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Original Article

Ozone exposure promotes melanogenesis through MAPK-dependent oxidative stress signaling: attenuation by resveratrol in zebrafish and melanoma cells

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Abstract: Ground-level ozone, a major air pollutant, primarily forms through photochemical reactions between nitrogen oxides (NOx) and volatile organic compounds (VOCs) in sunlight. Ozone is known to harm human health and skin, making it a significant environmental concern. In this study, we examined the effects of ozone exposure on melanogenesis and whether resveratrol could influence this process. Using zebrafish models and B16F10 melanoma cells, we assessed changes in melanin production. In B16F10 cells, we investigated the impact of 1 ppm ozone exposure on reactive oxygen species (ROS) production and its subsequent effect on mitogen-activated protein kinase (MAPK) signaling pathways, including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Our findings showed that ozone exposure significantly increased melanin levels in both zebrafish and B16F10 cells. Ozone exposure enhanced p38 and JNK phosphorylation, reduced ERK phosphorylation, and upregulated microphthalmia-associated transcription factor (MITF) and tyrosinase expression. Resveratrol was selected as a candidate agent for its ability to inhibit MAPK signaling. Resveratrol treatment effectively reduced ozone-induced melanin production by decreasing MITF and its downstream enzyme, tyrosinase. These results highlight the role of MAPK signaling in ozone-induced pigmentation and suggest that resveratrol could be a promising agent for protecting against ozone-related skin damage.

Keywords: Ozone, melanin, mitogen-activated protein kinase, zebrafish, resveratrol

Introduction

Ozone (O_3) is a significant air pollutant strongly linked to respiratory diseases and poses serious health risks, particularly for vulnerable groups such as children, the elderly, and individuals with pre-existing health conditions. Both ozone and its precursors, volatile organic compounds (VOCs), harm human health and overall quality of life [1]. More research is urgently needed to elucidate the biological mechanisms by which air pollutants cause disease and to develop effective strategies to mitigate their effects. Under high ambient temperatures, significant amounts of nitrogen monoxide (NO) and nitrogen dioxide (NO_2) are emitted into the atmosphere. NO rapidly oxidizes to NO_2, which then photolyzes to produce reactive oxygen atoms. These atoms react with molecular oxygen to form ozone [2]. Although ozone is not a VOC, it is classified as a secondary pollutant formed through photochemical reactions between VOCs and nitrogen oxides (NO_x). The contribution of individual VOCs to ozone formation depends on their chemical reactivity. Tropospheric ozone, commonly known as "bad ozone", is a major air quality concern because of its harmful effects on human and environmental health. Acute ozone exposure can worsen respiratory symptoms and increase the risk of death among people with asthma. In plants, higher ozone levels reduce photosynthetic effi-

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ciency and crop yields. Additionally, ozone is a potent greenhouse gas, and human-made emissions of ozone significantly drive global climate change. Ozone precursors include vehicle emissions, industrial processes, and biomass burning. In Taiwan, ground-level ozone has recently surpassed particulate matter (PM_{10} and $PM_{2.5}$) as the primary air pollutant affecting ambient air quality. Because of its known health risks, ozone is a crucial pollutant in global environmental regulations. Epidemiological studies have linked ozone exposure to increased risks of cardiovascular disease, ischemic heart disease, respiratory illnesses, and cardiopulmonary death [3]. Despite these associations, direct evidence from cellular and animal studies on the biological effects of ozone remains limited, and public awareness of its risks is low.

To bridge this gap, it is essential to examine ozone-induced biological responses and regulatory pathways using both *in vitro* and *in vivo* models. Such research can deepen mechanistic understanding and support the development of targeted interventions. As a highly reactive oxidant, ozone primarily damages the respiratory tract but may also affect other organ systems, especially with chronic or high-dose exposure. Long-term ozone exposure has been linked to the development of chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, hypertension, and neurodegenerative conditions [4]. This study examines the immediate biological effects of ozone, particularly its potential to alter skin pigmentation through melanogenesis. Melanin, the primary determinant of skin color, is produced by melanocytes through a tightly regulated process called melanogenesis. This process involves a series of enzymatic reactions, with tyrosinase playing a key role by converting tyrosine to levodopa (L-DOPA) and then oxidizing it to dopaquinone. Melanogenesis is also regulated by several internal signaling pathways, including Wnt/β-catenin, phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), and α-melanocyte-stimulating hormone (α-MSH) signaling through the melanocortin 1 receptor (MC1R) [5]. Among the main regulatory pathways, the mitogen-activated protein kinase (MAPK) pathways, specifically extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, are crucial for regulating genes associated with melanogenesis [6]. Phosphorylated ERK (P-ERK) typically suppresses melanogenesis by degrading the transcription factor MITF (microphthalmia-associated transcription factor), whereas phosphorylated JNK (P-JNK) and phosphorylated p38 (P-p38) usually promote melanin production by increasing MITF transcription and stimulating the production of melanogenic enzymes [7]. Disruption of these pathways can cause pigment-related disorders and provide insights into potential targets for treating hyperpigmentation or hypopigmentation.

In our experimental models, ozone exposure upregulated MITF and tyrosinase expression in mouse melanoma B16F10 cells and zebrafish embryos. Western blot analysis and melanin quantification assays showed that ozone enhanced melanogenesis, likely through activation of the MAPK signaling cascade. This pathway is crucial for melanocyte homeostasis, and its dysregulation may lead to pigmentary disorders, including hyperpigmentation. We used resveratrol, a naturally occurring polyphenol, as a potential intervention to assess its effectiveness in reducing ozone-induced melanogenesis. By targeting MAPK-related signaling pathways, resveratrol may confer protective effects against environmental pollutant-induced skin pigmentation.

