의학박사 학위논문

미세먼지(particulate matter)에 의한 피부 노화와 색소침착에 미치는 홍삼 유래 천연성분의 역할

Korean red ginseng extracts may protect human skin from oxidative damage and pigmentation resulting from particulate matter exposure

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Korean red ginseng extracts may protect human skin from oxidative damage and pigmentation resulting from particulate matter exposure

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이 논문을 의학박사 학위 논문으로 제출함

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Abstract

Background and Objectives: Exposure to airborne particulate matter (PM) is an ever-increasing concern worldwide. Strategies to counter the detrimental effects such as induction of pigmentation and ROS-associated aging and inflammation of cutaneous exposure to PM are being investigated actively. Among various naturally derived products, Korean red ginseng extracts and individual ingredients have been well demonstrated for their role in suppression of ROS, inflammation, and resultant skin aging. In addition, some saponin ingredients were reported to affect skin melanogenesis. Recently, Rg3 and Rf saponins were revealed to work as anti-melanogenic agents. In order to find ideal modality for protecting human skin from airborne PM-induced oxidative damage and hypermelanosis, we hypothesized that Korean red ginseng extracts may protect or reverse the PM-induced detrimental effects. Our aim was to design *in vitro* and *ex vivo* experiments to demonstrate the skin protective effects of red ginseng extracts after cutaneous exposure of the skin to particulate matter.

Methods: The biological effects of four types of particulate matter (PM_{10}) and Korean red ginseng extracts (saponin and non-saponin fractions) were evaluated both *in vitro* and *ex vivo*. Cell viability and intracellular reactive oxygen species (ROS) levels were determined in normal human epidermal melanocytes (NHMs), human epidermal keratinocytes (NHKs) and their cocultures using the DCF assay. Experiments with *ex vivo* skin explants also demonstrated the protective properties of saponins against the detrimental effects of exposure to particulate matter (PM). Melanin assay, quantitative real time PCR and western blotting were also performed to determine the effect on melanogenesis and the implicated molecular signaling pathways in *in vitro* and *ex vivo* experiments.

Results: Exposure to 100 μg/ml of PM resulted in decreased keratinocyte viability coupled with augmented oxidative stress indicated by DCF fluorescence. These observations were attenuated by treatment with saponins. Non-saponin fractions could decrease PM-induced oxidative stress, but could not improve keratinocyte viability. PM exposure also led to increased melanin production in a coculture model, which was mitigated by treatment with

saponin fractions. Treatment with both saponin fractions and non-saponin fractions resulted in a decrease in matrix metalloproteinase (MMP) levels after exposure to PM.

Conclusion: Korean red ginseng extracts could protect the skin from the harmful effects of PM exposure by reducing ROS, keratinocytic cell death and dermal matrix deterioration. Moreover, saponins successfully reduced PM-induced melanin accumulation thus can be utilized as safe and effective anti-melanogenic agents in real world when melanogenic PM is prevalent.

Keywords: particulate matter, melanogenesis, skin aging, oxidative stress, *Panax ginseng*, saponins

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INTRODUCTION

Growing public awareness of the harmful effects of particulate matter (PM) exposure on various organs and systems has led to active research to determine the mechanism of damage and prevention or treatment, with special focus on the respiratory system. It has been clinically and experimentally demonstrated that, because the respiratory system is inevitably exposed to airborne pollutants such as PM, underlying respiratory diseases such as asthma and chronic obstructive pulmonary diseases and lung cancer are exacerbated by PM exposure.(1, 2) Another organ that is at high risk of PM exposure is the eye. In fact, eye diseases such as keratoconjunctivitis, are shown to be induced by contact with PM.(3) The skin is also in direct contact with the environment, and atmospheric PM is thus thought capable of damaging the skin.

In the past decade, clinical reports have shown the correlation between atmospheric PM concentrations and the progression of skin aging. In previous studies, signs of skin aging composed of both pigmentary change and decreased skin laxity have been associated with PM exposure.(4, 5) Indeed, clinical investigations in China and Europe showed that increased atmospheric PM clinically resulted in more solar lentigines and more prominent wrinkles. However, the biological mechanistic link between PM and pigmentation and/or extrinsic skin aging remains largely uncertain. Most of studies suggested that the link is reactive oxygen species (ROS). It was demonstrated that PM increases oxidative stress in skin cells and induces ROS-dependent inflammation.(6) Another study suggested a role of PM-induced autophagy in skin aging.(7)

In addition to searching for a mechanistic link, there are urgent needs to develop strategies to prevent or reverse the detrimental effects such as induction of pigmentation and ROS-associated aging and inflammation by cutaneous exposure to PM. Especially, anti-PM and anti-melanogenic strategy need to be actively explored since people with dark skin type in Korea and other East-Asian countries are at high risk of exposure to PM produced domestically and PM coming from neighboring countries.

Among various naturally derived products, Korean red ginseng extracts and individual ingredients have been well demonstrated for their role in suppression of ROS, inflammation, and resultant skin aging. *Panax ginseng,* commonly known as Korean red ginseng, has

served as a key ingredient in Asian herbal medicine for more than 2,000 years. Owing to its popularity, researchers have aimed to identify the active substances related to the beneficial effects of Korean red ginseng. The main active substances of Korean red ginseng can be divided into two categories: saponin fractions and non-saponin fractions. The saponins in the saponin fractions comprise a heterogeneous group of compounds including ginsenosides, which are known as the pivotal substances of ginseng extracts. To date, more than 40 ginsenosides have been isolated and identified among the saponin fractions. Ginsenosides have been investigated for their antioxidant, anti-inflammatory, anti-neoplastic, and even hair growth-promoting effects in dermatologic indication.(8-12) In addition, some saponin (ginsenoside) ingredients and non-saponin ingredients were reported to affect skin melanogenesis, in either anti-melanogenic or pro-melanogenic way for the treatment of hyper- or hypopigmentation disorders. Particularly, Rg3 and Rf ginsenosides were revealed to work as anti-melanogenic agents. Although the non-saponins of Korean red ginseng extracts have not been investigated as thoroughly as saponins, anti-inflammatory and other biologic effects of non-saponins are continuously being reported.(13, 14)

In order to find effective oral or topical agents for the protection of human skin from airborne PM-induced oxidative damage and pigmentation disorders, we hypothesized that saponins or non-saponins of Korean red ginseng extracts may protect or reverse the PM-induced oxidative stress and melanin accumulation considering that Korean red ginseng extracts are known to reduce both ROS and inflammation which are main pathogenesis of PM-induced skin disorders.(6) For a simulating approach towards the real world PM-induced skin disorders, this study was designed to investigate the effect of exposure to 3 types of PM measuring between 2.5 μm and 10 μm in size (PM_{10}) and 1 type of local PM less than 10 μm from the outside of our research institution. Furthermore, as the skin is a paracrine organ and biologically interacting with other cells in responses to PM, skin constituent cells including human keratinocytes and melanocytes, keratinocyte conditioned media-treated dermal fibroblasts, cocultures of keratinocytes and melanocytes, and *ex vivo* skin cultures were used in this study.

