In vitro assays to study the effects of air pollutants on skin: exposure to urban dust and cigarette smoke extract.

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SUMMARY

Exposure of the skin to air pollutants is associated with skin aging and inflammatory or allergic skin conditions. Visible signs of aging and inflammation are a major concern for many people. There is a real need for identification and characterization of the effects of skin care products against air pollutants. We introduce here different *in vitro* assays on fibroblasts and 3D skin models which are appropriate for investigating the protective effects of skin care ingredients and formulas. Using these models exposed to standardized urban dust and cigarette smoke extracts, we addressed the effects of pollutants on the oxidative stress level, inflammation status, as well as detoxification and antioxidant pathways. The effects of the pollutants on the chosen biomarkers were compared with reference compounds known for their protective effects, including carnosic acid and sulforaphane. Our data demonstrate the efficacy of *in vitro* assays to assess the anti-pollution and detoxification potency of investigational compounds.

INTRODUCTION

Skin aging and inflammatory skin conditions are of primary concern for millions of people around the world. Such skin changes and disruptions do not only occur because of genetic or passing time but are also due to exposure to different environmental factors. Increase of air pollution over the years has been recognized to have major effects on the human skin. The impact of pollution on the quality of the skin becomes a subject of very important concern, especially in large industrial cities such as Mexico City, Shanghai, Sao Paulo, where exposure to air pollutants has been linked to skin aging, inflammation and allergy through population studies (Lefebvre *et al*. 2015, Drakaki *et al*. 2014, Pan *et al.* 2015, Mavrfrydi *et al.*2015, Vierkötter *et al.* 2010, Vierkötter *et al.* 2011, Pham *et al.* 2015).

In spite of these studies showing the harmful effects of air pollution on the skin, there is a lack of *in vitro* standard models to identify and characterize the protective or preventive effects of dermo-cosmetic products against air pollutants. We therefore established standardized assays relying on our *in vitro* 2D and 3D models and a selection of reference pollutants such as atmospheric particulate material collected in an urban area or smoke extracts from a reference cigarette program. We then used our reference models to characterize their negative effects on skin biology and assess the efficacy of protective compounds in modulating the biomarkers associated with skin aging and skin inflammation induced by air pollutants. We focused our interest on specific biomarkers associated with oxidative stress level, inflammation status, skin barrier function as well as detoxification and antioxidant pathways activation.

METHODOLOGY

Cell culture

Normal Human foreskin-derived Dermal Fibroblasts (NHDF) and Epidermal Keratinocytes (NHEK) were cultivated in monolayer in their appropriate medium. Reconstituted Human

Epidermis was cultured at the air-liquid interface in a suitable culture medium. All cultures were maintained in a humid atmosphere at 37°C with CO2 5%.

Cytotoxicity study

To determine the concentrations of pollutants and references, preliminary experiments were conducted on NHEK and NHDF in culture triplicates. The cells were seeded in 24-well plates in their respective media for either 24 or 72h. At the end of the treatment, cell viability was then assessed by a MTS assay (3- (4,5-dimethythiazol-2-yl) -5- (3-carboxy-methoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium).

Reactive oxygen species

Analysis of the effects of pollutants on the production of Reactive Oxygen Species (ROS) by NHEK keratinocytes and NHDF fibroblasts was carried out based on the use of the prefluorescent probe H2DCFDA (2'-7'dichlorodihydrofluorescein diacetate). The cells were incubated with the probe which diffused into the cells. Cleavage of the acetate group is achieved by intracellular esterases. The probe can then be oxidized by free radicals present in the cell, producing the fluorescent compound DCF (2'-7 'dichlorofluorescein). The production of free radicals can then be quantified according to the level of fluorescence emitted by the oxidized probe. The cells were irradiated or not with UVA using an illuminator. Then the fluorescence emitted following oxidation of the probe was quantified (excitation: 485 nm, emission: 520 nm).

