ABSTRACT

PAMBIANCHI, ERIKA. Topical Intervention to Prevent Pollution Induced Extrinsic Aging and Aberrant OxInflammatory Responses in Orthodox and Innovative Skin Models
(Under the direction of Dr. Giuseppe Valacchi).

The World Health Organization estimates that 91% of urban's world population breathes polluted air, exceeding the current guidelines. Different pollutants can interact directly or indirectly with several organs like gastro-intestinal, cardiovascular, neuronal and cutaneous systems. Pollution exposure has been associated with development/exacerbation of multiple inflammatory conditions like asthma, Alzheimer's disease, inflammatory bowel syndrome, as well as atopic dermatitis, acne, psoriasis and premature skin aging. Recently, the harmful effects of pollution on skin have been reported. Skin, as the major interface between the body and the environment, is the most exposed organ to the noxious effects of the environment; nevertheless, it is characterized by a strong defensive cellular system (enzymatic and non-enzymatic antioxidants) and antimicrobial peptides (AMPs), all molecules that aim to protect the skin from the outdoor-induced tissue damage.

Although recently increasing cutaneous diseases have been associated with pollution exposure, to date, the mechanistic effects of environmental stressors on skin are still not well understood. What it has been shown so far is that many challenges to which we are exposed daily are able to induce a cutaneous inflammatory response together with an oxidative stress damage (OxInflammation).

In the present work we aimed to elucidate the mechanisms of pollution-induced OxInflammatory skin damage, mainly focusing on the pollutants ozone (O_3) and diesel engine exhaust (DEE) by using several skin models, including 2D culture of keratinocytes, *3D* skin models, *ex vivo* human skin explants (cultured with or without physiological tension) and human

volunteers. Furthermore, we evaluated the topical application of different synthetic and natural extracts as a tool to prevent pollution-induced skin damage and aging.

In the first study we investigated the beneficial effects of topical application of blueberry extract in preventing ozone-induced damage, via the use of 2D, 3D and *ex vivo* human biopsies. We demonstrated that ozone induced oxidative stress and inflammation via the activation of the inflammasome, a recently discovered player in innate immune responses. Topical application of blueberries not only reduced the ozone-induced inflammasome activation, but also enhanced keratinocytes wound healing and normalized proliferation and migration processes previously altered by ozone.

In the second study we evaluated the OxInflammatory effects of DEE on *ex vivo* skin explants and we showed that DEE caused oxidative stress, inflammation and structural impairment of cutaneous barrier. However, the pre-treatment with a dual formulation containing rosemary and spirulina normalized the alterations induced by DEE on skin tissue.

In the third study we confirmed that DEE causes cutaneous damage via iron-induced peroxidation and we proposed the use of an iron chelator (deferoxamine, DFO), together with a radical scavenger formulation (CE Ferulic), as a way to counteract the DEE-induced damage via both ROS and Fenton reaction. Indeed, we observed that the topical treatment with DFO and CE Ferulic, alone and in combination prevented the upregulation of oxidative and inflammatory markers, as well as the loss of collagen fibers and cutaneous-barrier associated proteins downregulated by DEE.

Finally, in the last two studies we evaluated the modulation by ozone of cutaneous AMPs expression. Our study demonstrated that the ozone-induced increase of OxInflammatory markers and AMPs was strictly triggered by an unbalanced redox homeostasis suggesting AMPs redox

modulation. Furthermore, via the use of Ten Bio models (*ex vivo* skin explant resembling physiological skin tension), we demonstrated the importance of skin tension when investigating pollutants that target skin.

In conclusion, this work elucidates the harmful effects of pollution exposure on skin and provides important insights on the advantages and limitations of different skin models. Moreover, this work suggests the topical application of multiple compounds as a tool to prevent the different mechanisms of damage induced by environmental stressors.

Topical Intervention to Prevent Pollution Induced Extrinsic Aging and Aberrant OxInflammatory Responses in Orthodox and Innovative Skin Models

by
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DEDICATION

This dissertation is dedicated to my mother Patrizia, my father Daniele and my brother Matteo, who have been my source of strength and inspiration, who continually supported me in pursuing my dreams and even from the other side of the world provided me emotional and moral support every time I thought of giving up.

And to my husband Cory, who has been my beacon in the night, his love and support, his model of commitment and hard work empowered me to complete this path.

I owe it all to you.

BIOGRAPHY

Erika Pambianchi is from Ravenna, a small city located in Italy, known for its mosaics and good food.

Since the very first years of life she showed interest in understanding the fascinating world of the human body and of nature.

This affection moved her to pursue a Pharmacy Bachelor's and Master's degree, where she met Dr. Giuseppe Valacchi as her Physiology Professor at the University of Ferrara.

During the last year of Pharmacy school, as she was doing her experimental thesis in Dr. Valacchi's lab she fell in love with the world of research.

After her graduation in 2017, she decided to start a new adventure and move to North Carolina, at the Plants for Human Health Institute as a lab technician in Dr. Valacchi's lab.

Upon discovering his work in the field of skin biology, environmental pollution and OxInflammation, she started a doctorate majoring in both Animal Science and in Nutrition at North Carolina State University to further investigate the role of topically applied natural and synthetic extracts in preventing pollution-induced skin damage and premature aging.

Working in one of the greatest research institutions, Erika improved her learning, scientific skills and ability to solve problems creatively.

These experiences gained over the years have led her to continue her professional career working in the field of skin biology around the world.

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CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

1.1.1. Pollution

The World Health Organization (WHO) estimated that 91% of the urban world's population breathes polluted air and that over 4.2 million premature deaths due to pollution exposure have been reported in 2019 (1). Indeed, air pollution is now one of the leading causes of death in the world, surpassing extensively the number of deaths attributed to threating and deadly diseases such as HIV/AIDS, malaria, vector-borne and various infectious and parasitic epidemics (2).

Ambient air pollution refers to the diversified variety of mixtures of gas (nitrogen dioxide -NO_2-, nitric oxide -•NO-, sulfur dioxide -SO_2-, carbon monoxide -CO- and ozone -O_3) and particulate matter (PM) fractions, which present a heterogeneous composition as well (3–5).
Air pollutants can be classified based on their chemical origin as either primary (when directly emitted in the environment by combustion of fossil fuel) or secondary (when formed in atmosphere by chemical reactions with other pollutants like i.e. ultraviolet light -UV, hydrocarbons, etc) (6). Furthermore, environmental pollutants can also be classified based on their source, either natural (soil, volcanic emission, forest fire) or anthropogenic (particulate matter, industry emission, diesel engine exhaust -DEE, coal combustion and oil refineries (7, 8)).

It is well known that environmental pollutants can negatively affect the function and the structure of different target organs, including the heart, lungs, gut, and brain (9–12). However, only recently, skin has been shown to be another target tissue of pollution damage. In the context of pollution exposure, skin plays a cardinal role, since, due to its location and size, it represents the body's first defense and target against the external environment. Problems arise when prolonged or repetitive exposure to pollutants exceeds the cutaneous' defense capacity. Indeed,

multiple studies have found a correlation between pollution exposure and the development or the exacerbation of multiple skin ailments such as psoriasis, acne, eczema, atopic dermatitis and premature aging (13–15).

Different pollutants (O_3, DEE, cigarette smoke – CS- and UV), share similar mechanisms by which they exert damaging effects on the skin (oxidative stress, inflammation and depletion of antioxidant defenses), but at a molecular level each pollutant presents specific ways to affect the cutaneous tissue, based on its physical and chemical characteristics that are able to influence the interaction with the skin. In fact, these differences can explain why the concomitant exposure to multiple pollutants (which is unavoidable) exacerbates and enhances the noxious effects on skin of a single pollutant, as also showed by our group (16). For instance, while one pollutant can limit its interaction to the epidermis, this can further favor the entrance of other stressors such as pathogens and other pollutants (like PM and DEE) (17, 18), leading to a magnification of the cutaneous pollution-induced damage and contributing to systemic damage.

1.1.2. Ozone (O_3)

O_3 is an invisible gas with a strong oxidative potential due to its unstable chemical structure. Unlike the stratospheric layer (over 10 Km above ground level) of ozone that forms naturally and absorbs UV radiation before reaching the Earth; tropospheric ozone (ground level) originates from photochemical smog, the reaction between sunlight (UV), nitrogen oxides and hydrocarbons emitted by car exhaust. In less polluted environments, ozone is formed via the photolysis of NO_2 induced by sunlight (at wavelengths <424 nm) (19).

Ozone concentrations vary over the year depending on the seasons, altitude and geographical locations but reports evidenced that in most of polluted cities, ozone can reach concentrations of

0.5 and sometimes even 0.8 ppm (14, 20, 21). Of note, recent literature suggests that some damaging effects of ozone exposure on target organs are evident already at 0.1 ppm (16, 22–26).

In the last 3 decades many studies correlated ozone exposure with the development of respiratory and cardiovascular complications such as chronic obstructive pulmonary disease (COPD), lung cancer and ischemia (27–30). Only recently the effects of ozone on skin have been studied (13, 31–33).

Ozone is not a radical species *per se,* nevertheless it is very reactive and it has now been well proven that its effects on skin are mediated by free radicals. In fact ozone is not able to penetrate the skin and it interacts with the polyunsaturated fatty acids (PUFA) and lipids present in the stratum corneum (SC) leading to their oxidation and to generation of reactive oxygen species (ROS) including H_2O_2 and other secondary lipid oxidation products (LOPs), particularly unsaturated aldehydes (4-hydroxy-nonenal – 4HNE), capable of triggering inflammatory responses (34, 35) and modulating different important physio-pathological skin pathways (34).
The ozone-generated free radicals, ROS and LOPs (32, 36, 37) can interact with plasma membranes rich in lipids and initiate the cascade reaction known as lipid peroxidation (38–40). This chain reaction can induce the formation of other ROS and propagate the oxidative damage into the deeper layers of epidermis, leading to the activation of pro-inflammatory transcription factors (AP1, NFkB) (41) and intracellular protein kinases (ERK, JNK, p38, MAPK) (42) involved in cell growth and differentiation and in the degradation of the connective cutaneous tissues (43). Moreover chronic oxidative stress induced by ozone-generated ROS can overwhelm the skin's defenses leading to the depletion of antioxidant players like the nuclear factor erythroid-2-related factor 2 (Nrf2) (44), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (Cat) (45) and lead to cutaneous conditions (46, 47).

1.1.3. Particulate Matter (PM) and Diesel Engine Exhaust (DEE)

Unlike ozone that is a pollutant composed of only one element (oxygen), PM can be very heterogeneous in composition, since it is formed by organic and inorganic, solid, liquid, mixed-phase and organometallic elements, like transition metals, polycyclic aromatic hydrocarbons (PAHs) and endotoxins (3–5).

Among the anthropogenic-generated air pollutants, PM is considered one of the most noxious and it has been correlated with global increased mortality and morbidity (48, 49). The damaging effects of PM are intimately related to its chemical composition and size. Indeed, PM can be divided into 3 fractions based on its aerodynamic equivalent diameter (AED): coarse particles (circa 10 μm diameter), fine particles (ranging between 2.5 and 0.1 μm) and ultrafine particles (UFP) (smaller than 0.1 μm).

UFP are the most dangerous type of PM since, due to the micrometric size, toxins, microorganisms and other organic components can easily adsorb on the PM's surface and, in the context of lung damage, pass through the alveolar epithelial cells and enter the cardiovascular system leading to systemic damage of distal tissues and the activation of immune cells (50, 51).

Noxious effects of PM on cardiovascular and respiratory tissues have been extensively reviewed, indeed, it is well known that coarse particles are filtered by the proximal airway, and that fine particles and UFP can reach the gas exchange area and be absorbed systemically (52). However, the mechanisms of entry of PM into the skin are still under debate, although few studies suggested that PM can penetrate the skin via hair follicles or trans-dermally and promote oxidative stress not only at a more superficial level but also in the deeper cutaneous layers (53–55). In addition, it is also possible that PM are able to reach the skin from the blood flow once inhaled (56).

Furthermore, PM can directly damage skin barrier and functions leading to premature aging and inflammation (57, 58).

Another anthropogenic pollutant that has increased dramatically in the last few decades, due to the frenetic needs of modern society, is diesel engine exhaust (DEE) (59). Although the exact composition of diesel engine exhaust is strictly correlated with the engine, oil, fuel type, specific operation modes and additives, in general DEE is composed of gasses (nitric oxide, nitrogen dioxide, sulfur dioxide, carbon monoxide formaldehyde, acetaldehyde and acrolein), condensates (hydrocarbons, water, sulfuric acid) and a solid carbon core covered by different metals (iron, manganese, copper, zinc) and other compounds (PM, PAHs, endotoxins, microorganisms) (60).

Reported harmful effects of DEE are strictly related to its heterogeneous chemical composition and involve indeed, the presence of transition metals, especially iron (Fe) that can catalyze Fenton reactions leading to the generation of hydrogen radicals and to the activation of the aryl hydrocarbon receptor (AhR) which, in turn, via the regulation of the cytochrome P450 enzyme CYP1a1b, triggers the conversion of PAHs into redox active quinones (61). In addition, particles present in DEE can serve as organic carriers of lipophilic PAHs, capable to be absorbed by the skin and then enter the tissue and eventually penetrate the cells and affect mitochondria ROS production (62). Independently of the source generating ROS, these molecules are able to initiate and perpetuate oxidative damage and worsen the inflammatory state of skin tissue (63).

It is generally known that oxidative stress and inflammation are intimately associated and communicate in a positive-feedback loop, this cross-talk can lead to an aberrant vicious cycle defined as OxInflammation (64), where the oxidative stress caused by exogenous stimuli (like pollution) induces and, concomitantly, it is fueled by inflammatory responses (16, 65). Prolonged OxInflammation can induce damage to skin structure (collagen, elastin) and barrier function

(filaggrin, involucrin), leading to water loss, increased susceptibility to other external factors (pathogens, pollutants, etc.) to enter the skin and amplification of the OxInflammatory state itself. Furthermore, DEE and other pollutants that indirectly carry iron on their surfaces, can modify the cellular ferrokinetics by either chelating this transition metal or by displacing the metal from cardinal cellular sites (as discussed in more detail in Chapter 4) (66, 67).

Different studies performed in humans and rodents showed that DEE exposure, both its gaseous and solid factions (PM), are able to cause inflammation, increase susceptibility to infection and can develop/exacerbate cardiovascular and immune diseases (60, 68, 69). Moreover, recent evidence correlated the exposure to the solid fraction of DEE (specifically PM), to premature skin aging (70) and the worsening of cutaneous pigmentation, coarse wrinkles and elastosis (71).

1.1.4. Ultraviolet Light (UV)

Although exposure to UV light is important for the human body to produce vitamin D_3 and it can be therapeutically advantageous to ameliorate psoriasis, prolonged exposure to this natural pollutant is the primary cause of photoaging and can lead to harmful cutaneous consequences, such as inflammatory skin diseases up to malignant melanoma.

UVA radiation comprises more than 90-95% of total solar radiation that reaches the Earth's surface and although it is considered weak energy, it is able to penetrate the deep layers of dermis. On the other hand, UVB (which constitutes only around 5-10% of total ultraviolet radiation) is a high energy radiation that can be blocked primarily by the stratospheric ozone layer and once it comes into contact with skin, it is mainly absorbed by epidermal cells (32, 72).

The ability of UV to negatively affect the different layers of skin and trigger multiple interdependent cellular responses makes it a very dangerous stressor. In fact, when UV reaches the epidermal/dermal cells, it gets absorbed and results in direct damage to multiple biomolecules and organelles (proteins, lipids, DNA and mitochondria) and also in indirect activation of pro-inflammatory transduction pathways and production of ROS that in turn can cause a cascade of oxidative events leading to the progressive deterioration of cutaneous cellular structures and functions (73).

Indeed, it has been demonstrated that when UVA reaches the basal layers of epidermis, it can cause mutations of p53 gene, induce the development of skin carcinogenesis (74), immune suppression (75) and apoptosis in keratinocytes via the activation of death receptors on cellular membranes as a protective mechanism to eliminate the keratinocytes bearing DNA mutations induced by UV (76).

Even though human skin adapted to UV exposure via the production of the UV-absorbing pigment melanin, prolonged exposure to the strong oxidative potential of UV can trigger carcinogenic mutations in melanocytes as well (77).

1.1.5. Cigarette Smoke (CS)

Cigarette smoke is a highly complex aerosol composed by over 7000 different chemicals distributed between the particulate and the gas phases. CS has been classified as Group A carcinogen by the Environmental Protection Agency (EPA) (78).

Most of the chemicals present in CS are formed during the incomplete combustion process in the cigarette and are pro-oxidants (ROS, reactive nitrogen species -RNS- and electrophilic aldehydes) that can induce oxidative stress leading to peroxidation of cellular biomolecules. In particular, it

has been documented that the gas-phase of CS contains more than 10^{14} carbon and oxygen-based radicals per puff (79, 80).

A wealth of evidence demonstrated that the half-life of radicals present in the gas phase of CS is relatively short (80, 81), therefore, the general notion to explain CS toxicity resides in its ability to induce oxidative stress via radical species present in the tobacco smoke itself or by reactive oxidants produced indirectly through secondary oxidative events like lipid peroxidation and mitochondrial damage (82).

The negative effects of cigarette smoke on health have been extensively investigated, but the specific effects on skin have been brought to light only in the last few decades. Finally, in 1985 the facial features of cigarette smokers were clinically defined as "smoker's face" and comprehended accelerating and premature aging, wrinkles and sallow coloration (83). The mechanisms at the base of visible skin damage are due to the induction of matrix metallo-proteinases (MMPs) and elastosis, both enzymes that degrade the connective tissues' elements (collagen and elastin fibers) (84).

Other deeper and more dangerous effects are also caused by CS exposure in cutaneous tissue and they are intimately related to the oxidative stress and inflammatory status induced by the wide range of chemicals present in the different CS phases. Of note, many of these lipophilic compounds and also PAHs are able to penetrate the epidermal barrier and enter systemic circulation via the dermal capillaries, leading to systemic inflammatory responses (85, 86). Many studies showed indeed that the continuous exposure to CS over the years overwhelms the skin defense system (vitamin E, vitamin C, GSH, GPX, SOD, Cat) (87).

Recent evidence associated CS exposure to the development/exacerbation of different skin diseases (impaired wound healing, squamous cell carcinoma, melanoma, acne, eczema, hair loss

(88)) and in particular psoriasis due to the ability of CS to implement 3 different mechanisms at the base of the etiology of psoriasis itself: epidermal hyperproliferation, immune cells infiltration in epidermal and dermal layers and pathological angiogenesis (89, 90).

1.2. Skin Defense

1.2.1. Antioxidant system

Cutaneous cells are able to quench, to a certain extent, oxidative stress, thanks to the presence of an endogenous defense system composed of multiple antioxidant enzymes (such as glutathione -GSH, superoxide dismutase -SOD, glutathione peroxidase -GPX, catalase -Cat, thioredoxins -TRX, peroxiredoxins -PRX), non-enzymatic micronutrients (vitamin C, vitamin E, bilirubin) and metal-binding proteins (ferritin) (91). Among these compounds, some have direct antioxidant capabilities and others are able to regenerate the antioxidant potential of the first ones when they get oxidized during the oxidative-quenching reactions (92, 93).

Although the cellular defense system can be considered adequate in homeostatic conditions, its antioxidant potential could become overwhelmed and insufficient when the exposure to oxidative stimuli becomes prolonged and extensive, i.e. daily exposure to environmental pollution (92, 94).

In this context it becomes very important to help and improve the skin defense system. It is well known that nutrition is fundamental for skin health, since a healthy diet provides antioxidant micronutrients such as vitamins C, E and carotenoids able to counteract oxidative damage by acting as scavengers of singlet oxygen and hydroxyl radicals and by transforming them into non-reactive species that can be further detoxified by GSH and ascorbate itself. Moreover, healthy nutrition can also provide other essential vitamins, amino acids and trace

minerals that are necessary co-factors for the synthesis and correct function of antioxidant enzymes (cysteine for GSH, copper, zinc and manganese for SOD isoforms and selenium and cysteine for GPX and TRX) (47). In fact, many different skin conditions have been related to nutrient deficiencies, like skin fragility and wound healing impairment that are symptoms of scurvy, caused by ascorbic acid deficiency (95).

Unfortunately, dietary intervention alone may not be sufficient to prevent skin conditions induced by pollution exposure due to the fact that epidermis, which is the cutaneous layer most exposed to pollutants, lacks a direct blood and nutrients supply (47).

Therefore, in the context of skin disease and skin damage prevention, topical application assumes great importance to guarantee the delivery of necessary nutrients for skin defense.

As a matter of fact, topical intervention to improve skin diseases and skin defense against environmental pollution has been evaluated for years (96–100), in particular vitamin C and vitamin E (tocopherols) to prevent premature skin aging, cutaneous damage and ameliorate skin conditions (97, 101–103).

In the last few years, due to the rising levels of environmental pollutants, more natural compounds have gained attention for their antioxidant and antinflammatory properties. These natural compounds present polyphenolic fractions that are important for plant survival and protection from external stressors.

The last decades brought to light evidence of reduced cardiovascular disease, improvement of diabetes type 2, weight maintenance and neuroprotection, associated with blueberry (104–106) and rosemary consumption (107–109). Furthermore, recent studies evaluated the topical application of various blueberry extracts to stimulate collagen synthesis, reduce telangiectasias, wrinkle formation and premature skin aging (110–112).

Natural compounds present different phytochemical profiles, for instance blueberries mostly contain anthocyanins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin, malvidin), pro-anthocyanidin, flavonols and phenolic acids (113), while rosemary contains other phenolic compounds such as rosmarinic acid, carnosol and carnosinic acid (114).

The variety and properties of phenolic composition of different natural compounds attracted interest from global industries in the fields of nutrition, health, pharmaceutics and cosmetics (115) but a lot is still unknown regarding the beneficial properties and possible uses of natural compounds.

Due to the chemical features of these phytochemicals, their susceptibility to thermal and light exposure makes them susceptible to oxidative degradation, therefore technological improvements to preserve their activity and deliverability is of great interest. In this context, the concomitant use of other substances together with natural extracts can be beneficial not only to preserve them from degradation but also to bring other rheological properties to the topical application. Indeed, in this work the use of the alga Spirulina (*Arthrospira platensis)* has been evaluated together with rosemary extract (Rosmarinus officinalis) to improve the rheological features of the formulation and providing at the same time nutritional proteins (Chapter 3).

Another molecule that has been shown important to counteract oxidation, specifically induced by free iron via Fenton reaction, is the iron chelator deferoxamine (DFO) which, in this work has been studied in a topical formulation together with a cosmeceutical product (CE Ferulic) as an additive tool to prevent iron-induced oxidative stress (Chapter 4).

1.2.2. Antimicrobial peptides

The skin presents different ways to protect itself from external pathogens and one of them is represented by antimicrobial peptides (AMPs). AMPs are part of the innate immune response and prevent pathogens from spreading by disrupting their membrane integrity (116).

Antimicrobial peptides are small peptides (4 kDa) that can be divided in different classes: defensins and cathelicidins.

Defensins' cationic β-sheet structure is stabilized by three intramolecular disulfide bonds and based on their arrangement can be further classified into alpha (expressed by neutrophils and Paneth cells) or beta defensins (expressed by epithelial cells and keratinocytes).

Cathelicidins instead have an amphipathic α-helical structure with a conserved N-terminal cathelin domain and a variable C-terminal with antimicrobial properties (116). Humans express different types of defensins (hBD1, hBD2 and hBD3) but just one cathelicidin: LL-37, codified by the gene CAMP.

Recent studies demonstrated that AMPs, in particular hBD2, hBD3 and LL-37 can be induced in response to infection, inflammation and injury (117–123) via the regulation of the redox sensitive transcription factors NF-kβ and AP-1 (124, 125).

Once activated, AMPs not only act as antibacterial agents but they can also induce the activation and chemotaxis of immune cells, stimulate angiogenesis and upregulate the production of pro-inflammatory cytokines (126–133).

Although AMPs seem to have protective effects, their role in skin diseases is still controversial, since they have been found to be increased in inflammatory skin diseases such as in active lesions of psoriasis and atopic dermatitis (122, 134–137), even though this finding for atopic dermatitis is still under debate (136).

1.3. The Skin and its Models

The skin is the largest sensory organ in our body (approximately 2 m^2) and it is composed of three main layers: the epidermis, the dermis and the subcutaneous fat, which acts as an insulation layer to protect the internal organs from temperature and physical impacts. The dermis is the vascularized layer, embedded with free nerve endings, sebaceous glands, hair follicles and lymphatic vessels. This layer can be divided further into stratum reticulare (formed by thick collagen fibers) and stratum papillare which has a higher concentration of thin collagen fibers. The principal cell types present in the dermis are fibroblasts (responsible to produce both elastin and collagen fibers), followed by other resident cells like endothelial, nervous and dendritic cells. Above the dermis, in intimate contact with it, there is the epidermis: the outermost cutaneous layer that protects the skin itself from external stressors.

The epidermis is composed of keratinocytes that based on their differentiation status, form the different layers of epidermis. The lowest layer of the epidermis is the stratum basale, which contains transiently amplifying stem cells, precursors of keratinocytes, that, during their differentiation steps, withdraw from mitosis and start expressing differentiation-dependent markers (keratins) becoming, in the last cutaneous layer (stratum corneum -SC), densely keratinized corneocytes (138).

These corneocytes are held together by a lipid-laden extracellular matrix that acts as a protective shield against external chemical/physical stressors and excessive transepidermal water loss (TEWL).

In this context, the use of an appropriate skin tissue model has become fundamental to study and identify the mechanisms behind pollution-induced damage; therefore, several models have been developed over the years to study the skin and its responses, including *in vitro* models (2D

cell lines and 3D skin models), *ex vivo* skin biopsies and *in vivo* approaches (animals and human volunteers).

Considering the functional complexity of the skin and the many cells and layers that compose it, experimental models need to be chosen based on the endpoints of interest as well as the limitations of each experimental procedure and ethical, practical and economic reasons. It's important to know that there is no perfect model to study skin responses and that new knowledge about skin homeostasis and pathophysiology has to be gained using multiple different experimental approaches and cutaneous models.

Based on these premises, signal transduction pathways are better characterized in 2D models; topical intervention and percutaneous permeation can be preferably studied in 3D models and skin explants; while feeding studies can be better evaluated in animal and clinical intervention.

1.3.1. 2D Skin models

2D models represent the most economical, accessible, reproducible, easy to handle and fast cutaneous model available on the market.

Different immortalized cell lines can be employed and the reproducibility of their results derives from the fact that these populations are more homogenous compared to primary cell lines.

Different types of cutaneous immortalized cells can be used in 2D models: keratinocytes (of human origin like HaCaT and A431, or mouse origin like JB6 (139, 140)) and fibroblasts (like 3T3 cell line (141)). Due to their multiple and variegated functions and their different localization in skin layers, co-cultures of keratinocytes and fibroblasts represent a great model to reproduce the cellular cross-talk fundamental in specific cellular responses like wound healing (142).

Although primary cells (normal human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF)) present more donor-to-donor variability and limitations due to passage number, they are still considered the more biologically relevant because they are isolated directly from either healthy donors or subjects affected by specific pathologies. An important role for primary cells regards *in vitro* senescence studies and skin aging.

Generally, 2D models are the best model to study acute toxicity, signaling pathways and mechanisms since this model allows the silencing of specific proteins; however, in 2D models keratinocytes grow in monolayer cultures therefore they cannot undergo terminal differentiation and cannot form the outermost layer of the skin, the stratum corneum, which is the layer directly affected by many pollutants unable to penetrate the skin, like ozone.

To overcome the problem of the lack of stratum corneum in 2D models, recent technology progressed to the 3D models.

1.3.2. 3D Skin models

3D skin models are a more complete model compared to monolayer 2D cell lines and based on their complexity can be classified in Reconstructed Human Epidermis (RHE) (also called human epidermal equivalents) and Full Thickness (FT) 3D skin models also known as human skin equivalents.

Both RHE and FT 3D models consist of all epidermal layers present in human skin including the SC. Like 2D cell lines, both RHE and FT 3D models allow the silencing of single proteins, making them great tools to study signaling pathways in a multilayered model.

RHE are the best tool to investigate cutaneous stress responses in laboratories since it can either be created handmade or commercially purchased (143). In particular, RHE can become useful to mimic real life topical application of substances.

FT, instead, comprehends both epidermal and dermal layers, separated by a basement membrane. Epidermis is composed by fully stratified and differentiated keratinocytes and they are intimately associated with dermis-forming fibroblasts. This complex structure allows for a reliable crosstalk and signaling pathways important for the validation of skin homeostatic and pathological responses closer to real life (144).

1.3.3. In Vivo Skin models

Nowadays, porcine skin is the most used cutaneous animal model, due to the fact that pig skin structurally resembles human skin (145). Porcine skin can be a great model to study cutaneous inflammation and allergic responses since pigs can get rashes and eosinophilic esophagitis (EoE) (146), nevertheless this animal model presents thicker layers and higher content of lipids compared to human skin (147). Some models of rodents have also been employed in research being less expensive and easier to handle but the structural differences and higher hair follicle density of murine skin in comparison to human skin, make them the second choice when it comes to animal testing (even though models of hairless mice exist).

Due to ethical concerns, high variability, excessive cost and difficult in handling, animal testing has had multiple drawbacks and therefore, the use of non-invasive clinical procedures on human volunteers has become the preferable route to evaluate cutaneous responses in vivo, although donor-to-donor variation should be taken into consideration.

1.3.4. Ex Vivo Skin models

Although in the last 20 years the technology moved forward developing reliable skin models that closely simulate human skin in different aspects (3D cutaneous tissues), the skin model that better resembles skin complexity remains *ex vivo* human skin explants, usually obtained from elective abdominoplastic surgeries.

Even though this model presents some limitations like excessive costs, restricted access, donor dependency, cannot be maintained for a long period and lacks circulation and innervation which are present in *in vivo* models, *ex vivo* human skin biopsies are still generally chosen to study dermal absorption studies since the barrier properties of the skin are well preserved even after excision of skin.

One important feature lacking in all skin models that instead is important in human skin is the cutaneous tension. Skin tension has been shown to be a fundamental player in skin homeostasis, structure, functional properties and responses to external stimuli (148). To overcome this problem and bring forward the research in human skin response, a group of researchers patented "Ten Bio". (https://ten-bio.com/) the state of art in cutaneous models: *ex vivo* human skin explants cultured under physiological tension to maintain the cell-cell interaction and tissue-scale tensional homeostasis, to enable reliable evaluation of a multitude of skin responses and biomechanics.

1.4. Scope of Current Research

The human body is continuously exposed to environmental stressors including pollution and to date, avoiding these particular exposures is impractical and unfeasible.

In particular, the skin, due to its location and extent, is considered among the most exposed organs to pollution.

Although the effects of pollution on cardiovascular, respiratory and neuronal systems have been investigated extensively (14), the mechanisms and consequences of skin exposure to air borne pollution are still scarce.

Therefore, this work aimed to investigate the mechanisms of action and damaging effects of the exposure of skin to two specific pollutants: ozone (Chapter 2, 5 and 6) and diesel engine exhaust (Chapter 3 and 4).

Moreover, considering the need to find potential therapies and preventive treatments to quench/counteract OxInflammatory cutaneous damage induced by pollution exposure, we evaluated the topical application of natural (Blueberry extract in Chapter 2, Rosemary and Spirulina extract in Chapter 3) and synthetic compounds (CE Ferulic and Deferoxamine in Chapter 4), alone and in combination as new possible tools to prevent pollution induced skin damage and premature cutaneous aging.

One of the endogenous protective effectors in innate immune responses that helps defend our body from the entry of pathogens is represented by antimicrobial peptides (AMPs) (116).

Since AMPs have been found altered in many inflammatory skin conditions (122, 134–137) and since exposure to pollution has been associated with the development/exacerbation of these same cutaneous diseases (32, 149–151), in this work we wanted, also, to bring light to the relationship between ozone exposure and cutaneous AMPs expression (Chapter 5) in *in vitro*, *ex vivo* and *in vivo* models. As a proof of concept, we also investigated the ozone-induced redox regulation of antimicrobial peptides in a new cutaneous model (Ten Bio) cultured under physiological tension, to examine the role of skin tension in cutaneous responses to pollution (Chapter 6).

1.5. References

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CHAPTER 2: BLUEBERRY EXTRACTS AS A NOVEL APPROACH TO PREVENT OZONE-INDUCED CUTANEOUS INFLAMMASOME ACTIVATION

Pambianchi E, Ferrara F, Pecorelli A, Woodby B, Grace M, Therrien JP, Lila MA, Valacchi G. Blueberry Extracts as a Novel Approach to Prevent Ozone-Induced Cutaneous Inflammasome Activation. Oxid Med Cell Longev. 2020 Aug 13;2020:9571490.

2.1. Abstract

The World Health Organization estimates that 7 million people die every year due to pollution exposure. Among the different pollutants to which living organism are exposed, ozone (O_3) represents one of the most toxic, because its location which is the skin is one of the direct tissues exposed to the outdoor environment. Chronic exposure to outdoor stressors can alter cutaneous redox state resulting in the activation of inflammatory pathways. Recently, a new player in the inflammation mechanism was discovered: the multiprotein complex NLRP1 inflammasome, which has been shown to be also expressed in the skin. The topical application of natural compounds has been studied for the last 40 years as a possible approach to prevent and eventually cure skin conditions. Recently, the possibility to use blueberry (BB) extract to prevent pollution-induced skin toxicity has been of great interest in the cosmeceutical industry. In the present study, we analyzed the cutaneous protective effect of BB extract in several skin models (2D, 3D, and human skin explants). Specifically, we observed that in the different skin models used, BB extracts were able to enhance keratinocyte wound closure and normalize proliferation and migration responses previously altered by O_3. In addition, pretreatment with BB extracts was able to prevent ozone-induced ROS production and inflammasome activation measured as NRLP1-ASC scaffold formation and also prevent the transcripts of key inflammasome players such as CASP1 and IL-18, suggesting that this approach as a possible new technology to prevent cutaneous pollution damage. Our data support the hypothesis that BB extracts can effectively reduce skin inflammation and be a possible new technology against cutaneous pollution-induced damage.

2.2. Introduction

The last estimates of the World Health Organization (WHO) state that 9 out of 10 people living in urban areas are exposed to pollution levels above the healthy recommendations, leading to around 7 million deaths per year [1].

Pollution is a term used to describe a wide array of pollutants (ozone, diesel fuel exhaust, cigarette smoke, and heavy metals) to which living organisms are exposed, and among them, tropospheric ozone (O_3) is one of the most toxic [2].

O_3 concentrations can vary depending on altitude, seasonality, and the geographical location of the area (rural or urban); in some of the most polluted cities, O_3 concentration can reach concentration between 0.5 ppm and 0.8 ppm [2–4].

O_3 is a secondary pollutant because its formation is due to the interaction between the hydrocarbons and oxides of nitrogen released from car exhaust and sunlight (UV), leading to photochemical smog [2]. The effects of O_3 exposure on target organs such as the respiratory tract have been investigated over the last 3 decades, and a strong correlation was clearly revealed between the development of respiratory conditions and ozone exposure. On the other hand, the effects that O_3 has on the skin have been studied only recently [5– 8] suggesting a link between the development of skin conditions and ozone exposure [2, 9–11]. Although O_3 is not a radical per se, it is very reactive and its ability to induce tissue damage is mainly associated with its interaction with the cutaneous lipids present in the stratum corneum (SC), generating molecules such as hydrogen peroxide (H_2O_2) and lipid peroxidation products (4 hydroxynonenal (4HNE)) that can trigger an inflammatory response [12–16].

Recently, a new protein complex, the inflammasome, has been shown to be involved in several inflammatory tissue responses. It can be activated by a wide array of stimuli, such as pathogen and

danger-associated molecular patterns (PAMPs and DAMPs), ionic flux, lysosomal damage, mitochondrial dysfunction, and the production of reactive oxygen species (ROS) [17].

Different inflammasomes have been so far identified (NLRP1, NLRP3, NLRC4, and AIM2); they are all multimeric protein complex part of the innate immune system and which rely on pattern recognition receptors (PPRs) to sense extracellular stimuli and a variety of stress factors [17, 18]. In particular, stimulation of inflammasomes NLRP1 and NLRP3 leads to the assembly of the components of this receptor (NOD-like sensor receptor (NLRs), apoptosis- associated speck-like adaptor protein (ASC), and procaspase 1) yielding to caspase 1-mediated activation which induces the secretion of proinflammatory cytokines such as IL-18 and IL-1β [17]. Chronic activation of the inflammasome has been correlated with the development of different conditions: atherosclerosis, autoinflammatory disease, Parkinson's and Alzheimer's disease [19–24], and recently also with skin diseases such as vitiligo, atopic dermatitis, acne, melanoma, pigmentation, and psoriasis [25–27].

Previous studies provided evidences that the NLRP1 (rather than the NLRP3) inflammasome is involved in epidermis inflammation [28, 29]. Canonical activation of the inflammasome requires the oligomerization of 3 different proteins: sensor receptor NLRP1, the speck-like receptor ASC, and procaspase 1. Once these interactions are established, the autocatalysis of the procaspase 1 in active caspase 1 induces the cleavage of the inactive zymogens pro-IL-18 and pro-IL-1β into their active forms [17]. Our previous work unveiled the ability of ozone (ranging from 0.4 to 0.5 ppm) to activate cutaneous NLRP1 inflammasome [30].

