum was recorded immediately after the UV exposure. The signal intensities of the recorded spectra were analyzed by double integration and reported as a function of UV doses.

3. Results and discussion

In the following the radical sun protection factor (RSF) method will be applied to analyze both sunscreen products that protect against UV-induced free radicals as well as self-tanning agents that enhance the amount of UV-induced free radicals.

The nitroxyl probe PCA was used as a dye for the detection of free radicals. The nitroxyl probe is a suitable probe to monitor the biological redox reaction. The radical protection of sunscreens can be tested with the RSF method [2]:

$$\mathrm{RSF} = \frac{N(\text{free radicals})_{\text{unprotected}}}{N(\text{free radicals})_{\text{protected}}}$$

The RSF is a factor that presents the ratio between the number N of generated free radicals in the unprotected and protected/treated skin, assuming the same applied UV dose (constant irradiance, variable irradiation time) for both, or the increase/decrease in sun-exposure time when using a given skin product which results in the generation of the same amount N of free radical/ROS like for the unprotected skin. The amount of free radicals induced in the skin by UV radiation is determined by the reaction of primary FR with a semistable nitroxyl spin probe. The signal intensity of this spin probe is detected by ESR spectroscopy as a function of UV doses. This reaction between primary FR and the nitroxyl takes place only in the skin (or in biologically active tissues) and is limited to the time interval of UV irradiation (Fig. 1). Without UV irradiation the spin probe is stable inside the skin tissue, i.e. it is not reduced by the enzymatic and non-enzymatic antioxidants present.

Changing the UV irradiation dose (constant irradiation time, different irradiation intensities), the signal decay of the nitroxide changes (see Fig. 2). The variation in intensity of the UV irradiation can be achieved by applying optical density filters with

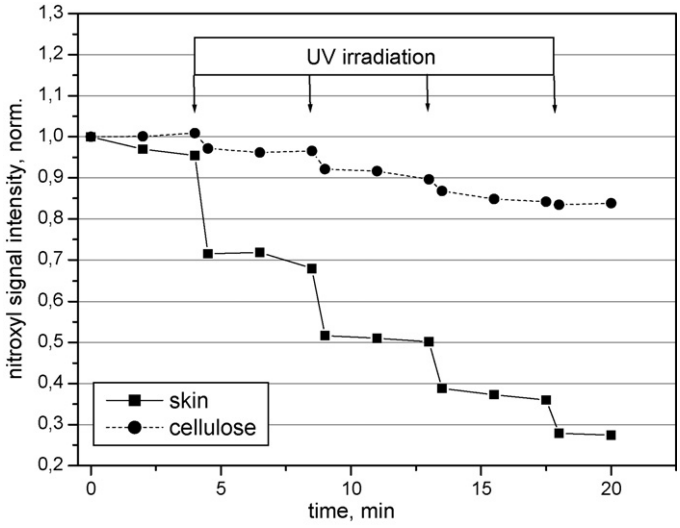

Fig. 1. ESR signal intensity decay of the nitroxyl probe in skin (■) or a cellulose support (●). The arrows indicate moments of UV irradiations at 28 J/cm^2 for 30 s periods.

different transmission (PF). The higher is the optical attenuation factor (PF) the lower is the transmission and the less free radicals are generated in the skin. For each PF the PCA signal intensity is recorded as a function of UV irradiation time and the decay is fitted monoexponentially. The rate constants of each decay are calculated. The rate constant of unprotected skin (k_1) over the rate constant of protected skin (by applying an optical density filter with a given PF (k_n) is a non-linear function of the UV intensity, from which a calibration curve can be constructed (Fig. 3). In this figure the k_1/k_n values are plotted against the ln of the PF and using the following equation the RSF can be calculated:

$$\mathrm{RSF} = \mathrm{PF} = \frac{\ln(k_1/k_n) - a}{b}$$

This RSF factor can quantitatively represents the protection effect of sun protection products such as sunscreens contain-

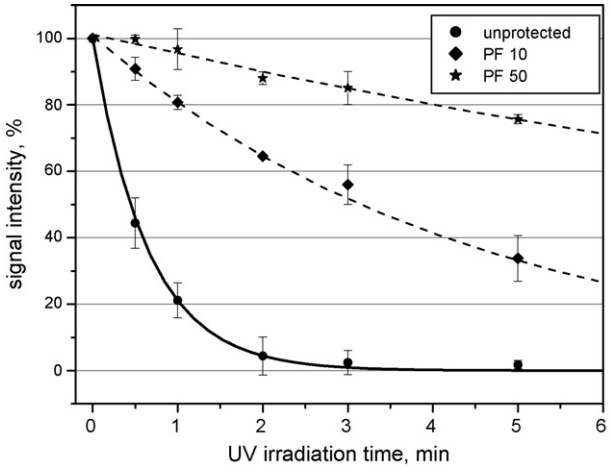

Fig. 2. Nitroxyl signal intensity decay in skin samples as a function of UV irradiation time with different density filters (PF 50: 2% transmission; PF 10: 10% transmission).

