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" Impact of Ozon exposure on the skin: new method to monitor in-vivo oxidative damages related to skin barrier alteration"

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Abstract

The increased ozone exposure is an environmental concern, which in certain urban centers is exceeding safe levels. Ozone is among the six categories of pollution, according to EPA (Environmental Protection Agency), responsible for skin alterations related to premature skin aging and inflammatory processes. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are responsible for oxidative damage in the skin which may lead to skin barrier disruption. Understanding these effects and how the skin can recover from those damages is necessary for the further development of methods to protect the skin against environmental pollution. In this study, we use *in-vivo* vibrational spectroscopic systems (FTIR and Confocal Raman) to investigate the effects of ozone on barrier function and permeation of the skin. These techniques could be used in the future to support anti-pollution claims for innovative cosmetic formulations.

In-vivo vibrational spectroscopy (Raman and FTIR) was used to evaluate the molecular and structural alterations related to ozone exposure throughout the whole stratum corneum and the viable epidermis layers and, how those damages can impact the skin barrier function. Those results were correlated to transepidermal water loss (TEWL) measurements. After ozone exposure we observed alterations in the lipid organization inside the stratum corneum and oxidative damages in the whole stratum corneum. Vibrational spectroscopy was used to evaluate these skin damages but also used to investigate the natural skin recovery process or related to topical application of skin products.

The constant exposure to external aggressors, as UV radiation, ozone, particle matter, is a growing concern for public health as well as for the cosmetic industry. Both are working to understand the complexity of these skin alterations with the aim of developing strategies to prevent or restore these skin damages. The findings of this study are of great value to support the development of cosmetic products with this purpose. Indeed, the methods used in this study can be used clinically to evaluate skin alterations and therefore can be used to support anti-pollution claims.

1. Introduction

The increased ozone exposure is an environmental concern, which in certain urban centers can exceed safe levels. Ozone is among the six categories of pollution, according to EPA (Environmental Protection Agency), responsible for skin alterations related to premature skin aging and inflammatory processes. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are responsible for oxidative damage in the skin which may lead to skin barrier

disruption [1], [2]. Understanding these effects and how the skin can recover from those damages is necessary for the further development of methods to protect the skin against environmental pollution [3].

Data from World Health Organization (WHO) estimates that 91% of the world's population live in areas with poor air quality, which can compromise their health. Not only respiratory alterations are related to the air quality, but also the skin can present several alterations related to excessive exposure to pollution [4], [5]. Among those, skin aging, skin pigmentation as well as inflammatory alterations are described in the literature to be related to pollution [2], [6], [7].

It is well known that the skin is the largest organ in the human body and presents as a barrier against the external effects, thus it is very important to understand how air pollution can interact with the skin, what are the alterations that are present after exposure to pollution and how can those alterations be prevented [8], [9]. The lipids present in the stratum corneum when exposed to pollution can present alterations in its conformation which leads to a disruption on the skin barrier and consequently increase the permeability of undesirable components [4].

One of the major components of air pollution is ozone – O3, a very potent oxidant that can lead to oxidative damages in the skin lipids and consequently disruption of the skin organized structure. Studies have shown that the skin component most prone to present oxidative effects after interaction with ozone is squalene, which forms squalene peroxides and consequently leads to inflammatory effects on the skin and further cause several skin disorders [1], [10]. Those oxidized lipids can be used as biomarkers for the evaluation of the effects of ozone on the skin and facilitate the study of the consequences of these interactions[11].

Spectroscopic methods are great tools to evaluate skin structural alterations related to ozone exposure as well as to evaluate the protective effect of specific formulations designed to support anti-pollution claims [11].

In the present study, our aim was to evaluate the skin after exposure to a controlled dose of ozone using spectroscopic measurements *in vivo* to correlate the data with our previous results recorded ex-vivo. The final objective being to develop suitable spectroscopic methods to clinically evaluate the damages related to pollution exposure and the application of those methods to test skin products efficacy to prevent or restore those alterations.

