Supplement-Photoprotection

Solar ultraviolet radiation induces biological alterations in human skin *in vitro*: Relevance of a well-balanced UVA/UVB protection

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ABSTRACT

Cutaneous damages such as sunburn, pigmentation, and photoaging are known to be induced by acute as well as repetitive sun exposure. Not only for basic research, but also for the design of the most efficient photoprotection, it is crucial to understand and identify the early biological events occurring after ultraviolet (UV) exposure. Reconstructed human skin models provide excellent and reliable *in vitro* tools to study the UV-induced alterations of the different skin cell types, keratinocytes, fibroblasts, and melanocytes in a dose- and time-dependent manner. Using different *in vitro* human skin models, the effects of UV light (UVB and UVA) were investigated. UVB-induced damages are essentially epidermal, with the typical sunburn cells and DNA lesions, whereas UVA radiation-induced damages are mostly located within the dermal compartment. Pigmentation can also be obtained after solar simulated radiation exposure of pigmented reconstructed skin model. Those models are also highly adequate to assess the potential of sunscreens to protect the skin from UV-associated damage, sunburn reaction, photoaging, and pigmentation. The results showed that an effective photoprotection is provided by broad-spectrum sunscreens with a potent absorption in both UVB and UVA ranges.

Key words: Skin equivalent, sunscreens, ultraviolet radiation, UVA/UVB protection

INTRODUCTION

Skin is the primary target of environmental stresses, in particular, of sun exposure. Biological and clinical consequences of sun exposure range from immediate sunburn reaction and tanning to long-term effects such as photoaging, photocancer, or hyperpigmented lesions. In these processes, two skin compartments are affected: the epidermis and the dermis. It is now well admitted that both UV wavelength ranges are involved. UVB rays (290-320 nm), the most energetic UV wavelengths reaching the earth's surface, can directly induce DNA lesions such as cyclobutane pyrimidine dimers or 6, 4 photoproducts,[1] whereas UVA radiations (320-400 nm) are less energetic but have higher penetration properties. Their major mode of action is the generation of reactive oxygen species (ROS).[2]

For basic research, and also for the design of the most efficient photoprotection, it is crucial to understand and identify the early biological events occurring after UV exposure. For practical and ethical reasons, *in vivo* human studies are often difficult. In contrast, classical skin cell cultures poorly reproduce physiological conditions such as epidermal differentiation or cell—matrix interactions. For all these reasons, *in vitro* organotypic skin models have been developed providing a three-dimensional tissue structure and a complete epidermal differentiation like *in vivo*. A first human skin model,[3] composed of fully differentiated epidermis built upon a living dermal equivalent

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including dermal fibroblasts, allowed us to investigate the biological effects of both UVB and UVA on epidermal keratinocytes and dermal fibroblasts. Direct and indirect effects, as well as interactions between the two skin cell types could be analyzed. A second model was used for studies related to skin pigmentation and its modulation by UV exposure. For this purpose, normal human melanocytes were successfully integrated into a reconstructed human epidermis, thus providing a three-dimensional pigmented epidermis.

In both skin models, due to the presence of horny layer, photoprotection assessment could be performed after topical application of sunscreen formulations like in real conditions to human skin prior to UV exposure. Protective efficiency against UVB- or UVA-induced damage could be monitored. Broad-spectrum photoprotection or the influence of sunscreen photostability could be assessed.

ORGANOTYPIC MODELS

Reconstructed skin

In vitro reconstructed skin was obtained as previously described using normal human epidermal keratinocytes and dermal fibroblasts.[3,4] Dermal equivalent was obtained after contraction of a mixture of collagen type I and human dermal fibroblasts. Human normal keratinocytes were seeded on this template and the culture was left for 7 days in submerged conditions at 37.2°C in the presence of 5% carbon dioxide in minimal essential medium containing 10% fetal calf serum,[5] allowing the cells to proliferate and form a basal layer. Then, the culture was raised upwards to air–liquid interface for an additional 7 days to allow the keratinocytes to differentiate completely and build a horny layer.[6]

Reconstructed pigmented epidermis

Epidermis was reconstructed according to the technique described by Régnier *et al*.[7-12] De-epidermized dermis (DED) was placed, with basement membrane side up, in a Petri dish. Normal human melanocytes and keratinocytes were co-seeded onto the DED at 10:1 ratio (total 5×10^5 cells) into a stainless steel ring. After 6 days of culture in keratinocyte growth medium, the DED was lifted on a stainless steel grid at the air–liquid interface and maintained in keratinocyte differentiation medium, DMEM/F12 (3:1) containing 10% fetal calf serum, 10 ng/ml Epidermal Growth Factor (EGF), 400 ng/ml hydrocortisone, and 5 μg/ml insulin.

