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Astragali Radix and its compound formononetin ameliorate diesel particulate matter-induced skin barrier disruption by regulation of keratinocyte proliferation and apoptosis

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Abstract

Ethnopharmacological relevance: Astragali Radix (AR), the root of *Astragalus mongholicus* Bunge, is widely applied in traditional medicine to promote skin health and tissue regeneration.

Aim of the study: This study investigated the effects of AR and its active compound, formononetin (FMT), on skin barrier defects in keratinocytes exposed to diesel particulate matter (PM).

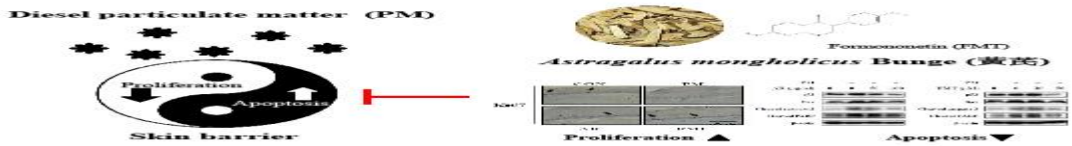
Materials and methods: HaCaT cells and three-dimensional (3D) human skin reconstructed model were pre-treated with AR (50, 100 μg/ml) and FMT (30, 50 μM), then treated with PM (200 μg/ml).

Results: AR and FMT significantly enhanced the expression of Keratin (KRT) 16 in PM stimulated HaCaT cells. PM increased p53 and Bax expression as well as the subsequent cleavage of caspase 3 and PARP in HaCaT cells, while this was inhibited by AR and FMT treatment. *In vitro* studies using the PM stimulated 3D human skin reconstructed model revealed that AR and FMT increased the

expression of KRT 16 and KRT 17. Histological examination of the 3D human skin reconstructed model showed that AR and FMT up-regulated the expression of Ki67, but down-regulated the expression of cleaved caspase 3. Both AR and FMT significantly inhibited phosphorylation of ERK, but not JNK and p38 MAPK in PM stimulated HaCaT cells.

Conclusions: These results suggest that AR and FMT act as anti-pollution agents and alleviate PM induced skin barrier defects through regulation of apoptosis and proliferation in keratinocytes.

Graphical Abstrcat:



Abbreviations:

AR, Astragali Radix; FMT, formononetin; PM, diesel particulate matter; CXCL-8, C-X-C motif ligand 8; CXCL-10, C-X-C motif ligand 10; ICAM-1, intercellular adhesion molecule 1; MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase

Key words:

Astragalus mongholicus Bunge; Formononetin; Diesel particulate matter; Skin barrier; Apoptosis; Proliferation

1. Introduction

Diesel particulate matter (PM) produced by internal combustion engines is believed to be a major constituent of atmospheric particulate matter in the environment (Mazzarella et al., 2007). Particulate

matter is a complex mixture of polycyclic aromatic hydrocarbons, metals, organic chemical compounds and other unidentified constituents (Guo et al., 2014). Exposure to PM can lead to serious health problems, such as pulmonary dysfunction, cancer, and cardiovascular disease (Lee et al., 2016; Li et al., 2017).

The main function of the skin is to act as a barrier, protecting the body against unwanted influences from the environment. Much of this barrier function is provided by the stratum corneum, the outermost layer of the skin. Disruption of the stratum corneum can be caused by disorders of barrier formation, infection, and prolonged inflammation (Wikramanayake et al., 2014). Recent study indicated that the effects of PM on keratinocyte proliferation and apoptosis are important to skin barrier disruption (Magnani et al., 2016; Pan et al., 2015). The balance between proliferation and apoptosis of keratinocytes is essential for maintenance of epidermal layers and stratum corneum formation. The rates of keratinocyte proliferation and apoptosis are well balanced under normal conditions, while ultraviolet (UV) radiation and other toxic stimuli can trigger apoptotic signaling pathways that lead to rapid cell death (Raj et al., 2006). Notably, results from *in vitro* and *in vivo* experiments have suggested that PM may induce keratinocyte cell death by overexpression of Bax or activation of the caspase cascade (Hu et al., 2017; Piao et al., 2018). Hence we evaluated the effects of standard reference PM issued by the National Institute of Standards and Technology (NIST) of the United States on keratinocyte apoptosis, proliferation and its underlying molecular mechanism (Chin et al., 1998; Upadhyay et al., 2003).

Astragali Radix (AR), the root of *Astragalus mongholicus* Bunge, is widely used in traditional medicine for tonifying immune response and promoting the growth of new tissues (Lee et al., 2017). Astragali Radix exerts potent cardioprotective effects through down-regulation of phospholamban phosphorylation and up-regulation of the myocardial cAMP content in isoproterenol induced myocardial injury in rats (Xu et al., 2008). The main compounds in AR are isoflavonoids (calycosin 7-O-β-glucoside, formononetin), triterpene saponins (astragaloside I, astragaloside IV) and polysaccharides (Lee et al., 2017). *In vivo* and *in vitro* studies have suggested that the isoflavonoids offer protection against wounds, UV radiation and age-related skin changes (Choi et al., 2007).

Additionally, formononetin (FMT) was reported to enhance bone regeneration in ovariectomized mice and in a drill-hole injury generated mouse model (Singh et al., 2017). Based on its protective effects in other organs and tissues, we predicted that AR containing FMT would exert beneficial effects on skin barrier disruption by PM. Therefore, the present study investigated the protective role and potential mechanisms of AR and FMT in prevention of skin disruption by PM stimulation.

2. Materials and methods

2.1. Plant materials.

Astragali Radix was purchased from Kyung Hee University Hospital (Seoul, Korea) and verified by Professor Heung-Mook Shin (College of Korean Medicine, Dongguk University, Gyeongju, Korea). A voucher specimen (2018-A-11) was deposited at the College of Korean Medicine, Dongguk University. Astragali Radix (15 g) was extracted with 200 ml of EtOH at 70°C for 3 h, after which the extract was filtered through Whatman #2 filter paper (Whatman International, Maidstone, UK), evaporated in a rotary vacuum evaporator, and freeze-dried (FD8508S, Busan, Korea) (yield 7.33% w/w). The dried material obtained (AR) was dissolved in distilled dimethyl sulfoxide and sterilized by passing it through a 0.22 μm syringe filter (Millipore, Burlington, MA, USA) before use (Table 1).

