IMPACT OF REAL-LIFE OZONE EXPOSURE ON SKIN *IN VITRO* AND *IN VIVO*

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Abstract

Background: Skin is one of the main organs directly exposed to environmental insults. At the ground level, Ozone (O_3) concentration can exceed 0.1 ppm during peaks. Ozone is an extremely reactive molecule and directly interacts with lipids of epidermis, impacting indirectly deeper layers of the skin. Ozone exposure has been correlated with dermatological disorders like atopic dermatitis. The present study focused on evaluating the *in vitro* impact of real-life concentrations of ozone on skin.

Methods: Ozone generator consisted in an ultraviolet lamp generating ozone by photolysis of oxygen from air. Ozone was dispersed in a chamber inside a cell culture incubator. Reconstructed human full-thickness skin model (T-skin™, Episkin SA, Lyon, France) was exposed to various concentrations of ozone (from 0.9 ppm to 0.1 ppm).

Results: Our results showed that real-life ozone exposure conditions induced lipid oxidation and protein carbonylation, without any major impact on skin histology. Inflammatory cytokines like Interleukins 6 and 8 (IL6 and IL8) were found increased after ozone exposure. Surprisingly, Filaggrin expression was differentially modulated after 0.9 ppm or 0.4 ppm of ozone.

Conclusion: Ozone is a worldwide urban concern and its concentration will increase with global climate warming. The current study brings for the first time new insights on impact of real-life ozone exposure conditions on reconstructed full-thickness skin model, that could

explain the increase of atopic dermatitis prevalence observed after ozone peaks. It is then key for consumers (i) to be aware of threats from ozone exposure for skin and (ii) to use daily topical application of specific cosmetical formulations that can protect cutaneous tissues against the impact of ozone exposure.

Keywords: pollution; ozone; full-thickness skin; real-life exposure.

1. Introduction

At the ground level, Ozone (O_3) is a gas produced by the action of ultraviolet radiations on primary pollutants mainly produced by human activities like nitrogen oxides and Volatile Organic Compounds (VOC) (Figure 1). The World Health Organization (WHO) recommends aiming for 8-hour daily maximum not exceeding 100 $\mu g/m^3$ (0.05 ppm) and the peak season mean 8-hour ozone concentration not exceeding 60 $\mu g/m^3$ (0,03 ppm) [1]. Unfortunately, ozone concentration can exceed 0.1 ppm, especially during peaks [2, 3]. Ozone is a very unstable molecule, with a high oxidation potential, that reacts with many materials like biological substrates. Long-term exposure to ground-level ozone is associated with the development of chronic obstructive pulmonary disease (COPD), a progressive and debilitating disease that makes it harder to breathe. Skin is one of the main organs directly exposed to environmental insults, especially pollution and ozone, that constitutes the strongest oxidizing agent in contact with the skin in daily life. This pollutant directly interacts with lipids of epidermal surface layers and especially with unsaturated lipids present in sebum and stratum corneum. This ozonolysis reaction leads to lipid oxidation, generating oxidized molecules, including very reactive aldehydes. Then follows a cascade of biochemical reactions and cellular responses impacting deeper layers of the skin like endogenous antioxidant depletion, protein carbonylation, cellular stress and inflammation [4-10]. Ozone exposure has been correlated with disruption of skin integrity and dermatological disorders like atopic dermatitis [11-15], but no causal link has been showed yet under real-life ozone exposure. To address this issue, the present study focused on evaluating the *in vitro* impact of real-life concentrations of ozone on skin.