UV exposure, biological analysis, and sunscreen application

In order to evaluate the impact of sun exposure on the reconstructed models, organotypic cultures were exposed to different types of UV radiation, i.e. UVB, UVA, complete Solar Simulated Radiation (SSR), and Daily UV exposure Radiation (DUVR), mimicking a realistic outdoor exposure.[13]

At different points of time after UV exposure, the samples were analyzed. General morphology as well as sunburn cell formation was monitored using classical histology.[6] Immunostainings were performed using monoclonal antibodies directed against thymidine dimers[14] to detect DNA lesions and against vimentin (Monosan) to label dermal fibroblasts. The amount of released matrix metalloproteinase-1 (MMP-1) was assessed using enzyme-linked immunosorbent assay (ELISA) technique on culture medium. Gene expression was evaluated using quantitative real-time polymerase chain reaction (Q-PCR) after extraction of tRNA from epidermal and dermal fibroblasts separately.[15] Epidermal pigmentation was assessed by colorimetric measurements with the Microflash Spectrocolorimeter (Datacolor). We used the L* parameter representing luminance (L* = 0 for absolute black and L* = 100 for absolute white) to determine skin color. To visualize melanocytes on detached epidermal sheets and to stain cellular melanin on histological sections, dihydroxyphenylalanine (DOPA) and Fontana–Masson stainings were performed, respectively.

Sunscreen formulations were applied topically to the surface of the skin model (2 mg/cm^2) prior to UV exposure.

RECONSTRUCTED SKIN MODEL WITH A LIVING DERMAL EQUIVALENT: PREVENTION OF UV-INDUCED BIOLOGICAL ALTERATIONS RELATED TO SUNBURN AND SKIN AGING BY EFFICIENT SUNSCREENS

Figure 1 shows that reconstructed skin *in vitro* resembles normal human skin *in vivo*. The main characteristic features such as a well-differentiated epidermis covered by corneocyte layers (a stratum corneum) and a fibroblast populated dermal equivalent could be observed in the *in vitro* model.

UVB exposure induces direct epidermal damage related to sunburn reaction

Since UVB radiation is almost fully absorbed by the different epidermal layers and penetrates poorly into the dermis, its damaging effects are mainly localized to the epidermis.

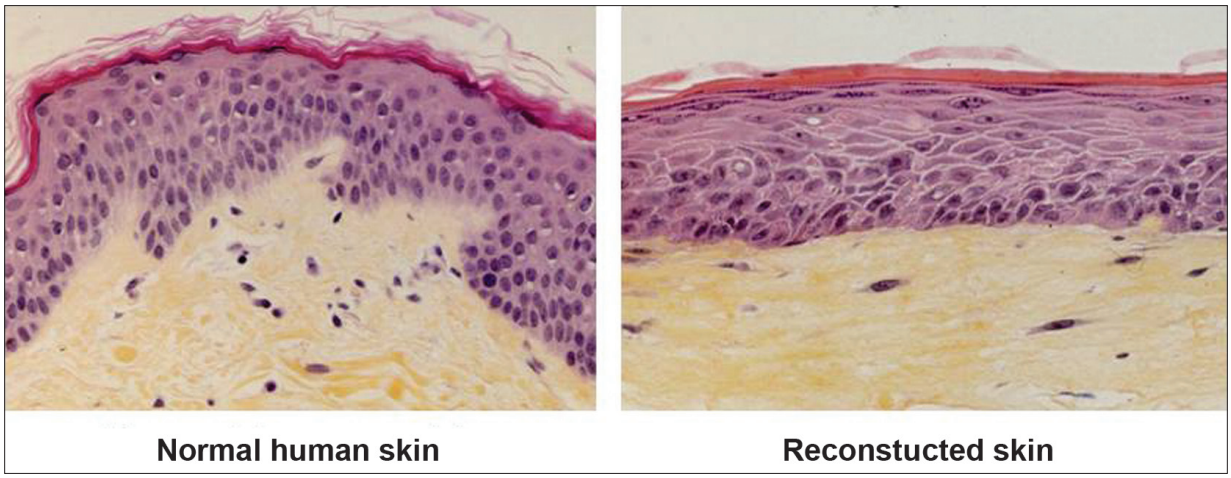

Figure 1: Histological sections of normal human skin and reconstructed skin *in vitro* (Hematoxylin-Eosin-saffron staining HES, × 200)

