A Thesis for the degree of Doctor of Philosophy

Potential mechanism for mitigating premature skin aging of sulforaphane using coculture system

Department of Veterinary Medicine

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List of Abbreviations

ACTH	adrenocorticotropic hormone
cAMP	cyclic adenosine monophosphate
CCN1	cysteine rich protein 61
COX	cyclooxygenase
EDN1	endothelin 1
ECM	extracellular matrix
IL	interleukin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
KGS	keratinocyte growth supplement
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B
MITF	microphthalmia-associated transcription factor
MMP-1	matrix metalloproteinase-1
α-MSH	α-melanocyte stimulating hormone
PGE2	prostaglandin E2
PGF2a	prostaglandin F2a
PM	particulate matter
RIPA	radio immune precipitation assay
ROS	reactive oxidative stress
RT-PCR	reverse transcription-polymerase chain reaction
SF-KGM	serum free-keratinocyte growth medium
SFN	sulforaphane
TNF	tumor necrosis factor
TRP-1	tyrosinase related protein 1
TYR	tyrosinase
UVB	ultraviolet ray B.
UVR	ultraviolet radiation

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General Introduction

1. Structure and function of human skin

The skin is the outermost layer covering the human body. It is an organ that provides protection against shocks, temperature changes, ultraviolet radiation (UVR), and particulate matter (Ichihashi et al., 2011). Human skin is a stratified epithelium. Each layer consists of different cell types with their own distinct functions. The skin can be broadly divided into the overlying epidermis, dermis, and underlying hypodermis (Fig. 1; Benítez and Montáns, 2017).

The epidermis is the outermost layer of the skin with a thickness of 0.2 mm on average. The epidermis can be further subdivided into four layers, beginning with the outermost layer; stratum corneum, granular cell layer, prickle cell layer and basal cell layer.

The outermost layer of skin, with an average thickness of 0.2 mm, is the epidermis. It can be further subdivided into four layers, beginning with the outermost: the stratum corneum, granular cell layer, prickle cell layer, and basal cell layer. Approximately 95% of the cells constituting the epidermis are keratinocytes. Keratinocytes divide and proliferate in the undermost layer, the basal cell layer, whereafter they mature and migrate to the surface. The epidermal turnover time is the time taken for the epidermis to replace itself. Starting from cell division in the basal cell layer, before proceeding to the division of daughter cells and, finally, to shedding from the epidermal surface, the turnover time is approximately 40−56 days (Iizuka, 1994; Koster, 2009). Other cell types exist within the epidermis: melanocytes, which are responsible for skin color, Langerhans cells, which have an immune function, and Merkel cells, which are mechanoreceptors (Ichihashi et al., 2011).

The dermis, with a thickness of 2.0−3.0 mm, is separated from the epidermis above by the basement membrane, and confers elasticity and strength to the skin.

The dermis primarily consists of fibroblasts within an extracellular matrix (ECM) made of structural proteins (mainly collagen and elastin). It also contains various immune cells, including macrophages and dermal dendritic cells (Ng and Lau, 2015). The main component of the ECM is collagen fiber (mainly type I−III), with other components including elastic fiber (formed from elastin) and proteoglycan (formed from hyaluronic acid and chondroitin sulfate, among others). Collagen accounts for 70% of the dry weight of the dermis and provides firmness to the skin. In contrast, elastin fiber, with its crosslinked structure, accounts for 1−2% and gives elasticity to the skin (Ichihashi et al., 2011). The dermis contains hair follicles, sweat and sebaceous glands, sensory nerve endings, lymphatic vessels, and blood capillaries that extend to the dermal side of the dermo-epidermal junction. The capillaries allow nutrient and oxygen delivery to, as well as waste removal from, the avascular epidermis via diffusion across the dermo-epidermal junction (Ng and Lau, 2015).

The hypodermis is the subcutaneous layer below the dermis; it largely consists of fat. It is the main source of structural support for the skin, and also insulates the body from cold and aids in shock absorption. It is interlaced with blood vessels and nerves (Lawton, 2019).

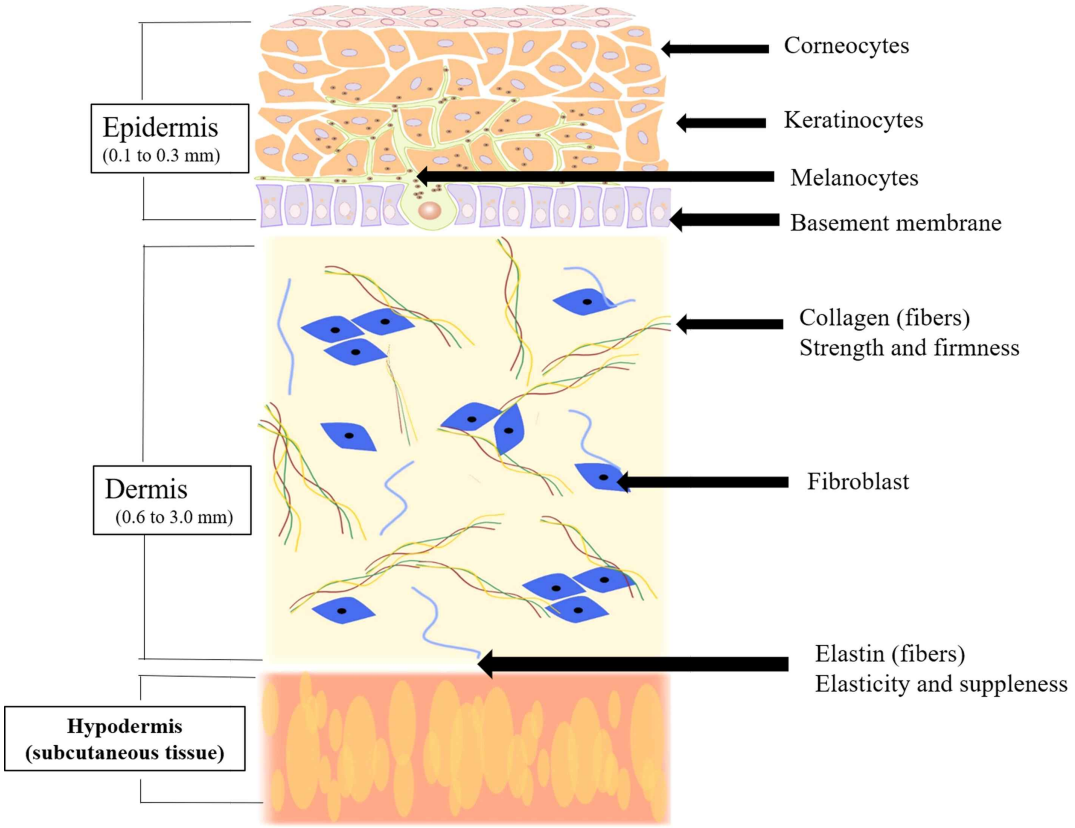

Fig 1. Schematic Illustration of the human skin structures. Human skin is broadly divied into the overlying epidermis, dermis and underlying hypodermis.