Gene expression analysis using qRT-PCR

Gene expression changes were analyzed using TaqMan qPCR assays. RNA extraction was performed according to the supplier's recommendations. The RNA collected were stored at -80°C. The RNA concentration was determined by spectrophotometric measurement and the RNA quality was analyzed by capillary electrophoresis. After reverse transcription, the TaqMan assays were processed as described by the manufacturer's instructions. In brief, cDNA were mixed in 96 well plates with a specific buffer and probes specific to the target genes. Plates were sealed and qPCR were run by a Real-Time PCR System. Relative quantification of gene expression was performed using the comparative Ct ($\Delta\Delta$Ct) method. The references of the TaqMan genes expression assays (*Applied Biosystems*) are listed below.

Table 1: References of TaqMan gene expression assays

TaqMan gene expression assay ID	*Catalog references*
18S	*Hs_9999990_s1*
COL1A1	*Hs-00164004-m1*
ELN	*Hs_00355783_m1*
MMP1	*Hs_00899658_m1*
NQO1	*Hs_00168547_m1*
PTGS2	*Hs_00153133_m1*

Quantification of the inflammatory mediators

The quantification of the inflammatory markers has been performed using specific ELISA kits, based on a standard curve and according to supplier's specifications.

Immunohistochemistry

RHE samples fixed in formaldehyde were dehydrated with ethanol and isopropanol before paraffin inclusion for histological assessment and immune-labelling. Paraffin sections were stained for histology with eosin and hematoxylin (H/E). Slides were mounted with specific medium and examined with a photomicroscope equipped with a digital camera. For immune-

labelling, paraffin-embedded sections were de-paraffined and re-hydrated. Antigen unmasking was performed and sections were incubated with primary antibody after blocking in serum. The products references are given in the table below.

Table 2: References of reagents and products used for immunohistochemistry

Produit	Firme	Référence
Ethanol	Klinipath	4098.9005
Isopropanol	Klinipath	4101.9005
Ultraclear	Klinipath	3905
PBS10X	Life Technologies	70011-036
Sodium citrate	VWR-Merck	1.06448.500
Tween®20	Sigma	P1379
Goat serum	Abcam	ab7481
Anti-filaggrin (mouse)	Acris Antibodies GmbH	AM00245PU-N
Anti-mouse Alexa-488	Molecular Probes	A11001
Dapi	Invitrogen	D1306
Mowiol	Sigma	32,459-0

Trans-epidermal electrical resistance

Skin barrier function of RHE samples was determined by measuring trans-epidermal electrical resistance (TEER) using the Ohmmeter Millicell ERS (*Millipore*). Empty insert was used as blank. All measures were performed on 12 wells plates filled with PBS.

Statistical analyses

Experimental data were analyzed for statistical significance using Student *t* tests unless otherwise specified. Differences were considered significant if the p value was below 0.05.

RESULTS AND DISCUSSION

An extensive literature search allowed us to select multiple reference compounds that represent real life air pollutants (fine particles, urban dust, diesel combustion particles, cigarette smoke extracts) (Alley et al. 2009, Yang et al. 2013, Ekstrand-Hammarström et al. 2013 Vattanasit et al. 2014). We present here some of the results obtained using urban dust (UBD) and cigarette smoke extracts (CSE). Similarly we also selected several protective reference compounds (sulforaphane, carnosic acid or vitamin C) to validate our models and assess the efficacy of such references to protect from deleterious effects of pollutants (Boddupalli et al., 2012, Abdull et al., 2012, Bauer et al., 2012, Park et al. 2013). Carnosic acid is a phenolic diterpene from rosemary extract, a potent antioxidant which prevents expression of matrix metalloproteinases in skin fibroblasts and keratinocytes (Park *et al*. 2013). Sulforaphane is a mild activator of the detoxification pathway which maintains the endogenous antioxidant enzymes as well as the antioxidant activity of vitamins A, C, and E (Boddupalli *et al.* 2012).

