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## **Bench approaches to study the detrimental cutaneous impact of tropospheric ozone**

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**Running title:** Experimental approaches to study the effects of ozone on skin health

## **Abstract**

Being exposed to ground-level ozone (O<sub>3</sub>), as it is often the case in polluted cities, is known to have a detrimental impact on skin. O<sub>3</sub> induces antioxidant depletion and lipid peroxidation in the upper skin layers and this effect has repercussions on deeper cellular layers, triggering a cascade of cellular stress and inflammatory responses. Repetitive exposure to high levels of O<sub>3</sub> may lead to chronic damages of the cutaneous tissue, cause premature skin aging and aggravate skin diseases such as contact dermatitis and urticaria. This review paper debates about the most relevant experimental approaches that must be considered to gather deeper insights about the complex biological processes that are activated when the skin is exposed to O<sub>3</sub>. Having a better understanding of O<sub>3</sub> effects on skin barrier properties and stress responses could help the whole dermato-cosmetic industry to design innovative protective solutions and develop specific cosmetic regime to protect the skin of every citizen, especially those living in areas where exposure to high levels of O<sub>3</sub> is of concern to human health.

**Keywords:** environmental pollution, skin, 3D skin tissue model, oxidative stress, skin protection.

## **1. Introduction**

### ***1.1 A protective though vulnerable barrier between the body and the surrounding environment***

The skin is the largest organ in the human body. Its outer layer, the epidermis, is a continuously renewing tissue mainly composed of keratinocytes<sup>1</sup>. Keratinocytes arise from the basal layer of the epidermis and migrate to the upper epidermal layers by changing their structure and composition until they differentiate into corneocytes in the stratum corneum (SC)<sup>2</sup>.

Below the epidermis, the dermis represents the thickest skin layer. It contains an extensive vessel network and it is mainly composed of fibroblasts that secrete collagen and elastin that increase skin mechanical strength and elasticity. Below the dermis, the hypodermis consists of mostly adipose tissue. It provides insulation and serves as an energy storage area<sup>2</sup>.

The SC is the outermost layer of the skin. It is predominantly composed of corneocytes that are surrounded by a crystalline lamellar lipid matrix composed of ceramides, fatty acids and cholesterol. The corneocytes membrane is additionally surrounded by a protein envelope, the so-called cornified envelope, which is mainly made of loricrin, involucrin and filaggrin<sup>3</sup>. Corneocytes are interconnected by transmembrane glycoproteins which bind each other on adjacent corneocytes and ensure SC integrity.

The barrier function of the SC is further ensured by other factors such a low surface pH, the colonization of the skin surface by bacterial flora and the antioxidant capacity of the skin's outermost layer<sup>1,4</sup>.

Although SC is the major contributor to the barrier function of the skin, it is continuously exposed to both natural environmental stress factors and pollutants of

anthropic origin. The ways those pollutants interact with skin and induce cutaneous damages are still not fully understood, especially since our skin is not exposed to only one type of pollutant at a time, but to a complex mixture of chemical entities that evolves with time and with our living habits. The major mechanism by which O<sub>3</sub> insults exert a detrimental effect on the skin is through the generation of oxidative stress. It has been reported that O<sub>3</sub> can cause lipid and protein oxidation especially in the outer layers of the SC<sup>5-7</sup>.

### ***1.2 Properties and effects of ozone on human health***

Ozone is an inorganic polar molecule consisting of three oxygen atoms (48 Da). The O<sub>3</sub> molecule is naturally present in the atmosphere. About 90% of the O<sub>3</sub> resides in the stratosphere at a concentration of approximately 10 parts per million (ppm). It forms a protective layer that absorbs the UV radiation coming from the sun<sup>8</sup>.

In the troposphere (ground-level), O<sub>3</sub> is formed by chemical reactions between oxides of nitrogen (NO<sub>x</sub>), volatile organic compounds (VOC), and other radicals (produced mainly from automobile traffic and industrial activities), O<sub>2</sub> and sunlight. The presence of nitric oxide (NO), also emitted from vehicles, contributes to O<sub>3</sub> depletion<sup>9</sup>. For this reason, high traffic areas often show lower O<sub>3</sub> levels compared to the sub-urban and rural areas. Moreover, O<sub>3</sub> concentration can vary from one area to another because of air mass flows that transport O<sub>3</sub> precursors far from their emission points and according to different environmental conditions (i.e., temperature, humidity, latitude and altitude)<sup>7,10,11</sup>. The concentration of O<sub>3</sub> in the troposphere, reported as the maximum hourly mean value, can reach levels up to 0.35 ppm in severe polluted episodes<sup>12</sup>.

O<sub>3</sub> is one of the most powerful known oxidants that quickly reacts with biological targets<sup>13</sup>. Hence, O<sub>3</sub> present in the troposphere is hazardous to all living organisms on

earth<sup>14</sup>. The Ambient Air Quality Directive of the European Parliament and of the Council (2008/50/EC) reports that the maximum daily 8-hour mean threshold to avoid negative health effects is 120  $\mu\text{g}/\text{m}^3$   $\text{O}_3$  (corresponding to  $\sim 0.06$  ppm  $\text{O}_3$ ). The World Health Organization (WHO) set the bar even lower and recommended 0.05 ppm  $\text{O}_3$  as maximum threshold. However, according to the 2019 report of the European Environmental Agency, the percentage of the EU-28 urban population exposed to  $\text{O}_3$  levels exceeding the WHO air quality guidelines value was 96 % in 2017, scarcely showing any fluctuation since 2000<sup>ref.15</sup>. The same trend is also observed for other densely populated regions in the rest of the world where maximum daily mean values up to 0.19 ppm  $\text{O}_3$  were reported<sup>16,17</sup>.