Materials and methods

Reagents, cell culture, and ozone exposure box

Resveratrol, SB203580, and SP600125 (Sigma-Aldrich, St. Louis, MO, USA) were used as experimental compounds. B16F10 murine melanoma cells were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (FBS), 50 μg/mL gentamicin, and 1% L-glutamine. Cultures were maintained at 37°C in an incubator with 5% CO_2. For ozone exposure, an enclosed acrylic system was designed to enable passive ozone introduction, thereby avoiding active injection. A commercial ozone generator was used to deliver ozone into the chamber, with airflow regulated via an inlet-outlet ventilation system. Ozone concentration was continuously tracked using a calibrated detector (Figure S1). Ozone concentration inside the chamber was continuously monitored using an ozone analyzer and maintained

at 1.0 ± 0.2 ppm. A low-speed circulation fan ensured homogeneous distribution of ozone within the chamber. The ozone concentration was measured at multiple points within the chamber to confirm uniform exposure. Cells or zebrafish were exposed to 1 ppm ozone for 3 hours per day over three days. Control groups were exposed to ambient air filtered under identical conditions. Post-exposure, protein lysates were collected for downstream analysis.

Zebrafish housing and embryo collection

Adult Danio rerio were obtained from a certified vendor and housed in 5-L acrylic tanks at 28.5°C under a controlled 14:10 h light/dark photoperiod. Fish were fed three times daily, six days a week, with a combination of dry flake food and live Artemia salina. Embryos were collected within 30 minutes of light onset after spontaneous mating, in accordance with established protocols [6]. All animal procedures were approved by the IACUC of National Sun Yat-sen University and Kaohsiung Medical University (Approval No. 114042).

Cell viability assessment

B16F10 cells were seeded into 96-well plates at 1×10^5 cells per well and exposed to ozone (1 ppm) for 3 hours per day over three days. Each experimental group was tested in sextuplicate. After exposure, cells were rinsed and incubated in fresh medium with 2% FBS. Cell viability was measured using the WST-1 colorimetric assay (Dojindo, Tokyo, Japan) per the manufacturer's instructions. Absorbance at 450 nm was recorded, and results were expressed relative to untreated controls [8].

Total reactive oxygen species (ROS) assay

B16F10 cells were seeded into 96-well plates at a density of 1×10^5 cells per well, pre-treated with resveratrol (20 μM) or not, then exposed to ozone (1 ppm) for 3 hours each day over three days. Each experimental group was tested in sextuplicate. The dichlorodihydrofluorescein diacetate (DCFH-DA) assay (ChekineTM reactive oxygen species detection fluorometric assay) was used as a cell-based assay to detect and quantify intracellular ROS, which are markers of cellular oxidative stress. A fluorescent compound that emits green light at approximately 530 nm when excited around 485 nm. This green fluorescence was measured using a fluorescence plate reader.

Quantification of melanin content

After treatment, cells or tissues were washed with PBS and lysed in cold lysis buffer containing 20 mM sodium phosphate (pH 6.8), 1 mM PMSF, 1 mM EDTA, and 1% Triton X-100. Melanin granules were isolated by centrifugation, resuspended in Soluene-350 (PerkinElmer, Waltham, MA, USA), and incubated at 100°C for 15 minutes. Absorbance at 500 nm was then measured using a microplate spectrophotometer to quantify melanin content [9]. Melanin content was normalized to the total protein concentration of each sample, as determined by BCA assay (Pierce Biotechnology, Rockford, IL, USA).

Western blot analysis

Protein concentration was determined via BCA assay. Cells were pretreated with resveratrol, SB203580 (25 μM) or SP600125 (10 μM) for 2 hours before ozone exposure. Following treatment, protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, UK). Primary antibodies against MITF, tyrosinase, and phosphorylated/total ERK, p38, and JNK were used. β-actin or GAPDH served as loading controls. HRP-conjugated secondary antibodies were applied, and signals were visualized using enhanced chemiluminescence (ECL). Band intensities were analyzed using ImageJ software (NIH, Bethesda, MD, USA) [10].

Zebrafish pigmentation analysis

Synchronized embryos were distributed into 96-well plates (3-4 embryos per well) containing embryo medium. Embryos were exposed to ozone (1 ppm) for 3 hours per day over three days. After exposure, embryos were anesthetized using tricaine methanesulfonate and embedded in 3% methylcellulose for microscopic imaging (MZ16, Leica Microsystems, Germany). Pigmentation was analyzed using ImageJ software as previously reported [6]. Pigmentation intensity was quantified using ImageJ software by measuring the integrated optical density of melanin-containing regions. All analyses were performed using identical

