



Cutaneous antimicrobial peptides: New “actors” in pollution related inflammatory conditions

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ABSTRACT

Ozone (O₃) exposure has been reported to contribute to various cutaneous inflammatory conditions, such as eczema, psoriasis, rash etc. via a redox-inflammatory pathway. O₃ 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 (H₂O₂). 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 O₃ 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 O₃) and observed increased levels of all the measured AMPs upon O₃ 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.

1. Introduction

Tropospheric ozone (O₃) is formed at the ground level through chemical reactions between nitrogen oxides (NO_x) and volatile organic compounds, when pollutants are emitted by cars, industrial boilers, refineries, and chemical plants in the presence of sunlight [1]. Exposure to O₃ has been associated with negative effects on respiratory, cardiovascular, and neurological functions [1]. However, only recently has the noxious effects of O₃ exposure on cutaneous tissues been studied. O₃ exposure has been correlated with premature skin aging, contact dermatitis, atopic dermatitis, and psoriasis [2–6]. O₃ 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 O₃ 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 O₃-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

Abbreviations: AMPs, antimicrobial peptides; hBDs, human beta defensins, ozone (O₃); CAMP, cathelicidin antimicrobial peptide; hCAP18, human cationic bacterial protein of 18 kDa; WHO, World Health Organization; NO_x, nitrogen oxides; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1, activator protein-1; H₂O₂, hydrogen peroxide; 4HNE, 4-hydroxy-nonenal; CFU, colony forming unit; VDR, vitamin D receptor; AhR, aryl hydrocarbon receptor; ROS, reactive oxygen species; COX2, cyclo-oxygenase 2; UV, ultraviolet; PM, particulate matter; TLR, toll-like receptor; PUFAs, polyunsaturated fatty acids.

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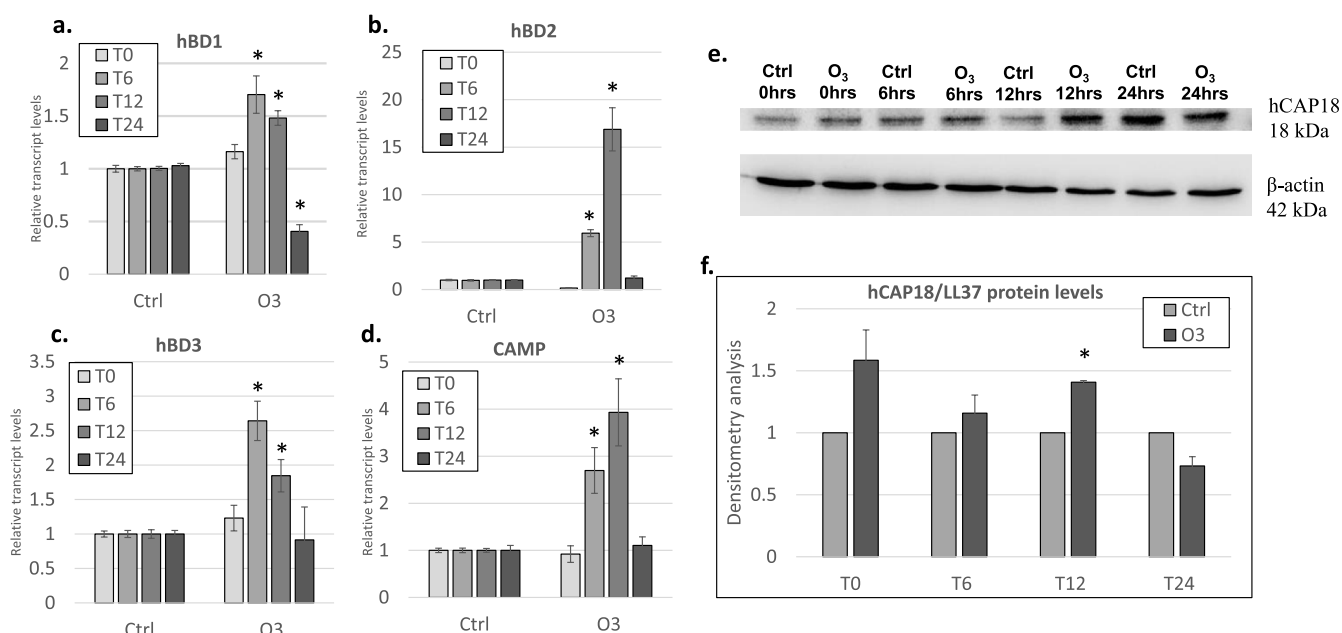
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* = p value < 0.05

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 O₃ 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.