.Table 1. Plant extracts used in the present study. Scientific name, used part, yield, quantification of formononetin content by HPLC

Latin name	Scientific name	Used part	Yield	Formononetin Content
Astragali Radix	*Astragalus mongholicus* Bunge	Root	7.33%	0.191 μg/mg

2.2. Chemicals and reagents.

High glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Welgene Inc. (Gyeongsangbuk, Korea). The serum and reagents for cell culture were obtained from Invitrogen Inc. (Carlsbad, CA, USA). The 3D skin reconstructed model (NeodermED) was purchased from Tego Science Inc. (Seoul, Korea). The standard reference material diesel particulate matter SRM 2975 was

obtained from the NIST (Gaithersburg, MD, USA). Human CXCL-8, CXCL-10, intercellular adhesion molecule (ICAM)-1, and ELISA kits were purchased from Koma Biotech Inc. (Seoul, Korea). The antibodies used for the analyses included anti-p-ERK, p-JNK, p-p38, PARP, and cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA), anti-loricrin, involucrin, KRT 16, KRT 17, and Ki67 (Abcam, Cambridge, MA, USA), anti-Bax, p53 (Santa Cruz Biotech, Paso Robles, CA, USA), anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), and Bcl-2 (Oncogene Research Products, La Jolla, CA, USA).

2.3. HPLC analysis.

Formononetin was purchased from ChemFaces Biochemical Co. Ltd. (Wuhan, China), and its purity was determined to be ≥ 98% by HPLC. The chemical structure of FMT is shown in Fig. 1A. The ethanol extract of AR analysis and quantification of the FMT were performed using a HPLC 1290 system (Agilent, Santa Clara, CA, USA) at the Korea Basic Science Institute (Seoul, Korea). The ethanol extract of AR (10 μl) was separated on a Poroshell 120 SB-C18 column (3.0 × 100 mm, 2.7 μm, Agilent) at a flow rate of 0.5 ml/min. Mobile phase A and B consisted of 0.1% formic acid and 0.1% formic acid in acetonitrile, respectively. The gradient was as follows: 5% (B) for 5 min, 5–90% (B) for 15 min, and equilibration for 5 min. Formononetin was detected at 248 nm and the column temperature was 40°C for elution of this compound.

2.4. 3D human skin reconstructed model culture and treatments.

NeodermED (Tegoscience, Seoul, Korea) is a commercially available 3D human skin reconstructed model in which keratinocytes and dermal fibroblasts are 3-dimensionally cultured to mimic the morphology and physiology of human skin (Lee et al., 2015; Park et al., 2015). The 3D human skin reconstructed model was placed in 12-well plates containing 1 ml of the medium provided by the manufacturer. Next, the 3D skin reconstructed model was stimulated with PM at 200 μg/ml every 2 days for 6 days. For therapy, the 3D skin reconstructed model was treated with AR (50, 100 μg/ml) or FMT (30, 50 μM). The model was then incubated at 37°C under 5% CO_2 atmosphere for 6 days, during which time the medium was changed every two days. At the end of the experiment,

reconstructed skin tissue was fixed in 4% paraformaldehyde and embedded in paraffin for serial sectioning (5 μm). The processed sections were subsequently stained with hematoxylin and eosin (H/E). For immunohistochemistry, sections were incubated with anti-Ki67 and cleaved caspase 3 for 72 h in a 4°C humidified chamber. Next, the sections were linked with the appropriate secondary antibody for 24 hours at room temperature. Finally, the slides were treated with an avidin-biotin complex kit (Vector Lab, Burlingame, CA, USA) and then developed with 0.05 M Tris-HCl buffer solution (pH 7.4) that consisted of 0.05% 3,3'-diaminobenzidine and 0.01% HCl, after which they were counterstained with hematoxylin. All sections were examined with a digital camera (Olympus UC30, Tokyo, Japan) mounted on a phase contrast microscope (Olympus CK40-32PH, Tokyo, Japan) using the DIXI image solution 2.89 software (DIXI Optics, Daejeon, Korea).

2.5. Cell culture and treatments.

HaCaT cells (a human keratinocyte cell line) were maintained in high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown at 37°C and 5% CO_2 in a humid environment. During incubation, the culture medium was changed every 2 days. The cells were made quiescent when the confluence was 70–80% by starvation in serum-free medium for 24 h, after which they were treated with different concentrations of AR or FMT.

2.6. Cell toxicity and proliferation assays

The cytotoxic effects of AR or FMT on HaCaT cells were examined using XTT assays. After treating cells with AR (50, 100 μg/ml) or FMT (30, 50 μM) for 24 h, 50 μl of XTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 4 h. The absorbance was then measured at 450 nm (using a reference wavelength of 670 nm) using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Proliferation of HaCaT cells was determined using a 5-bromo-2'-deoxy-uridine (BrdU) proliferation assay kit (Cell Biolabs, San Diego, CA, USA), which measured DNA synthesis. Cells were pretreated with the AR (50, 100 μg/ml) or FMT (30, 50 μM) for 1 h, then

stimulated with PM (200 μg/ml) for 24 h. These cells were incubated with 10 μl of BrdU-labeling solution for 4 h, after which the absorbance was measured at 450 nm using a microplate reader.

2.7. Enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were pre-incubated with AR (50, 100 μg/ml) or FMT (30, 50 μM) for 1 h, then stimulated with PM (200 μg/ml) for 24 h. The 3D skin reconstructed model was treated with AR (50, 100 μg/ml) or FMT (30, 50 μM) for 1 h, then stimulated with PM at 200 μg/ml every 2 days for 6 days. The levels of human CXCL-8, CXCL-10, and ICAM-1 in conditioned medium were determined using commercial kits according to the manufacturer's protocols. Absorbance was measured at 450 nm using an automated microplate reader (Molecular Devices, San Jose, CA, USA).

2.8. Western blot analysis.

HaCaT cells and 3D skin tissues were lysed with tissue extraction reagent (Thermo Fisher Scientific, Waltham, IL, USA). The protein was then extracted by centrifuging at 8,000 × g for 15 min at 4°C, after which the supernatant was collected. Next, 25–50 μg of proteins were resolved by 10–12% SDS-PAGE electrophoresis, then transferred onto polyvinylidene difluoride membranes (PVDF) (Merck Millipore, Carrigtwohill, Ireland). The membranes were subsequently blocked with 5% skim milk in 1× PBS for 2 h at room temperature, then incubated with primary antibodies followed by secondary antibody horseradish peroxidase-conjugated anti IgG. All membranes were detected by enhanced chemiluminescence (BioRad, Hercules, CA, USA) and the band intensities of proteins were quantified using GelPro V3.1 software (Media Cybernetics, Rockville, MD, USA).