Cells are able to quench, to a certain extent, the production of ROS via endogenous antioxidant enzymes (glutathione peroxidase, catalase, and superoxide dismutase, including the thioredoxin and peroxiredoxin systems) [31–33]; nevertheless, this defensive system can be insufficient when the

exposure to oxidative stimuli is particularly intense or long lasting, as can be the case when living in polluted environments [31, 34].

The use of topical interventions to improve skin defense against the outdoor environment has been studied for many years [15, 16, 35, 36]. Interventional studies indicate that it is possible to delay extrinsic skin aging and cutaneous damage and to improve skin conditions through the administration of specific natural compounds [37, 38] such as vitamin C and vitamin E (tocopherols) [14–16, 39–41], but more research needs to be done in providing more efficient compounds or even possibly mixture combining several natural extracts to be used against pollution-induced skin damage.

In the last few years, research on health benefits associated with blueberry (BB) dietary intake has risen sharply, due to evidence supporting blueberry's beneficial properties in reducing the risk of cardiovascular disease and type 2 diabetes, improving weight maintenance, and neuroprotection and most of all due to their significant antioxidant and anti-inflammatory properties thanks to their abundant polyphenolic compounds [42–44].

Blueberries are rich in polyphenolic compounds, which are widely distributed in nature and are important for plant survival since they attract pollinators and protect the fruit itself against various abiotic and biotic stress sources [45]. The main phytochemicals present in blueberry fruit are polyphenol compounds, including anthocyanins, proanthocyanidin, flavonols, and phenolic acids. Anthocyanins represent the major group of polyphenols in BB, including monoglycosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin [46]. For cultivated blueberries, anthocyanins are mostly concentrated in the blueberry skin, giving the characteristic indigo color; therefore, a small-sized blueberry would have a relatively higher skin surface (with respect to the berry volume) and consequently higher anthocyanin content compared to another bigger blueberry

[47]. Wild lowbush blueberry (*Vaccinium angustifolium* Aiton), typically smaller than most cultivated blueberries, contains anthocyanins in both the skin and the flesh of the berry fruit [48, 49].

The topical application of various blueberries has been studied to reduce telangiectasias, wrinkle formation, and skin aging [50–52], and its topical and medicinal use has been recorded in the Traditional Ecological Knowledge of Native American pharmacopoeia [53]; but the potential advantageous mechanisms of topical application against pollution-induced damage have never been evaluated, especially for Vaccinium species.

Since ozone exposure has already been associated with the development and exacerbation of inflammatory skin diseases ([2, 54, 55]), we hypothesized that the use of blueberry (BB) extracts would be able to quench the ozone-induced activation of cutaneous inflammasome through the prevention of redox imbalance.

In the present study, we have observed that BB extract was able to prevent ozone-induced inflammasome-related genes and proteins levels and also the oligomerization of the inflammasome components. In addition, BB extract pretreatment was efficient in improving epithelial wound healing and decreasing oxidative markers related to ozone exposure.

2.3. Materials and Methods

2.3.1. Ozone Generator

O_3 was generated via electrical corona arc discharge from O_2 and combined with ambient air to flow into a plexiglass box (ECO3 model CUV-01, Italy, Model 306 Ozone Calibration Source, 2B Technologies, Ozone Solution), as previously described [56] and constantly monitored by an ozone detector. The dose was based on our previous studies [15].

2.3.2. Plant Material

A uniform composite of ripe wild low-bush blueberries (*V. angustifolium*, Aiton) grown in maritime provinces of Canada (Quebec, Nova Scotia, Prince Edward Island, and New Brunswick) and the State of Maine (USA) was provided by the Wild Blueberry Association of North America (Old Town, ME, USA). Blueberries (BB) were individually quick-frozen (IQF) within hours of harvest and then stored at −70°C until they were shipped to North Carolina on dry ice, where they were then stored at -80°C. Frozen BB samples were lyophilized before extraction.

2.3.3. Extraction

Extraction of lyophilized BB was conducted according to our published procedures with minor modifications [57]. Ground freeze-dried BB (5 g) were placed in 50 ml centrifuge tubes embedded in ice and homogenized in 30 ml of cold extraction solvent (70% aqueous methanol, 0.5% acetic acid) using a Pro 250 homogenizer (Pro Scientific Inc. Oxford, CT, USA) for 4 min. The obtained slurry was centrifuged (Sorvall RC-6 Plus, Asheville, NC, USA) for 10min at 3452 × g force at 4°C. The supernatant was collected in a 100ml volumetric flask. The residue was then extracted twice with same solvent, and supernatants were collected all together and brought to a final volume

of 100ml with the extraction solvent. An aliquot was evaporated to an aqueous solution and lyophilized [58].

2.3.4. Blueberry Extract Preparation

BB frozen extract powder was solubilized in a volume of dimethylsulfoxyde (DMSO) (Thermo Fisher Scientific, USA, cat no. 20688 99.5%) needed to reach a primary concentration of 100 mg/ml (stock), aliquoted, and stored at -80°C. For each experiment, a new freshly made BB treatment was prepared in complete media from the stock, via serial dilutions: ranging from 0.1 µg/ml to 100 µg/ml based on the skin model utilized. The specific volumes utilized to prepare the serial dilutions of BB extract were also utilized to make the negative controls of DMSO (final concentration 0.01%) in complete media.

2.3.5. Keratinocytes Culture, BB Pretreatment, and Ozone Exposur.

HaCaT cells (AddexBio, USA) were cultured in high-glucose (4.5%) Dulbecco's modified Eagle's medium (Corning, USA) supplemented with 10% FBS (Sigma, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, USA) (complete media) and grown at 37°C in 5% CO_2 and 95% air. For LDH, BrdU, and DCFDH, 10,000 cells were seeded in 96-well plate dishes (Corning, USA); 250,000 cells were seeded into 12-well plates (Corning, USA) to perform the scratch-wound assay; and 1 million cells were instead seeded in 6cm^2 petri dishes (Corning, USA) for RT-PCRs. After 8 hours from the seeding, HaCaT were starved with high-glucose Dulbecco's modifies Eagle's medium supplemented with 1% FBS (Sigma, USA), 100U/ml penicillin, and 100µg/ml streptomycin (Gibco, USA). On the second day, in the morning, the media were removed and either control medium containing the preestablished volume of DMSO (0.01%) or BB extracts

were added at a dose of 10 μg/ml and left for 24 h while cells were incubated at 37°C in 5% CO_2 and 95% air. In the third morning, the BB pretreatment media and the control media were discarded and complete media were added; then, cells were placed in a plexiglass box connected to the ozone generator and exposed to ozone for 1h at the dose of 0.5ppm. After the ozone exposure, samples were harvested for the biochemical and immunochemical assays.

2.3.6. 3D Skin Model Treatment and Ozone Exposure

3D skin model "EpiDerm" (reconstructed human epidermis (RHE)) was purchased from MatTek corporation (EpiDerm, EPI- 200). Upon arrival, the 24 inserts containing 3D skin tissues were transferred into 6-well plates prefilled with 1ml of MatTek Assay medium (provided by MatTek corporation, USA), according to the manufacturer's instructions as previously described [59]. The plates were placed in the incubator overnight (5% CO^2, 37°C) for recovery. On the day after, either control medium containing the preestablished volume of DMSO or BB extracts were added at the dose 100 μg/ml and left for 24 h. On day 3, the media were discarded and complete media were added to the tissues and exposed to O_3 for 5 h at the dose 0.5 ppm. Protein and RNA were collected right after exposure (T0), 6h (T6), and 24 h (T24) postexposure.

2.3.7. Human Skin Explant Treatment and Ozone Exposure

Healthy human skin was purchased from Hunstad/Kortesis/Bharti Cosmetic Surgery clinic. 12mm punch biopsies were taken from the skin and excised using sterile scissors; subcutaneous tissue was removed with a scalpel, and the biopsies were rinsed with Phosphate-Buffered Saline (PBS) containing antibiotics/antimycotic using a sterile technique [30]. Then, the skin explants were transferred to 6-well plates and cultured in complete media, at 37°C in 5% CO_2 and 95% air.

The morning after, either control medium containing the preestablished volume of DMSO or BB extracts were added at the dose of 100 µg/ml and incubated for 24 h. On day 3, the tissues were placed in a plexiglass box connected to the ozone generator and exposed for 5 h at the dose of 0.5 ppm. Skin samples were collected, dehydrated, and embedded in paraffin to perform immunohistochemistry at 0h, 6h, and 24 h upon ozone exposure.

2.3.8. Lactate Dehydrogenase (LDH) Cytotoxicity Assay

After 24h of BB pretreatment at the doses of 0.1, 0.5, 1, 5, and 10 µg/ml for HaCaT and 10, 50, and 100 µg/ml for RHE, supernatants from both the 2D and 3D models were collected, and cytotoxicity was calculated by measuring the amount of the enzyme lactate dehydrogenase (LDH) released in the cytosol, according to manufacturer's instructions (Roche, USA, cat no. 11644793001). Levels of LDH released into the media from keratinocytes and RHE were normalized to the positive control Triton X100, considered 100% LDH release [60].

2.3.9. Scratch Wound Healing Assay

After seeding (250,000 HaCaT cells per 12-well plate), BB pretreatment, and ozone exposure, the adherent monolayer of human keratinocytes was mechanically scratched with a sterile p200 pipette tip and cellular debris were washed off with PBS (Corning, USA). Pictures of the wound area were taken at different time points (0 h, 18 h, and 36 h upon ozone exposure) in three different places, using AxioVision software (40x magnification). Quantification of the wound width was determined by analysis with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and compared to the wound area at 0 h, arbitrarily set at 100 [61, 62].

2.3.10. In Vitro Cell Migration Assay

HaCaT cells were seeded in 10 cm^2 petri dishes and pretreated with BB at the dose 10 μg/ml for 24 h; then, the cells were detached and 50,000 cells were resuspended in serum-free media and seeded in 8μm pore size transwells (QCMTM 24-well Colorimetric Cell Migration Assay kit, Millipore, USA) coated with 0.15 mg/ml bovine collagen IV. After 30 min, 650 μl of complete media was added at the bottom of each well, acting as a chemoattractant. Following ozone exposure (0.5 ppm for 1 h) the transwell inserts were fixed for 10 min with 70% ethanol, stained with 0.02% of Coomassie Blue for 15 min, and rinsed with double-distilled water. HaCaT cells left unmigrated in the upper part of the transwell were gently removed with a cotton swab, and pictures of 3 randomly selected fields were captured using AxioVision software (20x magnification). Automated quantification of the migrated cells was per- formed using ImageJ program as follows: conversion to gray-scale of the image, removal of noise, and adjustment of brightness and contrast (min = 87, max = 167); then, a Phansalkar threshold and watershed were applied [62].

2.3.11. In Vitro Cell Proliferation Assay

Cellular proliferation in HaCaT cells was evaluated by bromodeoxyuridine (BrdU) incorporation assay (Roche, USA, cat no. 11647229001). After seeding (10,000 cells/well in 96-well plate) and upon BB pretreatment and ozone exposure, 20 μl of BrdU labeling solution/well was added to the cells and incubated for 24 h (5% CO_2, 37°C); following, the cells were dried and fixed and the cellular DNA was denatured allowing a better detection by the antibody of the already incorporated BrdU [63]. Then, as indicated by the manufacturer, the monoclonal anti-BrdU peroxidase-conjugated antibody was added to each well and incubated at room temperature for 90

min. The cells were then rinsed with PBS, and the bound peroxidase was photometrically detected after 30 min via substrate reaction and quantified by measuring the absorbance produced at 370 nm (reference wavelength 492 nm).

2.3.12. Dichlorofluorescein (DCF) Assay

10,000 cells were seeded in a 96-well plate, starved overnight, and pretreated with BB 10 µg/ml for 24 h. Prior to ozone exposure, the BB pretreatment was removed and cells were washed with warm PBS. Then, 2′,7′ acetylated dichlorofluorescein (DCF) (Invitrogen, Thermo Fisher Scientific, USA, cat no. C2938) was resuspended in PBS to reach the concentration 10µM and incubated with the cells, in the dark, for 30 min at 37°C, to allow the internalization of the fluorescent probe in the cells. Following, the DCF was removed and 100 µl/well of Dulbecco's modified Eagle's medium without Red Phenol (Corning) supplemented with 1% of FBS (Sigma), 100 U/ml penicillin, and 100µg/ml streptomycin (Gibco) was added. The cells were then exposed to ozone (1 h at 0.5 ppm), and the fluorescence of oxidized DCF dye was evaluated 1 hour after ozone exposure as previously described [64].

2.3.13. ASC Oligomerization Assay

HaCaT cells (1 × 106) were grown in 6 cm^2 petri dishes, starved overnight, and pretreated with BB 10 µg/ml for 24 h. Just prior to ozone exposure, the BB pretreatment was removed, and cells were exposed to ozone for 1h at 0.5ppm and collected after 0h, 1h, and 3h. Cells were washed in cold PBS, gently detached with a scraper, and centrifuged for 5 min at 1500 × g. The cell pellet was resuspended in 500 µl of cold lysis buffer (containing Hepes, KOH 20 mM (pH 7.5), KCl 150 mM, NP-40 1%, 1% protease inhibitor cocktails (Sigma), and PMSF 0.1mM). Following, cell

lysates were centrifuged at 1800 × g at 4°C for 8min, and 30µl of the lysates was collected as input for Western blot analysis (later resuspended in 2X Laemmli buffer, 20% beta-mercaptoethanol), while the remaining volume was centrifuged again for 10 min at 5000 × g at 4°C. Upon centrifugation, to induce crosslinking of the oligomers, the lysates were resuspended in 500 µl of cold PBS containing disuccinimidyl suberate (DSS) (Thermo Fisher Scientific, USA, CAS 68528-80-3 Alfa Aesar) and incubated at RT for 30 min on a rotator. Following, the cellular samples were centrifuged for 10min at 2500 rpm at 4°C, and the crosslinked pellets were then resus- pended in 1X Laemmli buffer and 10% beta-mercaptoethanol. The input and crosslinked samples were boiled for 10 min at 95°C and then analyzed by running samples on a 4–12% SDS-PAGE gel. Bands were digitized, and densitometric analysis was performed using ImageJ software.

2.3.14. Immunocytochemistry

HaCaT cells were grown on coverslips (10,000 cells), starved overnight, and pretreated with BB 10 µg/ml for 24 h. Right before ozone exposure, the BB pretreatment was removed, and cells were exposed to ozone for 1 h, at 0.5 ppm, collected at the different time points, and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at 4°C. Permeabilization was performed with 0.25% Triton X100 in PBS and then blocked in PBS-BSA (Bovine Serum Albumin, Sigma) 1% at room temperature for 1 h. ASC and NLRP1 primary antibodies were then incubated overnight (ASC, cat NBP1-78977 NovusBio, USA 1 : 100 in 0.25% BSA/PBS and NLRP1 sc-166368 Santa Cruz, USA 1 : 50 in 0.25% BSA/PBS) at 4°C. The following day, the coverslips were incubated with the fluorochrome-conjugated secondary antibodies (A11004 Alexa Fluor 568, A11008 Alexa Fluor 488) for 1 h at room temperature. DAPI (D1306 Invitrogen, USA) was utilized to stain the nuclei (1 min at room temperature). Then, coverslips were mounted onto glass slides using PermaFluor

Aqueous Mounting Medium (TA-006-FM Thermo Fisher Scientific) and examined using a Zeiss Z1 AxioObserver LSM10 confocal microscope equipped at 40x magnification. ASC specks were analyzed via ImageJ, and ASC speck number was correlated with the number of nuclei present in the correspondent picture.

As for the skin explants, 4 μm sections were punched and deparaffinized with the use of xylene and rehydrated in decreasing alcohol gradients. 10mM sodium citrate buffer (AP-9003-500, Thermo Fisher Scientific) (pH6.0) was utilized at a subboiling temperature (microwave settings 500 W, 10 min) to induce antigen retrieval and then cooled off for 20 min. Following, 2 washes × 5 min with PBS were performed and then sections were blocked with 5% BSA in PBS at room temperature for 45 min, then incubated overnight at 4°C with primary antibodies for ASC (cat NBP1- 78977 NovusBio, USA) dil. 1:100 in PBS with 2% BSA, NLRP1 (sc-166368 Santa Cruz, USA) at 1:50 dilution in 2% BSA in PBS, and 4HNE (AB5605 Millipore Corp., USA) at 1:400 dilution in 2% BSA in PBS. The following day, 3 washes with PBS of 5 min each were performed, followed by the incubation in the dark with fluorochrome-conjugated secondary antibodies (A11004 Alexa Fluor 568, A11008 Alexa Fluor 488 and A11055 Alexa Fluor 488) at 1:500 dilutions in PBS with 2% BSA for 1h at room temperature. After 3 washes (5min each) with PBS, the sections were mounted onto glass slides using PermaFluor mounting media (Thermo Fisher Scientific) and images were collected by a Zeiss LSM10 microscope equipped with 40x magnification.

2.3.15. Protein Extraction

Cell lysates were extracted in ice- cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1mM EDTA, 0.1% SDS, 5mM nethylmaleamide (Sigma),

and protease and phosphatase inhibitor cocktails (Sigma). Lysates were then centrifuged for 15min at 4°C and 12700 rpm, supernatants were collected, and soluble protein concentration was measured via Quick Start Bradford protein method (Bio-Rad, USA). RHE were collected and harvested in Tissue Protein Extraction Reagent (T-PERTM) (Thermo Fisher Scientific) supplemented with 1% of protease and phosphatase inhibitor cocktails (Sigma, USA). Three cycles of freezing/thawing by moving from liquid nitrogen to 37°C were performed, and following, centrifugation at 12700 rpm for 15 min at 40°C was assessed. Protein content was evaluated on the RHE lysates via Bradford assay (Bio-Rad).

2.3.16. Western Blot Assay

4–12% polyacrylamide SDS gels were loaded with equivalent amounts of protein (previously denatured for 10min at 95°C), which were then separated by molecular size. The gel was electroblotted onto nitrocellulose membranes, and blocking was performed with Tris- buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% nonfat milk, for 1h at room temperature. After overnight incubation with the antibody caspase 1 (2225S cell signaling, USA) diluted 1:1000 in TBS-T with 1% nonfat milk (Bio- Rad, USA), the membranes were incubated for 1 h with the secondary antibody conjugated with horseradish peroxidase and the signal was detected by chemiluminescence (Bio-Rad, USA). Beta-actin (A3854 Sigma, USA) was used for loading control. Bands were digitalized, and densitometry analysis was evaluated via ImageJ software.

2.3.17. RNA Extraction and Quantitative Real-Time PCR (q- rtPCR)

For HaCaT cells and RHE, total RNA extraction was performed via the Aurum Total RNA Mini Kit with DNase digestion (Bio-Rad), according to the manufacturer's protocol. Specifically,

for the RHE, 700 µl of lysis buffer provided by the kit was added and the tissues were homogenized with Precellys tissue homogenizer (9 cycles of 30 s with a 30 s break at 8000rpm at 4°C). The same kit was utilized to extract total RNA from HaCaT samples. cDNA was then generated from 1µg of total RNA, using the iScript cDNA Synthesis Kit (Bio-Rad). Evaluation of the mRNA levels of ASC, Caspase 1, and IL-18 genes was assessed via quantitative real-time PCR using SYBR® Green Master Mix (Bio- Rad) on a LightCycler® 480 Real-Time PCR System (Roche), according to the manufacturer's protocol. Gene expression was quantified via the number of cycles obtained to reach a predetermined threshold value in the intensity of the PCR signal (CT value). Beta-actin was employed as the reference gene, and the samples were compared using the relative cycle threshold (CT). After normalization, the fold change was determined using the 2-ΔΔCT method. The primers used are listed here: (β-actin forward ATTGCCGAC AGGATGCAGA/reverse AGTACTTGCGCTCAGGAGGA, ASC forward ATGCGCTGGAGAACCTGA/reverse TCT CCAGGTAGAAGCTGACCA, Caspase 1 forward CCGTTC CATGGGTGAAGGTA/reverse TGCCCCTTTCGGAATAA CGG, and IL-18 forward TGCAGTCTACACAGCTTCG/ reverse ACTGGTTCAGCAGCCATCTT).

2.3.18. Statistical Analysis

Each of the variables tested is expressed as mean ± standard deviation (SD) of three independent experiments.

Statistical analysis was performed via GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were evaluated by analysis of variance (ANOVA) for single time point or by two-way ANOVA when different time points were included, followed by Tukey's post hoc test. A p value < 0.05 was considered statistically significant.

2.4. Results

2.4.1. Cytotoxic Evaluation of Blueberry (BB) Extract in 2D and 3D Cutaneous Models

The first step of our study was the evaluation of the cytotoxicity of the BB extracts in our 2D and 3D cell culture models. Human keratinocytes and skin 3D models (RHE) were pretreated for 24 hours with different doses of BB extracts (0.1, 0.5, 1, 5, and 10µg/ml for the HaCaT and 10, 50, and 100 µg/ml for the RHE), and cytosolic LDH released was evaluated in the supernatant. Our results showed that BB treatment did not affect cellular viability at all the doses tested in both the models. The average release of LDH for HaCaT cells was around 20% (Figure 1(a)) and 18% for the RHE (Figure 1(b)) with respect to the 100% cell death (Triton X100). Based on these results, we have decided to use the following doses of BB: 10µg/ml for HaCaT and 100 µg/ml for RHE models.

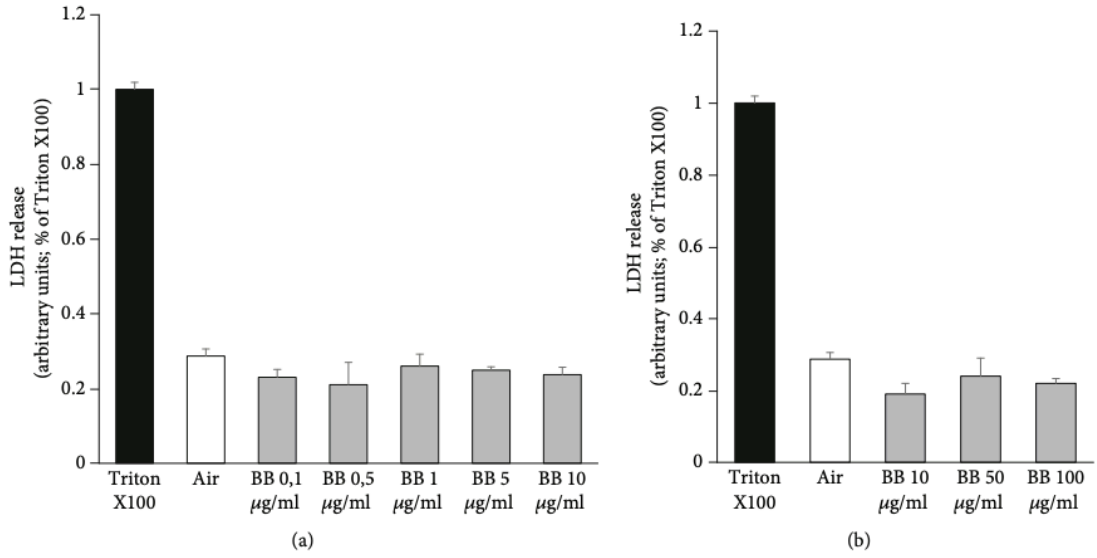

Figure 1: Cytotoxic evaluation of blueberry (BB) extract in 2D and 3D cutaneous models. (a) Keratinocytes cells were pretreated with different doses of BB (0.1, 0.5, 1, 5, and 10 µg/ml) for 24 h. (b) 3D models were pretreated with BB doses ranging from 10 to 100 µg/ml. Cytotoxicity was calculated into the supernatant of pretreated 2D and 3D models, by measuring the amount of LDH released from the cytosol. Data are the average ± SD of three independent experiments.

2.4.2. Effect of Blueberry (BB) Extract on Ozone Modulation of Keratinocyte Migration, Proliferation, and H_2O_2 Production

In our previous study, we demonstrated that O_3 and other pollutants are able to impair the skin repair abilities [65, 66]; therefore, next, we wanted to evaluate the potential properties of BB extract in improving the wound closure impairment induced by O_3.

As shown in Figure 2(a), exposure to O_3 (0.5 ppm for 1 h) significantly impairs the ability of the keratinocytes to recover the scratch wound. Indeed, after ozone exposure, at 18 h time point, the wound was still 75% open with respect to the 30% of the control. This difference was still evident after 36h of ozone exposure, where the wound was still 35% open while the control was completely recovered. Of notice, 24h pretreatment with BB extracts significantly improved the keratinocyte wound closure ability with 50% and 15% open wound at 18 h and 36 h, respectively.

Because the scratch-wound assay is not able to discriminate between a proliferative and migratory effect, we decided to further evaluate both of these cellular responses in our experimental conditions.

As depicted in Figure 2(b), O_3 exposure decreased by 50% the migratory property of the cells after 3 h of exposure and over 50% at 6 h time point. Also, in this case, BB extract pre-treatment was able to completely abolish the effect of O_3 exposure at 3 h time point and to improve the migratory efficiency of about 50% at 6 h.

Similar response was observed also for the proliferative assay as depicted in Figure 2(c). O_3 exposure reduced HaCaT proliferation by 15%, and BB pretreatment was able to rescue this effect.

Considering the antioxidant properties of BB, we have tested whether BB were able to prevent O_3-induced H_2O_2 formation. As shown in Figure 2(d), keratinocyte ROS production after 1 h post-

O_3 exposure was 5-fold higher com- pared to the control, and the pretreatment with BB extracts significantly suppressed this increase (circa 25%).

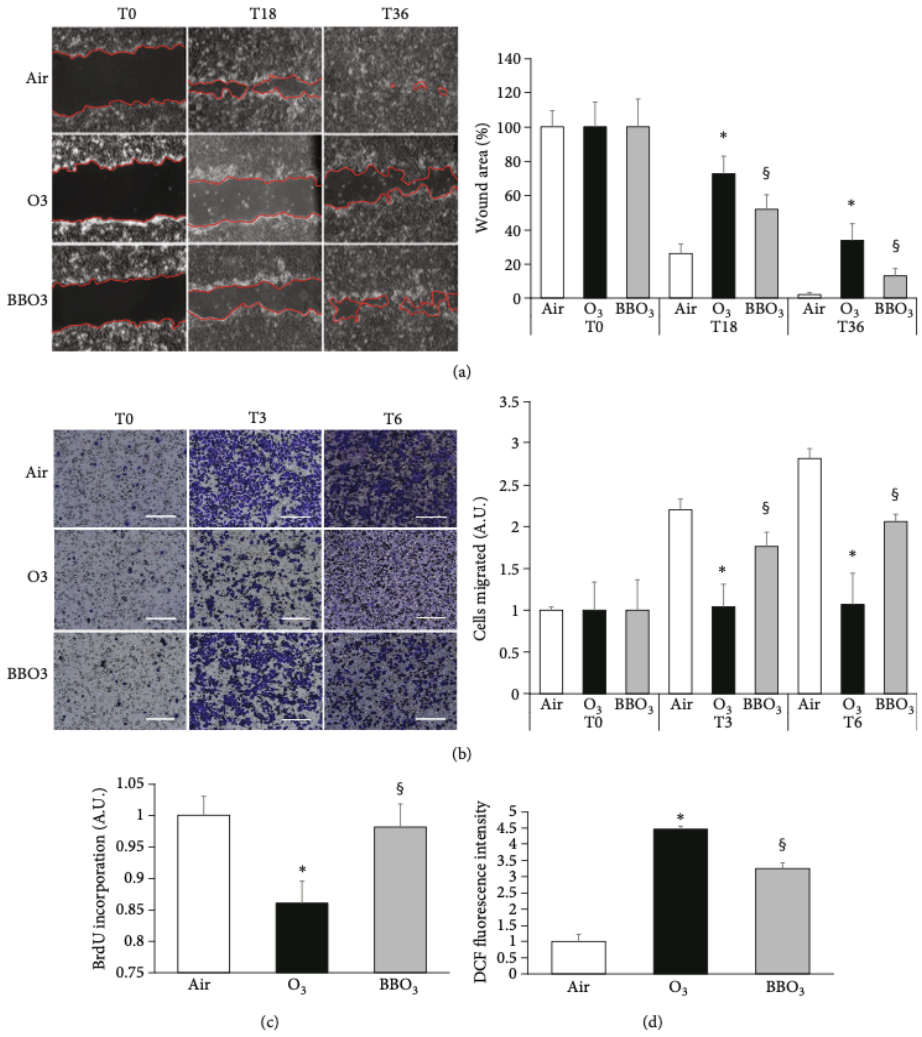

Figure 2: Effect of blueberry (BB) extract on ozone modulation of keratinocytes migration, proliferation, and ROS production. (a) Scratch was performed on confluent monolayer of HaCaT cells, and pictures were taken to measure wound area at different time points (0-18- 36 h). On the left, depiction of the wound after 0, 18, and 36 h. On the right, quantification of the wound area at each time point via ImageJ. Data are shown as percent of 0 h. (b) Representative depiction of migration experiment performed on HaCaT cells, scale bar 200 μm. Cells were seeded in 8 μm pore size transwells, exposed to O_3, and incubated for 0, 3, and 6 h. After fixation, migrated cells were stained with 0.02% Coomassie Blue. On the right, ImageJ quantification of the migrated cells after 0, 3, and 6 h from O_3 exposure. Data are shown as average of 6 picture fields (20x magnification). (c) The growth response of HaCaT cells pretreated with BB was assessed after 24 h after O_3 exposure by BrdU incorporation. (d) H_2O_2 production was evaluated via DCF after 1 h upon O_3 exposure (1 h at 0.5 ppm) in HaCaT cells pretreated with BB. Data are the results of three independent experiments. $*p < 0:05$ air vs. O_3, $§p < 0:05$ O_3 vs. BBO_3 by one- way or two-way ANOVA.

2.4.3. Blueberry (BB) Extract Prevents O_3-Induced Activation of Inflammasome in 2D Skin Models

Cellular proliferative and migratory alterations are phenomena that are present in several inflammatory skin conditions [67, 68]. We have recently showed the ability of O_3 to induce inflammasome activation and oligomerization [30]; now, we wanted to assess whether BB pretreatment could prevent this effect.

As showed in Figures 3, 24 h after O_3 exposure, there was a significant increase in the transcript levels of key players in the inflammasome activation in HaCaT cells. As depicted in Figure 3(c), IL-18, which is an end product of the inflammasome activation, increased around 5-fold at 24 h time point; while Caspase 1 (Figure 3(b)) and ASC (Figure 3(a)) were already induced right after the O_3 exposure (T0) and further increased at the later time point (T24). Of note, pretreatment with BB extracts was able to prevent the induction of ASC (Figure 3(a)), Caspase 1 (Figure 3(b)), and IL-18 (Figure 3(c)) in keratinocytes.

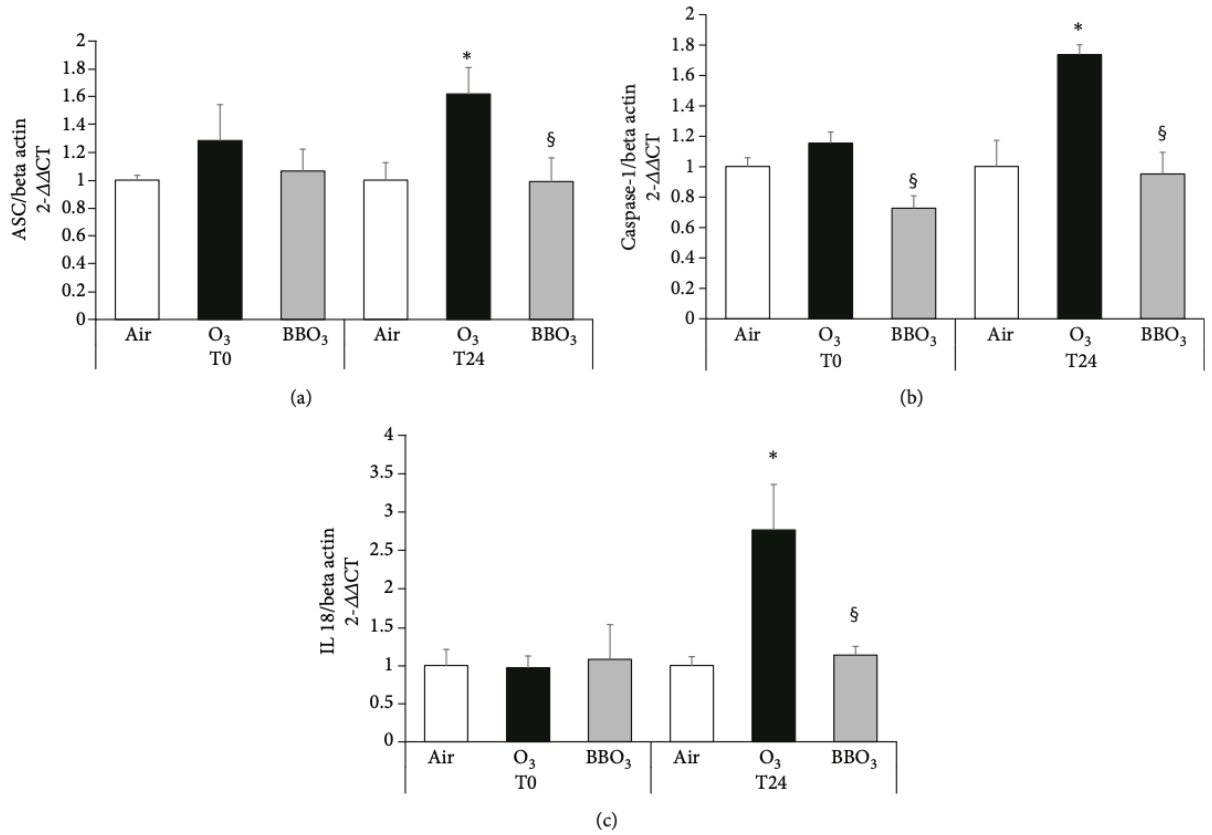

Figure 3: Blueberry (BB) extract prevents O_3-induced activation of inflammasome in 2D skin models. HaCaT transcript levels for (a) ASC, (b) Caspase 1, and (c) IL-18 at 0 and 24 h. Data are the results of three independent experiments. ∗p < 0:05 air vs. O_3, §p < 0:05 O_3 vs BBO_3 by two-way ANOVA.

2.4.4. Blueberry (BB) Extract Prevents O_3-Induced Activation of Inflammasome in 3D Skin Models

Since Caspase 1 is the cardinal player of the inflammasome, which actively cleaved the cytokine proforms, we evaluated its transcripts and protein levels also in the 3D model (RHE) [69].

As expected, the RHE confirmed the keratinocyte results. Indeed, O_3 exposure clearly induced a remarkable increase in Caspase 1 transcripts and protein levels at the different time points analyzed (6 h for mRNA and 0 h, 6 h, and 24 h for proteins). Also, in this case, BB extract pretreatment clearly decreased the O_3 effect, especially after 24 h, with a 4.5-fold decrease in caspase 1 protein levels (Figure 4(a) and 4(b)).

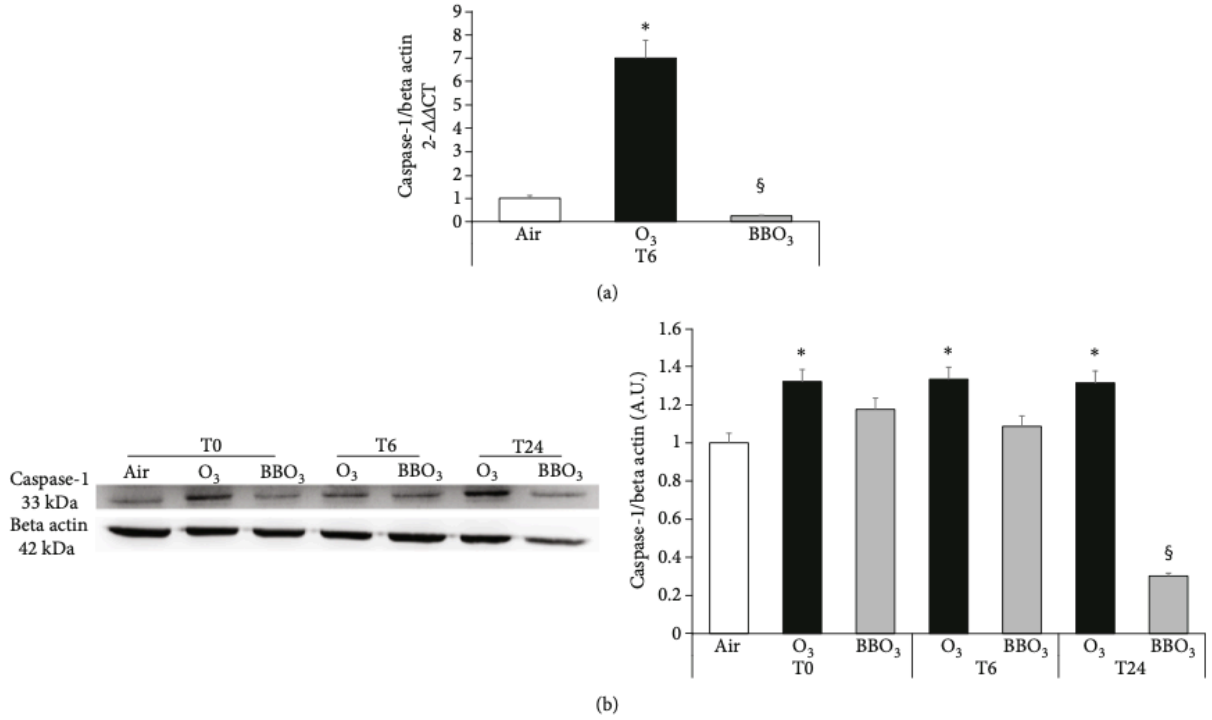

Figure 4: Blueberry (BB) extract prevents O_3-induced activation of inflammasome in 3D skin models. RHE transcript levels for Caspase 1 (a). Protein levels of Caspase 1 in RHE at 0, 6, and 24 h after O_3 exposure (b), on the right, relative quantification via ImageJ analysis. Data are the results of two independent experiments. ∗p < 0:05 air vs. O_3, §p < 0:05 O_3 vs. BBO_3 by two-way ANOVA.

