Biochemical and biological effects of air pollution on the function of human skin.

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Abstract

According to the World Health Organisation, 99% of the world's population live in areas where the air quality exceeds air quality guideline limits, this highlights a pressing need for the investigation into types of pollutant-induced damage [1]. In this study we aim to investigate the biological effects of particulate matter (PM) and ozone exposure on human skin equivalents. Human skin equivalents were exposed to 0.01μg/μl PM, 0.05μg/μl PM, 0.3ppm ozone, or a combination of 0.01μg/μl and 0.3ppm ozone for eight hours daily for a total of seven days, before harvesting skin equivalents and culture medium. Structural, gene and protein analyses were performed using histological staining, qPCR, Western blotting and ELISA assays. PM induced aberrant epidermal differentiation characterised by a concentration-dependent decrease in viable epidermal thickness, whereas ozone exposure induced an increase in keratinocyte proliferation characterised by an increase in viable epidermal thickness. Whilst PM showed no major damaging effects on any downstream marker, ozone was shown to upregulate the gene/protein expression of wrinkling-associated MMP-1 and MMP-3, and also induce the release of inflammatory marker PGE2. There were also some significant synergistic effects when comparing single pollutant exposure to combination pollutant exposure. This study presents the differential effects of various pollutants, and shows how exposure to multiple pollutants simultaneously may augment the

initial changes associated with ageing. However, further investigation into the precise mechanisms involved is necessary before preventative strategies can be implemented.

Keywords: pollution; ageing; skin; in-vitro.

1. Introduction

With the global population rapidly increasing comes the continuous demand for further urbanization, industrialization, and the mass upsurge in energy production and vehicle emissions. All of these anthropogenic actions have led to the increased production of ambient air pollutants which can be split into primary and secondary pollutants. Primary pollutants can be further subdivided into particulate matter (PM) and gases (CO, CO_2, NO, NO_2, NO_X, SO_2), whereas secondary pollutants refer to those that are formed from reactions with primary pollutants, such as ozone that is formed from photochemical reactions between primary pollutants, heat and ultraviolet (UV) radiation [2]. The increase in these pollutants has been implicated in the deaths of 4.2 million people annually, and although the majority of these deaths have been linked to cardiorespiratory conditions, the involvement of pollutants in the aetiology of conditions involving other organs has now become to be investigated [1]. One such organ brought into question due to its continual exposure to the environment is the skin, with a number of these pollutants already being implicated in the damage of skin structure and function. The pollutants that have gained the most attention due to their environmental abundance and their potential to be modified by human impact are particulate matter and ground-level ozone.

Particulate matter (PM) is a complex mixture of microscopic particles and liquid droplets suspended in air, these particles can originate from a number of different anthropogenic sources including motor vehicle missions, open fires and power plant byproducts [3]. It is thought that the smaller the PM particles the more danger they present due to their ability to travel further into internal systems. Ambient PM represents a global health risk, already being implicated as a risk factor for various cardiovascular and pulmonary diseases, as well as cancers. However, it is only in recent years that the role of PM in the development of skin conditions and skin ageing has been brought into question, with a number of studies indicating a significant positive correlation between air pollution and the development of skin conditions and/or skin ageing characteristics [4-6]. One study observed a 20% increase in pigment spots on the forehead and cheeks with increased PM exposure associated with traffic, whilst another showed smokers were almost five times more likely to have facial wrinkles than non-smokers [7, 8]. It is suggested that PM can exert its effects through two

different formats; the first is the effects exerted through the actual particle itself, with their large surface area making them highly reactive towards biological surfaces (such as skin) [9]. The second is their ability to act as carriers for other organic chemicals, such as polycyclic aromatic hydrocarbons (PAHs) which are capable of adsorbing on to the surface of PM and exerting their own damaging effects [10]. These damaging effects on the skin can not only be exerted by the parent compound of PAHs but also from the photo-modified products which have distinctive bioactivities in comparison to the parent compound. The aromatic rings that form PAHs are able to absorb solar light in both the visible and the entire UV spectrum, allowing PAHs to undergo photo-oxidation and transform into ROS-forming quinones; there has also been some evidence that with an increased number of aromatic rings comes a higher phototoxic potency [11]. Ozone is present in both the Earth's upper atmosphere (stratosphere), and also at ground level (troposphere) where it is found at high concentrations in smog. Tropospheric ozone, or "bad ozone", is mainly created by the chemical reactions between nitrogen oxides and volatile organic compounds (VOC) in the presence of sunlight [12]. These ozone forming chemicals come from various sources, however around 50% of the nitrogen oxides and 45% of the VOC originate from motor vehicle exhaust fumes, with the remaining percentage originating from industrial processes, such as those reactions used in power plants and oil refineries [13]. Alongside PM, ozone has also been implicated in the decline of skin health, with a higher incidence of hospital admissions for skin related conditions being seen in populations exposed to higher levels of ozone, and further studies confirming ozone-induced lipid peroxidation, inflammation and structural damage [14, 15].