Absence of acute cytotoxicity in presence of urban pollutants at usual concentrations

To determine the concentrations of pollutants and references that will be used in the assays, preliminary dose-response experiments were conducted on NHEK and NHDF. We conducted the same assays on reference compounds sulforaphane and carnosic acid. The concentrations of reference pollutants and protective compounds have been selected based

on their biological relevance and do not induce cytotoxicity as measured by MTS assays after 24h and 72h of culture (examples of data obtained on NHDF after 72h of treatment are given in the Table below). The chosen concentrations specific to each cellular model corroborate with previous studies (Alley et al. 2009, Yang et al. 2013, Ekstrand-Hammarström et al. 2013 Vattanasit et al. 2014).

Table 4: Concentration thresholds of references inducing cytotoxicity after 72h in NHDFs

Pollutant or protective agent	Concentration decreasing viability more than 20%
UBD (Urban Dust)	>100 µg/cm²
DEP (Diesel Exhaust Particles)	>100 µg/cm²
Benzo[a]pyrene	3 µM
CSE (Cigarette Smoke Extract)	>200 µg/ml
Carnosic acid	>20µM
Suldoraphane	10µM
Vitamin C	>500µg/ml

Urban pollutants at usual concentrations increase UVA-induced oxidative stress levels

In presence of the chosen concentrations, our data demonstrated that pollutants including urban dust (UBD) and cigarette smoke extract (CSE) do not induce significant oxidative stress *per se*, yet they increase significantly the oxidative stress levels of NHDF and NHEK cells exposed to UVA (Figure 1). This oxidative stress is inhibited in presence of vitamin C and to a lesser extent in presence of carnosic acid or sulforaphane.

Figure 1: Relative oxidative stress levels on human fibroblasts exposed to air pollutants

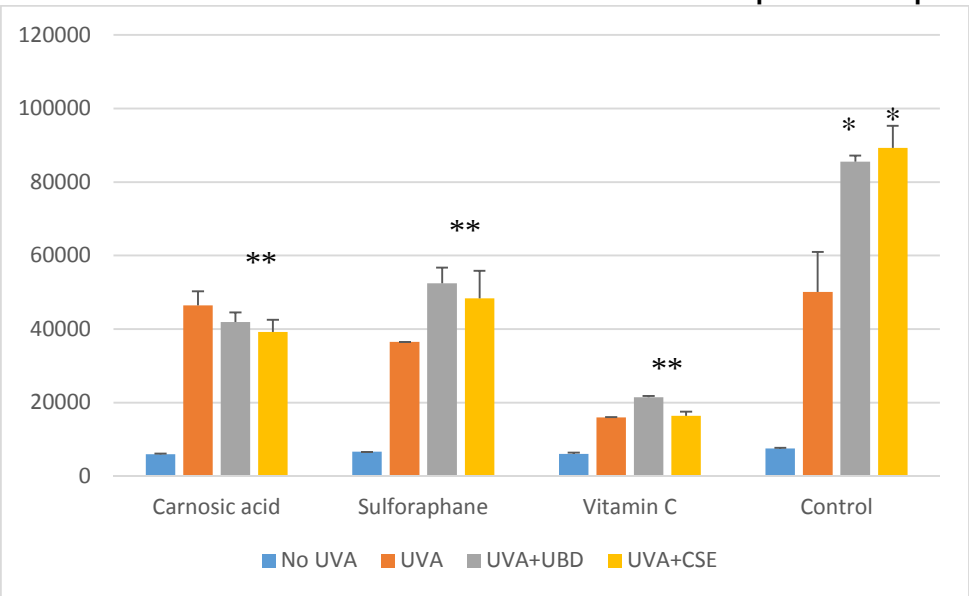

Figure 1: Relative measurement of the intensity of oxidative stress induced in NHDF cells after 48 hours of culture in presence of air pollutants (UBD: Urban Dust, CSE: Cigarette Smoke Extract) and after treatment with UVA. The figures shows comparisons of stress levels induced in the presence or absence of protective agents (carnosic acid, sulforaphane, vitamin C). There are significant statistical differences between UVA treated cells with and without exposure to pollutants (*) as well as between UVA treated cells exposed to pollutants and treated with and without protective references (**). The graph shows the mean and standard deviations of culture triplicates. Similar results were obtained on NHEK.