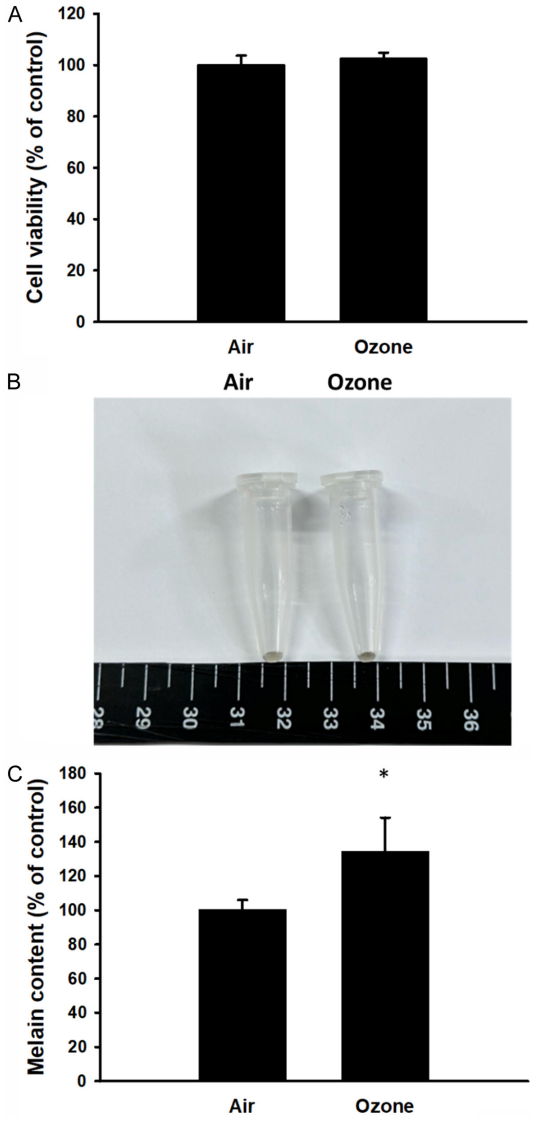

Figure 1. Effects of ozone on cell viability and melanin production in B16F10 cells. B16F10 cells were treated with ozone at 1 ppm for 3 consecutive days (3 hours per day). A. Cell viability was measured by the WST-1 assay. B. Photograph of precipitated B16F10 melanoma cells. C. Effect of ozone on cellular melanin content. Cells were incubated for 3 consecutive days (3 hours per day) with (1 ppm) and without ozone (*, $P < 0.05$) (mean ± SD, n = 6). Each experiment was repeated three times with similar results.

threshold settings and were conducted in a blinded manner. All experiments were conducted in triplicate, yielding reproducible results.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Homogeneity of variance was verified before conducting statistical comparisons. Student's t-test was used to evaluate group differences, with statistical significance set at $P < 0.05$.

Results

Ozone exposure enhances melanogenic response

Although the concentration used in this study (1 ppm) is higher than typical ambient levels, short-term peak ozone concentrations may reach several hundred ppb in highly polluted regions. Higher experimental concentrations are commonly used in mechanistic studies to reveal early cellular responses to oxidative stress. Similar concentrations have been employed in previous experimental ozone studies. To investigate the effect of ozone on melanin biosynthesis, B16F10 melanoma cells were treated with 1 ppm ozone for three consecutive days (3 hours/day). Cell viability and melanin content following exposure are presented in **Figure 1**. This exposure regimen did not cause significant cytotoxicity (**Figure 1A**), thereby enabling assessment of pigmentation effects. Notably, ozone exposure significantly elevated melanin production in B16F10 cells (**Figure 1B** and **1C**), indicating that ozone promotes melanogenesis *in vitro*.

MAPK pathway activation underlies ozone-induced pigmentation

Given that the MAPK signaling cascade plays a key role in stress-related melanogenesis, we assessed the expression of melanogenic proteins and MAPK activity after ozone treatment. Western blot analysis revealed a notable increase in MITF and tyrosinase protein levels in ozone-exposed cells (**Figure 2A**). Additionally, phosphorylation of p38 and JNK was elevated. In contrast, phospho-ERK levels declined, despite unchanged total protein levels. Densitometry confirmed significant modulation of these signaling proteins (**Figure 2B**) (*$P < 0.05$; **$P < 0.01$), supporting that ozone activates melanogenic signaling via the MAPK pathway. To evaluate the effect of ozone exposure on ROS production in B16F10 cells, we exposed cells to 1 ppm ozone for three hours. The total ROS assay showed a significant increase in intracellular ROS levels in B16F10 after ozone exposure (**$P < 0.01$; ***$P < 0.001$) (**Figure**