2.9. Statistical analysis.

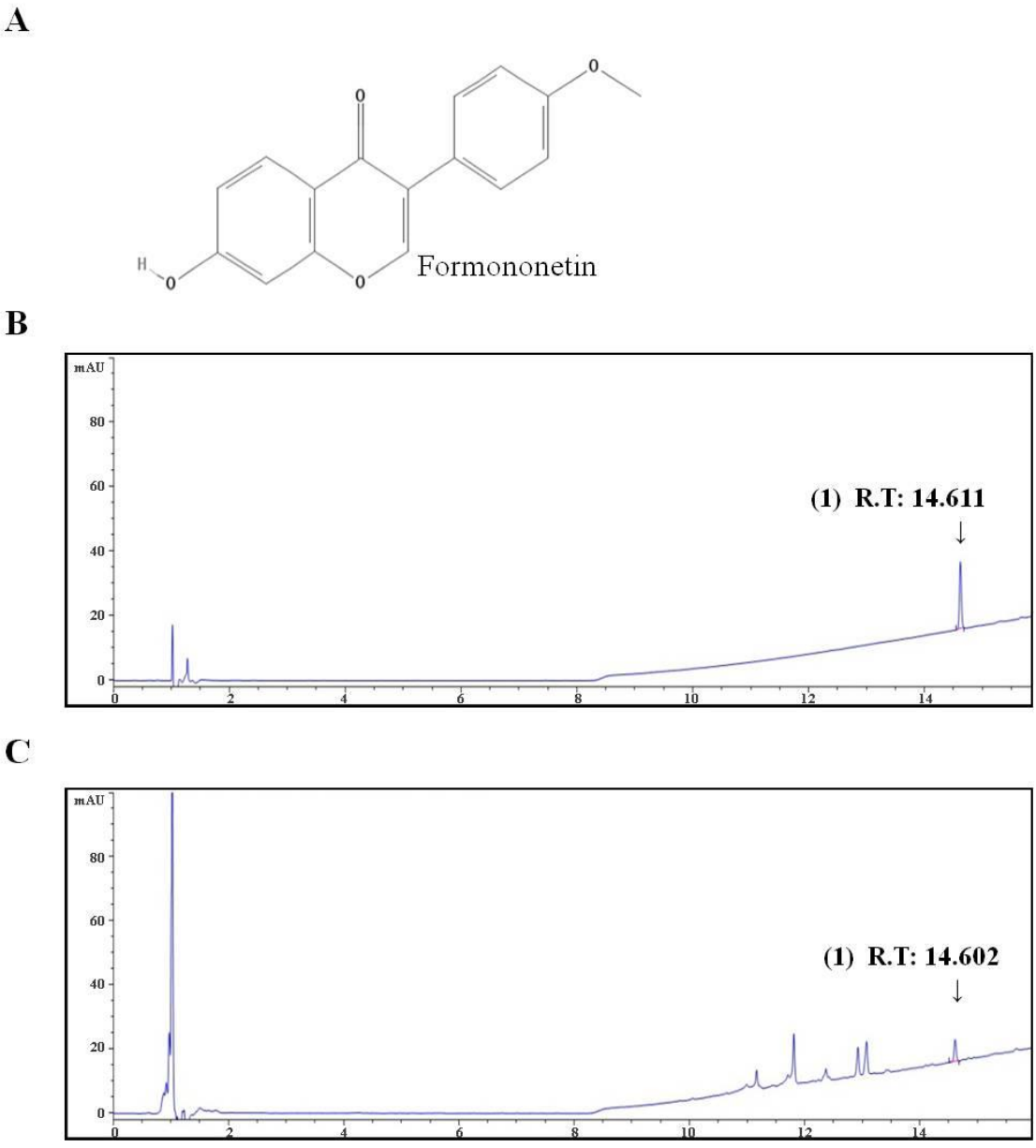
All analyses were performed using data from at least three independent experiments performed in duplicate. Data were reported as means ± SEM. Groups were compared by unpaired Student's t-test, with a p-value < 0.05.

3. Results

3.1. Quantitative determination of the FMT in ethanol extraction of AR.

The FMT in the ethanol extract of AR was determined by HPLC, and representative chromatogram patterns of the standard compounds and extract are shown in Fig. 1. The retention time of FMT was approximately 14.602 min (Fig. 1C), and the concentration in AR was 0.191 μg/mg (Table 1).

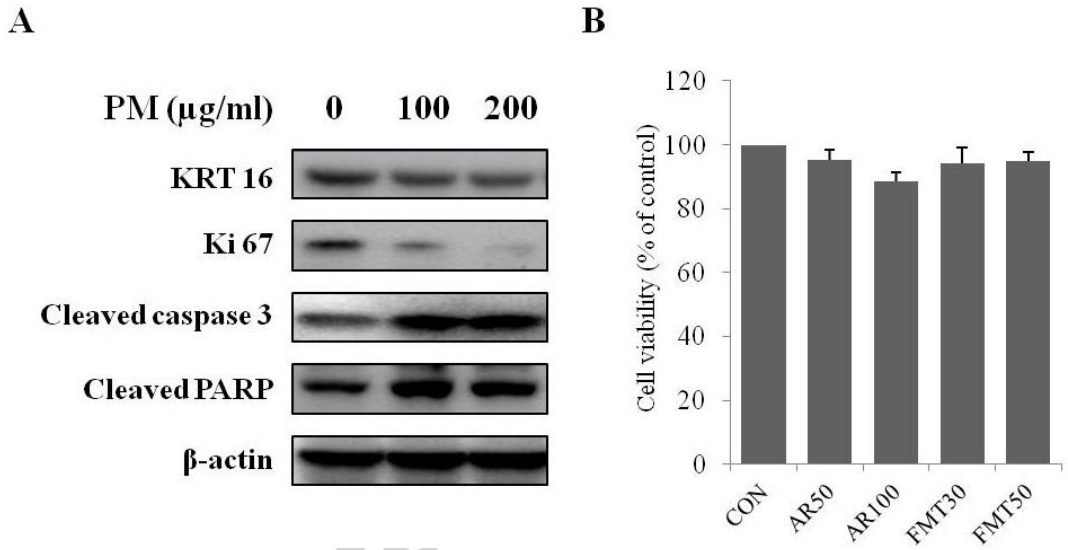
Figure 1.



3.2. Effects of AR and FMT on keratinocyte proliferation and differentiation in PM stimulated HaCaT cells.

To examine the effects of PM on proliferation and apoptosis of keratinocytes, HaCaT cells were stimulated with PM. As shown in Fig. 2A, 200 μg/ml of PM markedly decreased expression of KRT 16 and Ki67 and increased expression of cleaved caspase 3 and PARP. Hence, subsequent experiments were performed at this concentration of PM that inhibited proliferation and promoted apoptosis of keratinocytes. The XTT assay revealed that AR (50 and 100 μg/ml) and FMT (30 and 50 μM) did not affect HaCaT cell viability; therefore, all experiments were performed within that range (Fig. 2B).