Exposure of reconstructed skin to UVB radiation (50 mJ/cm^2) induces typical biological alterations that are similar to those on normal human skin following the same exposure. Immediately after exposure, immunostaining with an antibody directed against cyclobutane pyrimidine dimers (CPD) reveals the presence of these DNA lesions in the nuclei of keratinocytes of normal human skin and *in vitro* reconstructed skin.[14,16,17] Twenty-four hours later, the sunburn cells could be visualized with their typical histological features and their suprabasal localization.[17] Sunburn cells have been shown to correspond to apoptotic keratinocytes.[18,19] The sunburn apoptotic keratinocytes over-express galectin-7. Galectin-7 may be implicated in detachment of apoptotic keratinocytes from the surrounding cells.[20,21] Its high expression is directly linked to the stabilization and accumulation of p53 protein, induced by UVB exposure.[22] P53 protein accumulation is one of the major events occurring after UVB exposure, inducing cell growth arrest and allowing DNA repair. This process avoids delayed mutagenic events involved in tumor formation.[23,24]

It is therefore possible to detect direct DNA damage as well as subsequent cellular responses in the *in vitro* model. The comparative analysis between *in vivo* and *in vitro* conditions showed that the type of markers, their kinetics, as well as the dose of UVB inducing biological response are similar in both the systems [Figure 2].

UVA exposure induces direct dermal alterations related to photoaging process

Unlike UVB radiation, UVA radiation, due to its high penetration properties, can reach deeper parts of the skin and affects the dermal compartment. In the reconstructed skin model, UVA actually induces major alterations in the dermal compartment through the generation of ROS. As a result of exposure to UVA radiation (25 J/cm^2), the dermal fibroblasts located in the upper part of the dermal equivalent disappear within 48 h following exposure through an apoptotic process[25] [Figure 3]. On the other hand, the epidermal structure and organization are not morphologically affected, indicating that the survival ability of dermal fibroblasts after exposure to pure UVA is lower compared to that of epidermal keratinocytes. These results confirm previous experiments showing that dermal fibroblasts are more sensitive to UVA-induced oxidative stress than keratinocytes.[5,26]

Interestingly, a recent study has investigated the impact of oxidative stress induced by daily UV exposure in a reconstructed skin model.[15] Gene expression of 24 markers involved in oxidative cell response was assessed in fibroblasts and keratinocytes in parallel. The results showed a high sensitivity of dermal fibroblasts to oxidative stress [Figure 4]. Altogether, these phenomena may be implicated in early events occurring during photoaging that lead to drastic alterations of dermal structure and "solar elastosis."[27,28] Previous human *in vivo* studies have also shown that repetitive exposures to low UVA doses induced early morphological and biochemical alterations in the dermis.[13,29,30]

UV-induced MMP-1, a crucial biomarker of photoaging

Exposure of human skin to UVB or UVA, alone or combined such as SSR or DUVR, results in increased MMP-1 production.[15,26,31,32] MMP-1 is an interstitial