Environmental stressors have the ability to differentially affect the epidermal and dermal compartments within the skin, with epidermal cells showing the ability to adapt to protect against damage, and dermal cells modulating characteristic signs of skin ageing. Keratinocytes in the epidermal layer have the ability to adapt to different extrinsic stimuli, with damage inducing the activations of factors such as p53 which can regulate cell cycle arrest, DNA repair pathways and apoptosis. After damage has subsided, keratinocytes proliferate rapidly, and the increased epidermal thickness allows for superior protection against stressors like UV [16]. The involvement of keratinocytes in the formation of the stratum corneum also provides the body with a protective barrier against environmental assaults, preventing penetration of a wide range of chemicals and microbes. Environmental stressors also have the ability to induce

different ECM profiles via dysregulation of ECM degrading proteins MMPs and disruption to ECM protein expression, consequently impacting skin integrity [17]. This change also occurs with age, as skin cells undergo a genetic switch in expression patterns from matrix secreting to a matrix degrading phenotype, this is thought to be in part due to the induction of low level chronic inflammation termed "inflammaging" [18].

Given the recent findings on pollutant-induced effects on the skin, it was of interest to not only investigate the different biological effects of PM and ozone on the skin, but also how these pollutants may interact to augment damage.

2. Materials and Methods

2.1 Pollutant Exposure

Phenion full thickness skin equivalents (Henkel AG & Co. KGaA, Düsseldorf) were exposed to either 0.01µg/µl PM, 0.05µg/µl PM, 0.3ppm ozone or a combination of 0.01µg/µl PM and 0.3ppm ozone. PM was prepared in PBS to a concentration of 0.01µg/µl or 0.05µg/µl, and sonicated with a SONICS Vibra-Cell 505 sonication probe for 15-20 seconds, before vortexing and applying in a 25µl volume to the surface of each skin equivalent. The vehicle control group consisted of equivalents being exposed to 0.2% DMSO in PBS. After the 8-hour exposure period, all skin equivalents were washed with PBS, before repeating for seven days.

2.2 RNA Extraction

Skin equivalents were homogenized in pre-chilled RLT buffer using a Precellys Evolution tissue homogeniser (Bertin Instruments, UK). The tubes were centrifuged at 12,000 rpm for 5 minutes at 4°C before transferring the supernatant to pre-chilled RNase-free microcentrifuge tubes. The Qiagen RNeasy mini kit was used for the remainder of the RNA extraction, using their protocol for purification of total RNA from animal tissue with optional on column DNase digestion.

2.3 Reverse Transcription of RNA

RNA was used as a template to produce cDNA via reverse transcription in order to analyse relative expression of a gene transcript using quantitative PCR. Reverse transcription was

performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) following manufacturer's guidelines using the GeneAmp PCR System 9700 (Applied Biosystems, UK).

2.4 Quantitative PCR

Quantitative PCR (qPCR) was performed on cDNA to analyse any changes in the expression levels of genes associated with skin wrinkling. qPCR was performed with TaqMan Advanced Master Mix and 40ng cDNA using the QuantStudio 3 Real-Time PCR System. Primers were purchased from ThermoFisher; GAPDH (Hs02786624_g1), MMP-1 (Hs00899658_m1) and MMP-3 (Hs00968305_m1).

2.5 ELISA

Culture medium was harvested after 2 and 7 days of pollution exposure, centrifuged at 200 x*g* for 5 minutes at 4°C before removing the supernatant and analysing PGE2 secretion using enzyme-linked immunosorbent assays (ThermoFisher, UK) following manufacturer's guidelines. Background absorbance was subtracted from all datapoints, before using a 4-parameter algorithm to create a standard curve and interpolating unknown PGE2 concentrations.

2.6 Histological Analysis

Skin equivalents were placed in between two biopsy foam pads which was then placed in a tissue embedding cassette and placed in a Histotainer II specimen container prefilled with 10% neutral-buffered formalin (Sigma Aldrich, UK). Skin equivalent paraffin embedding, processing, sectioning and H&E staining were performed at the Newcastle Molecular Pathology Node, UK. The Leica SCN400 Brightfield Slide Scanner and Autoloader system were then used to acquire full section images of stained slides. For epidermal thickness analysis, individual lines were placed perpendicular to the skin around every 0.3µm for the entire skin equivalent, with around 150-350 lines being added to each equivalent and their measurements recorded.

2.7 Western Blotting

Skin equivalents were homogenized in pre-chilled RIPA buffer (Fisher Scientific, UK) with freshly supplemented cOmplete protease inhibitor cocktail (Roche, UK) using a Precellys

Evolution tissue homogeniser (Bertin Instruments, UK). Homogenates were centrifuged at 12,000 rpm for 20 minutes at 4°C, before using the supernatant for protein analysis. Bradford assay was used to quantify protein, and samples were prepared in NuPAGE Sample Reducing Agent and NuPAGE LDS Sample Buffer before heating at 70°C for 10 minutes. SDS-PAGE was performed, and proteins were then electro-transferred on to the nitrocellulose membrane using the iBlot 2 semi-dry transfer system (Invitrogen, UK). Immunodetection was subsequently performed using MMP-1 (R&D Systems, 1/1000), MMP-3 (R&D Systems, 1/1000), GAPDH (Santa Cruz, 1/2000), and appropriate IRDye 800CW and IRDye 680RD secondary antibodies. Membranes were then imaged using the LI-COR Fc Dual Mode Imaging System. Densitometric analysis was then performed to analyse protein expression.