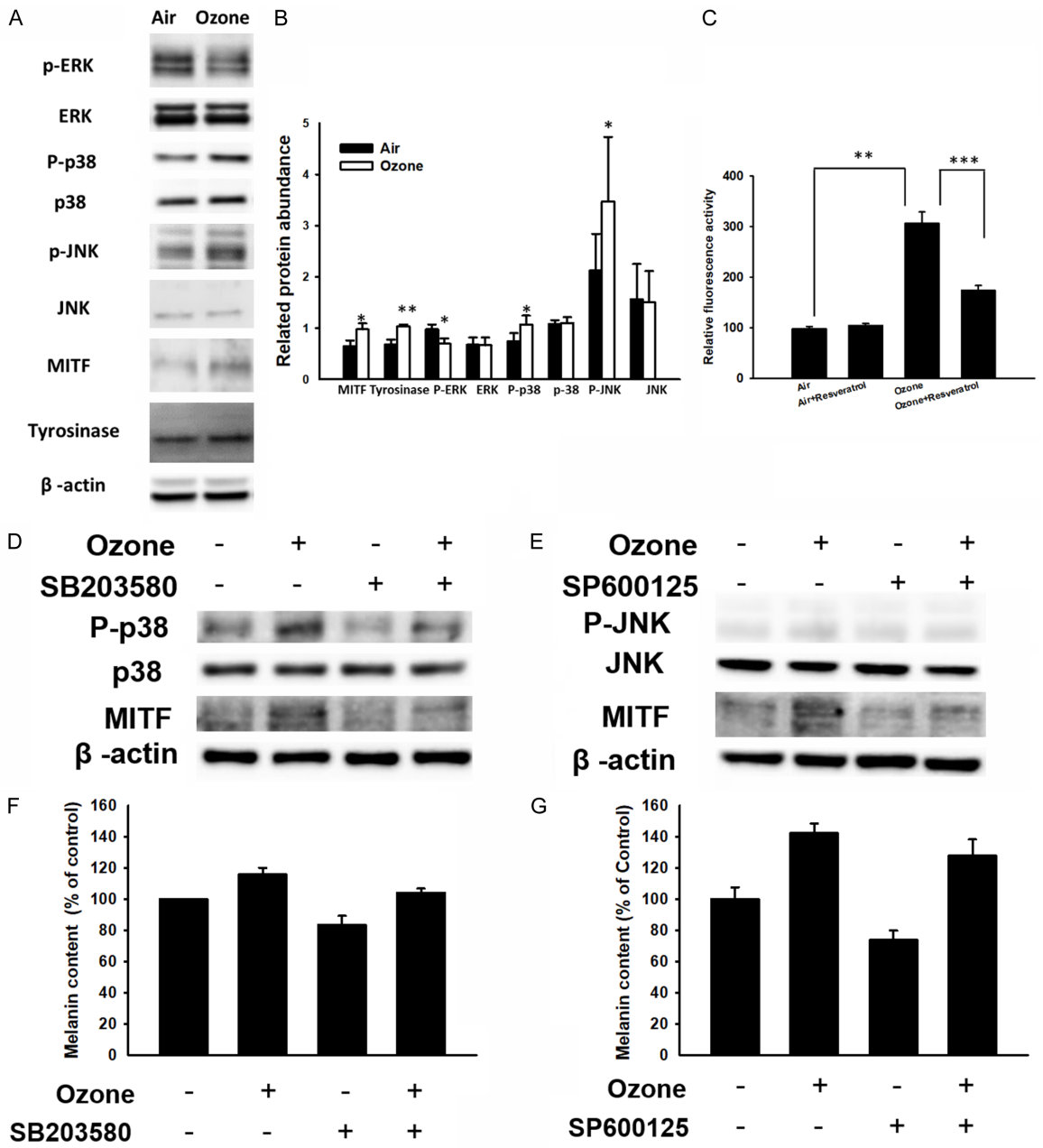

Figure 2. Expression levels of tyrosinase and MITF after ozone treatment. B16F10 cells were treated with ozone at 1 ppm for 3 consecutive days (3 hours per day). A. Protein expression was determined by immunoblotting. B. β-actin expression served as the quantitative control (*, $P < 0.05$; **, $P < 0.01$). Each experiment was repeated three times with similar results (mean ± SD, n = 3). C. ROS levels in B16F10 cells treated with air or ozone for three days, with or without resveratrol (20 μM). The assay was analyzed using the Chekine™ reactive oxygen species detection fluorometric assay. Protein expression levels after p38/JNK inhibitor treatment. B16F10 cells were pretreated with SB203580 (p38 inhibitor, 25 μM) or SP600125 (JNK inhibitor, 10 μM) for 2 hours before ozone exposure. D, E. Protein expression was assessed by immunoblotting. F, G. Effect of ozone on B16F10 melanin content (***, $P < 0.001$). Each experiment was repeated three times, yielding similar results (mean ± SD, n = 3).

2C). Ozone exposure was associated with activation of MAPK signaling pathways that correlate with increased melanogenic protein expression. The present results show that ozone-induced melanogenesis is associated with increased phosphorylation of p38/JNK and decreased phosphorylation of ERK. Additional inhibitor-based rescue experiments confirmed

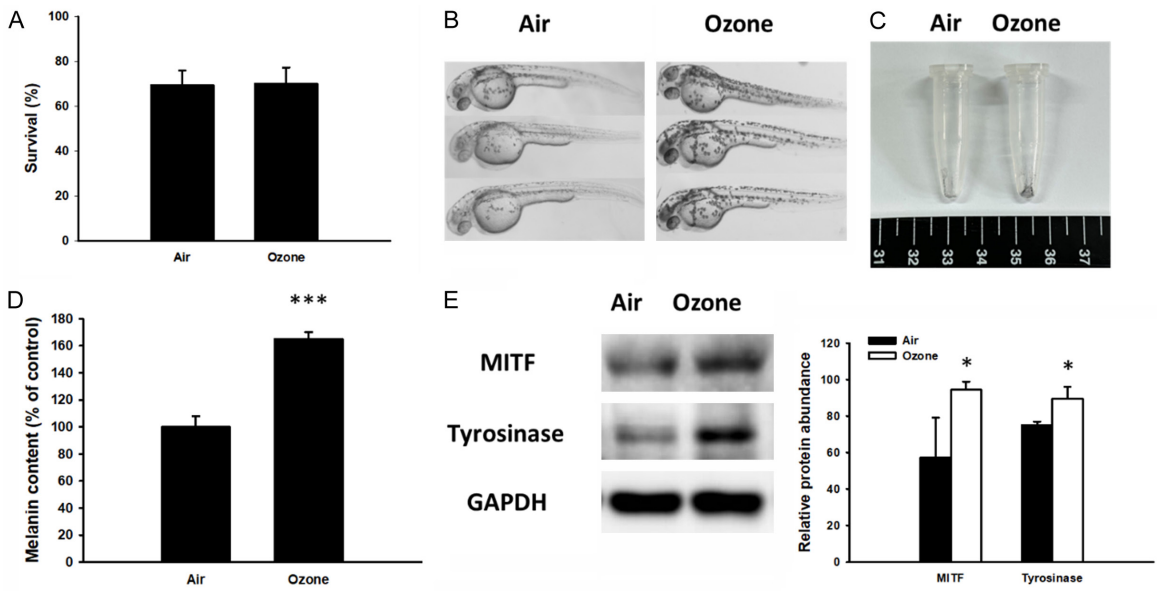

Figure 3. Effects of ozone on zebrafish pigmentation. Synchronized embryos were exposed to ozone at 1 ppm for 3 consecutive days (3 hours per day). A. Zebrafish viability was measured. B. Image of ozone-treated zebrafish. C. Photograph of precipitated zebrafish. D. Effect of ozone on zebrafish melanin content. E. Protein expression after ozone (1 ppm) treatment was assessed by immunoblotting in zebrafish. GAPDH expression served as the quantitative control (*, $P < 0.05$) (mean ± SD, n = 3). Each experiment was repeated three times with similar results.

the causal contributions of the individual p38/JNK phosphorylation pathways (**Figure 2D** and **2E**) and of melanin production (**Figure 2F** and **2G**).