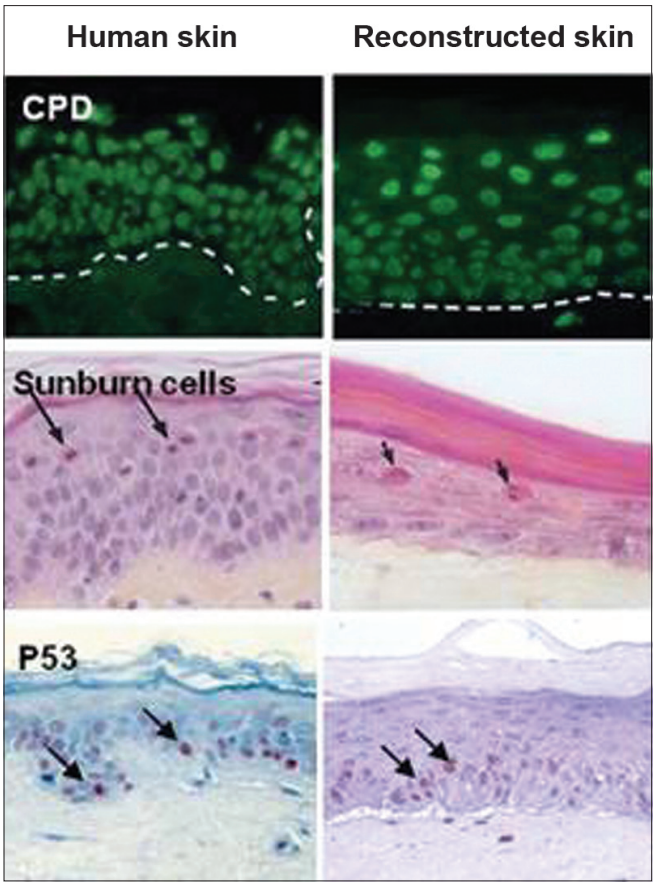

Figure 2: UVB- Typical biological markers induced by UVB exposure (50 mJ/cm²) in normal human skin or reconstructed skin *in vitro*. Detection of cyclobutane pyrimidine dimers (CPD) immediately after exposure, and observation of Sunburn cells on HES staining and p53 protein accumulation at 24 hours (arrows). (CPD and Sunburn cells pictures: ×400; p53 pictures: ×200)

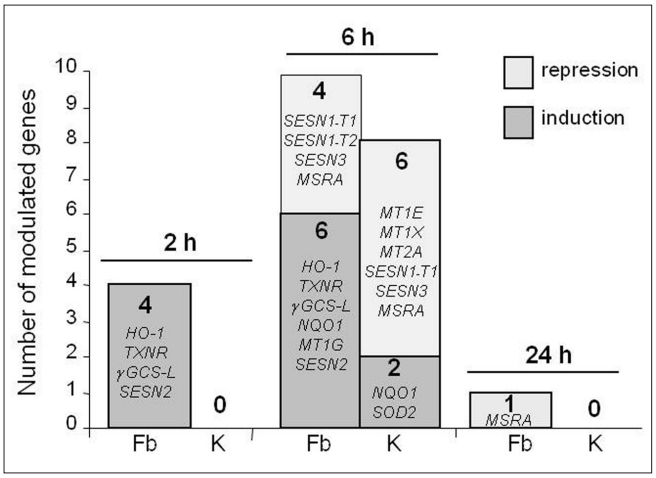

Figure 4: Distribution, type and mean of gene modulation after 7J/cm² DUVR exposure of human reconstructed skin. 2, 6 and 24 hours after exposure, mRNA levels of 24 oxidative stress markers were quantified by QPCR in fibroblasts (Fb) and keratinocytes (K) of reconstructed skin. Number of significantly modulated genes and type of modulation at each time point in both cell types

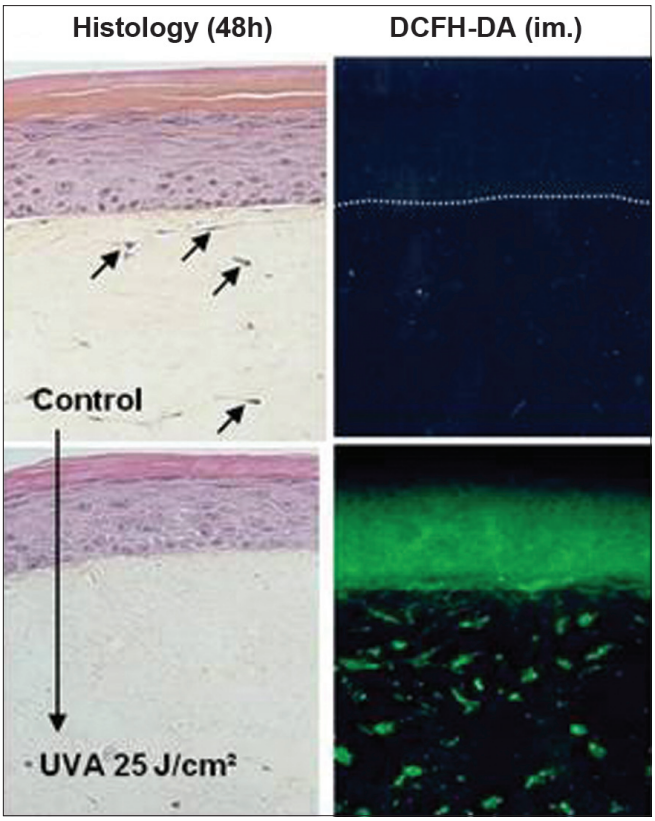

Figure 3: UVA - Induction of dermal fibroblast alterations within the dermal equivalent after UVA exposure of reconstructed skin. Disappearance of dermal fibroblasts (arrows) 48 hours after UVA exposure (25J/cm²) shown on reconstructed skin section stained by HES (×200)-Visualization of oxidative stress after incorporation of dichlorofluorescin diacetate DCFH-DA probe (green signal)