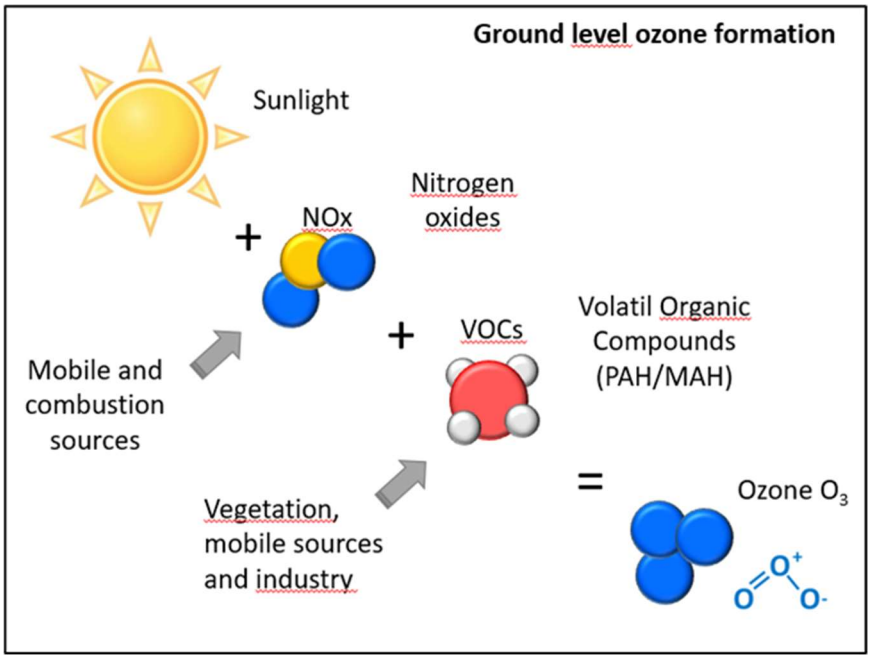

Figure 1. Formation of tropospheric ozone

2. Material and methods

2.1. Culture of reconstructed full-thickness skin model

Reconstructed human full-thickness skin model T-Skin™ (Episkin SA, Lyon, France) were shipped after 11 days of culture (D11, SKINJ11), receipt at D12 and cultured according to the manufacturer's instructions.

2.2. Topical reconstructed skin exposure to ozone (O_3)

Ozone exposure of reconstructed skin model was performed thanks to a system combining a cell culture incubator and an ozone generator (Figures 2B, 2C). This system allowed a controlled ozone generation, a homogeneous gaz dispersion and a reduction of ozone interaction with materials other than reconstructed skin model, while maintaining optimal skin culture conditions (Figure 2A). In this system, T-skin™ model culture conditions were optimized (Figure 2A). Ozone was generated with the 49i UV photometric O_3 Analyzer (Thermo Scientific, Mégatec), using a UV lamp photolyzing oxygen from air (Figure 2B). T-skin™ were exposed to various O_3 concentrations (0.9 ppm, 0.4 ppm or 0.1 ppm) for 6 or 18 hours (2 or 3 repeated exposures). Subsequently, the medium was daily replaced with fresh maintenance medium. Temperature, CO_2 percentage and humidity were monitored during exposures (37°C, 5% and 95%, respectively). T-Skin™ were exposed to O_3 between D18 and D21 and samples were collected at D22 (Figure 3).

2.3. Histological analyses

Morphologies of the skin equivalent were analyzed using hematoxylin, eosin and saffron (HES) staining.

2.4. Quantification of 8-isoprostane marker

8-isoprostane was measured in culture medium collected at D22, with the 8-isoprostane ELISA kit (Cayman Chemical). The ELISA was performed according to the manufacturer's instructions. The 8-isoprostane standards included in the ELISA kit were treated in the same way as the samples to obtain a calibration curve, which was used to estimate the 8-isoprostane levels in the samples.

2.5. Carbonylated proteins absolute quantification (Carbonyl Score)

Epidermis samples were homogenized using a lysis buffer and clarified by centrifugation. Total protein quantification was performed with the Bradford protein assay. Carbonylated proteins were labelled with a fluorescent probe (Ex = 480 nm / Em = 530 nm) functionalized with an amino-oxy group and separated by SDSPAGE (4-20%). Total proteins were post-stained with Coomassie Blue. Fluorescent scanning was performed using Ettan DIGE (Imager) system. Quantification of carbonylated proteins was performed on digitalized images by densitometric analysis using total protein staining as loading control. Results were expressed as the carbonyl score = carbonylated protein fluorescent signal/total protein fluorescent signal for each sample.

2.6. IL6 and IL8 cytokines concentration measurement

IL6 and IL8 concentrations were measured in the culture medium on day 22 using the R&D Systems kit (LXSAHM 96 tests-kit Luminex plex) according to the manufacturer's instructions with the BioPlex 200 Array Reader BioRad.

