Particulate matter 2.5 induced hyperpigmentation in reconstructed human epidermis model (MelaKutis®)

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To the Editor: Epidemiological studies have confirmed that fine particulate matter (PM2.5) is related to skin hyperpigmentation, but experimental evidence is lacking.[1] Here, we used normal human epidermal keratinocytes (NHEKs), melanocytes (NHEMs), and reconstructed human epidermis models (MelaKutis®) to investigate the impact of PM2.5 on skin melanogenesis. MelaKutis® is a 3D skin model with a stratified structure, which is composed of human keratinocytes and melanocytes. Its structure and metabolic features are highly similar to those of the natural human skin. Moreover, external factors can stimulate the melanocytes located in the basal layer to produce melanin, resulting in hyperpigmentation. In our study, to simulate the effect of PM2.5 on human skin, we utilized PM2.5 collected in Beijing, China. Zhang *et al*[2] previously analyzed the components of PM2.5 (collected in Beijing) and revealed that benzo[a]pyrene (BAP) was one of the most abundant and toxic components of PM2.5. Thus, we chose BAP as positive control.

PM2.5 samples were collected continuously for 24 h in haze days from November 2018 to March 2019. An HY-1000 intelligent large-flow TSP sampler (optional PM2.5 cutter, Qingdao Hengyuan Technology Development Co., Ltd.) was employed for quartz filter sampling at an average flow rate set at 1000 L/min. Then, the samples were immersed in 75% ethanol, followed by ultrasonically shaking for 60 min in a water bath for particle elution. Sterile water was used to prepare a high concentration stock solution, which was then stored at −20°C. BAP was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). NHEKs, NHEMs, and MelaKutis® were manufactured by Biocell Biotechnology (Dongguan, Guangdong, China). The other chemicals were of reagent grade. PM2.5 and BAP were diluted to different concentrations using certain cell culture media (KC2500, Medium 254 or M-TA medium). The effects of PM2.5 on the viability of NHEKs and NHEMs were determined by MTT assay. NHEKs were cultured in KC2500 (Guangdong Biocell Biotechnology), and NHEMs in Medium 254 (Gibco, Grand Island, NY, USA). Cells were incubated with various concentrations of PM2.5 (3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00, and 400.00 μg/mL) or BAP (0.50, 1.00, 2.50, 3.50, 5.00, 10.00, 20.00, and 50.00 μmol/L) at 37 °C in 5% CO_2 for 24 h. Then, we treated the cells with MTT dye for another 4 h in dark and recorded the absorbance (optical density) at 490 nm using a Spectrophotometer (BioTek, Winooski, VT, USA). To observe the effects of PM2.5 and BAP on cell morphology, NHEKs were treated with different concentrations of PM2.5 (3.13, 6.25, 12.50, 25.00, and 50.00 μg/mL) or BAP (1.00, 2.50, 3.50, 5.00, and 10.00 μmol/L). Next, 24 h later, the cell morphology was observed at 200× magnifications under an inverted microscope (Olympus Corporation, Tokyo, Japan). All the cell experiments above contain three repeat groups ($n = 3$). MelaKutis® were maintained in M-TA medium (Biocell Biotechnology, China) at 37 °C in 5% CO_2, according to the manufacturer's instructions. Further, we added 10 μL of different concentrations of PM2.5 (7.50 and 12.50 μg/mL) or BAP (3.00 and 5.00 μmol/L) to each model on the surface and changed the medium. Further, 24 h later (we defined this as Day 1), before repeating the drug administration as on Day 0, we wiped the sample residue gently using sterilized cotton swab. On Days 2 to 6, the procedures of Day 0 and Day 1 were repeated. On Day 7, all models were collected and subjected to appearance observation, apparent brightness (L *value), melanin content, melanin distribution, and tissue morphology (Hematoxylin-Eosin staining [H&E]) analysis. We divided the six experimental groups (each group contained six repeat models, $n = 6$) randomly into group A and group B, each of which contained $6 \times 3 = 18$ models (each group had three repeat models, $n = 3$). Group A: MelaKutis® images were subjected to appearance observation, and then harvested the models for immunohistochemistry and histology examinations. After paraffin embedding, 5 to 8-μm thick tissue blocks were sectioned, which were stained with Fontana-Masson and H&E staining to observe their

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melanin distribution and tissue morphology. Pictures of the slides were taken under an upright microscope (Olympus Corporation, Japan, 400×). Group B: The L* value was measured by a Colorimeter (DSM II, Denmark). Then, we transferred the models into 1.5-mL centrifuge tubes, followed by centrifugation (2000 r/min for 10 min). Next, we discarded the medium, washed the pellets with 1 mL of 1× phosphate buffered solution, and centrifuged again (3000 r/min for 5 min). Further, we dissolved the sediments in 1 mL of 1 mol/L NaOH containing 10%Dimethyl sulfoxide at 80 °C for 40 min. The melanin content in each sample was determined by measuring the absorbance at 405 nm. Each specimen was read three times. SPSS 24.0 was used for statistical analysis, and one-way analysis of variance was applied to analyze the results. $P < 0.05$ was considered to indicate a statistically significant difference.