2. Environmental factors influencing skin aging

Aging is a process in which intrinsic and extrinsic factors progressively lead to a loss of structural integrity and physiological function (Friedman, 2005). The general aging process, which is genetically determined and occurs solely du to the passage of time, is known ad the the intrinsic skin aging process, whereas the process of skin aging induced by environmental factors is the extrinsic skin aging process (Vierkötter and Krutmann, 2012).

Prominent manifestations of the extrinsic skin aging process are coarse wrinkles, solar elastosis and pigment irregularities (Fig 2). On an individual basis, the rate of extrinsic skin aging depends on genetic make-up and patterns of exposure to different enviromeental exposure than others. Indeed, vast differences hane been observed between ethnic groups in the manifestation of extrinsic skin aging (Halder and Ara, 2003; Tschachler and Morizot, 2006).

Three important harmful environmental factors affecting skin aging include UVR, smoking and pollutants. In the late nineteenth century, the suggestion ahta chronic sun exposure damages the skin and leads topremature skin aging and skin cancer was sdvanced by two dermatologists (Hollander, 1986). Extrinsic skin aging mainly results from UVR, which is known as photoaging. Photoaging acts synergistically with chronological aging, and they share common clinical features including wrinkle formation, skin laxity, and skin fragility, which are caused by dermal matrix alterations (Fisher et al., 2002). The dermal matrix contains ECM proteins, such as collagen, elastin, and proteoglycans, which are confer strength and resilience to the skin. Skin aging associated with dermal matrix alterations and atrophy can be caused by the senescence of dermal cells such as fibroblasts, and by the decreased synthesis and accelerated breakdown of dermal collagen fibers (Murakami et al., 2012).

Tobacco smoking, as the leading cause of morbidity, is responsible for more

than 3 million deaths each year worldwide (Smith and Fenske, 1996). Like UVR exposure, smoking can lead to extrinsic skin aging. Results from large epidemiological studies imply a link between tobacco smoking and premature skin aging (Koh et al., 2002; Morita, 2007). Skin damage from long-term smoking can result in a “smoker’s face” where, due to damage to collagen fibers and elastin in the dermis, the facial skin appears grayish, and lines develop around the eyes and mouth (Morita et al., 2009).

Exposure to air pollution is associated with increasing morbidity and mortality worldwide. Airborne pollutants may enter the human body via multiple routes, including direct inhalation and ingestion, as well as from dermal contact, and they have well-documented acute and long-term effects on human health (Kampa and Castanas, 2008). As the largest organ of the human body, as well as the boundary between the environment and the organism, the skin, unsurprisingly, is one of the major targets of air pollutants. Air pollution has considerable effects on human skin, and all pollutants are believed to have their own toxicological impact. Recent reports on the impact of outdoor air pollutants on skin have focused on particulate matter and ozone (Juliano and Magrini, 2018). Exposure to airborne particulate matter from traffic and industry is associated with an increased risk of extrinsic skin aging, specifically manifesting as pigmented spots and wrinkles among Caucasian women of European ancestry (Vierkötter et al., 2010).

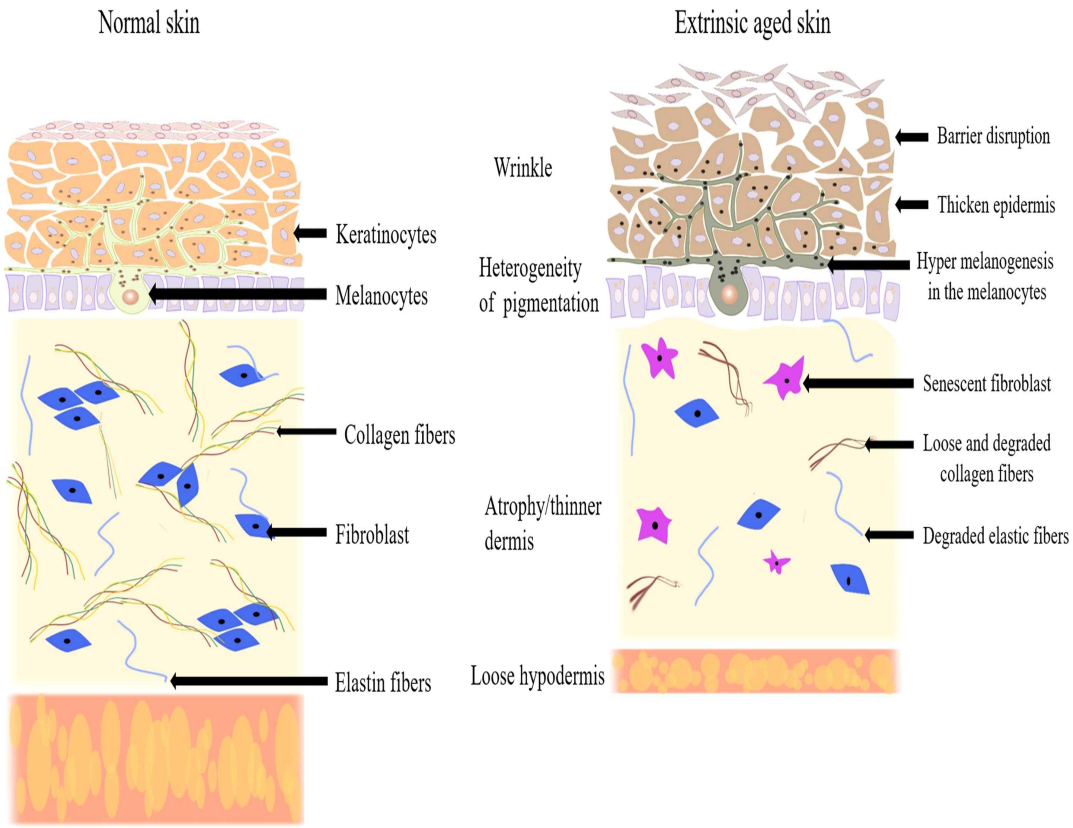

Fig 2. Schematic organization of the normal and extrinsic aged skin tissue. This illustration is a comparison of normal skin and extrinsically aged skin.

3. Paracrine signaling in extrinsic skin aging

3.1 Form of cell signaling

Cells typically communicate by releasing various types of chemicals that travel to target sites to elicit responses (Libretexts, 2020). The major forms of signaling in multicellular organisms are autocrine, juxtacrine, paracrine, and endocrine. In autocrine signaling, the cell produces ligands that bind to itself; i.e., the signaling cell and target cell can be the same or a similar cell (Libretexts, 2020). Juxtacrine signaling occurs when the signaling molecule is transferred across cell membranes via direct contact between neighboring cells (Libretexts, 2020). Paracrine signaling occurs between nearby cells; the signals elicit quick responses and, due to degradation of the paracrine ligands, last only for a short amount of time (Libretexts, 2020). Paracrine factors are diffusible proteins that act as ligands and are responsible for paracrine signaling (Gilbert, 2000). Endocrine signaling occurs between distant cells and is mediated by hormones released from specific endocrine cells that travel to target cells (Libretexts, 2020).