2.7. Protein extraction and Western Blot analysis

Upon O_3 exposure, at D22, tissue inserts were rinsed once with PBS. Tissues were transferred to tubes containing 130 µL of RIPA buffer (Sigma R0278), 1 µM de pepstatine (Roche 11 359 053 001) and protease inhibitors (Roche 04 693 159 002). Samples were grinded 2 times for 30 sec at 20 Hz with a Mixer Mill (Retsch MM301) and centrifuged 10 minutes at 10 000 g. Pellets were discarded and supernatants containing proteins were collected. Protein concentration was determined by the method with the Pierce BCA 562 nm kit (Thermo Scientific 23227).

Protein extracts were heated 2 min at 85°C and 8 µg were resolved under reducing conditions by SDS-PAGE on 8–16% NuPAGE Tris-Glycine gels (NOVEX, San Diego, CA). The separated proteins were electroblotted onto nitrocellulose membranes (NOVEX, San Diego, CA), blocked with 5 % d'ECL Prime Blocking Agent (Amersham RPN418) and 0.1% Tween 20 (Sigma) in PBS, and probed with an anti-FLG (Santa-Cruz sc66192) diluted at 1/500. Antibody bound to the membrane was detected with horseradish peroxidase-conjugated goat anti-mouse (Amersham NA931V) diluted at 1/5000. Anti-actin HRP-conjugated (Santa-Cruz Sc47778 HRP) was diluted at au 1/5000. Revelation was performed with ECL prime Western Blotting detect reagent (Amersham RPN2232) according to the manufacturer's instructions

2.8. Statistical analysis

One-way analysis of variance (ANOVA Dunnett corrected) or Wilcoxon tests were used for each of the tested variables. For ANOVA test, results were considered significant with a P-value<0.05. Data are expressed as mean ± S.D. of triplicate determinations obtained in 3 independent experiments. For Wilconxon test, results were considered significant with an Effect size >2. This parameter is similar to P-value but take into account the small samples size.

3. Results

3.1. Development of a system allowing ozone exposure of reconstructed skin model in real-life conditions

Literature reports many studies showing the impact of ozone on the skin. These studies are either based on models exposed to ozone generated by an electrical corona arc discharge in concentration ranges higher than realistic conditions, or through clinical or epidemiological studies on groups of people living in areas with various levels of pollutants including ozone. But in the latter case, ozone is not the only player of exposome, and other factors like UV, primary pollutants or lifestyle have to be taken into consideration. The current study presents a straightforward, convenient, and convincing *in vitro* ozone exposure system on reconstructed skin. To control the generation and distribution of ozone, an exposure chamber inside a cell culture incubator was developed (Figure 2C). Its configuration was designed to allow a uniform and controlled ozone diffusion flow on several biological samples, while providing optimal culture conditions (Figure 2A). The ozone generator consisted in an ultraviolet lamp generating ozone by photolysis of oxygen from air. This device was both an ozone generator and analyzer,

capable of ppb range measurement. It allowed a precise and controlled delivery of ozone concentration inside the chamber, even with real-life ozone concentrations (Figure 2B).