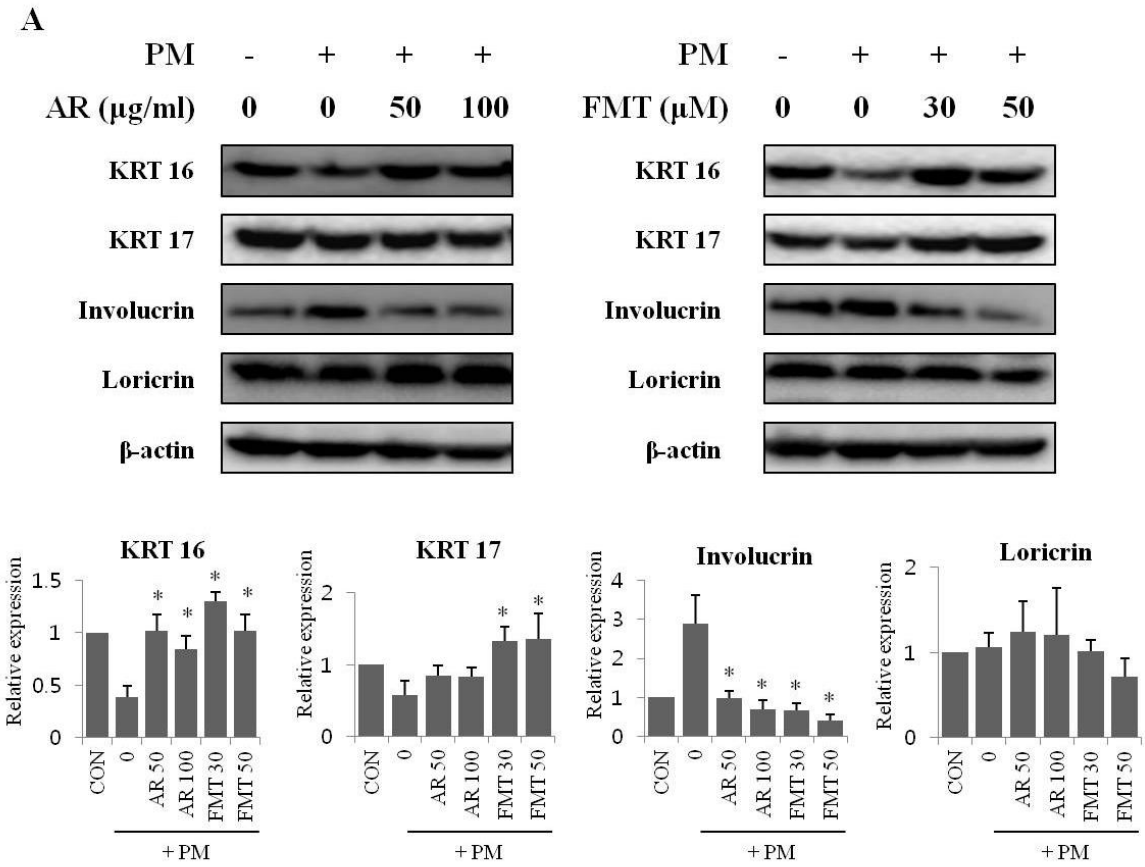
Figure 2.

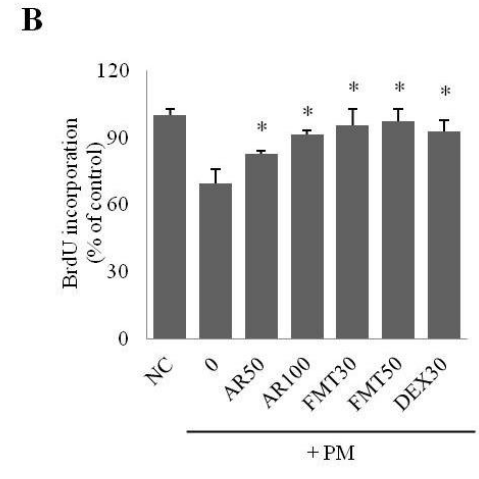


Treatment with AR and FMT significantly increased the expression of KRT 16 in PM stimulated HaCaT cells. Moreover, PM stimulation of HaCaT cells reduced KRT 17 expression, but addition of FMT significantly increased the KRT 17 expression. AR exerted no significant effect on KRT 17 expression when compared with cells treated with PM alone (Fig. 3A). To further evaluate whether AR and FMT may restore the cell proliferation, BrdU incorporation assay was performed. PM treatment caused a significant reduction in HaCaT cells proliferation in comparison with vehicle-treated cells. Treatment of HaCaT cells with AR and FMT significantly restored the cell proliferation, which had been decreased by PM (Fig. 3B). Next we assessed the effects of AR and FMT on the keratinocyte differentiation markers, involucrin and loricrin, in PM stimulated HaCaT cells. PM stimulation increased involucrin expression, but pre-treatment with AR and FMT decreased involucrin

expression in HaCaT cells. Loricrin expression was not altered by PM stimulation, and neither AR nor FMT had a significant effect on loricrin expression in PM stimulated HaCaT cells (Fig. 3A).

Figure 3.