3.2 Keratinocyte-derived paracrine factors and their signaling

Keratinocytes, which constitute 90% of the cells in the epidermis, communicate with other types of skin cells, such as fibroblasts, melanocytes, and immune cells, via paracrine factors (Than et al., 2019). This communication is facilitated by increased expression of paracrine factors, which regulate multiple biological processes in skin tissue including cell proliferation, cell migration, anti-apoptosis, pigmentation transfer, and ECM remodeling (Than et al., 2019). Harmful environmental factors including UVR, smoking, and pollutants enhance paracrine factor secretion from keratinocytes (Farage et al., 2008).

When exposed to UVR, keratinocytes generate various factors that increase melanogenesis, including proopiomelanocortin-derived peptides such as α-melanocyte

stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), both of which are key melanogenic stimulators (Rousseau et al., 2007). Secreted α -MSH/ACTH binds to the melanocortin 1 receptor on melanocytes to activate the cyclic adenosine monophosphate (cAMP)-protein kinase A pathway, which in turn activates the cAMP response element-binding protein transcription factor (Buscà and Ballotti, 2000).

Endothelins (EDNs) are secreted mainly from keratinocytes and bind to EDN receptors on melanocytes to regulate melanogenesis (Imokawa et al., 1992). In response to extrinsic stress, the expression of EDNs is greatly increased; this activates various signaling cascades, such as the EDN1-EDN receptor type B pathway. This pathway plays a significant role in ultraviolet B (UVB)-induced pigmentation by modulating microphthalmia-inducing transcription factor (MITF) phosphorylation in normal human melanocytes (Zhang et al., 2013).

Prostaglandins (PGs) are lipid hormones produced from arachidonic acid by cyclooxygenase. They are mainly released from keratinocytes, with additional release occurring from epidermal melanocytes (Gledhill et al., 2010). PGE2 and PGF2a, the main keratinocyte-derived PGs, rapidly proliferate after UVR exposure and enhance pigmentation by promoting melanocyte dendricity and tyrosinase expression via the activation of G-protein-coupled receptors (EP1, EP3 and FP) on melanocytes (Fig 3; Scott et al., 2004). Other important keratinocyte-derived factors that positively regulate melanogenesis include basic fibroblast growth factor, hepatocyte growth factor, granulocyte-macrophage colony-stimulating factor, and leukemia inhibitory factor (Yuan and Jin, 2018).

Cytokines play a central role in the visible clinical signs of aging (Borg et al., 2013). These small secreted proteins can affect the communication and interaction between cells. In the skin, cytokines are produced by immune cells, such as Langerhans cells, as well as by other cells types, including keratinocytes and

epithelial cells (Feliciani et al., 1996). Cytokine activity is usually initiated by a cascade involving several cytokines acting simultaneously or sequentially, and is ended by other inhibitory cytokines or by their own receptors (Feliciani et al., 1996).

In the skin, the cytokine tumor necrosis factor alpha (TNF-α) is produced by fibroblasts, macrophages, monocytes, and keratinocytes (Bashir et al., 2009a). UVB radiation promotes TNF-α release from dermal fibroblasts and epidermal keratinocytes (Bashir et al., 2009b). TNF-α is proinflammatory; it inhibits collagen synthesis and induces matrix metalloproteinase (MMP)-9 production (Youn et al., 2011). High concentrations of TNF-α are correlated with decreased collagen production in fibroblasts via the induction of collagenase activity (Chou et al., 1996).

Another proinflammatory cytokine, interleukin (IL)-6, increases after menopause and is associated with the formation of skin wrinkles (Kim et al., 2012). When exposed to TNF-α, interferon-γ, or IL-4, keratinocytes increase IL-6 production (Li et al., 1996). IL-6 levels are further upregulated upon exposure to UVR (Omoigui, 2007) and fine particulate matter (PM2.5; Ryu et al., 2019). In aged dermal fibroblasts, IL-6 also increases the expression of MMPs, which decrease collagen production, further unbalancing collagen homeostasis (Fig. 3; Maggio et al., 2006).