Gene expression analysis reveals key cellular functions modulated by air pollutants

In order to gain insight into the cellular pathways modulated by air pollutants and the potency of protective agents to reverse the disrupted skin functions, we performed a series of gene expression analyses on targets known to be involved in skin aging and inflammation.

Exposure to CSE for 72h does not induce a significant change in collagen or elastin mRNA expression; although a transient decrease is observed at 24h. Conversely, urban dust induced a decrease of ELN and COL1A1 mRNA levels which persists after 72h of culture (Figure 2). A positive effect of sulforaphane is observed after 24h of culture. However its protective function is not observed after 72h of culture. This could be explained by its degradation which would therefore require to change the culture media every 24h. This hypothesis remains to be confirmed. A beneficial effect is observed on ELN expression levels in presence of carnosic acid after 72h of exposure to UBD. However this effect is not observed on COL1A1 expression.

Figure 2: mRNA expression fold changes of COL1A1 and ELN upon exposure to air pollutants

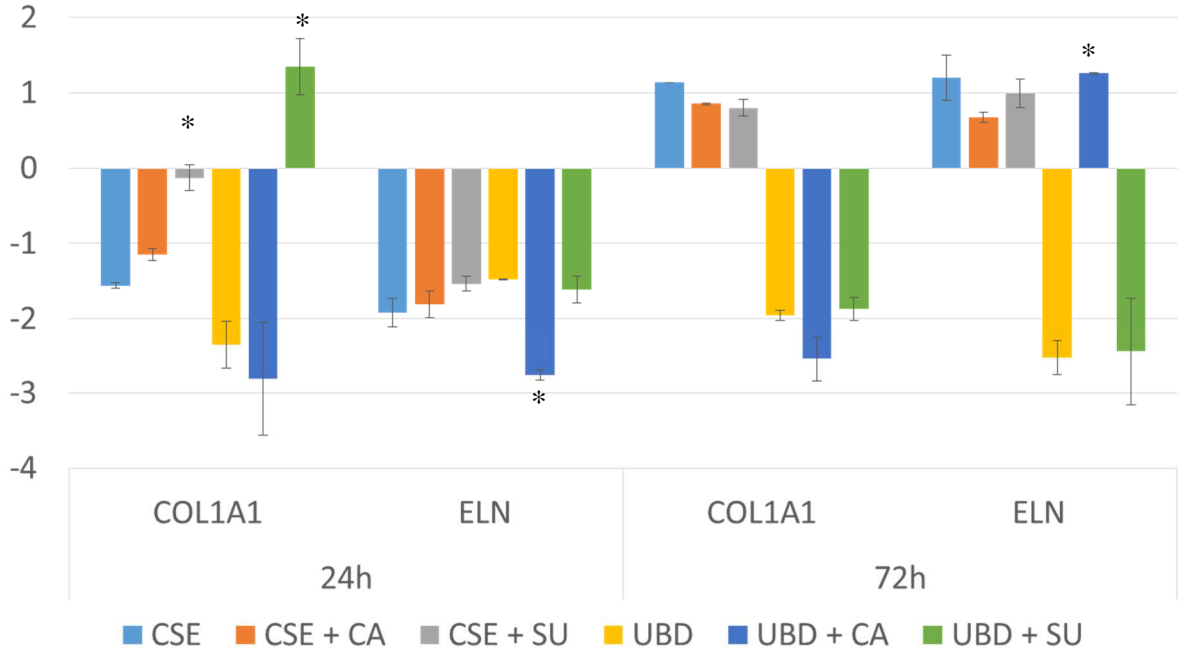

Figure 2: COL1A1 and ELN mRNA expression fold changes from NHDF cells exposed to UBD and CSE for 24 and 72h. qRT-PCR assays were performed as explained in the methods and average fold changes of duplicates are shown with standard deviations. Significant statistical differences are shown on the graph (*) between cells exposed to pollutants with and without protective reference (to be compared with their respective pollutant, CSE or UBD, and time of exposure). UBD: Urban dust, CSE: Cigarette smoke extract, CA: Carnosic acid, SU: Sulforaphane.