2. Materials and Methods

Ozone exposure:

Under a fume hood, ozone was generated using corona discharge (MP-3000 Multi-Purpose Ozone Generator, A2Z Ozone) with an output of 3g/h. A custom cap was positioned on top of the forearm skin area. The cap presents an input and output tube, in which the air or ozone can flow from the generator, over the skin. The skin areas were exposed to ozone for different time periods (10-30 minutes).

Confocal Raman measurements

For this study, forearm areas (2cm in diameter) were selected to evaluate *in-vivo* the impact of ozone exposure on human skin. For each condition a series of 3 Raman spectroscopic lines were obtained confocally for depth scans into the skin at 5-micron steps for 20

microns deep. Confocal Raman spectra were recorded using a SkinProbe system from Horiba. A confocal Raman microscope equipped with a 660 nm laser.
The Spectral parameters used to scan the skin samples were:
Laser frequency: 660 nm
Laser power: 100 mW
Acquisition time: 1 exposure at 5 seconds/spectrum
Spectral range: 4000 – 400 cm^{-1}

In vivo ATR-FTIR spectroscopic measurements

The skin was evaluated *in vivo* by ATR-FTIR spectroscopic measurements using the REMSPEC® equipment, to evaluate the molecular and structural alterations on the stratum corneum region after exposure to Ozone. For this purpose, 5 measurements were conducted on the panelist's arm region before and after ozone exposure.
The ATR-FTIR spectra was recorded in the mid-IR region range from 4000 to 950 cm-1 with a spectral resolution of 8 cm^{-1} and 128 scans accumulation.

Spectroscopy analysis

All the spectra as well as the hyperspectral images were analyzed using Isys Chemical Imaging analysis 5.0 software (Malvern Instruments Limited, Malvern Works, UK).
For statistical analysis the pixel values were extracted from the hyperspectral images and ANOVA test was applied using GraphPad Prism 8 software (San Diego, California, United States)

3. Results

In vivo skin evaluation using different spectroscopic techniques was performed, in order to understand the molecular and structural alterations that can occur in the stratum corneum after the ozone exposure and if the skin can recover from those damages. By ATR-FTIR spectroscopy, only the skin surface was evaluated and the markers used to evaluate the oxidative damages were the bands at 1278 cm^{-1} and 1716 cm^{-1} (Figure 1). After 15 minutes of ozone exposure, oxidative damages are already detected, being more pronounced after 30 minutes. The skin recovery was also monitored. The recovery process was observed 3 hours after exposure, with a return to the initial conditions after 24 hours.

