Preventive effects of antioxidants on changes in sebocytes, outer root sheath cells, and *Cutibacterium acnes*-pretreated mice by particulate matter: No significant difference among antioxidants

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Abstract

**Objectives:** Particulate matter (PM) is an air pollutant that can damage human skin; antioxidants have shown some efficacy in alleviating PM-induced skin inflammation. We investigated the antioxidant effects of punicalagin, epigallocatechin-3-gallate (EGCG), and resveratrol on PM-induced changes in cultured human sebocytes, outer root sheath (ORS) cells, and *Cutibacterium acnes*-pretreated mice.

**Methods:** Sebocytes and ORS cells were cultured with 100 μg/mL PM10 and 5 μM punicalagin, 1 μM EGCG, or 1 μM resveratrol for 24 h. In *C. acnes*-pretreated mice, inflammatory nodules were treated with 100 μg/mL PM10 and 5 μM punicalagin, 1 μM EGCG, or 1 μM resveratrol. Cell viability was measured using an MTT assay. Antioxidant effects were analyzed according to RNA expression, using real-time PCR, as well as reactive oxygen species (ROS) and sebum measurements.

**Results:** Antioxidants inhibited the upregulation of inflammatory cytokines, matrix metalloproteinase, aryl hydrocarbon receptor, and NF-kB as well as the production of ROS induced by PM10 in cultured sebocytes and ORS cells. The preventative effects of punicalagin and EGCG on biomarker expression in cultured sebocytes and ORS cells were slightly greater than those of resveratrol, though the difference was not significant. In *C. acnes*-pretreated mice, the antioxidants inhibited inflammatory cytokine and matrix metalloproteinase expression as well as sebum production.

**Conclusions:** Antioxidants effectively reduced the expression of inflammatory biomarkers and sebum production in cultured sebocytes, ORS cells, and *C. acnes*-pretreated mice.

**Keywords**
Acne, antioxidants, outer root sheath cells, particulate matter, sebocytes

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Introduction

In urban areas, human skin is exposed to various environmental stressors, including particulate matter (PM), which is a complex mixture of particles originating from several human industries and naturally windblown dust.\(^1\,^2\) PM of < 10 μm (PM10) is a major air pollutant consisting of solid and liquid particles, including toxic compounds, such as transitional metals, endotoxins, organic chemicals, and ultrafine components. PM10 excites alveolar macrophages as well as airway epithelial and endothelial cells to produce inflammatory mediators.\(^3\,^5\) It also induces the expression of monocyte adhesion molecules and synthesis of reactive oxygen species (ROS) in the skin. The continuous generation of oxidative stress can cause extrinsic skin aging and inflammatory disorders.\(^6\,^9\)

Antioxidants are widely used to inhibit oxidative stress and improve inflammatory skin conditions. Several polyphenolic compounds have antioxidant and anti-inflammatory effects.\(^10\,^12\) For example, punicalagin is a natural polyphenol found in pomegranates (Punica granatum), and pomegranate peel extract has been shown to relieve oxidative stress and inflammatory responses of monocytes exposed to PM10.\(^10\) Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea with diverse physiological activities, including the inhibition of inflammation in vivo and in vitro.\(^13\) Punicalagin and EGCG are also known to rescue cell viability and relieve the inflammatory response in human keratinocytes exposed to PM10.\(^14\) Resveratrol is a natural polyphenolic compound that is synthesized by plants in response to infection or other stressors. A recent study found that resveratrol also has many beneficial effects for skin.\(^15\)

In this study, we investigated the effects of punicalagin, EGCG, and resveratrol on PM10-induced changes in cultured human sebocytes and outer root sheath (ORS) cells. In addition, we assessed the response of PM10-induced inflammatory biomarkers to the antioxidant agents in Cutibacterium acnes-pretreated mice, an animal model of acneiform inflammation.

Methods

Sebocyte and ORS cell culture

Non-balding scalp specimens were obtained from male patients undergoing hair transplantation surgery at the Kyungpook National University Hospital (Daegu, South Korea). Occipital hair sebaceous glands were isolated using a binocular microscope and transferred to Biocoat collagen type I-coated tissue culture dishes (CORNING, Kennebunk, ME, USA) where they were cultured in human sebocyte basal medium containing sebocyte growth supplement (Cell Application, San Diego, CA, USA) at 5% CO₂ and 37°C. After 2 weeks of isolation, the cells were harvested with 0.25% trypsin/10 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA) in Hank’s balanced salt solution and subcultured with an EpiLife supplement (Gibco BRL, Rockville, MD, USA).

