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ORIGINAL ARTICLE

Particulate Matters Induce Apoptosis in Human Hair Follicular Keratinocytes

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Background: Particulate matters (PM) comprise a heterogeneous mixture of particles suspended in air. A recent study found that urban PMs may penetrate into hair follicles via transfollicular and transdermal routes in dorsal skin. **Objective:** To investigate the effects of PM on *ex vivo* cultured human scalp hair follicles and hair follicular keratinocytes *in vitro*. **Methods:** TUNEL staining was employed to check cells undergoing apoptosis in cultured hair follicles after PM treatment. MTT assay was employed to check cell viability after PM treatment. Quantitative real-time PCR analysis was employed to quantitate the expression of inflammatory genes, matrix metalloproteinases (MMPs), and Duox1. Inflammatory cytokine levels were measured by ELISA after PM treatment. The level of reactive oxygen species (ROS) production was measured using a chemical fluorescent probe by a fluorescence plate reader. **Results:** Abundant TUNEL-positive cells were observed in the keratinocyte region of hair including the epidermis, sebaceous gland, outer root sheath (ORS), inner root sheath (IRS), and bulb region. The viability of follicular cells, including the ORS, was found to be decreased upon PM exposure. mRNA expression and protein levels of inflammatory response genes and MMPs were upregulated in a dose-dependent manner by PM treatment. ROS levels were also increased by PM. **Conclusion:** These data strongly suggest that penetrated PMs from air pollution may cause apoptotic cell death to follicular keratinocytes by increased production of ROS and inflammatory cytokines, which could impair hair growth. **(Ann Dermatol 32(5) 388~394, 2020)**

-Keywords-
Apoptosis, Hair keratinocyte, Hair loss, Particulate matter, Reactive oxygen species

INTRODUCTION

Air born particulate matters (PMs) comprise a mixture of air particles composed of metals, organic compounds, biologically-originating ions, and a carbon particle core that varies in size. These chemical constituents are produced by various natural and industrial resources[1]. Concern regarding the health effects of urban air pollution has increased with the rapid industrialization of the modern era, and numerous epidemiologic studies have indicated that exposure to PM is associated with increased risk of diverse diseases such as cancers, pulmonary, respiratory, and cardiovascular diseases, as well as skin diseases[2-4]. Skin is the largest organ of human body and the outermost barrier between the environment and the body that provide powerful defensive capacity. Hair is a unique skin appendage that contains dermal cells and epidermal keratinocytes, which undergoes a sequential cycle of anagen (the active growth phase), catagen (the apoptotic regression phase), and telogen (the resting phase)[5].

Various studies have been performed from multiple centers to investigate the impacts and consequences of urban pollution on skin in areas of Mexico and Shanghai[6,7].

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These analyses demonstrated that urban pollution negatively impacted skin quality, and they identified biochemical parameters that caused changes in sebum secretion and the cutaneous structure. Moreover, Galliano et al.[8] used the deposit and adherence test of PM to explore the impact of urban pollution on the human hair surface, and Naudin et al.[9] suggested that polycyclic aromatic hydrocarbons, which co-exist with PMs in air pollutants, accelerate the ultrastructural degradation of hair fibers. These studies showed that pollution affects hair surface properties and alters hair quality *in vitro* and real-life condition. The signaling pathways related to skin barrier dysfunction upon exposure to PMs (standard reference material [SRM] 1649b) were explored *in vivo* and *in vitro* by Lee et al[10].
A recent study by Jin et al.[11] observed inflammation and detrimental effects upon PM penetration into barrier-disrupted skin *in vivo* via stimulation of inflammatory mediator and reactive oxygen species (ROS) production. In addition, a recent review suggested that systemic and direct dermal exposure and uptake of PMs can occur in specific populations[12]. These findings suggest that penetrated PMs might alert the hair growth mechanism. However there is no clear evidence of the effects of PM exposure on scalp skin and hair follicles, although recent studies have provided evidence that environmental cigarette smoke from air pollution is closely related with androgenetic alopecia[13]. Therefore, we explored the effects of PM on human hair using ex *vivo* organ culture and primary cultured human hair follicular cells.