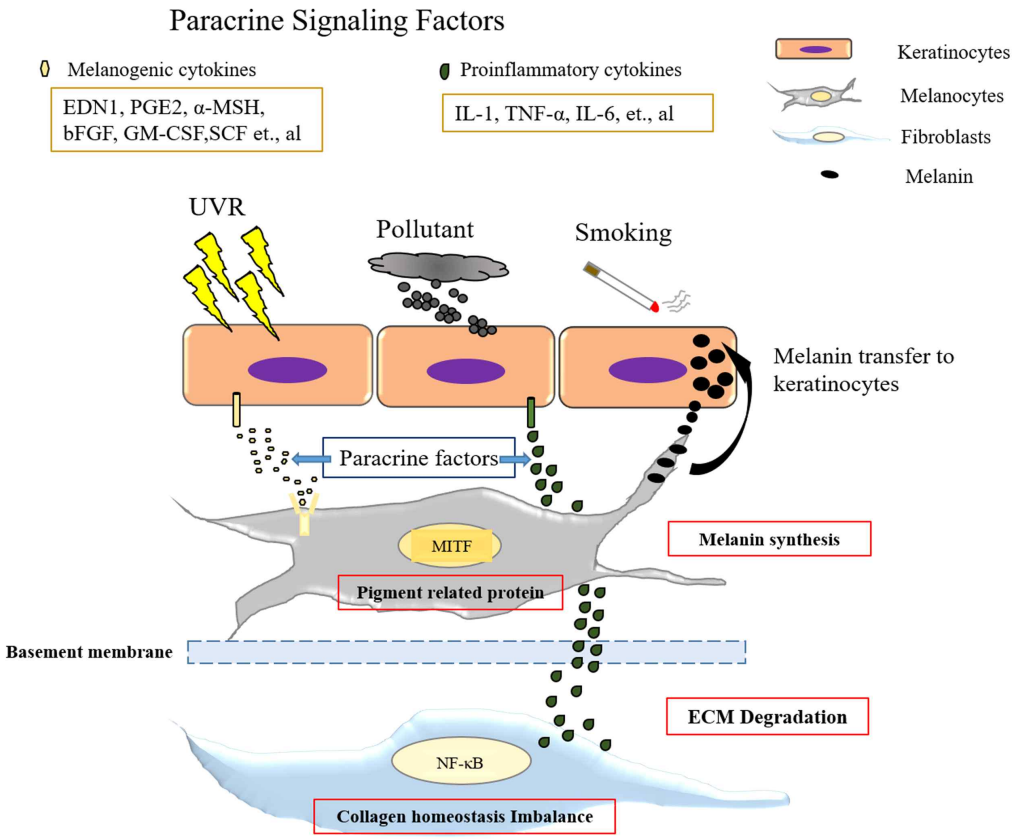

Fig. 3. Skin pigmentation and ECM degradation via keratinocyte paracrine release. Upon extrinsic stress, human epidermal keratinocytes generate various paracrine factors that increase melanogenesis, including α-MSH, EDN1, and PGE2, which are key melanogenic stimulators. Extrinsic stress also cause keratinocytes to increase th e production of IL-6 and TNF-α, which inhibits collagen synthesis and induces the production of MMPs in fibroblasts, which further increases ECM degradation. **Abbreviations**: EDN1: endothelin-1; PGE2: prostaglandin E2; α-MSH: α -melanocyte-stimulating hormone; bFGF: basic fibroblast growth factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; SCF: stem cell factor; IL: interleukin; TNF-α: tumor necrosis factor alpha; MITF: microphthalmia-associated transcription factor; ECM: extracellular matrix; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

4. Cell culture form for paracrine signaling testing

The growth of cells in controlled environments outside of an organism's body is referred to as cell culture (Teimouri et al., 2019). Cell cultures are available in monolayer and co-culture forms; those that grow in controlled, flat environments, such as a Petri dish, are monolayer cell cultures (Duval et al., 2017). For decades, monolayer cell cultures were the primary type of cell culture used (Duval et al., 2017). When assessing pharmaceutical compounds, it was traditional to first test them in vitro using monolayer cell cultures, before moving onto animal testing and clinical trials (Edmondson et al., 2014). However, the results obtained from in vitro monolayer cell culture studies are limited (Edmondson et al., 2014).

Co-cultures have long been used to study cell interactions, and are fundamental to cell-cell interaction studies (Goers et al., 2014). Co-cultures, which include at least two cell types to maintain the original tissue's spatiotemporal characteristics, are in vitro models that attempt to mimic in vivo anatomical conditions (Fig. 4; (De Simone et al., 2017)). Co-cultures are highly applicable for drug research because, compared to animal models, they can provide a quasi in vivo model of human tissue and allow for high-throughput testing and in-depth monitoring of a drug's effects on cell-cell interactions (Wu et al., 2010).

Depending on the state of cell adhesion, co-cultures are generally divided into two types: direct or indirect co-culture (Shimasaki et al., 2018). In direct co-culture, the cell types are mixed together; they are in direct contact with each other. Although easy to perform procedurally, the analytical results are difficult to interpret. In contrast, in indirect co-cultures, the cell types are placed in different environments, and intercellular interactions are meditated via humoral factors. Indirect co-culture systems are also divided into two types: conditioned medium transfer and filter separation (Fig. 4; Goers et al., 2014). Standard methods for filter separation of cells include using a Boyden chamber or a Transwell plate, which is a modified Boyden

chamber (Thomsen and Lade Nielsen, 2011). The Transwell plate is also known as a cell culture insert. Initially, the device was used to assess cell invasion and migration, but is now the standard method for many indirect co-culture procedures (Shimasaki et al., 2018).

The primary function of skin is to act as a barrier; it protects the internal organs from external insults while maintaining a stable internal physiology (Than et al., 2019). Skin development and function depend on numerous internal and external factors, and the effects of cell-tissue communication are critical (Than et al., 2019). In vitro co-culture models of skin cells, particularly of humans, allow biologically relevant cell-cell interactions to be established, leading to recapitulation of tissue microenvironments and better mimicry of the skin's physiological environment. For this reason, co-cultures have gained attention as tools for assessing skin cell interactions.

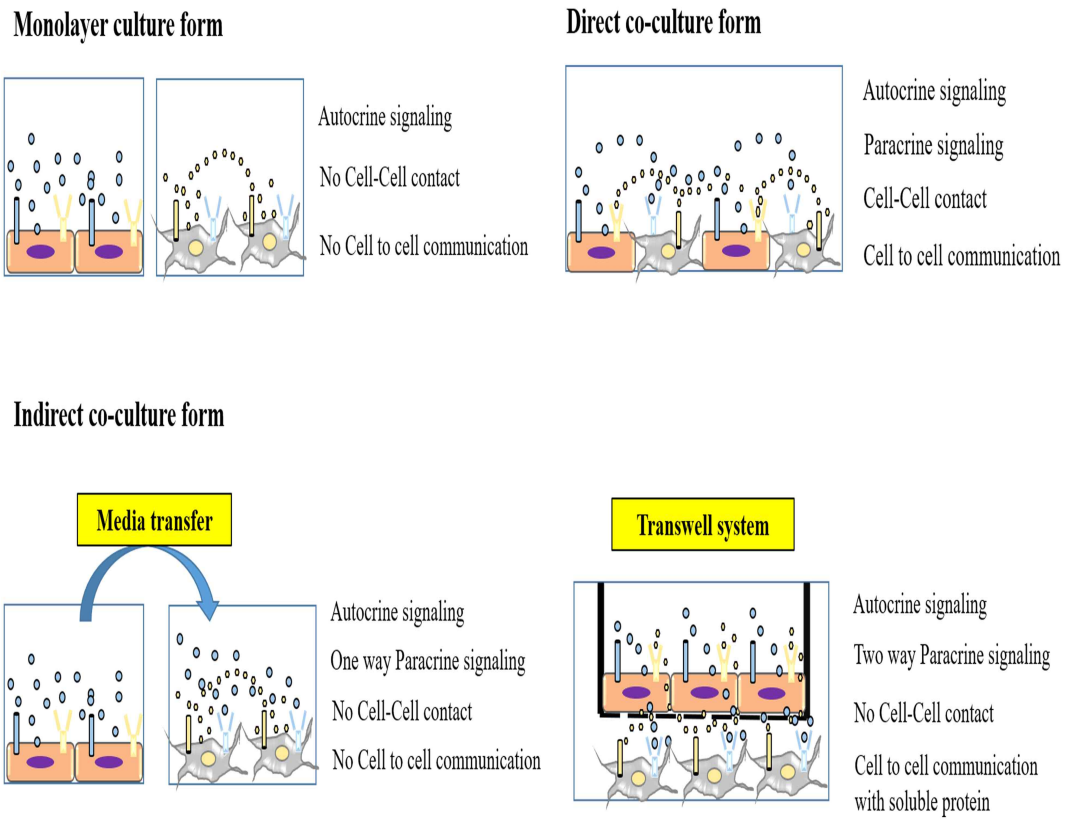

Fig 4. Different types of co-cultures. In direct co-culture, the cell types are mixed together, with the cells directly contacting each other. Indirect co-culture systems are subdivided into two types: conditioned medium transfer and filter separation, such as with a Transwell plate.