2.4.5. Blueberry (BB) Extract Prevents O_3-Induced Inflammasome Oligomerization in HaCaT Cells

The activation of the NLRP1 inflammasome occurs only following the oligomerization of the scaffold-forming components. To evaluate the effect of BB extract on the oligomerization and inflammasome activation in keratinocytes upon O_3 exposure, we performed immunofluorescence using different dyes (green for ASC, red for NLRP1).

We observed increased perinuclear colocalization of ASC and NLRP1 upon O_3 exposure at 0 h, 3 h, and 6 h, and the BB extract pretreatment significantly decreased the oligomerization of the scaffold (Figure 5(a)). Therefore, it is possible to hypothesize that BB extracts somehow are able

to prevent the formation of ASC oligomers which is the first step for the inflammasome activation [70].

ASC oligomerization assay confirmed these results as shown in Figure 5, where it is possible to appreciate an increase in ASC oligomer and dimer formation (15%) after O_3 exposure while this effect was almost completely rescued by BB extracts (Figure 5(b)).

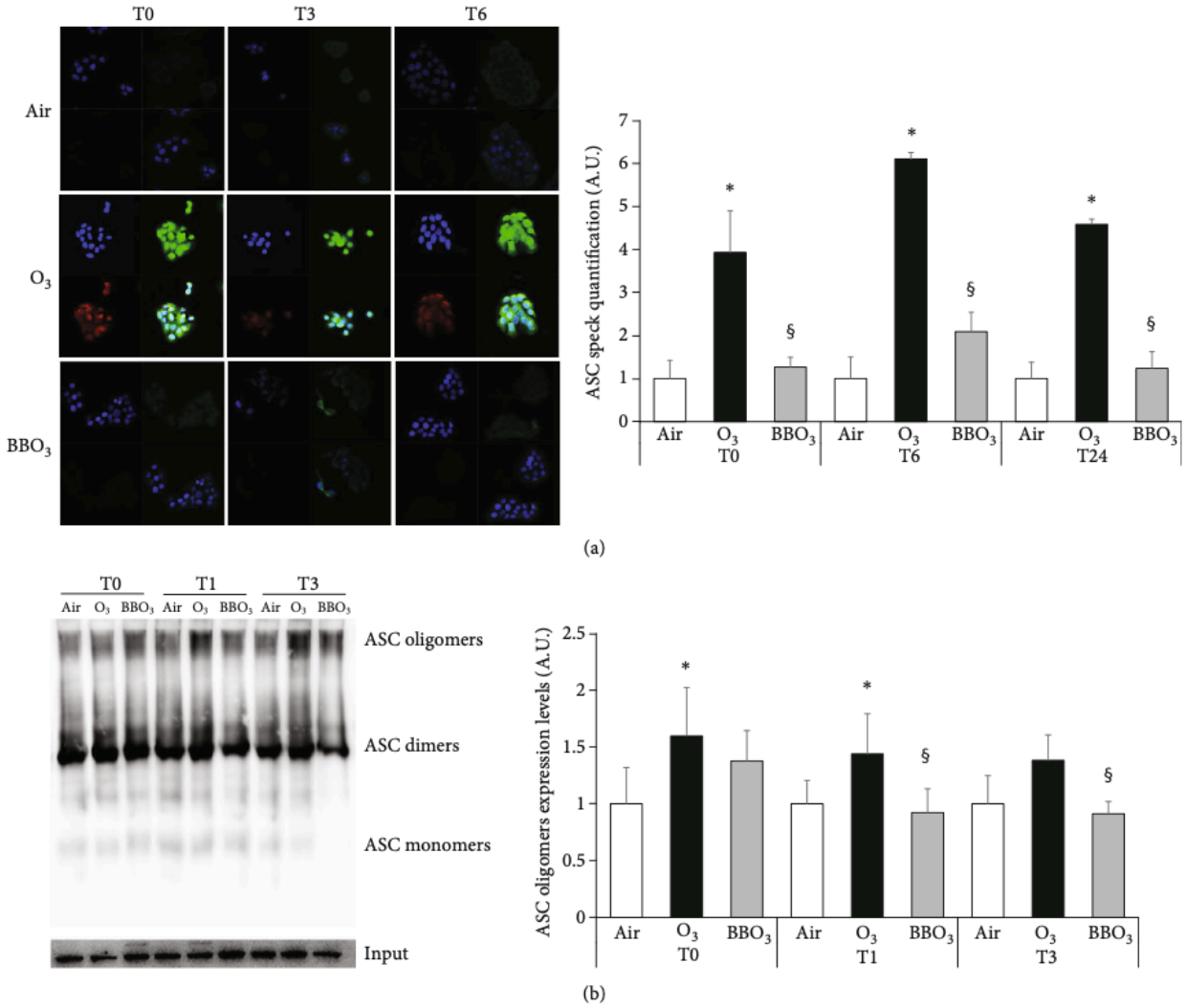

Figure 5: Blueberry (BB) extract prevents O_3-induced inflammasome oligomerization in HaCaT cells. (a) Immunofluorescence staining of ASC (green), NLRP1 (red), DAPI (blue), and merge in HaCaT cells pretreated with 10 μg/ml of BB for 24 h then exposed to 0.5 ppm of O_3 for 1h at 0, 3, and 6h postexposure (40x magnification). On the right, ASC speck formation was quantified using ImageJ. (b) ASC oligomers, dimers, monomers, and input protein levels in keratinocytes at 0, 1, and 3 h after O_3 exposure (1 h, 0.5 ppm). On the right, depiction of ASC oligomers and dimer quantification using ImageJ. Data are the results of three independent experiments. $*p < 0{:}05$ air vs. O_3, $§p < 0{:}05$ O_3 vs. BBO_3 by two-way ANOVA.

2.4.6. Blueberry (BB) Extract Prevents O_3-Induced Inflammasome Activation and Oxidative Stress in Ex Vivo Human Skin Biopsies

To further validate our previous data on 2D and 3D skin models, we decided to perform our experiments on a more complete cutaneous model represented by the ex vivo human skin explants. The biopsies were pretreated with 100 μg/ml of BB extracts for 24 h and exposed to 0.5 ppm of O_3 for 5 hours. As depicted in Figure 6(a); BB extract topical application was able to quench the increased protein levels of both ASC (green) and NLRP1 (red) and their nuclear colocalization 6h after O_3 exposure.

Since redox signaling is an important part of the inflammatory pathway [71], we also evaluated the level of 4 hydroxynonenal (4HNE) as a reliable marker of oxidative damage and lipid peroxidation. As depicted in Figure 6(b), O_3 significantly increases the 4HNE levels immediately after the exposure and this effect was still visible at the later time points. BB extract pretreatment was able to prevent the 4HNE formation.

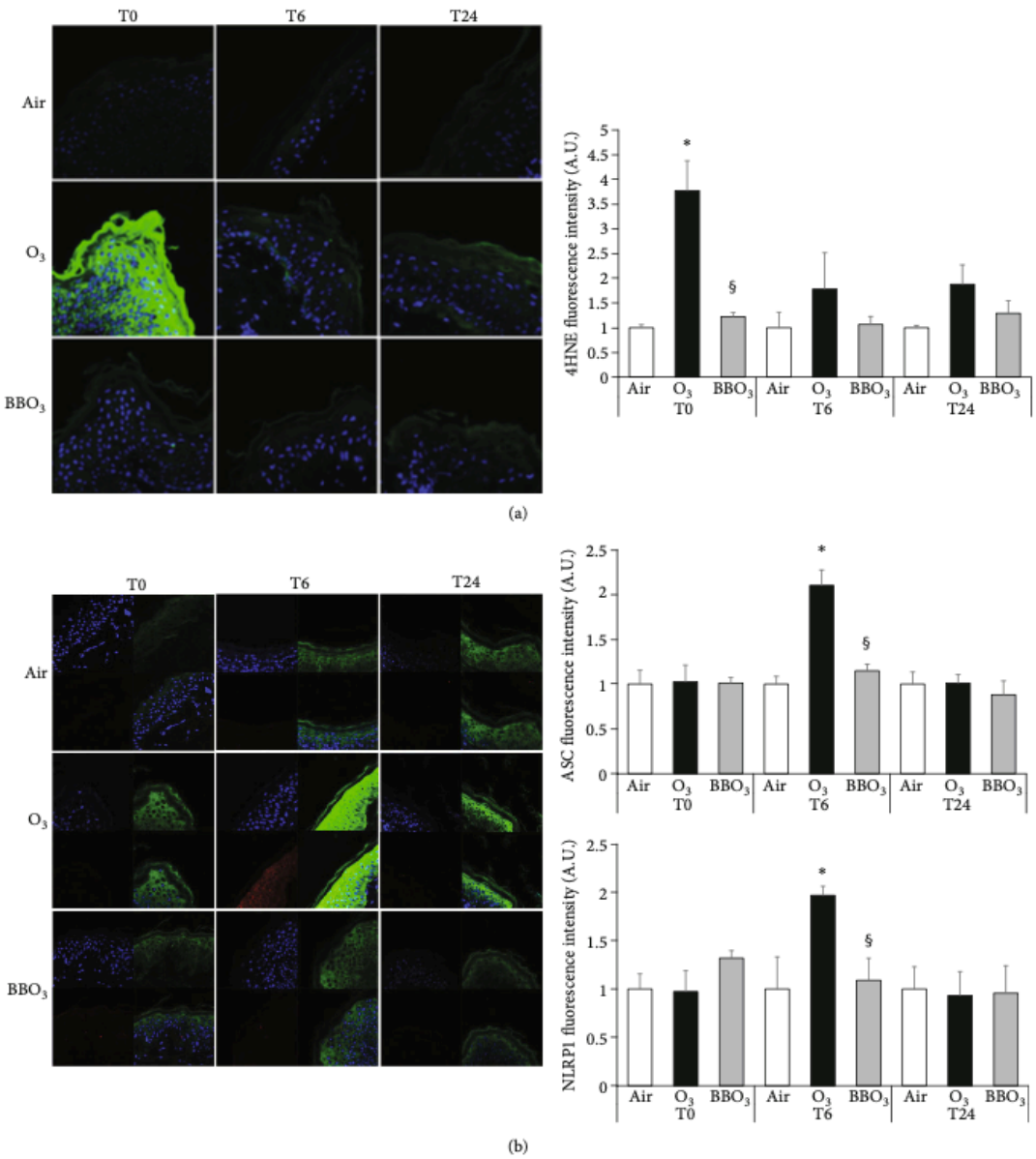

Figure 6: Blueberry (BB) extract prevents O_3-induced inflammasome activation and oxidative stress in ex vivo human skin biopsies. (a) Immunofluorescence staining for 4HNE (green), DAPI (blue), and merge in ex vivo human skin biopsies exposed to O_3 for 5 hours, 0.5 ppm, directly after exposure, 6 h and 24 h postexposure (40x magnification). Quantification of the fluorescence is depicted in the right panel. (b) Immunofluorescence staining of ASC (green), NLRP1 (red), DAPI (blue), and merge in ex vivo human skin explants pretreated with 100μg/ml of BB for 24h then exposed to 0.5ppm of O_3 for 5h at 0, 6, and 24h postexposure (40x magnification). On the right panels, semiquantifications of the intensities of the signals of 4HNE (a) and ASC and NLRP1 (b) using ImageJ. Data are the results of three independent experiments. $*p < 0{:}05$ air vs. O_3, $§p < 0{:}05$ O_3 vs. BBO_3 by two-way ANOVA.

2.5. Discussion

It is now well accepted and documented that exposure to environmental pollution affects our health and this phenomenon is not localized to few urban centers, but it is a global emergency that has been estimated to decrease human life span by 10-15 years [72]. There are several pollutants to which living organisms are daily exposed, and among them, O_3 has been shown to be one of the most toxic. It should be mentioned that the O_3 derived from the stratospheric–tropospheric exchanges accounts for 20% of its tropospheric level. Nowadays, ozone is mainly produced by complex photochemical reactions involving solar radiation and anthropogenic pollutants [73, 74]. Photochemical ozone is formed by reactions involving solar radiation and anthropogenic pollutants (methane, non-methane volatile organic compounds, and carbon monoxide) in the presence of nitrogen oxides while in less polluted environments, ozone is produced in the presence of sunlight (at wavelengths < 424 nm), through the photolysis of NO_2 [73].

In addition, as recently reported, the levels of O_3 are still increasing making the effect of this pollutant to our health a real concern not only for the present but even more for the future [75].

Being a gas, the toxicity of O_3 has been well documented in the respiratory tract [76–78] and in the last 2 decades, its effect on cutaneous tissues has been also investigated since the skin is another organ directly exposed to this agent [2, 5–8].

O_3 is a small molecule with strong oxidizing properties, with a redox potential of +2.07, able to oxidize a wide range of compounds including rubber. Indeed, it is too reactive to penetrate the tissues and as shown first for the lungs and more recently even for the skin [3], its ability to affect the tissues is mainly due to the generation of bioactive compounds that are formed by its interaction with the biological systems. Several studies have confirmed the ability of O_3 to oxidize cell membrane, generating radical species that can damage the tissues. The generation of redox-

mediated molecules in the stratum corneum can eventually affect the deeper layers of the skin, modulating important physio pathological skin pathways [12].

The cutaneous proinflammatory effect of O_3 is mainly driven by its ability to activate nuclear factor kappa-light-chain enhancer (NF-κB) which is a master regulator of proinflammatory responses. In the last few years, different players of inflammation have been studied and in particular, the inflammasome machinery has been recognized to play a key role in inflammatory skin conditions [25–27]. The activation of the inflammasome occurs in response to different stimuli including cell damage and pathogen-associated molecular patterns (DAMPs and PAMPS) as well as prooxidant stimuli such as O_3 [17, 18], and also, in this case, NF-κB activation plays an important role.

In our previous work, we have shown the inflammatory and oxidative effect of O_3 on the skin and its ability to activate NF-κB and increase oxidative damage [30]. The aim of the present study was to evaluate the eventual protective effect of BB extracts against O_3-induced skin damage. Indeed, the exponential increase of pollution levels has aroused the need to find effective molecules that can be used as defensive agents against pollution-induced skin damage and premature cutaneous aging.

The antiaging research has been focused on an enormous range of products (natural or synthetic) with the aim to prevent, postpone, or reverse cutaneous aging signs. In general, those molecules can act in 2 ways, either quenching directly the radicals or by activating the cellular endogenous defensive system nuclear factor (erythroid-derived 2)-like 2 (NRF2) [79–81].

BB has been shown to be able to activate the nuclear factor erythroid-2-related factor 2 (NRF2) [82] and also quench radical formation [83] in several tissues, and it is now a general belief that BB beneficial effects are not limited to the “chemical” antioxidant properties, but mainly to its

ability to induce an active cellular defense [84]. Therefore, as a follow-up of our recent study [30], in the present work, we were interested in evaluating the ability of BB extracts to prevent O_3-induced skin inflammasome activation. In addition, BB topical application has been shown to stimulate collagen synthesis and prevent chronological skin aging [50–52, 85]. Among the wide array of natural antioxidant substances, we decided to focus our attention on blueberries because of their complex phytochemical profiles that have already shown to quench free radicals [42, 43]. The ability of BB to activate the NRF2 pathway could also indirectly affect the inflammasome activation as altered redox homeostasis can be also a trigger for this inflammatory pathway [18, 86]. We should also mention that the crosstalk between NRF2 and NF-κB is crucial for maintaining the cellular responses and to resolve an inflammatory status. An imbalance between NRF2 and NF-κB pathways can lead to chronic inflammation; therefore, the activation of NRF2 by BB extracts can prevent NF-κB activation and modulate the tissue inflammatory status [87].

We were able to show that BB extracts were not toxic in the tested in vitro and ex vivo models. We found that BB extracts improved the recovery of scratch wound closure in O_3-treated cells. The impairment of O_3 on cutaneous wound healing has also been described previously by Lim et al., in aged mice [66], showing that the combination of aging and O_3 exposure is able to reduce the levels of TGFβ, a key player in tissue would healing [66, 88]. Considering that in vitro wound healing is mainly a test to evaluate the proliferative and migratory properties of the cells, we assessed whether O_3 could affect any of those pathways and the eventual role of BB extracts. Our data showed that O_3 was able to decrease both proliferation and migration of the cells while the BB pretreatment prevented these effects probably either via the activation of a cellular defensive system or less probably, by a direct interaction with the free radicals generated by O_3.

It is possible that the inhibition of proliferation is a consequence of their ability to activate NRF2 which has an inhibitory influence on NF-κB that is one of the regulators of cyclin D1 expression, a key protein for cellular proliferation [61, 85, 89]. Although we did not evaluate the effect of BB on NFR2 activation, we showed a significant decrease in H_2O_2 by BB after O_3 exposure, although the use of the probe DCF to measure H_2O_2 is controversial [90].

The overproduction of ROS and NF-κB activation has often been linked to the development of inflammatory responses related to skin and other tissues. Recently, the involvement of the inflammasome machinery has been studied in relation of several skin conditions. The activation of the inflammasome requires two steps: a "priming" step that induces transcriptional upregulation of NLRP1, pro-IL-18, and pro-IL-1β, via NF-κB and AP-1 signaling and post- translational modifications of NLRP1 (phosphorylation, ubiquitination), followed by a second signal that yields to conformational changes in the NOD-like receptor structure which allows for the binding with the adaptor ASC and assembly of the whole complex [17].

To better understand the molecular mechanisms responsible for the BB effect on O_3-induced inflammasome activation and oligomerization, we pretreated the keratinocytes with BB extracts, before exposing them to O_3 and then, we evaluated transcript levels of IL-18, ASC, and Caspase 1 as well as ASC specks and oligomers. Our data evidenced the remarkable activity of BB in preventing the increased aforementioned mRNA levels and the oligomerization of the proteins NLRP1 and ASC, upon O_3 exposure. These data confirmed the study by Wang et al. which demonstrated reduced gene expression levels of NLRP, Caspase 1, ASC, and proinflammatory cytokines IL-1β, TNF-α, IL-6, and iNOS in macrophages pretreated with BB extracts and then challenged with lipopolysaccharide [91].

As keratinocytes grown in monolayer culture do not undergo terminal differentiation, which results in the formation of the outermost layer of the skin, the stratum corneum (SC), and since this layer is the main target of O_3 [92], we have performed our experiments also in RHE (reconstructed human epidermis). The use of RHE is strongly recommended to study in vitro cutaneous protection, since is not only represented by all the living epidermis layers but it also includes the SC [69], and our data confirmed the ability of BB extracts to prevent O_3-induced Caspase 1 increase at both transcripts and protein levels. Although, RHE is a very reliable in vitro model, it still does not present all the SC layers making it more accessible to outdoor stressors. In addition, the lack of other skin cells such as melanocytes, immunity cells, might compromise skin responses [69].

For this reason, we further confirm our data in an ex vivo human skin explant which presents a normal skin barrier, functional basal layer, a mature SC, and all the cell types and cutaneous appendages present in in vivo human skin [93].

As demonstrated in 2D and 3D models, O_3 was able to activate the inflammasome machinery also in the human skin explants, and BB extracts prevented this induction. In the specific, O_3 exposure not only induced the oligomerization of the inflammasome components but also increased the levels of lipid peroxidation as detected by 4HNE protein adduct formation (as previously reported [16]) while the pretreatment with BB extract was able to prevent the O_3-induced increase of 4HNE, ASC, and NLRP1 and their oligomerization. These data are in line not only with the theory that O_3 is able to affect skin via the formation of lipid peroxidation products but also with the theory that this mechanism is redox mediated.

In conclusion, we have demonstrated, by the use of three different skin models, that BB extracts are able to prevent the inflammatory and oxidative skin damage induced by O_3, making blueberries

a possible innovative natural ingredient for new technologies against cutaneous pollution-induced damage.

2.6. References

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CHAPTER 3: NOVEL SPRAY DRIED ALGAE-ROSEMARY PARTICLES ATTENUATE POLLUTION-INDUCED SKIN DAMAGE

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Novel spray dried algae-rosemary particles attenuate pollution-induced skin damage. Molecules. 2021 Jun 22;26(13):3781.

3.1. Abstract

The present study investigated the effect of spray-dried algae-rosemary particles against pollution-induced damage using ex-vivo human biopsies exposed to diesel engine exhaust (DEE). For this, the complexation of hydroalcoholic rosemary extract with Chlorella (RCH) and Spirulina (RSP) protein powders was conducted. The process efficiency and concentration of rosmarinic acid (RA), carnosic acid (CA), and carnosol (CR) phenolic compounds of both products were compared. The RSP spray-dried production was more efficient, and RSP particles presented higher CR and CA and similar RA concentrations. Therefore, spray-dried RSP particles were prioritized for the preparation of a gel formulation that was investigated for its ability to mitigate pollution-induced skin oxinflammatory responses. Taken altogether, our ex-vivo data clearly demonstrated the ability of RSP gel to prevent an oxinflammatory phenomenon in cutaneous tissue by decreasing the levels of 4-hydroxynonenal protein adducts (4HNE-PA) and active matrix metalloproteinase-9 (MMP-9) as well as by limiting the loss of filaggrin induced by DEE exposure. Our results suggest that the topical application of spirulina-rosemary gel is a good approach to prevent pollution-induced skin aging/damage.

Keywords: cosmeceuticals; pollution; phytochemicals; herbs

3.2. Introduction

Striking evidence has demonstrated that air pollution can cause severe damage to human skin, triggering disorders such as inflammatory reactions, allergies, skin aging and cancer, due to alterations in physiological parameters that impact skin health [1,2,3,4]. In this regard, there is increasing consumer demand for cosmeceuticals based on natural products with the aim to improve skin beauty and protect skin from environmental insults [5,6,7,8]. A common pathway to identify promising sources of active phytochemicals involves the search for plant species traditionally used in folk or traditional medicine. For example, rosemary (Rosmarinus officinalis) is an aromatic plant that has long been used in herbal remedies due to its multiple biological activities including antioxidant, antimicrobial, anti-inflammatory [9,10,11] and skin renewal properties [12]. It is recognized as a major source of bioactives, mainly phenolic compounds such as rosmarinic acid, carnosic acid, and carnosol [13] and has attracted interest from food, pharmaceutical, and health-related industries worldwide [10,14]. In addition, algae-derived products are now part of a growing market, because algae can substitute for chemical and synthetic components in eco-friendly cosmeceuticals [15,16].

However, health-promoting phytochemicals are susceptible to thermal and oxidative degradation. Therefore, technological solutions are required to produce smart products with preserved natural phytoactives able to deliver active molecules in an easy-to-handle, stable format. In this context, we demonstrated that spray drying microencapsulation applied to the production of protein–polyphenol particles is an efficient tool to produce natural dried products with preserved characteristics and enhanced attributes [17,18,19]. This straightforward strategy complexes safe, wholesome proteins to a wide range of natural polyphenol-rich sources to create functional, clean-label products with concentrated levels of phytochemicals for multiple applications.

In this study, we developed spray-dried algae-rosemary particles using a hydroalcoholic rosemary extract complexed to algae-derived protein powders. Algae have recently been highlighted as natural rich sources of value-added metabolites with beneficial skin applications—in particular, Spirulina (*Arthrospira platensis*) and Chlorella (*Chlorella sp.)*-derived products [15]. Hence, by combining two recognized natural sources of metabolites with skin protective properties, our hypothesis is that we can achieve highly efficient formulations against pollution-induced skin damage. For this, spray-dried Spirulina-rosemary and Chlorella-rosemary particles were produced, and the process efficiency and concentration of rosmarinic acid, carnosic acid, and carnosol phenolic compounds of both products were compared. These two parameters were used as the selection criteria to prioritize a candidate experimental group that was used for the preparation of a gel formulation further investigated for its ability to mitigate pollution-induced skin oxinflammatory responses [20]. To the best of our knowledge, this is the first report that evaluates the effect of rosemary phenolics complexed to algae protein on the attenuation of pollution-induced skin damage.

3.3. Results

3.3.1. Solids Recovery and Characterization of Spray-Dried Algae-Rosemary Particles

Chlorella and Spirulina proteins were tested for their ability to mitigate spray drying stickiness and enhance production efficiency when complexed to hydroalcoholic rosemary extract. As shown in Figure 1, both experimental groups RCH and RSP achieved solids recovery higher than 50%, considered the threshold of successful spray drying operations [21]. However, the solids recovery of Spirulina-rosemary particles was higher ($p < 0.05$) when compared to Chlorella-rosemary spray drying process.

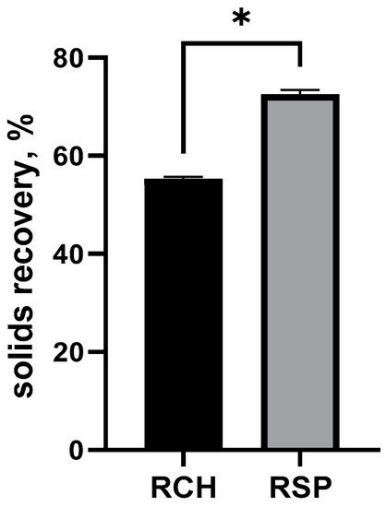


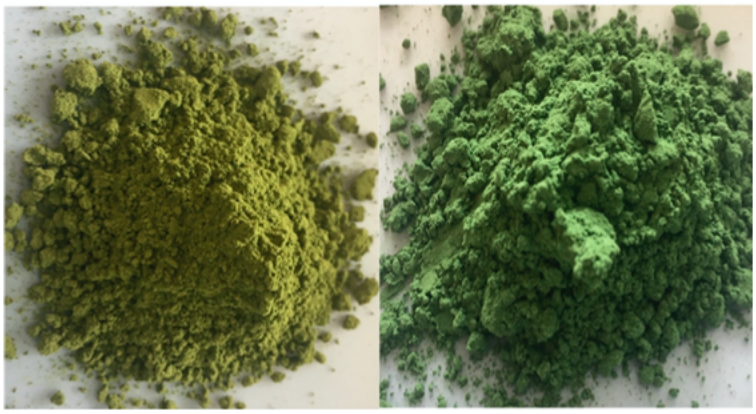
Figure 1. Solids recovery (%) of spray-dried algae-rosemary particles. RCH: Chlorella-rosemary particles and RSP: Spirulina-rosemary particles. Bars indicate standard deviation. Samples marked with an asterisk are significantly different: * $p < 0.05$.

RCH and RSP presented similar ($p > 0.05$) water activity (aw) levels (Table 1), which coincide with typical values of stable spray-dried products [22]. In addition, both RCH and RSP particles presented visually intense, attractive green color (Figure 2), confirmed by spectrophotometer color parameters that demonstrate samples with similar lightness L*, on the green side of the color spectrum (Table 1).

Table 1. Water activity and color parameters of spray-dried algae-rosemary particles [1].

	RCH	RSP
Water activity	0.323 ± 0.001	0.314 ± 0.001
L*	50.12 ± 1.54	50.96 ± 0.31
a*	−5.93 ± 0.63 [b]	−7.34 ± 0.06 [a]
b*	14.73 ± 1.55 [a]	8.42 ± 0.10 [b]

[1] **RCH: spray-dried Chlorella-rosemary particles and RSP: spray-dried Spirulina-rosemary particles.** Superscripts with different letters ([a,b]) in the same row are significantly different ($p < 0.05$). Results are shown as mean ± standard deviation. CIELAB parameters: L*: lightness; color coordinates: a*—green to red; b*—blue to yellow.



(a) (b)

Figure 2. Visual aspect of spray-dried (a) Chlorella-rosemary (RCH) and (b) Spirulina-rosemary (RSP) particles.

3.3.2. Concentrations of Rosmarinic Acid, Carnosol and Carnosic Acid

Figure 3 shows that RSP particles captured significantly higher concentrations of carnosic acid and carnosol ($p < 0.001$), and a similar concentration of rosmarinic acid when compared to RCH particles. Thus, considering the increased solids recovery, similar RA and higher CR and CA concentrations, RSP was selected for gel formulation.

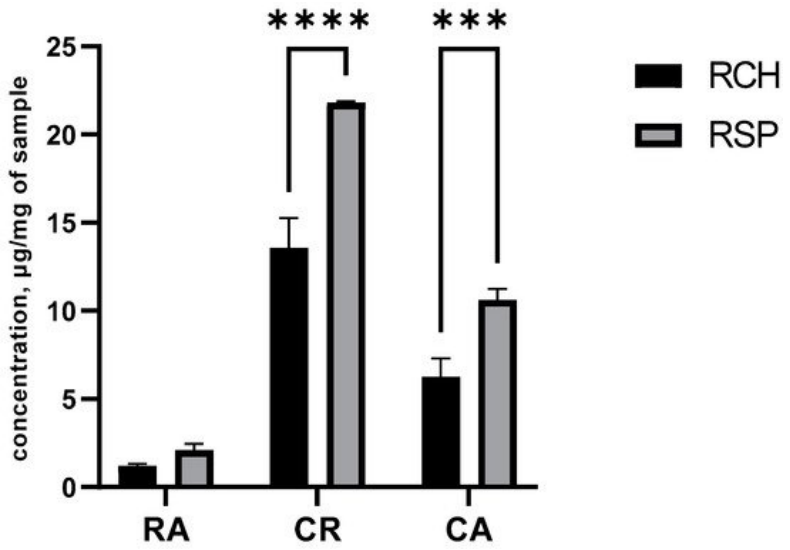


Figure 3. Concentrations of rosmarinic acid (RA), carnosol (CR) and carnosic acid (CA) in spray-dried algae-rosemary particles. RCH: Chlorella-rosemary particles and RSP: Spirulina-rosemary particles. Concentrations were calculated as µg/mg of spray-dried sample. Bars indicate standard deviation. Samples marked with asterisk are significantly different: *** $p < 0.001$; **** $p < 0.0001$.

3.3.3. Evaluation of Pollution-Induced Skin Damage

As depicted by the immunofluorescence assay in Figure 4a, DEE significantly promoted the formation of cutaneous 4HNE-PA, a well-known marker of lipid peroxidation-mediated protein damage [23]. In particular, the 4HNE-PA signal was evident in the stratum corneum and epidermis, which are the main targets of pollution-mediated damage [24]. Of note, 4HNE-PA immunofluorescent reaction was also slightly detected in the dermal layer of DEE-exposed skin (Figure 4a). Quantification of fluorescence intensity confirmed the increase of 4HNE-PA levels after DEE exposure (T0, 30′) and, although with a declining trend, remained significantly higher in pollutant-exposed tissues than in air-exposed skin for up to 24 h after DEE insult (Figure 4b). The topical pretreatment for 24 h with RSP gel significantly prevented 4HNE-PA formation in DEE-exposed skin tissues, to a similar extent for all time points tested (Figure 4b). No effect of RSP gel and vehicle (Veh) was noticed in air-exposed skin explants (Figure 4a,b).

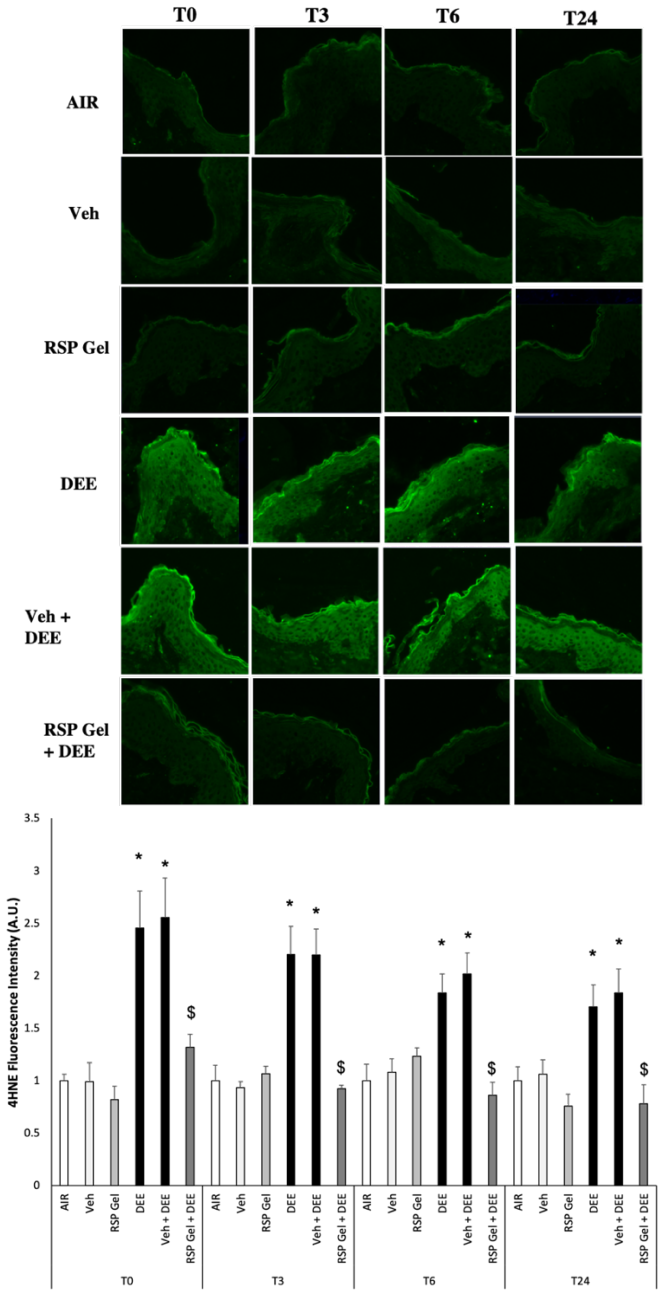


Figure 4. Skin exposure to DEE increases protein damage mediated by lipid peroxidation (i.e., 4HNE), while topical application of RSP gel prevents this effect. Levels of 4HNE-PA in ex vivo human skin explants untreated or pre-treated with RSP gel (a) Green fluorescence staining represents 4HNE-PA immunoreactivity. Original magnification 40×. (b) Semi-quantification of the immunofluorescence intensities performed by ImageJ is shown in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), * $p \leq 0.05$ Air vs DEE or Veh + DEE; $ $p \leq 0.05$ DEE or Veh + DEE vs RSP Gel + DEE, by ANOVA.

Accumulation of 4-HNE-modified proteins in the skin tissue can trigger cascades of proinflammatory signals and mediators including extracellular matrix-degrading metalloproteinases (MMPs) [25]. Therefore, we next evaluated the activation of MMP-9 by DEE exposure in the presence or not of the RSP gel. As depicted in Figure 5a, the dermal layer of DEE-exposed skin showed a higher immunoreaction for the active form of MMP-9 than that observed in skin tissues not exposed to DEE. The quantification of fluorescence signal demonstrated that the increase of MMP-9 active form was more evident 30 min after DEE exposure, and its increase was still significant at 3, 6 and 24 h (Figure 5b). As observed for 4HNE-PA, 24 h pretreatment with algae-rosemary gel was able to inhibit the increase in active MMP-9 levels in skin tissues exposed to DEE (Figure 5a,b). No effect of the vehicle (Veh) only treated tissues was noticed.