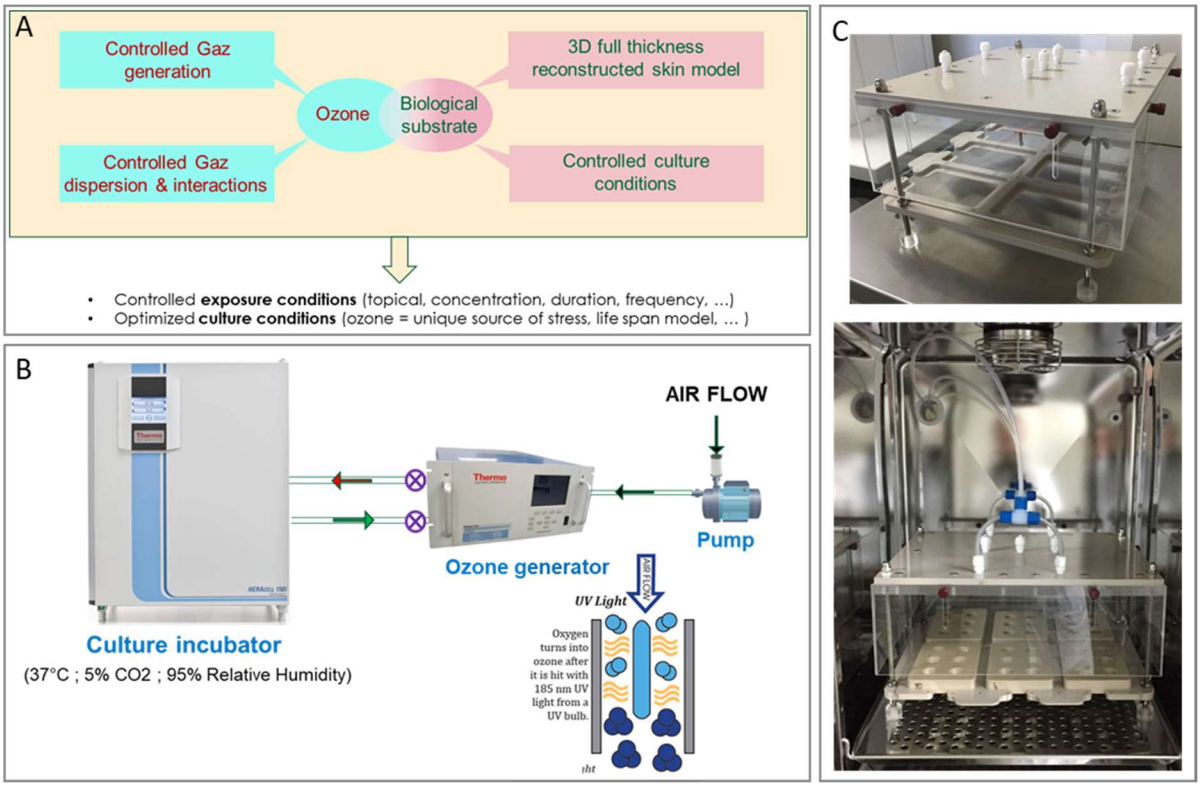

Figure 2. **Ozone exposure system on the reconstructed human full-thickness skin model T-skin™.** (A) Critical points to control on ozone generation and exposure and on biological substrates. (B) Schematic view of the system with cell culture incubator, ozone generator and analyzer and pump. (C) Ozone exposure chamber on the bench and in the cell culture incubator.

3.2. Ozone exposure alters morphological properties in a full-thickness reconstructed skin model

In order to study the impact of various range of ozone concentrations, three concentrations were chosen according to previous results published in the literature [5, 9] and real-life concentrations that can occur during ozone peak season (0.1 ppm) [2, 3]. Repeated ozone exposures were chosen instead of a single exposure to mimic a chronic exposure. Full-thickness reconstructed skin model T-skin™ was exposed 2 or 3 times with 0.9 ppm, 0.4 ppm or 0.1 ppm either for 6 hours (0.9 ppm) or 18 hours (0.4 ppm and 0.1 ppm) (Figure 3). Histological analyses showed that repeated ozone exposures, at the three concentrations tested, had a low impact on skin (Figure 4). Major morphological modifications were observed at 0.9 ppm with a decrease of epidermal thickness, a less organized basal layer and a less differentiated model with a decrease of the granular layer thickness. Depending on the T-skin™ batch, a parakeratosis could be observed (Figure 4A, 4B).

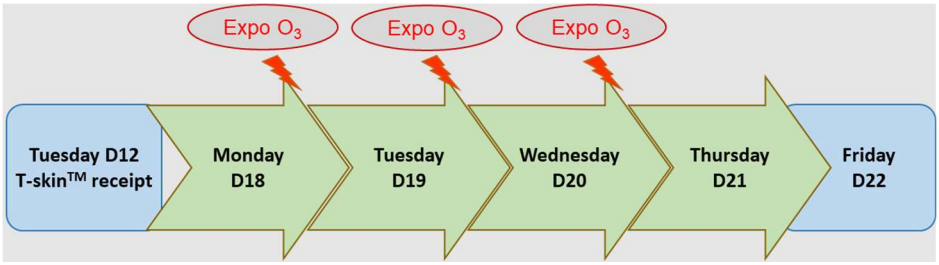

Figure 3. Ozone exposure protocol on the reconstructed human full-thickness skin model T-skin™.