collagenase able to hydrolyze type I collagen, the major component of the dermis, and it seems to play a crucial role in the disorganization and progressive degeneration of dermal extracellular matrix.[31,33,34]

The increase in MMP-1 after UV exposure was also observed in reconstructed skin model,[26,35] which allowed to better understand the role of keratinocyte and fibroblast in MMP-1 induction. Under UVA exposure, MMP-1 production was directly induced in the dermal fibroblasts. Removal of epidermis immediately after UVA exposure did not alter this induction. These results confirmed other data on UVA-induced MMP-1 in cultured fibroblasts.[32,36] In contrast, UVB-induced MMP-1 production required the presence of the epidermis. The use of monolayered cultured keratinocytes and fibroblasts, as well as reconstructed skin, demonstrated that UVB-induced MMP-1 resulted from a paracrine mechanism involving the release of epidermal soluble factors such as cytokines, interleukin (IL)-1α and IL-6.[26,35]

Protective effects of well-balanced sunscreens

The wavelength-specific biological damage induced in both epidermis and dermal equivalent allows the photoprotection afforded by various sunscreen formulations to be assessed with regard to damage induced by UVB, UVA, SSR, or DUVR exposure.[37-40] Since the *in vitro* model has a horny layer, it is possible to apply the products topically on skin surface, thus mimicking real life conditions. The protecting effects against UV-induced epidermal or dermal damage in reconstructed skin model can be evaluated at various time points after exposure.

The importance of UVB–UVA transmission profile in photoprotection was evaluated, thanks to those models. Products that absorb both UVB and UVA radiation were shown to provide better protection with regard to photoaging markers than preparations that absorb mostly in the UVB range. Regarding these biological parameters, the value of the Sun Protecting Factor (SPF), which evaluates mostly UVB protection, seemed not to be sufficient to predict the photoprotective effect of the sunscreen in solar-simulated exposure conditions.[37]

A recent study using a skin reconstructed model was conducted to assess the protection afforded by two different sunscreens under standard daily ultraviolet radiation exposure conditions.[38] The two sunscreens had the same SPF value but different profiles of UVA protecting factor or UVA-PF [ratio of SPF/UVA-PF (Persistent Pigment Darkening PPD) <3 for sunscreen A and >3 for sunscreen B]. The efficiency of these sunscreens was evaluated with regards to their ability to protect against UVB and UVA biological damage induced by SSR exposure. Dose response experiments showed that the sunscreen with the highest UVA-PF (A) provided a better protection against dermal damage. The results showed that the sunscreen having the ratio SPF/UVA-PF (PPD) <3 gave a higher protection than the sunscreen with a ratio >3 as regards photoaging-related biomarkers, i.e. dermal fibroblast alteration [Figure 5] (photoprotection is higher with sunscreen A compared to sunscreen B) and MMP production. It thus demonstrates that for a given SPF value, efficient photoprotection required a significant UVA absorption potency.

In order to characterize the protection afforded by a broad-spectrum sunscreen [SPF: 67.5 ± 6.2 and UVA-PF (PPD method): 31.1 ± 6.4] at the molecular level, a semi-global gene expression analysis was performed. Two hundred and forty-four genes in keratinocytes and 227 in fibroblasts were analyzed separately in the reconstructed skin after UVA exposure with and without prior application of the sunscreen. In both skin compartments, UVA radiation induced modulation of several genes involved in extracellular matrix, oxidative stress response, heat shock response, cell growth, inflammation, and epidermal differentiation. Sunscreen pre-application abrogated these effects or reduced them significantly. This revealed a very high photoprotective activity of the sunscreen that could be evidenced using an unsupervised clustering analysis or a gene by gene comparison approach[40] [Figure 6]. This data indicated that a broad-spectrum sunscreen