### ***1.3 Mechanism of action of ozone on skin***

Being a highly reactive molecule,  $\text{O}_3$  cannot penetrate human skin. The biological adverse effects induced by  $\text{O}_3$  stem from its ability to cause oxidation of biomolecules by a direct reaction or through the formation of free radicals and reactive intermediates. Biologically active products formed by ozonolysis can act as signalling molecules, penetrate in the lower skin layers and cause further damages<sup>18</sup>. It has already been extensively demonstrated that a prolonged oxidative stress status can lead to chronic inflammation and be responsible for premature skin aging and other several skin diseases such as atopic dermatitis, psoriasis and skin cancer<sup>19–24</sup>. Epidemiological studies suggested that exposure to ambient  $\text{O}_3$  might be related to wrinkle formation<sup>25</sup> and other adverse skin disorders<sup>26</sup> which can be eventually linked to a chronic oxidative imbalance. Skin biomolecules prone to react with  $\text{O}_3$  and reactive intermediates include: SC lipophilic (tocopherols) and hydrophilic (ascorbate, urate, glutathione) antioxidants, sebum constituting unsaturated lipids (e.g. squalene), cholesterol as well as

polyunsaturated fatty acids (PUFAs) present in both cell membranes and SC extracellular lipid matrix. In particular, the ozonation of olefins present in the SC leads to the formation of reactive oxygen species (ROS) and a variety of small and diffusible lipid peroxidation products such as lipoperoxyl radicals, malondialdehyde (MDA), isoprostanes and alkenals<sup>27</sup>. Those lipid peroxidation products trigger skin pro-inflammatory responses and cause additional damage in the epidermal and dermal skin layers. Moreover, the interaction of reactive molecules with skin amino acids and proteins can cause the oxidation of lysine, arginine, proline and threonine residues involved in several skin functions. The toxic effects of O<sub>3</sub> on skin involve thus the formation of free radicals, the formation of lipid peroxidation products, the oxidation of functional groups, the alteration of membrane permeability and the induction of inflammatory responses<sup>9</sup>.

Researchers are currently focusing on understanding the underlying molecular and cellular pathways involved. Targeting those pathways would be a possible way to counteract the detrimental effects of O<sub>3</sub> on skin.

## **2. Towards understanding the effects of ozone on skin**

### ***2.1 Ozone generation and exposure testing***

Three main methods can be used to generate O<sub>3</sub> in an experimental setting: UV radiation (by photochemical oxygen molecules dissociation), electrolysis (by direct current application across two electrodes in an aqueous electrolyte) and lastly corona discharge. The first two are characterized by a low O<sub>3</sub> output and the production is difficult to control, making the corona discharge the most frequent process used for research purposes. In the corona discharge system, O<sub>3</sub> is produced when oxygen passes through an intense energy field created by two electrodes with a large difference of potential

(corona). The energy from the electric discharge causes the oxygen molecules to break into oxygen atoms which, in turn, form the ozone molecule in presence of an excess of oxygen (O<sub>2</sub>)<sup>ref.13</sup>. In an experimental setting, the obtained O<sub>3</sub> mixture (95% O<sub>2</sub>, 5% O<sub>3</sub>) can also be combined with ambient air and allowed to flow into an exposure chamber. The entire exposure system needs to be built using ozone-inert materials such as 316 L stainless steel, Pyrex<sup>®</sup> glass, polytetrafluoroethylene (PTFE) and polyurethane, to avoid the release of any substance that could be produced from O<sub>3</sub> oxidation<sup>13</sup>. During an exposure experiment, it is of paramount importance to continuously monitor O<sub>3</sub> concentration, temperature and relative humidity within the exposure chamber to ensure reproducible results. The concentration of O<sub>3</sub> can be monitored by an O<sub>3</sub> analyser and the mean exposure value adjusted according to the goals of the experiment. O<sub>3</sub> analysers can be divided into two main categories: UV analysers and electrochemical sensors. Both require frequent maintenance and calibration (at least twice a year) to provide accurate measurements. Figure 1 shows a scheme of an experimental set-up that can be used to study the effects of O<sub>3</sub> on skin.

## ***2.2 Experimental approaches: from in vivo to in vitro studies***

To date, numerous *in vivo* and *in vitro* studies were conducted to assess the effects of O<sub>3</sub> exposure on skin (Table 1). All these investigations, revealed that O<sub>3</sub> is responsible for ROS generation, antioxidant depletion, as well as lipid and protein oxidation<sup>7-9,18,28-30</sup>. In addition, the damages induced by O<sub>3</sub> can further initiate an inflammatory response. The first experiments used to investigate the effects of O<sub>3</sub> on skin were mainly conducted *in vivo* with murine skin as well as *in vitro* with two-dimensional (2D) cell cultures<sup>6,31-33</sup>. However, murine skin and 2D cell cultures do not comprehensively mimic human skin features. It is only later, thanks to the *in vitro* engineering of three-

dimensional (3D) skin tissue models representing human skin<sup>34</sup>, that additional studies were performed to further understand the detrimental effects of O<sub>3</sub> on cutaneous tissue. Moreover, the ban of animal testing in the cosmetic industry and the recommendation to follow the 3Rs principle (Replacement, Reduction, and Refinement) in research, further supported the use of alternative skin tissue models<sup>35</sup>.

3D skin tissue models can be divided into two main categories: reconstructed human epidermis (RHE) and full-thickness (FT) models. RHE models closely mimic the morphological, biochemical and physiological properties of the human epidermis, whereas FT models consist of both dermal and epidermal tissue layers. The main advantage of the latter skin tissue model is that it allows one to study the interaction between keratinocytes and dermal fibroblasts, which can affect the mechanical properties and expression of essential skin proteins as well as skin homeostasis<sup>36</sup>.

To build RHE models, normal human epidermal keratinocytes (NHEKs) are seeded into specific trans-wells that separate the wells into two distinct compartments, the apical and basolateral compartments. The medium in the apical compartment can be removed after a few days to achieve the air-liquid interface culture condition to promote cell differentiation. The main difference between RHE and FT models is that in the latter NHEKs are seeded on top of a hydrogel used as the extracellular matrix for the proliferation of normal human dermal fibroblasts (NHDFs).

Both RHE and FT models have structures and functions similar to that of human cutaneous tissue<sup>34,37</sup>. Thus, over the years they became preferred or have even been enforced alternative to animal testing for several agents. For example, such bioengineered 3D skin tissue models are nowadays regularly used in the cosmetic and personal care industry to confirm the lack of irritative or corrosive properties of



cosmetic ingredients as well as finished formulations before they are launched into the market. The Organization for Economic Co-operation and Development (OECD) has already approved several RHE models (EpiDerm™, EpiSkin™ and SkinEthic™) and related test protocols specifically for the evaluation of skin irritancy and corrosivity<sup>38-42</sup>. An advantage of reconstructed 3D skin tissue models is the opportunity to tune the tissues according to the main target of the study. It is indeed possible to integrate additional epidermal cell types such as melanocytes, immune cells (i.e. Langerhans cells) and adipocytes, as well as skin appendages like hair follicles or even microbial communities. Obviously the complexity of the reconstruction process increases as well<sup>34,35,37,41</sup>.