MATERIALS AND METHODS

Cell culture

Hair biopsy specimens were obtained from the non-balding occipital scalp region of patients with androgenic alopecia during hair transplantation at Kyungpook National University Hospital (Daegu, Korea) with the patients' written informed consent. The Medical Ethical Committee of the Kyungpook National University Hospital approved all of the study protocol (IRB No. KNUH 2013-02-001-007). Hair follicles were isolated and cultured by the method described previously[14]. Dermal papilla (DP), dermal fibroblast (DF), dermal sheath (DS), and outer root sheath (ORS) cells were isolated from dissected anagen hair follicles and cultured as described previously[15]. Briefly, isolated primary human hair cells were cultured in keratinocyte growth medium for ORS and in DMEM low-glucose medium for DP, DS, and DF (Clonetics Inc., San Diego, CA, USA). The cells were maintained in a humidified incubator at 37°C under 5% CO_2. We used primary cultured cells at passage 2 to 3 in this study.

All data were analyzed using Prism 6 for Windows Version 6.01 (GraphPad Software Inc., San Diego, CA, USA). *p*-values of less than 0.05 were considered significant.

PM10 preparation and treatment

For the *in vitro* study, PM10 (PM10-like, European reference material ERM-CZ120) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml and diluted to a range of concentrations with the basal medium of each cell type. The PM10 suspension was then sonicated for 30 minutes to prevent aggregation. Experiments were conducted within 1 hour of PM preparation to avoid variability in PM components at different concentrations between replicates.

Cell viability test

Cells were incubated with PM at the indicated concentration for 24 hours. Cell viability was assayed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) according to the manufacturer's protocol. Viability was compared with the value of the non-PM-treated control.

Quantitative real-time PCR

Total RNA was extracted from cultured primary human DP and ORS cells using RNAiso Plus (Takara Bio Inc., Shiga, Japan). Three micrograms of total RNA was used to synthesize cDNA using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Gene-specific forward and reverse primers were used in each well of a 96-well plate for quantitative real time-PCR (qPCR) using a Step OnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Takara Bio Inc.) according to the manufacturer's instructions. The PCR conditions were 95°C for 2 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. qPCR primer information is shown in Supplementary Table 1. The data were calculated using the comparative Ct method ($\Delta\Delta$Ct method) for to normalization.

ELISA

The protein levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, IL-8, and IL-6 were determined by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, DP and ORS cells were seeded in 6-well plates (1.2×10^6 cells/well). After 24 hours starvation in serum-free media, cells were exposed to different concentrations of PM10 suspension for the indicated time. Then, the culture supernatant was collected at 4°C, centrifuged for 20 minutes at $1,000\times g$ at 4°C, and stored at −20°C for subsequent assays. Serial dilutions of recombinant human proteins were used to es-