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 mammals, 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 O₃ 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]; Kortjng 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 O₃ 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 O₃. We observed increased levels of these AMPs in response to O₃ 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 O₃ 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.

2. Results

2.1. Ozone (O₃) increases AMPs levels in human keratinocytes

Since both, O₃ exposure and increased AMPs levels are independently associated with cutaneous inflammatory conditions, we want to evaluate whether O₃ 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 O₃ 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 O₃ 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 O₃ 12 h post-exposure (Fig. 1e and f). These data confirm the hypothesis that O₃

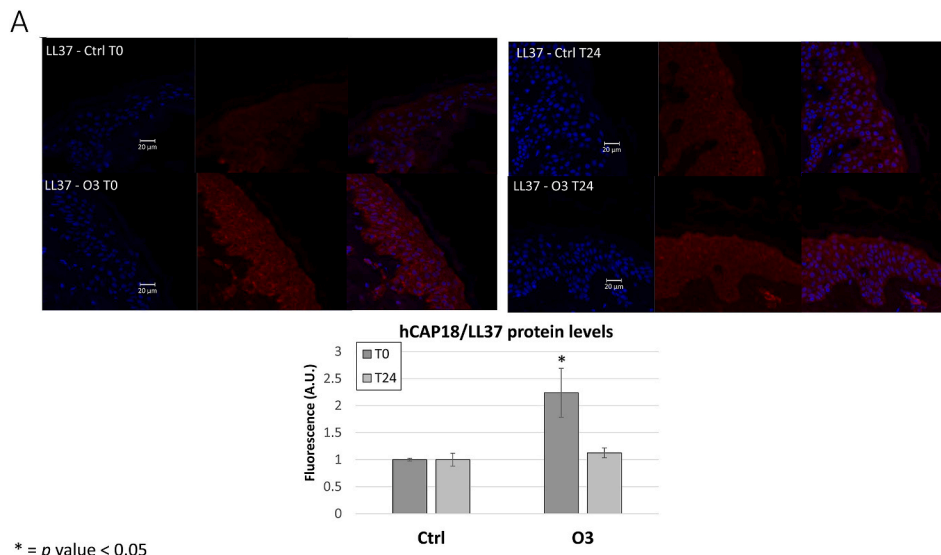
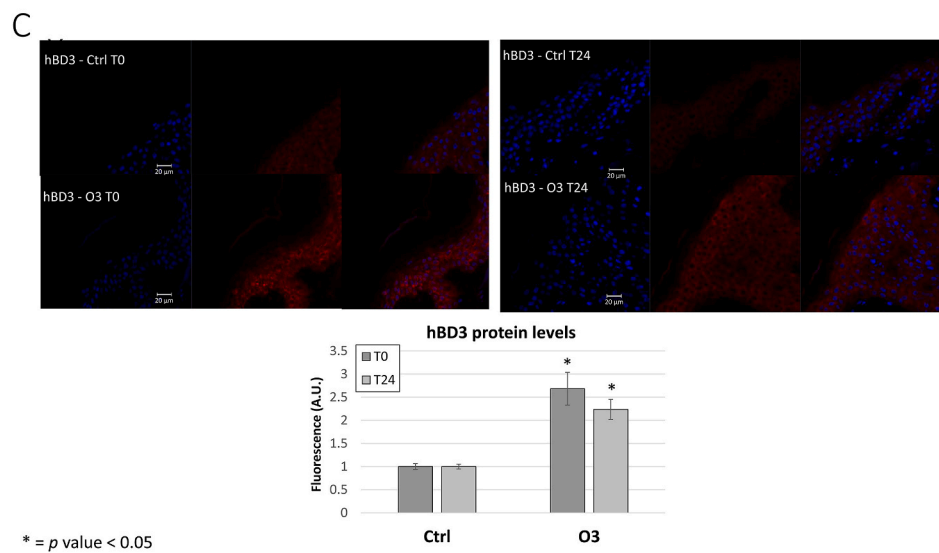
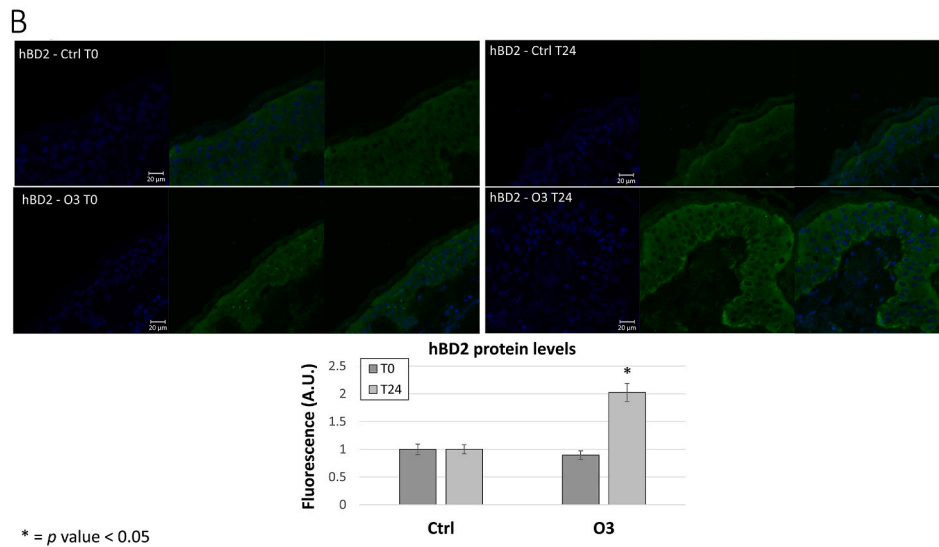