Pollutants under study increased expression of inflammation inducers such as cyclooxygenase-2 and 5-lipoxygenase, while carnosic acid and sulforaphane were not able to hamper this effect. The same conclusions were obtained looking at IL-8 levels in cell culture supernatants (data not shown). On the contrary, carnosic acid was able to inhibit the expression of matrix metalloproteinase 1 (MMP1), an extracellular matrix degradation enzyme of which expression is induced by air pollutants. The decrease of MMP1 is a valuable protective effect to reduce the extracellular matrix degradation induced by air pollutants (Figure3).

While an endogenous protective response induces a slight increase of antioxidant enzymes HMOX1 and NQO1 in presence of pollutants, treatments with carnosic acid or sulforaphane induced a drastic increase of expression of such antioxidant enzymes, therefore boosting endogenous cellular ability to resist to oxidative stress (Figure 3). These results corroborate the reduction of reactive oxygen species shown on Figure 1.

Air pollutants also induced the expression of CYP1A1, a member of the cytochrome P450 superfamily of detoxification enzymes of which expression is dependent on the activation of the aryl hydrocarbon receptor (AhR). This enzyme family is involved in detoxification processes by increasing the reactivity of exogenous molecules to facilitate further conjugations with charged species such as glutathione. These mechanisms facilitate their excretion and are part of the bioactivation and detoxification of xenobiotic chemicals (Guengerich 2001). However CYP1A1 activation of xenobiotics also results in accumulation of highly reactive compounds including carcinogens (Ono *et al.* 2013, Androutsopoulos *et al*. 2009). This is the case in presence of polycyclic aromatic hydrocarbons which represent a significant fraction of air pollutants. We observed a decrease of CYP1A1 expression levels in presence of carnosic acid, although the expression induced by air pollutants was not totally abolished (Figure 3). It may be of great importance for protective agents to be able to control the extent of AhR target genes expression in order to benefit from their protective functions while not inducing accumulation of highly reactive compounds. Another hypothesis would be that protective agents such as carnosic acid are able to inhibit xenobiotic activity upstream of AhR activation hence decreasing its activity and the expression of its target genes.

Figure 3: mRNA expression fold changes of CYP1A1, MMP1, NQO1 and HMOX1 after treatment