For ORS cell cultures, the hair follicle shaft and bulb regions were removed to prevent contamination by other cells. The trimmed hair follicles were immersed in DMEM supplemented with 20% fetal bovine serum. On the third culture day, the medium was changed to EpiLife (Gibco BRL) keratinocyte growth medium containing supplement. Sebocytes and ORS cells after the second passage were used in our experiments. Immunofluorescence was performed to confirm sebocyte and ORS cell types using antibodies against CK1-3, CK15, and CK19. Sebocytes were positive only for CK1-3 (Supplemental material Figure 1). In vitro experiments were conducted in the Basic Lab at the School of Medicine, Kyungpook National University (Daegu, South Korea). The study design was approved by the Medical Ethical Committee of Kyungpook National University (IRB Number KNU 2021-03-006), and informed consent was obtained from all participants.

Animal study

Female 6-week-old HR-1 mice were purchased from SLC (Japan) and acclimatized for 1 week. C. acnes was obtained and isolated from the pustules of Korean patients with acne. This study was conducted using our previously reported inflammatory acne mouse model.\(^16\) C. acnes were injected at 10⁹ colony-forming units/20 μL into four sites on a mouse’s back using a 30-gauge needle. After 1 week, 100 μL of 100 μg/mL PM10 (PM10-like, European reference material ERM-CZ120; Sigma-Aldrich, St Louis, MO, USA) and 5 μM punicalagin, 1 μM EGCG, or 1 μM resveratrol (Sigma-Aldrich) were applied to the treated back skin for 2 weeks. All mice (3 for punicalagin, 3 for EGCG, and 3 for resveratrol) were sacrificed by CO₂ inhalation to obtain dorsal skin samples. Tissue samples, including the inflammatory nodules, were cut to 1 × 1 cm for RNA isolation and tissue staining. The experiment was conducted to contain the same amount of RNA after RNA isolation. The experimental scheme is presented in Supplemental material Figure 2. Animal experiments were conducted from 2020 at the animal experiment center managed by the School of Medicine, Kyungpook National University. Our study design was approved by the Institutional Animal Care and Use Committee of Kyungpook National University (IRB Number KNU 2021-0068) and followed the Guide for the Care and Use of Laboratory Animals.
MTT assay for cell viability

We used an MTT assay to assess cell viability. Sebocytes and ORS cells were seeded in 96-well collagen-coated plates at a density of 5000 cells per well (Becton Dickinson, Franklin, LJ, USA) and incubated for 24 h. Subsequently, various concentrations of punicalagin, EGCG, and resveratrol (Sigma-Aldrich) were added to the wells for 3 days. MTT solution (3-[4,5]-dimethylthiazol-2,5-diphenyltetrazolium bromide) was added at 70 μg/well for 3 h. The formazan produced was solubilized with DMSO, and optical density was measured at 570 nm.

Real-time polymerase chain reaction for biomarker expression

Subsequent to treatment with PM10 and 5 μM punicalagin, 1 μM EGCG, or 1 μM resveratrol for 24 h, total RNA was obtained from sebocytes and ORS cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany); cDNA was synthesized from 3 μg total RNA with a cDNA synthesis kit containing ImProm-II reverse transcriptase and oligo-dT primers based on the manufacturer’s protocol (Promega, Madison, WI, USA).

Real-time polymerase chain reaction (PCR) was conducted with a Step One Plus real-time PCR assay (Applied Biosystems, Foster City, CA, USA). All reactions were conducted with Power SYBR Green premix (Applied Biosystems) using 50 ng cDNA and 10 pM primers. PCR primer sequences are summarized in Supplemental material Table 1. The amplification cycling conditions were as follows: 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 60 s. The PCR products were evaluated using Step One Plus real-time PCR software (Applied Biosystems).