2.8 Statistical Analyses

Statistical analysis was conducted using GraphPad Prism 9. Percentage change to control data and qPCR results (calculated using $2^{-\Delta\Delta Ct}$ method) were first logged as fractions before performing one sample t-tests to assess whether average percentage changes differ significantly from the hypothetical logged mean of the control. Unpaired t-tests were used to assess synergism between single and combination pollution exposure groups. Epidermal thickness measurements were assessed using Kruskal-Wallis test and corrected for multiple comparisons using Dunn's test. Statistical significance was defined as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Results are presented as mean with standard deviation (SD) represented by the error bars.

3. Results

3.1 Pollutant Exposure Induces Changes in Epidermal Thickness

Upon visual assessment, it was noted that PM seemed to alter stratum corneum structure to the point of reducing viable epidermal thickness, indicating an increase in keratinocyte turnover rate (Figure 3.1). This is more apparent in the higher concentration 0.05µg/µl PM group, where the stratum corneum lacks typical structure, with no compact layer and an increased basket-weave layer. On the other hand, ozone and combination pollution induced thickening of the viable epidermal layer, and a reduction in the stratum corneum layer. In terms of dermal layer changes, PM exposed skin equivalents seem to have an increased papillary dermal content, whereas ozone exposed skin equivalents seem to have a decreased papillary dermal content. Analysis of epidermal thickness confirmed visual assessment of pollutant-induced changes (Figure 3.2). Viable epidermal thickness was decreased by 13.4 (p=<0.001) and 20.6% (p=<0.001) for 0.01µg/µl and 0.05µg/µl PM, respectively. Ozone induced an increase of 70.6% (p=<0.001) in viable epidermal thickness when applied alone, and an increase of 69.4% (p=<0.001) in viable epidermal thickness when applied in combination with 0.01µg/µl PM. A significant concentration-dependent decrease in viable epidermal thickness was observed between the lower concentration of 0.01µg/µl and the higher concentration of 0.05µg/µl PM (p=<0.001). There were no significant synergistic changes in viable epidermal thickness when comparing ozone to ozone with the addition of 0.01µg/µl PM.