5. Anti-pollution skin care ingredient

"Anti-pollution" is one of the most widely used words in the personal care and cosmetics industry. The number of anti-pollution skin care products and cosmetics has significantly grown over the past few years, as air quality deteriorates worldwide and awareness and concerns regarding the adverse effects of environmental pollutants on the skin increase (Mistry, 2017). There are various signs of pollution-induced skin damage, including dryness, dark spots, fine lines and wrinkles, dull and uneven skin tone, loss of elasticity, inflammation, and exacerbation of acne (Juliano and Magrini, 2018). No consensus exists on which pollution biomarkers are best able to assess the efficacy of anti-pollution products. Many suppliers have developed compounds comprising a mixture of active ingredients with different mechanisms of action (Mistry, 2017). Few active ingredients have been demonstrated to alleviate pollution-induced skin damage. Recently tested active ingredients are summarized in Table 1. Studying the various active ingredients available, and their mechanisms of action, can help guide their selection.

Table 1. Recently tested active ingredient in anti-pollution skin care products

Mechanism	Ingredient	Target cells	Refer
Strengthening skin barrier	Astragali Radix extract	HKC RHE model	(Nguyen et al., 2019)
	Glycofullerenes	HKC	(Lee et al., 2020)
Anti-Oxidant	Niacinamide	HKC	(Zhen et al., 2019c)
	Fermented Fish Oil	HKC	(Hyun et al., 2019)
	Eckol	HKC	(Zhen et al., 2019a)
	DPHC	HKC Animal	(Zhen et al., 2019b)
Anti-inflammatory	Alginic acid	HKC / MMC	(Fernando et al., 2018)
	Resveratrol	HKC	(Shin et al., 2020)
	Glycofullerenes	HKC	(Lee et al., 2020)
	Afzelin	HKC	(Kim et al., 2019a)
	Eupafolin	HKC	(Lee et al., 2016)
	2'-Fucosyllactose	HKC	(Lee et al., 2019)
Prevent Degradation of Collagen/Elastin	DPHC	HDF	(Wang et al., 2020)
	Camellia japonica flower extract	HKC, Human living skin explants	(Kim et al., 2019b)
	Fucosterol	HKC / HDF	(Fernando et al., 2019)

Abbreviations: DPHC, Diphlorethohydroxycarmalol; HKC, Human keratinocytes; HDF, human dermal fibroblasts; MMC, Mouse macrophage; RHE, Reconstructed human epidermis;

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Potential mechanism for mitigating premature skin aging of sulforaphane using coculture system

Footnote) This study has been published by *Phytomedicine* (volume 77, October 2020, 153276)

1. Abstract

Skin aging, potentially caused by exposure to PM2.5, is characterized by wrinkling, abnormal pigmentation, and skin dryness triggered by several keratinocyte-derived paracrine factors. Sulforaphane (4-methylsulfinylbutyl isothiocyanate, SFN), commonly found in cruciferous vegetables, has diverse biological effects on skin tissue. In the present study, we have investigated whether SFN may alleviate PM2.5-induced premature skin aging. We used keratinocyte/melanocyte or keratinocyte/fibroblast coculture models of skin cells and measured the parameters of melanogenesis, collagen homeostasis and inflammation. SFN inhibited the development of reactive oxygen species in keratinocytes exposed to PM2.5. In keratinocyte/melanocyte cocultures, it significantly inhibited the upregulation of melanogenic paracrine mediators (including EDN1 and PGE2) in keratinocytes exposed to PM2.5; the synthesis of melanogenic proteins including MITF, TRP-1, and TYR; and the levels of melanin in melanocytes. SFN treatment of keratinocyte/fibroblast cocultures significantly reduced the PM2.5-induced expression of NF-κB-mediated cytokines including IL-1β, IL-6, TNF-α, and COX-2. In fibroblasts of the keratinocyte/fibroblast coculture system, the expression levels of phospho-NF-κB, CCN1, and MMP-1 were significantly decreased whereas procollagen type I synthesis was significantly increased. Collectively, our results suggest that SFN mitigates PM2.5-induced premature skin aging by suppressing melanogenesis and maintaining collagen homeostasis. It acts by regulating the release of paracrine factors from keratinocytes.

Keyword : Coculture system, Collagen homeostasis, Melanogenesis, Particulate matter 2.5, Premature skin aging, Sulforaphane

2. Introduction

Ambient particulate matter (PM) constitutes a major environmental threat and a serious public health issue. A recent epidemiological study suggested that PM negatively affects human skin and exacerbates preexisting skin disease (Li et al., 2015). It triggers skin disorders and pathologies. Skin may become xerotic or sensitive, and age prematurely and/or more rapidly (Hüls et al., 2016; Kim et al., 2013; Li et al., 2015; Vierkötter et al., 2010). Premature skin aging, which is the principal skin disorder of those exposed to PM, is characterized by coarse wrinkles, irregular pigment spots, and elastosis (Vierkötter et al., 2010; Yaar et al., 2002). The mechanisms by which PM has these damaging effects are thought to include oxidative stress and inflammation, both of which are important contributors to skin aging (Puri et al., 2017). PM may penetrate the dermis via hair follicles or sweat ducts, thus bypassing the stratum corneum (Heather, 2005). PM per se perturbs the skin barrier (Lee et al., 2016; Pan et al., 2015).

Recent strict regulations state that animals cannot be used to screen anti-pollution agents identified as medical cosmetics. There is an urgent need for a human skin model allowing the paracrine interactions between $PM_{2.5}$-exposed keratinocytes and other skin cells (melanocytes and fibroblasts) to be evaluated in detail. A recent study by Connell et al. used an integrated keratinocyte/fibroblast cell culture model to mimic viable skin (Connell et al., 1992). $PM_{2.5}$-exposed keratinocytes initiated molecular crosstalk (using diffusible signaling molecules such as pro-inflammatory cytokines and paracrine factors) with skin cells including melanocytes and fibroblasts (Borg et al., 2013; Ryu et al., 2019). However, the effects of these paracrine factors on melanocytes and fibroblasts were not studied.

Sulforaphane (SFN), an isothiocyanate, is widespread in cruciferous vegetables and has anti-cancer (Xu et al., 2006), antioxidant (Mahéo et al., 1997), detoxification (Mahéo et al., 1997), and anti-inflammatory (Shirasugi et al., 2010) effects. SFN

removes ROS by inducing the expression of detoxification enzymes (Zhu, 2008) and also inhibits UVB-induced inflammatory gene expression (Shibata et al., 2010). However, no effects of SFN on $PM_{2.5}$-induced premature skin aging have yet been reported. The aim of this study was to evaluate the effect of SFN in keratinocytes on $PM_{2.5}$-induced melanogenesis and alteration of collagen homeostasis using keratinocyte/melanocyte or keratinocyte/fibroblast coculture systems.