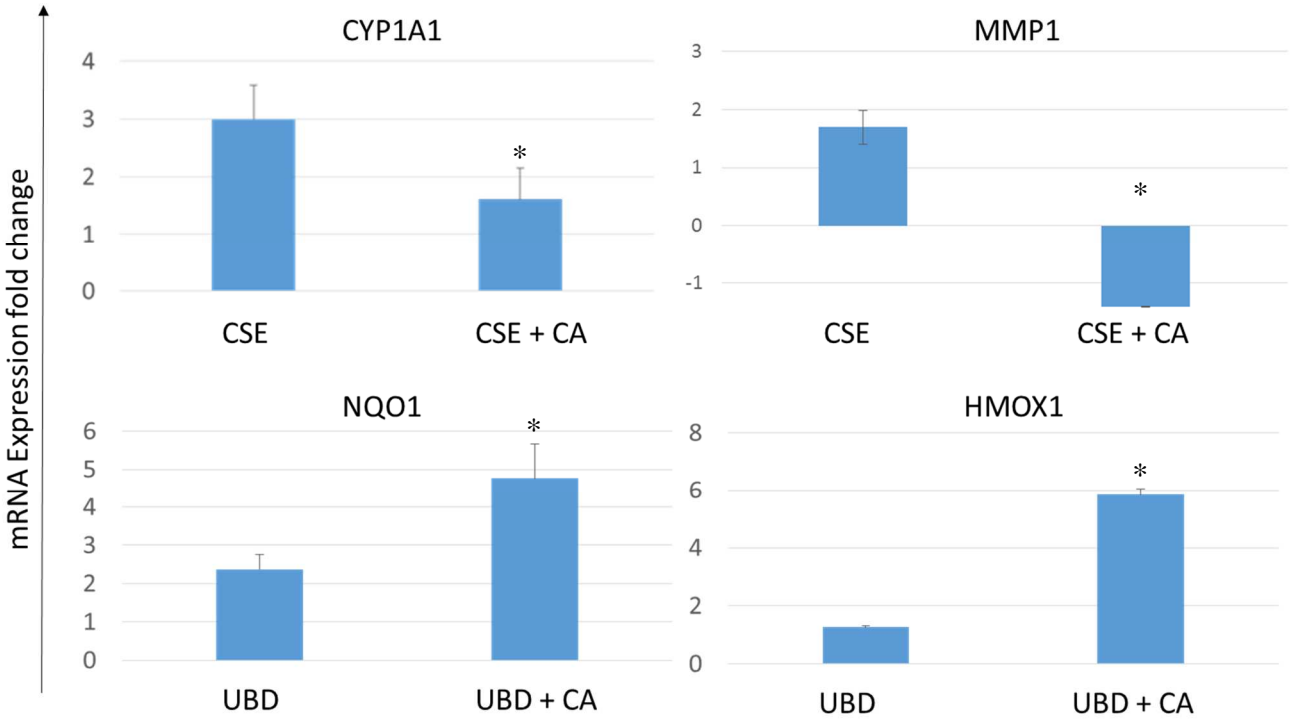

Figure 3: mRNA expression fold changes of 4 target genes of the detoxication pathway, from NHDF cells exposed to UBD and CSE for 24h. See methods and Figure 2 legends for details. Significant statistical differences are shown on the graph (*) between cells exposed to pollutants with and without carnosic acid.

Air pollutants impede the skin barrier function of reconstructed human epidermis

In order to further study *in vitro* the impact of air pollutants on the integrity of the skin biology, RHE samples were exposed topically to UBD and CSE for 4 days and the quality of skin barrier function was determined through measurement of transepidermal electrical resistance. This method showed a significant decrease of skin barrier function upon exposure to these air pollutants (Figure 4).

Figure 4: Transepidermal electrical resistance of RHE exposed to pollutants

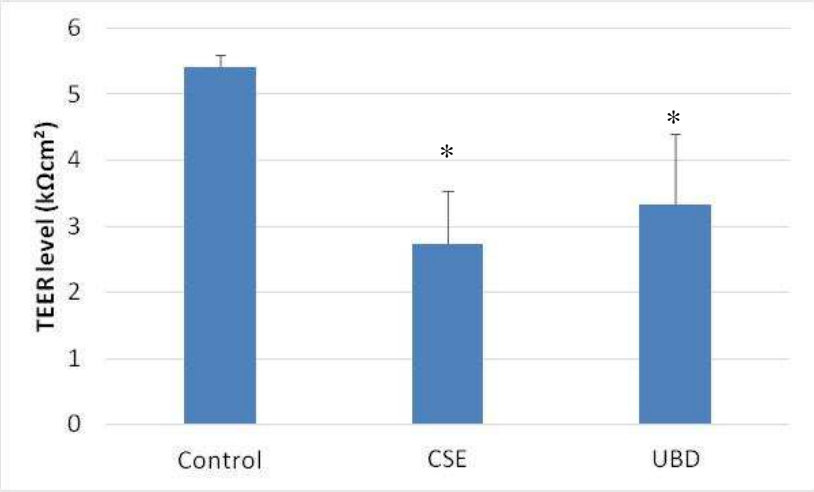

Figure 4: Transepidermal electrical resistance of RHE exposed to pollutants for 4 days. Significant statistical differences are shown on the graph (*) between controls and cells exposed to pollutants.