Fig. 2. Exposure of human biopsies to ozone increases AMP protein levels in the skin. Ex vivo human abdominoplasty biopsies from three different donors were exposed to 0.4 ppm O_3 . Sections were collected directly after exposure (T0) and 24 h (T24) post exposure and embedded in paraffin. Immunofluorescence 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 \pm SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



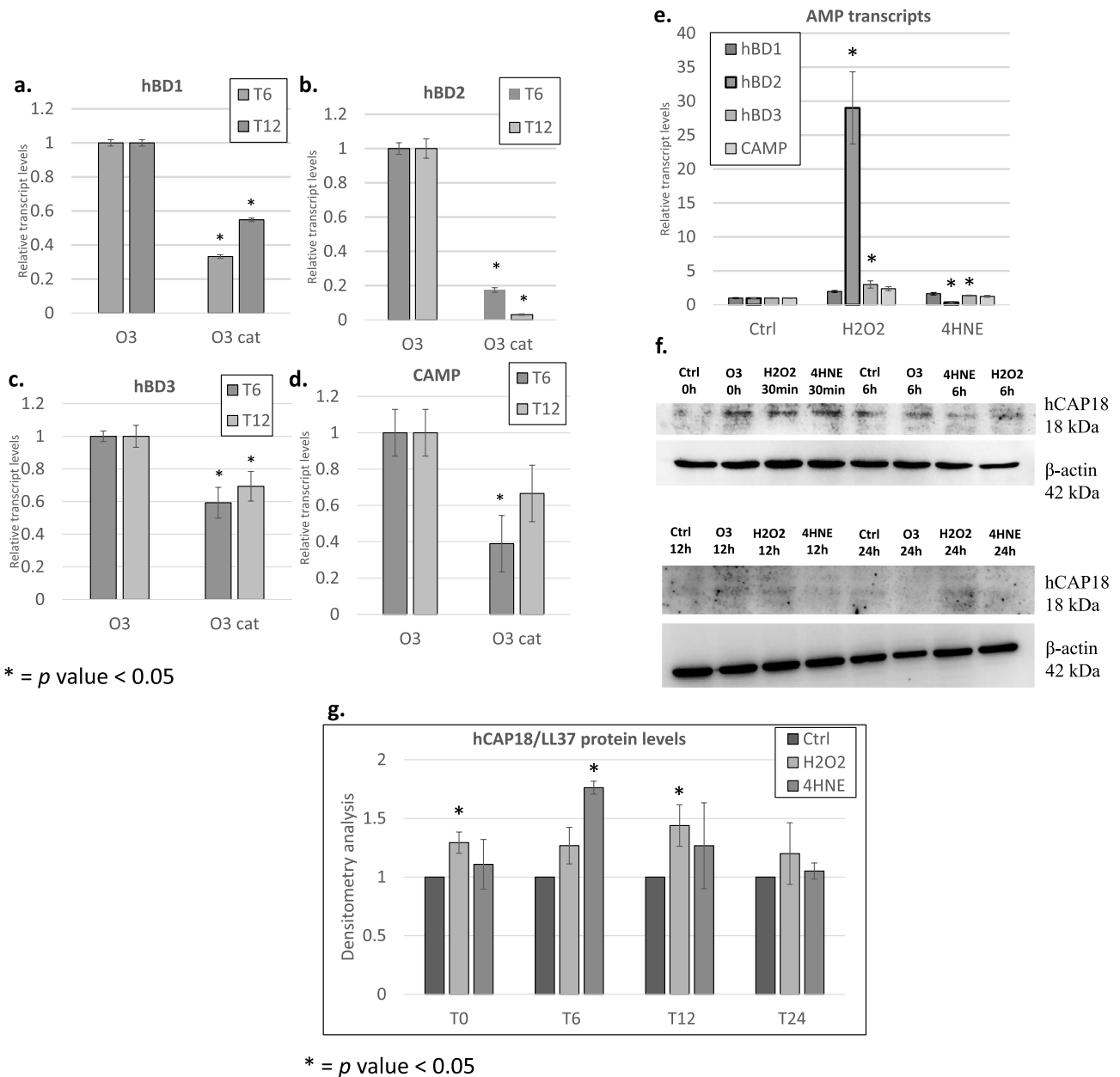


Fig. 3. Ozone-induced signaling mediator H₂O₂ regulates AMP levels in keratinocytes. Transcript levels of hBD1 (a), hBD2 (b), hBD3 (c), and CAMP (d) were assessed in keratinocytes pretreated with 20 μ g/mL of catalase (cat) then exposed to O₃ at different time points. AMPs transcript levels in keratinocytes treated with 50 μ M H₂O₂ 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 H₂O₂ or 4HNE (f). Quantification is depicted in panel (g). *p-value<0.05, compared to control. Error bars = mean \pm SEM.

exposure is able to affect cutaneous AMPs expression and levels.

2.2. O₃ 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 O₃ 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 O₃ 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.

2.3. Redox regulation of cutaneous AMPs

As mentioned before, O₃ is not able to penetrate the skin as it reacts instantaneously with the polyunsaturated fatty acids (PUFAs) present in the stratum corneum, producing several byproducts, including hydrogen peroxide (H₂O₂) and aldehydes, such as 4-hydroxynonenal (4HNE) [39]. To determine whether O₃-induced H₂O₂ 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 O₃ for 30 min and collected samples 6 and 12 h post-exposure. As depicted in Fig. 3, pretreatment with cat prevented O₃-induced hBD1, hBD2, hBD3, and CAMP transcripts (Fig. 3a–d).