Fluorescence microplate assay for ROS production

Cellular ROS production was assessed using 2′,7′-dichlorodihydrofluorescein diacetate. For 24 h, cells were incubated in six-well collagen-coated plates (Becton Dickinson) at $5 \times 10^5$ cells/well, and for 30 min, the cells were prelabeled with 10-μM DCF-DA (Invitrogen, Carlsbad, CA, USA). The plates were washed with PBS, treated with 5 μM punicalagin and 100 μg/mL PM10, and incubated at 37°C for 2 h in the dark. Cells were extracted with 20 mM Tris-Cl buffer containing 1% sodium dodecyl sulfate and 2.5 mM EDTA. The extracts were centrifuged for 15 min at 13 000 r/min, and the supernatants were detected using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 485-nm excitation and 538-nm emission wavelengths.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) for three independent experiments. Differences between groups were assessed by analysis of variance (ANOVA), with $p < 0.05$ considered to denote significance.

Results

MTT assay to determine antioxidant concentrations

An MTT assay was conducted to assess the concentration-dependent effects of the antioxidative agents on the viability of human sebocytes and ORS cells over 3 days (Figure 1). Optimal cell viability was observed at 5 μM punicalagin, 1 μM EGCG, and 1 μM resveratrol, respectively.
Preventative effects of antioxidants against PM10-induced upregulation of inflammatory biomarkers in sebocytes and ORS cells

According to real-time PCR, the expression of interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α (Figure 2(a)); matrix metalloproteinase (MMP) 1, MMP3, and MMP12 (Figure 2(b)); as well as the aryl hydrocarbon receptor (AhR) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) was significantly increased in PM10-treated sebocytes and ORS cells after 24 h (Figure 2(c)) (*p<0.05). This upregulation in biomarker expression levels was inhibited by punicalagin, EGCG, and resveratrol (*p<0.05). The preventative effects of punicalagin and EGCG on the expression of IL-1α, IL-1β, and IL-8 in sebocytes and IL-1β, IL-6, and TNF-α in ORS cells were slightly greater than those of resveratrol. Similarly, the expression of AhR in sebocytes was more strongly inhibited by punicalagin and EGCG than resveratrol, though the difference was not significant. ROS production in sebocytes and ORS cells increased with PM10 treatment. However, the upregulation of ROS production was inhibited by punicalagin, EGCG, and resveratrol (*p<0.05) (Figure 2(d)). Punicalagin and EGCG had greater preventative effects on ROS production in ORS cells compared to resveratrol, though there was no significant difference.

Preventative effects of antioxidants against PM10-induced increases in inflammatory nodule size and thickness

PM10 increased C. acnes-induced inflammatory nodule diameter and thickness; punicalagin, EGCG, and resveratrol inhibited the aggravation of C. acnes-induced inflammatory nodule diameter by PM10 after 1 and 2 weeks and thickness after 2 weeks only (Figure 3(a) and (b)) (*p < 0.05). Treatment groups were compared to a vehicle group treated with antioxidants dissolved in DMSO. There was no significant difference in nodule response between the antioxidant groups.

Preventative effects of antioxidants on PM10-induced sebum production in sebocytes and inflammatory nodules

PM10 upregulated the expression of peroxisome proliferator-activated receptor (PPAR)-γ, stearoyl-CoA desaturase (SCD), sterol regulatory element-binding protein (SREBP)1a, and SREBP1c in sebocytes; punicalagin, EGCG, and resveratrol downregulated PPARγ, SCD, SREBP1a, and SREBP1c expression in sebocytes...
The preventative effects of punicalagin on SREBP1c expression in sebocytes were slightly greater than those of EGCG and resveratrol, though there was no significant difference among groups. In addition, PM10 upregulated sebum production in C. acnes-induced inflammatory nodules, and punicalagin, EGCG, and resveratrol inhibited the upregulation of sebum production by PM10 after 2 weeks (Figure 5(b)).

**Discussion**

Inflammatory dermatoses, such as acne, could be aggravated by environmental factors, including PM10. Acne is associated with the elevated expression of proinflammatory cytokines. Activation of AhR by PM10 exacerbates proinflammatory cytokine expression via ROS production. Furthermore, oxidative stress induces complex biological processes that promote the expression of...
transcription factors, such as activator protein 1 (AP-1) and NF-κB.\(^{17}\)

Antioxidants are expected to alleviate oxidative stress and inflammatory injury due to PM. In a previous study on urban air pollution, chocolate inhibited TNF-α, IL-6, and IL-1β upregulation in a mouse model.\(^{18}\) Another study showed that the ethanol extract of *Eucheuma cottonii* reduced alveolar macrophage deposition and malondialdehyde serum levels in rats exposed to PM10 coal dust.\(^{19}\)

Punicalagin, an active constituent of pomegranate peel extract, is a well-known polyphenolic antioxidant, and its anti-inflammatory properties have been demonstrated in various experimental models.\(^{20–22}\) Park et al.\(^{10}\) found that this extract attenuated PM10-induced ROS production and TNF-α, IL-1β, MCP1, and ICAM-1 expression in THP-1 monocytic cells. Additionally, Seok et al.\(^{14}\) showed that punicalagin effectively alleviated PM10-induced cytotoxicity and inflammatory reactions in human primary epidermal keratinocytes.