Moreover, the reduced barrier function of those bioengineered 3D skin tissues compared to native human skin and the difficulty of reproducing a proper vascularized network at large scale limit somehow the extrapolation of experimental data to humans. This fact has motivated researchers to conduct *ex vivo* studies on skin biopsies and studies on humans to further gain a better understanding of O<sub>3</sub> effects on skin. The different skin tissue models used to investigate the effects of O<sub>3</sub> on skin are shown in Figure 2.

### ***2.2.1 In vivo murine studies***

As mentioned before, the first studies to investigate O<sub>3</sub> effects on skin were conducted *in vivo* using hairless mouse. The main advantages of using mice, compared to other rodents and animals, are the easy availability and the possibility to use genetic engineering for the study of different pathological features<sup>35</sup>. Murine *in vivo* studies were used to understand the oxidative effects of O<sub>3</sub> on skin and to characterize which skin layers are affected after O<sub>3</sub> exposure. Thiele *et al.* observed a significant depletion

of the antioxidant levels and an increase in MDA formation when murine skin was exposed to acute doses of O<sub>3</sub> (10 ppm for 2h)<sup>43</sup>. They also demonstrated that the decrease of endogenous α-tocopherol was found only in the upper epidermis, whereas the lower layers remained unaltered<sup>32</sup>. Similar studies on hairless mice showed that other endogenous skin antioxidants such as glutathione, uric acid and vitamin C are also sensitive to depletion when exposed to high and acute O<sub>3</sub> concentrations (1 and 10 ppm for 2h)<sup>44,45</sup>. In addition, the O<sub>3</sub> oxidative power within SC on other targets (such as proteins and lipids) was also confirmed<sup>32</sup>. Interestingly, Thiele *et al.* also demonstrated that topical application of vitamin E attenuates the O<sub>3</sub>-induced oxidative damage to cutaneous lipids<sup>43</sup>. They concluded that exogenous vitamin E acts as a first line of defence against acute short-term exposure to O<sub>3</sub> (10 ppm for 2h), reducing to some degree the oxidative damage caused by O<sub>3</sub> to skin lipids and proteins.

Other studies performed *in vivo* with murine skin demonstrated that O<sub>3</sub> not only affects antioxidant levels and oxidation markers in the SC, but also induces stress responses in the living layers of the skin, most likely through indirect mechanisms<sup>46</sup>. For the first time, it was shown that exposing hairless SKH-1 mice to acute levels of O<sub>3</sub> (8 ppm for 2h) produces a cascade of stress-related cellular responses. These include the induction of heat shock proteins (HSPs, in particular HSP27 and HSP70), known to be produced in response to cellular challenges.

The induction of the HSPs was also observed in another study where hairless SKH-1 mice were exposed to a 10-fold lower concentration of O<sub>3</sub> (0.8 ppm for 6h/day during 6 days)<sup>47</sup>.

A repeated exposure to 0.8 ppm of O<sub>3</sub> was also found to induce the expression of pro-inflammatory cytokines, as well as of heme oxygenase 1 (HO-1) and cyclooxygenase 2

(COX-2), as well as the expression of the proliferating cell nuclear antigen (PCNA)<sup>47</sup>. HO-1, also known as HSP32, is a stress protein involved in the protective cellular response against environmental stressors. HO-1 gene expression can be triggered by mitogen-activated protein kinases (MAPKs) or directly regulated by transcription factors, including NF- $\kappa$ B<sup>48</sup>. COX-2 is induced in response to inflammation and, in turn, is responsible for the induction of prostaglandin E2 (PGE2) that regulates epidermal homeostasis and repair<sup>49</sup>. PCNA is known to be involved in DNA replication and repair, and it is induced as stress responses to DNA damage. In the abovementioned study, an increase in keratin 10 (K10) expression was recorded together with PCNA induction, suggesting that O<sub>3</sub> triggers keratinocyte proliferation and differentiation affecting skin biology and structure<sup>47</sup>.

Another study testing a single exposure to a lower O<sub>3</sub> concentration (0.8 ppm for 6h) resulted in a cascade of biological reactions in the living layers of the murine skin, involving the activation of matrix metalloproteinase 9 (MMP-9), an enzyme related to extracellular matrix injury and repair<sup>50</sup>. The observed effect of O<sub>3</sub> on MMPs levels indicated that O<sub>3</sub> could also affect wound healing, accelerate skin ageing and wrinkle formation. However, it is unclear from this study if the expression of MMP-9 was directly triggered by O<sub>3</sub> or if it resulted from the release of pro-inflammatory cytokines (e.g. TNF- $\alpha$ , IL-1 and IL-8). Indeed, the regulation of MMP-9 levels can also be mediated by nuclear factor kappa-light-chain-enhancer of activated B cell (NF- $\kappa$ B), the transcription factor controlling the production of pro-inflammatory cytokines.

Mice were also used to investigate the effect of age on skin responses to pollutants<sup>51,52</sup>. Sticozzi *et al.* exposed young (8-week old) and old (18-month old) mice to O<sub>3</sub> (0.25 ppm for 6h/day during 4 days) and showed a difference in the oxidative stress and

inflammatory marker responses. Old mice had a higher baseline level of oxidative stress markers compared to younger mice. This work further confirmed that O<sub>3</sub> exposure activates NF-κB (as measured by I<sub>κ</sub>-Bα phosphorylation) and enhances the levels of pro-inflammatory interleukins such as IL-6 and IL-8 that are under the control of NF-κB<sup>52</sup>. The activation of NF-κB observed in the study could be a consequence of MAPK activation by peroxidation products. NF-κB is a key regulator in apoptosis signalling pathways, proliferation, and inflammation. Its activation plays a crucial role in the expression of numerous pro-inflammatory and adaptive inflammatory responses<sup>53,54</sup>. Previously, Lim *et al.* also studied the effects of O<sub>3</sub> exposure (0.5 ppm for 6h/day during 9 days) on cutaneous wound healing according to the age of the mice<sup>51</sup>. They found that O<sub>3</sub> significantly delayed wound closure in old mice, whereas in young mice, it had no significant effect. Moreover, a strong association was observed between NF-κB activation, transforming grow factor (TGF-β) signalling and wound repair upon O<sub>3</sub> exposure.