Based on the cell viability of the PM2.5-treated/BAP-treated NHEKs and the cell viability curves we drew [Supplementary Figure 1, http://links.lww.com/CM9/A873], we found that the lower concentrations of PM2.5 (≤12.50 μg/mL) or BAP (≤5.00 μmol/L) did not cause a significant decrease in cell viability or cell morphology change. The change of cell viability of NHEMs was similar to that of NHEKs. Therefore, we defined PM2.5 = 12.50 μg/mL/BAP = 5.00 μmol/L as the maximum safe concentrations (C_{max}) of NHEKs and NHEMs. We selected PM2.5 (7.50 and 12.50 μg/mL) and BAP (3.00 and 5.00 μmol/L) for subsequent studies. Continuous stimulation on MelaKutis® with PM2.5/BAP for 7 days caused no obvious abnormality in the tissue morphology. Compared with the control groups, the 12.50 μg/mL PM2.5-treated MelaKutis® and 5.00 μmol/L BAP-treated MelaKutis® became darker, and melanin particles increased mainly in the lower parts of the sections. Furthermore, fissures were observed in the 5.00-μmol/L-BAP group [Figure 1]. The L* values of MelaKutis® treated by both 12.50 μg/mL PM2.5 and 5.00 μmol/L BAP decreased ($P = 0.000$, $P = 0.000$), whereas the changes in the 7.50 μg/mL PM2.5 and 3.00 μmol/L BAP groups were not statistically significant ($P = 1.000$, $P = 1.000$). Meanwhile, the melanin contents of both the 12.50 μg/mL PM2.5 and 5.00 μmol/L BAP groups increased ($P = 0.001$, $P = 0.047$), but the changes in the 7.50 μg/mL PM2.5 group and 3.00 μmol/L BAP group were not statistically significant ($P = 0.948$, $P = 1.000$) [Supplementary Table 1, http://links.lww.com/CM9/A873].

In our study, we found that lower concentrations of PM2.5 (≤12.50 μg/mL) did not cause a significant decrease in the cell viability or a change in the cell morphology of keratinocytes/melanocytes, whereas higher doses led to cell death and cell deformation in a dose-dependent manner. Since this was a qualitative experiment, the selection criteria for PM2.5/BAP concentrations were not so strict. However, at selected concentrations that were too close to C_{max}, the results were not considered representative. Thus, we chose 7.50 μg/mL of PM2.5 and 3.00 μmol/L of BAP for the subsequent experiments, whose cytotoxicity was negligible. Our results showed that 12.50 μg/mL PM2.5 induced hyperpigmentation in the reconstructed human epidermis model, suggesting that low concentrations of PM2.5 may lead to skin hyperpigmentation. Since no significant change in the cell numbers of the keratinocytes or melanocytes was observed in the cell or MelaKutis® experiments, we speculated that PM2.5 induced hyperpigmentation mainly by increasing the melanin synthesis. Clear corneum, the strongest barrier against environmental stressors, including PM, was observed. Therefore, little possibility exists for PM2.5 to enter the epidermis and directly contact with melanocytes to cause melanogenesis. We preferred to explain our results using the scheme PM2.5 → keratinocyte → melanocyte → increased melanin production. The epidermal-melanin unit was composed of keratinocytes and melanocytes, which is essential to melanogenesis. This unit responded rapidly to a variety of external stimuli through paracrine and autocrine pathways leading to melanin production.[3-5] A large number of studies have been conducted on the impact of environmental PM on keratinocytes, including polycyclic aromatic hydrocarbons (PAHs) and PM2.5. The existing evidence includes the activation of the aryl hydrocarbon receptor (AhR) signaling pathway, oxidative stress, and the induction of inflammatory cascade.[1,5] As an external sensor, AhR is expressed in all skin cell types and has been found to induce the expression of several target genes after binding to its ligands (such as PAHs). One of the target genes, cytochrome P450 family enzyme, metabolized PAHs, and the formed metabolites induced reactive