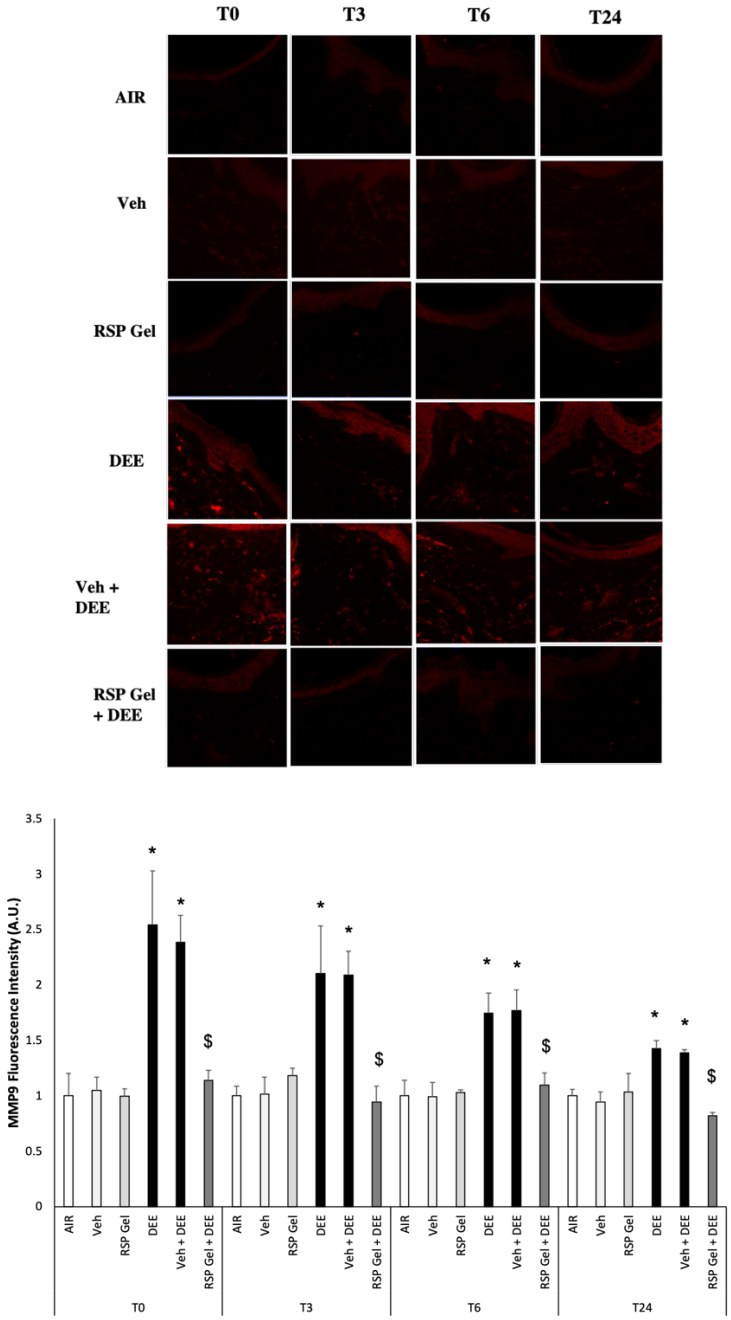


Figure 5. Skin exposure to DEE increases the levels of matrix metalloproteinase-9 (MMP-9), while topical application of RSP gel prevents this effect. Levels of MMP-9 in ex vivo human skin explants untreated or pre-treated with RSP gel (a) Red fluorescence staining represents MMP-9 immunoreactivity. Original magnification 40×. (b) Semi-quantification of the immunofluorescence intensities performed by ImageJ is shown in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), * $p \leq 0.05$ Air vs DEE or Veh + DEE; $ $p \leq 0.05$ DEE or Veh + DEE vs RSP Gel + DEE, by ANOVA.

Skin exposure to pollution can also impact cutaneous barrier functions [26]. As shown in Figure 6a, DEE exposure clearly decreased the filaggrin levels (a key protein involved in maintaining the stratum corneum properties). Indeed, we observed a clear decrease in immunofluorescence staining for filaggrin in the stratum corneum of DEE-exposed skin (Figure 6a), and this was evident already 30 min after DEE exposure and persist also after 3 and 24 h post-exposure (Figure 6b). Notably, 24 h-pretreatment with RSP gel prevented the stressor-mediated decline in filaggrin levels (Figure 6a,b).

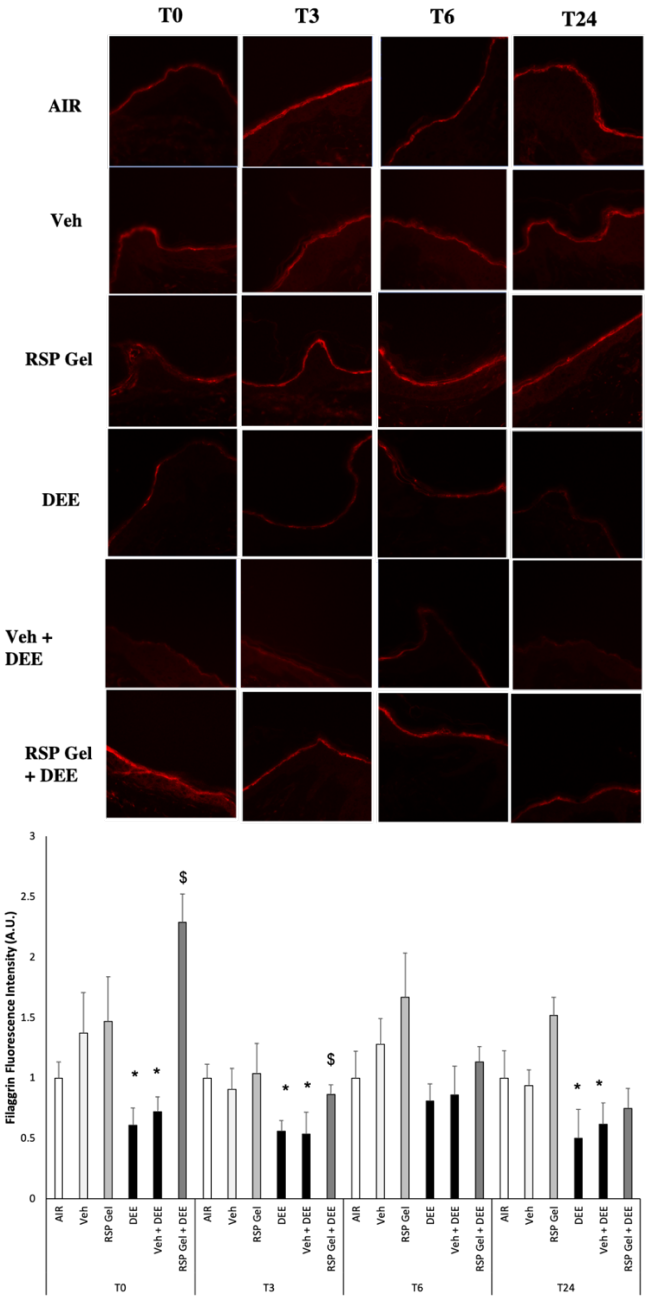


Figure 6. Skin exposure to DEE decreases filaggrin levels, while topical application of RSP gel prevents this effect. Levels of filaggrin in ex vivo human skin explants untreated or pre-treated with RSP gel (a) Red fluorescence staining represents filaggrin immunoreactivity. Original magnification 40×. (b) Semi-quantification of the immunofluorescence intensities performed by ImageJ is shown in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), * $p \leq 0.05$ Air vs DEE or Veh + DEE; $ $p \leq 0.05$ DEE or Veh + DEE vs RSP Gel + DEE, by ANOVA.

3.4. Discussion

Spray drying is extensively used in the food and pharmaceutical industries to produce plant-derived products in a scalable, cost-effective manner. It presents several advantages such as short drying time, lower electricity consumption, and allows the production of regularly shaped particles obtained in a single-step procedure [27]. The solids recovery (%) is an important spray drying parameter defined as the relationship between the solids content of the final powder and the initial solid content in the feed mixture. This parameter can be affected by heat-induced reactions that lead to stickiness and consequent particle adhesion to chamber walls, causing product losses and drying difficulties. In other words, higher solids recovery translates into more powder and increased efficiency, and for this reason, it is frequently referred to as drying efficiency or drying yield [22].

One of the main factors affecting drying efficiency is the type and concentration of wall material [28]. Indeed, because proteins are efficient film-forming molecules with important surfactant activity, they preferentially migrate to the air–water interface and envelope bioactive compounds, forming a physical barrier that enables higher drying efficiency [29]. In this study, we showed that the solids recovery of Spirulina-rosemary particle production was higher ($p < 0.05$) when compared to Chlorella-rosemary spray drying process. This higher recovery might result from enhanced surface-active properties and film-forming capacity caused by the higher protein content of Spirulina protein powder used in this study [30,31].

Water activity is an important attribute of powdered products since it dictates the storage stability and susceptibility to degradation reactions [32]. Both RCH and RSP presented similar ($p < 0.05$) aw levels within the range typically found for stable spray-dried powders [33], besides displaying attractive, vibrant green colors (Figure 2). The spray dried algae-rosemary particles

were also characterized regarding the concentration of rosemary phenolic diterpenes, carnosic acid (CA), carnosol (CR), and rosmarinic acid, recognized as potent antioxidant molecules with superior performance when compared to synthetic antioxidants used in the industry [9] and several biological activities [14]. Our results (Figure 3) show that RSP particles captured significantly higher concentrations of rosemary antioxidants when compared to RCH particles. We previously demonstrated that protein forms a protective layer during spray drying that may serve as a protective shell around core phytocompounds and avoids potential degradation during drying and storage [17,19]. In this case, spirulina protein powder exerted a more efficient role and therefore, this group was selected for further pollution-induced damage evaluation using human skin biopsies.

In particular, to explore the potential efficacy of the RSP formulation in modulating an oxinflammatory event, i.e., a phenomenon characterized by a harmful interplay between an imbalance redox homeostasis and a subclinical inflammation [26], a gel formulation containing 100 µg/mL RSP particles was tested in an ex vivo human skin model exposed to DEE. Taken altogether, our ex vivo data clearly demonstrated the ability of RSP gel to prevent pollution-induced 4HNE-mediated protein damage, extracellular matrix degradation and epidermal barrier dysfunction in cutaneous tissue. Several mechanisms could underlie these protective effects, mainly related to the high concentration of carnosic acid and carnosol present in the Spirulina-rosemary gel tested in this study. In fact, in vitro experimental results revealed the ability of both carnosic acid and carnosol of preventing lipid oxidation through different processes [34]. For example, carnosic acid directly quenches reactive pro-oxidant compounds, whereas carnosol is resistant to oxidation by reactive oxygen species (ROS), but has an inhibitory effect on the lipid peroxidation process by reacting directly with lipid radicals [34]. Furthermore, in different cell

types including neuronal and hepatic cell lines, rosemary extract and its polyphenolic diterpenes demonstrated to activate the Nrf2 antioxidant response pathway by inducing the transcription and expression of several cytoprotective enzymes such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and hemeoxygenase-1 (HO1) [35,36,37,38]. Therefore, our hypothesis is that the cutaneous topical application of RSP gel was able to prevent DEE-induced 4HNE-PA formation through the combination of both mechanisms. On one hand, carnosic acid and carnosol can directly quench reactive oxidants and limited lipid peroxidation reactions and, thus, 4HNE generation during DEE exposure. Secondly, the pretreatment with RSP gel for 24 h may have induced the activation of the Nrf2-mediated antioxidant response, boosting the cutaneous defensive antioxidant enzymes. In line with this hypothesis, it was demonstrated the hormetic effects of carnosic acid and carnosol in inducing an Nrf2-dependent antioxidant defense in normal human skin fibroblasts. The phenolic diterpenes exhibited potential antiaging effects in human skin fibroblasts by preventing hydrogen peroxide-induced premature senescence. In addition, carnosol ameliorated several features of cells undergoing replicative senescence (i.e., aging) [39].

Moreover, carnosic acid was effective against deleterious effects of pollutants including urban dust and cigarette smoke extract in normal human skin fibroblasts and epidermal keratinocytes. In particular, carnosic acid induced a significant increase in HO1 and NQO1 gene expression in normal human skin fibroblasts exposed to urban dust, thus enhancing the cellular endogenous defenses against an oxidative insult. In addition, carnosic acid significantly prevented the MMP-1 mRNA upregulation induced by cigarette smoke extract in normal human skin fibroblasts [40]. Recently, the inhibition of TNFα-induced MMP-1 secretion has also been attested in dermal fibroblasts treated either with carnosic acid or rosemary extract [41]. Similarly, pretreatment with carnosic acid inhibited expression and release of multiple MMPs induced by ultraviolet irradiation

in both primary human dermal fibroblasts and primary human epidermal keratinocytes, demonstrating that carnosic acid can counteract UV-induced skin photoaging [42].

Consistently with previous findings, our study indicated the ability of algae-rosemary gel to inhibit the increase in active MMP-9 levels induced by DEE exposure in the skin tissue. These effects could be related to the well-recognized immunomodulatory functions of rosemary extract and its active constituents [43]. Specifically, the mechanism by which RSP gel ameliorated the DEE-induced skin inflammation could be explained through the inhibition of the nuclear factor kappa B (NF-κB) signaling pathway. Indeed, in a dose-dependent manner, carnosol inhibited IL-1β-stimulated nuclear translocation of NF-κB-p65, decreasing the gene expression of inflammatory genes in human chondrocytes [44]. Another study showed similar results in monocyte/macrophage-like cells, confirming the ability of a methanol extract of rosemary to inhibit LPS-induced MAPKs and NF-kB activation [45].

This effect could also justify our results on the filaggrin expression. Indeed, a downregulated expression of filaggrin was also observed in human keratinocytes exposed to urban particulate matter and the underlying mechanisms involved ROS-mediated activation of the transcription factors NF-κB and AP-1 [46]. Therefore, the combined ability of algae-rosemary gel to limit the 4HNE-mediated protein damage through a probable enhancement of the skin defense functions and to restrain the DEE-stimulated inflammatory state by modulating the NF-kB signaling pathway could contribute to its protective effects against DEE-induced loss of filaggrin, as already demonstrated for another plant-derived phenolic compound [47]. In addition, it should be mentioned that the loss of filaggrin could not only affect skin barrier functions and possibly promote skin conditions related to pollution exposure, but it could even facilitate the entrance of exogenous pathogens and allergens that can further damage the cutaneous tissues [48,49]. Finally,

it should be mentioned that the present study was performed in an “ex vivo” model, which, although is one of the most reliable approaches to represent skin tissue in a laboratory, it is still a model that has some limitations. One impediment of existing ex vivo tissue culture systems is that viability and integrity of the tissue can generally not be maintained for a long period; in addition, the lack of circulation and innervation, which are present in in vivo models, preclude the possibility to evaluate a complete inflammatory response. Therefore, these aspects need to be considered before translating ex vivo results to human subjects.

3.5. Materials and Methods

3.5.1. Chemicals

All organic solvents were HPLC grade and obtained from VWR International (Suwanee, GA, USA). Organic Chlorella protein powder (50% protein, Triquetra Health, Tampa FL, USA), organic Spirulina protein powder (66% protein, Zazzee Naturals, Austin, TX, USA) and xanthan gum (MakingCosmetics, Snoqualmie, WA, USA) were used in this study.

3.5.2. Preparation of Rosemary Hydroalcoholic Extract

Fresh rosemary shoots with leaves (Rosmarinus officinalis, variety Spice Mountain) were locally obtained (John Weddington Greenhouse, Salisbury, NC, USA). Fresh leaves (moisture content 71.3% ± 2.0%) were stripped off and extracted with aqueous ethanol under vacuum to minimize the oxidation of phenolic constituents. Batches of 200 g were processed for 15 min using a blender (Vita-Mix Corp, Cleveland, OH, USA) attached to a vacuum accessory. The mixture was sonicated for 15 min and the supernatant was collected and centrifuged at 4 °C. The rosemary solid fraction was re-extracted with 500 mL of 80% aqueous ethanol using a similar protocol, followed by a third extraction using 500 mL of 50% aqueous ethanol. The combined supernatant (approx. 2.5 L) was vacuum evaporated to remove ethanol. A final hydroalcoholic rosemary extract containing 7.3 ± 0.2% dry matter was obtained (final volume of approx. 400 mL). Several batches extracted over a period of 48 h were kept under refrigeration at 4 °C and mixed to form one single batch used for all spray drying experiments.

3.5.3. Spray Drying

Two experimental algae-rosemary groups were investigated in this study: rosemary extract complexed with organic Chlorella protein powder (RCH) and rosemary extract complexed with organic Spirulina protein powder (RSP). Before each production batch, the algae protein powders were dispersed directly into the hydroalcoholic rosemary extract by vigorous mixing (PRO Scientific Bio-Gen PRO200, Oxford, CT, USA) for 2 min at 15,000 rpm until complete dissolution. The prepared feed solution was atomized using a spray dryer (B-290, Buchi Labortechnik AG, Switzerland) at 110 °C (outlet temperature 58–61 °C) following preliminary experiments (data not shown). The spray drying system operated using air in co-current flow under the following optimized conditions: 1.5 mm diameter nozzle, 10 mL/min of feed flow (controlled by peristaltic pump) kept under constant magnetic stirring during drying. For each algae-rosemary sample, 10% (w/v) of algae protein powder was added to the hydroalcoholic rosemary extract. The resulting spray-dried particles were collected from the collection chamber only, and all the other particles adhering to the drier walls and/or pipes were discarded. The final powdered product was weighed and immediately sealed in Ziploc® bags. The solids recovery (also referred to as production yield) of spray-dried algae-rosemary samples was calculated as a percentage (%) according to the ratio [total solids content of resulting particles (algae-rosemary particles)/total solids content in the feeding mixture (before spray drying)] × 100 according to our previous protocol [17].

3.5.4. Water Activity and Color Measurements

The water activity of spray-dried algae-rosemary samples was measured using an Aqualab water activity meter (Decagon, Pullman, WA, USA). A reflectance spectrophotometer (CR-400,

Konica, Minolta, Japan) previously calibrated with white and black standards was used to determine the color parameters lightness (L*), greenness (−a*) or redness (+a*), and blueness (−b*) or yellowness (+b*).

3.5.5. HPLC-DAD Analysis for Rosmarinic Acid, Carnosol, and Carnosic Acid

HPLC-DAD was conducted on an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array detector (DAD) set at 230 nm. The chromatographic separation was performed on Phenomenex Synergi 4 µm hydro-RP 80A column (250 mm × 4.6 mm × 5 µm, Torrance, CA, USA) thermostatted at 25 °C according to our previously described method [50]. Rosmarinic acid (RA), carnosol (CR) and carnosic acid (CA) were quantified based on standard curves constructed with corresponding reference standards and expressed as µg/mg DW.

3.5.6. Preparation of Gel Samples Containing Algae-Rosemary Particles

Initially, a solution of 0.25% (w/v) of xanthan gum was prepared and mixed for 30 min using a magnetic stirrer until complete dissolution. This xanthan gum gel was used to prepare a hydrocolloidal solution containing 100 µg/mL of Spirulina-rosemary particles (selected in the first part of the study) that was used for all skin tissue experiments.

3.5.7. Ex Vivo Human Biopsies Treatment and Exposure to Diesel Engine Exhaust (DEE)

Healthy human skin, purchased from a local hospital (Hunstad/Kortesis/Bharti Cosmetic Surgery clinic), was obtained from three different donors undergoing elective abdominoplasty, as approved by the IBC at NC State University (USA). Subcutaneous fat was trimmed from 12 mm

punch biopsies, and biopsies were rinsed with PBS containing antibiotics/antimycotic. Next, biopsies were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 ug/mL streptomycin at 37 °C in 5% CO2 with the upper part of the epidermis exposed to the outside environment.

The next day, medium was changed, and the algae-rosemary gel (RSP) or the only vehicle (Veh) (xantan guam 0.25%; w/v solution) were topically applied. After 24 h of pre-treatment, biopsies were exposed for 30 min to DEE generated by a Kubota RTV-X900 diesel engine (3-cylinder, 4-cycle diesel with overhead valves, 1123 cc that has 24.8 HP at 3000 rpm) and cultured in fresh medium for the different time points (3, 6 and 24 h) following the DEE exposure.

3.5.8. Immunofluorescence

Paraffin-embedded 4 µm sections of skin biopsies were deparaffinized in xylene and rehydrated in decreasing alcohol gradients. Antigen retrieval was achieved using heat-based epitope retrieval with sodium citrate buffer (Thermo Fisher Scientific, Waltham, MA, USA) (pH 6.0) at a sub-boiling temperature in a 500-watt microwave for 10 min. After cooling, sections were washed in PBS, blocked with 5% BSA in PBS, and incubated with primary antibodies for 4HNE (dil. 1:400; AB5605, Millipore), MMP9 (dil. 1:200; NBP2-13173, Novus Biologicals), filaggrin (dil. 1:50; sc-66192, Santa Cruz Biotechnology, Inc.), in 2% BSA in PBS. Sections were then washed in PBS and incubated with fluorochrome-conjugated secondary antibodies (dil. 1:1,000) (Alexa Fluor 568 A11004 or Alexa Fluor 488 A11055) in 2% BSA in PBS at RT, and then washed with PBS. Nuclei were stained with DAPI (1874814, Invitrogen) in PBS, and sections were then washed with PBS. Sections were mounted using PermaFluor mounting medium (Thermo Fisher Scientific) and imaged on a Zeiss LSM10 microscope. Images were quantified using ImageJ.

3.5.9. Statistics

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). For comparisons between groups, analysis of variance (ANOVA) followed by Bonferroni's post-hoc test was conducted. All data were expressed as means ± standard deviations and statistical differences between means were determined using $p \leq 0.05$ significance level.

3.6. Conclusions

In this study, we investigated a strategy to take advantage of phenolic-rich hydroalcoholic rosemary extract complexed with algae-derived protein for skin health applications. The present study demonstrates the protective effect of these novel spray-dried algae-rosemary particles against one of the most noxious pollutants, diesel exhaust, to which our skin is daily exposed. Collectively, our results suggest that the topical strategy using rosemary–spirulina gel is a good approach to prevent pollution-induced skin aging/damage.

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CHAPTER 4: DEFEROXAMINE TREATMENT IMPROVES ANTIOXIDANT COSMECEUTICAL FORMULATION PROTECTION AGAINST CUTANEOUS DIESEL ENGINE EXHAUST EXPOSURE

Pambianchi E, Ferrara F, Pecorelli A, Benedusi M, Choudhary H, Therrien JP, Valacchi G. Deferoxamine Treatment Improves Antioxidant Cosmeceutical Formulation Protection Against Cutaneous Diesel Engine Exhaust Exposure.

4.1. Abstract

Skin is one of the main targets of outdoor stressors. Considering that pollution levels are rising progressively, it is not surprising that several cutaneous conditions have been associated to its exposure. Among the pollutants, diesel engine exhaust (DEE) represents one of the most toxic as it is composed of a mixture of many different noxious chemicals generated during the compression cycle, for ignition rather than an electrical spark as in gasoline engines. The toxic chemicals of most concern in DEE besides the oxides of nitrogen, sulfur dioxide, and various hydrocarbons are metals that can induce oxidative stress and inflammation.

The present study aims to evaluate the effects of topical application, singularly or in combination, of the iron-chelator deferoxamine and a commercially available formulation, CE Ferulic, in up to 4 days of DEE-exposed skin.

DEE induced a significant increase in the oxidative marker 4-hydroxy-nonenal (4HNE) and matrix-metallopeptidase-9 (MMP-9), the loss of cutaneous-barrier-associated proteins (filaggrin and involucrin), and the decrease in collagen-1, while the formulations prevented the cutaneous damage in an additive manner.

In conclusion, this study suggests that iron plays a key role in DEE-induced skin damage and its chelation could be an adjuvant strategy to reinforce antioxidant topical formulations.

4.2. Introduction

The World Health Organization (WHO) estimated that circa 90% of the urban population around the world lives in area with pollutant levels that exceed WHO guideline limits and this has been linked to premature death of 7 million people yearly (1).

The use of the word "pollution" can be misleading given that are several different pollutants to which living organisms are exposed. Based on their chemical and physical properties the USDA has divided them in 6 main groups: ozone (O_3), particulate matter (PM), carbon monoxide (CO), lead, sulfur dioxide (SO_2), and nitrogen dioxide (NO_2) (2).

Among them, the role of PM on human health has been investigated for several years and many pathologies have been associated to its exposure. Indeed, it has been shown that PM can impact the structure and functionality of many organs, including lungs, brain, hearth, lungs and gut (3–6). Only more recently the association between PM exposure and skin conditions has been analyzed and now there is solid evidence suggesting the role of PM in accelerating skin aging, increasing cutaneous spots and inducing skin inflammation (7, 8).

Among the PM, diesel engine exhaust particles (DEE) have been shown to be among the most noxious to human health and among the most predominant worldwide (9). Of all existing internal combustion engines, the diesel engine is among the most popular and is therefore of great concern with respect to the environment and public health. Invented in the late 1800s the diesel engine became very popular in the 1980s and since then, its popularity has continuously expanded and occupied the urban centers increasing the spread of these toxic compounds in the environment.

Although most of the attention of airborne PM has focused on the impact on human respiratory and cardiovascular systems (10, 11) and other organs such as gut, liver and kidney (12, 13), recently the effect of DEE on skin has been investigated. In fact, as a consequence of its peculiar

location, cutaneous tissue acts as a biological shield against air pollutants and prolonged and repetitive exposure to high levels of airborne PM have been shown to have profound adverse effects on the integumentary apparatus (8, 14).

Diesel exhaust is a complex mixture composed of a solid core where, based on its source, several elements including metals are present. Harmful effects of DEE exposure have been suggested to involve local Reactive Oxygen Species (ROS) production which could, in part, be generated from the particles themselves. On the other hand, particles can serve as organic compounds carriers like polycyclic aromatic hydrocarbons (PAHs), which are highly lipophilic, and capable of localizing in mitochondria contributing to the evidenced ROS production (15).

The oxidative capacity of PM has been attributed to its transition metal constituents, especially iron (Fe), which can catalyze Fenton-like reactions and generate ROS, initiating oxidative damage mechanisms(16) by the formation of hydroxyl radicals. Therefore, the presence of iron can be considered as one of the possible triggers of the noxious effect of DEE exposure.

Consequently, to reduce the excess of pro-oxidant metal, iron-chelators, such as deferoxamine (DFO) have been widely used to prevent iron damage. As of today, only few studies have investigated the role of iron as part of the possible harmful effect of DEE exposure on cutaneous tissues and the possible novel uses of the iron-chelators as adjuvant for already established anti-pollution formulations [18,19].

Our study aims to evaluate the use of the iron chelator DFO, as a topical treatment, to be combined with antioxidant topical interventions. Specifically, the present work focuses on the evaluation of the possible additive effect of the topical combination of DFO with the commercially available antioxidant cosmeceutical formulation, CE Ferulic, against DEE-induced skin damage.

4.3. Material and Methods

4.3.1. Human specimens

Human skin explants were purchased from Hunstad/Kortesis/Bharti Cosmetic Surgery clinic and derived from three different healthy subjects undergoing routine elective abdominoplasties. For each tissue sample, collected following informed consent, skin biopsies were taken with a 12 mm full thickness punch and subcutaneous fat was trimmed using sterile scissors. The skin biopsies were then rinsed with Phosphate Buffer Solution (PBS), containing antibiotics/antimycotic and then transferred into 6 well plates pre filled with 1 ml of DMEM High Glucose supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin, using sterile technique (19). The plates were incubated at 37°C in a 5% CO_2/95% air atmosphere and left undisturbed for overnight recovering.

4.3.2. Application of CE Ferulic, Deferoxamine (DFO) and vehicles

After overnight recovering, skin biopsies medium was replaced with fresh complete medium. Then, 5 µl of CE Ferulic composed by 15% L-ascorbic acid, 1% α-tocopherol and 0.5% ferulic acid (CE Ferulic, SkinCeuticals Inc), 20 µl of DFO 100 µM (DM533, Sigma Aldrich), alone or in combination and corresponding vehicles (CE Ferulic vehicle and PBS) were applied topically to the skin explants, in triplicate for each condition. For the combination CE Ferulic + DFO, first 20 µl of DFO were applied and evenly spread with a sterile glass rod, then after 15 minutes, 5 µl of CE Ferulic were added and uniformly distributed. Plates were incubated in humidified 5% CO_2/95% air atmosphere for 24 hours. The experiment was performed, for each donor and at least in triplicates for each condition.

4.3.3. Diesel Engine Exhaust (DEE) exposure

On the following day, the skin biopsies were treated again with the above-mentioned compounds before the exposure to DEE for 30 minutes. DEE was generated by a Kubota RTV-X900 diesel engine (3-cylinder, 4-cycle diesel with overhead valves, 1123 cc that has 24.8 HP at 3000 rpm) as previously reported (20). The engine was let run for 10 seconds allowing the diesel exhaust to be delivered to an exposure chamber. The skin explants were then left in the chamber for 30 minutes in the presence of DEE. A set of skin explants, controls, were exposed to purified and HEPA-filtered air. After DEE exposure, media was replaced and tissue samples were collected either 24 h after the first exposure (Day 1) or after four days (Day 4). Treatment and exposure were performed daily.

4.3.4. Tissue collection and Immunohistochemical analysis

At the end of each time point (Day 1 and Day 4), skin explants were fixed in 10% neutral buffered formalin for 48 h at 4°C, dehydrated and embedded in paraffin. 4 μm thick sections were deparaffinized in xylene and then rehydrated through a series of decreasing alcohols to water. Antigen retrieval was performed by boiling slices in 10 mM sodium citrate buffer solution (Thermo Fisher Scientific, USA) (pH 6.0) at a sub-boiling temperature in a 500 W microwave for 10 min. After cooling, slices were washed in PBS, blocked with 5% Bovine Serum Albumin (BSA) in PBS, and incubated with primary antibodies for 4HNE (dil. 1:500) (AB5605, Millipore Corporation), Collagen 1 (dil. 1:400) (AB34710, Abcam), pro-MMP-9 (dil. 1:50) (MAB9111, Novus Biologicals) MMP-9 (dil. 1:200) (NBP2-13173, Novus Biologicals), Involucrin (dil. 1:50) (sc-21748, Santa Cruz Biotechnology, Inc.), or Filaggrin (dil. 1:50) (sc-66192, Santa Cruz Biotechnology, Inc.) in 2% BSA in PBS. Sections were then washed three times in PBS and

incubated with fluorochrome-conjugated secondary antibodies (dil. 1:500) (Alexa Fluor 568, A11004 or Alexa Fluor 488, A11055) in 2% BSA in PBS at room temperature, and then washed with PBS. Nuclei were stained with DAPI (D1306, Invitrogen) in PBS. After a triple series of washings with PBS, slices were mounted using PermaFluor mounting media (ThermoFisher Scientific) and imaged on a Zeiss LSM10 microscope, equipped at 40X magnification. Images were quantified using ImageJ (21).

4.3.5. Statistical analysis

Statistical analyses were performed via GraphPad Prism 6 software (GraphPad Software Inc.). Differences between groups were evaluated by analysis of variance (ANOVA) considering time points (Day 1 and Day 4) separately, followed by Tukey's post-hoc test. A p-value ≤ 0.05 was considered statistically significant. All variables tested are expressed as mean ± standard deviation (SD) of three independent experiments.

4.4. Results

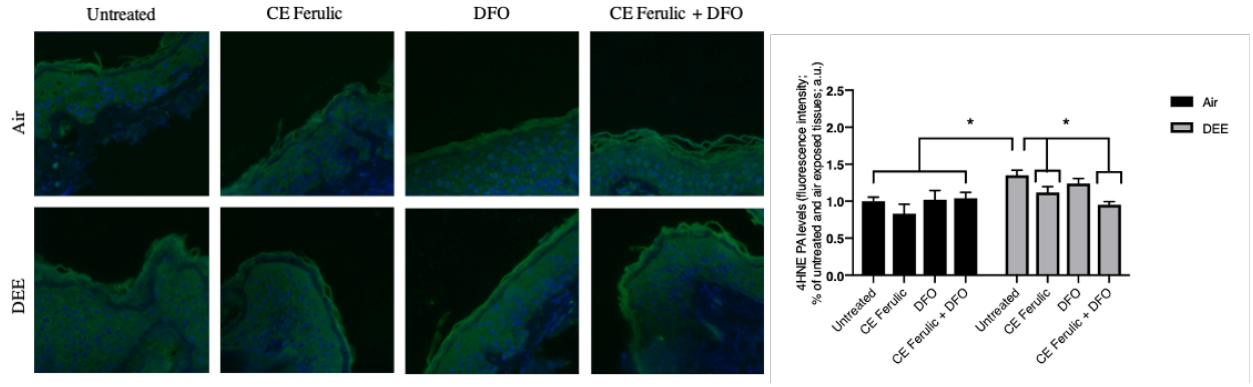
4.4.1. Evaluation of CE Ferulic and DFO pre-treatment in preventing DEE exposure-induced cutaneous 4HNE Protein Adducts formation

Considering that the outermost layer of the skin, the stratum corneum, is rich in lipids, it is a perfect substrate for the interaction with DEE that leads to the formation of several lipid oxidation products (LOP) among which 4-hydroxy-nonenal (4HNE) is one of the most reactive.

As shown in Fig. 1, DEE exposure induces the formation 4HNE protein adducts, with a moderate increase at Day 1 (Fig.1A) and with a more evident and significant induction at Day 4 (Fig.1B). DFO treatment was able to clearly prevent 4HNE formation at Day 4 and CE Ferulic showed the ability to prevent 4HNE formation from Day 1 (Fig.1A) to Day 4 (Fig.1 B). The combined application of CE Ferulic and DFO seemed to promote, at both time points, a moderate additive effect in reducing DEE exposure induced 4HNE levels (Fig. 1A and 1B).

Collectively, these results confirmed the harmful effect of DEE exposure on cutaneous tissue and propose beneficial additive properties of the combined topical application of the iron chelator DFO and the cosmeceutical formulation CE Ferulic, in preventing the formation of lipid peroxidation in exposed skin.

A.



B.

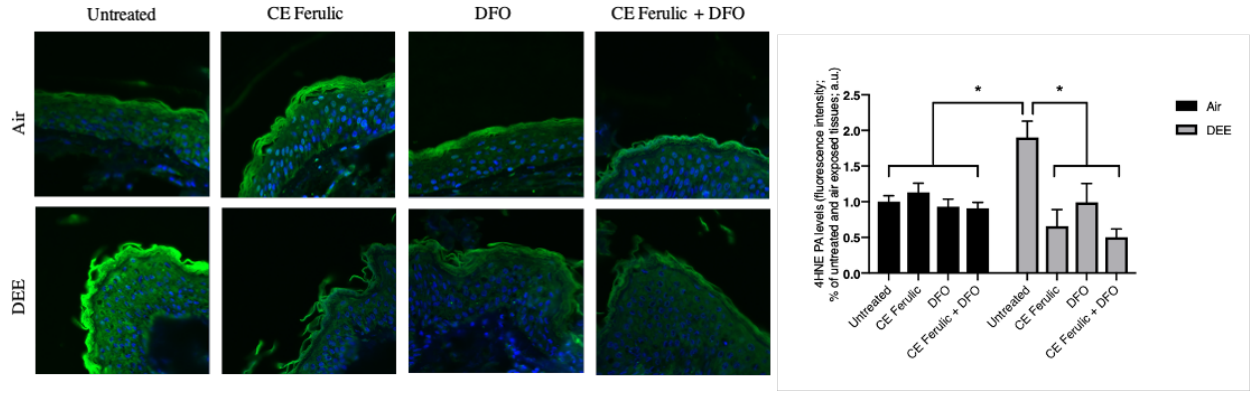


Fig 1. Skin exposure to DEE increases lipid peroxidation related damage, while topical application of CE Ferulic and its combination with DFO can prevent this effect. Levels of 4HNE PA in *ex vivo* human skin explants untreated or pre-treated with the cosmeceutical formulation mixture and DFO, alone or in combination, and exposed to air or DEE at Day 1 (A) and Day 4 (B). Green fluorescence staining represents 4HNE PA immunoreactivity, while cell nuclei are stained in blue with DAPI. Original magnification 40x. On the right, semi-quantification of the immunofluorescence intensities performed by ImageJ are showed in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), $*p \leq 0.05$, by ANOVA.

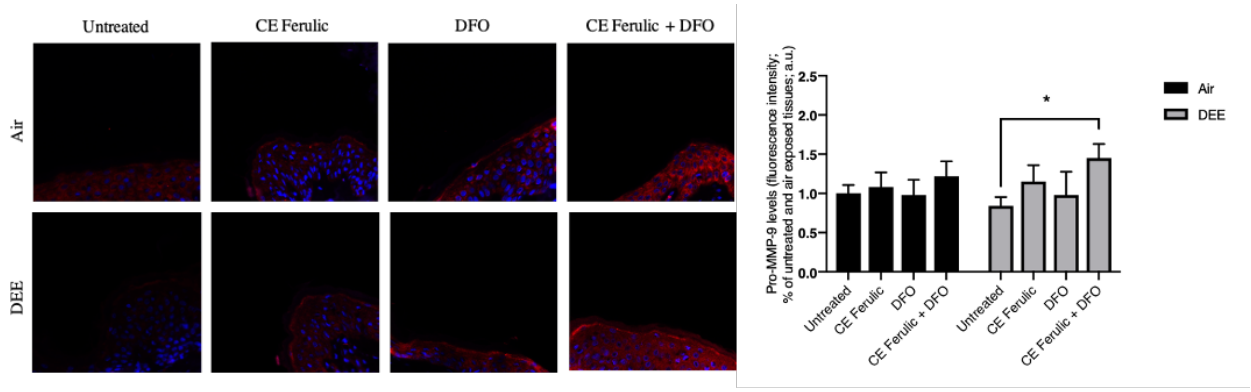
4.4.2. Evaluation of CE Ferulic and DFO pre-treatment in preventing DEE exposure modulation of pro-MMP-9 and MMP-9 levels

Different pollutants have been shown to be both the cause and the effect of inflammatory responses, therefore fueling a vicious circle that has been defined as OxInflammation (20, 22). Matrix metallopeptidase 9 (MMP-9) is an enzyme responsible for the degradation and turnover of extracellular matrix components (EMC) and tissue remodeling. It has been shown that MMP-9 is a key player in cutaneous inflammation when its activation is aberrantly overexpressed (23–25).