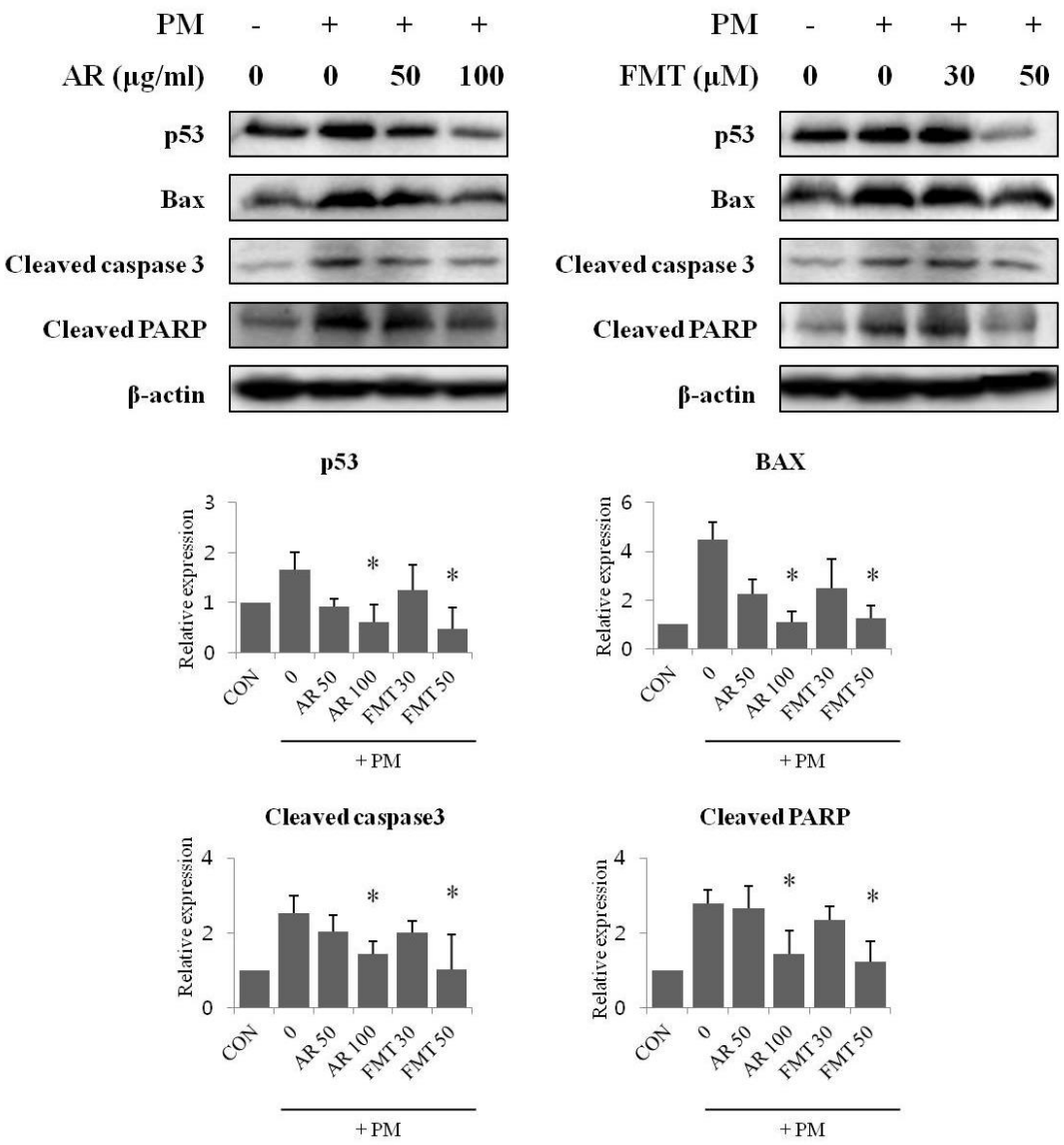


3.3. Effects of AR and FMT on apoptotic protein expression in PM stimulated HaCaT cells.

Given that AR and FMT increased proliferation marker expression, we next investigated the inhibitory effects of AR and FMT on PM induced apoptosis of HaCaT cells. As shown in Fig. 3C, PM stimulated HaCaT cells significantly increased p53 and Bax expression levels compared with vehicle-treated cells. In contrast, AR (100 μg/ml) and FMT (50 μM) suppressed PM induced p53 and Bax expression when compared to cells treated with PM alone. Along with these results, PM stimulation significantly increased expression of cleaved caspase 3 and cleaved PARP when compared with vehicle in HaCaT cells. In contrast, AR (100 μg/ml) and FMT (50 μM) not only reduced cleaved caspase 3, but also blocked PARP cleavage (Fig. 3C).

Figure 3.