3. Material and methods

3.1. Chemical and reagents

SFN (L-sulforaphane, S6317) and $PM_{2.5}$ (diesel particulate matter, NIST 1650b) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A monoclonal antibody targeting phospho-nuclear factor kappa-light-chain-enhancer of activated B cells (pNF-κB) was purchased from Cell Signaling Technology (Beverly, MA, USA). Monoclonal antibodies targeting tyrosinase (TYR) and cysteine-rich protein 61 (CCN1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies targeting tyrosinase-related protein 1 (TRP-1) and microphthalmia-associated transcription factor (MITF), a prostaglandin E_2 (PGE_2) ELISA kit, Interleukin 6 (IL-6) ELISA kit and Tumor necrosis factor α (TNF-α) ELISA kit were obtained from Abcam (Cambridge, UK). The endothelin 1 (EDN1) ELISA kit and the human matrix metalloproteinase-1 (MMP-1) Quantikine ELISA kit were purchased from R&D Systems (Minneapolis, MN, USA). A procollagen type I C-peptide ELISA kit was purchased from Takara Bio (Shiga, Japan).

3.2. Coculture

Normal human melanocytes isolated from the foreskin of a moderately pigmented donor were obtained from Cascade Biologicals (Portland, OR, USA) and maintained in medium 254 (Cascade Biologicals) supplemented with HMGS-2 (Cascade Biologicals) and 1% (w/v) antibiotics (Gibco, Life Technologies Co., Grand Island, NY, USA) at 37°C in a humidified atmosphere of 95% air/5% CO_2 (v/v). The HaCaT human keratinocyte cell line from Amore Pacific Company (Gyeonggi-do, Republic of Korea) and human epidermal fibroblast cells from Cascade Biologicals were maintained in DMEM:F12 medium (3:1 v/v) supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) antibiotics (Gibco, Life Technologies Co).

The coculture system (Fig. 5) was a modification of that previously described

(Jung et al., 2013). Briefly, melanocytes and fibroblasts (both at 2.5 × 10^5 cells/well) were seeded into six-well plates and keratinocytes (5 × 10^5 cells/well) or vehicle were seeded into six-well cell culture inserts (Corning, USA); a membrane with pores 0.4 μm in diameter separated the upper and lower chambers. After 24 h, keratinocyte were incubated with different concentrations of PM2.5 (25, 50, 100, and 200 ppm) for 72h or keratinocytes in the upper chambers were pretreated with coculture medium containing $PM_{2.5}$ (50 ppm) for 24 h and then exposed to SFN (at 0, 1, 2.5, and 5 μM) in coculture medium for 72 h. Upon initiation of keratinocyte/melanocyte coculture, the medium in the upper and lower chamber was replaced with serum free-keratinocyte growth medium (SF-KGM) with keratinocyte growth supplement (KGS; Invitrogen). For keratinocyte/fibroblast coculture, standard growth medium was used.

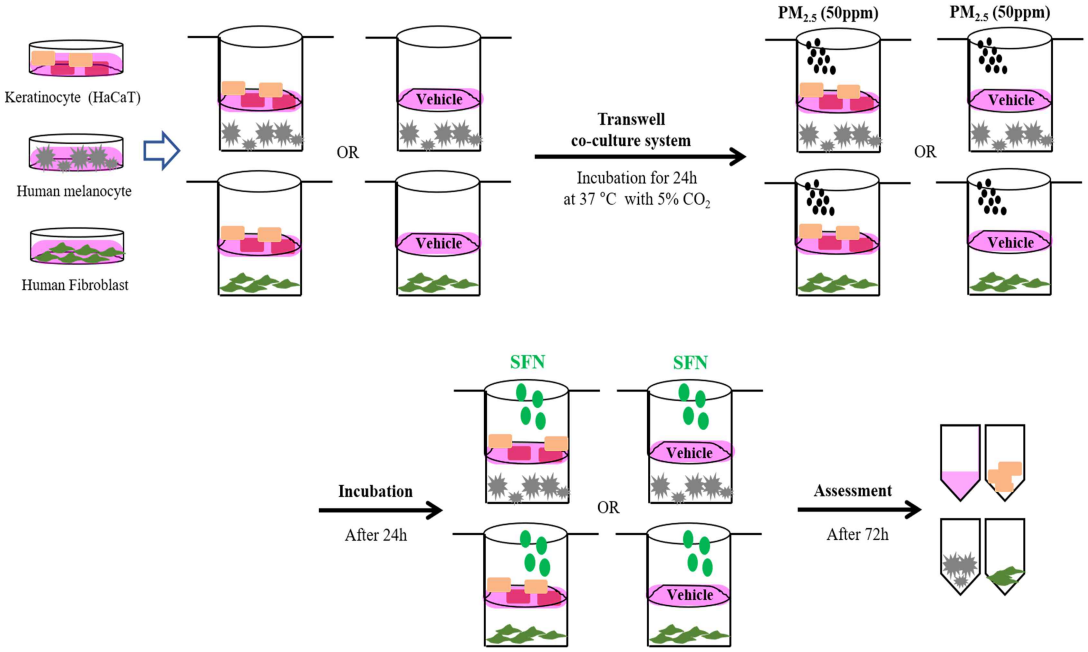

Fig 5. Coculture systems containing inserts of keratinocytes and melanocytes or fibroblasts. The coculture systems were incubated for 24 h at 37°C under 5% (v/v) CO_2. Keratinocytes or vehicle in the upper chamber were stimulated with $PM_{2.5}$ for 24 h. Stimulated keratinocytes or vehicle with or without sulforaphane (SFN) were cocultured with melanocytes or fibroblasts. The culture media and cells were harvested after the incubation periods.

Figure is excerpted from "***Phytomedicine*, volume 77, 2020, 153276**"

3.3. Cell viability and ROS levels

We used the MTT method (Choi et al., 2017) to explore whether $PM_{2.5}$ and SFN were detrimental to keratinocytes; we assessed ROS levels as described previously (Chaiprasongsuk et al., 2016). Briefly, cells were seeded into 96-well plates, cultured with SFN (0–40 μM) for 72 h or cultured with $PM_{2.5}$ (25, 50, 100, and 200 ppm) for 72 h, and then incubated with MTT solution (100 μg/well) for 4 h. The formazan precipitate was dissolved in DMSO. MTT reduction was quantified by measuring absorbance at 570 nm using a microplate reader (MULTISCAN GO; Thermo). We assayed intracellular ROS levels and the viability of keratinocytes treated with or without SFN (1, 2.5, and 5 μM) and $PM_{2.5}$ (50 ppm) for 72 h. To assay the ROS level, the cells were incubated in DPBS with 10 μM H_2DCFDA at 37°C for 30 min and 10,000 cells were analyzed per experimental condition by fluorescence-activated cell sorting (FACS Calibur; BD). The ROS level was normalized to the number of cells.