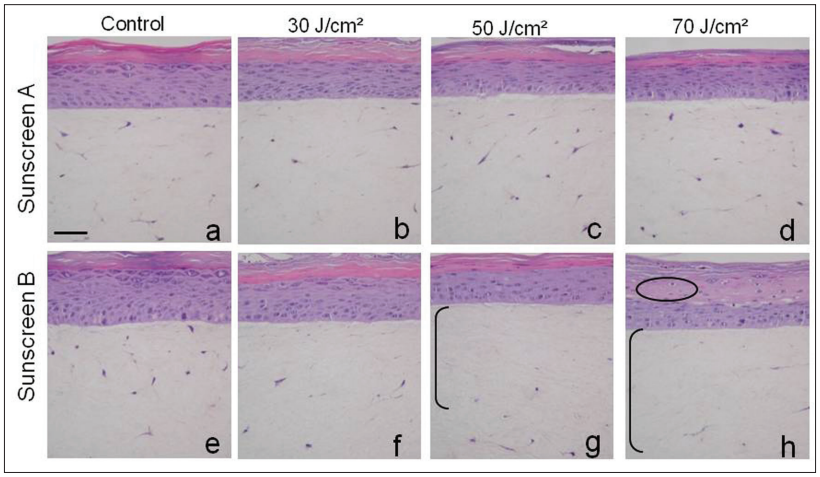

Figure 5: Evaluation of protection by sunscreens A and B in reconstructed skin exposed to increasing doses of DUVR. Sunscreens A and B had same SPF (15) but different SPF/UVA-PF ratio, < and >3, respectively. Products were applied onto samples before exposure to DUVR (0, 30, 50 or 70 J/cm²). Note a good protection where product A has been applied compared to alterations observed in samples with product B. Scale bar: 50 and #956;m. Ovals :alterations in the epidermis. Bracket: the depth of dermal alterations. (Hematoxylin-Eosin-saffron staining, ×200)

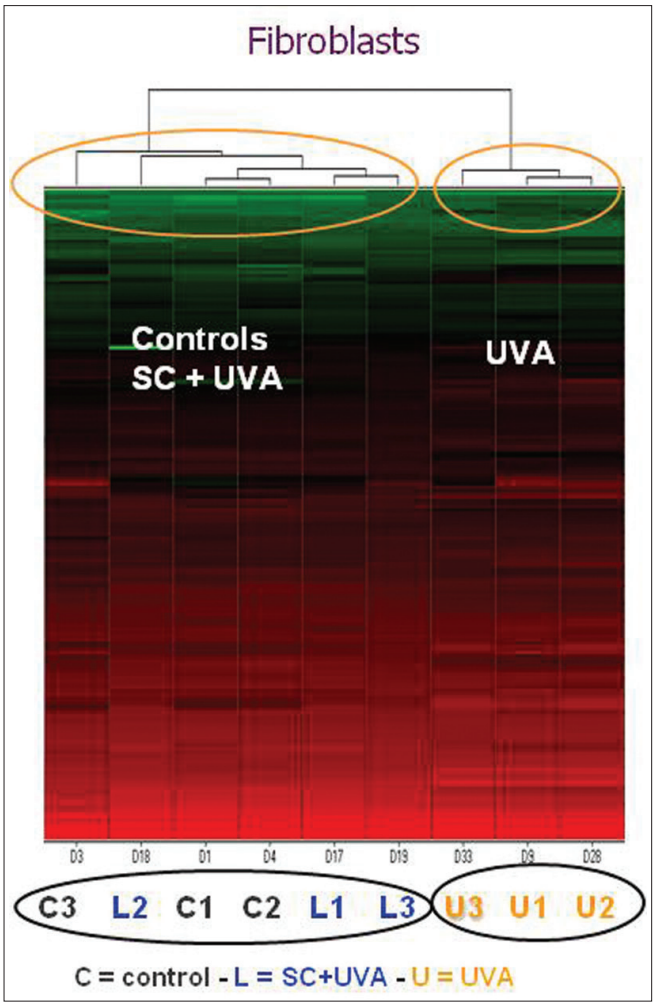

Figure 6: Heat map of gene expression in reconstructed skin exposed to 30 J/cm² UVA. Expression of 191 transcripts was detected by Q-PCR in fibroblasts of control reconstructed skins (C1-3), samples exposed to UVA (U1-3) and exposed to UVA after sunscreen application (L1-3). The length of the vertical lines of the dendrogram represents the similarity of the samples. The circles group the closest conditions

was able to prevent UVA-induced gene responses corresponding to cellular events beyond the *in vivo* protection factor determination.