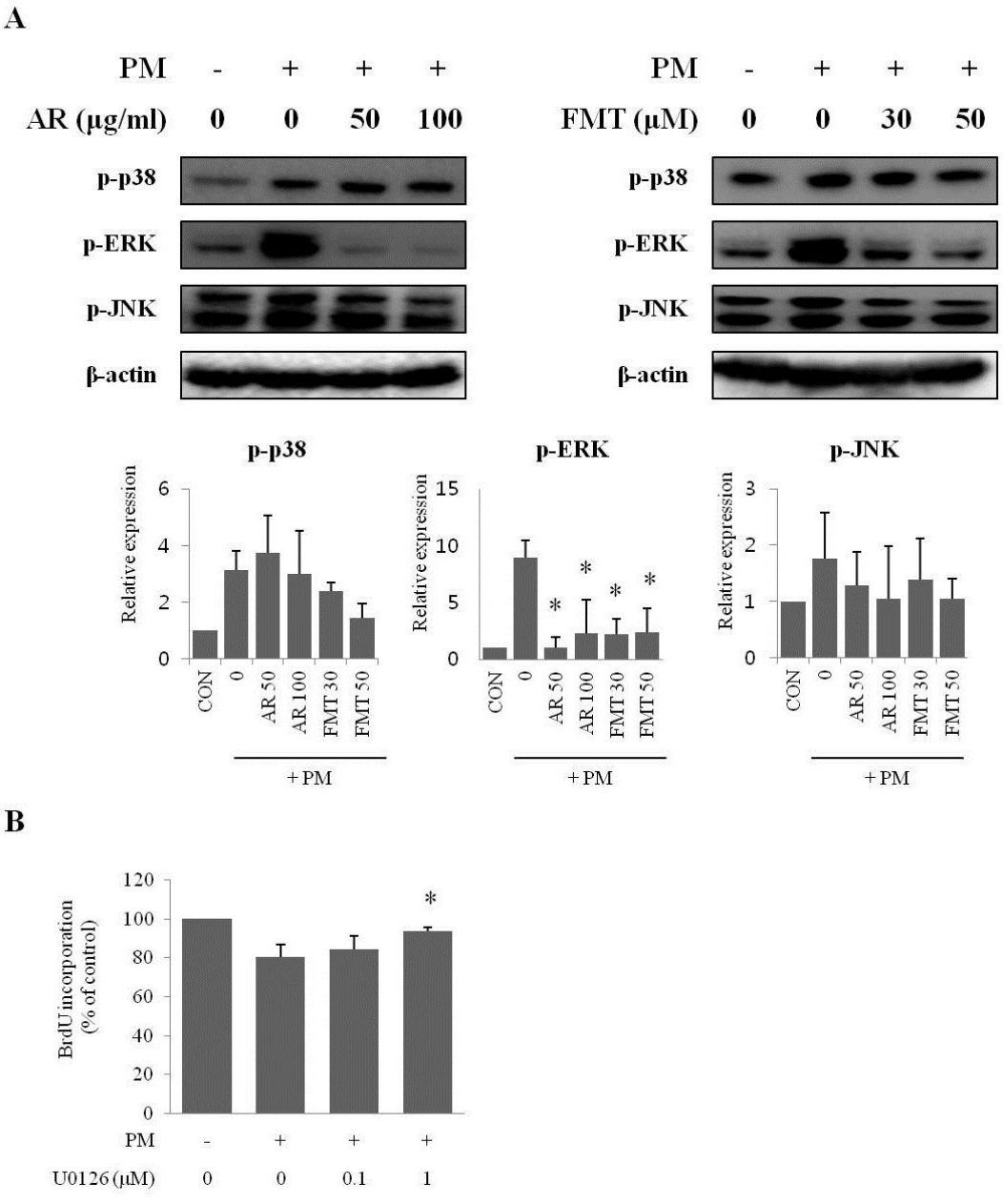
C



3.4. Effects of AR and FMT on the MAPK pathway in PM stimulated HaCaT cells.

To understand the molecular mechanism underlying the effects of AR and FMT on PM induced apoptosis, we examined the MAPK signaling pathway, which is known to be activated in response to PM stimulation (Chin et al., 1998). We evaluated the phosphorylation of ERK, JNK and p38 in HaCaT exposed to PM. AR and FMT significantly suppressed PM induced phosphorylation of ERK in HaCaT cells (Fig. 4A). To further confirm the role of ERK, we added U0126, a specific inhibitor of ERK in PM stimulated HaCaT cells. U0126 (1 μM) significantly restored the cell proliferation, which had been decreased by PM (Fig. 4B).

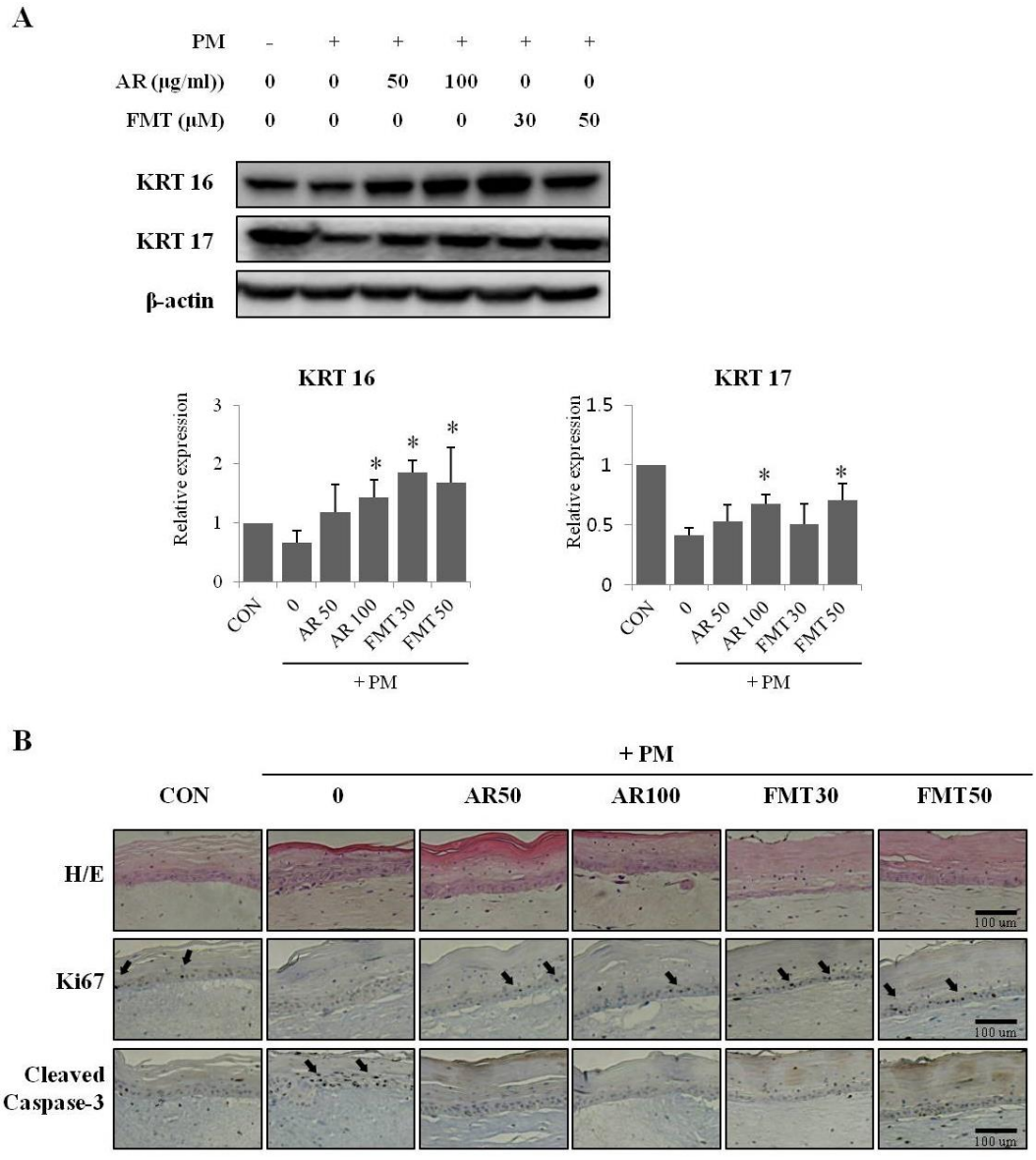
Figure 4.



3.5. Effects of AR and FMT on PM stimulated 3D human skin reconstructed model.

We next investigated the effects of AR and FMT on keratinocyte proliferation in the PM stimulated 3D human skin reconstructed model. After exposure to PM for 6 days, the proliferation of keratinocytes was impaired as measured by the reduced expression of KRT 16 and KRT 17. In contrast, AR (100 μg/ml) and FMT (50 μM) treatment increased KRT 16 and KRT 17 expression in the PM stimulated 3D human skin reconstructed model (Fig. 5A). Immunohistochemical observation revealed that AR and FMT treatment increased Ki67 expression in the PM stimulated 3D human skin reconstructed model (Fig. 5B). Additionally, PM stimulation significantly increased the expression of cleaved caspase 3, while its expression was decreased by AR and FMT treatment (Fig. 5B).