3.4. Assay of melanogenic paracrine mediators

The upper-chamber culture medium and lower-chamber culture medium of keratinocyte/melanocyte cocultures or The lower-chamber culture medium of vehicle chamber/melanocyte cocultures were collected and centrifuged at 13,000 rpm for 15 min to remove debris. Medium EDN1 and PGE_2 levels were measured via ELISA, following the kit manufacturers' instructions.

3.5. Melanin assay

Melanin production was evaluated as described previously (Panich et al., 2010). Lower-chamber melanocytes were harvested at 72 h after exposure to SFN and then the cell pellets were solubilized in 1 N NaOH for 1 h to dissolve melanin, which was measured spectrophotometrically at 475 nm. The melanin level was the absorbance/μg protein in each cell extract.

3.6. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously reported (Choi et al., 2017; Han et al., 2007), with slight modifications. Total RNA was prepared using a total RNA extraction kit (Qiagen, Hilden, Germany) and RT-PCR was performed using 200ng amounts of total RNA and the One-Step RT-PCR PreMix kit (Intron Biotechnology, Korea) employing appropriate sense and antisense primers. The RT-PCR conditions were those recommended by the manufacturer. The primers and annealing temperatures are listed in Table 2. PCR products were separated on 1.2% (w/v) agarose gels stained with Dyne STAR (Dyne Bio, Korea) and the bands were visualized and photographed using a UV transilluminator (Davinch-k, Korea). Relative mRNA levels were quantified using ImageJ software (NIH, Bethesda, MD, USA). The target PCR levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1. Sequences of primers the investigated genes in RT-PCR analysis.

Primer		Sequence	Annealing
COX-2	Sense	5'- ACC CCC GCC ATG GAG AGT GT-3'	60°C
	Antisense	5'-GAG GCA GGC AGG CAA GGT GA-3'	
GAPDH	Sense	5'- ATT GTT GCC ATC AAT GAC CC - 3'	52°C
	Antisense	5'- AGT AGA GGC AGG GAT GAT GT -3'	
IL-1β	Sense	5'- ACT GCA CGC TCC GGG ACT CA- 3'	57°C
	Antisense	5'- AAG GGC TGG GGA TTG GCC CT- 3'	
IL-6	Sense	5'- AGC GCC TTC GGT CCA GTT GC-3'	57°C
	Antisense	5'-GCT TCG TCA GCA GGC TGG CA-3'	
TNF-α	Sense	5'- ATG AGC ACT GAA AGC ATG ATC –3'	60°C
	Antisense	5'- TCA CAG GGC AAT GAT CCC AAA GTA GAC CTG CCC –3	

Abbreviations: COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, Interleukin; TNF, tumor necrosis factor.

Table is excerpted from "***Phytomedicine*, volume 77, 2020, 153276"**

3.7. Western blotting

Proteins were extracted as previously described (Chaiprasongsuk et al., 2016). Whole cells were incubated for 10 min at 4°C with radioimmune precipitation assay (RIPA) buffer containing 10% (v/v) NP40, 5 M NaCl, 1 M HEPES (pH 7.4), 0.5 M EDTA (pH 8.0), and a proteinase inhibitor cocktail. Proteins were quantified using the Bradford method (Bio-Rad, Germany). We used the Wes system (ProteinSimple, San Jose, CA, USA) to detect pNF-κB, MITF, TYR, TRP-1, CCN1, and β-actin, as described in the "Simple Western User Manual" (Chen et al., 2013). All electrophoresis and immunoblotting steps were performed using a fully automated capillary system. The levels of target proteins were normalized to those of β-actin.

3.8. Enzyme immunoassays for IL-6, TNF-α, MMP-1 and procollagen Type I

The lower-chamber culture media of keratinocyte/fibroblast cocultures and lower-chamber culture media of vehicle/fibroblast cocultures were collected. IL-6, TNF-α, human MMP-1 and procollagen type I levels assayed using a IL-6 ELISA kit (Abcam ,Cambridge, UK), TNF-α ELISA kit (Abcam, Cambridge, UK), human MMP-1 Quantikine Elisa kit (R&D Systems) and a procollagen type I C-peptide Elisa kit (Takara Bio) (Yatsushiro et al., 2011), according to the manufacturers' instructions. Absorbances at 450 nm were measured using a microplate reader (Thermo-Fisher, MA, USA) and normalized to the cell number.

3.9. Statistical analysis

All data are expressed as means ± standard deviations (n = 3). The results were analyzed using one-way analysis of variance followed by the Student–Newman–Keuls *post hoc* test for multiple comparisons. A p-value < 0.05 was considered statistically significant.

4. Results

4.1. Determination of $PM_{2.5}$ and SFN concentrations in keratinocytes

We investigated the optimal concentration of SFN prior to addition to coculture (Fig. 5). $PM_{2.5}$ induced significant death of keratinocytes in a dose-dependent manner (Fig. 6A). The level of melanin, the final product of melanogenesis, in cocultured melanocytes was significantly increased by $PM_{2.5}$ in a dose-dependent manner (Fig. 6B). SFN (Fig. 6C) did not induce significant keratinocyte cell death, SFN was nontoxic at a concentration of 5 μM (Fig. 6D). Therefore, we used $PM_{2.5}$ at 50 ppm and SFN at 0–5 μM in further experiments.