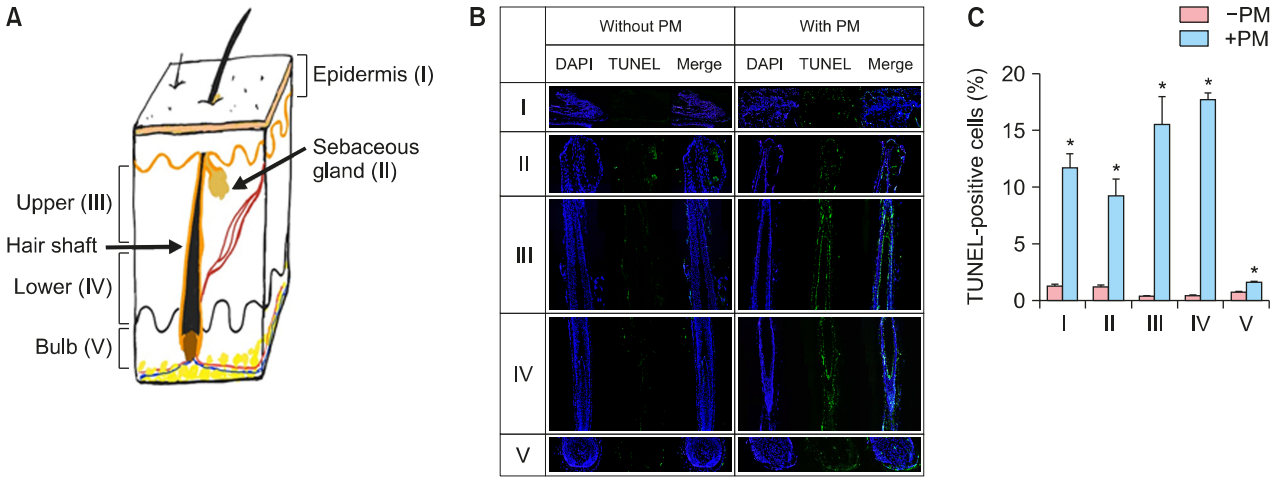

Fig. 1. Apoptotic cell death in *ex vivo* cultured human hair follicles after PM10 treatment. (A) Diagram of longitudinal dissection of skin with hair follicles. (B) Organ cultured hair follicles were treated with PM10 solution (100 μg/ml) for 5 days and subjected to TUNEL assay. Representative images from each hair follicle region are shown. DAPI (blue), TUNEL (green), and merged (cyan). (C) TUNEL-positive cells were quantified, and the data are presented as the mean±standard deviation from three independent experiments with hair follicles from three different individuals. *$p<0.05$ compared with the control by Student's t-test. PM: particulate matter.

tablish a standard curve for optical density measurements by an ELISA reader at 450 nm.

Reactive oxygen species measurement

Cells were seeded in a 96-well pate (4.5×10^4 cells/well) and serum-starved for 24 hours. After discarding the supernatant, the indicated concentration of PM10 suspension was applied in serum-free media. Plates were washed with PBS after 1 hour of exposure and then filled with 25 μM DCF-DA solution diluted in pre-warmed phenol-free HBSS (Gibco®; ThermoFisher, Waltham, MA, USA) and incubated at 37°C for 1 hour protected from light. ROS was detected using a fluorescence plate reader excitation at 490 nm and emission at 525 nm. As a positive control of this experiment, cells were incubated with freshly prepared 0.5 mM tert-Butyl hydroperoxide (TBHP) for 20 minutes at 37°C.

TUNEL assay

A TUNEL kit (EMD Millipore, Billerica, MA, USA) was used according to the manufacture's protocol.

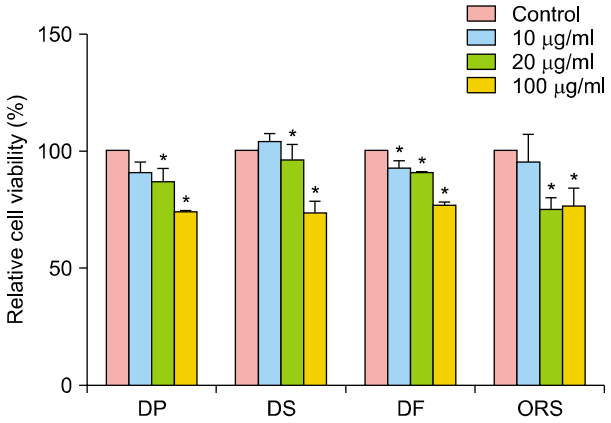

Fig. 2. Effect of PM10 on the cell viability of follicular cells. MTT assay was performed in cultured primary human dermal papilla (DP), dermal sheath (DS), dermal fibroblast (DF), and outer root sheath (ORS) cells. After 24 hours of serum starvation, cells were treated with suspended PM10 solution at the indicated concentrations for 24 hours. Data are presented as the mean± standard deviation from three independent experiments using cells from three individuals. *$p<0.05$ compared with the control by Student's t-test. PM: particulate matter.

RESULTS

First, to investigate overall effects of PM10 on human hair, TUNEL assay was carried out using *ex vivo* organ culture in the presence of PM10 solution. Surprisingly, as compared to the control group, cells undergoing apoptosis were widely observed from nearly all keratinocyte regions in the hair follicle including the epidermis, sebaceous gland, ORS and inner root sheath (IRS) layer in both the upper and lower follicle (Fig. 1). In addition, some apoptotic cells were occasionally detected in the DP and DS in all three cases.