MATERIALS AND METHODS

Materials

Both saponin fractions and non-saponin fractions of Korean red ginseng were provided by the Korea Ginseng Corporation (Daejeon, Korea). The antibodies for tyrosinase, MITF, and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) were purchased from Thermo Fisher Scientific (Invitrogen, Waltham, MA, USA). The antibody for MMP-1 was obtained from GeneTex (Irvine, CA, USA). H2O2 and the antibodies for beta-actin and N-Acetyl-L-cysteine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Particulate matter collection

We collected and selected PM (we call it as local PM in this manuscript) less than 10 μm by reference to previous publication of Chung et al.[15] at outside of Asan research institution building located in an urban area in Seoul, Korea from January 2019 to March 2019. The collection site was 200 meters away from a two-way street with total of eight lanes. Han River, which is more than one kilometer wide, locates 500 meters away from the collection site. In addition, 3 commercially available types of PM measuring between 2.5 μm and 10 μm in size (PM10) were purchased and tested which PM increases melanogenesis to a more degree in compared to local PM. Urban aerosols (CRM No. 28) were purchased from the National Institute for Environmental Studies (NIES), Ibaraki, Japan. Certified and reference values for elements and polycyclic aromatic hydrocarbons (PAHs) constituting the material are available online on the NIES webpage (https://www.nies.go.jp/labo/crm-e/aerosol.html). The standard reference materials 1648a (pmA) and 1649b (pmB) were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). These both consist primarily of PAHs and heavy metals, and their constitutions are available on the NIST webpage (http://www.nist.gov/srm).

Cell culture and Coculture

Normal human epidermal melanocytes (NHM; neonatal-moderately pigmented) were cultured in Medium 254 supplemented with human melanocyte growth supplement (HMGS; Cascade Biologics, Invitrogen) at 37 and 5% CO2. NHM were used at passages between 3 and 5. Normal human epidermal keratinocytes (NHK; neonatal) were cultured in Epilife medium supplemented with human keratinocyte growth supplement (HKGS; Cascade Biologics, Invitrogen) at 37 and 5% CO2. NHK were used at passages between 3 and 5. A coculture of human keratinocytes and melanocytes was generated in keratinocyte medium, at a seeding ratio of 1:5 (for melanin assay) or 1:1 (for melanin assay, western blotting and intracellular signaling assay). NHM were seeded into a 6-well plate at a density of 6 × 104 or 3 × 105 cells per well. On the next day, NHK were added to each well at a density of 3 × 105 cells for the coculture. After 24 h, particular matter (PM) was added and melanin content was measured after 3 or 5 days.

Cell viability assay

Cell viability was measured using MTT assays. After starvation for 24 h, cells were treated with PM for 24 h. MTT solution (2.5 mg/ml) was added to the culture medium and incubated for 4 h. The resulting formazan crystals were dissolved in DMSO. Absorbance was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

Measurement of melanin contents

Cells were dissolved in 1 N NaOH at 100°C for 30 min and centrifuged at 13,000 rpm for 1 min. The optical densities (OD) of the supernatants were measured at an absorbance of 405 nm using a microplate reader. The protein content of the samples was determined using the Bradford assay (Bio-Rad. Hercules, CA, USA). Melanin content was calculated by normalizing with the protein concentrations.

Intracellular ROS assay

Intracellular ROS levels were measured using the DCF-DA assay. Normal human epidermal keratinocytes (NHEK) were seeded into a 96-well plate at 1.8×10^4 cells per well and were treated with 100 μg/ml PM and 50 μg/ml saponins or 50 μg/ml non-saponins for 24 h. After 24 h, the medium was removed and cells were washed twice with PBS. Cells were then incubated with 10 μM DCF-DA for 30 min at 37°C in dark and washed twice with PBS. Fluorescence intensity was then measured at 485 nm excitation and at 535 nm emission using a microplate reader (VICTOR Multilabel Plate Reader, PerkinElmer, Waltham, MA, USA). For short time ROS assay and to examine reversal reduction of ROS by a known anti-oxidant N-Acetyl-L-cysteine, NHEK were seeded into a 96-well plate at 3×10^4 cells per well and were pretreated with 50 μg/ml saponin, 50 μg/ml non-saponin or 1 mM N-Acetyl-L-cysteine for 1 h and then treated with 100 μg/ml PM for 3 h. For comparison of the effect between pretreatment or simultaneous treatment, the cells were simultaneously treated with 50 μg/ml saponin, 50 μg/ml non-saponin or 1 m N-Acetyl-L-cysteine and 100 μg/ml PM for 3 h. After indicated time, the medium was removed and cells were washed twice with PBS. Cells were then incubated with 10 μM DCF-DA for 30 min at 37°C in dark and washed twice with PBS. Fluorescence intensity was then measured at 485 nm excitation and at 535 nm emission using a microplate reader (VICTOR Multilabel Plate Reader, PerkinElmer, Waltham, MA, USA).

Quantitative real-time polymerase chain reaction (PCR)

Total cellular RNA was extracted from cells using FavorPrepTM Total RNA Purification Mini Kit according to the manufacturer's instructions (Favorgen, Ping Tung, Taiwan). Following extraction, the quantity and quality of the RNA were determined using a NanoDrop® ND-1000 spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Single stranded cDNA was synthesized from 1 μg of total RNA using a Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). Quantitative real time PCR (qRT-PCR) was performed using the LightCycler® 480II system coupled with SYBR Green chemistry (Roche Applied Science, Penzberg, Germany). In terms of qRT-PCR settings, initial denaturation was

performed at 95°C for 5 min, followed by 55 cycles of amplification at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The cDNA was amplified using the following primers:

Name	Forward (5' to 3')	Reverse (5' to 3')
Interleukin (IL)-1 α	AGGGCGTCATTCAGGATGAA	CGCCAATGACTCAGAGGAAGA
Interleukin (IL)-1 β	TCCCCAGCCCTTTTGTTGA	TTAGAACCAAATGTGGCCGTG
IL-8	AACCCTCTGCACCCAGTTTTC	ACTGAGAGTGATTGAGAGTGGAC
TNF-α	AGCTGCCCCTCAGCTTGAG	CCCAGGGACCTCTCTCTAATCA
MMP1	CTCTGGAGTAATGTCACACCTCT	TGTTGGTCCACCTTTCATCTTC
MMP2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC
MMP3	CGGTTCCGCCTGTCTCAAG	CGCCAAAAGTGCCTGTCTT
MMP9	TCGTCATCGTCGAAATGGGC	GGGACGCAGACATCGTCATC
RPLPO	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC

Western blotting

Cells were lysed in protein lysis buffer (Intron Biotechnology, Seongnam, Korea) and centrifuged at 13000 rpm for 10 min. Protein concentrations in the supernatant were determined using a bicinchoninic acid protein assay kit. Next, 20 μg of proteins were separated per lane by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA). Image analysis was used to determine the relative band densities, using Image J software (https://imagej.nih.gov/ij/).

Ex vivo skin explant model

Discarded full-thickness skin was obtained from four women 30~50 years old after abdominoplasty at Asan Medical Center. Informed patient consent and approval by the Institutional Review Board were obtained. The whole-skin samples were treated PM (1648a) or none (control). A sterilized stainless steel grid was placed on a six-well plate and the skin specimens were placed on the stainless steel grid. DMEM medium supplemented with 10% FBS and 10% antibiotics was filled up to the stainless steel grid. After 6 days of culture in an incubator at 37 °C with 5% CO2, the specimens were fixed in 10% formalin and embedded in paraffin section. Melanin was detected with Fontana-Masson staining.

Production of preconditioned media

Preconditioned media from culture of HaCaT and HDF cells was produced according to the method previously described by Fernando et al.(15) To prepare PM samples, PM was dissolved in DMSO, emulsified and diluted using PBS. CPM preparation was done by suspending in DMEM medium. HaCaT cells were seeded at 1×10^5 cells/ml concentration and incubated for 24 h. Then, they were treated with different concentrations of PM. After 1 h, the cells were induced with CPM (125 μg/ml) and incubated for half an hour. To remove CPM, the cells were washed twice with new culture media and replaced by new media. After 24 h of the incubation period, the cell media was collected, filtered and stored at −80 °C for future use. In the meantime, HDF cells were seeded at 1×10^5 cells/mL concentration for 24 h in well plates. The preconditioned media from corresponding HaCaT cells treatment groups were treated to the HDF cells and incubated for 30 min. Then, the wells were emptied and washed twice with new culture media to remove the remaining preconditioned media and replaced with new culture media. This was done only to stimulate the cells. The stimulated HDF cells were then incubated for 48 h. After, the cell media was collected for evaluating MMPs and inflammatory cytokines.