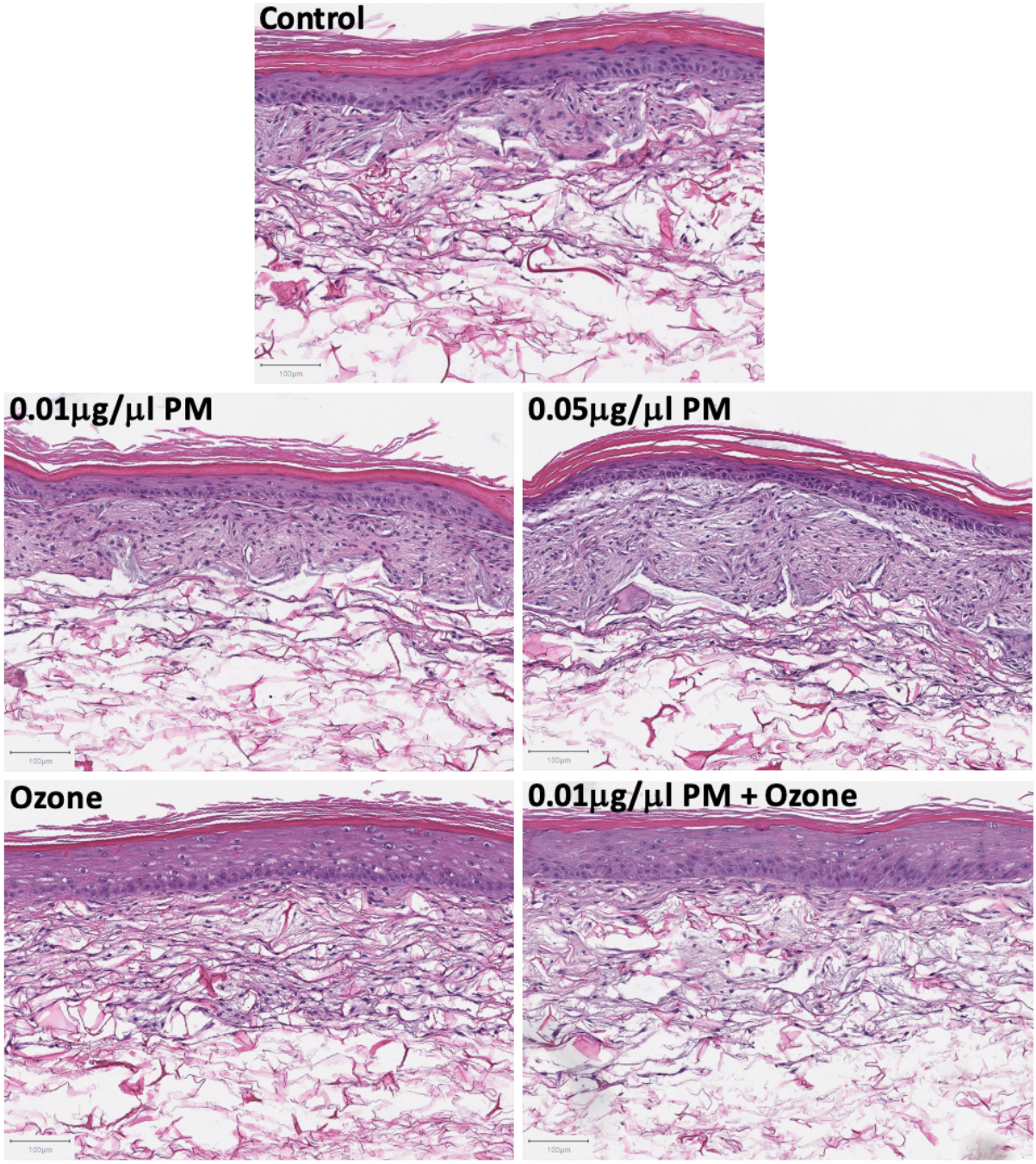

Figure 3.1: Haematoxylin and eosin staining of Phenion FT skin equivalents after single and combination pollutant exposure for seven days.
After the seven-day exposure period, skin equivalents were harvested, fixed in 10% neutral buffered formalin, paraffin embedded and processed before H&E staining. Full H&E stained Phenion FT skin equivalent sections were scanned using a Leica SCN400 Brightfield Slide Scanner at 40X, with a representative image for each skin model presented. Scale bars: 100µm.

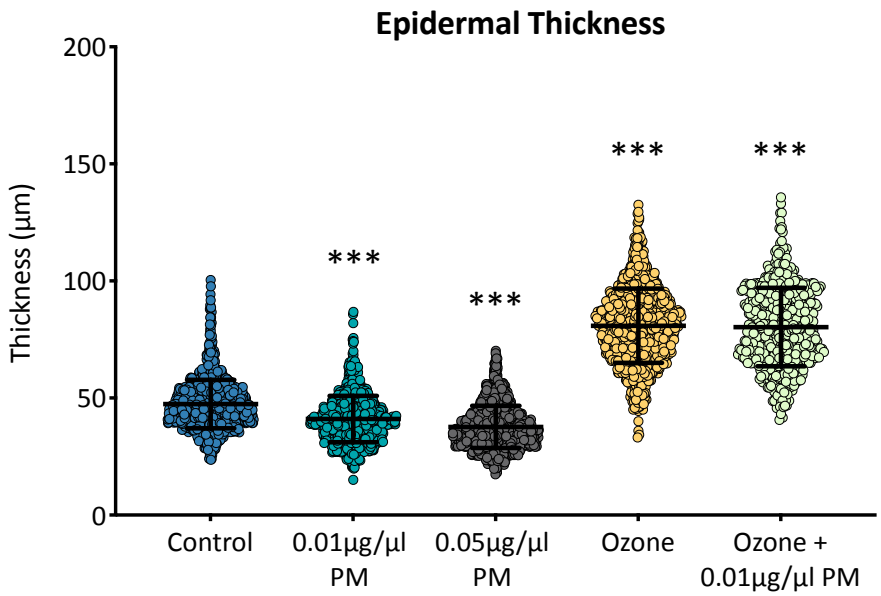

Figure 3.2: Analysis of viable epidermal thickness after single and combination pollution exposure.

Viable epidermal thickness (stratum basale to the stratum granulosum) was measured around every 20µm for the entire skin equivalent using QuPath with around 300-400 individual measurements for each equivalent obtained, n=4. Dermal thickness was also measured, with around 100 individual measurements for each equivalent obtained, n=4. Kruskal-Wallis test was performed and corrected for multiple comparisons using Dunn's test with *** p <0.001 representing significance when compared to the vehicle control and brackets showing significance when assessing synergism between ozone and ozone + 0.01µg/µl PM.

3.2 Pollutant Exposure Induces Changes in MMP Expression

Neither 0.01µg/µl (0.96±0.42), nor 0.05µg/µl (1.20±0.37) PM exposure induced any significant changes in MMP-1 gene expression (Figure 3.3). Ozone seemed to be the main driver of damage, with ozone (2.67±0.42, p=0.0011) and combination pollution (6.46±2.72, p=0.0053) causing significant increases in MMP-1 gene expression. Synergistic effects were also observed when comparing single ozone exposure to a combination of ozone and 0.01µg/µl PM (p=0.02).

MMP-3 gene expression showed the same pattern of induction as MMP-1 however with a lower level of induction (Figure 3.3). Exposure to 0.01µg/µl PM (0.45±0.42, p=0.018) and 0.05µg/µl PM (0.59±0.33, p=0.1041) induced a downregulation in MMP-3 gene expression. Ozone induced no significant changes in expression when applied alone (1.10±0.59), however when in combination with 0.01µg/µl PM induced an upregulation of (2.90±1.59, p=0.0339). A significant synergistic effect was also observed when comparing ozone to a combination of ozone and 0.01µg/µl PM (p=0.04).