Subsequently, to confirm cellular cytotoxicity in the major component cells of hair follicles, we tested cell viability in the presence of PM10 in cultured human hair cells including DS, DF, DP, and ORS cells. All cells showed dose-dependent increased cytotoxicity upon PM10 treatment (Fig. 2); 100 μg/ml PM10 showed the maximum effect and

was used for further study.

Several reports have highlighted the importance of cytokines such as TNF-α, IL-1α, IL-1β, IL-6, and IL-8, which have been implicated in the inflammatory response of HaCaT cells exposed to PM[16]. To determine the mRNA expression levels of these cytokines, qPCR was performed after PM10 exposure to cultured ORS cells. The expression levels of all inflammatory factors were increased (Fig. 3A). Subsequently, the protein levels of TNF-α, IL-1β, IL-6, and IL-8 were measured by ELISA. Interestingly, IL-6 and IL-8 protein were significantly increased by PM10 treatment as compared to the control group in ORS cells (Fig. 3B).

Numerous studies have reported that PM causes inflammatory mediator productions through the generation of ROS and free radicals. NADPH oxidase (NOX) family members

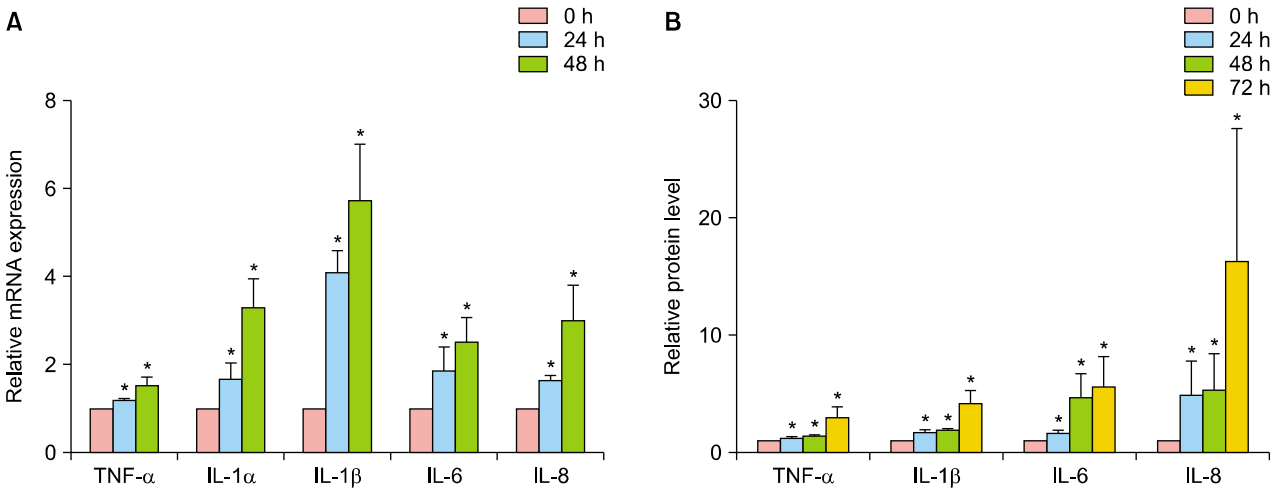

Fig. 3. Upregulation of inflammatory cytokines by PM10 treatment in cultured human outer root sheath (ORS) cells. Cells were treated with suspended PM10 (100 μg/ml) solution for the indicated times after 24 hours serum starvation. Inflammatory cytokine genes including tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, IL-6, and IL-8 mRNA expression levels were examined by quantitative real-time PCR (A), and their protein levels were measured from culture supernatants by ELISA (B). Data are shown as relative levels compared to the control group. Data are presented as means±standard deviation from three independent experiments using cells from three individuals. PM: particulate matter. *$p<0.05$ compared with the control by Student's t-test.