Hairless mice represent one of the most accepted skin tissue to study exogenous effects on skin. However, there are several limitations that need to be taken into account when translating results from murine studies to humans: epidermis and SC are around 4 times thinner, the intercellular lipid composition of the SC is different and the corneocyte surface is lower than in human skin<sup>55</sup>.

### ***2.2.2 Human skin and epidemiologic studies***

Although most of the research carried out to date to study the impact of O<sub>3</sub> on cutaneous tissues involved either cell cultures or murine skin, there have been some studies describing experiments on humans. One of the first studies was conducted by He *et al.*, who tested the effects of high levels of O<sub>3</sub> (0.8 ppm for 2h) on vitamin E depletion and

oxidized lipids production in human SC, which was collected after O<sub>3</sub> exposure by tape stripping and cup-scrub extraction methods<sup>56</sup>. They demonstrated that O<sub>3</sub> causes a 50% reduction in skin microflora, confirming O<sub>3</sub> bactericidal effects. It is worth emphasizing that acute doses of O<sub>3</sub> (e.g. 5-10 ppm for 2h) were required to induce a similar increase in lipid peroxidation markers in mice<sup>43</sup>. This observation likely indicates that human skin is more sensitive to the effects of O<sub>3</sub> than murine skin, probably due to some differences in lipid composition<sup>57</sup>.

To address the paucity of data on human skin, Valacchi *et al.* recently conducted studies with human volunteers to investigate the effects of O<sub>3</sub> exposure (0.8 ppm for 3h/day during 5 days)<sup>58</sup>. Through the analysis of skin biopsies, they found that O<sub>3</sub> exposure increases epidermal levels of 8-iso prostaglandin F2 alpha (8-iso PGF2a) and 4-hydroxy-2-nonenal (4-HNE). Moreover, it induced a significant increase in NF-κB (p65 subunit) activation, as well as in COX-2 and MMP-9 levels.

Even if skin biopsies preserve most of the human skin physiological characteristics, inter-subject variability remains one of the main limitations of studies conducted on human skin. In addition, the possibility of studying the effects of O<sub>3</sub> on diseased skin models will often be limited by ethical concerns and availability issues. More recently, Curpen *et al.* developed a new testing method to assess the oxidative power of O<sub>3</sub>. They analysed the sebum of patients exposed to 0.1 up to 1 ppm of O<sub>3</sub> for 2 hours. Results showed a decrease of squalene and a corresponding increase of MDA compared to unexposed samples. They also indicated an ideal sebum sampling time between 1 and 3 hours after exposure, when the most significant differences were observed<sup>59</sup>.

Lately, it was shown that O<sub>3</sub> exposure is also linked to the inflammasome activation. The inflammasomes are multiprotein complexes that activate an inflammatory caspase

(i.e., caspase-1), and their activation ultimately leads to the processing and release of pro-inflammatory cytokines. Inflammasomes NOD-like receptors 1 and 3 (i.e., NLRP1 and NLRP3) are the main inflammasome complexes present in the skin. Their activation has been linked to several disease including skin diseases including atopic dermatitis and psoriasis<sup>60-63</sup>.

A recent study was conducted on human skin biopsies that were cultured *ex vivo* and exposed to 0.4 ppm of O<sub>3</sub> for 4h. Results showed that O<sub>3</sub>, besides affecting the redox homeostasis of the skin tissues (measured in terms of H<sub>2</sub>O<sub>2</sub> levels and 4-HNE expression), induces inflammasome activation. Both transcript and protein levels of NLRP1 and caspase-1 significantly increased after O<sub>3</sub> exposure. Consequently to the inflammasome activation, also IL-1 $\beta$  and IL-18 expression significantly increased<sup>64</sup>. The contribution of O<sub>3</sub> to skin diseases was already suggested based on epidemiological studies. It was demonstrated an exposure-response relationship between increased O<sub>3</sub> concentration and adverse skin conditions such as urticaria, atopic eczema, contact dermatitis, rash/other nonspecific eruption, and infected skin diseases<sup>26,65</sup>.

Interestingly, following the recent evidence that air pollution contributes to skin aging, Fuks *et al.* investigated the association of long-term exposure to ambient O<sub>3</sub> levels with markers of extrinsic skin aging in Caucasian men and women<sup>25</sup>. They found a positive association of O<sub>3</sub> exceedance days with more coarse wrinkles on the forehead, independently from UV exposure.

### ***2.2.3 In vitro 2D cell cultures studies***

The simplest experimental approach to study the effects of O<sub>3</sub> on skin is to use 2D cell cultures grown in culture plates. Although primary keratinocytes and fibroblasts isolated from new-born or adult donors are the most common cell types used in those 2D

cultures<sup>33,66,67</sup>, immortalized cell lines, such as human adult low calcium high temperature (HaCaT) and DK7-NR human keratinocytes, were also considered in some studies to evaluate the impact of O<sub>3</sub> on cutaneous cellular responses<sup>68</sup>.

McCarthy *et al.* exposed primary human keratinocytes (i.e., NHEKs) to moderate and high concentrations of O<sub>3</sub> (0.4 and 0.8 ppm for 0.5h). They found that 0.8 ppm O<sub>3</sub> exposure can compromise mitochondrial functions, which can ultimately impact NHEK cellular viability<sup>67</sup>. Other studies conducted with keratinocytes monolayers showed changes in the expression of scavenger receptor B1 (SR-B1), a protein involved in cellular cholesterol uptake and of extreme importance for skin homeostasis<sup>69</sup>, and in the expression of aryl hydrocarbon receptor (AhR), a protein involved in cellular detoxification system<sup>33,70</sup>.