RESULTS

Exposure to PM reduced keratinocyte viability and concomitant treatment of saponin fractions rescued cell viability

Normal human epidermal keratinocytes (NHKs) were cultured with varying concentrations of local PM from 50 to 400 μm/ml for 24 h, and cellular viability was assessed by MTT assay. Keratinocyte viability decreased significantly and dose-dependently after exposure to PM compared to that of the control (Fig. 1A). Microscopic images revealed cellular damages with fragmentation and reduced number of cells after treatment with PM (Fig. 1B). Then, NHKs were treated with different concentrations of saponin fractions. Interestingly, at moderate doses of 25 and 50 μg/ml of saponin fractions, a significant increase in cell viability was observed (Fig. 2A). Treatment with varying concentrations of non-saponin fractions revealed no particular increase in cell viability up to a concentration of 25 μg/ml, whereas treatments with 50 and 75 μg/ml both rather resulted in a decrease in cell viability. (Fig. 2B) Next, measurement of NHK viability after concomitant treatment with 100 μg/ml local PM and different concentrations of either saponin or non-saponin fractions was performed. Concomitant treatments with 50 and 75 μg/ml of saponin fractions rescued NHKs from PM-induced decrease in cell viability significantly (Fig. 3A). On the contrary, concomitant treatment with non-saponin fractions did not promote cell viability. Instead, cell viability was further compromised by treatment with 75 μg/ml of non-saponin fractions. (Fig. 3B)

Exposure to PM increased intracellular oxidative stress and concomitant treatment with non-saponin fractions attenuated ROS levels

DCF assay was performed using NHKs to assess intracellular oxidative stress. A dramatic increase in fluorescence levels was observed 24 h after treatment with varying concentrations of local PM. (Fig. 4) This increase was more pronounced than that observed with hydrogen peroxide treatment, whose ROS-producing effect is expected to be high in earlier time points. Up to 200 μg/ml of local PM, the PM concentration and the increase in

intracellular oxidative stress showed a positive correlation. Having observed a clear increase in intracellular oxidative stress induced by local PM treatment, we investigated the effect of both saponin and non-saponin fractions on the PM-induced oxidative stress. The concentration of PM was kept at 100 μg/ml throughout this experiment and NHKs were treated with 100 μg/ml PM together with either saponin or non-saponin fractions. Compared to the PM-treated control, treatment with 100 μg/ml saponin fractions and 50 and 100 μg/ml non-saponin fractions resulted in significantly decreased intracellular oxidative stress as determined by the DCF assay. (Fig. 5) Treatment with 100 μg/ml of non-saponin fractions showed a marked decrease in oxidative stress compared to that with 50 μg/ml of non-saponin fractions, which was still able to attenuate oxidative stress better than 100 μg/ml of saponin fractions. It is well known that antioxidants better reduce ROS when they are pretreated before being subject to oxidative stress. Thus, we investigated the difference in outcome between pretreatment and simultaneous treatment. Saponin fractions more effectively attenuated ROS when simultaneously treated whereas non-saponin fractions and N-acetyl-L-cysteine 1 mM were more effective in reducing ROS when pretreated (Fig. 6).

Exposure to PM increased melanin production and saponin fractions attenuated PM-induced melanogenesis

To investigate the effects of PM exposure on the production of melanin pigments, normal human melanocytes (NHMs) were treated with various concentrations of local PM and the melanin contents were measured on day 3. All tested concentrations of local PM (50, 100, 200, and 400 μg/ml) did not significantly affect the melanin content. (Fig. 7) However, when the same experiment was performed with keratinocyte/melanocyte cocultures, a significant increase in melanin content was observed at a PM concentration of 100 μg/ml and higher. (Fig. 8) This increase showed a dose-dependent relationship with PM concentration. A similar trend was observed when another PM (China urban dust) was used, but the increase in melanin content was more pronounced. (Fig. 9A) Treatment with varying concentrations of up to 200 μg/ml of metallic constituents of urban particulate matter (SRM1648A) proportionally increased the melanin content in the cocultures. (Fig. 9B) However, treatment with 400 μg/ml SRM1648A did not increase the melanin content compared to that with 200

μg/ml of SRM1648A. Keratinocyte/melanocyte cocultures were concomitantly treated with 100 μg/ml of local PM and either saponin or non-saponin fractions at various concentrations to determine their effects on melanin production. Concomitant treatment with saponin fractions at concentrations of 50 and 100 μg/ml, could attenuate the increase in melanin content by PM. On the contrary, treatment with non-saponin fractions did not bring down the melanin content. (Fig. 10A) We then examined whether saponin fractions could reduce the melanin contents at lower concentrations (from 6.25 to 50 μg/ml). Even at the lowest tested concentration of 6.25 μg/ml, concomitant treatment with saponin fractions significantly reduced the melanin content. (Fig. 10B) Western blot analysis was performed to evaluate the effect of saponin fractions on the expression of melanogenesis-related genes. Treatment with 100 μg/ml of local PM upregulated the expression of MITF, tyrosinase, and MMP-1 compared to the control. This upregulation was reversed, though not completely, by treatment with 50 μg/ml of saponin fractions. (Fig. 11)

Saponin fractions reversed PM-induced increase in the mRNA expression of pro-inflammatory cytokines

The changes in mRNA expression levels of pro-inflammatory cytokines in keratinocytes after exposure to PM were evaluated by qRT-PCR. Exposure to 100 μg/ml of local PM led to a notable increase in IL-2β, IL-8, and TNF-α, whereas no significant change in IL-1α was observed. Treatment with 50 μg/ml of saponin fractions led to significant reduction in the mRNA levels of all four cytokines. (Fig. 12)

Both saponin and non-saponin fractions attenuated PM-induced upregulation of dermal matrix metalloproteinases

Human dermal fibroblasts were incubated in keratinocyte-conditioned media and the levels of matrix metalloproteinases (MMPs) were measured. Incubation in keratinocyte-conditioned media obtained in the presence of PM resulted in increased levels of MMP1, 2, and 3. Addition of either saponin or non-saponin fractions decreased the levels of all four tested MMPs. Though not so significant, non-saponin fractions showed a more notable

decrease in MMP levels compared to saponins. Ursodeoxycholic acid (UDCA) as well as other ginsenosides were also tested, but their effects on MMPs were variable. (Fig. 13)

DISCUSSION

The effects of air pollution on human health and their prevention have long been a global issue. Among other pollutants, airborne particulates or particulate matter (PM) are classified as Group 1 carcinogens by the World Health Organization. Their microscopic size allows them to deeply penetrate organs and blood streams when inhaled. However, it was not clear whether PM could penetrate the skin barrier.

Recent reports have demonstrated that both PM smaller than 2.5 μm ($PM_{2.5}$) and PM measuring 2.5 to 10 μm (PM_{10}) can penetrate the stratum corneum of the skin and subsequently cause harmful reactions.(16) The results of *ex vivo* experiments in this study confirm that PM_{10} can penetrate the skin as well as alter cutaneous physiology including melanogenesis. *In vitro* investigations demonstrated that PM_{10} decreases keratinocyte viability at elevated concentrations. The exact mechanism by which PM_{10} compromises the viability of keratinocytes is uncertain; however, the dose-dependent increase in oxidative stress, as shown in this study, is suggested to be a key pathway. Other biomolecular pathways that have been indicated responsible for the cytotoxicity of PM include intracellular organelle dysfunction, mitochondrial damage, and autophagy.(6, 7, 16) The results of this study indicate the ability of Korean red ginseng extracts to protect keratinocytes from PM-induced cytotoxicity by attenuating intracellular oxidative stress. We focused on the level of oxidative stress because of the well-established antioxidant effect of Korean red ginseng extracts. Nonetheless, it is still possible that Korean red ginseng extracts act on keratinocytes by other mechanisms, which would require further research.