In order to correlate this lost of TEER with the level of differentiation achieved in the reconstructed epidermal tissue we looked at the filaggrin abundance of RHE exposed for 48h to either CSE or UBD (Figure 5). As expected by the TEER observations, we noticed a significant decrease in filaggrin abundance after exposure to air pollutants.

Figure 5: Abundance of filaggrin on fully differentiated RHE exposed to pollutants

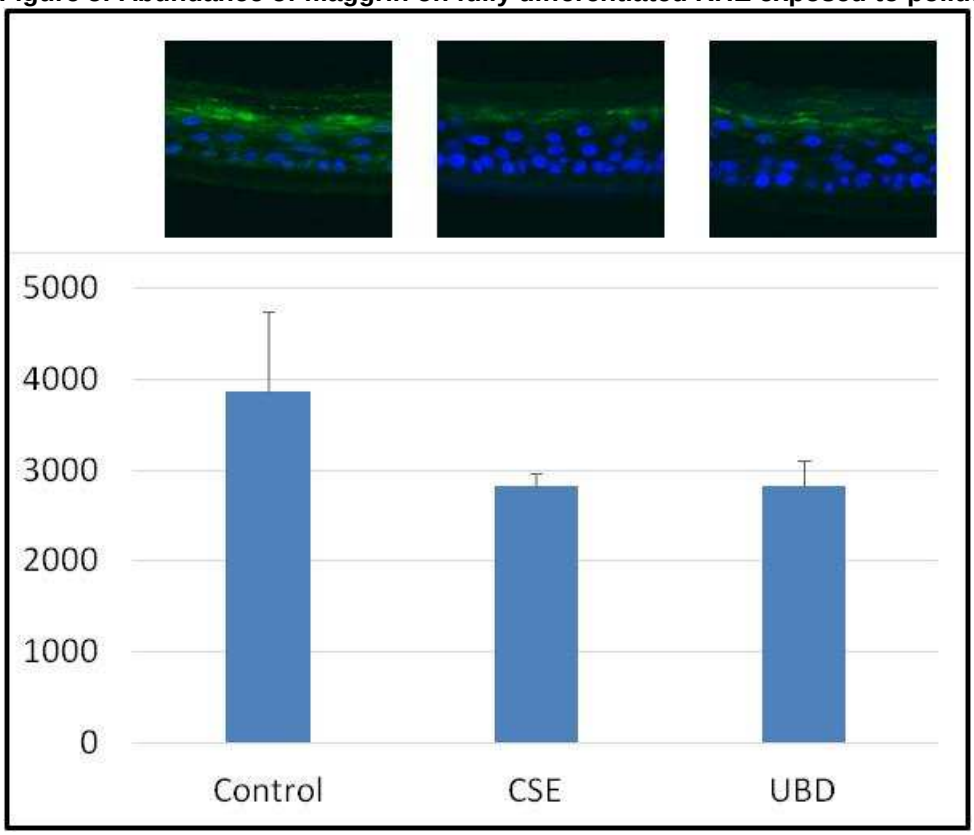

Figure 5: RHE were exposed topically to air pollutants for 48h then processed for immunohisto-chemistry and stained with anti-filaggrin antibody. The abundance of filaggrin (representative pictures on the figure) was quantified and normalized to the surface area and the number of nucleated cells. Significant statistical differences are shown (*) between controls and cells exposed to pollutants.

Surprisingly, further experiments treating the RHE for a longer period from an earlier differentiation stage with CSE induced the opposite effect, showing more filaggrin expression in presence of chronic exposure to CSE, especially in the stratum corneum (Figure 6). Of note, this increase of filaggrin abundance was mainly due to a thicker stratum corneum. Nevertheless, this increase of expression corroborates with previous studies that have shown the potency of cigarette extracts to induce filaggrin expression in keratinocytes (Renò et al. 2011). These observations demonstrate a clear difference between UBD and CSE exposure in terms of biological impact on the skin barrier integrity and differentiation. Nevertheless, exposure to both pollutants leads to similar disruption concerning transepidermal electrical resistance. Further experiments are therefore required to fully understand the different impacts of these pollutants on the skin barrier functions.