As a proof of concept, we exposed human keratinocytes to H₂O₂ to understand whether this molecule could be a possible messenger of O₃ affects AMPs transcript levels. As shown in Fig. 3e, H₂O₂ significantly

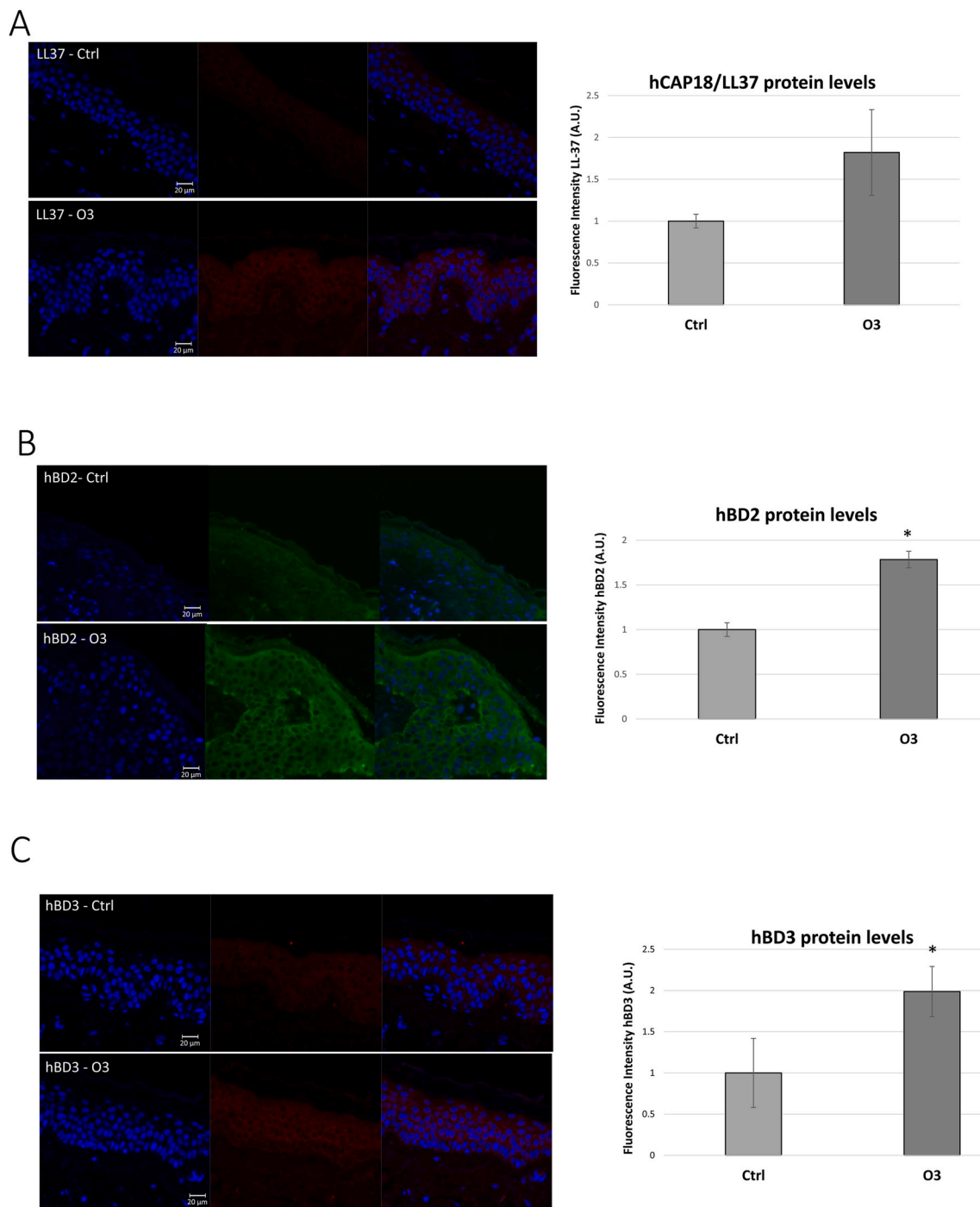


Fig. 4. Exposure of human volunteer subjects to ozone increases AMP protein levels in the skin. Forearms of human volunteer subjects were exposed to 0.8 ppm O_3 for 3 h a day for 5 consecutive days. We examined protein levels of hCAP18/LL37 (a, c) and hBD3 (b, d) using immunofluorescence in 3 mm punch 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 \pm SEM.

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.