Melanin production is a strictly regulated process involving skin-resident cells, and is orchestrated by multiple factors including inflammatory responses as well as environmental stimuli. Although melanin pigments play an indispensable role in skin physiology by protecting the skin from ultraviolet irradiation, aberrantly increased production of melanin pigments is one of the top reasons for dermatologic consultations. In addition to chemicals such as fragrances, which may lead to inflammation-associated hyperpigmentation, environmental exposure to air pollutants can also result in cutaneous hyperpigmentation by provoking inflammatory reactions. Although stimulation of melanin production by PM through aryl hydrocarbon receptors (AhR) has been recently postulated, direct evidence that

PM exposure leads to increased melanin synthesis was lacking.(17) The results of this study provide experimental evidence that exposure to PM_{10} promotes melanin synthesis in a dose-dependent manner. The results of western blotting and qRT-PCR suggest that PM stimulates melanin production via induction of MITF and a subsequent increase in tyrosinase activity. Furthermore, changes in the inflammatory cytokine profile after PM exposure seem to play an additional role. Increased mRNA expression of proinflammatory cytokines including IL-1α, IL-1β, IL-8, and TNF-α after exposure to PM_{10} was noted. This increase in pro-inflammatory cytokine levels was reversed by concomitant treatment with saponin fractions. Because inflammatory reactions tend to promote melanin production, downregulation of inflammation by saponin fractions is thought to be an important if not the only, mechanism by which saponin fractions prevent PM-induced hyperpigmentation. (18, 19) Another possible pathway implicated in the anti-melanogenic property of Korean red ginseng extracts experimented in this study is by reduction of oxidative stress. It has been postulated that oxidative stress exerted by reactive oxygen species (ROS) activates certain members of MAPK pathway such as p38, JNK and ERK.(20) This subsequently leads to MITF nuclear translocation, eventually leading to increased melanin synthesis. Therefore, the anti-melanogenic property of both saponin and non-saponin fractions of Korean red ginseng extracts is suspected to be based on their anti-oxidant action at some degree.

We demonstrated that exposure to PM resulted in an increased level of matrix metalloproteinases (MMPs). In the skin, MMPs has a major function of extracellular matrix (ECM) degradation in the dermis, which is an essential process of ECM remodeling. In youthful skin, this remodeling process takes place in a fine balance between degradation and production of ECM, maintaining the amount of ECM in the dermis within a narrow homeostatic range. However, both increased ECM degradation by MMPs and decreased ability to regenerate the components of ECM result in reduced elasticity of the skin and skin laxity, which are the main signs of skin aging. Thus, the increase in MMPs observed after exposure to PM partly explains the association of PM exposure and signs of skin aging such as skin wrinkling. One of the pivotal findings of this study include the observation that Korean ginseng extracts can suppress this increase in MMP levels following PM exposure. Because MMP-induced dermal collagen fragmentation is directly affected by oxidative stress, we suspect that this phenomenon is based on the ability of Korean red ginseng

extracts to reduce oxidative stress caused by PM to epidermal keratinocytes.(21) Anti-oxidative effects were observed for both saponin and non-saponin fractions in our *in vitro* experiments. Likewise, the degree of MMP attenuation was greater for the non-saponin fractions. Another mechanism implicated in the suppression of MMPs by Korean red ginseng extracts is the modulation of inflammatory cytokines as observed in this study. Although regulation of MMP isoenzymes is a very complex process, pro-inflammatory cytokines including IL-1 and TNF-α are known to promote MMP production in response to various triggers.(22) Hence, we suggest that the observed decrease in MMPs by treatment with Korean red ginseng extracts involves both anti-oxidant and anti-inflammatory actions of saponin and non-saponin fractions.

The present study indicates that the constituents of Korean red ginseng extracts have different biological activities. Experiments were carried out separately for saponin fractions and non-saponin fractions, both of which are important active compounds of Korean red ginseng extracts. Although saponin fractions have been investigated in much detail, studies on non-saponin fractions are limited. This study indicates a preferential antioxidant effect of non-saponin fractions. Because all experiments were performed separately for saponin and non-saponin fraction, the possibility of a synergistic effect in terms of cutaneous protection from PM-induced damage needs to be considered. Further investigations are thus necessary to confirm this possibility.

Certain limitations of this study need to be addressed. First, in our *ex vivo* skin model, PM was applied on the skin in a liquid suspension form, which is a non-standard mode of contact. Reproducing the actual environmental exposure to PM requires a well-contained chamber as well as a nebulizer, and determining the concentration or amount of PM that adheres to the skin is a challenging process. The actual physiological reactions to PM exposure could not be identified in these experiments. Second, the exact composition of Asan PM was not defined. However, we suggest that its composition should not differ greatly from that used in the study by Jin et al.(16) considering the proximity of the collection sites (16 km apart). Lastly, although the ex vivo skin model is expected to closely reflect cutaneous physiology, systemic factors could not be thoroughly evaluated in this study owing to the intrinsic nature of the model. Therefore, future in vivo assessment of cutaneous exposure to PM is warranted.

In this study, we observed an increase in melanin production induced by exposure to PM. Moreover, this study provides evidence that Korean red ginseng extracts have a potent anti-oxidant and anti-melanogenetic effects on PM-exposed skin. Upon separate evaluation of saponin and non-saponin fractions, saponin fractions could inhibit PM-induced melanogenesis far better than non-saponin fractions, whereas non-saponin fractions showed significant anti-oxidant effects. As exposure to air pollutants including PM is virtually unavoidable, there is a need for strategies to protect the skin from the harmful effects of environmental exposure to pollutants. Future studies are thus required to determine the optimal mode of delivery as well as effective formulations to derive clinical benefits from Korean red ginseng extracts.

국문요약

배경 및 목적: 미세먼지로 불리는 대기 중에 부유하는 미세입자들은 호흡기계를 비롯해 대기와 직접적으로 접촉하는 인체 기관들에 다양한 건강문제를 야기한다. 기존의 연구들은 피부가 미세먼지에 노출되면 피부 내 활성산소가 증가하고 피부의 탄력 소실로 인한 주름의 생성과 흑자와 같은 과색소 병변의 발생이 증가함을 보였다. 이러한 미세먼지에 의한 악영향으로부터 피부를 보호하기 위한 다양한 방향의 연구들이 진행되고 있는데, 그 중에서 천연 유래 성분의 국소 도포 또는 복용에 대한 연구도 활발히 이루어지고 있다. 홍삼(Panax ginseng)에서 얻어지는 다양한 생물학적 유효성분들에 대한 연구가 활발하게 이루어지고 있으며, 산화 스트레스를 효과적으로 줄여준다는 연구 결과들이 존재한다. 또한 최근 Rg3와 Rf로 대표되는 사포닌분획 성분들은 피부의 멜라닌 생성과정의 조절에 관여하는 것으로 밝혀진 바 있다. 따라서 미세먼지에 의한 피부 노화와 색소변화에 대응할 수 있는 방법을 확인하기 위해 홍삼 유래 천연성분이 미세먼지에 노출된 피부 세포와 피부 조직에 미치는 영향을 알아보고자 한다.