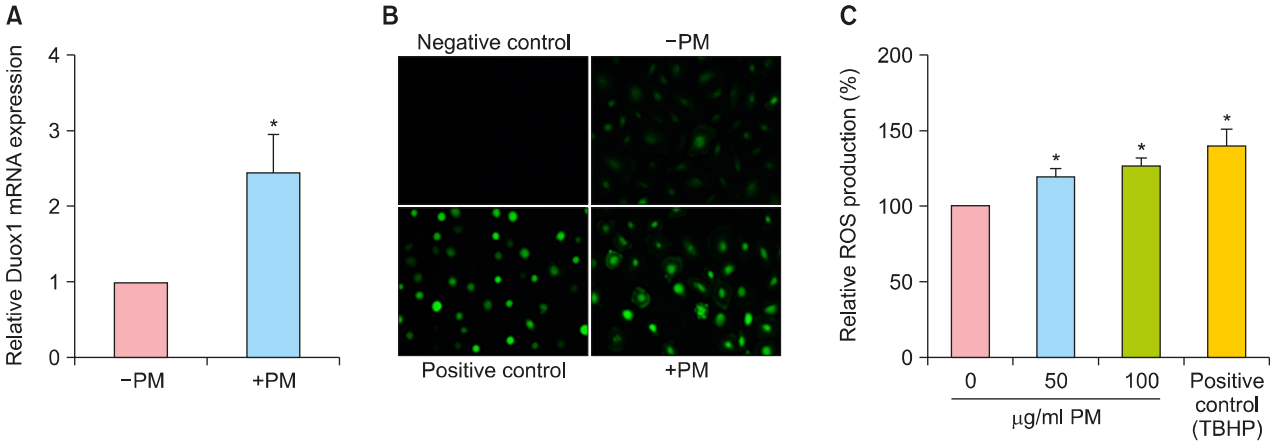

Fig. 4. Effect of PM10 on reactive oxygen species (ROS) production in cultured human outer root sheath (ORS) cells. (A) Cells were treated with PM10 (100 μg/ml) for 48 hours, and Duox1 mRNA expression levels were examined by quantitative real-time PCR. (B) Cells were treated with the fluorescent probe CM-H_2DCFDA (20 μM) for 1 hour in the absence or presence of PM10, and ROS production was visualized by fluorescent microscopy. The negative control is without CM-H_2DCFDA. For the positive control, cells were treated with TBHP (0.5 mM) for 20 minutes. (C) Intracellular ROS levels were measured, and data are shown as the relative levels compared to the control group. Data are presented as means±standard deviation from three independent experiments using cells from three individuals. PM: particulate matter. *$p<0.05$ compared with the control by Student's t-test.

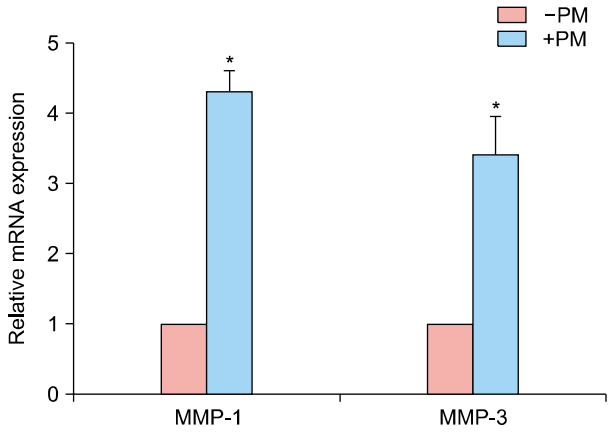

Fig. 5. Matrix metalloprotease mRNA expression after PM10 treatment in cultured human outer root sheath (ORS) cells. Cells were serum-starved for 24 hours prior to PM10 (100 μg/ml) treatment for 48 hours. Data are the relative mRNA expression of metalloprotease-1 (MMP-1) and metalloprotease-3 (MMP-3) and are presented as means±standard deviation from three independent experiments using cells from three individuals. *$p<0.05$ compared with the control by Student's t-test. PM: particulate matter.