To further evaluate the effect of DEE and topical application of CE Ferulic and DFO we also measured the levels of the pro-form (pro-MMP-9) and its active form (MMP-9). Pro-MMP-9 is an inactive zymogen with an un-cleaved pro-peptide domain that requires further processing by other MMPs or serine proteases to become fully active and yield to EMC degradation. As shown in Fig.2, DEE exposure decreases the levels of pro-MMP-9 at Day 1 (Fig.2A) and this effect becomes even more significant at Day 4 (Fig.2B) suggesting the activation of this enzyme. As a proof of concept, we also measured the levels of the active form of MMP-9. As shown in Fig.3, DEE exposure clearly induced MMP-9 activation in skin at both Day 1 (Fig.3A) and Day 4 (Fig.3B). This effect was completely quenched by the topical application of both, DFO and CE Ferulic over time.

The topical application of CE Ferulic and DFO, and even more their combination was able to prevent the cleavage of pro-MMP9 and preventing its activation.

A.



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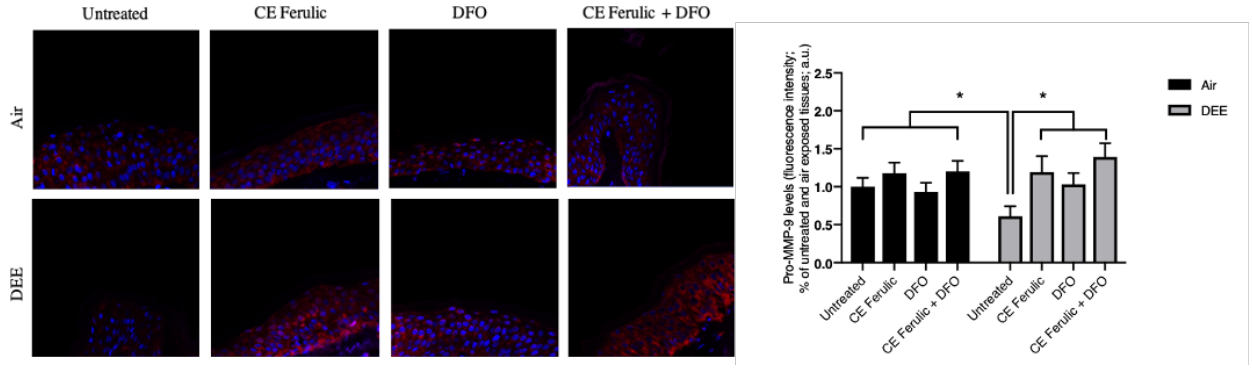
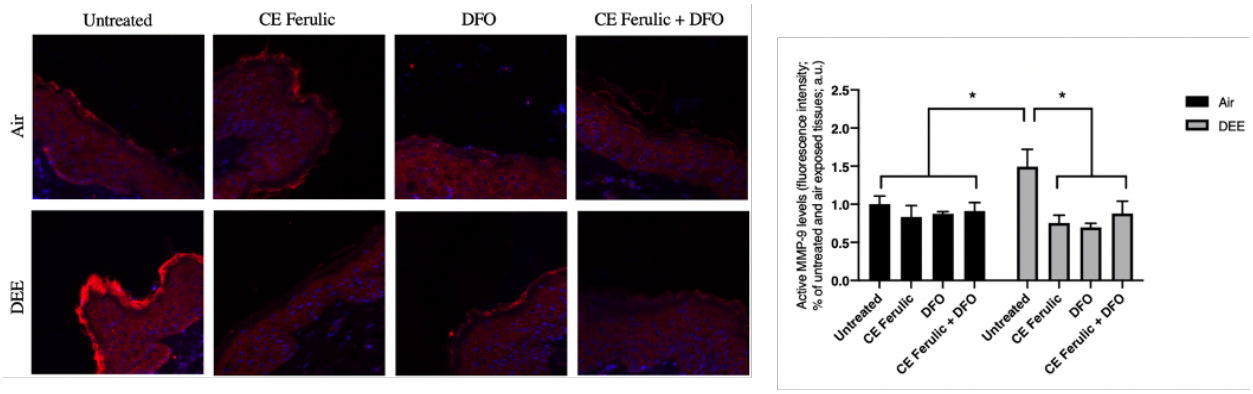


Fig 2. Skin exposure to DEE induces a decrease in the levels of pro-MMP-9, while topical application of CE Ferulic and DFO prevents this effect. Levels of pro-MMP-9 in *ex vivo* human skin explants untreated or pre-treated with the cosmeceutical formulation mixture and DFO, alone or in combination, and exposed to air or DEE at Day 1 (A) and Day 4 (B). Red fluorescence staining represents pro-MMP-9 immunoreactivity, while cell nuclei are stained in blue with DAPI. Original magnification 40x. On the right, semi-quantification of the immunofluorescence intensities performed by ImageJ are showed in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), $*p < 0.05$, by ANOVA.

A.



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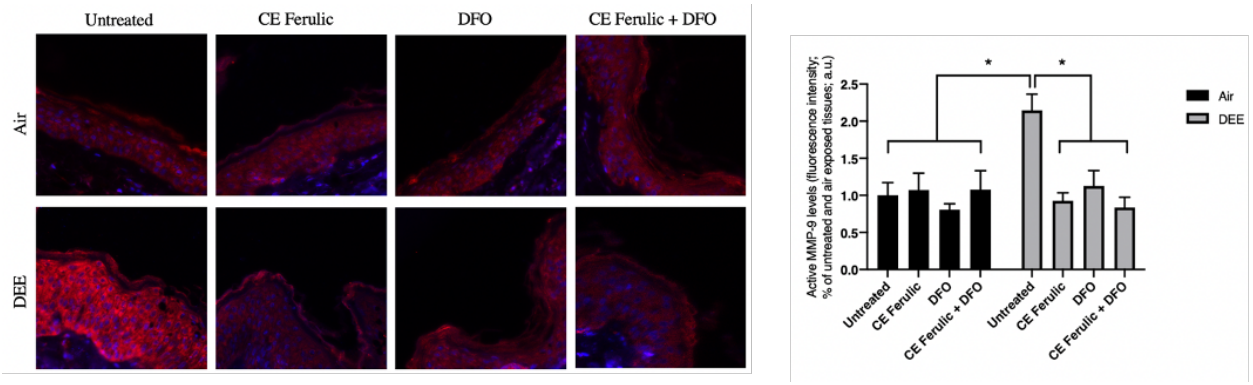


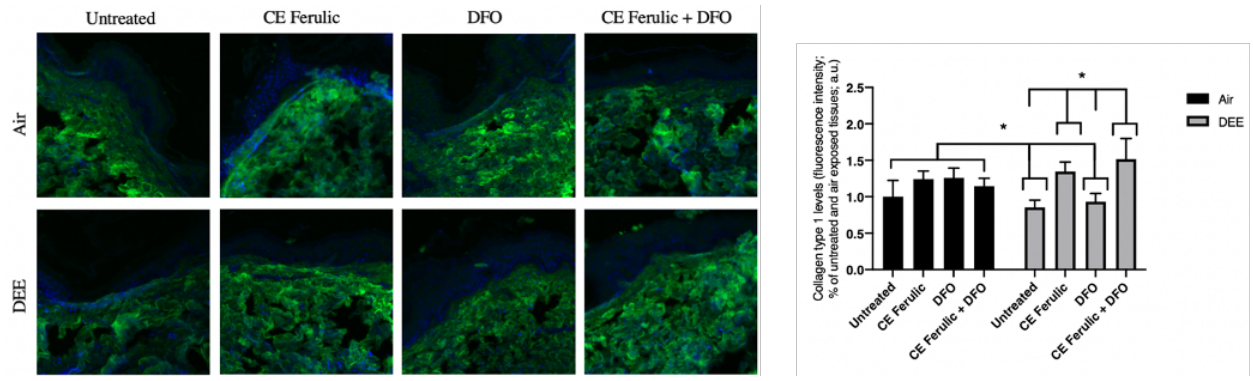
Fig 3. Skin exposure to DEE induces the levels of MMP-9 enzyme, while topical application of CE Ferulic and DFO counteracts this effect. Levels of MMP-9 in *ex vivo* human skin explants untreated or pre-treated with the cosmeceutical formulation mixture and DFO, alone or in combination, and exposed to air or DEE at Day 1 (A) and Day 4 (B). Red fluorescence staining represents MMP-9 immunoreactivity, while cell nuclei are stained in blue with DAPI. Original magnification 40x. On the right, semi-quantification of the immunofluorescence intensities performed by ImageJ are showed in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), *$p < 0.05$, by ANOVA.

4.4.3. Evaluation of CE Ferulic and DFO pre-treatment in preventing DEE exposure induced effects on cutaneous Collagen 1

Type I collagen is one of the most abundant molecules in the body, and it is particularly important in skin and in the connective tissue. It is an interstitial matrix collagen organized in

fibrils, which are essential for the competence of the skin. Collagen 1 depletion is one of the most evident signs of skin aging and it has been linked with the cutaneous premature aging induced, among the many factors, also by pollution exposure. As depicted in Fig. 4 exposure to DEE significantly decreased the levels of Collagen 1 at both time points (Day 1 and Day 4). This effect was prevented by CE Ferulic and only slightly by DFO at Day 1 (Fig. 4A). After 4 days of exposure, both the compounds were able to significantly prevent the loss of Collagen 1, although no additive effect was noticed when DFO and CE Ferulic were applied simultaneously (Fig.4B).

A.



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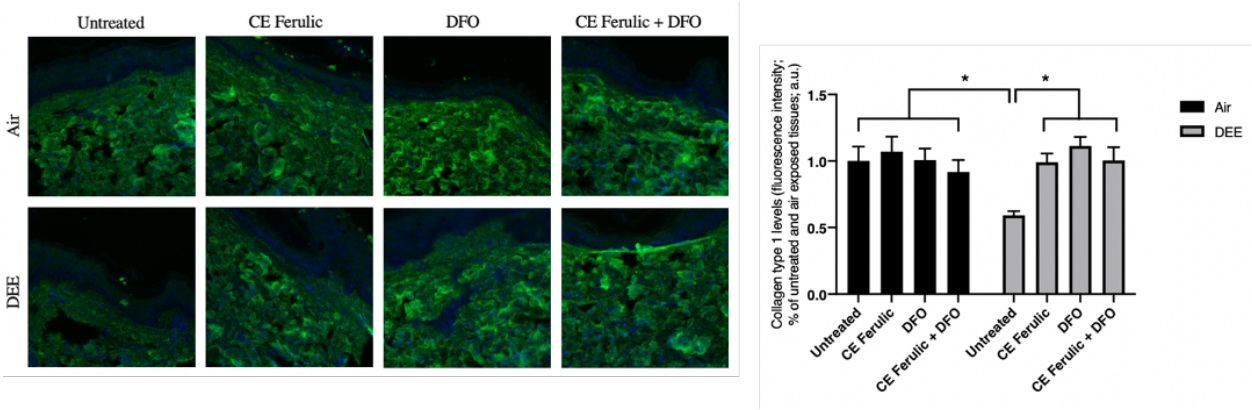


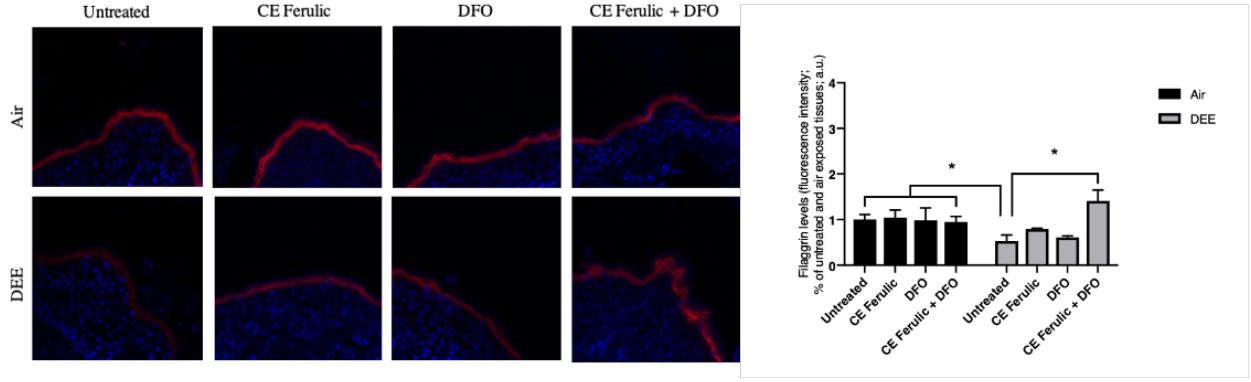
Fig 4. Skin exposure to DEE decreases levels of collagen 1, but topical application of CE Ferulic and DFO prevents this effect. Levels of collagen 1 in *ex vivo* human skin explants untreated or pre-treated with the cosmeceutical formulation mixture and DFO, alone or in combination, and exposed to air or DEE at Day 1 (A) and Day 4 (B). Green fluorescence staining represents collagen 1 immunoreactivity, while cell nuclei are stained in blue with DAPI. Original magnification 40x. On the right, semi-quantification of the immunofluorescence intensities performed by ImageJ are showed in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), $*p < 0.05$, by ANOVA.

4.4.4. Evaluation of CE Ferulic and DFO pre-treatment in preventing DEE exposure induced decrease of cutaneous Filaggrin levels

The skin barrier is fundamental to ensure healthy skin and previous studies demonstrated that exposure to pollutants can exacerbate inflammatory skin diseases by compromising the cutaneous

barrier itself and its associated proteins such as filaggrin (20, 26), a protein involved in epidermal hydration and keratin matrix formation (26). As shown in Fig 5A and 5B, at both time points (Day 1 and Day 4), there was a significant decrease in filaggrin protein levels after DEE exposure when compared to the untreated tissues exposed to air. The treatments with CE Ferulic and DFO alone prevented filaggrin loss in DEE exposed tissues at Day 4; moreover, their combined topical application resulted in a clear additive effect in counteracting DEE-induced filaggrin loss. In addition, the combined treatment further induced filaggrin levels over the baseline, suggesting a possible new mechanism to induce this important protein when its levels is seriously compromised (26, 27).

A.



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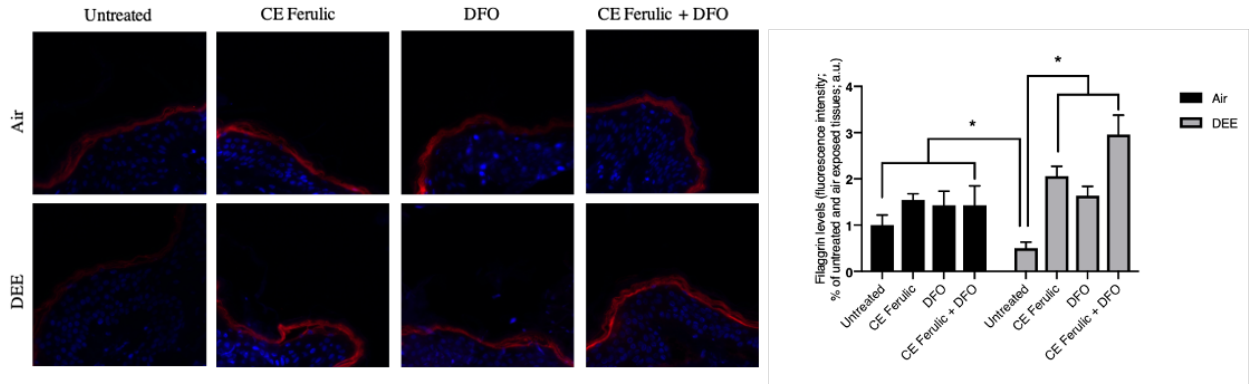


Fig 5. Skin exposure to DEE induces a decrease in filaggrin levels, while topical application of CE Ferulic and DFO prevents this effect. Levels of filaggrin in *ex vivo* human skin explants untreated or pre-treated with the cosmeceutical formulation mixture and DFO, alone or in combination, and exposed to air or DEE at Day 1 (A) and Day 4 (B). Red fluorescence staining represents filaggrin immunoreactivity, while cell nuclei are stained in blue with DAPI. Original magnification 40x. On the right, semi-quantification of the immunofluorescence intensities performed by ImageJ are showed in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), $*p < 0.05$, by ANOVA.

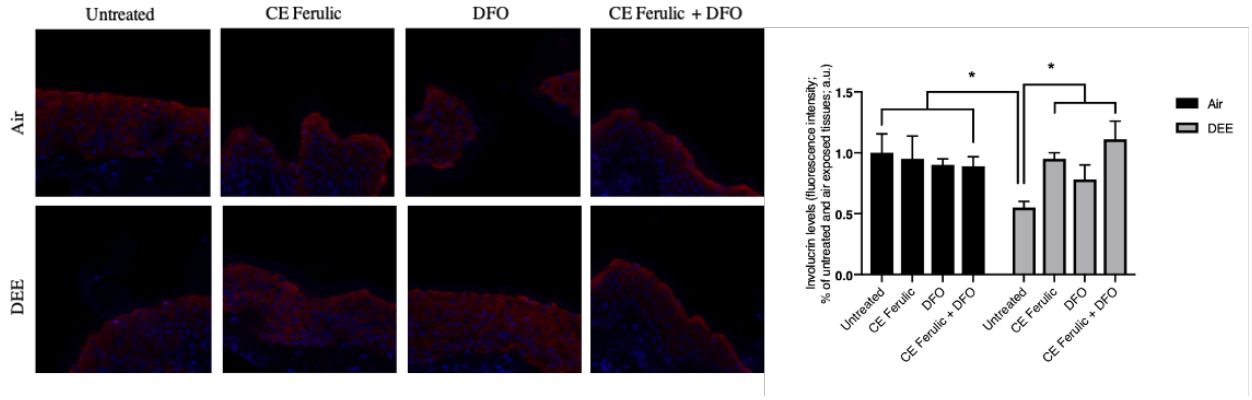
4.4.5. Evaluation of CE Ferulic and DFO pre-treatment in preventing DEE exposure induced decrease of cutaneous Involucrin levels

Together with filaggrin, involucrin is another important protein involved in the formation of keratinocytes cornified cell envelope, being therefore a key component of fully differentiated epidermal layer and playing an essential role in skin barrier.

Similarly, to the effect of DEE on filaggrin levels, DEE at both time points (Day 1 and Day 4) remarkably downregulated the levels of involucrin when compared to untreated air-exposed skin explants (Fig 6A and 6B). This harmful effect was prevented by the topical application of CE Ferulic and DFO. Noteworthy, the combined topical treatment was not only able to prevent DEE decreased involucrin levels, but as shown also for the filaggrin (Fig.5), it was able to further stimulate its levels over the baseline.

Taken together, these data corroborate the damaging effects induced by DEE on cutaneous tissues and suggest that the combined topical application of CE Ferulic and the iron chelator, in our case DFO, can strongly improve skin barrier functions by increasing involucrin and filaggrin levels in DEE-exposed skin.

A.



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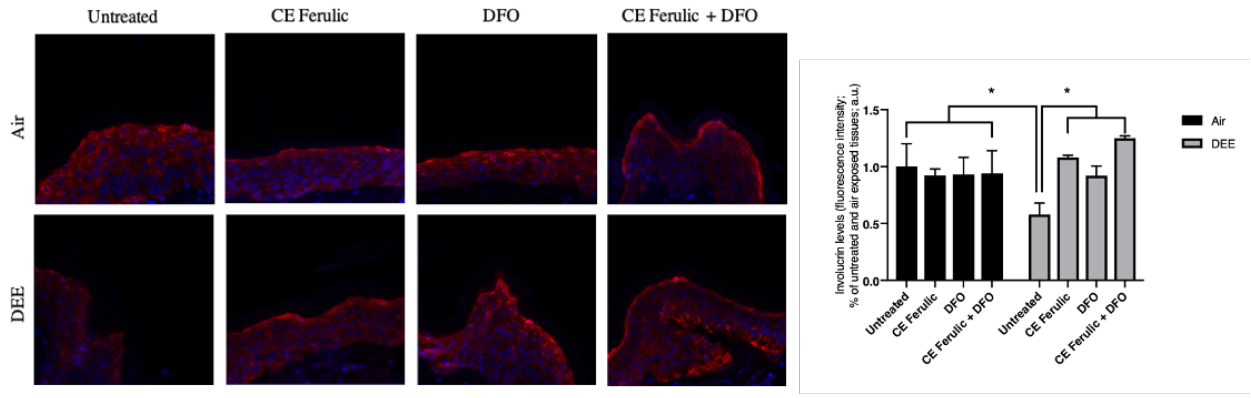

Fig 6. Skin exposure to DEE induces a decrease in involucrin levels, while topical application of CE Ferulic and DFO prevents this effect. Levels of involucrin in human skin explants untreated or pre-treated with the cosmeceutical formulation mixture and DFO, alone or in combination, and exposed to air or DEE at Day 1 (A) and Day 4 (B). Red fluorescence staining represents involucrin immunoreactivity, while cell nuclei are stained in blue with DAPI. Original magnification 40x. On the right, semi-quantification of the immunofluorescence intensities performed by ImageJ are showed in the histograms. Data are expressed as arbitrary units (averages of three independent experiments), *$p < 0.05$, by ANOVA

4.5. Discussion

Diesel engine emissions have increased significantly in the last few decades, particularly in densely populated urban cities and areas. Among the different environmental pollutants, DEE is the most prevalent anthropogenic source of pollution worldwide (9). Diesel exhaust is a complex mixture, and even if its exact composition is intimately related to the engine type, operation mode, type of fuel, lubrication oil and their respective additives (28, 29), 3 primary fractions can be identified: solid, condensates (hydrocarbons, water, sulfuric acid) and gaseous. The solid fraction is mainly composed of the elemental carbon, metals (such as magnesium, iron, manganese, platinum, copper, zinc, cerium) and non-metal oxides, mainly generated by normal engine wear, lubrication and fuel additives.

In general, exposure to airborne particulate matters (PM) is associated with several skin conditions including accelerated extrinsic aging (30) pigment spot formation, coarse wrinkle development, and elastosis (31).

The mechanisms involved in PM-associated skin disorders result from increased oxidative stress due to PM exposure. It is still under debate the ability of PM to penetrate the skin, although it has been suggested that eventually particulate matter is able to move through the skin via hair follicles or trans-dermally, generating oxidative stress (32–34).

In addition, polycyclic aromatic hydrocarbons (PAHs), which are components of diesel particles, can be absorbed through the skin and eventually damage the mitochondria, resulting in intracellular ROS production (35). These damaged mitochondria produce superoxide anions, which can be converted into H_2O_2 that can then undergo the Fenton reaction (as a consequence, for instance, of the iron present in the particles), to produce hydroxyl radicals, resulting in increased ROS and activation of redox sensitive transcription factors, such as AP1 and NFκB.

Therefore, the role of metals, and in particular of iron as mediator of diesel particle toxicity has been postulated. For this reason, the possible usage of iron chelators together with other topical antioxidants mixtures could be a good approach to prevent harmful effects of PM on skin health.

Our data confirmed this hypothesis, as the increase in lipid peroxidation measured by 4HNE levels was induced by DEE exposure and prevented by DFO treatment, and this effect was even more evident when DFO was added together with CE Ferulic. This was in line also with previous findings where the induction of an oxidative stress status in the skin was the consequence of pollutants exposure (alone or in combination) (19, 20).

In addition to this oxidative damage, DEE and other environmental pollutants have been demonstrated to exhibit another more specific harmful mechanism, via the disruption of iron homeostasis. Air pollutants can induce alterations in the cellular ferrokinetics by either a) complexing/chelating this micronutrient (via double bonds or the presence of electronegative functional groups) or b) by displacing it from pivotal cellular sites (36, 37).

The first mechanisms can induce the formation of complexes between the pollutant and iron itself, with the ability of reacting as a Fenton's reagent and catalyzing electron exchange and oxidative stress (38, 39). The same excess iron associated with pollution exposure can complex with available hydrogen and lipid peroxides to generate hydroxy and lipid radicals resulting in the cellular organelles and structures damage.

At the same time, the cell tries to restore intracellular iron by generating superoxide to allow for the chemical reduction step of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) required for the intracellular trace mineral entry (40–42). This reduction step triggers a vicious cycle that can generate more ROS, which consequently enhances the release of iron from protein carriers (ferritin) (43). This second wave of iron restoration could explain the more evident increase in

4HNE after 4 days of exposure, compared to early time points, as the increase of iron released from ferritin could further augment the cutaneous oxidative status and the formation of LOP.

Increased oxidative stress has been often associated with inflammatory processes, and this cross-talk can lead to a positive vicious cycle defined as OxInflammation (44), where oxidative stress induced by exogenous factors i.e. pollution, triggers and, at the same time, is fueled by aberrant inflammatory responses (20, 22).

This was confirmed in our study by the increased levels of MMP-9 after DEE exposure. Of note, DFO and CE Ferulic were able to quench MMP-9 activation in an additive manner suggesting that both iron and ROS are involved in its enzymatic activity.

MMP-9 expression is regulated by redox sensitive transcription factors (NFkB and AP1) and this enzyme is involved in the turnover and degradation of extracellular matrix components such as collagen contributing to wrinkles formation and premature skin aging. Therefore, it was not surprising that DEE exposure was able to affect collagen I levels and that the pre-treatment with DFO and CE Ferulic could prevent this damaging effect. These data confirmed the anti-pollution effect of CE Ferulic, as also noticed in a human study aimed to evaluate the cutaneous noxious effect of O_3 (25).

The oxidation and inflammation induced by DEE exposure can directly harm the skin structure and barrier function which, on the other hand, can fuel the perpetuation and amplification of the cutaneous OxInflammatory state itself.

In support of this idea, our study demonstrated that filaggrin (an important marker of differentiation and healthy skin barrier functions) (45), was decreased upon 1 and 4 days of DEE exposure. These data are in line with previous work by Lee et al. [43] who suggested that an inflammatory status can downregulate filaggrin levels. In addition, the same authors have recently

shown how the activation of NFkB is involved in keratinocytes filaggrin loss (46). Our group and others have indeed demonstrated the activation of NFkB by PM in skin models, confirming the hypothesis that the loss of filaggrin could be in part due to the inflammatory status (14).

Our data are also corroborated by a recent work by Li et al (47) where PM exposure not only decreased filaggrin levels in keratinocytes but it also significantly affected involucrin levels. The ability of PM to affect important cutaneous markers of differentiation can be interpreted as the ability of pollution to compromise skin barrier properties and possibly further allow external factors (i.e. pathogens, pollutants, etc.) to enter the skin.

Even if several studies demonstrated that skin health can be improved by diet (48), the local topical application should still be considered as a potent ally to prevent pollution-induced cutaneous damage. Supporting this theory is the extensive use of commercially available antioxidant formulation mixtures that have proven to have the ability to protect against single and combined pollutants exposure in preclinical and clinical studies (20, 25, 49, 50).

One of the treatments applied in these studies is represented by the CE Ferulic which is composed of 15% L-ascorbic acid, 1% α-tocopherol and 0.5% ferulic acid. This mixture, when topically applied, has shown to penetrate the stratum corneum (therefore possibly activating skin defensive mechanisms such as Nrf2 as demonstrated in 2D and 3D models [45-46]), preventing the UV-induced erythema, p53 activation, sunburn cells and DNA damage (51), and surprisingly exerting an additive beneficial photo-protective effect compared to the one mediated by the single elements (20, 52–54).

The suggested additive mechanism of action of this formulation is that ferulic acid acts as a reducing agent for vitamin E and C, therefore, as suggested by Pinnel's work, protecting and extending their reducing potential (52).

The aim of this study was the evaluation of a possible additive effect of two combined protective elements: a radical scavenger like CE Ferulic and an iron chelator, such as DFO. At the base of this idea is that, as pollution can induce extreme levels of oxidative stress and consequently lipid peroxidation, the use of antioxidant alone may not be enough to provide protection against DEE-induced skin damage (55). In fact, the dual action of DEE that is able to induce an unbalanced oxidative status directly and indirectly (via iron), suggests the protection through a bifunctional tool that targets both ROS and iron.

To date, many studied proved iron chelators, and DFO in particular, to be valuable allies in preventing iron-mediated oxidative damage. DFO is a hydrophilic drug with a short half-life and delivery *in vivo* into the hydrophobic stratum corneum can be somehow difficult, therefore more work needs to be done in trying to develop an efficient transdermal delivery system as suggested by Rodrigues et al. (56). They were able to uses reverse micelles to deliver DFO and improve wound healing in diabetic models.

Many other scientific groups demonstrated DFO to be suitable for topical application, specifically to ameliorate cutaneous chronic wounds (57), radiation-induced soft tissue injury (58), alleviate skin injury (59) and to prevent pressure-induced diabetic ulcers (60).

Consistent with these insights, are also our data that demonstrated how topically applied DFO, either alone or in combination with CE Ferulic, was able to counteract not only the DEE-induced lipid peroxidation and prevent MMP-9 activation, but also showed protective properties of the skin barrier, specifically by reverting the DEE-induced decrease in the involucrin and filaggrin levels.

However, new scientific insights showed that prolonged treatment with DFO or other effective iron chelators such as arolhydrazone chelators (61) can provoke severe cellular iron starvation,

leading to clinical complications (62–64). Therefore, other innovative iron chelator molecules could be taking the place of the most known DFO.

In particular, novel “smart pro-drugs” iron chelators have demonstrated effective capacities in preventing iron-induced oxidative damage upon UVA exposure and at the same time have shown physicochemical properties that allow them to be selectively activated *in situ* within the skin, only upon UVA irradiation (65). These molecules, known as photo-activable and photo-controller “caged” iron chelators could be the next step of topical anti-pollution protection.

In conclusion, although our research can be considered preliminary, it still allows us to corroborate previous data demonstrating the protective properties of CE Ferulic against pollution-induced OxInflammation and furthermore showed that the combined topical application of a cosmeceutical mixture containing CE Ferulic and an iron chelator such as DFO, could have potential additive effects in counteracting DEE-mediated skin damaging effects.

4.6. References

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CHAPTER 5: CUTANEOUS ANTIMICROBIAL PEPTIDES: NEW "ACTORS" IN POLLUTION RELATED INFLAMMATORY CONDITIONS

Woodby B, Pambianchi E, Ferrara F, Therrien JP, Pecorelli A, Messano N, Lila MA, Valacchi G.

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5.1. Abstract

Ozone (O_3) exposure has been reported to contribute to various cutaneous inflammatory conditions, such as eczema, psoriasis, rush etc. via a redox-inflammatory pathway. O_3 is too reactive to penetrate cutaneous tissue; it interacts with lipids present in the outermost layer of skin, resulting in formation of oxidized molecules and hydrogen peroxide (H_2O_2). Interestingly, several inflammatory skin pathologies demonstrate altered levels of antimicrobial peptides (AMPs). These small, cationic peptides are found in various cells, including keratinocytes, eccrine gland cells, and sebocytes. Classically, AMPs function as antimicrobial agents. Recent studies indicate that AMPs also play roles in inflammation, angiogenesis, and wound healing. Since altered levels of AMPs have been detected in pollution-associated skin pathologies, we hypothesized that exposure to O_3 could affect the levels of AMPs in the skin. We examined levels of AMPs using qRT-PCR, Western blotting, and immunofluorescence *in vitro* (human keratinocytes), *ex vivo* (human skin explants), and *in vivo* (human volunteer subjects exposed to O_3) and observed increased levels of all the measured AMPs upon O_3 exposure. In addition, *in vitro* studies have confirmed the redox regulation of AMPs in keratinocytes. This novel finding suggests that targeting AMPs could be a possible defensive strategy to combat pollution-associated skin conditions.

5.2. Introduction

Tropospheric ozone (O_3) is formed at the ground level through chemical reactions between nitrogen oxides (NOx) and volatile organic compounds, when pollutants are emitted by cars, industrial boilers, refineries, and chemical plants in the presence of sunlight [1]. Exposure to O_3 has been associated with negative effects on respiratory, cardiovascular, and neurological functions [1]. However, only recently has the noxious effects of O_3 exposure on cutaneous tissues been studied. O_3 exposure has been correlated with premature skin aging, contact dermatitis, atopic dermatitis, and psoriasis [2-6]. O_3 interacts with lipids present in the outermost layer of skin and initiates formation of bioactive products, such as aldehydes and free radicals, causing cutaneous redox imbalance (Valacchi et al., 2002). Oxidative stress induced by O_3 exposure is believed to contribute to the development and/or exacerbation of inflammatory skin disorders, but the mechanisms remain unclear. In this study, we have identified a potential role for AMPs as mediators of O_3-associated oxinflammation [7].

AMPs are key effectors in innate immune responses and protect from pathogens by disrupting pathogen membrane integrity [8]; they are divided into different groups, depending on their secondary structures. Defensins are small (~4 kDa), cationic peptides, characterized by three intramolecular disulfide bonds that stabilize two or more β-sheets, and are further classified as either beta or alpha, depending on arrangement of disulfide bonds [8]. Alpha defensins are primarily expressed by neutrophils and Paneth cells. Beta defensins are expressed in epithelial cells. Types of beta defensins found in human skin include human beta defensin 1 (hBD1), hBD2, and hBD3. Cutaneous hBD1 is constitutively expressed [9]; transcription of hBD2 and hBD3 is induced in response to infection, inflammation, or injury [10-14]. Cathelicidins are amphipathic, alpha-helical AMPs that contain a conserved N-terminal cathelin domain and a variable C-terminal

antimicrobial domain [8,10]. Although 30 members have been found in mammalians, humans only express one cathelicidin (CAMP gene). The precursor is called human cationic bacterial protein of 18 kDa (hCAP18), and the mature antibacterial peptide, generated through extracellular proteolytic cleavage, is named LL37 [15]. In skin, transcription of CAMP is induced in response to infection, inflammation, and injury [16-18]. We believe that oxidative stress, a consequence of O_3 exposure [19], can regulate AMP transcription, due to regulation of AMP-associated redox sensitive transcription factors (NF-κB and AP-1) [12,20,21].

Classically, AMPs function as antimicrobial agents. Recent research has indicated that AMPs can also regulate activation/chemotaxis of immune cells, stimulate angiogenesis, and induce production of proinflammatory cytokines [22-29]. Furthermore, the concentrations required for antibacterial activity of hBD3 are ~100–1000 fold excess, compared to its immunomodulatory activity [30]. Interestingly, increased levels of AMPs are detected in active lesions of inflammatory skin diseases, such as psoriasis and atopic dermatitis [17, 31-34], although this finding is still controversial for atopic dermatitis [33] both hBD2 and hBD3 have been originally isolated from psoriatic patients [11,35]; Korting et al., 2012). Higher genomic copy numbers of hBD3 also correlate with an increased risk in developing psoriasis [36], and self-DNA and LL37 complexes, as well as self-RNA and LL37 complexes, have been detected in psoriatic lesions, contributing to immune cell activation [37,38].

Since exposure to pollutants as well as altered AMPs levels are associated with the development/progression of inflammatory skin conditions, we hypothesized that exposure to the pollutant O_3 could affect cutaneous AMPs levels through a redox mechanism. In the present work we examined levels of hBD1, hBD2, hBD3, and CAMP *in vitro* using human keratinocytes, *ex vivo* using human skin biopsies, and *in vivo* in human volunteer subjects exposed to O_3. We

observed increased levels of these AMPs in response to O_3 in all models analyzed, likely due to redox regulation of AMPs.

This novel finding brings new insights on the possible role of pollutants in redox regulation of AMPs and how O_3 could affect the development/progression of inflammatory skin conditions, suggesting eventual new therapeutic anti-pollution strategies. This study could contribute to explain the increase of AMPs levels in inflammatory skin disorders (like psoriasis), creating a currently unexplored niche in pollution-induced skin pathologies.

5.3. Results

5.3.1. Ozone (O_3) increases AMPs levels in human keratinocytes

Since both, O_3 exposure and increased AMPs levels are independently associated with cutaneous inflammatory conditions, we want to evaluate whether O_3 exposure could modulate AMPs expression. Therefore, we determined the levels of various cutaneous AMPs including hBD1, hBD2, hBD3, and CAMP (LL37) in keratinocytes exposed to 0.2 ppm of O_3 for 30 min. We observed a similar trend for hBD1 and hBD3 with the highest response at 6 h post-exposure and a decline at the following time points (12 and 24 h) (Fig. 1a, and c); while the transcript levels of hBD2 and CAMP in O_3 exposed cells increased 6 h post-exposure and reach the highest expression 12 h post-exposure (Fig. 1b and d). As expected, we did not observe a large induction for the constitutively expressed hBD1 mRNA [9]. On the other hand, the response for hBD2, hBD3, and CAMP were very robust, confirming their susceptibility to different challenges (inflammation, injury, etc) [10-14,16-18]. We also assessed hCAP18 (LL37 precursor) protein levels (Fig. 1e) and observed a clear induction of hCAP18 by O_3 12 h post-exposure (Fig. 1e and f). These data confirm the hypothesis that O_3 exposure is able to affect cutaneous AMPs expression and levels.