재료 및 방법: 총 4가지 종류의 미세먼지를 이용해 실험을 진행했다. 이들은 모두 입자의 크기가 2.5-10 μm의 PM_{10}에 해당하는 미세입자들로 구성되어있으며, 정상 사람 표피 각질형성세포와 정상 사람 표피 멜라닌세포를 각각 따로 또는 공생배양하여 *in vitro* 실험을 진행했다. MTT 방법을 이용한 세포 독성 실험과 세포 내 활성산소를 측정하기 위해 DCF 시험을 실시했다. 실제 사람 피부 조직에서 미세먼지가 미치는 영향과 홍삼 유래 천연 성분의 역할을 확인하기 위해 *ex vivo* 실험을 진행했다. 멜라닌 생성 과정에 미세먼지와 홍삼 유래 천연 성분이 미치는 영향을 확인하기 위해 멜라닌 색소 측정 및 RT-PCR와 Western blot 방법을 이용했다.

결과: 100 μg/ml의 미세먼지 처리 후 각질형성세포의 생존력 저하와 산화 스트레스의 증가를 확인하였다. 이와 같은 미세먼지의 영향은 홍삼 유래 천연성분 중 사포닌 분획물 처리에 의해 효과적으로 역전되었다. 비사포닌 분획물은 미세먼지에 의한 산화 스트레스를 효과적으로 감소시켰으나 각질형성세포의 생존력을 증가시키지는 못했다. 각질형성세포와 멜라닌세포의 공생배양 모델에 미세먼지를 처리한 결과 멜라닌 색소

의 생산 증가를 확인했다. 이러한 멜라닌 증가가 사포닌 분획물 처리에 의해 역전되는 것을 확인했다. 또한 사포닌과 비사포닌 분획물 처리에 의해 미세먼지 처리 후 증가한 matrix metalloproteinase 농도가 감소하는 것을 확인했다.

결론: 홍삼 유래 천연성분은 피부의 미세먼지 노출에 의한 산화 스트레스 증가와 과다 멜라닌 색소 생성을 효과적으로 역전시킴을 보였다. 아울러 홍삼 유래 천연성분 처리를 통해 진피의 노화 과정에 중요한 matrix metalloproteinase의 증가로부터 피부를 보호할 수 있음을 확인할 수 있었다. 미세먼지에 의한 사회적 부담이 커지는 시대에 홍삼에서 유래한 천연성분들을 이용한 치료제의 개발이 가능 할 것으로 기대한다.

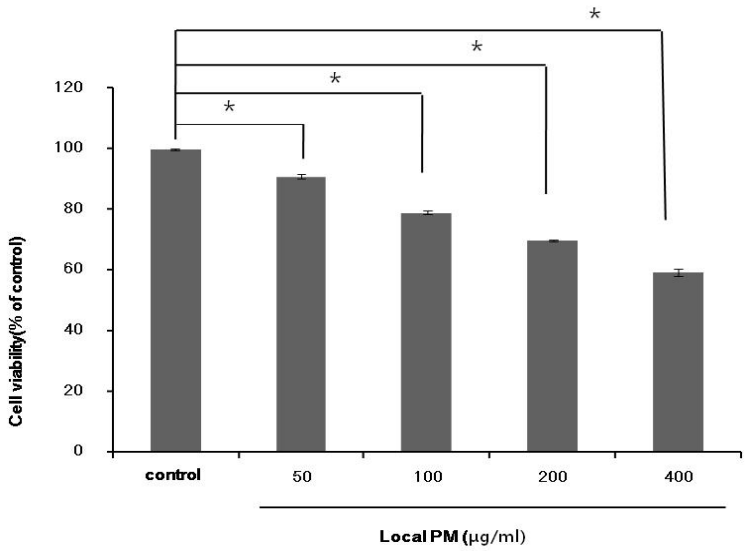
중요단어: Particulate matter, melanogenesis, skin aging, oxidative stress, Korea red ginseng

REFERENCES

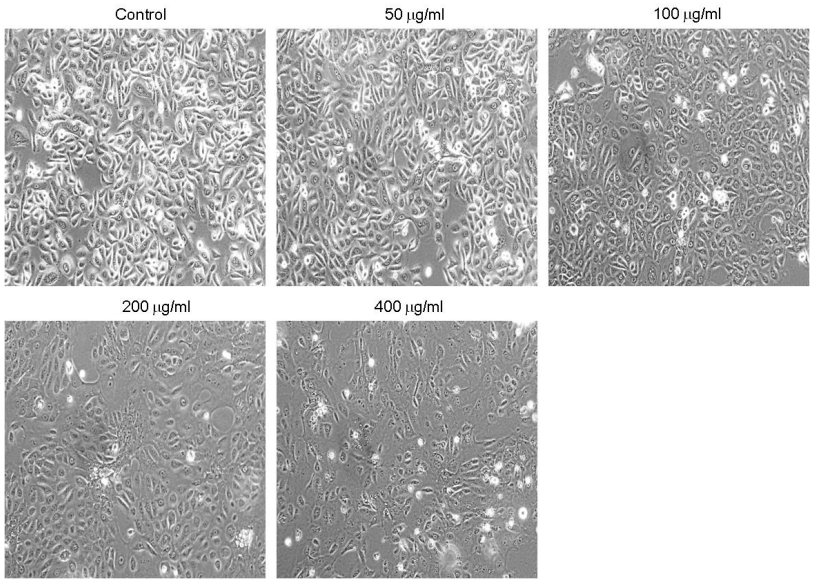
1. Huang YC, Li Z, Harder SD, Soukup JM. Apoptotic and inflammatory effects induced by different particles in human alveolar macrophages. Inhalation toxicology. 2004;16(14):863-78.
2. Soukup JM, Becker S. Human alveolar macrophage responses to air pollution particulates are associated with insoluble components of coarse material, including particulate endotoxin. Toxicology and applied pharmacology. 2001;171(1):20-6.
3. Li J, Tan G, Ding X, Wang Y, Wu A, Yang Q, et al. A mouse dry eye model induced by topical administration of the air pollutant particulate matter 10. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2017;96:524-34.
4. Vierkotter A, Schikowski T, Ranft U, Sugiri D, Matsui M, Kramer U, et al. Airborne particle exposure and extrinsic skin aging. J Invest Dermatol. 2010;130(12):2719-26.
5. Peng F, Xue CH, Hwang SK, Li WH, Chen Z, Zhang JZ. Exposure to fine particulate matter associated with senile lentigo in Chinese women: a cross-sectional study. J Eur Acad Dermatol Venereol. 2017;31(2):355-60.
6. Piao MJ, Ahn MJ, Kang KA, Ryu YS, Hyun YJ, Shilnikova K, et al. Particulate matter 2.5 damages skin cells by inducing oxidative stress, subcellular organelle dysfunction, and apoptosis. Arch Toxicol. 2018;92(6):2077-91.
7. Park SY, Byun EJ, Lee JD, Kim S, Kim HS. Air Pollution, Autophagy, and Skin Aging: Impact of Particulate Matter (PM10) on Human Dermal Fibroblasts. Int J Mol Sci. 2018;19(9).
8. Lee SJ, Lee WJ, Chang SE, Lee GY. Antimelanogenic effect of ginsenoside Rg3 through extracellular signal-regulated kinase-mediated inhibition of microphthalmia-associated transcription factor. Journal of ginseng research. 2015;39(3):238-42.
9. Kang HJ, Oh Y, Lee S, Ryu IW, Kim K, Lim CJ. Antioxidative properties of ginsenoside Ro against UV-B-induced oxidative stress in human dermal fibroblasts. Biosci Biotechnol Biochem. 2015;79(12):2018-21.
10. Yang X, Zou J, Cai H, Huang X, Yang X, Guo D, et al. Ginsenoside Rg3 inhibits colorectal tumor growth via down-regulation of C/EBPbeta/NF-kappaB signaling. Biomed Pharmacother. 2017;96:1240-5.
11. Li L, Wang Y, Qi B, Yuan D, Dong S, Guo D, et al. Suppression of PMA-induced tumor cell invasion and migration by ginsenoside Rg1 via the inhibition of NF-kappaB-dependent MMP-9 expression. Oncol Rep. 2014;32(5):1779-86.
12. Park GH, Park KY, Cho HI, Lee SM, Han JS, Won CH, et al. Red ginseng extract