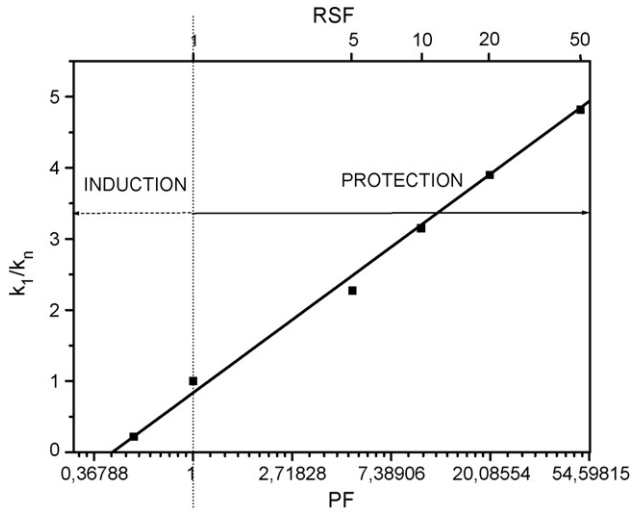

Fig. 3. Calibration curve: rate constant relation k_1/k_n as a function of the PF of optical density filters and the calculated RSF.

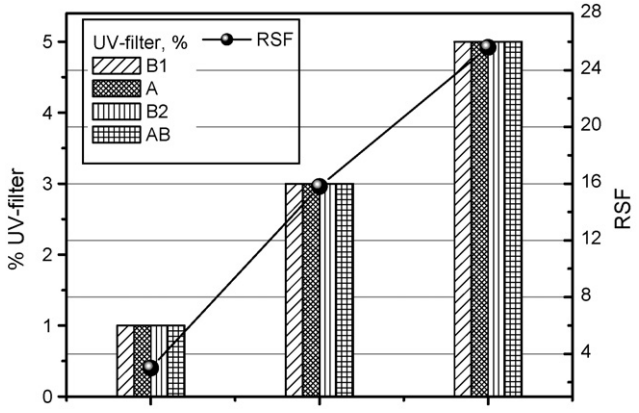

Fig. 4. RSF values (scattered data) of three sunscreens containing 1%, 3%, and 5% each of four UV-filters.

ing chemical or physical UV filters. A RSF > 1 indicates that an applied sunscreen provides a UV protection in terms of reduction of the UV-induced free radicals. On the other hand, a RSF factor < 1 indicates that a chemical product applied to the skin enhances the processes of UV-induced free radical production, and, consequently, the radical injury inside the skin caused by UV radiation.

The calibration curve (Fig. 3) allows to correlate the RSF factor to the UV intensities applied to the skin. A RSF factor of 2 means, that only 50% of the original UV intensity has penetrated into the skin and was able to produce free radicals. On the other hand, a factor of 0.5 means that inside the skin a free radical injury was measured that would have occurred if the skin was irradiated with twice the UV dosis of the control.

Examples of both the protection and induction processes are reported below.

3.1. Protection

Three sunscreens containing different concentrations of four commonly used UV filters were analyzed regarding to their capacity to protect skin against UV-induced free radicals. All products contained two chemical UV-B filters (B1 and B2), one UV-A filter (A) and one broadband filter (AB) at 1%, 3%, and 5% concentration (w/w) in a common W/O formulation. The products were applied according to the COLIPA standard to the stratum corneum of pig skin samples and the RSF method was used to determine the protective effect. The results are shown in Fig. 4. The RSF (scatter data) is directly proportional to the concentration of UV filters. The relationship between the RSF and the filter concentration strongly depends on the type of filters used (mainly UV-A filters are effective in reducing the amount of free radicals); the combination between different filters (UV-B filters protect UV-A filters from photodegradation); the photostability of the filters, and the homogeneity of the filters on the skin's surface [2]. The four UV filters in the sunscreen used are combined to "cover" the whole UV spectral range and in this case the RSF is a linear function of the UV-filter concentration until saturation is reached.

3.2. Induction

Generation of oxygen-derived free radicals by glycated proteins is widely believed to be one of the causes of oxidative stress in aging processes.