PROTECTIVE EFFECT OF SUNSCREENS AGAINST UV-INDUCED PIGMENTATION ON SKIN RECONSTRUCTED MODELS

The successful integration of normal melanocytes into reconstructed human epidermis provided a further improvement and opened new possibilities to study pigmentation in a three-dimensional structure close to normal human epidermis.

Figure 7a shows the pigmented reconstructed epidermis model. A macroscopic pigmentation could be observed and confirmed by histological analysis after Fontana–Masson staining. In the basal layer of the epidermis, differentiated melanocytes synthesize and transfer melanin into neighboring keratinocytes. By using normal melanocytes from different origins, pigmented epidermis can reproduce the original phenotype of donor's skin [Figure 7b].

Effect of UV exposure and sunscreen application

When this reconstructed model is exposed to UV radiation, an increase in pigmentation (tanning) is produced. Exposure of pigmented reconstructed human epidermis to SSR resulted in a dose-dependent stimulation of pigment production inducing a visible tanning of the epidermis. The melanin content and DOPA reactivity after irradiation increased accordingly [Figure 8]. The tanning response is quantified by colorimetric measurements. This delayed

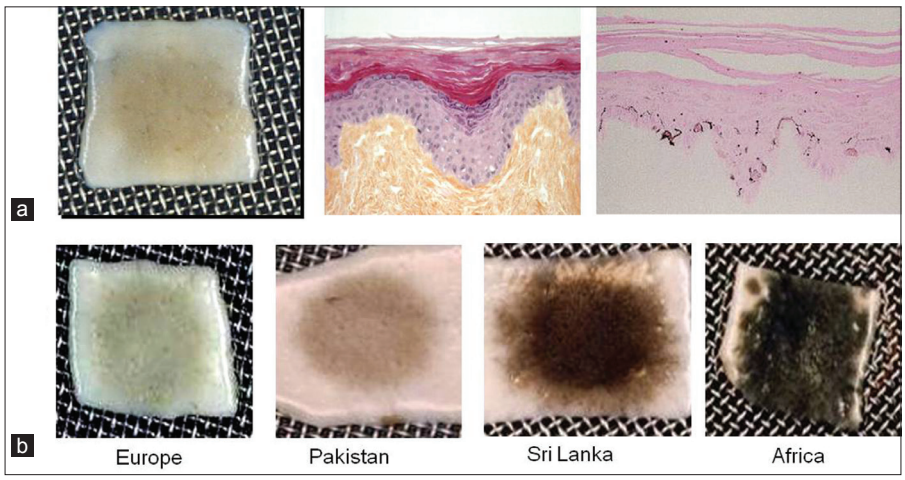

Figure 7: (a) Pigmented reconstructed epidermis observed macroscopically, histologically using Hematoxylin-Eosin-saffron and Fontana Masson stainings (×200). The organization of the epidermis is correct with the presence of melanocytes and melanin granules within the epidermis, (b) Pigmented epidermis obtained using melanocytes from donors originating from different countries or continents as indicated

hyperpigmentation involves a neo-melanogenic process that could previously be set off by UVB and UVA radiation separately.[10] On pigmented epidermis model, immediate pigmentation-darkening related to photo-oxidation of preexisting melanin and its precursors was only observed after UVA radiation.[10] This model which exhibits a pigmentary response similar to that of normal skin exposed to UV provides an excellent and reliable tool for studying the UV-induced changes in pigmentation, to evaluate the antipigmenting effect of applied ingredients or products and to test the efficacy of sunscreens.