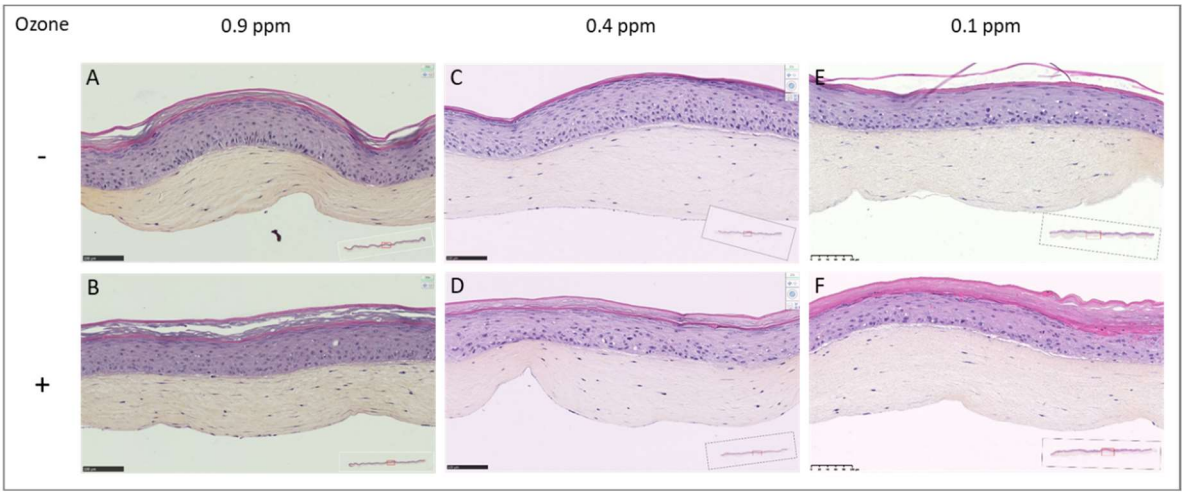

Figure 4. **Histological analysis of reconstructed skin exposed or not to ozone at 0.9 ppm, 0.4 ppm and 0.1 ppm**. (A, C, E) HES staining of control tissues not exposed to ozone. (B, D, F) HES staining of tissues exposed respectively to 0.9 ppm, 0.4 ppm and 0.1 ppm of ozone.

3.3. Real-life ozone exposure alters lipid oxidation and protein carbonylation in a full-thickness reconstructed skin model

To better elucidate the mechanisms involved in skin in response to ozone exposure, lipid oxidation and protein carbonylation were studied. To quantify lipid oxidation, 8-isoprostane marker released into culture medium was measured. Ozone exposure showed an increase of the level of 8-isoprostane marker, independently of the ozone concentration used (Figure 5A, 5B, 5C). Experiment with 0.1 ppm ozone concentration must be confirmed with two other independent experiments.

To measure protein carbonylation on epidermis, a carbonyl score was calculated. This method allows to quantify all the carbonylated proteins, resulting either from direct or indirect oxidation. Ozone exposure showed an increase of the level carbonyl score, independently of the ozone concentration used (Figure 5D, 5E, 5F). At 0.9 ppm, the increase was not significant: one explanation could be that the samples dispersion observed may be due to a higher impact of

ozone compared to lower ozone concentrations, like in histological analysis. Moreover, keratinocytes used for these experiments were obtained from two different donors. Experiment with 0.1 ppm ozone concentration must be confirmed with two other independent experiments.