Dihydroxyacetone (DHA) is the most frequently used self-tanning agent. It is used for cosmetic applications since 1950 and is accepted by FDA in concentrations up to 15%. Currently, 10% of the U.S. population, or nearly 30 million people, tan indoor. Other self-tanning agents used in combination with DHA are erythrulose, glyceraldehyde, or hydroxymethylglyoxal—all have in common the characteristics of reducing sugars. The Maillard browning reaction between carbohydrates and amines is part of an extensive series of reactions that is the basis for the brown color caused by the "sunless tanning" agent dihydroxyacetone in self-tanning products. The initial stages of the reaction are quite complex, but the ultimate products are brown polymers known collectively as melanoidins. The first step in the reaction of these sugars and the amino acids of the cell components in the stratum corneum and epidermis of the skin lead to ketoamines called Amadori products. The enolization processes of these Amadori products and their oxidation products are known to produce free radicals. Particularly, numerous fructose–amino acids (Amadori compounds) contribute to the formation of oxygen-derived free radicals and their subsequent oxidative damage to proteins [6]. There are also evidences that Amadori products trigger oxidative modification of lipids via the generation of superoxide, and imply the involvement of lipid glycation along with membrane lipid peroxidation in the pathogenesis of diabetes and aging [7].

In order to show that radicals are produced in the skin by the Maillard reaction, electron spin resonance studies have been used in vitro and in vivo [8,9]. In the present work we used the RSF method to quantify the radical generation in the early steps of Maillard reaction in skin.

In Fig. 5 the relative signal intensity of the nitroxyl spin marker is reported as a function of time. In the absence of

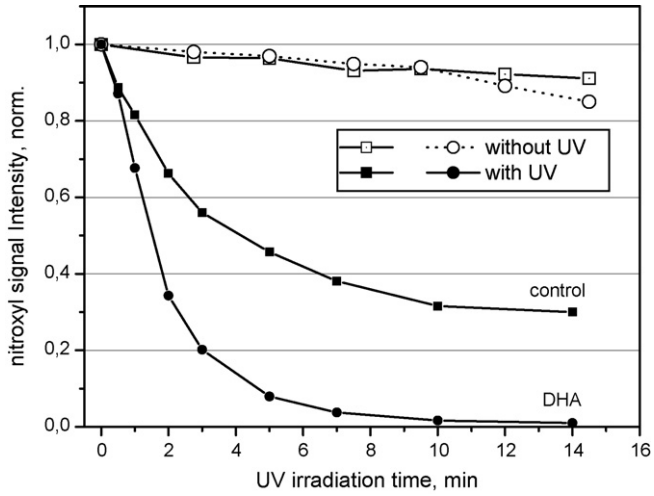

Fig. 5. ESR signal intensity of the nitroxyl spin marker in the skin. Open symbols (◯; □) refer to the absence of UV radiation. Full symbols (●; ■) refer to the signal intensity as a function of UV irradiation time in untreated skin (squares) and in skin treated with 10% of DHA (circles).

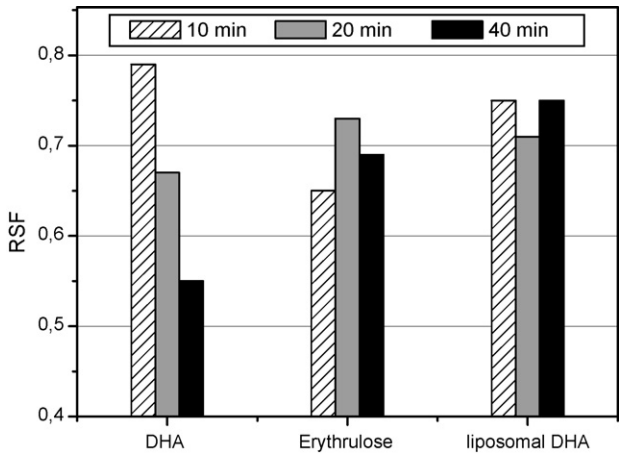

Fig. 6. RSF values of skin treated with 20% (w/w) water solution of DHA, erythrulose, and liposomal encapsulated DHA (ROVISOME DHA).