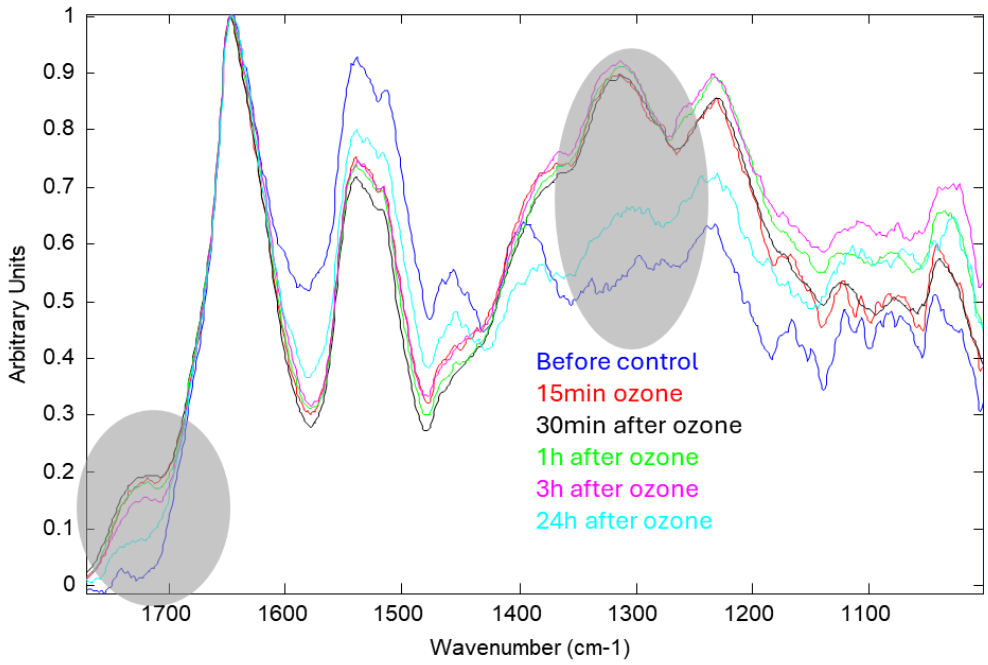

Figure 1: In vivo results showed that after short ozone exposure (15 min), oxidative damages can be detected. The oxidative damages remain few hours after ozone exposure and after 24 hours the skin recovers to baseline level.

To evaluate the depth profile of these oxidative damages inside the skin, *in vivo* measurements were recorded using a Confocal Raman system developed by HORIBA. This equipment is a non-invasive method that can monitor those alterations inside the stratum corneum and beyond in viable epidermis, being a complementary evaluation of the one obtained at the skin surface by ATR-FTIR spectroscopy. After exposure to ozone, a sharp band at 1044 cm^{-1} is observed which was not present in the control sample. This band indicates lipid oxidation damages. These damages evaluated on the skin depth profile were detected up to 10 µm deep into the skin.

4. Discussion

Pollution exposure as UV radiation, ozone, particle matter, among others, on a daily basis is inevitable. The oxidative damage in the stratum corneum lipids significantly compromise the skin barrier function and in consequence allows the skin permeation of undesirable molecules or particles [5], [12].

The findings of this research allowed to validate spectroscopic markers for oxidation products after ozone exposure using both IR and Raman. These markers were used to investigate the oxidative damage on the skin surface but also inside the skin using Confocal Raman, as well as the natural recovery of these oxidative damages, using *in vivo* ATR-FTIR spectroscopy.

The confocal Raman experiments showed that the oxidative damages occurred not only at the surface, but also deeper inside the skin. Indeed, the alterations were detected up to 10 µm below the skin surface. The stratum corneum presents a highly organized composition and structure, which confers its function of protecting the skin against external aggressors.

Understanding changes in the stratum corneum lipid organization is of great importance to understand the changes in the skin barrier function related to pollution exposure [13]. In this context, the alterations observed up to 10 µm can impact the skin barrier and consequently allows the penetration of undesirable molecules.

The IR markers illustrate the distribution of oxidation damage on the skin surface, as an effect from the ozone exposure. Previous *ex-vivo* experiments performed in our group using ATR-FTIR imaging spectroscopy combined to tape stripping method highlighted the depth profile of these oxidation damages, with oxidative damages observed inside the whole startum conreum and not only at the surafce. The same IR markers were also applied to *in vivo* measurements on forearm which were used to characterize the oxidation products on the skin [11].

In our *in vivo* study it was possible to observe the appearance of oxidative damages quickly, just after 15 minutes of exposure, being more pronounced after 30 minutes. Even thought it was possible to observe skin recovery, this process is not as quick as the damaging process, with the skin returning to initial conditions only after 24h. It is known that tropospheric ozone (O3) is a very powerful oxidant in the air and that concentration of ozone has raised in the past years [4].

Our results undoubtedly indicates that spectroscopic methods are relevant tools to evaluate skin damages related to pollutant exposure. The next step will be to develop protocols to test skin products that can prevent or restore the pollution effects on the skin, mostly related to ozone exposure products as ROS and RNS. Understanding how those alterations occurs and if there are better methods to prevent them, can facilitate the development of safe and effective skin products to improve the skin conditions and maintain the skin integrity.

5. Conclusion

Our study made possible to comprehend the complexity of the molecular and structural alterations related to oxidative damage induced by ozone exposure, which further can lead to a better approach to their prevention. The use of antioxidant substances in cosmetic products is already well stablished, so the study of the application of these substances can present several benefits. This can lead to the development of products that can contribute to the dermatological field in the prevention of several inflammatory conditions and in the maintenance of the skin in a healthier state. Our main objective being to develop suitable spectroscopic methods to evaluate clinically the damages related to pollution exposure and use these methods to tests efficacy of skin products to prevent or restore those alterations.

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