Studies involving 2D cell cultures were also carried out to evaluate important biomarkers of O<sub>3</sub>-induced oxidative stress and inflammation<sup>66,68</sup>. For instance, it was demonstrated that cells exposed to more relevant O<sub>3</sub> concentrations (0.1 and 0.2 ppm for 1h) have a dose-dependent increase in p65 subunit nuclear expression which can be used as a marker of NF-κB activation<sup>66</sup>. The same study also showed a significant transient induction of the nuclear factor erythroid 2-related factor 2 (Nrf2) following O<sub>3</sub> exposure. Nrf2 regulates cellular defensive mechanisms (e.g. it induces the production of antioxidant enzymes) and it can control the transcription of the cellular proteasome to eliminate the damaged molecules due to O<sub>3</sub> exposure.

More recently, it was also shown that exposure to moderate O<sub>3</sub> concentrations (0.4 ppm for 1h) induces the activation of the inflammasome complexes in HaCaT cells<sup>64</sup>.

2D cell cultures were used in a significant number of O<sub>3</sub> exposure experiments.

However, they are far from mimicking real conditions. The cells do not have a skin-like

structure and most importantly the outermost SC layer is missing, making them not optimal for many research activities, such as those aiming at evaluating the efficacy of a cosmetic ingredient that is applied topically on skin. In addition, under real-life conditions, the high reactivity of O<sub>3</sub> will hardly allow a direct interaction of this pollutant with living cells. 2D cell cultures become, however, a much more relevant model for studies aiming at investigating the biological effects of lipid oxidation products, which are produced in the SC following the oxidation of PUFAs by O<sub>3</sub>.

#### ***2.2.4 In vitro 3D skin tissues studies***

Early experiments to study the impact of O<sub>3</sub> on cutaneous responses using engineered 3D human skin tissues were done in 1999 by Weber *et al.*<sup>71</sup>. They exposed the EpiDerm™ RHE model to acute O<sub>3</sub> concentrations (5 ppm O<sub>3</sub> for 2h) topically treated with a vitamin E solution. They observed a significant decrease of exogenous vitamin E levels suggesting that specific cellular changes might be induced by a single acute exposure to O<sub>3</sub> in an organotypic skin model. More recently, the same RHE model was also used by Muresan *et al.*, who exposed it to 0.8 ppm O<sub>3</sub> for either 1h or 4h. They showed the capability of O<sub>3</sub> to alter cellular redox homeostasis through the generation of ROS<sup>70</sup>. In another study, the exposure of the EpiDerm™ RHE model to moderate O<sub>3</sub> concentrations (0.4 ppm for 4h) led to increased levels of inflammasome components<sup>64</sup>. This result was in accordance with the observations made in the same study in both HaCaT cells and *ex vivo* skin explants.

The EpiSkin™ RHE model was also considered to study the detrimental effects of O<sub>3</sub> on skin. Cotovio *et al.* showed that acute O<sub>3</sub> concentrations (10 ppm for 1h) induces a significant increase in the content of oxidized proteins compared to unexposed RHE tissues<sup>68</sup>. In another study, the same RHE model was exposed to moderate and high O<sub>3</sub>



levels (0.4 and 0.8 ppm for 4h)<sup>72</sup>. O<sub>3</sub> was responsible for both the release of pro-inflammatory mediators (e.g. interleukins, inducible nitric oxide synthase - iNOS, and COX-2) and the activation of signal transduction pathways (e.g. Nrf2 and NF-κB) that are involved in the cellular defensive system and pathogenesis of inflammation, respectively.

3D skin tissue models are also effective tools for evaluating the benefits of dermatocosmetic ingredients as protective and curative solutions against environmental stressors<sup>34,73</sup>. The presence of a cornified layer at the outermost surface of the stratified skin construct allows the topical application of semi-solid formulations, making those 3D skin models as the most appropriate tool to test the efficacy of a cosmetic ingredient. The presence of a cornified layer makes those 3D skin models also suitable to study the interaction of the residual skin microbiome with the human immune system<sup>74</sup>. This option is of importance in the context of O<sub>3</sub> exposure. It was in fact demonstrated that O<sub>3</sub> can decrease residual skin microflora by about 50%<sup>ref.56</sup>, and as a result promote the colonization of the SC with pathogenic strains of bacteria<sup>75</sup>.

Moreover, the possibility to design 3D diseased skin models (e.g. by genetic modifications or through inflammatory stimuli) is a very promising approach to identify the contribution of O<sub>3</sub> to the development or aggravation of inflammatory and autoimmune skin diseases<sup>34</sup>.

### **3. Benefits and drawbacks of the different experimental approaches in the context of ozone exposure**

The first *in vivo* studies on murine skin have promoted our understanding of the O<sub>3</sub> effects on skin. *In vivo* murine exposure experiments also allow to evaluate possible systemic effects of skin exposure to O<sub>3</sub><sup>ref.76</sup>. In addition, genetically modified mice can

help investigating the impact of O<sub>3</sub> exposure on skin diseases aggravation<sup>77,78</sup>. Murine specific gene silencing can also be used to highlight the role of specific proteins activated upon O<sub>3</sub> exposure<sup>79</sup>.

However, *in vivo* experiments on murine skin should not be the preferred approach to study the impact of O<sub>3</sub> on skin. First, murine skin only partly reproduces human skin features and pathologies. The differences in SC and epidermis structure and SC lipid composition may lead to misleading findings. Second, the ban on animal testing in the cosmetic industry does not allow to test protective cosmetic ingredients and formulations against this pollutant.

Studies with humans have the advantage of being the most physiologically relevant. Technically, it is possible to expose to O<sub>3</sub> the whole human body (while protecting the airways) or just part of it (e.g. the forearms), and to investigate the impact on healthy donors or donors with skin diseases. The effects of O<sub>3</sub> can be quantified by collecting either the sebum residing at the skin surface or skin biopsies.

However, human studies must follow several ethics requirements and are very expensive<sup>80</sup>. For this reasons O<sub>3</sub> exposure studies on humans are scarce. It should be noted that there exists another way to perform experiments on human skin samples, which is to collect the biopsies and maintained them in culture (i.e., *ex vivo* explants) for O<sub>3</sub> exposure experiments. This method is the closest to the *in situ* condition and it is often used for drug absorption studies because of the barrier properties of the skin explants<sup>34,80,81</sup>. Nevertheless, high inter-subject variability, restricted access, limited availability and excessive costs strongly discourage the use of native human skin, both *in vivo* and *ex vivo*, for O<sub>3</sub> exposure experiments.