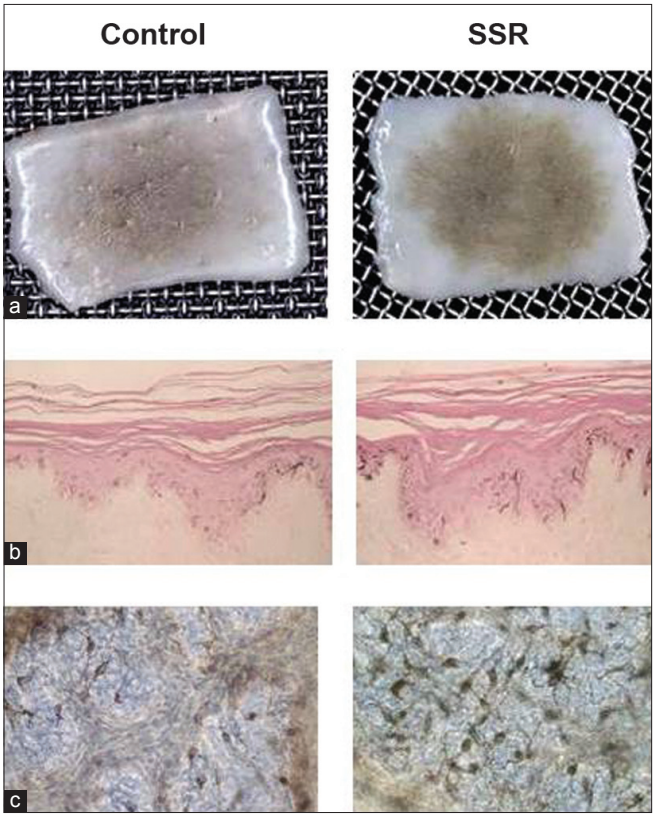

Figure 8: Solar simulated radiation (SSR)- induced pigmentation in reconstructed human epidermis, (a) control and exposed epidermis, (b) histology after Fontana-Masson staining (x200) and (c) Dopa reaction on epidermal sheet (x200)

Since many hyperpigmented skin lesions, such as melasma or actinic lentigines,[41,42] are associated with exposure to UV radiation, the pigmented reconstructed skin model has been used to evaluate the antipigmenting potential of sunscreens. A product containing 4% Mexoryl SX (bis-benzylidene camphosulfonic acid derivative), a photostable UV filter covering most of the UV spectrum, was applied onto pigmented reconstructed epidermis prior to UVA or SSR exposure. Mexoryl SX totally inhibited UVA-induced pigmentation and strongly reduced SSR-induced melanogenesis [Figure 9], showing its strong efficiency to prevent UV-induced hyperpigmentation.

CONCLUSION

The reconstructed human skin models described above provide excellent and reliable tools to study *in vitro*, the UV-induced alterations of the different skin cell types, keratinocytes, fibroblasts, and melanocytes in a dose- and time-dependent manner. Those models are also highly adequate and useful to assess the potential of sunscreens to protect the skin from UV-associated damage, sunburn reaction, photoaging, and pigmentation. Altogether, the results showed that an effective photoprotection is only provided by a real broad-spectrum sunscreen providing potent absorption in both UVB and UVA ranges and referred to as well balanced. Our data emphasized the fact

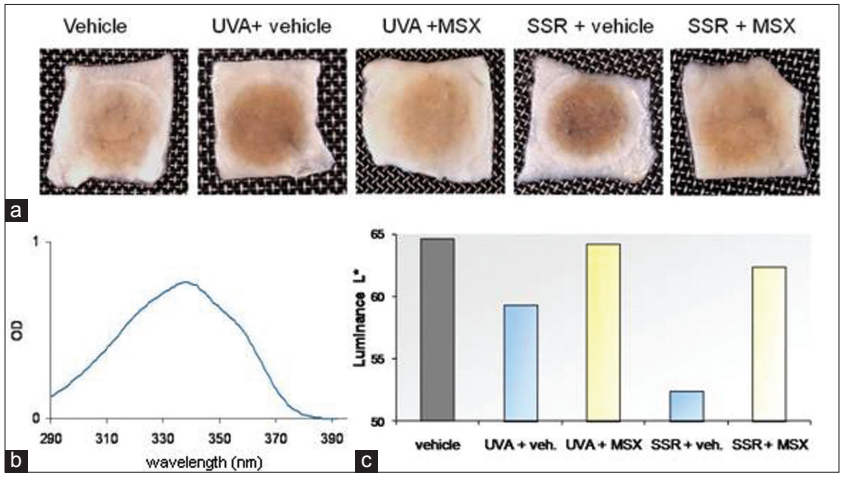

Figure 9: Protective effect of Mexoryl® SX against UVA- and SSR-induced pigmentation in reconstructed epidermis, (a) Macroscopic pictures (b) UV absorption spectrum of Mexoryl® SX and (c) Luminance values (L*) of the reconstructed epidermis

that for a given SPF value, efficient photoprotection required a significant UVA absorption potency.

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