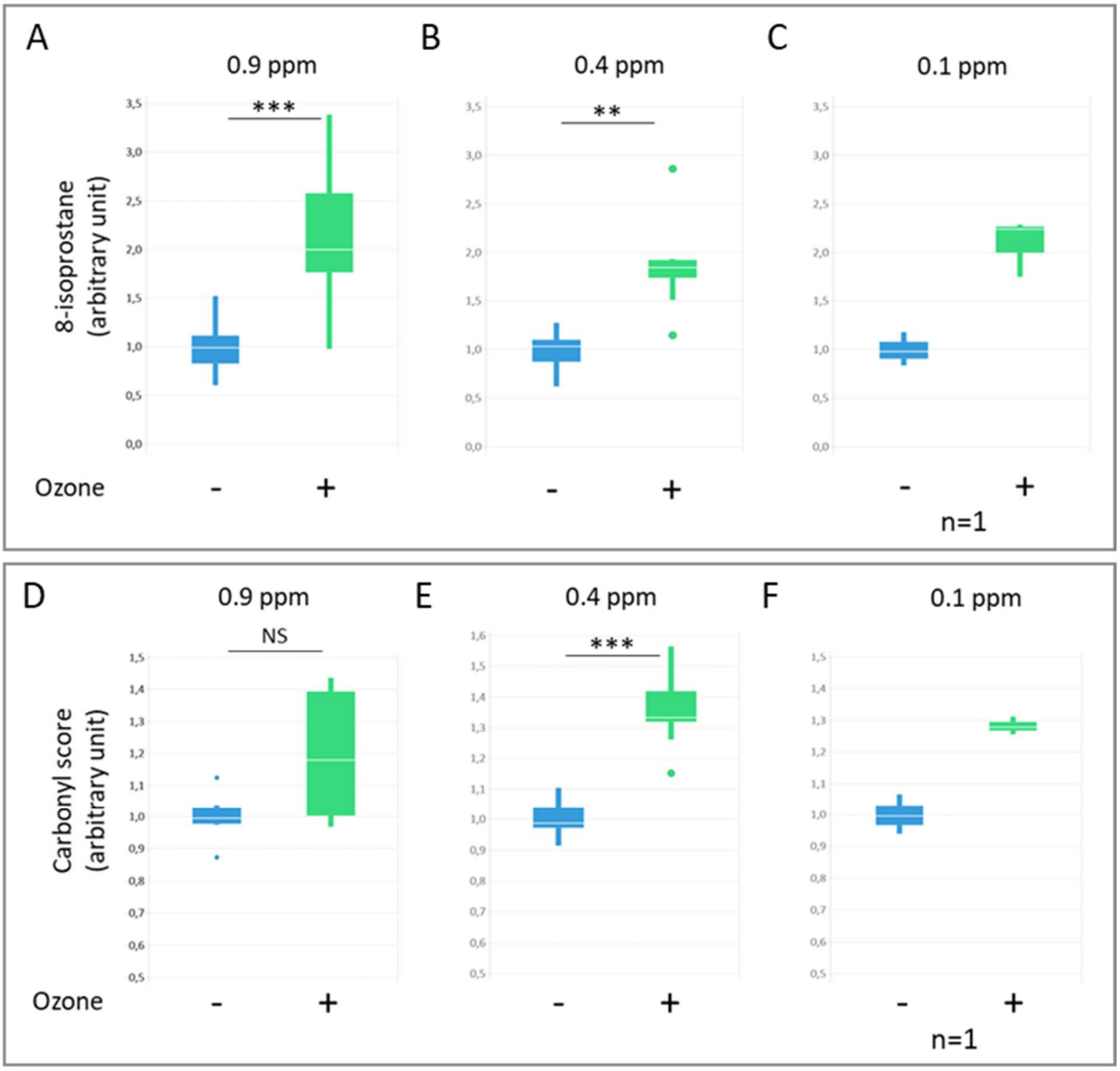

Figure 5. **Analysis of lipid oxidation and protein carbonylation on reconstructed skin exposed or not to ozone at 0.9 ppm, 0.4 ppm and 0.1 ppm.** (A, B, C) Lipid oxidation was quantified with 8-isoprostane marker. Results are expressed in arbitrary unit due to the normalization of samples compared to each control (D, E, F) Carbonylated proteins were quantified and results were expressed as the carbonyl score = carbonylated protein fluorescent signal/total protein fluorescent signal for each sample. Results are expressed in arbitrary unit due to the normalization of samples compared to each control.

NS = Not Significant, * P<0.05, ** P <0.01, *** P <0,001

3.4.Real-life ozone exposure induces inflammation

Literature reports an increase of inflammation after ozone exposure [6, 7, 10]. Inflammatory cytokines secretion was then quantified. Ozone exposure showed an increase of Interleukins 6 and 8 (IL6 and IL8) secretion in the culture medium, at 0.4 ppm (Figure 6A, 6C) and 0.1 ppm (Figure 6B, 6D). Experiment with 0.1 ppm ozone concentration must be confirmed with two other independent experiments.