Epidemiological studies are advantageous because they provide data on the long-term effects (i.e., chronic exposure) of O<sub>3</sub> on a large human population. They are powerful tools to understand the correlation between O<sub>3</sub> exposure and skin diseases development or aggravation.

However, exposure conditions are less precise and controlled. Paramount attention needs to be paid on how data are collected, modelled and analyzed to avoid exposure measurement errors and wrong evaluations<sup>82</sup>.

*In vitro* studies have several advantages over *in vivo* and *ex vivo* experiments: higher availability, better reproducibility, more experimental conditions flexibility, controlled exposure conditions, and are in general less expensive. However, sterile conditions must be respected during *in vitro* experiments, hence specific facilities are required. In addition, long-term repeated exposure experiments (i.e., several weeks to months) that mimic a more realistic chronic exposure to an air pollutant cannot be achieved.

2D cell cultures are easy and convenient to set up, which makes them a popular method for pollutants exposure *in vitro* studies<sup>66,83,84</sup>. When exposing 2D cell cultures to O<sub>3</sub>, it is crucial to substitute cell culture medium with proper solutions (i.e., Dulbecco's Phosphate Buffered Saline) that do not contain components sensitive to O<sub>3</sub> oxidation (i.e., cell nutrients and growth factors). However, the absence of nutrients that are essential to functions and viability of the cells, limits the amount of time they can be exposed to O<sub>3</sub>. In addition, 2D cell culture systems do not properly mimic the complexity of skin structure and functions.

Compared to 2D cell cultures, reconstructed 3D skin tissue models, such as RHE and FT models, are physiologically more representative of the human skin. Using specifically designed exposure chambers, it is possible to expose only the apical part of

the tissues to O<sub>3</sub> (i.e., not the cell culture medium underneath). In this way, 3D skin tissue models can be exposed to O<sub>3</sub> while maintained in the proper cell culture medium, allowing longer exposure experiments. Another benefit with respect to 2D cell cultures is that culture conditions can be set to mimic different skin diseases features<sup>85–88</sup>. The increased interest in the field of skin microbiome and its role in skin barrier function<sup>89,90</sup> have given rise to the development of 3D skin models including different skin microbiota species<sup>74,91</sup>. However, to date and to the best of our knowledge, no data about the effects of O<sub>3</sub> on 3D skin tissue models populated with skin microbiota were reported in the literature. The known bactericidal O<sub>3</sub> power<sup>8</sup> and previous *in vivo* experiments<sup>56</sup> suggest that further studies should be performed to understand how O<sub>3</sub> action on skin microbiota could also influence skin barrier function<sup>92</sup>.

Several *in vitro* assays that use RHE and FT models have been developed to test both the biological response of skin to environmental stressors and the efficacy of cosmetic protective solutions<sup>83,93,94</sup>. However, no standard exposure conditions to evaluate protective or preventive effects of dermato-cosmetic solutions against air pollutants have been yet defined.

3D skin tissue models are not perfect reproduction of human native skin: they are more permeable than human skin (i.e., less effective barrier function), do not include all human skin cells types and they lack of a proper vascular and nervous system<sup>95,96</sup>.

Although more complex 3D skin tissue models were successfully developed over the last years, they might lead to loss of reproducibility, robustness and may be less suitable for parallelized research.

The pros and cons of the different experimental approaches are summarized in Table 2.

#### **4. Conclusion and future perspectives**

This review gives an overview of the knowledge concerning ground-level O<sub>3</sub> exposure on skin health and the different experimental approaches available for those studies. The studies reported in the literature are in accordance. O<sub>3</sub> can trigger a cascade of oxidative stress responses leading to a chronic inflammation status and, as demonstrated in epidemiological studies, consequently, to reinforced skin aging and several cutaneous tissue pathologies. To study chronic exposure to O<sub>3</sub>, most of the experimental approaches described in this review, tested O<sub>3</sub> concentrations ranging from 0.4 ppm up to 10 ppm. Thus, this is mimicking O<sub>3</sub> levels present only in extreme pollution episodes or even higher exposure conditions. The effects observed post-exposure are however in accordance with the results obtained in the epidemiological studies in which individuals are exposed to relevant O<sub>3</sub> concentrations for longer period of time compared to bench experimental approaches.

Additional data regarding the positive feedback loop between oxidation by-products and inflammatory mediators should be generated. Further research is also needed to understand the biological and mechanistic effects of repeated exposure of relevant O<sub>3</sub> concentrations on skin, as well as the effects of O<sub>3</sub> when combined with other environmental stressors such as UV, cigarette smoke and particulate matter. Even if bioengineered 3D skin tissue models (i.e., RHE and FT models), lack of some structural and molecular aspects of native human skin, they appear to be the most adequate experimental approach to study the noxious effects of tropospheric O<sub>3</sub>. However, exposure procedures still need to be standardized, also for the purpose of testing protective solutions against this pollutant.

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Not applicable.

### **Conflict of Interest**

Marc Eeman and Benedetta Petracca are employees of Dow Silicones Belgium SRL.  
The authors have no conflict of interest to declare.