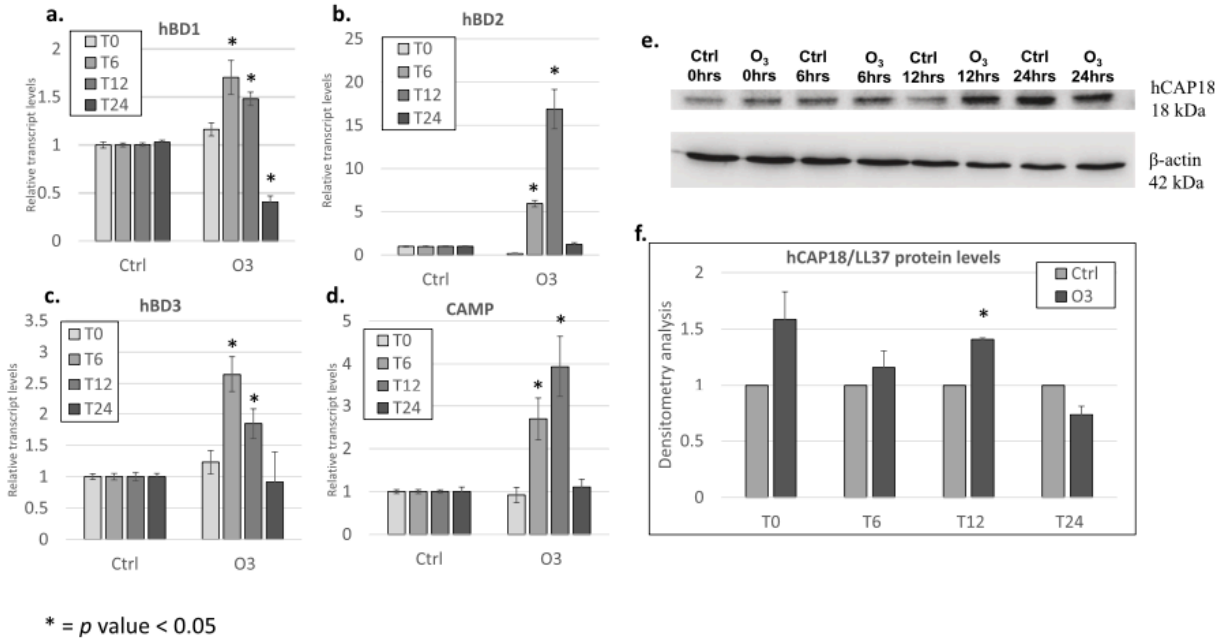


Fig. 1. AMPs are increased in keratinocytes exposed to ozone in vitro. qRT-PCR analysis of transcript levels of hBD1 (a), hBD2 (b), hBD3 (c), and CAMP (d) in keratinocytes exposed to 0.2 ppm of O_3 directly after exposure (T0), 6 h (T6), 12 h (T12), and 24 h (T24) post-exposure. (e) Representative immunoblot for hCAP18 levels in keratinocytes at different time points (T0, T6, T12, and T24), quantification is depicted in panel (f). Results from three independent experiments and three replicates. * = p-value<0.05. Error bars are defined as mean±SEM.

5.3.2. O_3 exposure increased AMPs protein levels in human skin biopsies

Considering the limitation of the 2D cell culture models, we want to confirm our data in *ex vivo* human skin biopsies, which incorporate all skin appendages such as hair follicles and sweat glands (produce AMPs). As depicted in Fig. 2, after O_3 exposure (T0) there was a clear and significant increase of hCAP18/LL37 staining throughout exposed epithelia (1.25 fold) (Fig. 2A) and this increment was lost 24 h post-exposure. As depicted in Fig. 2B, a clear increase in hBD2 (2 fold) was noticed after 24hr post exposure. In addition, we were able to also detect a significant increase in hBD3 levels immediately after O_3 exposure (T0) (1.6 fold) (Fig. 2C) and 24 h post-exposure (1.2 fold). These data confirm the results observed in 2D keratinocytes model.

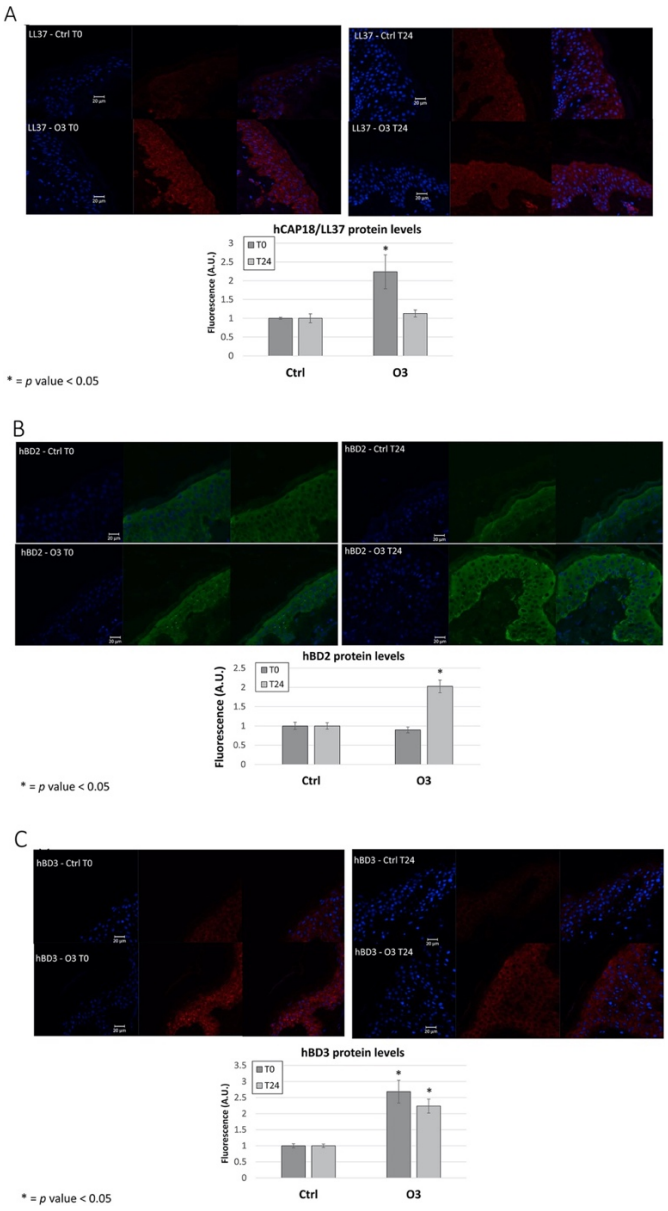


Fig. 2. Exposure of human biopsies to ozone increases AMP protein levels in the skin. Ex vivo human abdominoplasty biopsies from three different donors were exposed to 0.4 ppm O_3. Sections were collected directly after exposure (T0) and 24 h (T24) post exposure and embedded in paraffin. Immunofluorescence was performed analyzing protein levels of AMPs hCAP18/LL37 (A) hBD2 (B) and hBD3 (C). Images quantification was performed using ImageJ. Graphs depicted are the results of the quantification of three independent experiments. The images reported in this figure were chosen as being most representative of the quantification. Red staining indicates either hCAP18/LL37 or hBD3, green staining indicates hBD2. Blue staining represents DAPI. * = p-value<0.05. Error bars are defined as mean±SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

5.3.3. Redox regulation of cutaneous AMPs

As mentioned before, O_3 is not able to penetrate the skin as it reacts instantaneously with the polyunsaturated fatty acids (PUFAs) present in the stratum corneoum, producing several byproducts, including hydrogen peroxide (H_2O_2) and aldehydes, such as 4-hydroxynonenal (4HNE) [39]. To determine whether O_3-induced H_2O_2 is responsible for increased AMPs transcription, we pre-treated keratinocytes with 800 U/ml of catalase (cat) for 1.5 h then exposed to 0.2 ppm of O_3 for 30 min and collected samples 6 and 12 h post-exposure. As depicted in Fig. 3, pretreatment with cat prevented O_3-induced hBD1, hBD2, hBD3, and CAMP transcripts (Fig. 3a–d).

As a proof of concept, we exposed human keratinocytes to H_2O_2 to understand whether this molecule could be a possible messenger of O_3 affects AMPs transcript levels. As shown in Fig. 3e, H_2O_2 significantly increased transcript levels of all tested AMPs after 6 h with hBD2 the most upregulated (over 20-fold) (Fig. 3e). In addition, we observed a striking increase in protein levels of hCAP18 immediately after exposure to O_3 and after 30 min of treatment with H_2O_2 and 4HNE (the other main bio-product formed by O_3 interaction with the epidermis fatty acids) (Fig. 3f and g). We observed no clear differences between hCAP18 levels in keratinocytes treated with either 4HNE or H_2O_2 after 6 h suggesting that both molecules are able to affect hCAP18 levels. Of note is that the effect of H_2O_2 was evident up to 12–24hrs post exposure (Fig. 3f). These data suggest that AMPs are increased in response to O_3 in skin due to generation of mediators, such as H_2O_2 and 4HNE.

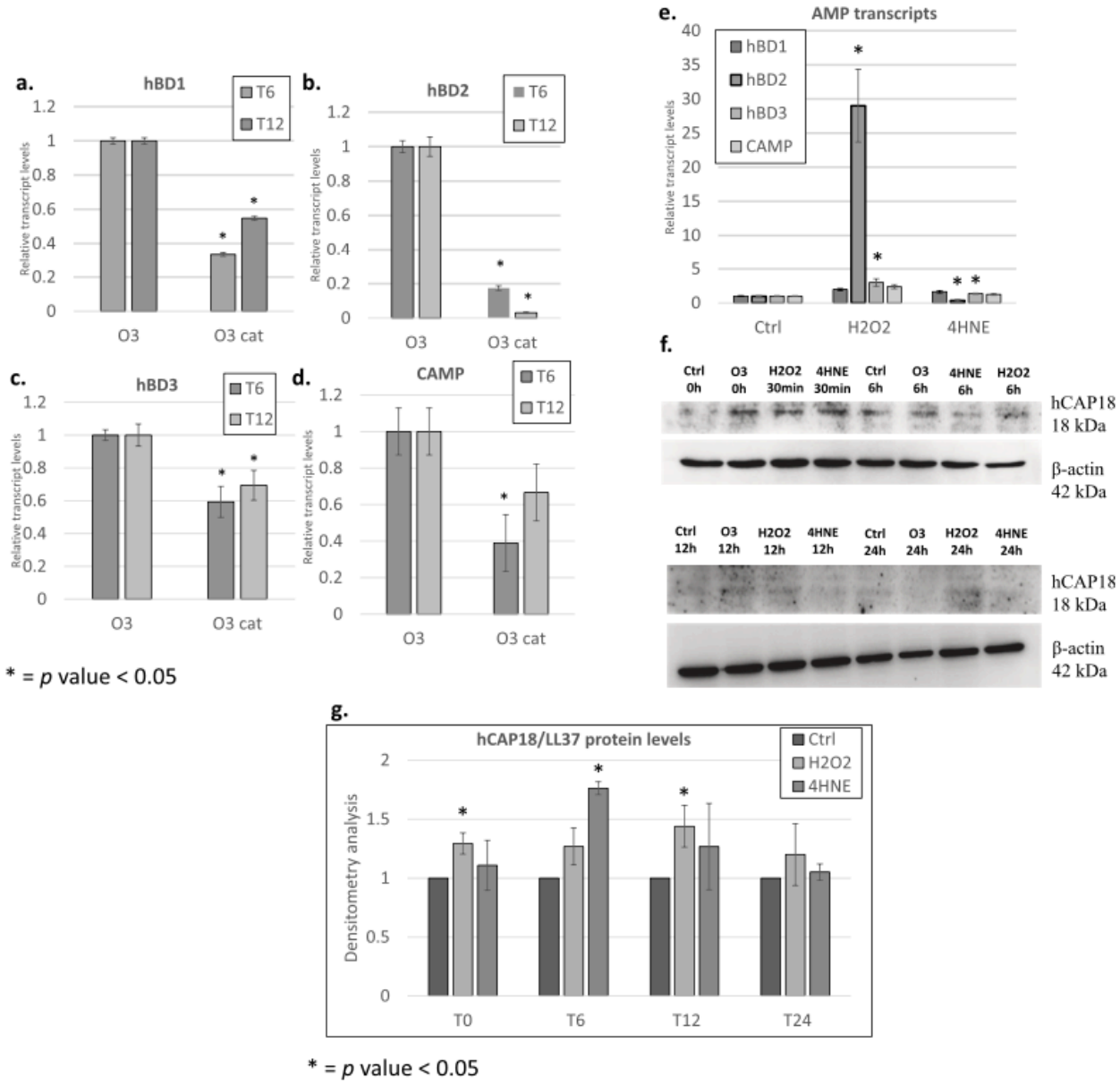


Fig. 3. Ozone-induced signaling mediator H2O2 regulates AMP levels in keratinocytes. Transcript levels of hBD1 (a), hBD2 (b), hBD3 (c), and CAMP (d) were assessed in keratinocytes pretreated with 20 µg/mL of catalase (cat) then exposed to O_3 at different time points. AMPs transcript levels in keratinocytes treated with 50 µM H_2O_2 for 6 h (e). Protein levels of hCAP18 in keratinocytes after 30 min, 6 h (T6), 12 h (T12), and 24 h (T24) of treatment with either H_2O_2 or 4HNE (f). Quantification is depicted in panel (g). *p-value<0.05, compared to control. Error bars = mean ± SEM.

5.3.4. Exposure of human volunteers to O_3 increases AMPs levels in skin

To further confirm our data, we determine the levels of AMPs after O_3 exposure in human volunteers. We exposed forearms of subjects to O_3 for 3 h a day for 5 consecutive days (Allendale Institutional Review Board; 7015-090-104/106-002; August 10, 2015) [40]. We examined levels of hCAP18/LL37, hBD2 and hBD3 in 3 mm punch biopsies taken from both unexposed (control) and exposed lateral forearms and observed increases in both LL37 (Fig. 4A), hBD2 (Fig. 4B) and hBD3 (Fig. 4C) positive staining throughout the epithelia of exposed forearms. As depicted in Fig. 4 the increase was statistically significant for all the AMPs analyzed, indicating that O_3-induced their upregulation also *in vivo* and substantiating the *in vitro* and *ex vivo* findings (Fig. 1, Fig. 2, Fig. 3).

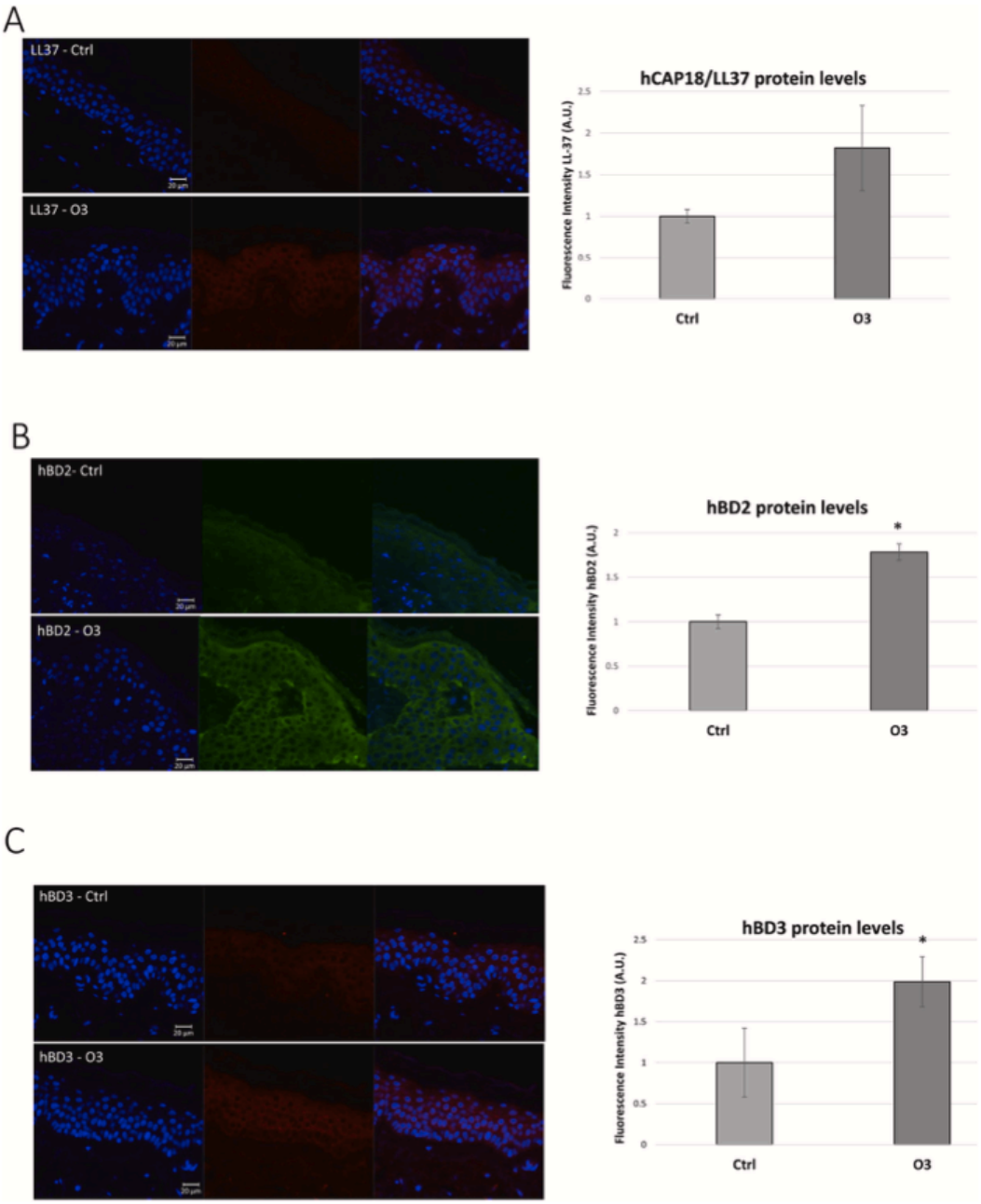


Fig. 4. Exposure of human volunteer subjects to ozone increases AMP protein levels in the skin. Forearms of human volunteer subjects were exposed to 0.8 ppm O_3 for 3 h a day for 5 consecutive days. We examined protein levels of hCAP18/LL37 (a, c) and hBD3 (b, d) using immunofluorescence in 3 mm punch biopsies taken from the aforementioned subjects from both unexposed (control) and exposed lateral forearms. Images were quantified using ImageJ. Data shown in (c–d) are the results of averages of multiple images from at least three different subjects. Images depicted in Fig. 5 were chosen as being the most representative of results after quantification. * = p-value<0.05. Error bars are defined as mean±SEM.

5.4. Discussion

O_3 exposure has been associated with the development/exacerbation of inflammatory skin conditions, such as atopic dermatitis, acne, and psoriasis [[2], [3], [4],77]. Inflammatory skin conditions are also associated with altered AMPs levels, indeed multiple AMPs were originally isolated from human skin pathologies [11,35]. Since O_3 exposure as well as increased levels of AMPs are associated with cutaneous inflammation [1,4,17,[31], [32], [33], [34]], we hypothesized that cutaneous AMPs upregulation due to O_3 exposure could be linked to the development/exacerbation of inflammatory skin conditions.

To test this, we used a variety of skin models, ranging from *in vitro* cell culture, *ex vivo* human skin explants, and human volunteer subjects. We utilized *in vitro* cell culture mainly for the mechanistic aspects of our study and ex vivo human skin explants, since this model incorporates skin appendages such as hair follicles and sweat glands (both apparatuses involved in AMPs production) to better evaluate cutaneous responses. In addition, human volunteer subjects were also exposed to O_3 and AMPs were evaluated *in vivo*. At this stage we have avoided the use of murine models because the classes of AMPs encoded in mice differ from humans [8,10], the transcriptional regulation of CAMP is different in mice vs. humans, due to presence of a primate-specific retrotransposable element [41,42] and the mechanisms involved in epigenetic regulation also differ between mice and humans [43]. Importantly, in all the skin models analyzed, *in vitro*, *ex vivo*, and *in vivo*, we observed that O_3 exposure increased levels of the cutaneous AMPs: hBD1, hBD2, hBD3, and CAMP, and this to our knowledge has never been demonstrated before.

Interestingly, we observed that transcript levels of hBD1 (constitutively produced by all epithelia) is increased in response to O_3 exposure. Schroeder et al. demonstrated that reduction of disulfide bonds of hBD1 increases its antimicrobial activity against pathogenic and commensal

bacteria and fungi [44]. Raschig et al. showed that the ability of hBD1 form bacteria-entrapping nets depends on reduction of its disulfide bonds [45], which can be mediated by the thioredoxin system [44,45]. In our study the transcript levels of hBD1 increased in keratinocytes exposed to O_3, although not evident as other cutaneous AMPs and this was expected considering that hBD1 is the constitutive form. Due to lack of a commercially available antibodies, we could not investigate whether O_3 exposure altered redox-dependent antimicrobial activity of hBD1, although it is likely that pollutant exposure affects hBD1 activity, as O_3 induces oxidative stress and hBD1 has been shown to be redox sensitive [46].

We also observed increased transcript levels of hBD2, hBD3, and CAMP, in response to O_3 exposure in keratinocytes and that hBD2 transcript levels are increased to a much higher level than either CAMP or hBD3, in response to both O_3 and H_2O_2. Transcription of these AMPs is regulated by different signaling pathways. CAMP transcription is regulated by vitamin D, due to presence of a vitamin D response element in the promoter. This promoter is also responsive to vitamin D receptor (VDR)-independent transactivation by AP-1 and NF-κB [42,47,48]. In addition, Steubesand et al. demonstrated that infection with Candida albicans increases hBD2, but not hBD3, transcript levels in an NF-κB-dependent manner in epithelial cells [21]. Further studies by Tsutsumi-Ishii [49] confirmed the susceptibility of hBD2 to NFkB regulation. This could explain the evident increase of hBD2 mRNA in response to H_2O_2 which has been demonstrated for more than 20 years a very specific and strong NFkB activator via IkB alpha phosphorylation [50]. In silico analyses indicate the hBD2 promoter has multiple potential NF-κB and AP-1 binding sites which is in agreement with several others studies [12,20]. The hBD3 promoter is covered in potential AP-1 binding sites and has three potential AhR binding sites (data not shown). Rademacher et al. demonstrated that blocking AhR signaling decreases Staphylococcus

epidermidis-induced increases in hBD3 transcription in skin [51]. Future studies include investigating whether we can detect NF-κB, AP-1, and AhR binding to the hBD2, CAMP, and hBD3 promoters, in response to O_3 exposure in skin, since these transcription factors have been shown to be all modulated by O_3 [1,52,53]

Based on our results, H_2O_2 alone is likely not fully responsible for O_3-mediated increases in hBD3 and CAMP transcription. It is possible that lipid-derived peroxides or other lipid-derived aldehydes such as 4HNE, affect cutaneous AMPs levels. In skin, O_3 exposure induces production of H_2O_2, as well as lipid peroxides and aldehydes, through interaction with PUFAs [54]. In addition to direct stimulation and/or modifications by lipid peroxides or aldehydes, these products could potentially alter AMPs levels indirectly through further increasing reactive oxygen species (ROS) levels. It has also been demonstrated that O_3 exposure increases levels of cyclo-oxygenase 2 (COX2) [55], which is also involved in ROS production. The idea that AMPs regulation is at least partially redox modulated is confirmed by the catalase experiments, suggesting that H_2O_2 can be one trigger for AMPs transcription.

In vivo O_3 exposure could also regulate AMPs levels by affecting the brain-skin axis. In this axis, stress induces production of hormones, neurotransmitters, and secreted factors that can regulate proinflammatory pathways in skin through regulation of NF-κB as well as lamellar body secretion (encapsulates AMPs), resulting in alterations in AMPs mRNA and/or protein levels [[56], [57], [58]].

Whether other pollutants affect AMPs levels has been sparsely investigated. For instance, Ultraviolet Radiation (UV) induces expression of AMPs (hBD1, hBD2, CAMP) in human keratinocytes, which could potentially alter the skin microbiome [[59], [60], [61]]. Exposure to particulate matter (PM) decreases the ability of respiratory epithelial cells to increase AMPs levels

in response to infection [62,63]. Vargas Buonfiglio et al. demonstrated that ambient PM (coal fly ash) binds to cationic AMPs, decreasing levels of active AMPs [64]. Whether PM decreases AMPs activity in skin is still unknown.

Activity of many AMPs is regulated by cleavage of signal sequences at N-terminal, which holds the COOH-terminal AMP domain in an inactive form. In skin, hCAP18 is cleaved by kallikrein-related serine proteases to LL37 [8]. LL37 can then be further cleaved into RK-31, KS-30, and K20, which exhibit different antimicrobial and immunomodulatory activities than LL37 [15]. Whether O_3 exposure regulates AMPs cleavage in skin is unknown. Another mechanism by which O_3 exposure could regulate AMPs activity is through inducing post-translational modifications, such as protein oxidation. Furthermore, O_3 exposure, which depletes cellular lipid content, could affect packaging of AMPs into lamellar bodies [8,65]. Disturbance of AMPs levels in skin could alter composition of the skin microbiome [30] and make the skin more susceptible to develop inflammatory conditions.

Independent of antimicrobial activities, AMPs can regulate chemotaxis, activation, and differentiation of immune cells, modulate cellular toll-like receptor (TLR) responses, inflammasome activation, stimulate angiogenesis, and regulate the expression/activity of proinflammatory cytokines/chemokines [8,10,22,23,27-29,66,67]. In inflammatory skin conditions, including psoriasis, atopic dermatitis, and rosacea, altered levels of AMPs have been detected, as well as self-RNA/LL37 and self-DNA/LL37 complexes (in psoriasis), which can regulate activity of immune cells involved in these conditions [17,31-34,37,38]. The consequences of AMPs induction in response to pollutants could result in development/exacerbation of inflammatory skin disorders, due to the ability of AMPs to regulate adaptive immune responses. Our lab has previously demonstrated that O_3 exposure can activate cutaneous inflammasome [68]

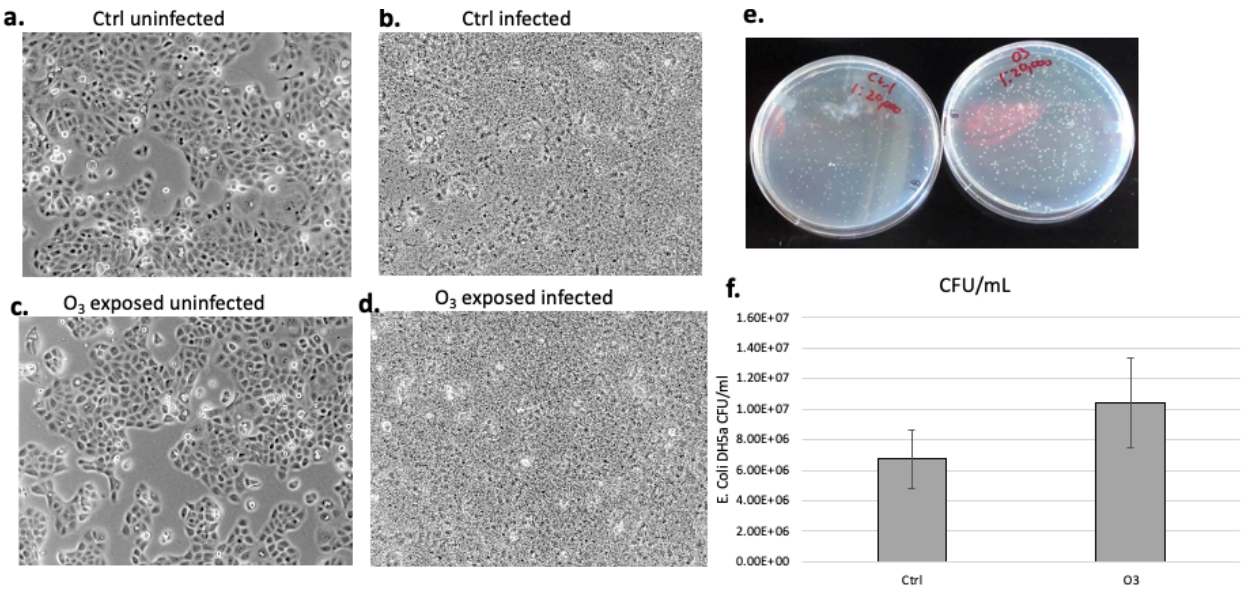
which become even more relevant considering that LL37 can stimulate skin inflammasome activation [66].

However, AMPs can also act as anti-inflammatory agents, depending on context [69]. In fact, AMPs have recently been utilized as cosmeceuticals to improve the structure and function of aged skin [70-76]. It is currently unclear whether increased levels of AMPs in response to pollution exposure inhibits or promotes inflammation in skin. Their increase upon pollution exposure could be also interpretated as a non-specific response to unknown xenobiotic leading to an altered skin homeostasis.

Despite the fact that AMPs have the function to protect our body, several studies have suggested that these molecules can contribute to the pathogenesis of various skin conditions such as psoriasis, atopic dermatitis, rosacea, acne vulgaris, systemic lupus erythematosus and systemic sclerosis. Thus, AMPs induction and activation can also be considered a double-edged tool where from one side can promote cutaneous immunity and from the other side trigger the pathogenesis of some skin disorders [71]. Therefore, at this stage is hard to understand whether cutaneous AMPs upregulation in response to O_3 is a "good" or a "bad" event; it is for sure a response to an oxinflammatory insult that leads to an alteration of skin homeostasis. Further studies need to be performed to better understand whether this induction is a transient response to an acute challenge or can lead to prolonged tissue responses. It is also possible that the induction of AMPs by pollution could affect cutaneous microbiome and therefore affect skin homeostasis.

The further step will be to evaluate whether the AMPs secreted are fully functioning as anti-inflammatory molecules. Our attempts using E-*coli* (see supplementary Figure 1), showed that infected cells exposed to O_3 increase their proliferation, suggesting their use in cosmeceuticals as

a new possible technology against pollution-induced skin damage although, in a long run the eventual development of bacterial resistance should also be taken in consideration.



Supplementary Figure 1. O_3 exposure increases bacterial growth on exposed cells. Since we observed that O_3 exposure increased cutaneous AMPs levels, we want to evaluate its effect on bacterial growth. To test this, we infected keratinocytes exposed to O_3 with $1x10^5$ CFU *Escherichia (E.) coli* immediately after exposure (Krasnodembskaya et al., 2010). After infection, we collected the media, serially diluted it, and plated bacteria on LB agar plates (Figure 1a-d). As a readout of bacterial growth, we assessed colony forming units (CFU). As shown in Figure 1e-f, we observed an increase in bacterial growth in keratinocytes exposed to O_3.

5.5. Materials and Methods

5.5.1. Cell culture

Human keratinocytes (HaCaTs) were cultured, as previously described [72] (see Supplemental Methods). 1 × 106 (RNA) or 9 × 105 (protein) keratinocytes were seeded onto 60 mm or 6-well plates and exposed the following day. For catalase experiments, keratinocytes were pre-treated with 20 µg/mL of catalase (C4963, Sigma, St. Louis, MO) for 1.5 h. For exposure, keratinocytes were exposed for 30 min at 0.2 ppm of O_3, as previously described [73]. For 4HNE and H_2O_2 experiments, keratinocytes were treated with either 50 µM concentration of H2O2 (H1009-100 ML, Sigma, St. Louis, MO) dissolved in water or 20 µM concentration of 4HNE (CAS 75899-68-2, Santa Cruz, Dallas, TX) dissolved in ethanol. Protein and RNA were collected at the indicated time points using protein lysis buffer (Supplemental Methods) or TRIzol (Ambion) [74].

5.5.2. Ozone generator

O_2 was used to generate O_3 via electrical corona arc discharge and combined with ambient air to flow into a plexiglass box (ECO3 model CUV-01, Torino, Italy Model 306 Ozone Calibration Source, 2B Technologies, Ozone Solution), as previously described [40,73]. Concentration of O_3 in the chamber was adjusted, depending on the model used, and continuously monitored by an O_3 detector.

5.5.3. Preparation of ex vivo human biopsies

Healthy human skin was obtained from elective abdominoplasties from 3 different donors. 12 mm punch biopsies were taken from the human skin. Next, subcutaneous fat was trimmed. Biopsies were rinsed with phosphate-buffered saline (PBS, Gibco, NY, USA) containing 1%

antibiotics/antimycotics using sterile technique and then immediately transferred to 6-well dishes and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, NY, USA) at 37 °C in 5% CO_2 humidified atmosphere. Experiments were performed the following day, as previously described by Ref. [68].

5.5.4. Ex vivo O_3 exposure

Biopsies from donor skins were placed in a plexiglass box connected to the ozone generator and exposed to 0.4 ppm O_3 for 4 h [68] N = 3. Samples were collected directly after exposure and 24 h post-exposure in 10% neutral buffered formalin and then dehydrated using alcohol gradients, followed by immersion in xylene for paraffin embedding.

5.5.5. Immunofluorescence

Immunofluorescence analysis was performed using 4 µm thick paraffin-embedded sections of *ex vivo* human biopsies or *in vivo* biopsies from human subjects, as previously described [40]. Briefly, sections were deparaffinized in xylene and rehydrated using alcohol gradients. Antigen retrieval was achieved using heat-based epitope retrieval with 10 mM sodium citrate buffer (AP-9003-500, Thermo, Waltham, MA) (pH 6.0) with 0.05% Tween 20 at a sub-boiling temperature in pressure cooker (Instant Pot Duo using the steam setting – Kanata, ON, Canada) for 15 min. After cooling for 30 min, the sections were washed 2 times for 5 min in PBS, blocked with 5% BSA in PBS at RT for 45 min, and incubated overnight at 4 °C with primary antibodies for hCAP18/LL37 (sc-166770, Santa Cruz, Dallas, TX) or hBD2 (ab63982 ABCAM Cambridge MA), hBD3 (sc-59495, Santa Cruz, Dallas, TX) at 1:50 dilutions in 2% BSA in PBS. The next day, sections were washed 3 times in PBS for 5 min, followed by a 1 h incubation with fluorochrome-conjugated

secondary antibodies (Alexa Fluor 568 cat. A11004, Invitrogen, Waltham, MA) at 1:1000 dilutions in 2% BSA in PBS at RT, and then washed with PBS 3 times for 5 min. Nuclei were stained with DAPI (cat. 1874814, Invitrogen, Waltham, MA) for 1–2 min in PBS at RT, and sections were then washed 3 times for 5 min with PBS and then with water. The sections were mounted onto glass slides using PermaFluor aqueous mounting medium (TA-006-FM, Thermo, Waltham, MA) and imaged via epifluorescence on a Zeiss LSM10 confocal microscope equipped at 40X magnification. Quantification of raw images was performed using ImageJ.

5.5.6. Immunoblotting

Total protein lysates were extracted in ice-cold RIPA buffer (cat. AAJ62524AD, Alfa Aesar, Tewksbury, MA) with 1% protease (cat. 78430, Thermo, Waltham, MA) and 1% phosphatase (cat. 1862495, Thermo, Waltham, MA) inhibitor cocktails. Lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. Protein content in supernatants was measured using the Quick Start Bradford Protein Assay Kit (cat. 5000201, Bio-Rad, Hercules, CA). Equivalent amounts of proteins were ran on 15% SDS-PAGE gels, electro-transferred onto nitrocellulose membranes, and blocked in TBS containing 0.1% Tween 20 and 5% not-fat milk (cat. 1706404XTU, BioRad, Hercules, CA). Membranes were incubated with 1:1000 dilution of hCAP18/LL37 (cat. sc-166770, Santa Cruz, Dallas, TX or cat. ab80895, Abcam, Cambridge, UK) or beta actin (cat. A3854, Sigma, 1:50,000 dilution) antibodies in 2% milk TBST. The membranes were then incubated with 1:10,000 dilutions of horseradish peroxidase-conjugated secondary antibodies (cat. 170–6516, BioRad, Hercules, CA) in 2% milk TBST for 1 h at RT, and the bound antibodies were detected in a chemiluminescent reaction (ECL, BioRad, Hercules, CA). Chemiluminescence was detected on ChemiDoc imager (BioRad, Hercules, CA), and the signal was quantified using Image J. For

quantification analysis, levels of hCAP18 were first normalized to beta actin then to the control sample for each time point for each experiment (n = 3). Next, we compiled densitometric values of blots from individual experiments together to generate the graph depicted in Fig. 1e.

5.5.7. RNA extraction and quantitative real time PCR

RNA samples were extracted using TRIzol (Ambion), and RNA was isolated using phenol-chloroform extraction using a modified version of the protocol described by Ref. [74]. Reverse transcription-PCR was performed using iScript cDNA Synthesis Kit (cat. 1708841, BioRad, Hercules, CA), according to the manufacturer's instructions. To investigate the mRNA expression of AMPs, quantitative real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (cat. 1725271, Biorad, Hercules, CA), according to the manufacturer's protocol on a Roche LightCycler 480 machine. Quantitative relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, using the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) to normalize, and control samples as internal calibrators. The primers used are listed in Table 1.