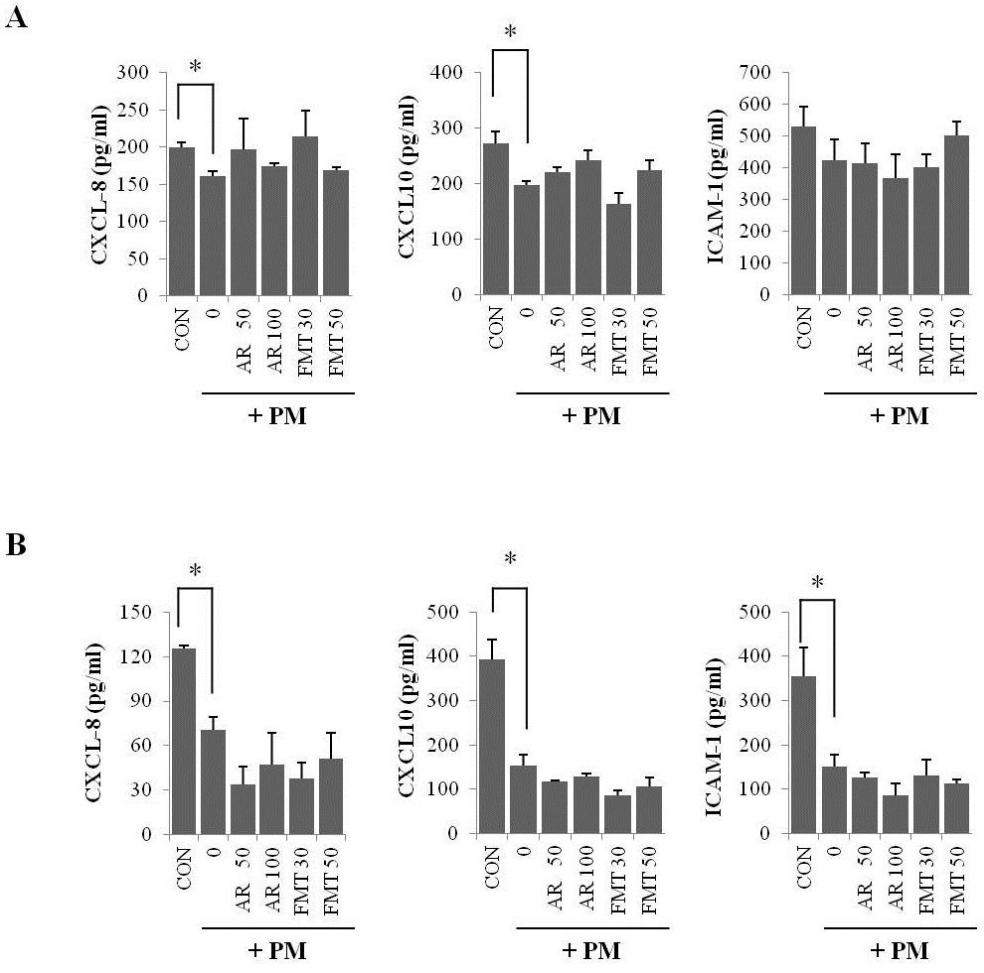
Figure 5.



3.6. Effects of AR and FMT on inflammatory chemokine and adhesion molecule production in PM stimulated 3D human skin reconstructed model and HaCaT cells.

Previous studies demonstrated that PM induced skin barrier disruption via high production of PGE2, a key mediator of the inflammatory process (Lee et al., 2016); therefore, the present study was conducted to identify additional associations between pro-inflammatory chemokine/adhesion molecule and skin barrier disruption in a 3D human skin reconstructed model. Based on ELISA, the PM stimulation significantly decreased production of CXCL-8 and CXCL-10 and moderately decreased production of ICAM-1 (Fig. 6A). To confirm the effects of PM on pro-inflammatory chemokine/adhesion molecule production, HaCaT cells were stimulated with PM. As shown in Fig. 6B, treatment with 200 μg/ml of PM reduced production of CXCL-8, CXCL-10, and ICAM-1 in HaCaT cells (Fig. 6B). However, the decreased levels of these cytokines were not significantly affected by treatment with AR and FMT (Fig. 6).

Figure 6.



4. Discussion

Epidermal keratinocytes are specialized epithelial cells that form a tight barrier for protecting against toxins and infectious microbes entry into the body. Skin barrier morphogenesis requires a balance between apoptosis and proliferation of keratinocytes. In the normal skin barrier developmental program, keratinocytes rapidly proliferate and undergo a program of apoptosis-driven terminal differentiation. However, toxic chemicals or physical stimuli alter balance between apoptosis and proliferation, enhancing apoptosis and suppressing proliferation of keratinocytes, ultimately leading to delayed wound healing and skin repair (Raj et al., 2006).

AR has been traditionally used for tonifying qi and strengthening the body (Wu et al., 2016). Experimental studies have also shown that AR has a positive effect on tissue regeneration and fibroblast proliferation (Lau et al., 2012). The results of the present study showed that AR and its active compound, FMT, increased the expression of Ki67, a nuclear protein that serves as a marker of proliferation in PM stimulated 3D skin reconstructed models. Keratins (KRT 16, KRT 17), another marker of proliferation, are filament proteins responsible for the mechanical integrity of keratinocytes (Pan et al., 2015). We showed that PM reduced the expression of KRT 16 in both PM stimulated HaCaT cells and the 3D skin reconstructed model. However, AR (100 μg/ml) and FMT (50 μM) significantly enhanced the expression of KRT 16 in PM stimulated HaCaT cells and the 3D skin reconstructed model. Moreover, FMT (50 μM) significantly increased KRT 17 expression in PM stimulated HaCaT cells and the 3D skin reconstructed model. FMT is an isoflavone that has been shown to display estrogenic properties and exhibit many biomedically useful properties, such as angiogenesis and wound healing activities (Huh et al., 2011; Li et al., 2015). Therefore, FMT may contribute to the therapeutic effects of AR in PM stimulated skin keratinocytes.

Apoptosis is a specific process that leads to cell death under various pathological conditions. The activation of effector caspases such as caspase 3 leads to downstream cleavage of nuclear substrates, including PARP. Activation of Bax by p53 also mediates mitochondrial membrane permeabilization and apoptosis. Importantly, such apoptotic activity was completely abolished by AR (100 μg/ml) and FMT (50 μM) in PM stimulated HaCaT cells. Moreover, AR and FMT decreased the expression of cleaved caspase 3 in a 3D skin reconstructed model based on IHC analysis. These findings

demonstrate that AR and FMT may have a positive impact on the prevention of PM induced skin disruption by inhibiting apoptosis and promoting proliferation of keratinocytes.

Keratinocyte differentiates from cuboidal shaped cells to flat, anucleated cells to create a cornified cell envelope structure (Xu et al., 2013). This cornified cell envelope structure, which is the outermost layer of the skin, provides a physical barrier against the external environment and epidermal water loss. The main process in the formation of the cornified cell envelope is expression of cornified cell envelope precursor proteins and cross-linking of these proteins by transglutaminase (Ishida Yamamoto and Iizuka, 1998). Involucrin and loricin are major precursor proteins to the cornified cell envelope expressed in keratinocyte differentiation. Involucrin, which is adjacent to the cell membrane, forms the exterior surface of the cornified cell envelope. Loricrin functions as the main reinforcement protein for the cornified cell envelope and is deposited onto a scaffold of involucrin (Kim et al., 2008).