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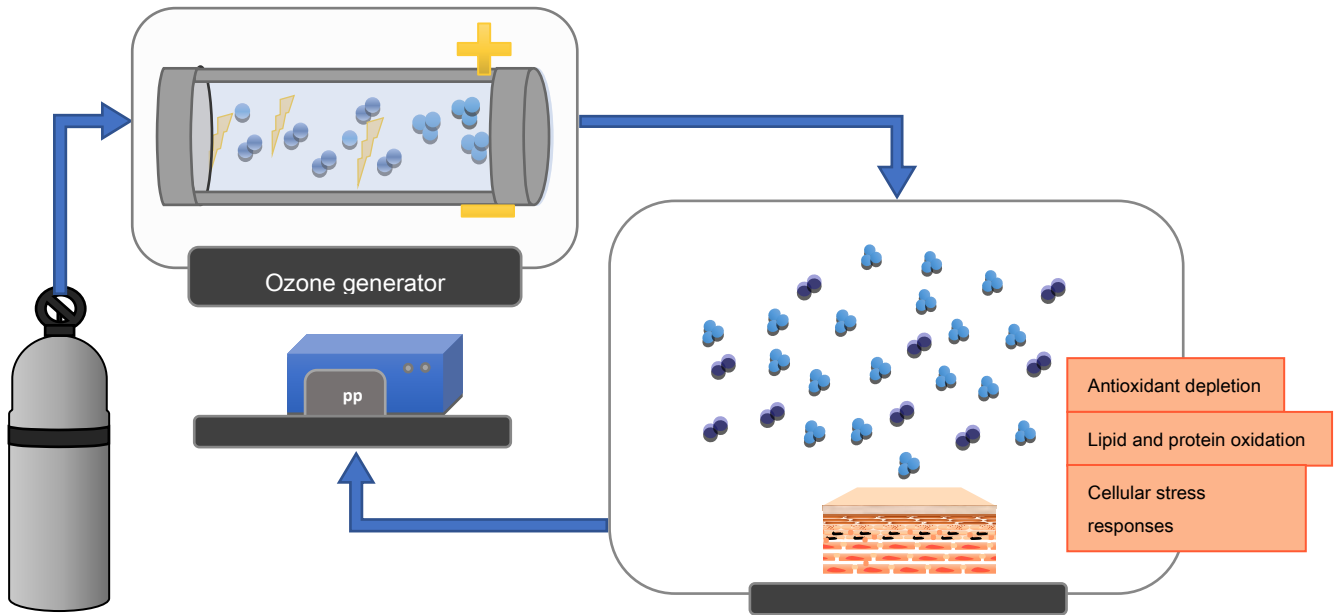
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## Figures

**Figure 1. Example of an O<sub>3</sub> exposure experimental set-up.** O<sub>3</sub> is produced when dehumidified air or oxygen passes through the corona discharge system of a high voltage generator. O<sub>3</sub> flows into an exposure chamber where its ambient concentration is monitored by an in-line O<sub>3</sub> analyser.



**Figure 2. Skin tissue models used to assess the effects of O<sub>3</sub> on skin:** *in vivo* murine skin, human skin, *in vitro* 2D cultures, *in vitro* 3D tissue models.

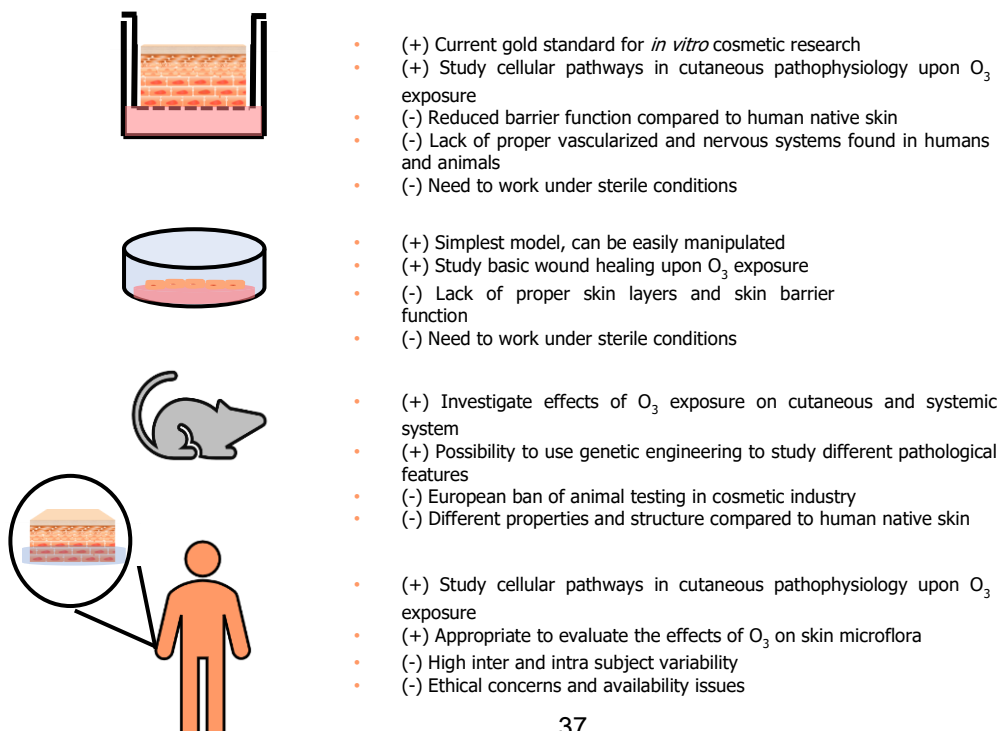


Table 1: Overview of the studies used to date to investigate the effects of O<sub>3</sub> on cutaneous tissues.