3.5. Real-life ozone exposure alters terminal differentiation markers linked with atopic dermatitis

Several publications in the literature report a correlation between increased concentrations of ozone and increase of dermatology consults for various dermatological disorders, like urticaria, atopic dermatitis, rash or eruption and infections. Moreover, some authors point out a particular correlation between ozone exposure and higher rates of atopic dermatitis, especially in children [13-15]. As Filaggrin is either mutated or its expression decreased in patients with atopic dermatitis, Filaggrin expression was quantified by Western Blot in reconstructed skins exposed to 0.9 ppm or 0.4 ppm of ozone. Surprisingly, 0.9 ppm of ozone showed an increase of Filaggrin expression protein (Figure 6E, 6F), while 0.4 ppm of ozone showed a decrease of Filaggrin expression protein (Figure 6G, 6H). Similarly, histological analysis of reconstructed skins exposed to 0.9 ppm showed a more important impact on terminal differentiation than 0.4 ppm. In order to better elucidate the mechanisms involved in the effect of ozone on Filaggrin expression, the maturation process of Filaggrin should be studied in more detail.

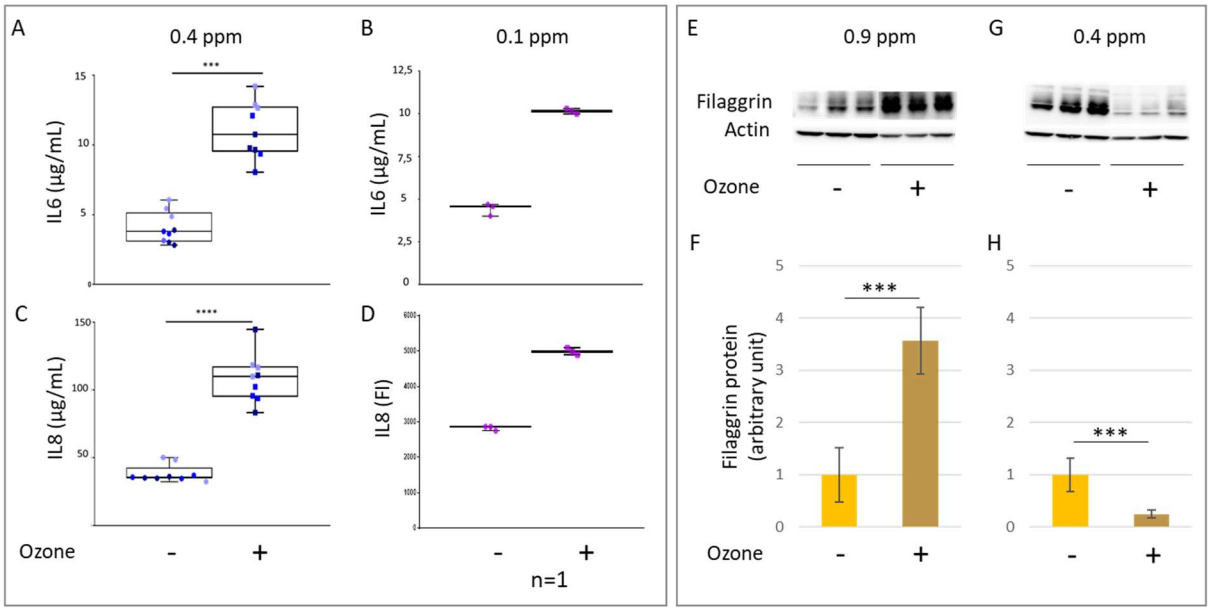

Figure 6. Analysis of inflammation and epidermal differentiation markers on reconstructed skin exposed or not to ozone at 0.9 ppm, 0.4 ppm and 0.1 ppm. (A, B) Interleukin 6 (IL6) was quantified by Luminex in the culture medium of reconstructed skins after exposure or not to 0.4 ppm or 0.1 ppm of ozone. (C, D) Interleukin 8 (IL8) was quantified by Luminex in the culture medium of reconstructed skins after exposure or not to 0.4 ppm or 0.1 ppm of ozone. FI = Fluorescence Intensity. (E, F) Western blot analysis (E) of Filaggrin protein expression levels on reconstructed skins after exposure or not to 0.9 ppm of ozone and quantification (F). Results are expressed in arbitrary unit due to the normalization of samples compared to the control (G, H) Western blot analysis (G) of Filaggrin protein expression levels on reconstructed skins after exposure or not to 0.4 ppm of ozone and quantification (H). * P<0.05, ** P <0.01, *** P <0,001, **** P<0.0001