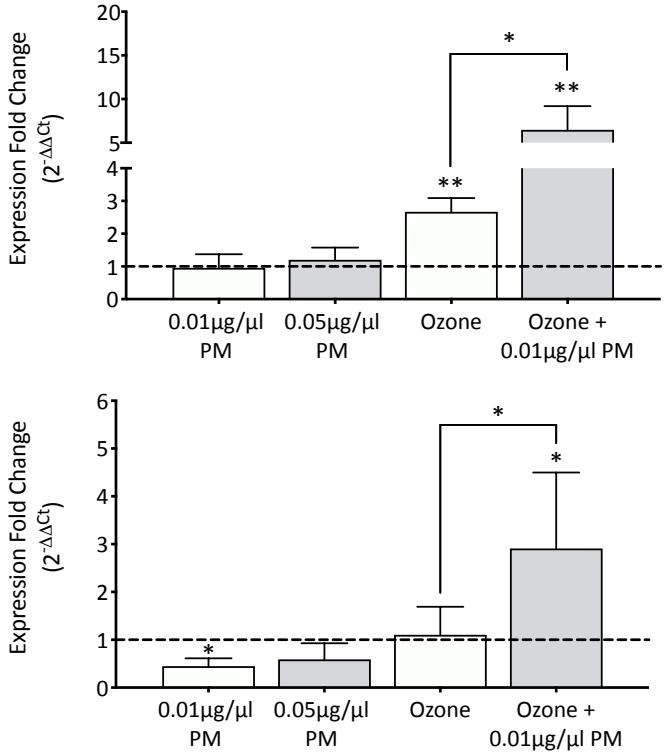

Figure 3.3: Analysis of MMP-1 and MMP-3 gene expression in Phenion FT skin equivalents after single and combination pollutant exposure for seven days.
Phenion FT skin equivalents were exposed to 0.2% DMSO in PBS (vehicle control), 0.01µg/µl PM, 0.05µg/µl PM, 0.3ppm ozone or a combination of 0.01µg/µl PM and 0.3ppmm ozone for eight hours daily for a total of seven days before extracting RNA and reverse transcribing into cDNA. Relative changes in gene expression were evaluated using the $2^{-\Delta\Delta Ct}$ method with the dashed line representing the mean control expression levels, n=4. One sample t-test was performed, with * p <0.05 and ** p <0.01 representing significance when compared to the vehicle control and an unpaired t-test was performed to assess synergism between ozone and ozone + 0.01µg/µl PM with brackets showing significance.

MMP-1 protein expression followed the same pattern as that of MMP-1 gene expression, with PM alone causing no changes, and ozone and combination pollution causing increases in protein expression (Figure 3.4). Ozone caused a significant increase in both glycosylated (125.2%, p=0.0159) and unglycosylated (133%, p=0.0082) proMMP-1 expression. When ozone and 0.01µg/µl PM were applied in combination, both glycosylated (267.4%, p=<0.001) and unglycosylated (247.7%, p=<0.001) proMMP-1 protein expression increased more dramatically than single ozone exposure. A significant synergistic effect was in fact observed for both glycosylated (p=0.03) and unglycosylated (p=0.03) proMMP-1 when comparing single ozone to combination pollution.