In vivo evidence of ozone-induced pigmentation in zebrafish

Zebrafish were used to evaluate the *in vivo* pigmentation response to ozone. Embryos treated with 1 ppm ozone (3 hours/day for 3 days) showed no signs of toxicity (**Figure 3A**). However, prolonged exposure increased pigmentation in developing larvae (**Figure 3B**). Quantitative and photographic evidence showed increased melanin content in adult zebrafish after ozone treatment (**Figure 3C** and **3D**). Protein expression analysis revealed significant upregulation of MITF and tyrosinase in ozone-exposed zebrafish (**Figure 3E**), confirming that ozone enhances pigmentation *in vivo*.

Resveratrol counteracts ozone-induced pigment induction

To examine whether resveratrol can suppress ozone-stimulated pigmentation, B16F10 cells were co-treated with resveratrol. As illustrated in **Figure 4A**, resveratrol reduced MITF and tyrosinase expression in a concentration-dependent manner, with marked suppression at 20 μM. Additionally, it diminished phosphorylation of p38 and JNK while increasing phospho-ERK levels. These effects were associated with decreased melanin accumulation, as confirmed by image analysis and pigment quantification (**Figure 4B** and **4C**), indicating the potent anti-melanogenic activity of resveratrol.

Resveratrol suppresses ozone-activated melanogenic signaling

To confirm that these effects depended on ROS, we added 20 μM resveratrol, a known ROS scavenger (**Figure 2C**), to counteract ozone-generated ROS. To further characterize resveratrol's inhibitory effects, we evaluated levels of melanogenic proteins and MAPK signaling under combined treatment. Resveratrol reversed ozone-induced upregulation of MITF and tyrosinase and decreased p-p38 and p-JNK levels (**Figure 5A**, **5B**), while upregulating phospho-ERK. These molecular changes coincided with a reduction in melanin content (**Figure 5C**). The protective effects were validated in zebrafish, where resveratrol reduced ozone-induced melanogenesis (**Figure 6**), confirming its efficacy in vivo.

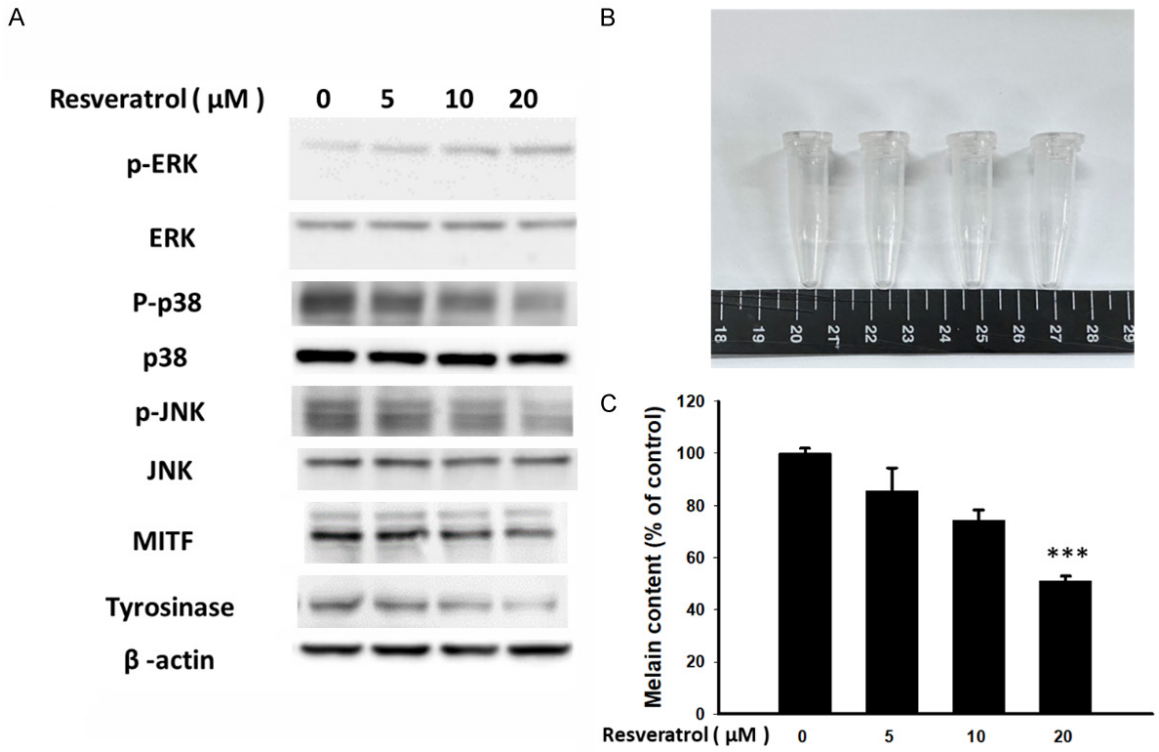

Figure 4. Protein expression levels after resveratrol treatment. B16F10 cells were treated with resveratrol (0-20 μM) for 2 hours. A. Protein expression was assessed by immunoblotting. B. Effect of resveratrol on cellular melanin content. C. Effect of ozone on B16F10 melanin content (***, $P < 0.001$). Each experiment was repeated three times, yielding similar results (mean ± SD, n = 3).