2.4. 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 O₃-induced their upregulation also *in vivo* and substantiating the *in vitro* and *ex vivo* findings (Figs. 1–3).

3. Discussion

O₃ exposure has been associated with the development/exacerbation of inflammatory skin conditions, such as atopic dermatitis, acne, and psoriasis [2–4,77]. Inflammatory skin conditions are also associated with altered AMPs levels, indeed multiple AMPs were originally isolated from human skin pathologies [11,35]. Since O₃ exposure as well as increased levels of AMPs are associated with cutaneous inflammation [1, 4,17,31–34], we hypothesized that cutaneous AMPs upregulation due to O₃ 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 apparatus involved in AMPs production) to better evaluate cutaneous responses. In addition, human volunteer subjects were also exposed to O₃ 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 differs 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 O₃ 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 O₃ 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 O₃, 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 O₃ exposure altered redox-dependent antimicrobial activity of hBD1, although it is likely that pollutant exposure affects hBD1 activity, as O₃ 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 O₃ exposure in keratinocytes and that hBD2 transcript levels are increased to a much higher level than either CAMP or hBD3, in response to both O₃ and H₂O₂. 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 NFκB regulation. This could explain the evident increase of hBD2 mRNA in response to H₂O₂ which has been demonstrated for more than 20 years a very specific and strong NFκB activator via IκB 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 O₃ exposure in skin, since these transcription factors have been shown to be all modulated by O₃ [1,52,53]

Based on our results, H₂O₂ alone is likely not fully responsible for O₃-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, O₃ exposure induces production of H₂O₂, 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 O₃ 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 H₂O₂ can be one trigger for AMPs transcription.

In vivo O₃ 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 [56–58].

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 [59–61]. 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 O₃ exposure regulates AMPs cleavage in skin is unknown. Another mechanism by which O₃ exposure could regulate AMPs activity is through inducing post-translational modifications, such as protein oxidation. Furthermore, O₃ 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 O₃ 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 interpreted 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 O₃ 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 O₃ 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.

4. Materials and methods

4.1. Cell culture

Human keratinocytes (HaCaTs) were cultured, as previously described [72] (see Supplemental Methods). 1×10^6 (RNA) or 9×10^5 (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 O₃, as previously described [73]. For 4HNE and H₂O₂ 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].

4.2. Ozone generator

O₂ was used to generate O₃ 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 O₃ in the chamber was adjusted, depending on the model used, and continuously monitored by an O₃ detector.

4.3. 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% CO₂ humidified atmosphere. Experiments were performed the following day, as previously described by Ref. [68].

4.4. *Ex vivo* O₃ exposure

Biopsies from donor skins were placed in a plexiglass box connected to the ozone generator and exposed to 0.4 ppm O₃ 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.

4.5. 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.

4.6. 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](#).

Table 1
Primers used in the study.

Gene	Forward	Reverse
GAPDH	TCGGAGTCAACGATTGGT	TCCCGTTCCTCAGCCTTGAC
hBD1	TTGTCTGAGATGGCTCAGGTGGTAAC	ATACTTCAAAGCAATTTTCCTTTAT
hBD2	GCATTGCACCAATACCAGT	CCAAAAACACCTGGAAGAGGCA
hBD3	TATCTTCTGTTTGCTTTGCTCTTCC	CGCCTCTGACTCTGCAATAA
CAMP	CGGTGTATGGGGACAGTGAC	TGGGTACAAGATTCCGCAAA

4.7. 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 $2^{-\Delta\Delta C_t}$ method, using the house-keeping gene glyceraldehyde-3-phosphate (GAPDH) to normalize, and control samples as internal calibrators. The primers used are listed in Table 1.

4.8. 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 O₃ 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.

4.9. Antibacterial experiments

E. coli strain DH5 α (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 O₃ for 30 min. Directly after exposure, cells were infected with 1 × 10⁵ CFU of bacteria and incubated for 6 h in 5% CO₂ 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.

4.10. 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.

Data availability

No large datasets were generated or analyzed in this study; however, all data will be shared by open request to the corresponding author.

Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2021.101952>.

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