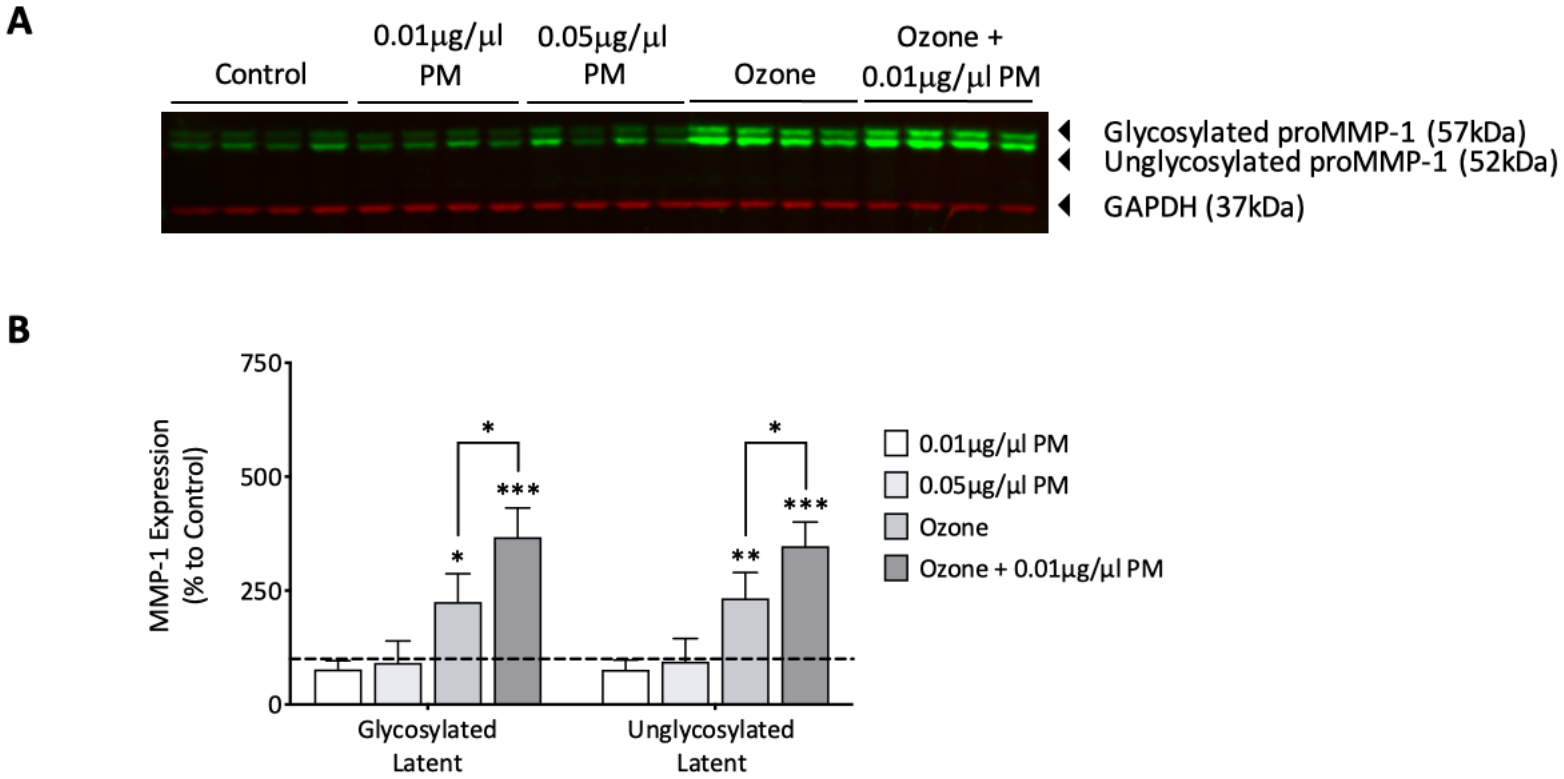

Figure 3.4: Analysis of MMP-1 protein expression in Phenion FT skin equivalent homogenate after single and combination pollutant exposure for seven days.

Phenion FT skin equivalents were exposed to either 0.2% DMSO in PBS (vehicle control), 0.01µg/µl PM, 0.05µg/µl PM, 0.3ppm ozone or a combination of 0.01µg/µl PM and 0.3ppmm ozone for eight hours daily for a total of seven days before homogenising and analysing MMP-1 protein expression using Western blotting (A). MMP-1 protein was normalised to GAPDH using densitometric analysis, before presenting as mean percentage changes to the vehicle control+SD, with the dashed line representing vehicle control mean, n=4 (B). Multiple one sample t-tests were performed, with * p <0.05, ** p <0.01 and *** p <0.001 representing significance when compared to the vehicle control and an unpaired t-test was performed to assess synergism between ozone and ozone + 0.01µg/µl PM with brackets showing significance.

MMP-3 protein expression also followed that of MMP-3 gene expression, with PM alone causing decreases, and ozone and combination pollution causing increases in protein expression (Figure 3.5). Downregulations of 17.8 (p=0.0694) and 34% (p=0.0736) in glycosylated proMMP-3 and a similar downregulation of 20.7 (p=0.0412) and 46.4% (p=0.046) in unglycosylated proMMP-3 were observed after exposure to 0.01µg/µl and 0.05µg/µl PM, respectively. Ozone caused an increase in both glycosylated (56.8%, p=0.0815) and unglycosylated (63.3%, p=0.0085) proMMP-3 expression. When ozone and 0.01µg/µl PM were applied in combination, both glycosylated (139.6%, p=0.0039) and unglycosylated (136.2%, p=<0.001) proMMP-3 protein expression increased more dramatically. A significant synergistic effect was in fact observed for unglycosylated (p=0.008) proMMP-3 and a trending significance for glycosylated proMMP-3 (p=0.06) when comparing ozone to combination pollution.