Discussion

Air pollution has long been recognized as a major public health concern, particularly in industrialized countries since the Industrial Revolution. Air pollutants arise from urban development, vehicle emissions, and municipal waste processing. Both short- and long-term exposure to these pollutants has been linked to numerous health problems, including stroke, chronic obstructive pulmonary disease (COPD), heart conditions, tracheal inflammation, and even lung cancer [11]. Beyond systemic effects, growing evidence indicates that air pollutants can significantly affect human skin health. Pollutants such as PM, ozone, and polycyclic aromatic hydrocarbons (PAHs) can penetrate the skin barrier, trigger oxidative stress, accelerate skin aging, worsen inflammatory skin conditions such as eczema and acne, and may lead to skin cancer [12]. Because the skin serves as the first line of defense, this study aims to investigate whether ozone can activate melanogenesis as a biological response to environmental stress, thereby helping protect the skin [13]. As a reactive air pollutant, ozone may disrupt normal cellular signaling and induce melanogenesis, potentially leading to abnormal skin pigmentation. Elucidating the signaling mechanisms involved is critical for developing preventive measures against ozone-related pigmentary damage.

Taiwan's Environmental Protection Agency (EPA) uses a more lenient definition of pollution, classifying it as "unhealthy for sensitive groups" only when the average level exceeds 0.125 ppm over eight hours. Not only is the indicator's response slower, but the permissible value is 1.7 times higher than in the United States. Therefore, we estimated Taiwan's hazardous level as exceeding 0.55 ppm over eight hours. For this study, we used a daily maximum ozone exposure of 1 ppm for 3 hours, which is lower than the Taiwan EPA's hazardous ozone pollution level.

Melanogenesis is a complex biochemical process controlled by melanocytes in the basal layer of the epidermis. It serves as a defense

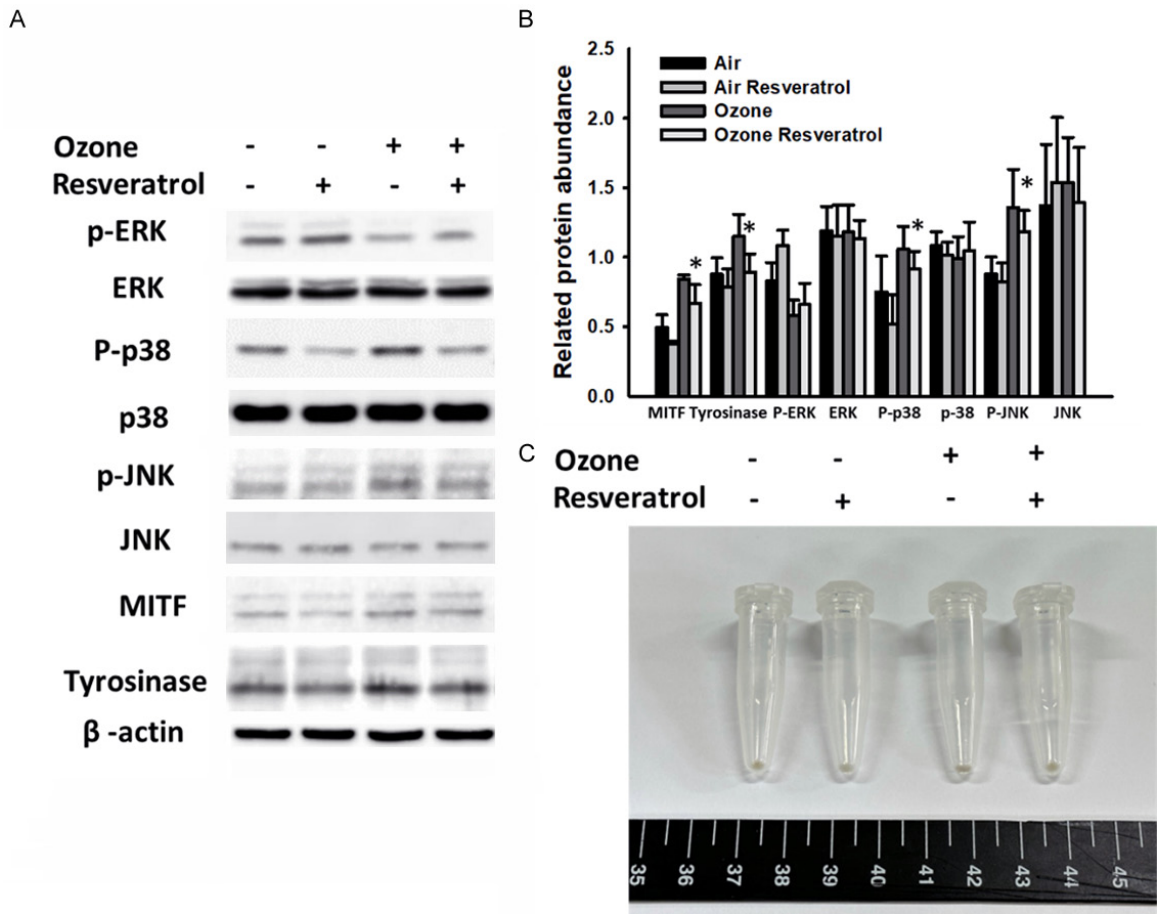

Figure 5. Protein expression levels after ozone or resveratrol treatment. B16F10 cells were treated with resveratrol (0-20 μM) for 2 hours. The cells were exposed to ozone at 1 ppm for 3 consecutive days (3 hours per day). A. Protein expression was determined by immunoblotting. B. β-actin expression served as the quantitative control (*, $P < 0.05$). Each experiment was repeated three times with similar results (mean ± SD, n = 3). C. Photograph of precipitated B16F10 melanoma cells treated with ozone or resveratrol.

against ultraviolet (UV) radiation by absorbing harmful rays and preventing UV-induced DNA damage. In addition to its protective role, melanin contributes to the pigmentation of skin, hair, and eyes. Several intrinsic and extrinsic factors influence melanin production. These include UV exposure, hormonal changes during pregnancy, inflammation from acne or skin injuries, certain medications, and age-related factors, all of which affect the regulation and distribution of melanin. Among these, chronic environmental exposures are increasingly recognized as possible drivers of pigmentary disorders [14].