EGCG attenuated inflammatory responses in human epidermal keratinocytes exposed to PM10. However, at the same concentration, EGCG is more potent than punicalagin in reducing PM10’s proinflammatory effects, while punicalagin exhibits a wider therapeutic range.\(^{14}\) In the present study, punicalagin and EGCG suppressed the proinflammatory effects of PM10 in sebocytes and ORS cells to a comparable degree. EGCG is also an antioxidant that effectively scavenges ROS.\(^{23}\)

Skin MMPs break down macromolecules in the extracellular matrix, including type I collagen.\(^{24–26}\) MMP-1, secreted by dermal fibroblasts and epidermal keratinocytes, degrades skin collagen.\(^{27,28}\) PM10-induced MMP-1 expression was effectively reduced by punicalagin and EGCG.\(^{14}\) Our study showed that punicalagin, EGCG, and resveratrol inhibited the upregulation of MMPs in sebocytes, ORS cells, and *C. acnes*-induced inflammatory nodules caused by PM10. Future studies should examine the in vivo effects of polyphenolic antioxidants on human skin.

**Figure 3.** Preventative effects of antioxidants against PM10-induced increase in the size and thickness of inflammatory nodules. (a). According to H&E staining, PM10 increased the size of *C. acnes*-induced inflammatory nodules, and punicalagin, EGCG, and resveratrol inhibited the aggravation of nodule size. (b). PM10 increased the diameter of *C. acnes*-induced inflammatory nodules, and punicalagin, EGCG, and resveratrol inhibited the aggravation of nodule diameter after 1 and 2 weeks. (c). PM10 increased the thickness of *C. acnes*-induced inflammatory nodules, and punicalagin, EGCG, and resveratrol inhibited the aggravation of nodule thickness after 2 weeks. Data are presented as the mean ± SD from three independent experiments (*p < 0.05*). *C*: *Cutibacterium*, PM: particulate matter, EGCG: epigallocatechin-3-gallate, SD: standard deviation.
Figure 4. Preventative effects of antioxidants against PM10-induced upregulation of inflammatory biomarkers in inflammatory nodules. (a). PM10 upregulated the expression of TLR2 in C. acnes-induced inflammatory nodules, and punicalagin, EGCG, and resveratrol inhibited the upregulation of TLR2 expression by PM10 after 2 weeks. (b). PM10 upregulated the expression of IL-1α, IL-1β, IL-6, IL-8, and TNF-α in C. acnes-induced inflammatory nodules; punicalagin, EGCG, and resveratrol inhibited the upregulation of IL-1α, IL-1β, IL-6, IL-8, and TNF-α by PM10 after 2 weeks. (c). PM10 upregulated the expression of MMP1, MMP3, and MMP12 in C. acnes-induced inflammatory nodules; punicalagin, EGCG, and resveratrol inhibited the upregulation of MMP1, MMP3, and MMP12 by PM10 after 2 weeks. Data are presented as the mean ± SD from three independent experiments (*p < 0.05). C.: Cutibacterium, PM: particulate matter, TLR: toll-like receptor, EGCG: epigallocatechin-3-gallate, SD: standard deviation, IL: interleukin, TNF: tumor necrosis factor, MMP: matrix metalloproteinase.
skin inflammation caused by airborne particles and the potential anti-aging effects of antioxidants in highly polluted environments.

Various in vivo and in vitro studies have reported the benefits of resveratrol on skin health.\textsuperscript{15,29} Keratinocyte treatment with resveratrol induced a dose-dependent increase in transglutaminase activity required for the generation of cornified envelopes (structures that determine epidermal permeability), while simultaneously inhibiting DNA synthesis.\textsuperscript{29-31} These results indicated that resveratrol prevents keratinocyte proliferation, while prompting differentiation. Moreover, Shin et al.\textsuperscript{15} found that resveratrol effectively inhibited PM10-induced inflammatory reactions in human primary keratinocytes.