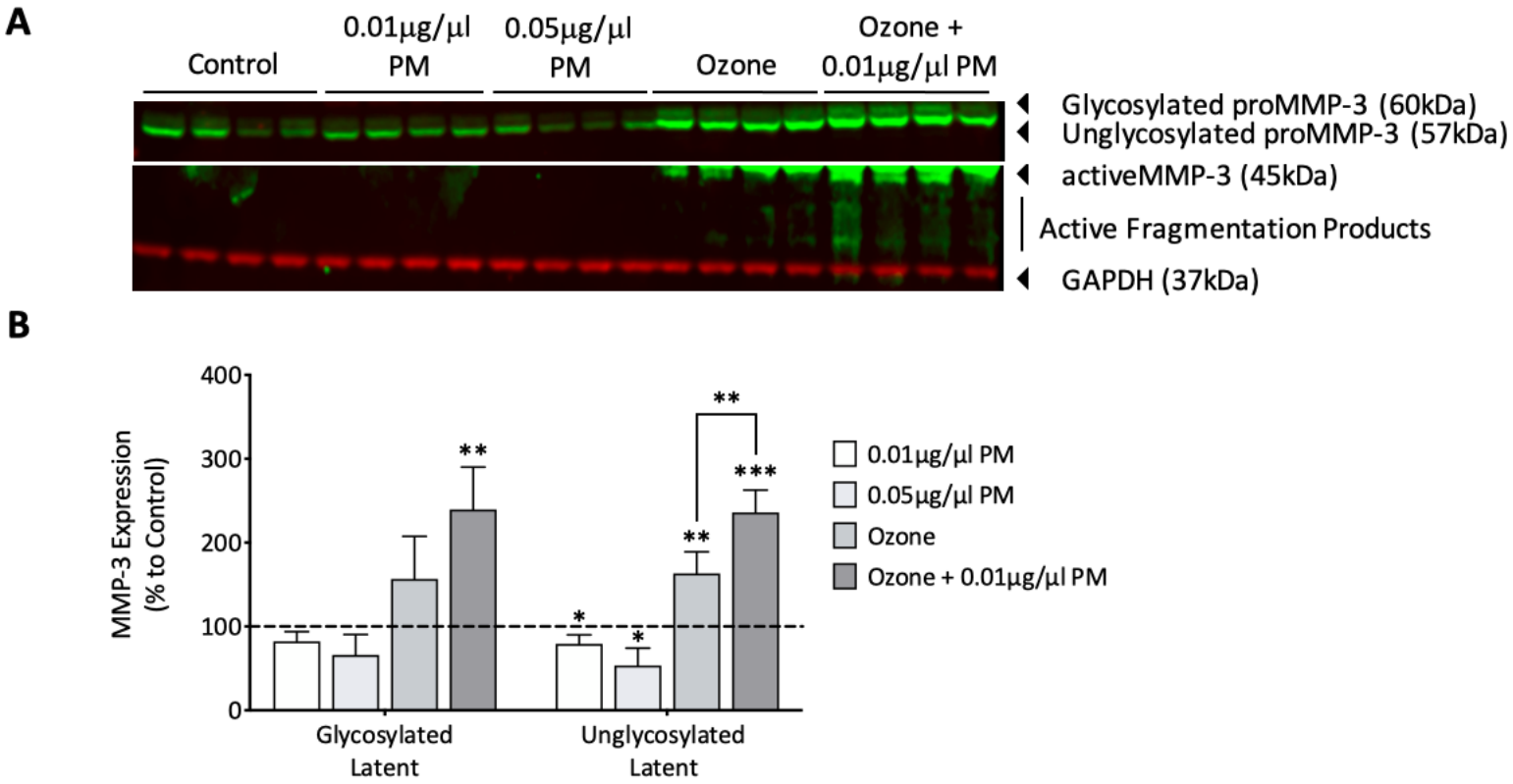

Figure 3.5: Analysis of MMP-3 protein expression in Phenion FT skin equivalent homogenate after single and combination pollutant exposure for seven days.

Phenion FT skin equivalents were exposed to either 0.2% DMSO in PBS (vehicle control), 0.01µg/µl PM, 0.05µg/µl PM, 0.3ppm ozone or a combination of 0.01µg/µl PM and 0.3ppmm ozone for eight hours daily for a total of seven days before homogenising and analysing MMP-3 protein expression using Western blotting (A). MMP-3 protein was normalised to GAPDH using densitometric analysis, before presenting as mean percentage changes to the vehicle control+SD, with the dashed line representing vehicle control mean, n=4 (B). Multiple one sample t-tests were performed, with * p <0.05, ** p <0.01 and *** p <0.001 representing significance when compared to the vehicle control and an unpaired t-test was performed to assess synergism between ozone and ozone + 0.01µg/µl PM with brackets showing significance.

3.3 Pollutant Exposure Induces Changes in Inflammatory Profile

Exposure to 0.01µg/µl PM induced no change in PGE2 release, however 0.05µg/µl PM induced an increase of 81.3% (p=0.0112) when compared to the vehicle control (Figure 3.6). In contrast, ozone induced more significant increases in PGE2 after both 2 (136%, p=0.005) and 7 (456.4%, p=0.0028) days of exposure, and when combined with 0.01µg/µl PM induced larger increases after both 2 (218.5%, p=0.0073) and 7 (834.3%, p=<0.001) days. Synergistic effects were observed when comparing combination pollution to ozone alone at both 2 (p=0.23) and 7 (p=0.07) days, however neither reach statistical significance.

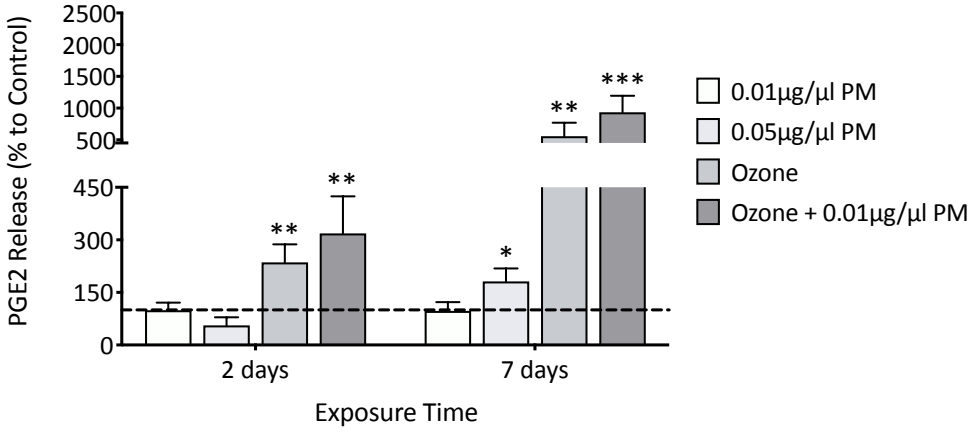

Figure 3.6: Analysis of PGE2 release in conditioned medium from Phenion FT skin equivalents after single and combination pollutant exposure for seven days.
Phenion FT skin equivalents were exposed to to 0.2% DMSO in PBS (vehicle control), 0.01µg/µl PM, 0.05µg/µl PM, 0.3ppm ozone or a combination of 0.01µg/µl PM and 0.3ppmm ozone for eight hours daily for a total of seven days, with the medium removed at days 2 and 7 and analysed for PGE2 release using ELISA. PGE2 was presented as mean percentage change to the vehicle control+SD, with the dashed line representing vehicle control mean, n=4. Multiple one sample t-tests were performed, with * p <0.05, ** p <0.01 and *** p <0.001 representing significance when compared to the vehicle control and an unpaired t-test was performed to assess synergism between ozone and ozone + 0.01µg/µl PM with brackets showing significance.