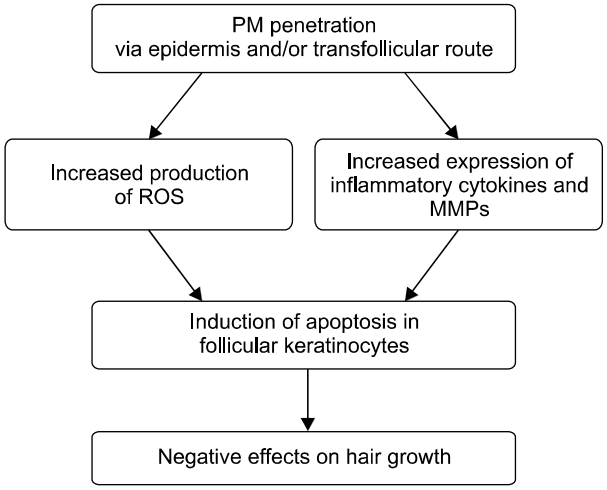

Fig. 6. Proposed model based on this study. Penetrated PM enters hair component cells via epidermal and transfollicular routes to increase ROS production and upregulate inflammatory cytokines and MMPs in follicular keratinocytes, which in turn results in apoptotic cell death and inflammation. Thereby, penetrated PMs from air pollution may have negative effects on hair growth. PM: particulate matter, ROS: reactive oxygen species, MMPs: matrix metalloproteinases.

produce ROS, and Duox1 is predominant NOX family member in ORS cells[17]. qPCR showed that Duox1 expression increased more than two-fold after 48-hour PM treatment compared to control cells (Fig. 4A). The level of ROS production was visualized by fluorescent microscopy (Fig. 4B) using a chemical fluorescent probe, CM-H_2DCFDA[18]. Consistent with the above findings, intracellular ROS production was increased in ORS cells after PM10 treatment in a dose-dependent manner (Fig. 4C). Collectively, these data show that PM10 induced Duox1 and increased ROS production in human hair keratinocytes. In addition to proinflammatory cytokines, the expression levels of matrix metalloproteinases (MMPs) are increased by ROS[19]; therefore, qPCR was conducted after PM exposure in ORS cells. MMP-1 and MMP-3 were upregulated more than three-fold upon PM treatment (Fig. 5).

DISCUSSION

In this study, we observed that PMs induce apoptotic cell death, especially in follicular keratinocytes in *ex vivo* cultured human scalp hair follicles (Fig. 1). In agreement with this, PM treatment decreased the viability of follicular cells, including ORS keratinocytes (Fig. 2). In addition, inflammatory cytokine expression was increased after PM10 treatment in cultured keratinocytes (Fig. 3). Considering that high-dose proinflammatory cytokines induce apoptosis of hair bulb keratinocytes *in vivo*[19] and our previous research showed that IL-6 inhibited hair shaft elongation in cultured human hair follicles and caused premature onset of catagen in mice[20], these data suggest that PM may impair hair growth by inducing inflammatory cytokines.

We also observed that PM treatment increased Duox1, an important NOX family member in ORS cells, as well as ROS production (Fig. 4). In addition, PM treatment increased MMP-1/3 expression in ORS cells (Fig. 5). ROS are chemically powerful molecules, and major biomolecules are susceptible to modification by ROS stress[16]. It is known that ROS can regulate redox-modulated matrix degradation via MMP gene expression and activation through keratinocyte signaling pathways[21]. Therefore, PM-generated ROS not only cause biomolecule modifications but may also activate MMPs, which together can cause inflammation and apoptosis in the skin, especially in epidermal keratinocytes.

Whether PMs are able to penetrate the skin via the hair follicle remains under debate[4,11,22]. Considering that the diameter of a scalp hair is generally larger than the size of all PMs and that a recent finding of *in vivo* skin distribution of rhodamine B after SRM 1649b treatment showed that dye delivery occurred via the follicle rather than via intracellular or transcellular means, PMs may accumulate in the aperture of the hair follicles and penetrate into follicular cells via the transfollicular route[23,24]. Despite the complexity of PM10 components and the moderate variability in donor hair samples, our *ex vivo* organ culture and *in vitro* cell culture studies suggest that penetrated PMs from air pollution may cause apoptotic cell death to fol-

licular keratinocytes through increased production of ROS and inflammatory cytokines, resulting in hair loss (Fig. 6).

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-32-388-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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