Based on previous findings, the treatment of PM10 should be considered in clinical disorders, including alopecia or hair disorders. PM was found to induce apoptosis through the upregulation of ROS and inflammatory cytokines in human hair follicular keratinocytes, leading to hair growth disorders.\textsuperscript{32} Indeed, Lee et al.\textsuperscript{33} showed that environmental factors could promote the pathogenesis of alopecia areata. We propose that antioxidants, such as punicalagin, EGCG, and resveratrol, could aid in the therapy of PM-related disorders.

Several studies related to the beneficial effects of antioxidants have recently been conducted. Costa et al.\textsuperscript{34} reported the protective effect of honokiol, a phenolic component of Magnolia officinalis, against cigarette smoking-induced inflammation in human foreskin fibroblasts and keratinocytes. Nobile et al.\textsuperscript{35} assessed the effects of polyphenol-enriched dietary supplements against air pollution by directly measuring antioxidant capacity in the subject’s saliva and skin in vivo. Randhawa et al.\textsuperscript{36} confirmed the beneficial effects of antioxidants from Terminalia chebula fruit extract on inflammatory cytokine

**Figure 5.** Preventative effects of antioxidants on PM10-induced production of sebum in sebocytes and inflammatory nodules. (a). PM10 upregulated the expression of PPAR\(\gamma\), SCD, SREBP1a, and SREBP1c in cultured sebocytes; punicalagin, EGCG, and resveratrol inhibited the upregulation of PPAR\(\gamma\), SCD, SREBP1a, and SREBP1c by PM10. (b). PM10 upregulated sebum production in C. acnes-induced inflammatory nodules, and punicalagin, EGCG, and resveratrol inhibited the upregulation of sebum production by PM10 after 2 weeks. Data are presented as the mean ± SD from three independent experiments (*\(p<0.05\)). C: Cutibacterium, PM: particulate matter, TLR: toll-like receptor, EGCG: epigallocatechin-3-gallate, SD: standard deviation, IL: interleukin, TNF: tumor necrosis factor, MMP: matrix, PPAR: peroxisome proliferator-activated receptor, SCD: stearoyl-CoA desaturase, SREBP: sterol regulatory element-binding protein.
production in a cell-based in vitro study and in a clinical study, applied the antioxidant directly to the subject’s skin. Similarly, antioxidants from Deschampsia antartica extract and antioxidant serum were evaluated in vivo through direct skin treatment in clinical subjects. These research models have proven to be robust and useful in elucidating the effects of antioxidants against various pollutants and can thus be adopted in future studies.

We acknowledge some limitations in our study design. We determined the antioxidant concentration that can be used in the animal experiments as the maximum concentration that did not affect cell proliferation in the in vitro experiments, which we considered a safe concentration. However, this method of selection requires further validation to support its applicability in an animal model. Additionally, we only considered PM10 without evaluating other substances that are closely related in their adverse effects and/or origins. Furthermore, the calculation and justification of the sample size were not done in this study.

In our assessment of sebocytes and ORS cells, punicalagin, EGCG, and resveratrol inhibited PM10-induced ROS production; reduced the expression of AhR and NF-kB; and downregulated the expression of proinflammatory markers, including IL-1α, IL-1β, IL-6, IL-8, and TNF-α, and MMPs. Furthermore, punicalagin, EGCG, and resveratrol reduced the expression of sebum production-related biomarkers in the cultured sebocytes. In mice with C. acnes-induced inflammatory nodules, punicalagin, EGCG, and resveratrol inhibited the PM10-induced upregulation of proinflammatory mediators and MMPs and also reduced sebum production.

Conclusions
Punicalagin, EGCG, and resveratrol should be clinically evaluated for their anti-inflammatory and antioxidative effects in the treatment of PM-related skin disorders. Specifically, punicalagin and EGCG performed slightly better than resveratrol in their inflammation- and oxidation-preventative effects.

Declaration of conflicting interests
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Ethics approval
Ethical approval for this study was obtained from The Medical Ethical Committee of the Kyungpook National University approved this study (IRB Number KNUH 2021-03-006).

Informed consent
Written informed consent was obtained from all subjects before the study.

Animal welfare
The present study followed the use of Laboratory Animals. The study was approved by The Institutional Animal Care and Use Committee of Kyungpook National University (IRB Number KNU 2021-0068).

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