promotes the hair growth in cultured human hair follicles. J Med Food. 2015;18(3):354-62.
13. Ahn H, Han BC, Kim J, Kang SG, Kim PH, Jang KH, et al. Nonsaponin fraction of Korean Red Ginseng attenuates cytokine production via inhibition of TLR4 expression. Journal of ginseng research. 2019;43(2):291-9.
14. Baek KS, Yi YS, Son YJ, Yoo S, Sung NY, Kim Y, et al. In vitro and in vivo anti-inflammatory activities of Korean Red Ginseng-derived components. Journal of ginseng research. 2016;40(4):437-44.
15. Fernando IPS, Jayawardena TU, Kim HS, Vaas A, De Silva HIC, Nanayakkara CM, et al. A keratinocyte and integrated fibroblast culture model for studying particulate matter-induced skin lesions and therapeutic intervention of fucosterol. Life Sci. 2019;233:116714.
16. Jin SP, Li Z, Choi EK, Lee S, Kim YK, Seo EY, et al. Urban particulate matter in air pollution penetrates into the barrier-disrupted skin and produces ROS-dependent cutaneous inflammatory response in vivo. J Dermatol Sci. 2018.
17. Peng F, Tsuji G, Zhang JZ, Chen Z, Furue M. Potential role of PM2.5 in melanogenesis. Environ Int. 2019;132:105063.
18. Prunieras M. Melanocytes, melanogenesis, and inflammation. Int J Dermatol. 1986;25(10):624-8.
19. D'Mello SA, Finlay GJ, Baguley BC, Askarian-Amiri ME. Signaling Pathways in Melanogenesis. Int J Mol Sci. 2016;17(7).
20. Darley-Usmar V. The powerhouse takes control of the cell; the role of mitochondria in signal transduction. Free Radic Biol Med. 2004;37(6):753-4.
21. Dasgupta J, Kar S, Liu R, Joseph J, Kalyanaraman B, Remington SJ, et al. Reactive oxygen species control senescence-associated matrix metalloproteinase-1 through c-Jun-N-terminal kinase. J Cell Physiol. 2010;225(1):52-62.
22. Sardy M. Role of matrix metalloproteinases in skin ageing. Connect Tissue Res. 2009;50(2):132-8.

Figure 1. Effect of particulate matter (PM) exposure on human keratinocyte viability. (A) Normal human epidermal keratinocytes were treated with various concentrations of Asan PM. MTT assay was performed 24 hours later. * $P < 0.05$ (B) Microscopic demonstration of the effects of PM on keratinocyte viability. Normal human epidermal keratinocytes were treated with various concentrations of local PM and photographs were taken 24 h later. Numbers on top indicate PM concentration in μg/ml. (original magnification x 100)

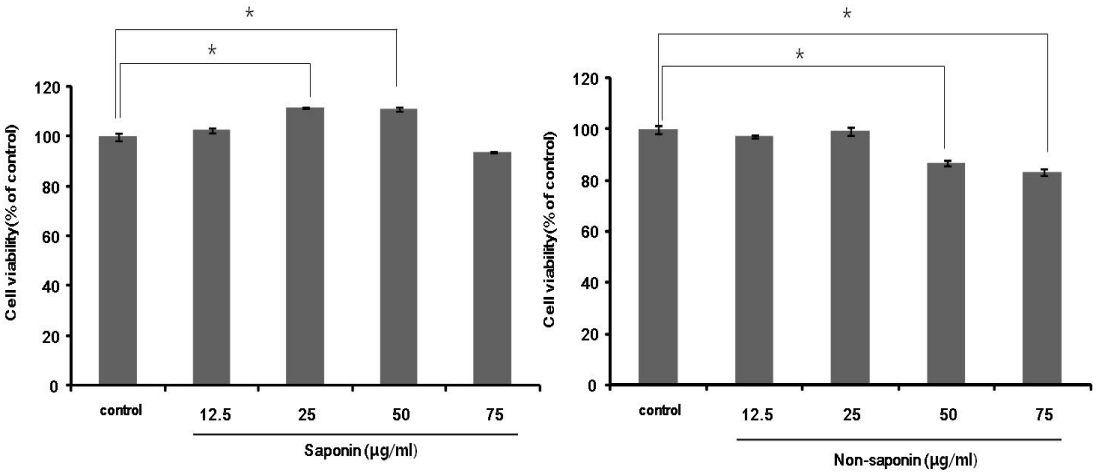


(A)



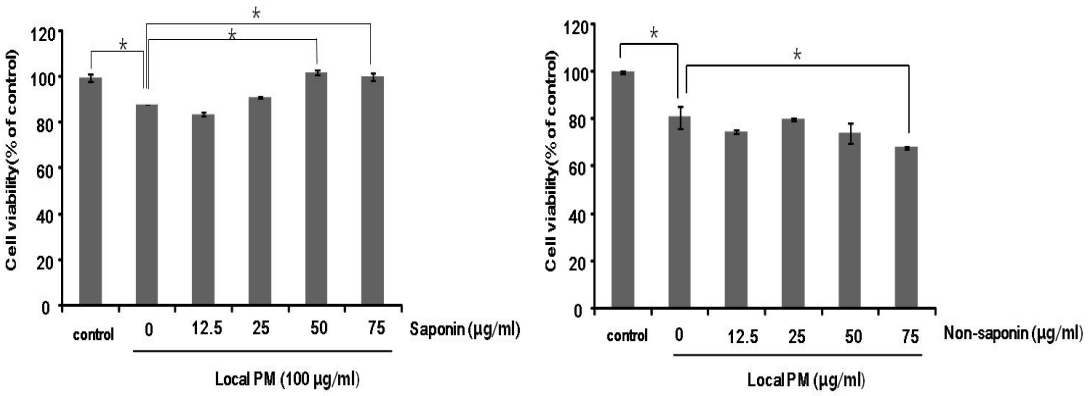
(B)

Figure 2. The effect of saponin and non-saponin fractions of Korean red ginseng extracts on keratinocyte viability. Normal human epidermal keratinocytes were treated with various concentrations of either (A) saponin or (B) non-saponin fractions. Cell viability was tested using MTT assay 24 h later. * $P < 0.05$



(A) (B)

Figure 3. Effects of concomitant treatment with 100 μg/ml of local PM and either (A) saponin or (B) non-saponin fractions on keratinocyte viability. * $P < 0.05$



(A) (B)

Figure 4. Change in intracellular oxidative stress following exposure to local PM. Normal human epidermal keratinocytes were treated with various concentrations of local PM and intracellular oxidative stress was quantified using CSF fluorescence. Hydrogen peroxide (H_2O_2) was used as a positive control. ** $P < 0.05$ in comparison to negative control

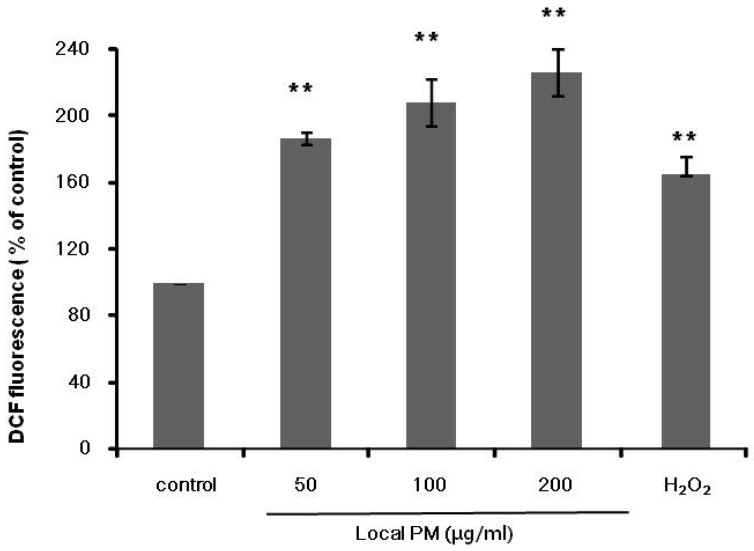


Figure 5. Change in intracellular oxidative stress following exposure to local PM and concomitant treatment with either saponin fractions or non-saponin fractions. Normal human epidermal keratinocytes were treated with 100 μg/ml of local PM and various concentrations of either saponin or non-saponin fractions. Hydrogen peroxide (H_2O_2) was used as a positive control. * $P < 0.05$