Recent studies have shown that delicate particulate matter can increase melanin production by activating inflammatory and oxidative stress pathways [14]. However, despite ozone being a major reactive component of urban air pollution, its direct effects on melanogenesis have not been thoroughly studied. This creates a significant gap in understanding the skin-related impact of air pollutants.

In this context, the current study provides essential insights into how ozone may influence melanin biosynthesis by modulating key intracellular signaling pathways. Specifically, the MAPK signaling cascade, including p38, ERK, and JNK, regulates melanogenic responses, likely by modulating MITF, a central regulator of pigment gene expression. Because these kinases are involved in inflammation, stress responses, and cell survival, their activation during ozone exposure suggests that melanogenesis may serve as a stress-adaptive mechanism in response to oxidative damage.

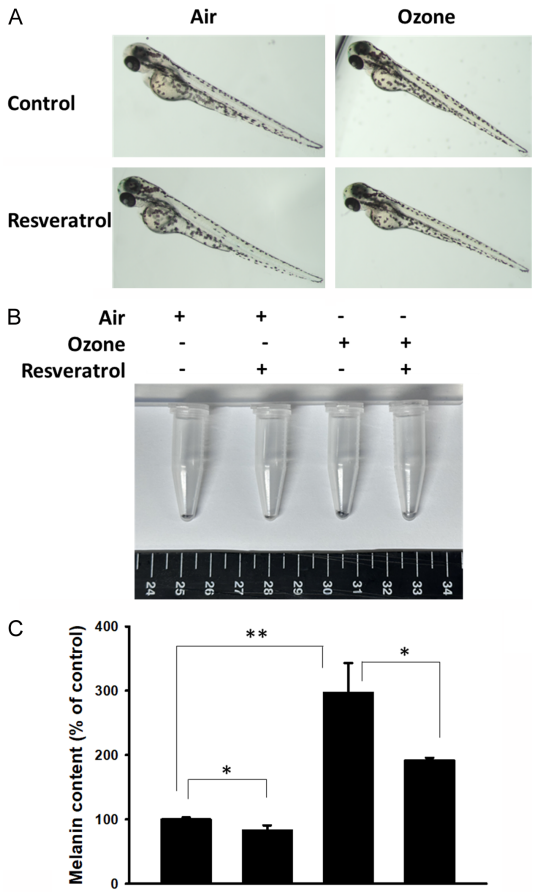

Figure 6. Effects of ozone on zebrafish pigmentation after resveratrol treatment. Synchronized embryos were pre-treated with resveratrol (20 μM) for 2 hours. Subsequently, the embryos were exposed to 1 ppm ozone for 3 consecutive days (3 hours per day). A. Image of zebrafish treated with ozone or resveratrol. Synchronized embryos were treated with ozone. B. Effect of ozone on zebrafish melanin content after resveratrol treatment. C. Effect of ozone on zebrafish melanin content after resveratrol treatment. Synchronized embryos were pre-treated with resveratrol (20 μM) for 2 hours. Subsequently, the embryos were exposed to 1 ppm ozone for 3 consecutive days (3 hours per day). *, $P < 0.05$; **, $P < 0.01$ (mean ± SD, n = 6). Each experiment was repeated three times with similar results.

Significantly, because the skin is the body's primary defense barrier, ongoing exposure to high ambient ozone levels, especially in urban areas such as Taiwan, raises public health concerns. The findings of this study underscore the need to further investigate pollutant-induced changes in signaling and to explore potential interventions to prevent ozone-related pigmentation. Future research should also examine whether ozone-driven melanogenesis contributes to long-term skin damage or pigmentation in exposed populations.

This study provides new insights into how ozone exposure affects melanogenesis, highlighting the skin as a key target of environmental oxidative stress. Our results show that ozone exposure significantly increases melanin content in both in vitro (B16F10 cells) and in vivo (zebrafish) models. At the molecular level, this increase in melanogenesis was associated with upregulation of key melanogenic regulators, including MITF and tyrosinase, supporting the hypothesis that ozone may promote melanogenesis via specific signaling pathways.

Importantly, we found that exposure to 1 ppm ozone strongly activated MAPK signaling pathways in B16F10 cells, including phosphorylation of p38 and JNK and downregulation of ERK phosphorylation. These kinases are well-known upstream regulators of MITF transcriptional activity and are widely linked to pigment regulation under UV or stress conditions. The increase in MITF expression after ozone exposure supports the hypothesis that MAPKs influence melanogenesis, suggesting that this process is likely mediated through a common pathway. Additionally, resveratrol treatment effectively reduced phosphorylation of p38 and JNK, while the subsequent increase in ERK phosphorylation suppressed MITF and tyrosinase expression in a dose-dependent manner. These results suggest that resveratrol may be a useful strategy for reducing pollutant-induced pigmentation. Sunscreen lotions containing resveratrol may reduce air-pollutant-induced melanin production and could be a promising product.