4. Discussion

Ozone is a worldwide urban concern and it has been established that ozone concentration has been increasing since last years in many countries and will continue to increase with global climate warming [3]. Maximal health thresholds recommended by the World Health Organization (100 µg/m^3 (0.05 ppm) for 8-hour daily maximum and 60 µg/m^3 (0,03 ppm) for the peak season mean 8-hour ozone concentration) [1] are widely exceeded in a large part of the world [2, 3].

It has been well established that long-term exposure to ground-level ozone is associated with the development of pulmonary diseases. As skin is directly exposed to environmental insults, especially pollution and ozone, and as ozone constitutes the strongest oxidizing agent in contact with the skin in daily life, it is of particular interest to study the impact of ozone on the skin. And a crucial point is to evaluate this impact with real-life conditions of ozone exposure in order to be as close as possible to *in vivo* conditions. As a matter of fact,

ozone exposure has been correlated with disruption of skin integrity and dermatological disorders like atopic dermatitis [13-15], but no causal link has been showed yet under real-life ozone exposure. The challenge of this study was to design a system of ozone exposure of reconstructed skins, managing controlled conditions of generation, dispersion and interaction with materials of ozone and optimized reconstructed skin culture conditions (temperature, CO_2 percentage and pH, humidity). The exposure system presented in this study (chamber inside a cell culture incubator and ozone generator and analyzer) (Figure 2) allowed to fulfill all these parameters and to expose reconstructed skins with low concentrations of ozone, with repeated exposures and during several-hour periods. The reconstructed human full-thickness skin model T-Skin™ was chosen in order to be able to study the impact of ozone in epidermis and dermis. Moreover, the protocol used was designed with various ozone concentrations, from 0.9 ppm to 0.1 ppm. This latter concentration is regularly reached even not exceeded in a large part of the world, particularly during ozone peaks [2, 3]. Two or three repeated exposures were performed in order to mimic a chronic exposure and to be as close as possible to *in vivo* conditions. This protocol performed during several days allowed ozone to react with epidermal surface layers and skin cells to implement all the biochemical reactions and biological mechanisms in deeper cell layers.

The current study brings for the first time new insights on impact of real-life ozone exposure conditions on skin. The results obtained in our study showed, on the one hand, that realistic concentrations of ozone induced the same effect as higher concentrations on lipid oxidation, protein carbonylation and inflammation, on epidermal surface and in the deeper layers of the skin. On the other hand, other parameters like histological analysis or Filaggrin expression showed a modulation depending on ozone concentration. This latter point highlights the interest to study realistic concentrations of ozone, in order to be as close as possible to *in vivo* conditions of ozone exposure. Another critical point is that repeated exposures showed a global exacerbated impact compared with a single exposure (data not shown). Results on Filaggrin expression were of particular interest. Compensation mechanisms could explain these discrepancies between exposures at 0.9 ppm and 0.4 ppm. The decrease of Filaggrin after 0.4 ppm of ozone could explain the increase of atopic dermatitis prevalence observed after ozone peaks [13-15], as Filaggrin is either mutated or its expression decreased in patients with atopic dermatitis.

5. Conclusion

The current study presents a straightforward, convenient, and convincing *in vitro* ozone exposure system on the reconstructed human full-thickness skin model T-skin™, allowing to be as close as possible as *in vivo* ozone exposure conditions. Our results showed that real-life ozone exposure conditions induced lipid oxidation and protein carbonylation, without any major impact on skin histology. Inflammatory cytokines like Interleukins 6 and 8 (IL6 and IL8) were found increased after ozone exposure. Surprisingly, Filaggrin expression was differentially modulated after 0.9 ppm or 0.4 ppm of ozone and could explain the increase of atopic dermatitis prevalence observed after ozone peaks. It is then key for consumers (i) to be aware of threats from ground-level ozone exposure for health but also for skin and (ii) to use daily topical application of specific cosmetical formulations that can protect cutaneous tissues against the impact of ozone exposure.

Conflict of Interest Statement. NONE.

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