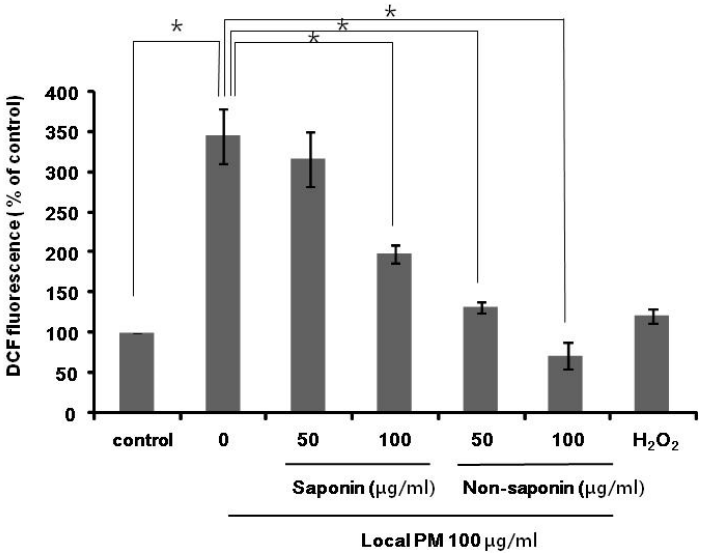


Figure 6. Change in intracellular oxidative stress after either pretreatment or simultaneous treatment with local PM and saponin or non-saponin fractions, N-acetyl-L-cysteine (NAC), and hydrogen peroxide (H_2O_2). * $P < 0.05$, ** $P < 0.01$ in comparison to negative control

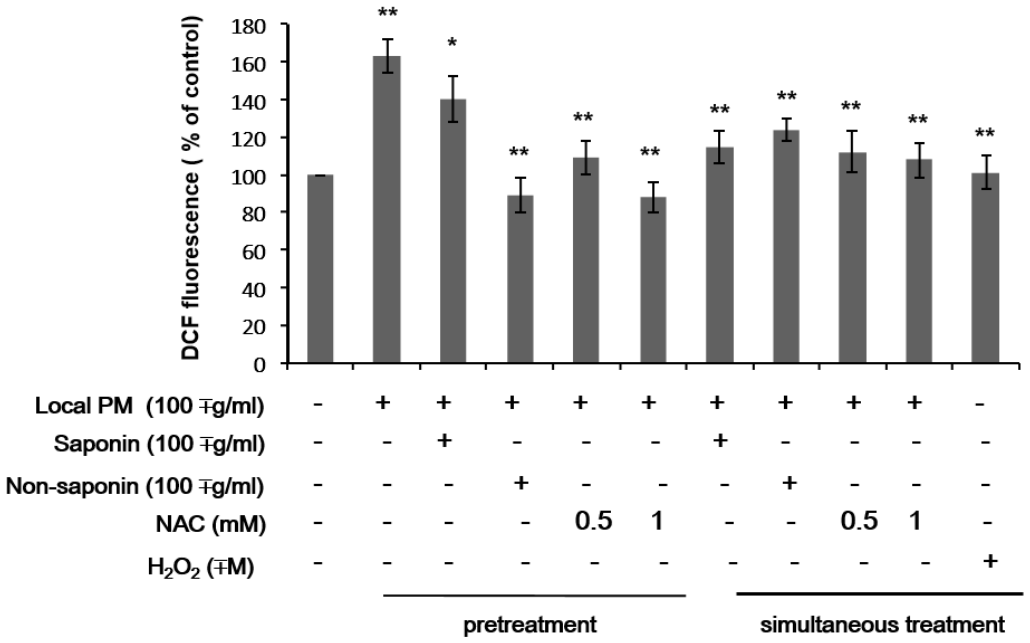


Figure 7. Effects of PM exposure on the melanin content of melanocyte. Melanocyte were exposed to various concentrations of local PM and the melanin content were measured on day 3.

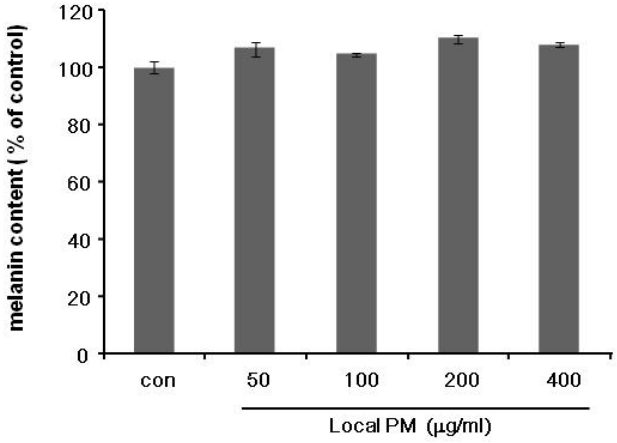


Figure 8. Effects of PM exposure on the melanin content of keratinocyte/melanocyte (1:5) coculture model. Cocultures were exposed to various concentrations of local PM and the melanin contents were measured at day 3. SCF/ET-1 (SE) treatment was used as the positive control. ** $P < 0.05$ in comparison to negative control

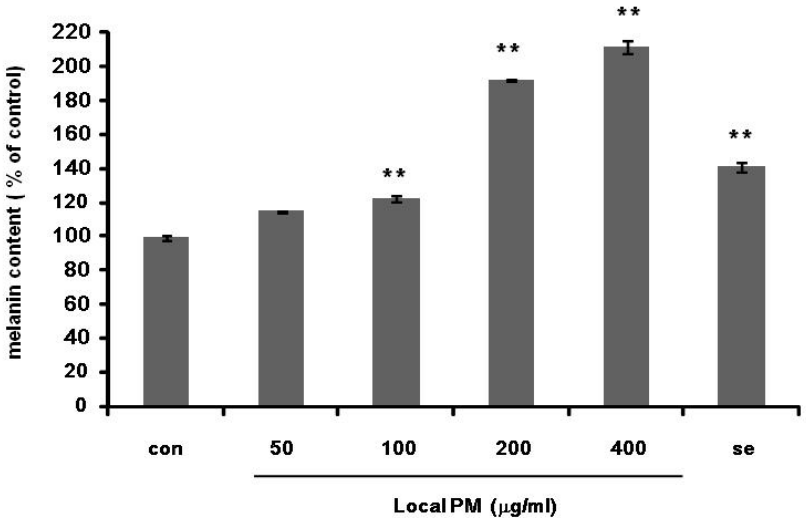
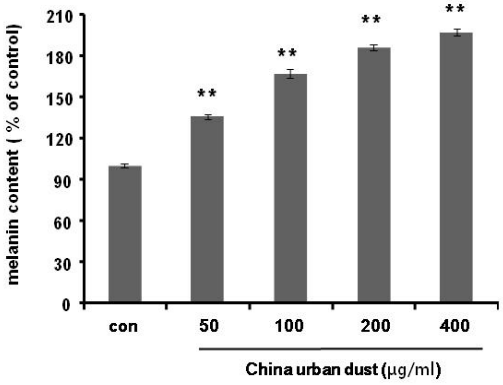
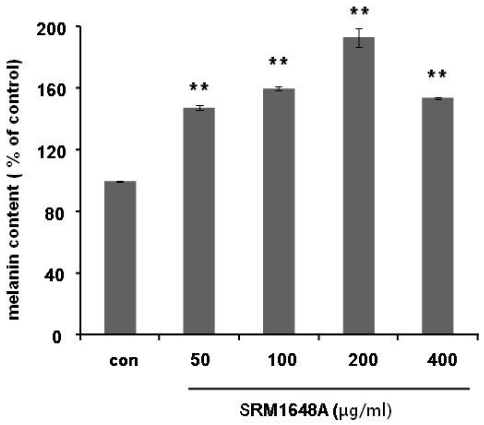