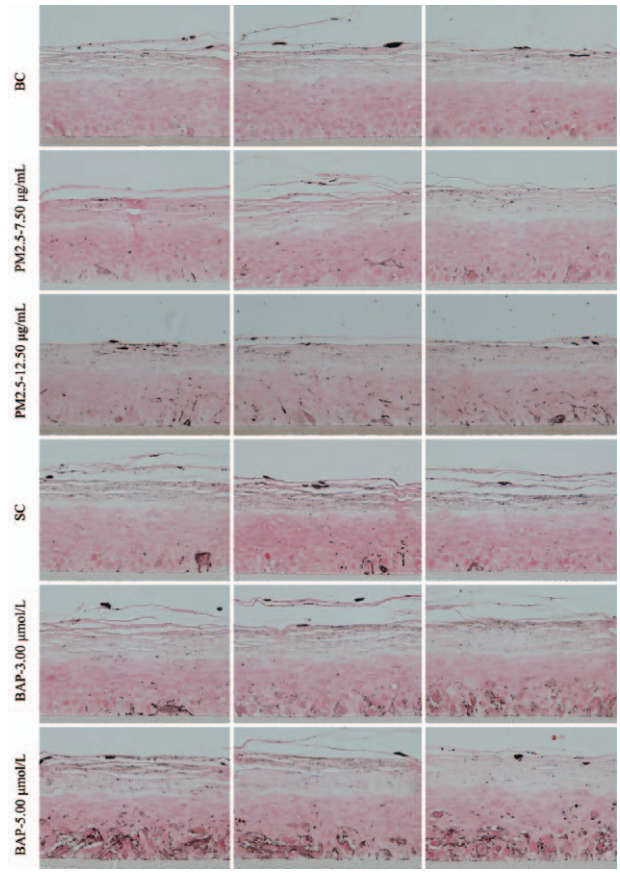

Figure 1: Sections showing the melanin distribution. The number of melanin particles of PM2.5-/BAP-treated MelaKutis® increased, mainly in the lower part of the slices. Fissures were observed in the 5.00 μmol/L-BAP group. (BC: blank control, SC: solvent control, and SC:BC + 0.01% DMSO, $n = 3$). BAP: Benzo[a]pyrene; DMSO: Dimethyl sulfoxide; PM2.5: Particulate matter 2.5.

oxygen species (ROS) production.[5] PM2.5 also triggered the production of ROS by keratinocytes through various pathways, which promoted higher melanin production by melanocytes.[1,5,6] Moreover, PM exposure induced keratinocytes to produce a series of inflammatory factors, such as melanocyte stimulating hormone (α-MSH), Interleukin (IL)-1α, IL-1β, IL-6, IL-8, Matrix Metalloproteinase (MMP)-1, MMP-2, MMP-9, and Tumor necrosis factor-α,[5,6] which are considered to be at least partly related to ROS.[5] α-MSH was established as one of the main paracrine cytokines secreted by keratinocytes that contributed to melanogenesis.[6] IL-1 promoted the secretion of endothelin-1 in keratinocytes, enhancing the migration and differentiation of melanocytes.[3,4] We speculate that PM2.5 could directly act on melanocytes in people with damaged skin barrier, inducing melanin production. Some metals (such as Fe) in PM2.5[1] could combine with sulfhydryl groups in epidermal cells and enhance the activity of tyrosinase.[3] In addition to melanocytes and keratinocytes, the interactions between other cells in the human skin should also be considered.[1,3]

We also found that the increased melanin was mainly distributed in the lower part of the slices, which was consistent with the metabolism of melanin.[3] MelaKutis® successfully simulated the process of melanogenesis: the melanosomes were converted into melanocytes, and then they were transmitted to the surrounding keratinocytes by endocytosis/exocytosis. In the process of epidermal replacement, the melanosomes moved up with keratinocytes and were gradually digested and absorbed.[3,4] In our investigation, the function of melanocytes was promoted by external stimuli, leading to an increased ability for synthesis and transportation of melanosomes.[3] Our experimental duration was only seven days, whereas the turnover time was 28 days. This may explain why melanin was distributed mainly in the lower part of the slices.

Furthermore, we found fissures in the 5.00 μmol/L-BAP group, which might have been related to the inflammatory response and the increased dendritic degree of the melanocytes. An earlier study confirmed that PM damaged the skin barrier by adjusting or even destroying the tight junction of the epithelial cells.[5] As mentioned above, PM2.5 stimulated the keratinocytes to produce a variety of inflammatory factors. Some of these cytokines further promoted the secretion of cytokines and adhesion molecules such as IL-8 and IL-1 by epithelial cells, fibroblasts, and endothelial cells.[7] All these cell types can induce skin inflammation, cause barrier disruption, and increase the damage of PM2.5 to the skin. Interestingly, a previous investigation established that the acantholysis of pemphigus vulgaris (PV) was strongly associated with MMPs, especially MMP-9.[8] The presence of PV was similar to that of the 5.00 μmol/L-BAP group. As MMPs showed wide proteolytic activities and overlapping specificities, and PM2.5 upregulated the levels of MMP-1, MMP-2, and MMP-9 in cultured keratinocytes,[5] we speculated that MMPs were associated with the fissures in the sections.

In summary, we propose that low concentrations of PM2.5 may cause skin hyperpigmentation. To the best of our knowledge, this is the first study to investigate the effect of PM2.5 on melanogenesis, in which a 3D epidermis model has been applied. Further *in vitro* and *in vivo* studies are warranted to clarify the mechanism involved.

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