Table 1. Primers used in the study.

Gene	Forward	Reverse
GAPDH	TCGGAGTCAACGGATTTGGT	TTCCCGTTCTCAGCCTTGAC
hBD1	TTGTCTGAGATGGCCTCAGGTGGTAAC	ATACTTCAAAAGCAATTTTCCTTTAT
hBD2	GCATTGCACCCAATACCAGT	CCAAAAACACCTGGAAGAGGCA
hBD3	TATCTTCTGTTTGCTTTGCTCTTCC	CGCCTCTGACTCTGCAATAA
CAMP	CGGTGTATGGGGACAGTGAC	TGGGTACAAGATTCCGCAAA

5.5.8. Human subjects' exposure

Human subjects consisted of Caucasian male and female (60% male and 40% female) human volunteers aged 18–55, free of systemic or dermatological disorders (N = 15). Written consent was obtained from the subjects. Subject forearms were exposed to 0.8 ppm O_3 for 3 h a day for 5

consecutive days. 3 mm punch biopsies were collected immediately after the last exposure (IRB approval - Allendale Institutional Review Board; 7015-090-104/106-002; August 10, 2015), as described by Ref. [40]. We did not observe sex-specific differences in upregulation of inflammatory markers in response to exposure.

5.5.9. Antibacterial experiments

E. coli strain DH5α (gifted from Hsieh lab at Plants for Human Health Institute (PHHI)) was used for these experiments. Colonies were seeded from frozen stocks and grown overnight at 37 °C in Luria-Bertani (LB) broth with slight agitation. Before antibacterial experiments, the bacterial cells were washed with PBS, and optical density at 600 nm was measured. For these experiments, bacteria were used that had exhibited absorbance of 0.4 at OD600 (in exponential growth phase). Number of CFU to use for infection was calculated using the following formula: OD600 = 4 × 108 CFU/ml of E. coli [75]. The day prior to exposure, human keratinocytes (HaCaTs) were washed three times with PBS and cultured in DMEM with 10% FBS and no antibiotics. 100,000 cells were seeded in 24-well plates, and, the following day, cells were exposed to 0.2 ppm of O_3 for 30 min. Directly after exposure, cells were infected with 1 × 105 CFU of bacteria and incubated for 6 h in 5% CO_2 humidified incubator at 37 °C. Microscopy images were taken on a Zeiss LSM 710 microscope 6 h post-infection. Aliquots were taken from culture media and serially diluted in LB broth and then plated on LB agar plates. Colonies were counted after overnight incubation at 37 °C. Representative experiment is depicted in Supplementary Figure 1. Quantification was based on counts from multiple serial dilutions that were averaged together to produce graph shown in Fig. 5.

5.5.10. Statistical analysis

Welch's unequal variance t-test was used to test results for significance. Statistical significance was considered at $p < 0.05$. Data are expressed as mean ± SEM.

5.6. References

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CHAPTER 6: TENSION AS A KEY FACTOR IN SKIN RESPONSES TO POLLUTION

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Tension As A Key Factor In Skin Responses To Pollution.

6.1. Abstract

Because it is the most evident organ exposed to the outdoor stressors, the effect of pollution to the skin has been widely studied in the last few decades. Although UV light is clearly the most aggressive stressor to which our cutaneous tissue is daily exposed, other components that are part of the tropospheric pollution have been shown to affect skin health and functionality. Among them, ozone has been proven to be one of the most toxic due to its high reactivity with the lipids present in the epidermis.

Studying the cutaneous effect of pollution in a laboratory is very challenging and it is important to develop and use the appropriate model based on the questions that we intend to answer.
Several skin models are available nowadays, in vitro models (2D cell lines and 3D cutaneous tissue), *ex vivo* skin biopsies and *in vivo* approaches (animals and human volunteers).
Although in the last 20 years the technology developed skin models that closely resemble human skin (3D cutaneous tissues), one of the best approaches to study cutaneous responses still remains the use of *ex vivo* human skin explants.

Unfortunately, one important cutaneous property not present in *ex vivo* human skin biopsies is the physiological tension, which has been shown to be a cardinal player in skin structure, homeostasis, functional properties and responses to external stimuli.
For this reason, to confirm and further evaluate the effect of ozone exposure in our integumentary system, we have performed experiments using the state of art in cutaneous models: the innovative model TenBio in which *ex vivo* human skin explants are cultured under physiological tension during the whole experimental procedure.

Specifically, we were interested in confirming and corroborating previous findings showing that ozone exposure modulates cutaneous antimicrobial peptides (AMPs) expression.

Indeed, we were able to demonstrate that ozone induced AMPs gene and protein expression (CAMP/LL-37, hBD2, hBD3) and that physiological tension could further modulate their expression when compared to matching skin biopsies cultured in non-tension conditions.
Moreover, different responses between tension and non-tension cultured skin were also observed for OxInflammatory markers like cycloxygenase-2 (COX2), matrix-metallo-proteinase 9 (MMP9), aryl hydrocarbon receptor (AhR) and 4-hydroxy-nonenal (4HNE).

This current study supports our previous findings confirming the ability of pollution to affect skin AMPs expression and that skin biopsies are a good and reliable model to study skin responses although, before extrapolating the data to real life, it is better to take into consideration the eventual role of skin tension.

6.2. Introduction

Air pollution is now one of the leading causes for premature death, followed distantly by HIV/AIDS infections, parasitic diseases like malaria and other vector-borne infectious ailments (1). This is a consequence of the latest estimates from World Health Organization (WHO) which state that 91% of urban world's population breathes polluted air, leading to 4.2 million annual premature deaths (2).

Environmental pollution is a generic term that encompasses a wide array of compounds present at the ground level that based on the United State Department of Agriculture (USDA) can be regrouped into 6 main contaminants, based on their chemical and physical characteristics: carbon monoxide (CO), lead (Pb), nitrogen dioxide (NO_2), ozone (O_3), particulate matter (PM) and sulfur dioxide (SO_2) (3).

Ozone stand out as one of the most dangerous and toxic airborne pollutants for human health (4), due to its high reactivity with biological membranes.

Around 90% of ozone naturally exists in the stratosphere where it plays a key role in absorbing UV radiation before reaching the ground level (5). The anthropogenic-formed ozone is present at the ground-level (troposphere) and it originates in the presence of UV sunlight from photochemical smog: the reaction between nitrogen oxides and hydrocarbons present in car exhaust (6).

Even though ozone levels can vary based on various conditions (humidity, seasonality, temperature, latitude, altitude), high concentrations have been reported in urban centers, ranging between 0.5-0.8 ppm during severe polluted episodes (4, 7–9). Of note, to avoid noxious health effects, the WHO set the threshold of ozone exposure to 0.05 ppm (10), however, the last reports from WHO itself estimated that 9 out of 10 people breathe air exceeding the recommended guidelines (9, 10).

Although O_3 is not a radical species *per se*, its noxious effect is mainly mediated by the formation free radicals. (11).

The skin is the second largest organ (after the respiratory tract) to be targeted by ozone exposure.

The mechanism by which ozone induces cutaneous damage is via the direct interaction and oxidation of poly unsaturated fatty acids (PUFAs) and lipids present in the outermost layer of skin, the stratum corneum leading to the direct generation of aldehydes (i.e. 4-hydroxynonenal -4HNE) and reactive oxygen species (ROS) including H_2O_2 all molecules able to further carry on ozone toxicity and damage skin barrier properties (12, 13).

In addition, the production of ROS can trigger inflammatory responses propagating oxidative damage through indirect harmful pathways, such as the activation of pro-inflammatory transcription factors (NFkβ, AP-1, AhR) (14) and intracellular protein kinases (MAPK, JNK,) involved in degradation of the connective dermal tissue (15) via the production of metallo-proteinases (MMP9) and the release of pro-inflammatory mediators (COX2, IL-18, IL-1β, etc.).

Our previous study evidenced *in vitro* that the production of these secondary messengers is not only one of the causes of the development/exacerbation of skin conditions, but is also the trigger of the production of antimicrobial peptides (AMPs) (16).

AMPs are small peptides (12-100 amino acid residues), key effectors in innate immune responses (17).

AMPs have as a general antimicrobial mechanism the electrostatic interaction and consequent disruption of negatively charged microbial cell membranes (18–20). Conventional antibiotics are synthesized enzymatically by microorganisms, inhibit enzymes involved in the synthesis of bacterial protein walls and have limited targets that can easily mutate to induce resistance (21,

22). Of note is the fact that AMPs, instead, are encoded by distinct genes, translated from mRNA templates and since no specific receptors are involved, AMPs are able to kill a broad spectrum of pathogens without the development of resistance (21, 22).

In humans two main categories have been identified: defensins (the constitutive hBD1 and the inducible hBD2 and hBD3) and cathelicidins (LL-37).

Interestingly, increased levels of AMPs have been detected in active lesions of inflammatory skin diseases such as psoriasis and atopic dermatitis (23–27).

Our previous study confirmed the hypothesis that the link between the development of inflammatory skin conditions and the exposure to ozone is the redox-sensitive upregulation of AMPs (16).

To study the effects of pollution exposure on skin, two approaches can be employed: retrospective studies, that analyze for example the air quality index of a particular period/area and the emergency admissions for cutaneous symptoms collected in that same area and period (28); or via the use of skin models that are directly exposed to pollution followed by endpoint data analysis. Multiple skin models have been developed over the years, including *in vitro* models (2D cell lines and 3D cutaneous models) and *ex vivo* skin biopsies; mostly to replace *in vivo* approaches that can be expensive and rise ethical concerns (animal testing) or presenting high donor-to-donor variability (human volunteers).

2D cell lines represent the most economical, accessible, reproducible and easy to handle cutaneous model available on the market; it can employ immortalized and primary cells however, this monolayer culture of keratinocytes does not undergo terminal differentiation, resulting in the absence of the stratum corneum (SC) which is the layer mostly affected by ozone exposure (10).

To overcome this problem 3D models have been developed: they are formed by differentiated keratinocytes able to form the SC, even though not all SC layers are present, making it more accessible to outdoor stressors (29). Although a lot of effort and resources have been put towards the development of 3D cutaneous tissues, one of the best approaches to study cutaneous responses still remains the use of *ex vivo* human skin explants because, despite the high donor-to-donor variability, they present all the skin layers and incorporate multiple skin appendages such as hair follicles and sweat glands (both important to produce AMPs) (16).

Considering that no perfect model to study skin responses exists, cutaneous models need to be chosen based on the endpoint of interest as well as their advantages and limitations. Based on these premises, 2D cell lines should be employed to evaluate signal transduction pathways and their mechanisms; 3D models and skin explants should be used to assess topical intervention and percutaneous permeation; while feeding studies can be better investigated in animal and clinical interventions.

Unfortunately, the most used skin models (2D cell cultures and *ex vivo* human skin biopsies) lack an important feature present in human skin: cutaneous tension.

Tensional homeostasis has been defined as the balance between the extracellular forces exerted on skin cells by neighboring cells and by the extracellular matrix (ECM) and the traction forces generated by the cells themselves (30). Skin tension manifests as internal tension-distribution patterns known as Langer's cleavage lines which are structures fundamental for the maintenance of skin integrity and flexibility (31).

Skin tension has been shown to play cardinal roles in many aspects of skin homeostasis not only during organ morphogenesis but also later in time to maintain skin structure and functions

such as ECM production, proliferative and migratory cellular processes and response to external stimuli (32).

Tensional homeostasis is important at an organ level as well as at a cellular level since cells can sense tension by being in contact with the actin cytoskeleton and adhesion molecules (33).

The importance of cutaneous tension and the need to have skin models that can closely resemble human skin, gave rise to the development of a new skin model called Ten Bio: *ex vivo* human skin explants that are cultured under physiological tension (34).

For this reason, in the present study we performed the experiments using Ten Bio to mimic *in vivo* skin tension, and confirmed that ozone exposure induces upregulation of cutaneous AMPs in tissues with and without tension, reasonably due to oxidative stress (as we previously reported *in vitro* (16)).

Furthermore, we observed that the presence of tensional homeostasis in skin either anticipated, delayed and/or prolonged the cutaneous response to ozone compared to non-tension skin exposed to ozone.

These findings corroborate our previous results, confirming the ability of ozone to induce OxInflammatory damage in skin and therefore induce AMPs expression in the presence and absence of cutaneous tension. Moreover, these results bring new insights about the importance of skin tension when investigating the effects of environmental stressors that interact and affect skin surface.

6.3. Materials and Methods

6.3.1. Ten Bio Ex Vivo Human Skin Biopsies and Ozone (O_3) Exposure

Ex vivo human skin explants, coming from 3 different healthy subjects who underwent elective abdominoplasties, were used to set up Ten Bio skin models: half of them were cultured under physiological tension (tension skin, Ten), while the other half was cultured without physiological tension (non-tension skin, NT) (Ten Bio https://ten-bio.com/). Ten Bio models were then transferred into 6 well plates pre-filled with standard medium provided by the company, using sterile technique. The plates were incubated, for overnight recovery, at 37 °C, 5% CO_2/95% air atmosphere.

The following morning, the skin explants were placed in semi-solid shipping media provided by the company. Following, half of the Ten Bio models (cultured in the presence and in the absence of physiological tension) were placed in a plexiglass box connected to the ozone generator (ECO3 model CUV-01, Torino, Italy, Model 306 Ozone Calibration Source, 2B Technologies, Ozone Solution), as previously described (35), and exposed for 4 hours at a dose of 0.4 ppm.
Skin samples were then collected at 0 hours (T0), 3 hours (T3), 6 hours (T6) and after 24 hours after ozone exposure (T24), for protein, RNA and immunohistochemical analysis.

The experiment was performed, for each donor, in triplicates for each condition.

6.3.2. RNA extraction and quantitative real time PCR

First, Ten Bio skin explants were homogenized using TRIzol (Ambion) with the help of a tissue homogenizer (Precellys 24 homogenizer, 10 cycles 6500 rpm 3 X 30 seconds, at 4°C) and RNA was then isolated using phenol-chloroform extraction method, using a modified version of the protocol described previously by Toni et al. (35). cDNA was generated from 1 µg of RNA, via

reverse-transcription, using iScript cDNA Synthesis kit (1708841, BioRad), according to manufacturer's protocol. To investigate transcription levels of AMPs, quantitative real-time PCR was assessed, using SsoAdvanced Universal SYBR Green Supermix (1725271, BioRad), according to the manufacturer's instructions on a LightCycler 480 machine (Roche).

Gene expression was quantified as the number of cycles needed to reach a threshold value in the intensity of the PCR signal (CT value). Glyceraldehyde-3-phosphate (GAPDH) was employed as housekeeping gene to normalize, and control samples were used as internal calibrators. After normalization, quantitative relative gene expression, expressed in fold changes, was calculated using the $2^{-\Delta\Delta Ct}$ method (36).

The primers used are listed here: GAPDH (forward TCGGAGTCAACGGATTTGGT / reverse TTCCCGTTCTCAGCCTTGAC), CAMP (forward CGGTGTATGGGGACAGTGAC / reverse TGGGTACAAGATTCCGCAAA), hBD2 (forward GCATTGCACCCAATACCAGT / reverse CCAAAAACACCTGGAAGAGGCA), hBD3 (forward TATCTTCTGTTTGCTTTGCTCTTCC / reverse CGCCTCTGACTCTGCAATAA).

6.3.3. Hematoxylin and Eosin (H&E) staining

After collection of the tissue samples, Ten Bio skin explants were fixed in 10% neutral buffered formalin for 48 hours at 4 °C, then dehydrated using increasing alcohol gradients, followed by immersion in xylene and paraffin embedding.

For H&E observation, 4 µm thick sections were deparaffinized in xylene and then rehydrated in decreasing alcohol gradients and then stained with Mayer's hematoxylin solution for 10 minutes (26043-06, Electron Microscopy Sciences). After rinsing the section in tap water for 15 minutes, sections were stained with aqueous Eosin Y solution (786-1072, Biosciences) for 3 minutes and

then dehydrated in increasing alcohol solutions, terminating with the xylene step. Following, the sections were mounted onto glass slides using a toluene-based solution (SP15-100, Fisher Chemical) and images were taken with (EVOS FL Auto, Life Technologies).

6.3.4. Immunohistochemical analysis

For histological observations, 4 µm thick sections were deparaffinized first on a hot plate (60°C for 30 minutes) and then in xylene; next they were rehydrated in decreasing alcohol gradients. Antigen retrieval was achieved via heat-based epitope retrieval with 10 mM sodium citrate buffer (AP-9003-500, Thermo), pH 6.0 with 0.05% Tween 20, at a sub-boiling temperature in water bath set at 95 °C for 8 minutes. After cooling for 20 minutes, the sections were washed 2 x 5 minutes with 1% Phosphate Buffered Saline (PBS) and then blocked with 5% Bovine Serum Albumin (BSA) in PBS for 45 minutes. After blocking, sections were then incubated overnight with primary antibodies prepared in 2% BSA/PBS for the following markers: LL-37 (sc-166770, Santa Cruz, dilution 1:50), hBD2 (ab63982, AbCam, dilution 1:500), hBD3 (sc-59495, Santa Cruz, dilution 1:50), 4HNE (AB5605, Millipore, dilution 1:500), AhR (NB 100-128, Novus, 1:150), COX2 (NB 100-868, Novus, 10 µg/ml), MMP9 (NBP2-13173, Novus, 1:200). The next day, sections were washed with 1% PBS 3 x 5 minutes, followed by 1 hour incubation at room temperature with fluorochrome-conjugated secondary antibodies diluted 1:500 in 2% BSA/PBS (AlexaFluor 568 A11004, AlexaFluor 568 A11057, AlexaFluor 488 A11055, AlexaFluor 488 A11008 Invitrogen).

Following, the sections were washed with 1% PBS 3 x 5 minutes, and then nuclei were stained with DAPI (1874814, Invitrogen, dilution 1:20’000) prepared in PBS, for 2 minutes at room temperature. Finally, sections were washed one last time with 1% PBS 3 x 5 minutes and then 1 x 5 minutes in double distilled (DDI) water, then mounted onto glass slides using PermaFluor

aqueous mounting media (TA-006-FM, Thermo) and imaged via epifluorescence on a Zeiss LSM10 confocal microscope equipped at 40X magnification. Quantification of at least 4 images per each condition was performed using ImageJ software.

6.3.5. Statistical analysis

Each of the variables tested was expressed as mean (in arbitrary units) ± standard deviation of three independent experiments, performed on 3 different donors.

Within each timepoint, the condition of non-tension skin exposed to ozone (O3 NT) was compared to the respective non-tension skin exposed to air (Air NT), similarly, the condition of tension skin exposed to ozone (O3 Ten) was compared to the respective tension skin exposed to air (Air Ten). Statistical analyses were performed via GraphPad Prism 6 software (GraphPad Software Inc.).

The differences between groups were evaluated by analysis of variance (ANOVA) considering the timepoints (T0, T3, T6, T24) separately, followed by Tukey's post-hoc test. A p-value <0.05 was considered statistically significant.

6.4. Results

6.4.1 Ozone exposure increases LL-37 levels in human skin biopsies in the presence and absence of tension

To confirm our previous data obtained in *in vitro, in vivo and ex vivo* skin models (16), we first evaluated the skin response to ozone exposure in the presence and absence of physiological tension, in terms of antimicrobial peptides expression.

In skin, the transcription of CAMP can be induced by injury, inflammation and infection (23, 37, 38), leading to the production of the pro-peptide hCAP18, that, once cleaved, releases the mature and active peptide LL-37: a 37 amino acid-long peptide comprising two N-terminal leucines (39).

As shown in Fig.1A, ozone exposure, in the presence and absence of tension, affected the cutaneous transcript levels of the gene CAMP. While tension-skin responded to ozone exposure at T3 with a 5-fold increase of CAMP transcripts and was still upregulated at T24; ozone exposure in non-tension skin induced upregulation of CAMP mRNA levels only at T6 (Fig. 1A).

In addition, as depicted in Figure 1B, ozone exposure induced a significant increase in LL-37 protein levels in skin tissues compared to air, in both the presence and absence of tension at T0 and T3. Of note is that while this upregulation was lost at T6 and T24 post-exposure in non-tension skin exposed to ozone (Fig. 1B, 1C) tension-skin was still able to respond to ozone stimulation upregulating LL-37 levels even at later timepoints (T6 and T24) (Fig. 1B, 1C).

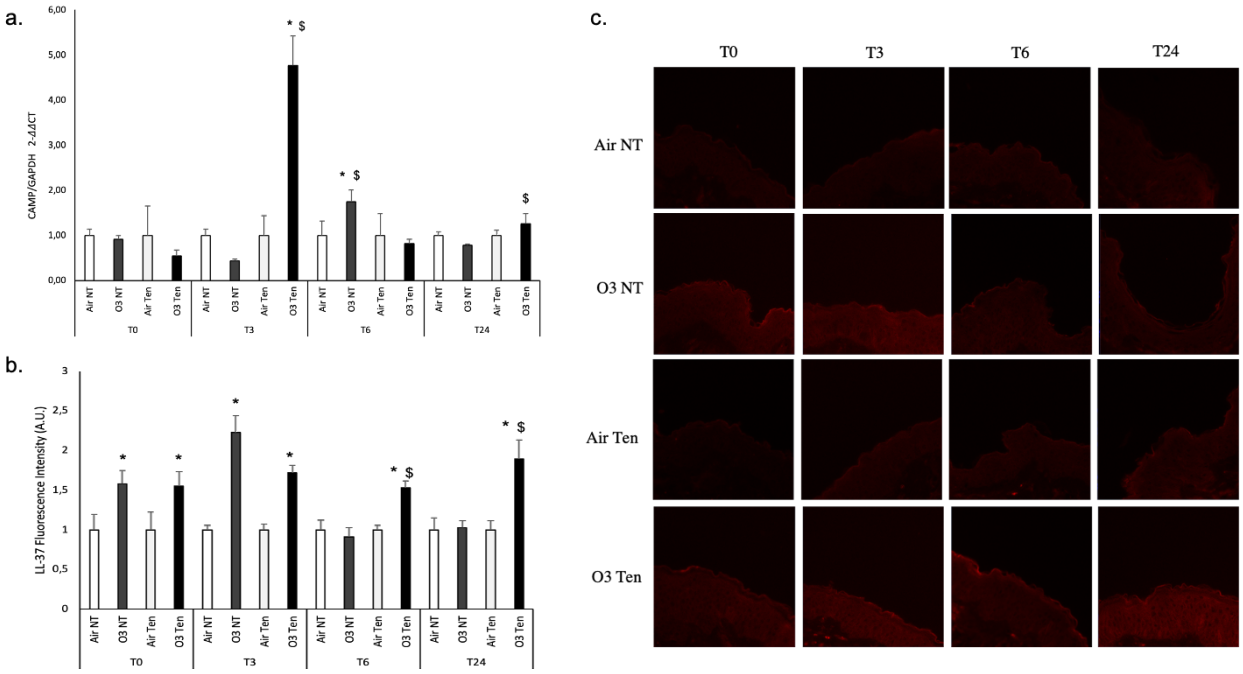

Figure 1. Transcripts and protein levels of CAMP/LL-37 were increased upon ozone exposure and the presence of physiological tension differently modulated their expression. *Ex vivo* human skin biopsies were cultured with (tension – Ten) or without (non-tension – NT) physiological tension (Ten Bio models) and then exposed for 4 hours to either air or 0.4 ppm of O_3. Samples were collected directly after exposure (T0), after 3 hours (T3), 6 hours (T6) and 24 hours (T24). a) qRT-PCR analysis of transcripts levels of CAMP. b) Semi-quantification of the LL-37 immunofluorescence intensities performed by ImageJ. c) Levels of LL-37, red fluorescence staining represents LL-37 immunoreactivity. Original magnification 40X Results from three independent experiments and three replicates are expressed as arbitrary units ± standard deviation. * = p-value < 0.05 by ANOVA.

6.4.2. Effect of tension on cutaneous hBD2 levels upon ozone exposure

As shown in Figure 2, ozone exposure clearly induced the levels of hBD2 in both tension and non-tension skin models, and also in this case the presence of tension affected the timing of hBD2 level increase. As depicted in Fig.2A, ozone induced hBD2 transcript levels only in tension skin at T6 and T24 compared to its control air and to the non-tension skin exposed to ozone (Fig. 2A), confirming that physiological tension plays an important role in skin responses.

In addition, as shown in Fig.2B and 2C right after O_3 exposure (T0), there was a greater increase in hBD2 levels in tension skin compared to both control air and non-tension skin exposed to ozone.

Interestingly, at T3 this trend between tension and non-tension skin response to ozone was reversed. In addition, 6 hours after ozone exposure (T6), we observed a second wave of hBD2 upregulation in tension skin exposed to ozone compared to its respective air and also to O_3 exposed non-tension skin. Surprisingly, at T24, we detected upregulated protein levels in both tension and non-tension skin exposed to ozone compared to their respective air treatment, although hBD2 levels in tension skin exposed to ozone were still significantly higher than the non-tension O_3 exposed skin (Fig, 2B, 2C).

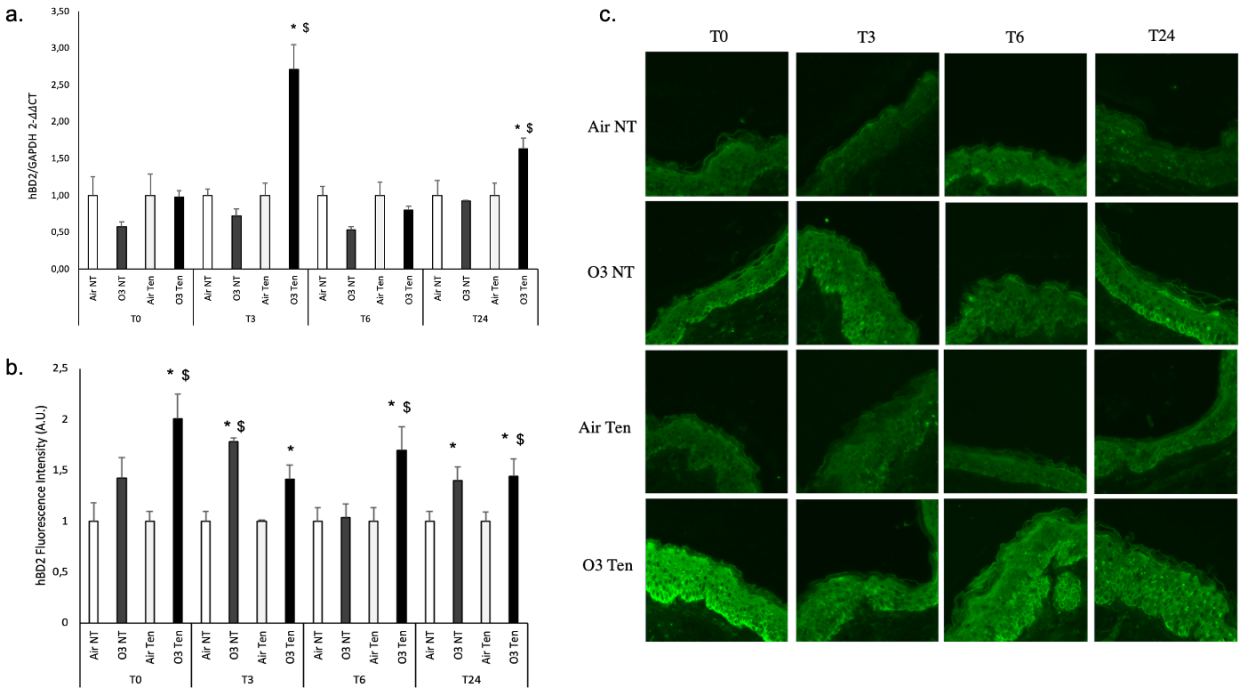

Figure 2. Transcripts and protein levels of hBD2 were increased upon ozone exposure and the presence of physiological tension differently modulated their expression. *Ex vivo* human skin biopsies were cultured with (tension – Ten) or without (non-tension – NT) physiological tension (Ten Bio models) and then exposed for 4 hours to either air or 0.4 ppm of O_3. Samples were collected directly after exposure (T0), after 3 hours (T3), 6 hours (T6) and 24 hours (T24). a) qRT-PCR analysis of transcripts levels of hBD2. b) Semi-quantification of the hBD2 immunofluorescence intensities performed by ImageJ. c) Levels of hBD2, green fluorescence staining represents hBD2 immunoreactivity. Original magnification 40X. Results from three independent experiments and three replicates are expressed as arbitrary units ± standard deviation. * = p-value < 0.05 by ANOVA.

6.4.3. Effect of tension on cutaneous hBD3 levels upon ozone exposure

To get a more complete picture of the cutaneous AMPs levels upon ozone exposure in the presence or not of physiological tension we evaluated also the levels hBD3.

As shown in Figure 3A, we detected significantly higher hBD3 mRNA levels in non-tension skin exposed to ozone compared to the same model exposed to air and also to tension skin exposed to ozone at T0. Tension skin instead recovered after 3 hours upon ozone exposure, by increasing the

expression of hBD3 transcripts to a level higher than both tension air and also non-tension skin exposed to ozone.

At T6, only non-tension skin exposed to ozone showed increased levels of hBD3 transcripts compared to its respective air and also to tension skin exposed to ozone, while instead after 24 hours from ozone exposure, although an upregulated trend is visible in tension skin, both skin models exposed to ozone, showed hBD3 transcript levels comparable to air (Fig. 3A).

As shown in Fig.3B and 3C, ozone exposure induced increased hBD3 protein levels in both tension and non-tension skin models, compared to air at T0. Of note, after 3 hours, tension skin exposed to ozone showed a significantly higher upregulation of hBD3 levels compared to non-tension skin and to the respective air condition (Fig. 3B, 3C).

At later timepoints (T6 and T24), only tension skin was able to respond to O_3 exposure with a significant hBD3 upregulation compared to its respective air and also to non-tension skin (Fig. 3B, 3C).

These results corroborate our previous findings observed in 2D, *ex vivo* human skin biopsies and *in vivo* (forearms of human volunteers) (16) confirming that ozone exposure clearly and significantly increases AMPs expression at both protein and gene levels. In addition, tensional homeostasis differentially regulated expression

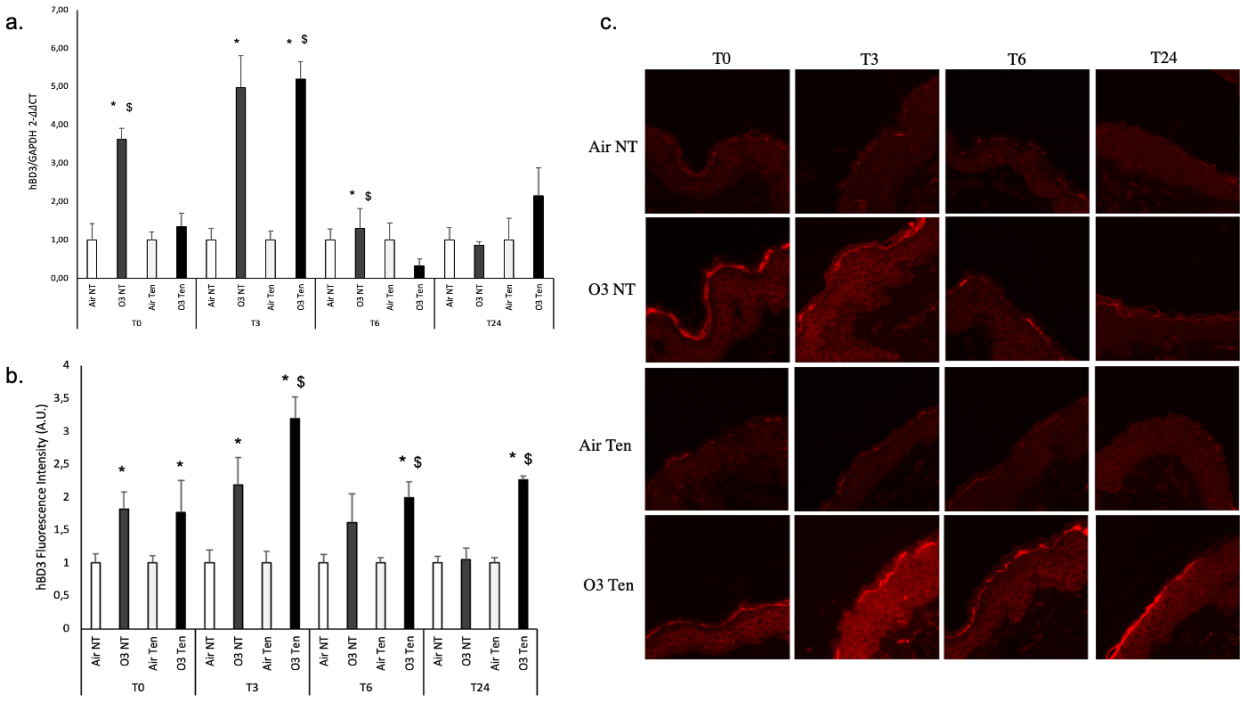

Figure 3. Transcripts and protein levels of hBD3 were increased upon ozone exposure and the presence of physiological tension differently modulated their expression. *Ex vivo* human skin biopsies were cultured with (tension – Ten) or without (non-tension – NT) physiological tension (Ten Bio models) and then exposed for 4 hours to either air or 0.4 ppm of O_3. Samples were collected directly after exposure (T0), after 3 hours (T3), 6 hours (T6) and 24 hours (T24). a) qRT-PCR analysis of transcripts levels of hBD3. b) Semi-quantification of the hBD3 immunofluorescence intensities performed by ImageJ. c) Levels of hBD3, red fluorescence staining represents hBD3 immunoreactivity. Original magnification 40X. Results from three independent experiments and three replicates are expressed as arbitrary units ± standard deviation. * = p-value < 0.05 by ANOVA.

6.4.4. Oxidative stress is associated with cutaneous AMPs regulation

As shown in Figure 4, ozone exposure induced increased levels of 4HNE in tension skin at all timepoints evaluated (T0, T3, T6, T24) compared to air. Interestingly, these levels are also significantly higher compared to non-tension skin exposed to ozone at all timepoints but T24, when only tension skin exposed to ozone appear higher than its respective air condition (Fig. 4A, 4B).

A similar trend is visible in Figure 4C, 4D where it is shown that the levels of AhR are augmented upon ozone exposure at T0 and T3, only in tension skin exposed to ozone compared to its respective air. A delayed response was detected in non-tension skin exposed to ozone which induced a significant increase in AhR protein levels compared to both non-tension air and tension skin exposed to the pollutant. At this stage, we observed no clear differences between AhR levels in both models exposed to either ozone or air after 24 hours (Fig 4C, 4D).

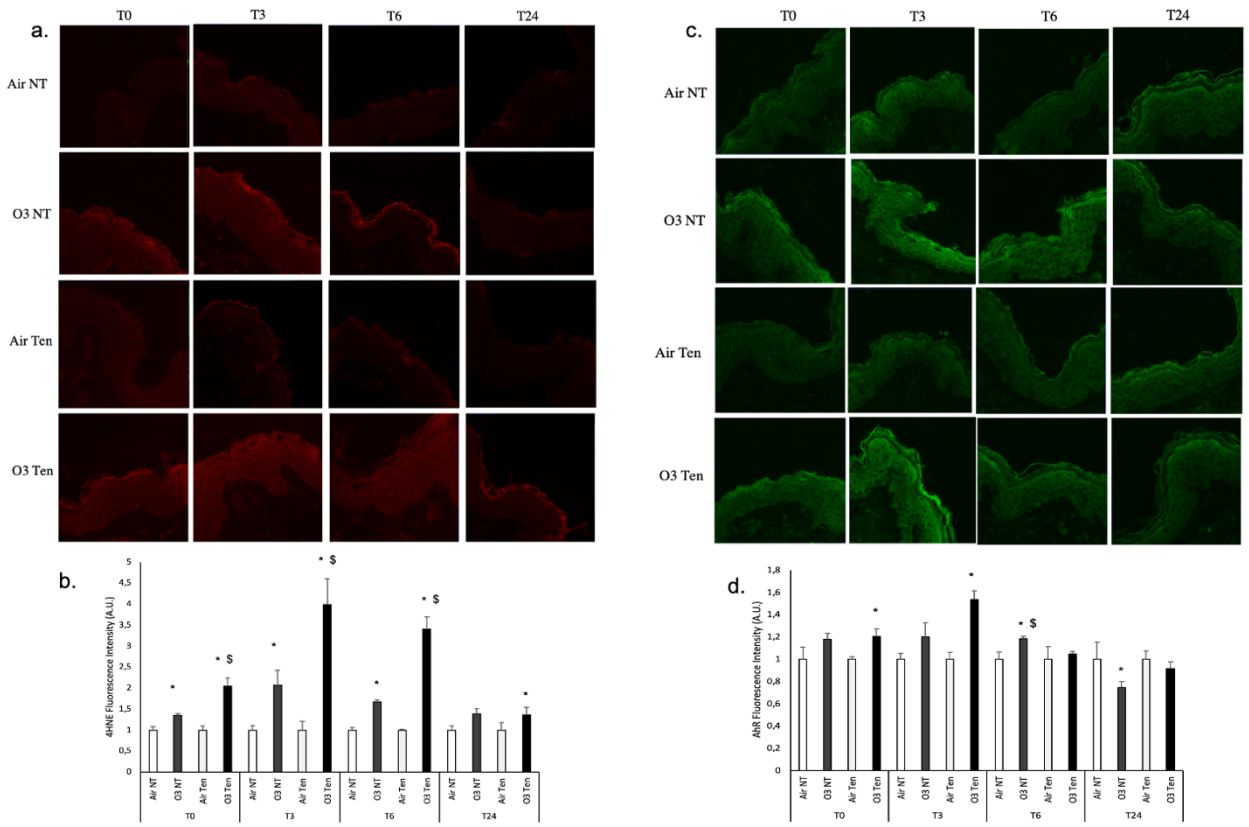

Figure 4. Ozone exposure upregulated protein levels of 4HNE and AhR, while the presence of physiological tension affected the timing of the response. *Ex vivo* human skin biopsies were cultured with (tension – Ten) or without (non-tension – NT) physiological tension (Ten Bio models) and then exposed for 4 hours to either air or 0.4 ppm of O_3. Samples were collected directly after exposure (T0), after 3 hours (T3), 6 hours (T6) and 24 hours (T24). a) Levels of 4HNE, red fluorescence staining represents 4HNE. Original magnification 40X. b) Semi-quantification of the 4HNE immunofluorescence intensities performed by ImageJ. c) Levels of AhR, green fluorescence staining represents AhR immunoreactivity. Original magnification 40X. d) Semi-quantification of the AhR immunofluorescence intensities performed by ImageJ. Results from three independent experiments and three replicates are expressed as arbitrary units ± standard deviation. * = p-value < 0.05 by ANOVA.