Previous studies of differentiation markers in HaCaT cells stimulated by PM produced inconsistent results (Lee et al., 2016; Li et al., 2017); hence, the present study further confirmed the effects of PM on the expression of epidermal differentiation marker protein in HaCaT cells. In the present study, we found that PM stimulation increased the level of loricrin expression, but did not induce significant changes in involucrin expression levels as compared to control cells. Our experimental results are consistent with those of a previous report showing that PM increased the level of loricrin expression (Li et al., 2017). Taken together, these results suggest that PM has very little or a minimal effect on the differentiation of keratinocytes.

MAPK is involved in apoptosis induced by PM (Cao et al., 2016; Chin et al., 1998). Specifically, MAPK increases apoptosis rates via regulation of Bax and caspase 3 in PM stimulated cardiomyocyte cells (Cao et al., 2016). Increased apoptosis was also shown to be accompanied by ERK phosphorylation in PM stimulated macrophages (Chin et al., 1998). To investigate the mechanism of action of AR and FMT on PM induced apoptosis, we examined the effects of AR and FMT on ERK, JNK and p38 phosphorylation. Astragali Radix and FMT significantly suppressed ERK phosphorylation in PM stimulated HaCaT cells, suggesting that AR and FMT exerts anti-apoptotic effects through the suppression of ERK phosphorylation. Using a specific inhibitor of ERK increased BrdU-positive cells in PM stimulated HaCaT cells, indicating that PM induced apoptosis through

ERK.

Inflammation itself may be able to induce a functional skin barrier disruption and thereby aggravate the eczematous reaction (Hänel et al., 2013). Previous studies have reported that PM induced COX-2 expression and PGE2 production were the prominent mechanisms in skin barrier disruption, but the associations between specific chemokines/adhesion molecules and PM induced skin barrier disruption remain unclear (Lee et al., 2016). Although PM is expected to increase chemokines/adhesion molecules production, PM reduced the production of CXCL-8 and CXCL-10 in both the 3D human skin reconstructed model and HaCaT cells. Additionally, PM significantly reduced the production of ICAM-1 in HaCaT cells. Our results indicated that defects in the skin barrier are not primarily linked to chemokines and adhesion molecules production. These findings are consistent with those of another report showing that PM can suppress the production of pro-inflammatory chemokines such as CXCL-8 and MCP-1 in human epithelial cells (Becker et al., 2005; Gioda et al., 2011). Further investigations are needed to gain a better understanding of immune response correlated to PM exposure; nevertheless, the results presented herein provide evidence that skin barrier disruption by PM may be induced through mechanisms other than up-regulation of the production of chemokines and adhesion molecules in keratinocytes.

5. Conclusions

AR and FMT increased expression of the proliferation marker proteins in a PM-stimulated HaCaT cells and 3D human skin reconstituted model. Moreover, treatment with AR and FMT inhibited apoptosis in keratinocytes. These results also showed both AR and FMT treatment to down-regulate the ERK1/2 phosphorylation in PM-stimulated HaCaT cells. Taken together, the results of this study suggest that AR and FMT act as anti-pollution agents and alleviate PM-induced skin barrier disruption through regulation of apoptosis and proliferation in keratinocytes.

Acknowledgements

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Author's contributions

Ly TH Nguyen and Uy T Nguyen performed the experiments and analyzed the data; YH Kim coordinated technical support; HM Shin analyzed and interpreted the data; IJ Yang wrote the manuscript and discussed data. All authors participated in the preparation of the manuscript and approved the final manuscript.

Conflict of interests

All authors declare that they have no conflicts of interests.

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Fig. 1. HPLC profile of Astragali Radix (AR) extract. (A) Chemical structures of formononetin (FMT). HPLC fingerprint of FMT (B) and AR (C). (1) FMT. AR, Astragali Radix; FMT, formononetin

Fig. 2. (A) Effects of PM on proliferation and apoptosis of HaCaT cells. Cells were treated with PM (100 or 200 μg/ml) for 24 h. The expression of KRT 16, Ki67, cleaved caspase 3, and cleaved PARP was assessed by western blotting analysis. (B) Cytotoxic effects of AR and FMT in HaCaT cells. HaCaT cells were treated with AR and FMT for 24 h at the indicated concentration. Cell viability was assessed using a XTT kit. Data were expressed as the means ± SEM of three independent experiments.

Fig. 3. Effects of AR and FMT on PM stimulated HaCaT cells. Cells were pre-treated with AR and FMT for 1 h, then treated with PM for 24 h. (A) Effects of AR and FMT on proliferation and differentiation markers expression in HaCaT exposed to PM. The expression of KRT 16, KRT 17, involucrin, and loricrin was assessed by western blotting analysis. (B) Cell proliferation was assessed using a BrdU kit. Data were expressed as the means ± SEM of three independent experiments. * $p < 0.05$ vs. PM alone. (C) Effects of AR and FMT on apoptotic markers expression in HaCaT exposed to PM. The expression of p53, Bax, cleaved caspase 3, and cleaved PARP was assessed by western

blotting analysis. Data were expressed as the means ± SEM of three independent experiments. * $p < 0.05$ vs. PM alone.

Fig. 4. (A) Effects of AR and FMT on the MAPK pathway in HaCaT exposed to PM. Cells were pre-treated with AR and FMT for 1 h, then treated with PM for 30 min. The expression of p-p38, p-ERK, and p-JNK was assessed by western blotting analysis. (B) Role of ERK in the proliferation of PM stimulated HaCaT cells. U0126 was used as a positive control for ERK inhibition. Cells were pre-treated with AR and FMT for 1 h, then treated with PM for 24 h. Cell proliferation was assessed using a BrdU kit. Data were expressed as the means ± SEM of three independent experiments. * $p < 0.05$ vs. PM alone.

Fig. 5. Effects of AR and FMT on PM stimulated 3D human skin reconstructed model. The 3D human skin reconstructed model was either unstimulated or stimulated with PM and treated with AR and FMT for 6 consecutive days. (A) Effects of AR and FMT on proliferation markers expression in PM stimulated 3D human skin reconstructed model. The expression of KRT 16 and KRT 17 was assessed by western blotting analysis. (B) H&E staining and immunohistochemical analysis of the expression levels of Ki67 and cleaved caspase 3. Data were expressed as the means ± SEM of three independent experiments. * $p < 0.05$ vs. PM alone.

Fig. 6. Effects of AR and FMT on the production of pro-inflammatory chemokine/adhesion molecule production in PM stimulated 3D human skin reconstructed model (A) and HaCaT cells (B). 3D human skin reconstructed model and HaCaT cells were pre-treated with AR and FMT for 1 h, then treated with PM for 6 days and 1 day, respectively. ELISA was conducted to determine chemokine/adhesion molecule levels in culture supernatants. Data were expressed as the means ± SEM of three independent experiments. * $p < 0.05$ vs. PM alone.