The purpose of using 1 ppm ozone was to induce measurable oxidative stress and early melanogenic signaling responses within an experimentally feasible time frame. Recently, we used the same exposure system, and proliferating cell nuclear antigen (PCNA) was measured to assess whether ozone exposure affects proliferative activity under identical conditions. Exposure to 1 ppm ozone for three hours daily can induce profound changes, but exposure over three consecutive days produces more noticeable and detectable physiological responses [15]. Ozone may first interact with cell membrane lipids, generating lipid per-

oxidation products that subsequently activate intracellular signaling pathways. Ozone is unlikely to act only through direct intracellular ROS generation. Because ozone is highly reactive, it may initially interact with membrane lipids, producing lipid peroxidation-derived mediators, such as 4-hydroxynonenal or malondialdehyde, which can subsequently activate stress-related signaling pathways. In addition, pollutant-responsive receptors, such as the aryl hydrocarbon receptor (AhR), may help link environmental exposure to melanogenic signaling. Resveratrol, a natural AhR antagonist [16], also counteracted ozone-induced melanogenesis. Resveratrol might inhibit AhR signaling pathways induced by ozone. In summary, our findings establish that the protective effects of resveratrol against melanogenesis are mediated by ozone, which markedly inhibits AhR activity. N-acetylcysteine (NAC) was the most effective at reducing melanin content and inhibiting tyrosinase activity. To determine whether ROS are associated with ERK activation leading to MITF degradation, ERK phosphorylation, and MITF protein levels were measured in rotenone-treated cells with or without N-acetylcysteine (NAC). Rotenone-mediated ERK activation was inhibited by NAC co-treatment [17]. The results above indicate that the signal transduction pathway by which NAC inhibits melanin is different from that of resveratrol, although both are considered antioxidants.

Although research links $PM_{2.5}$ to skin hyperpigmentation, few studies have examined ozone's role in this context. Our findings expand this line of research by identifying ozone as a potential factor that promotes melanin production. Given rising outdoor ozone levels in places such as Taiwan, our data raise essential concerns about the skin-related effects of long-term ozone exposure, particularly for individuals with high levels of outdoor activity or occupational exposure. These findings underscore the importance of understanding the molecular mechanisms underlying pollutant-induced skin pigmentation. Further research using human skin models and clinical studies is needed to assess long-term effects and develop effective protective strategies. Future studies should also examine the combined effects of multiple air pollutants on pigmentation and other skin-related outcomes.

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Disclosure of conflict of interest

None.

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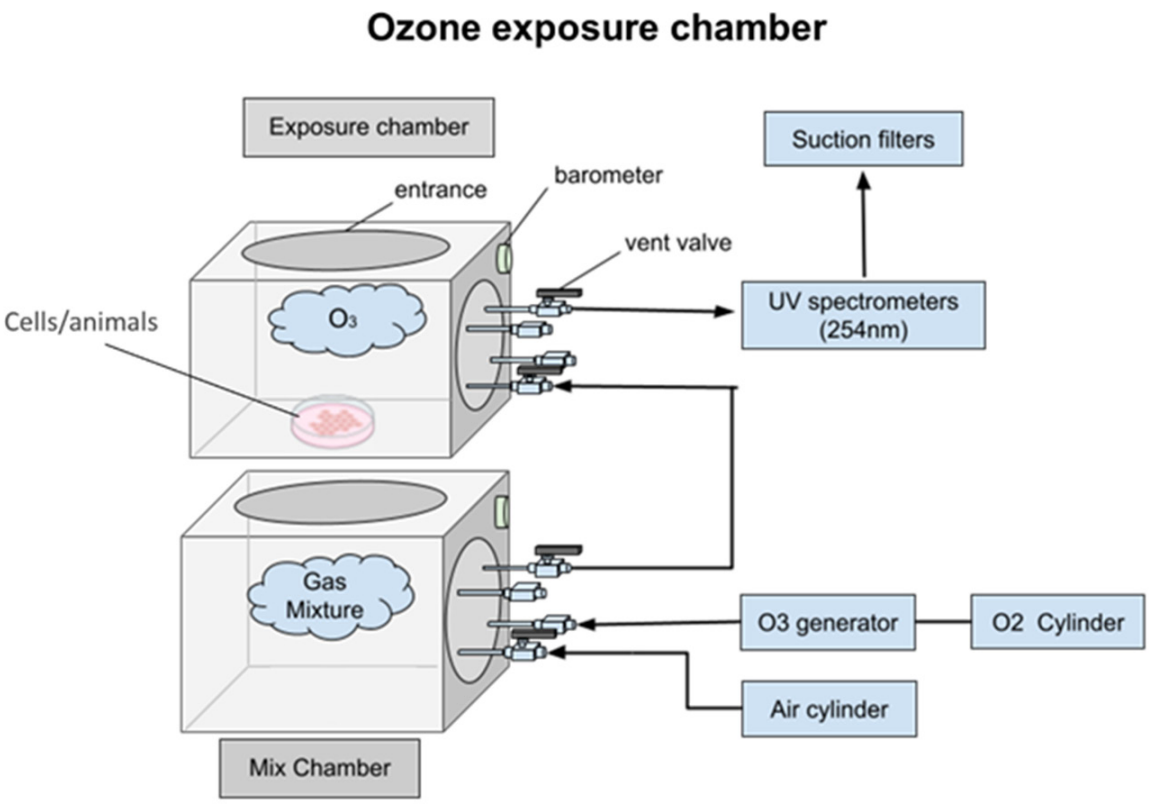

Figure S1. Schematic diagram of the ozone exposure system. A sealed acrylic exposure chamber was constructed to simulate environmental ozone exposure. Ozone was generated by an ozone generator and delivered into the chamber, while excess air was vented through an exhaust tubing system connected to the outside. An ozone detector was installed inside the chamber to continuously monitor and maintain the target ozone concentration at 1 ppm. Cells or zebrafish embryos were placed inside the chamber and exposed to ozone for 3 hours per day for three consecutive days. Control groups were exposed to ambient air under identical conditions.