Figure 9. Effects of anotherPM exposure on the melanin content of keratinocyte/melanocyte (1:1) coculture model. (A) China urban dust (B) SRM1648A * P < 0.05

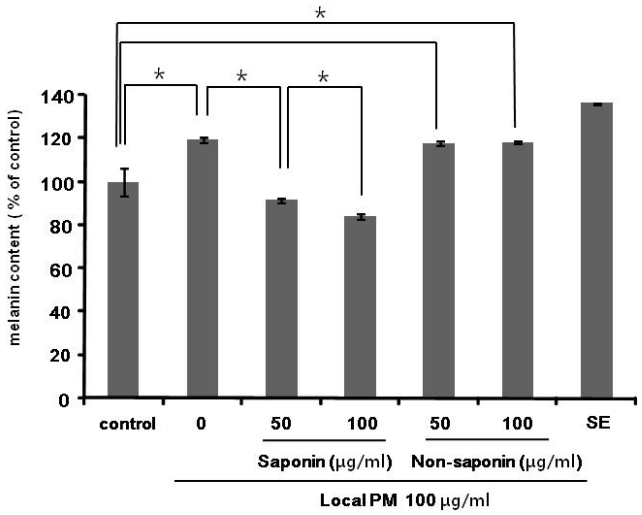


(A)

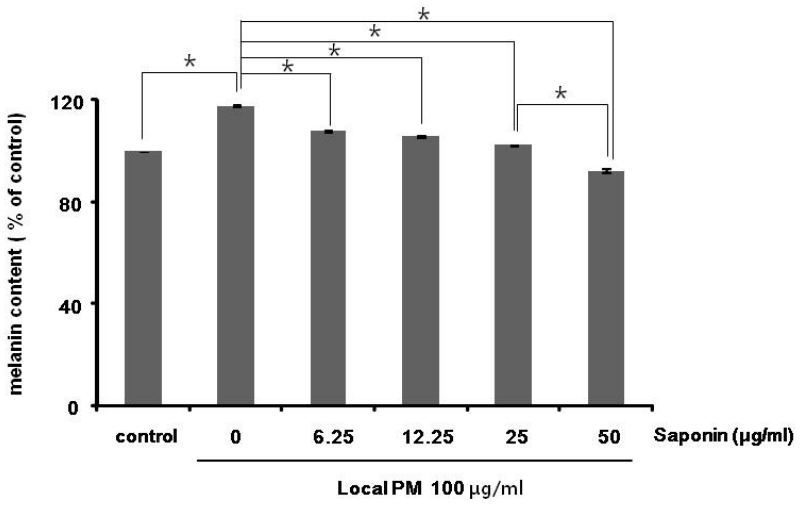


(B)

Figure 10. Attenuation of PM-induced increased melanin production by saponin and non-saponin fractions. Keratinocyte/melanocyte (1:1) coculture models were treated with 100 μg/ml of local PM and (A) either saponin or non-saponin fractions at different concentrations and (B) lower concentration of saponin fractions. Melanin contents were measured on day 3. SCF/ET-1 (SE) treatment was used as positive control. * $P < 0.05$



(A)



(B)

Figure 11. Western blotting showing changes in expression of MITF, tyrosinase and MMP-1 following exposure to 100 μg/ml of local PM in the absence and presence of saponin fractions. The numbers at the bottom indicate densitometry values.

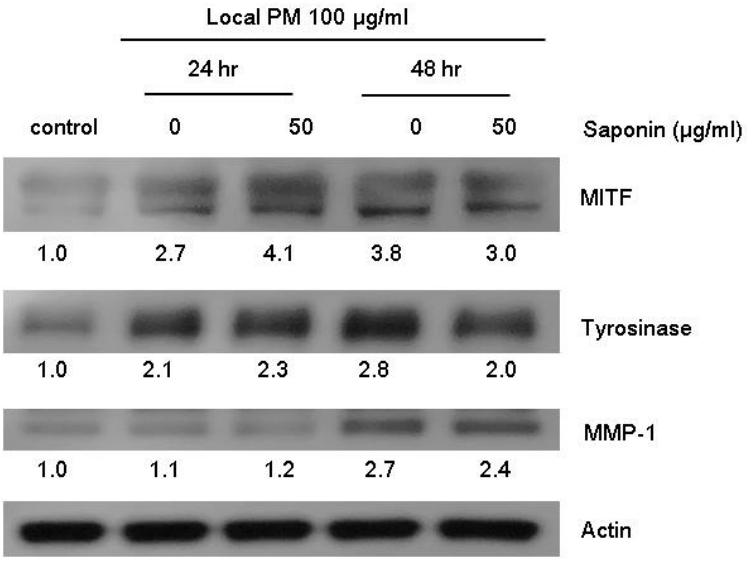
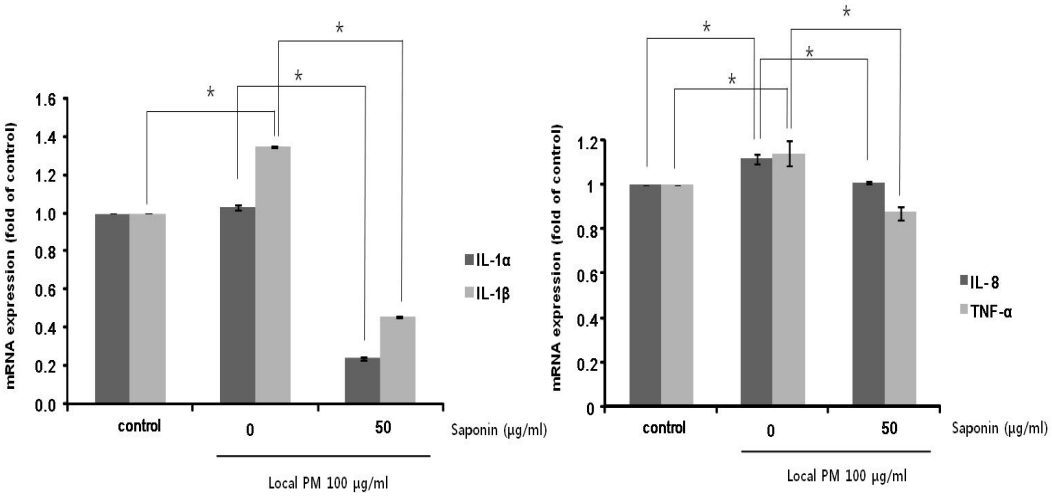


Figure 12. The effect of treatment with saponin fractions on the PM-induced increase in pro-inflammatory cytokine expression. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess mRNA levels of (A) IL-1α and IL-1β, and (B) IL-8 and TNF-α.



(A) (B)

Figure 13. Effects of saponin and non-saponin fractions on dermal matrix metalloproteinases (MMPs) after exposure to PM. Human dermal fibroblasts were incubated in conditioned media (CM) obtained from keratinocyte culture with or without 100 μg/ml of local PM. The effects of other ginsenoids (Rb2, RC, RE, RG3) and ursodeoxycholic acid (UDCA) were also investigated.