<i>Skin tissue model</i>	<i>Skin tissue model type</i>	<i>Cell type</i>	<i>O<sub>3</sub> exposure conditions</i>	<i>Main observations</i>
<i>In vivo</i>	Murine	n.a.	<ul style="list-style-type: none"> <li>• 1, 5 and 10 ppm for 2h single exposure and 1 ppm for 2h/day for 6 days <sup>6</sup></li> <li>• 8 ppm for 2h <sup>31</sup></li> <li>• 10 ppm for 2h <sup>32,43</sup></li> <li>• 10 ppm for 2h <sup>43</sup></li> <li>• 0.8 ppm for 6h/day for 6 days <sup>47</sup></li> <li>• 0.8, 1 and 10 ppm for 2h <sup>44</sup></li> <li>• 2 ppm twice per day for 7 days <sup>97</sup></li> <li>• 1 ppm for 2h <sup>45</sup></li> <li>• 8 ppm for 2h <sup>46</sup></li> <li>• 0.8 ppm for 6h <sup>50</sup></li> <li>• 0.5 ppm for 6h/day for 9 days <sup>51*</sup></li> <li>• 0.25 ppm for 6h/day for 4 days <sup>52</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↓ antioxidants (α-tocopherol and ascorbic acid) <sup>6,31,32,43–45,47,97</sup></li> <li>• ↑ oxidative stress markers <sup>6,32,43,46,50–52,97</sup></li> <li>• ↑ inflammatory markers (NF-κB -p65 nuclear translocation-, iNOS, COX-2, HO-1, IL-6, IL-8) <sup>46,47,50–52</sup></li> <li>• ↑ HSPs (HSP27, HSP70) <sup>46,50</sup></li> <li>• ↑ PCNA, K10, MMP <sup>47,50</sup></li> </ul>
<i>In vivo</i>	Human	n.a.	<ul style="list-style-type: none"> <li>• 0.8 ppm for 3h/day for 5 days <sup>58</sup></li> <li>• 0.8 ppm for 2h <sup>56</sup></li> <li>• 0.1 to 1 ppm for 2 h <sup>59</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (8-iso PGF2a, 4-HNE, MDA) <sup>58,59</sup></li> <li>• ↑ inflammatory markers (NF-κB -p65 nuclear translocation-, COX-2) <sup>58</sup></li> <li>• ↓ antioxidants (vitamin E) <sup>59</sup> and microflora population <sup>56</sup></li> </ul>
<i>Ex vivo</i>	Human	n.a.	<ul style="list-style-type: none"> <li>• 0.4 ppm for 4h <sup>64</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (H<sub>2</sub>O<sub>2</sub>, 4-HNE) <sup>64</sup></li> <li>• ↑ inflammasome activation <sup>64</sup></li> </ul>
2D	Human cell line	DK7-NR, HaCaT	<ul style="list-style-type: none"> <li>• 10 ppm for 20 min <sup>68</sup></li> <li>• 0.1 ppm for 30 min <sup>70*</sup></li> <li>• 0.4 ppm for 1h <sup>64</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (ROS, H<sub>2</sub>O<sub>2</sub>) <sup>64,68,70</sup></li> <li>• ↓ SR-B1 receptor, affected wound healing <sup>70*</sup></li> <li>• ↑ inflammasome activation <sup>64</sup></li> </ul>
2D	Human primary cells	NHEK	<ul style="list-style-type: none"> <li>• 0.1 and 0.2 ppm for 1h <sup>66</sup></li> <li>• 0.4 and 0.8 ppm for 30 min <sup>67</sup></li> <li>• 0.3 ppm for 20 min <sup>33</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (ROS, protein carbonyls, 4-HNE and Nfr2) <sup>66,67</sup></li> <li>• ↑ inflammatory markers (NF-κB -p65 nuclear translocation-, IL-1α and IL-8) <sup>66</sup></li> <li>• ↑ CPY1 isozymes and AhR receptor expression <sup>33</sup></li> <li>• ↓ mitochondrial activity (ATP levels) <sup>67</sup></li> </ul>
3D RHE	EpiSkin™ human epidermis model (Episkin)	NHEK	<ul style="list-style-type: none"> <li>• 10 ppm for 1h <sup>68</sup></li> <li>• 0.4 and 0.8 ppm for 4h <sup>72</sup></li> <li>• 0.3 ppm for 4h <sup>98</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (ROS, protein carbonyls, 4-HNE and Nfr2) <sup>68,72,98</sup></li> <li>• ↑ inflammatory markers (NF-κB -p65 nuclear translocation-, COX-2, HO-1) <sup>72,98</sup></li> </ul>

3D RHE	EpiDerm™ human tissue model (MatTek Corporation)	NHEK	<ul style="list-style-type: none"> <li>• 0.8 ppm for 1h and 4h <sup>70</sup></li> <li>• 5 ppm for 2h <sup>71</sup></li> <li>• 20 ppm for 1h <sup>93</sup></li> <li>• 0.4 ppm for 4h <sup>64</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (protein carbonyls, H<sub>2</sub>O<sub>2</sub>) <sup>64,70,71</sup></li> <li>• ↑ inflammatory markers (IL-1<math>\alpha</math> and E2-PGE2) <sup>93</sup></li> <li>• ↓ SR-B1 receptor <sup>70</sup></li> <li>• ↑ inflammasome activation <sup>64</sup></li> </ul>
3D FT	StrataTest® human skin model (Stratatech)	NIKS® + NHDF	<ul style="list-style-type: none"> <li>• 0.8 ppm for 30 min <sup>99</sup></li> </ul>	<ul style="list-style-type: none"> <li>• ↑ oxidative stress markers (ROS) <sup>99</sup></li> </ul>

n.a.: not applicable, \*: wound model

Table 2: Advantages and disadvantages of the different exposure methods and skin tissue models in the context of O<sub>3</sub> exposure.

Exposure method	Type	Advantages	Disadvantages
<i>In vivo</i>	Murine	(+) Investigate effects of O <sub>3</sub> exposure on cutaneous and systemic system (+) Possibility to use genetic engineering to study different pathological features	(-) European ban of animal testing in cosmetic industry (-) Different properties and structure compared to human native skin
	Human	(+) Most physiologically relevant (+) Evaluate the effects of O <sub>3</sub> on skin microflora (+) Study cellular pathways in cutaneous pathophysiology upon O <sub>3</sub> exposure	(-) High inter and intra subject variability (-) Ethical concerns and availability issues
	Human Epidemiological	(+) Data on long term effects and on large human population (+) Powerful tools to study the correlation between O <sub>3</sub> exposure and skin diseases development or aggravation	(-) Reduced controlled exposure conditions
<i>Ex vivo</i>	Human	(+) Study cellular pathways in cutaneous pathophysiology upon O <sub>3</sub> exposure (+) Closest to <i>in vivo</i> condition and proper skin barrier function, all skin appendages are present	(-) High inter and intra subject variability (-) Reduced availability and cost issues (-) Limited cutaneous areas are available
<i>In vitro</i>	2D cell cultures	(+) Simplest model, high reproducibility (+) High availability, easier manipulation, reduced cost	(-) Need to work under sterile conditions (-) Lack of proper skin layers and skin barrier function
	3D skin tissue models	(+) Validated alternative method to assess irritation and corrosion potential of chemical ingredients and cosmetic formulations (+) High reproducibility (+) Study cellular pathways in cutaneous pathophysiology upon O <sub>3</sub> exposure (+) Can be manipulated to integrate different skin cell types, microbial components and/or skin appendages (+) Can be designed to mimic skin pathological features	(-) Reduced barrier function compared to human native skin (-) Lack of proper vascularized and nervous systems found in humans and animals (-) Need to work under sterile conditions