Figure 6: Abundance of filaggrin on RHE exposed to pollutants during late differentiation

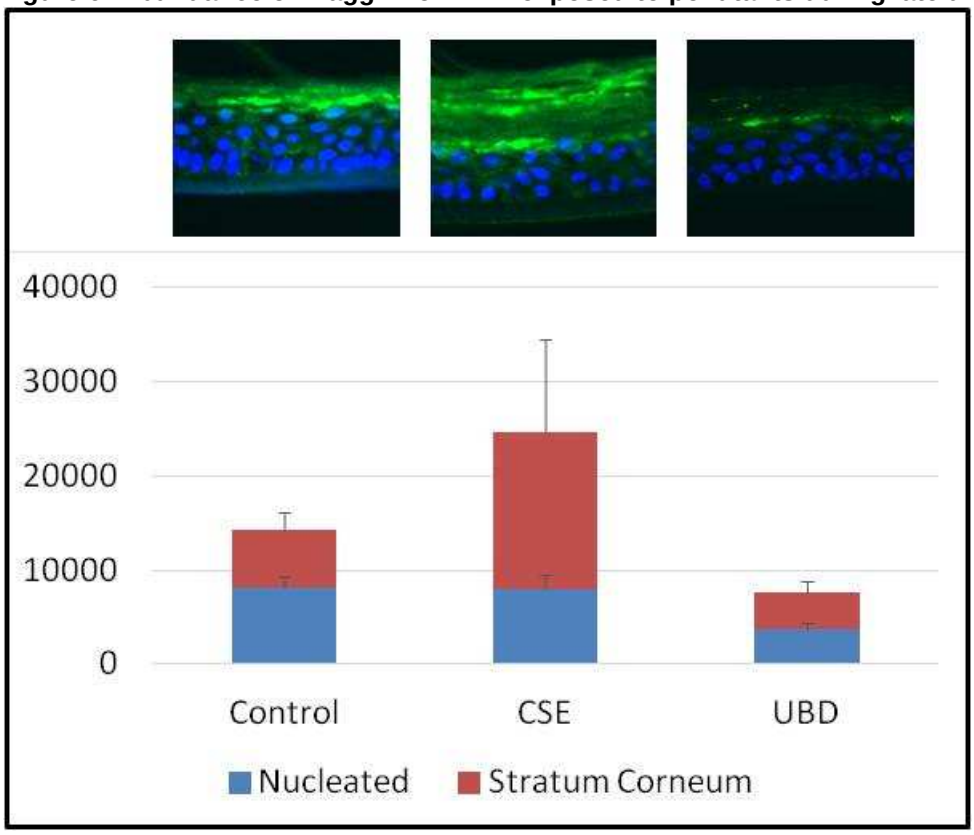

Figure 6: RHE were exposed topically to air pollutants during 4 days during late differentiation then processed for immunohistochemistry and stained with anti-filaggrin antibody. The abundance of filaggrin (representative pictures on the figure) was quantified and normalized to the surface area and the number of nucleated cells. Significant statistical differences are shown (*) between controls and cells exposed to pollutants.

CONCLUSION AND PERSPECTIVES

This study allowed us to characterize different biological responses from keratinocytes and dermal fibroblasts exposed to specific air pollutants. We observed pollutant-specific particularities such as the different impact of CSE exposure during RHE late differentiation or the different responses to carnosic acid and sulforaphane depending on the type of pollutant.

These assays shed light for a better *in vitro* characterization of the effects of air pollutants on skin biology and offer powerful tools for standardized evaluation of protective compounds for skin exposed to air pollution. Our data demonstrated the efficacy of *in vitro* assays to assess the anti-pollution and detoxification effectiveness of investigational compounds. We currently perform these assays on normal human dermal fibroblasts, epidermal keratinocytes, skin

explants, and reconstituted human epidermis and we are in the process of validating and optimizing additional assays to further assess the effects of anti-pollution compounds on the air pollution-induced disruption of the skin barrier function, inflammation and skin aging.

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