6.4.5. Ten Bio is a better model to study OxInflammatory response to ozone exposure

The term OxInflammation (40) underlines the close link between oxidative stress and inflammation leading to an aberrant vicious cycle in which oxidative stress induced by external stimuli generates and is fueled by inflammatory responses (41, 42). Therefore, after evaluating

the oxidative stress responses, we were interested in comparing the eventual inflammatory status between tension and non tension skin upon O_3 exposure (43–45).

As depicted in Figure 5A and 5B, the exposure of Ten Bio models to ozone induced, at T0, increased cyclooxygenase-2 (COX2) protein levels in both tension and non-tension skin compared to air conditions; of note, the levels of COX2 protein expression in non-tension skin exposed to ozone were significantly higher than the ones in tension skin exposed to O_3.

As for all the other timepoints (T3, T6, T24), ozone exposure induced a clear upregulation of COX2 levels solely in tension skin upon ozone exposure compared to all the other conditions evaluated (tension skin exposed to air and non-tension skin exposed to ozone). Surprisingly, tension skin was able to respond to ozone exposure by inducing the levels of COX2, even after 24 hours from ozone exposure (Fig. 5A, 5B).

A similar trend was visible also for the enzyme metallo-proteinase-9 (MMP9). Indeed, as shown in Figure 5C and 5D, a clear increment in MMP9 levels was noticed right after ozone exposure (T0) in both tension and non-tension skin models exposed to ozone, compared to the respective air conditions.

However, as already seen for COX2 levels, after 3 and 6 hours from ozone exposure (T3 and T6), only the skin model that was cultured under physiological tension showed induced MMP9 protein levels compared to air and to non-tension skin exposed to ozone, and this increment was lost 24 hours post-exposure in all the conditions examined (Fig. 5C, 5D).

Furthermore, as shown in Figure 6, we did not observe any morphological alterations in Ten Bio models cultured in the presence or in absence of physiological tension and exposed to 4 hours of either air or ozone at 0.4 ppm, suggesting that the physiological conditions used during the experimental procedure and the dose of ozone used are not overly aggressive (Fig. 6).

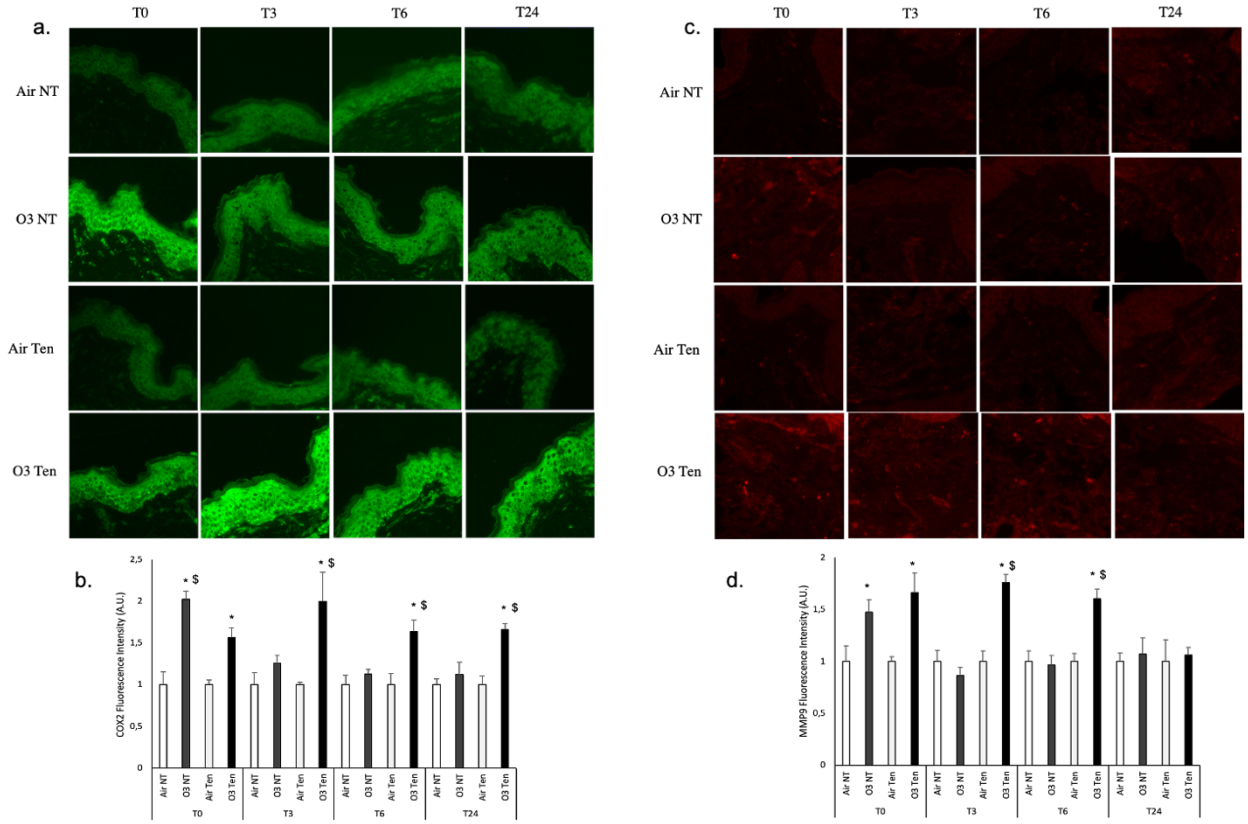

Figure 5. Ozone exposure upregulates protein levels of COX2 and MMP9, while the presence of physiological tension affects the timing of the response. *Ex vivo* human skin biopsies were cultured with (tension – Ten) or without (non-tension – NT) physiological tension (Ten Bio models) and then exposed for 4 hours to either air or 0.4 ppm of O_3. Samples were collected directly after exposure (T0), after 3 hours (T3), 6 hours (T6) and 24 hours (T24). a) Levels of COX2, green fluorescence staining represents COX2 immunoreactivity. Original magnification 40X. b) Semi-quantification of the COX2 immunofluorescence intensities performed by ImageJ. c) Levels of MMP9, red fluorescence staining represents MMP9 immunoreactivity. Original magnification 40X. d) Semi-quantification of the MMP9 immunofluorescence intensities performed by ImageJ. Results from three independent experiments and three replicates are expressed as arbitrary units ± standard deviation. * = p-value < 0.05 by ANOVA.

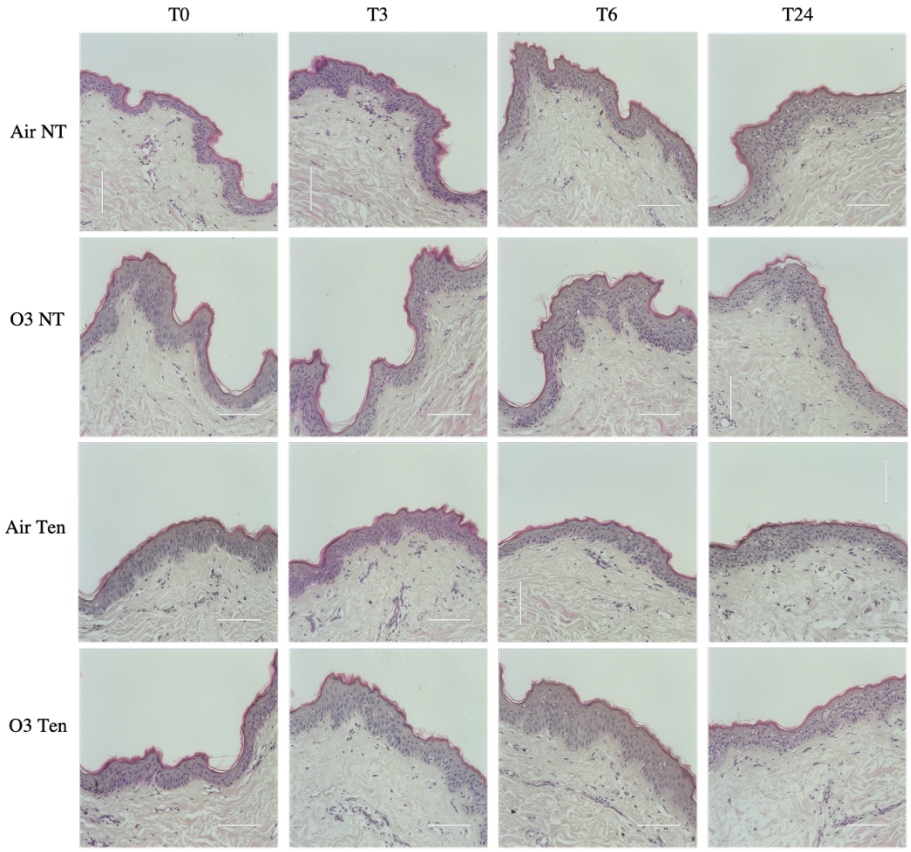

Figure 6. Presence or absence of physiological tension and ozone exposure does not alter tissue morphology.

Hematoxylin and Eosin (H&E) staining of Ten Bio *ex vivo* human skin biopsies cultured in presence (tension – Ten) or in absence (non-tension – NT) of physiological tension and exposed to 4 hours of either air or ozone at 0.4 ppm. Samples were collected directly after exposure (T0), after 3 hours (T3), 6 hours (T6) and 24 hours (T24). Original magnification 40X, scale bar 100 µm.

6.5. Discussion

Exposure to environmental pollutants is inevitable and it has been estimated to decrease human life span by 10-15 years (46). In particular, recent reports evidenced that ozone levels are still rising, making the exposure to this pollutant even more of a concern for future generations (47).

Ozone exposure has been associated both with the triggering of new inflammatory skin conditions, the worsening of pre-existing ones (such as atopic dermatitis, psoriasis, acne (48–51)) and also with induced AMPs levels (16).

AMPs constitute a ubiquitous and important component of innate immunity by protecting our bodies against pathogen infiltration. New evidence, moreover, found them capable to act as anti-inflammatory molecules in different circumstances (52), indeed, new cosmeceutical formulations targeting skin aging, list antimicrobial peptides within their ingredients to ameliorate cutaneous functions and structure (53, 54).

However, several studies suggested a potential negative involvement of AMPs in the pathogenesis of multiple skin conditions like atopic dermatitis, psoriasis, acne vulgaris, rosacea, systemic lupus erythematosus and systemic sclerosis (23–27). Therefore, AMPs induction could represent a double-edge sword: a friend that assists in skin immunity and a foe that contributes to inflammatory skin pathologies (54).

Altered AMPs levels have been recently observed upon different pollutant exposures (ozone, UV, particulate matter (55–60)) however it is currently unclear if this response to pollution inhibits or promotes cutaneous inflammation.

We believe that the physiological rationale behind AMPs upregulation after ozone exposure could be explained in 2 ways. First, it could be interpreted as a non-specific response of skin to external stimuli and secondly, AMPs could be triggered presumably to activate an antibacterial

response to prevent secondary infections caused by ozone that, by perturbing skin structure, makes it more exposed to the entry of other pollutants and pathogens.

Even though the reason why AMPs increase upon ozone exposure is still unclear, we have identified that the mechanism of its occurrence is via redox regulation (16). In fact, as we previously demonstrated and here confirmed, ozone, via lipid peroxidation of cutaneous lipids, induces the formation of oxidative mediators (4HNE and H_2O_2) that activate pro-inflammatory transcription factors (NFkβ, AP-1, AhR) (14) and intracellular protein kinases (MAPK, JNK) leading to the release of inflammatory cytokines like COX2, IL-18, IL-1β (61), and the cleavage of inactive metallo-proteinase zymogens (pro-MMP9) into active ones (MMP9) able to degrade the extra cellular matrix components (15).

Increased levels of 4HNE, AhR, COX2 and other inflammatory markers have been found elevated and involved in the pathogenesis of chronic skin conditions (62–64), while augmented levels of matrix MMP9 and reduced levels of collagen and elastin are known to be hallmarks of skin aging (65).

On the other hand, the activation of pro-inflammatory transcription factors has also been associated with the triggering of AMPs, since AMPs promoters present multiple binding sites for NFkβ, AP-1 and AhR.

In fact, *in silico* analysis brought to light the presence of multiple binding sites for both NFkβ and AP-1 in hBD2 promoter (66, 67) and three different sites for AhR, in hBD3 promoter (16). This is in line with a recent study from 2019 showing that the levels of hBD3 transcripts, induced in the first place by the cutaneous infection with *Staphylococcus epidermis*, were reduced after the blocking of AhR signaling (68).

To confirm the theory that oxidative stress regulates AMPs transcription, in our previous work we showed that, in human keratinocytes (*in vitro* model), the oxidative molecules H_2O_2 and 4HNE, originating from the interaction of ozone with the PUFAs present in the stratum corneum, were the responsible mediators of the effect of ozone in upregulating cutaneous AMPs levels (16).

The correlation between AMPs and markers of oxidative stress has become clearer in the present work, as we were able to show increased transcript and protein levels for all the AMPs evaluated (CAMP/LL-37, hBD2, hBD3) upon ozone exposure in both tissues cultured with and without tensional homeostasis, corroborating the previous findings from *in vitro*, *ex vivo* and human volunteer models (16). The induction of the transcript and protein levels of antimicrobial peptides after ozone exposure, is indeed explained by the activation of the redox-sensitive transcription factors NFkβ and AP-1, confirmed moreover by the increased levels of COX2 (that is under the regulation of NFkβ) (69) and MMP9 that instead depends on AP-1 activation (70).

In this current work we were not just interested in confirming the previous hypothesis, we also aimed to investigate the possible roles that skin tension plays in the cutaneous responses to ozone.

To test this, we used Ten Bio models cultured in presence or absence of tension, and observed that the timing of the response to ozone, evidenced by the increased AMPs levels, was strictly and solely related to the presence or absence of skin tension.

We suggest that this difference was due to the reduction of the micro folds in skin cultured under tension, therefore, in this case, more skin per unit of surface area was exposed to ozone, mimicking the real-life cutaneous exposure.

We theorize that this more acute response of skin tension exposed to ozone, caused an earlier formation of ozone mediators, compared to non-tension skin which, consequently, induced an earlier activation of transcription factors and earlier formation of OxInflammatory response. Upon

ozone exposure, we occasionally observed an apparently anticipated increase of some protein levels (hBD3, COX2) in non-tension skin compared to tension skin, however, this could be explained by the even earlier response of tension skin that was missed during the 4 hours of ozone; while the activation of these pathways in non-tension skin was triggered after ozone exposure allowing us to detect increased protein levels at T0 and T3. We believe that shorter exposures to ozone would allow us to observe an anticipated response of tension skin compared to non-tension skin.

This work validates the knowledge that the use of skin biopsies, even if not cultured under tension, is still one of the best and complete models to study skin responses. Furthermore, these findings emphasize the importance of keeping in account skin tension when investigating the effects of outdoor stressors that interact with cutaneous tissues. In fact, although the use of Ten Bio may not significantly affect the results of studies evaluating substances or pollutants that are able to penetrate the skin (like UV), the use of skin tension should be an essential feature present when investigating pollutants like ozone and particulate matter that have as a major mechanism of direct damage the interaction with lipids and PUFAs in the stratum corneum.

For these reasons, considering all the technological improvements that the research has been able to realize in the field of skin models in the last decade, it becomes even more important to choose skin models based on the endpoint of interest and the limitations and advantages of the models themselves. For instance, Ten Bio model would be beneficial not only to study pollutants that interact with the stratum corneum but also to perform physiological tests, such as drug delivery, to evaluate specific aspects that closely resemble biological and real-life responses, since cutaneous tensional homeostasis has been proven fundamental for skin structure, and functions at many stages of life (32).

Further work should focus on the investigation of the molecular mechanisms at the basis of cutaneous tension responses in the context of pollution-induced oxidative stress and antimicrobial peptides, to be able to obtain data that can be reliably extrapolated into real-life.

6.6. References

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CHAPTER 7: GENERAL CONCLUSIONS

Due to the frenetic lifestyle that society imposes, people are always exposed to environmental pollution.

Exposure to environmental pollution has been estimated to shorten human life span by 10-15 years (1) and to date, despite strict regulations to contain/reduce environmental pollution (2), there is no sign of stopping the advancement of this concerning global emergency.

Our whole body is subjected to external stressors like air borne pollution, nevertheless, skin represents the organ that is mostly affected by it, due to its peculiar location and its extensive surface area of 2 m^2 (22 ft^2) (3). Indeed, human skin acts both as a biological shield against air pollution and as a target, therefore, prolonged and repetitive exposure to pollution can overwhelm its defense system and induce profound adverse effects (2, 4, 5). As a matter of fact, the appearance of the skin is the mirror of our internal and external health.

Cutaneous tissue faces the direct and indirect effects of pollution exposure. In fact, pollution can directly affect some tissues and create local damage (eye, respiratory system) via direct induction of oxidative stress and inflammation, but it can also induce indirect systemic damage to the same and other organs via paracrine and endocrine mechanisms like the activation of transcription factors (NFkβ, AP-1, AhR) and production of secondary pro-inflammatory messengers.

This is true in the case of particulate matter (PM), a component highly present in diesel engine exhaust (DEE) that is able to reach the gastro-intestinal tract either via direct ingestion of contaminated food/water or via indirect inhalation of particles (6). When PM enters the lungs, based on its size, can be cleared and taken up by alveolar macrophages and then, via mucocillary clearance, can be carried to the oropharynx and swallowed, reaching the intestinal mucosa (6).

Once the PM is in the intestine, it can induce oxidative stress and inflammation, causing a disruption of the structure and functionality of the mucosa and also affecting negatively the intestinal microbiota (6).

Another important aspect that determines the skin susceptibility to indirect damage induced by pollutants is the presence of multiple axes between the cutaneous tissue and multiple organs, such as gut-skin axis (7). In this context, multiple studies demonstrated the existence of a link between the gut microbiota and skin health (8). Indeed, the DEE-induced modulation of the intestinal microbiome, composed of commensal bacteria, fungi, protozoa, viruses, can negatively influence the metabolism and the immunity of the host.

The gut microbiome, responds to external stimuli (that can directly or indirectly reach the intestinal mucosa), generating products called short chain fatty acids (SCFA) (acetate, butyrate and propionate), via the fermentation of the non-digestible fibers and produces other secondary effector molecules like neurotransmitters, hormones and secretory factors. All these products are able to be absorbed by the intestinal mucosa, reach the peripheral circulation and, via the interaction with G-protein-coupled receptors (GPCRs), affect the immunological functions of distal organs, such as skin, liver, brain and lungs (9–13).

However, the presence of a gut-skin axis does not only negatively affect the skin, indeed, it can also represent a potential tool to improve skin health. In fact, the positive effects of the gut-skin axis are based on a healthy nutrition since, by providing micronutrients like vitamin C, E, D, carotenoids, amino acid and trace minerals that are co-factors for the synthesis and function of antioxidant enzymes, the skin gathers from the diet all the essential factors required for a strong defense system. Of note, the type and amount of non-digestible fibers present in the diet, their transit time and the substrate source transitioning in the gastro-intestinal tract, can positively affect

the amount and rate of SCFA production. This has been confirmed by multiple studies which associated the SCFAs-induced activation of GPCRs expressed on keratinocytes, immune and endothelial cells with the downregulation of the redox sensitive signaling pathway NFkβ, stimulation of collagen synthesis in fibroblasts, modulation of histone deacetylases and T-reg accumulation in skin, therefore ameliorating the overall skin health and appearance (14–16).

Due to the structure of the integumentary organ, dietary intervention may not be enough to provide the skin with a defense system able to counteract the damage induced by pollution.
In fact, the skin epidermis, and in particular its outermost layer, the stratum corneum, are the main targets of many pollutants (4, 17–20) however, the epidermis, unlike the dermis, is not embedded with nutrients-providing blood vessels and therefore barrier function and antioxidant defense in surface layers cannot be guaranteed (8).

Thus, in the context of improving skin health, the topical application of natural or synthetic compounds becomes much more important to ensure an equal distribution of essential and antioxidant nutrients to all the skin layers, providing them the means to quench pollution-induced damage. Validation of this was given by different studies investigating topical application of vitamin C and E to prevent pollution-induced skin damage and premature skin aging (21–24).

Based on this premise, in the present work we focused our attention on the topical application of products as a way to prevent the damage caused by the exposure to ozone (O_3) (Chapter 2, 5, 6) and diesel engine exhaust (DEE) (Chapter 3, 4). In particular we evaluated the application of different natural extracts such as lowbush blueberry extract (*Vaccinium angustifolium*) (Chapter 2), and the combination of rosemary (*Rosmarinus officinalis*) and Spirulina extract (Chapter 3), considering that the possibility of combined multiple natural products, with the aim to improve skin health and protect it from environmental insults, has gained much attention from a cosmetic

point of view (22, 25–27). Of note, the combination with different natural extracts, as for the algae-derived products (like Spirulina), can be of a great use in cosmeceutical formulations to enhance phytochemicals delivery, without affecting rheological characteristics (28, 29).

A common way to identify potential sources of active phytochemicals is based on the folk and traditional medicine, indeed, the topical application of natural extracts for medicinal use has been documented already in the Traditional Ecological Knowledge of Native American Pharmacopoeia (30). Multiple evidence associated both blueberry and rosemary with antioxidative and anti-inflammatory activities and they appear to be in significant part due to their phenolic constituents. Blueberry extract contains multiple phytochemicals which are all classified as polyphenol compounds, such as proanthocyanidin, flavonols, phenolic acids and anthocyanins which are the main constituent of polyphenols and are mostly concentrated in the blueberry skin (31). Among anthocyanins, the prevalent ones in blueberry, *Vaccinium* species, are monoglycosides of delphinidin, cyanidin, petunidin, peonidin and malvidin (32). Rosemary instead presents a different phenolic composition, being made by rosmarinic acid (hydroxycinnamic acid), carnosic acid and carnosol (diterpenes) (33, 34).

The antioxidant activity of phenolic compounds resides in a double mechanism: the activation of the nuclear erythroid-2-related factor 2 (Nrf2) and the direct ability to quench radicals (35) although this aspect is mainly proven *in vitro*. This is the case of both rosemary and blueberries, indeed it has been shown that they are able to quench radicals via their phenolic chemical structure but also trigger Nrf2 defense at a cellular level (36, 37). This double protecting effect assumes more importance considering that pollutants have multiple mechanisms to damage skin and other organs.

In particular, in our current work we evidenced that O_3 and DEE share some harmful mechanisms and, on the other hand, exhibit specific ones. For example, O_3 and DEE present many differences, beginning from their chemical structure: unlike ozone that is a simple molecule composed by only 3 atoms of oxygen, DEE is formed by different fractions: gaseous, condensates and a solid carbon core covered by different metals (iron, manganese, copper, zinc) and other compounds (PM, PAHs, endotoxins, microorganisms) (38–41). The particulate matter fraction can also be very heterogenous in composition by being formed by organic and inorganic, solid, liquid and mixed phases that agglomerate in particles of different sizes: coarse particles (circa 10 μm diameter), fine particles (ranging between 2.5 and 0.1 μm) and ultrafine particles (UFP) (smaller than 0.1 μm). Due to their micrometric size, UFP can carry on their surface organic components, pathogens and toxins able to be absorbed by alveolar epithelial cells in the lungs and via the cardiovascular system, reach distal organs and trigger the activation of immune cells, causing systemic damage (42, 43) that can be sensed also by the skin.

Although still controversial, different studies proposed the capability of UFP to enter the skin via hair follicles and trans-dermally, therefore not only inducing a more superficial harm but also perpetuating a direct damage in deeper cutaneous layers (44–46). On the other hand, instead, ozone has been shown to be unable to enter the skin and induces damage only via the formation of superficial free radicals, a mechanism shared with DEE as well. Both of these pollutants can in fact interact directly and oxidize PUFAs and lipids present in the stratum corneum and epidermis causing a lipid peroxidation cascade, leading to the generation of aldehydes (like 4HNE) and ROS (like H_2O_2). These secondary messengers can directly affect the skin structure (47, 48) via downregulation of structural proteins like loricrin, filaggrin and involucrin as well as indirectly trigger signaling pro-inflammatory pathways (NFkβ, AP-1, MAPK, JNK) (49) in deeper skin

layers, perpetuating and worsening oxidative and inflammatory damage and inducing more structural damage via the activation of metalloproteinases that degrade collagen fibers (50).

Of note, the structural epidermal damage induced by exposure to pollutants not only induces a general impairment of skin barrier function and consequent excessive transepidermal water loss (TEWL) but it can also favor the entry of pathogens and facilitate the access of PM via hair follicles and trans-dermally, allowing them to reach the dermis contributing to systemic damage (51, 52).

In our work we demonstrated that both the natural extracts topically applied (blueberry and rosemary with spirulina extracts) prevented pollution-induced skin damage and aging, independent of the type of pollutant used (skin pre-treated with blueberry was exposed to ozone and DEE exposure was instead performed after topical application of rosemary and spirulina).
In particular we observed that topical application of blueberry extracts reduced ozone-induced inflammasome activation.

Inflammasomes are newly discovered cytosolic receptors which play important roles in inflammation via the sensing of extracellular stimuli like pathogens and danger-associated molecular patterns (PAMPs and DAMPs). Lately their chronic activation have been associated with the development of multiple skin conditions such as vitiligo, atopic dermatitis, acne, melanoma, hyper-pigmentation and psoriasis (53–55). Inflammasomes are generally formed by different subunits that require oligomerization to be activated: a NOD-like sensor receptor (NLR), an apoptosis-associated speck-like adaptor protein (ASC) and the pro-caspase-1. Upon sensing of extracellular stimuli, the oligomerization of the subunits leads to the formation of caspase-1 which induces the maturation of inactive zymogens into active pro-inflammatory cytokines (IL-18 and IL-1β).

In our work (Chapter 2) we demonstrated in three different skin models (*in vitro* keratinocytes, *3D* reconstructed human epidermis and *ex vivo* human explants) that ozone exposure not only induced oxidative stress (increased 4HNE levels) but also caused the upregulation of the inflammasome-related proteins (NLRP1, ASC, Caspase-1, IL-18) as well as their oligomerization/activation. Furthermore, we observed that pre-treatment with blueberry extracts prevented inflammasome activation and normalized 4HNE levels.

In Chapter 3 we showed that DEE exposure, like ozone, was able to induce 4HNE levels in epidermis and stratum corneum, indicating lipid peroxidation-mediated protein damage. Accumulation of 4HNE protein adducts is known to initiate secondary oxidative cascades and trigger inflammatory mediators like matrix-degrading metalloproteinases (MMPs). Indeed, we observed elevated MMP9 levels in the dermis of skin explants exposed to DEE as a sign of extracellular matrix degradation. Furthermore, DEE exposure affected the epidermal skin barrier function via the reduction of Filaggrin expression, a structural protein fundamental to maintain the stratum corneum properties. Importantly, the pre-treatment with rosemary and spirulina counteracted all these damaging effects induced by DEE.

We believe that rosemary and blueberry were able to prevent 4HNE-pollution induced levels via direct quenching of ROS and lipid peroxides. Consistent with our hypothesis, previous studies performed on different cell lines showed that the bioactive compounds in blueberries could directly quench ROS via their phenolic structure (35) as well as carnosic acid that was able to counteract reactive pro-oxidant species, while carnosol suppressed lipid peroxidation via interaction with lipid radicals (56–59).

We also hypothesize that part of the mechanism of action of both blueberry and rosemary extracts resides as well in the ability to activate the transcription factor Nrf2 with consequent

induction of its signaling pathway leading to the formation of cytoprotective enzymes such as NAD(P)H:quinone oxidoreductase-1 (NQO1) and hemeoxygenase-1 (HO1) (56–59).

Our hypothesis is based on the blueberry-induced Nrf2 activation as the indirect reason for the reduced oligomerization and upregulation of the inflammasome which is known to be triggered also by an altered redox homeostasis (60, 61). We believe that the same is true for rosemary extract which, because it contains carnosol, rosmarinic and carnosic acid, can induce Nrf2-mediated antioxidant response. To corroborate this hypothesis, it was demonstrated that carnosic acid and carnosol prevented hydrogen peroxide-induced senescence in cutaneous fibroblasts, via the activation of Nrf2 pathway (62) and that the consequent induction of HO1 and NQO1 counteracted the upregulation of MMP1 transcript levels (63) in human skin fibroblasts and keratinocytes protecting them from the noxious effects of urban dust, cigarette smoke (63, 64) and UV light (65).

It is important to underline that Nrf2 cross-talks with the redox-sensitive transcription factor NFkβ, therefore activation of Nrf2 signaling not only induces an antioxidant response, but at the same time inhibits NFkβ pathway which is involved in the tissue inflammatory responses (66). Together with the indirect inhibitory effect of NFkβ via the activation of its antagonist Nrf2, both blueberry and rosemary have also been shown able to inhibit directly NFkβ pathway, therefore protecting the skin from pollution-induced OxInflammatory events, characterized by a noxious interplay between subclinical inflammation and an imbalance redox homeostasis (67). This could explain the activation of MMP9 and the loss of filaggrin after exposure to DEE and a restoration of their levels to homeostasis after pre-treatment with rosemary and spirulina extract, as well as the inflammasome activation and the higher levels of H_2O_2 in skin models exposed to ozone that normalized upon topical application of blueberry extracts.

In line with this hypothesis, previous studies demonstrated the ability of rosemary to inhibit nuclear translocation of the subunit p65 of NFkβ induced by IL-1β in a chondrocyte cell lines (68) and also in monocyte/macrophages previously stimulated with LPS (69).

Another important role of NFkβ is its involvement in the regulation of cyclin D1 expression, a key protein associated with the cellular proliferation cycle (70–72). In fact, we demonstrated that ozone exposure affected the proliferation rate of keratinocytes, as well as their migration and wound closure ability. However, the treatment with blueberry extract was able to improve keratinocyte wound closure and normalized and rescued the proliferative and migratory responses previously altered by ozone exposure via NFkβ activation.

The OxInflammatory phenomenon in skin is known to be mediated by molecules able to induce peroxidation.

Important effectors of peroxidation, together with hydrogen peroxide, are transition metals, especially iron, which can participate into Fenton-like reactions leading to the generation of ROS like hydroxyl radicals and ions (73). Moreover, the polycyclic aromatic hydrocarbons (PAHs) (components of some pollutants like PM and DEE), can be absorbed through the cutaneous tissue and reach the mitochondria where they exacerbate ROS production generating superoxide anion that can be converted in H_2O_2 and fuel peroxidation via Fenton reaction (74).

Considering the key role that iron plays as one of the triggers of Fenton reaction, we hypothesized that by preventing the peroxidation via the use of an iron chelator such as deferoxamine (DFO) we would counteract the harmful effects of diesel engine exhaust on skin (Chapter 4).

To confirm our hypothesis, we pre-treated human skin explants with either DFO and a cosmeceutical formulation radical scavenger (CE Ferulic) (containing vitamin C, E and ferulic

acid) alone or in combination and we exposed the skin biopsies to DEE for either 1 or 4 consecutive days.

As anticipated, the exposure to DEE induced lipid peroxidation in skin, measured by upregulated 4HNE levels and both DFO and CE Ferulic alone and even more significantly in combination, where able to reduce 4HNE levels, presumably via the combinatory effects of iron chelation and direct neutralization of oxidative species.

As previously mentioned, lipid peroxidation can lead to aberrant inflammatory responses mediated by effectors like MMP9 that can further induce degradation of extracellular matrix (collagen and elastin fibers) and structural damage of cutaneous barrier function. Therefore, to confirm the downstream effects induced by peroxidation we measured MMP9 levels and we observed them increased upon DEE exposure and furthermore, we distinguished an additive effect of DFO and CE Ferulic in normalizing MMP9 levels, suggesting a dual involvement of iron and ROS in MMP9's enzymatic modulation.

In line with our hypothesis, we observed also reduced levels of Collagen type 1 as well as decreased levels of Filaggrin and Involucrin, structural proteins involved in skin barrier functions, demonstrating the ability of DEE to induce peroxidative damage as well as secondary inflammatory responses and structural impairment. These effects were completely prevented by the treatment with the iron chelator and the ROS scavenger formulation, underling the additive potential of DFO and CE Ferulic in preventing DEE-induced skin damage.

These data confirmed the idea that the use of one substance alone may not be enough to counteract the multiple damaging mechanisms activated by different pollutants. In fact, as shown previously by the topical application of both rosemary and Spirulina extracts, here we corroborated the concept that the additive action of multiple compounds (like an iron chelator and a radical

scavenger) can provide a broader protection through a bifunctional tool that targets both iron and ROS, fundamental point considering that pollutants are able to induce damage via both oxidative stress and inflammation.

Indeed, we suggest that a future cosmeceutical formulation should contain different components (natural and synthetic) to make the topical application a more efficient and better-performing tool in preventing pollution-induced skin damage.

Another important reason to give the skin multiple means to protect itself is because the cutaneous tissue responds to environmental stressors in different ways; in fact, in Chapter 5 and 6 we demonstrated that ozone is able to induce a non-specific response in skin through the activation of antimicrobial peptides (AMPs) which are usually activated by different stimuli like pathogens (Gram-positive and -negative bacteria, fungi, and viruses) and cytokines as part of the host defense response. We believe that skin responds to environmental pollutants by modulating AMPs' gene expression because pollution exposure can facilitate the entrance of external pathogens, allergens and other pollutants, leading to increased susceptibility to infection (51, 52).

To validate our hypothesis, we exposed multiple skin models (*in vitro* keratinocytes, *ex vivo* human skin explants, forearms of human volunteers and Ten Bio skin explants) to ozone and investigated the cutaneous response in terms of redox regulation of antimicrobial peptides.

We confirmed our previous findings demonstrating that ozone exposure induced oxidative stress and an inflammatory cascade measured via increased levels of 4HNE protein adducts, AhR, COX2 and MMP9. More importantly, for the first time we revealed the redox regulation of AMPs: we observed increased AMPs levels (hBD2, hBD3, CAMP/LL-37) upon ozone exposure as well as after treatment with 4HNE and H_2O_2, the oxidative mediators produced by the interaction of ozone with PUFAs and lipids present in the stratum corneum. As a proof of concept, we pre-treated

human keratinocytes with catalase prior to ozone exposure and showed that catalase treatment counteracted the increase of ozone-induced AMPs levels.

Moreover, we showed that the presence of tension in skin explants induced a different time response upon ozone exposure, exhibiting the important role of physiological tension when evaluating the effects of pollutants targeting skin surface. In fact, although both skin biopsies cultured with and without tension responded to ozone exposure with augmented AMPs and OxInflammatory markers levels, we suggest that the differences observed between tension and non-tension skin are the result of micro folds reduction in tension skin due to its stretching to homeostatic tension, therefore more skin per unit of surface area was exposed to ozone and its effects were observed at earlier timepoints or presumably even lost during the 4 hours of exposure.

As suggested in our present work, AMPs modulation is triggered and regulated by redox imbalance and considering that altered AMPs levels have been found in inflammatory skin diseases such as in active lesions of psoriasis and atopic dermatitis (75–79), we recommend the topical application of a mixture of antioxidant and antinflammatory products as a possible innovative tool to prevent pollution-induced skin damage and therefore preventing the triggering/exacerbation of inflammatory skin conditions.

This work carries significant importance in light of the great risk that our skin faces everyday being continuously exposed to pollution as well as the need to find new safe molecules to prevent the pollution-induced skin damage and aging. Moreover, our approach with Ten Bio technology should be considered pioneering since, for the first time, the role of cutaneous homeostatic tension has been shown fundamental to investigate skin responses to environmental stressors.

7.1. References

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