UV radiation the signal is stable over a time interval of more than 15 min for both untreated skin and skin treated with 10% DHA. During UV irradiation the signal intensity of the nitroxyl decreases rapidly, and for DHA treated skin this decrease is more pronounced. This decrease is due to the reaction of oxygen-derived free radicals induced during UV radiation with the semistable nitroxyl dye. During the Maillard reaction occurring in DHA treated skin, ketoamine intermediate products are formed, which are susceptible to UV irradiation leading to a dramatic increase in free radical production. In order to quantify this radical formation, the RSF method has been applied for three self-tanning ingredients: dihydroxyacetone dissolved in water; dihydroxyacetone encapsulated in liposomes; erythrulose dissolved in water. Both the applied concentration and the penetration time of the products before UV radiation have been changed for all three products. Table 1 and Fig. 6 show the results of the RSF investigation. It is worth noting, that the coloring effect of all products appears only after ca. 12 h after the application, but the Maillard reaction begins to take place within the very first minutes. DHA and the liposomal DHA result in an orange-brown color; erythrulose gives a lighter yellowish color. As shown in the results, the RSF factor of all products was determined to be <1. That means, that all products induce free radical formation and enhance the free radical injury during UV irradiation, compared to untreated skin. As a tendency, the higher is the self-tanning agent concentration, the higher is the radical induction. The highest effects were observed for DHA (Table 1). The liposomal encapsulation seems to attenuate the radical injury to some amount, at least when high concentrations (10–20%) are applied for a longer penetration time (>20 min). This can be due to different penetration kinetics of the liposomes into the epidermal layers of the skin and to a lower reaction capacity of the sugars with peptides and amino acids due to the liposomal encapsulation. At higher concentrations also erythrulose enhance the radical formation, although its coloring efficacy is well below that of DHA.

Table 2
Additional free radicals produced in skin treated with 20% of self-tanning agents compared to untreated skin

Penetration time (min)	Additional free radicals in skin (%)		
	DHA	Liposomal DHA	Erythrulose
10	127	133	154
20	149	141	137
40	182	133	145

In Table 2 these additional free radicals generated by presence of the self-tanning agents with respect to non-treated skin, expressed in percentage are reported for the three tanning products at different concentrations and penetration times.

After 40 min of application of a solution of 20% DHA on the skin, more than 180% of additional free radicals are generated during UV irradiation with respect to untreated skin. Sun

Table 1
RSF values for DHA-, erythrulose-, and liposomal DHA-treated skin

Concentration (%)	RSF								
	DHA			Liposomal DHA			Erythrulose		
	10[a]	20[a]	40[a]	10[a]	20[a]	40[a]	10[a]	20[a]	40[a]
5	0.93	0.95	1.00	1.00	0.98	0.86	0.98	0.83	0.72
10	0.81	0.74	0.64	0.92	0.82	0.76	0.97	0.81	0.72
20	0.79	0.67	0.55	0.75	0.71	0.75	0.65	0.73	0.69

[a] Penetration time (min)

exposure duration is shortened by the use of self-tanning agents. After applying self-tanners one can stay less time in the sun to reach the amount of UV-induced free radicals that are formed in untreated/unprotected skin. In order to avoid a pronounced photoaging process, sun exposure should be avoided after the application of self-tanning products containing reducing sugars. Paradoxically, also skin-lightening agents as kojic acid seem to induce the generation of ROS during sun exposure [10].

4. Conclusion

The quantitative determination of ROS/FR in skin during UV irradiation is an important requirement when the biological consequences of sun exposure are under study. Particularly, skin aging, wrinkling, dermatosis and other pathological conditions are strongly thought to be primarily caused by UV-induced free radicals in the epidermal and dermal layer of the skin. Electron spin resonance (ESR) spectroscopy is a valid tool for the determination of free radical reactions and the herein presented RSF method (radical skin protection factor) is a standardized and validated tool for the quantification of radical injury in skin biopsies. By this method both the protection effect of sunscreens and UV filters and the radical induction of self-tanning agents can be investigated and quantitatively determined. The efficacy of sunscreen formulations in protecting the skin against UV-induced free radicals depends on the composition of UV filters. Mainly UVA filters, that absorb or scatter the radiation in the UVA region (280–400 nm) contribute largely to high RSF values. The higher is the RSF, the longer is the resting time in the sun. On the other hand, RSF values < 1 indicate an additional free radical injury in the skin, as it was found in skin treated with self-tanning agents. During UV radiation of the treated skin the first reaction products and intermediates of the Maillard reaction are susceptible to UV and generate a huge amount of additional free radicals inside the skin. After 40 min treatment with 20% DHA solution an RSF factor of 0.55 was found. That means that more than 180% of additional free radicals are generated in the skin during UV exposure. The activity of antioxidants in neutralizing the ROS induced by UV irradiation of skin treated with self-tanners or skin lightener is currently under study.

The following facts can be seen as important results of the performed study:

- DHA shortens considerably the persistence time in the sun characterized by a RSF < 1. That means for a formulation with 20% DHA and a measured RSF = 0.55 the exposure duration is shortened by half.
- The detrimental effect of DHA during sun exposure can be reduced by the encapsulation of DHA in liposomes.
- A prior enhancement of the intrinsic antioxidative potential (AOP) [11] of the skin or a simultaneous application of DHA with a potent antioxidant can minimize the additional generation of free radicals during UV irradiation.

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