4. Discussion

Ozone exposure was shown to induce viable epidermal thickening, this is most likely a protective mechanism to protect the more viable cells from further damage, in a mechanism similar to that observed after UV exposure. The induction of cell damage and the resulting activation of damage response pathways can subsequently lead to the increase in keratinocyte proliferation rate, allowing keratinocytes to not only replace those lost as a consequence of damage, but also to provide transient protection against stressors [16, 19]. PM exposure induced a concentration-dependent decrease in viable epidermal thickness and increase in stratum corneum thickness. These characteristics are typical of a chloracne phenotype and can be explained by the early onset of keratinocyte differentiation, a phenotype that has been shown to be induced by TCDD-induced AHR activation [20]. The PM mixture used in this study does contain TCDD, in addition to a number of other AHR ligands, which could explain how PM is inducing a chloracne phenotype. PM also seemed to induce changes in the structure of the stratum corneum, showing a concentration-dependent decrease in the thickness of the lower compact layer, and an increase in the upper basket-weave layer. A basket-weave pattern within the upper stratum corneum is a hallmark of a healthy epidermis, being shown to be involved in skin softness and also maintaining skin hydration via increased water barrier function, however an excessive basket weave formation may indicate dysregulation [21]. The intercellular spaces formed between corneocytes allows for the formation of lipid layers that contribute to the skins water barrier. This basket-weave formation is instigated by the controlled degradation of the adhering junctions between corneocyte sheets (corneodesmosomes) by proteases such as kallikreins or cathepsins [22]. The use of xylene in histological staining extracts the lipids between corneocytes leading to the appearance of the basket-weave pattern due to the lack of corneodesmosomes [21]. The increased level of basket-weave formation observed in the PM exposure group could be due to the increased degradation rate of corneodesmosomes, which could be a possibility given published research showing PM-induced decreases in desmocollin-1, one of the major proteins involved in corneodesmosome structure, as well as a number of kallikreins [23, 24]. Another possibility is that PM exposure increases lipid production, resulting in a larger lipid

volume between corneocytes and therefore an increased basket-weave formation after xylene extraction.

PM caused decreases in both MMP-1 and MMP-3 gene and protein expression, this could be a result of the reduction in epidermal thickness, with the lower number of keratinocytes either secreting less MMPs themselves, or limiting the crosstalk with fibroblasts to induce expression of MMPs. This indicates PM has limited penetrative potential to increase MMP expression in the dermal layer. In contrast, ozone exposure was shown to induce increases in the gene expression of both MMP-1 and MMP-3, as well as the protein expression of both glycosylated and unglycosylated proMMP-1 and proMMP-3. Whilst increases in proMMPs are of concern, the activation status should also be one to note, with active MMPs showing functional degradation of ECM proteins. MMP-3 showed signs of activation-associated fragmentation intracellularly after ozone exposure, and could be attributed to the induction of oxidative stress, with ROS shown to modulate both expression and activation of MMPs [25]. Since higher levels of activation occurs extracellularly, analysis of the levels of MMP-1 and MMP-3 in culture medium would provide further insight into the activation status after pollution exposure.

With inflammation and wrinkling showing a general tendency to be mutually induced and shown to be signature characteristics in senescent phenotypes, inflammatory marker levels were also investigated. Skin equivalents exposed to ozone showed a significant increase in PGE2 secretion, and would implicate ozone in modulating the metabolism of arachidonic acid from phospholipids, possibly through lipid peroxidation [26]. Whether the increase in inflammatory cytokine synthesis is linked to the increased cell proliferation, or whether the increased cell proliferation is a result of the increased inflammatory cytokine synthesis is something that needs to be elucidated, however studies are favouring the latter [27]. It would be of interest to note how the induction of these inflammatory cytokines would influence inflammatory cells residing in the skin if they were to be incorporated into skin equivalents.

Synergistic properties of ozone and PM were also investigated, with the study being the first of its kind to do so, to my knowledge. Combination pollution did not induce any significant synergistic changes in epidermal structure or PGE2 secretion when compared to ozone exposure alone, however did with MMP-1 and MMP-3 gene/protein expression. Since ozone most likely exerts its effects through oxidative stress, it's possible that the addition of PM could cause cumulative oxidative stress, or that the cellular ATP pool isn't sufficient to facilitate adaptive mechanisms for both ozone and PM-induced damage. Synergistic effects should be taken into consideration when assessing pollution-induced damage since individuals are rarely exposed to just one type of air pollutant, therefore further investigation into how these and other air pollutants may interact with each other is necessary. The synergism of these pollutants with UV in particular should be a priority to investigate, as UV has been shown to photoactivate components of pollution such as PAHs and augment their damaging effects [28].

Conclusion

PM seemed to show very little effect on downstream markers and only showed surface level effects on epidermal structure, characteristic of a chloracne phenotype. In contrast ozone was shown to upregulate the gene/protein expression of wrinkling-associated MMP-1 and MMP-3, and also induce the release of inflammatory marker PGE2. It must be noted that although PM alone was not enough to induce any downstream effects, when combined with ozone significant synergistic effects were observed, which resulted in the acceleration of the ageing phenotype in skin equivalents. This study presents the differential effects of various pollutants, and shows how exposure to multiple pollutants simultaneously may augment the initial changes associated with ageing, which may need to be acknowledged during investigation of the effects of pollution and during compound screening.

Acknowledgments

The work in this study was funded by UK Research and Innovation/Biotechnology and Biological Sciences Research Council (UKRI/BBSRC, BB/S506837/1) in collaboration with Croda, awarded to Mark A. Birch-Machin as principal investigator.

Conflict of Interest Statement

None.

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