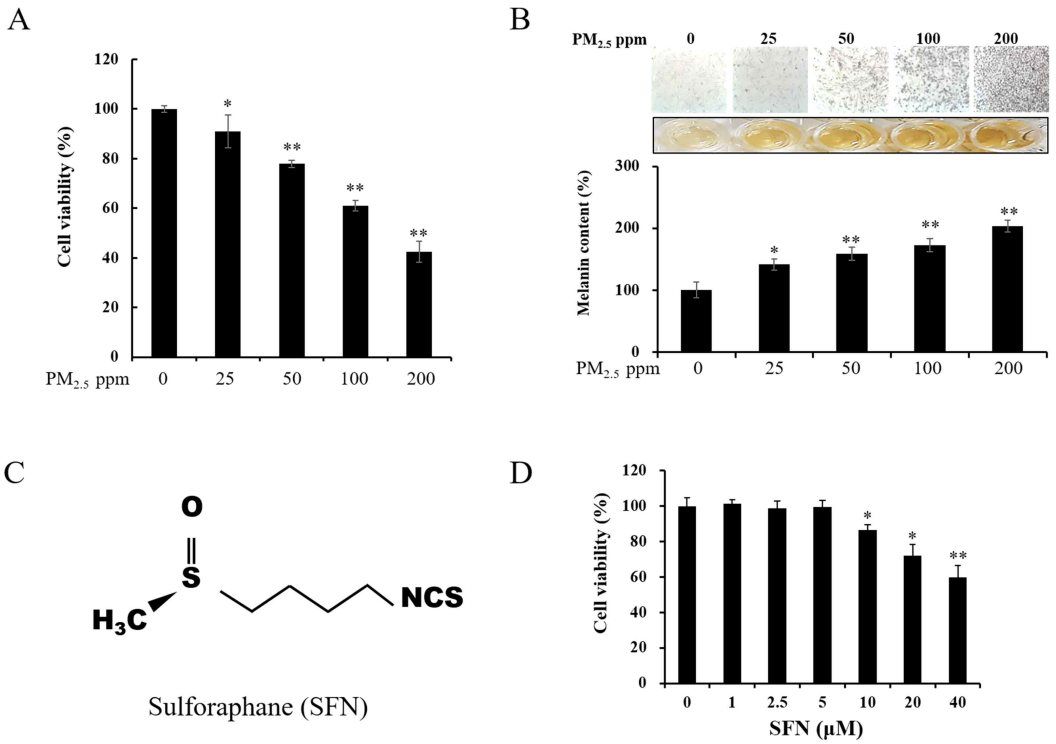

Fig. 6. Determination of PM2.5 and SFN concentration in keratinocytes. (A) Dose-dependent effect of PM2.5 in keratinocytes on the melanin content of melanocytes. Keratinocytes were treated with PM2.5 at the indicated concentrations for 72 h in coculture. (B) Dose-dependent effect of PM2.5 (0, 25, 50, 100, and 200 ppm) on the viability of monocultured keratinocytes by MTT assay. (C) Chemical structure of SFN. (D) Effect of SFN (0,1, 2.5, 5, 10, 20, and 40 μM) on the viability of monocultured keratinocytes by MTT assay. Keratinocytes were treated with or without SFN (1, 2.5, and 5 μM) and PM2.5 (50 ppm) for 72 h. Bars are means ± standard deviations (SDs) of data from three independent experiments (n = 3). #p < 0.01 vs. negative control; *p < 0.05, **p < 0.01 vs. PM2.5-treated sample.

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4.2. The antioxidant effect of SFN

The intracellular ROS level (as revealed by the DCF fluorescence intensity) was higher in $PM_{2.5}$-treated keratinocytes compared to the negative controls. However, keratinocytes treated with SFN (1, 2.5, or 5 μM) had a significantly reduced ROS level compared to $PM_{2.5}$-treated keratinocytes ($p < 0.05$, Fig. 7A). $PM_{2.5}$-induced cytotoxicity was reduced by 1, 2.5, or 5 μM SFN ($p < 0.05$, Fig. 7B).

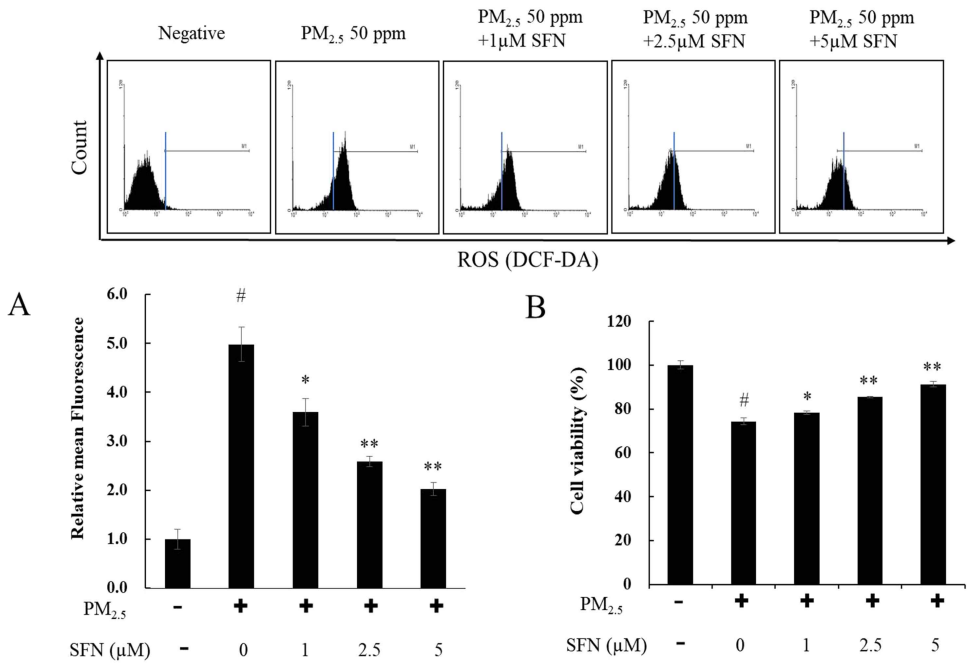

Fig. 7. The effects of SFN on oxidative stress following PM2.5 exposure in keratinocytes. (A) $PM_{2.5}$ increased the intracellular ROS level in monocultured keratinocytes by FACS. (B) Effect of SFN (1, 2.5 and 5 μM) on $PM_{2.5}$-induced cytotoxicity of monocultured keratinocytes by MTT assay. Keratinocytes were treated with or without SFN (1, 2.5, and 5 μM) and PM2.5 (50 ppm) for 72 h. Bars are means ± standard deviations (SDs) of data from three independent experiments (n = 3). #p < 0.01 vs. negative control; *p < 0.05, **p < 0.01 vs. PM2.5-treated sample.

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4.3. SFN inhibits melanogenesis in the keratinocyte/melanocyte coculture system

The expression levels of EDN1 (Fig. 8A) and PGE_2 (Fig. 8B) in keratinocyte culture medium significantly increased following keratinocyte exposure to $PM_{2.5}$ ($p < 0.01$ vs. negative controls). SFN significantly inhibited EDN1 and PGE_2 upregulation in a dose-dependent manner. The levels of EDN1 (Fig. 9A) and PGE_2 (Fig. 9B) in melanocyte culture medium with keratinocytes were significantly increased in the $PM_{2.5}$-treated groups compared to the negative controls (both $p < 0.01$ vs. negative controls). By contrast, the levels of EDN1 and PGE_2 were decreased in the SFN-treated group ($p < 0.05$ vs. $PM_{2.5}$-treated sample; Fig. 9A and 9B). However, the levels of EDN1 and PGE_2 in melanocyte culture medium without keratinocytes were not significantly increased in the $PM_{2.5}$-treated groups compared to the negative controls (Fig. 9A and 9B). Furthermore, it (1, 2.5, and 5 μM) inhibited melanin production by melanocytes after the keratinocytes of keratinocyte/melanocyte cocultures were exposed to $PM_{2.5}$ (Fig. 9C). The melanocytes of keratinocyte/melanocyte cocultures treated with SFN also expressed significantly lower levels (compared to the $PM_{2.5}$ treated sample) of melanogenic proteins including MITF, TYR, and TRP-1 (Fig. 9C). Thus, SFN inhibits melanin synthesis by blocking the release of $PM_{2.5}$-induced melanogenic paracrine mediators from keratinocytes (Fig. 12).

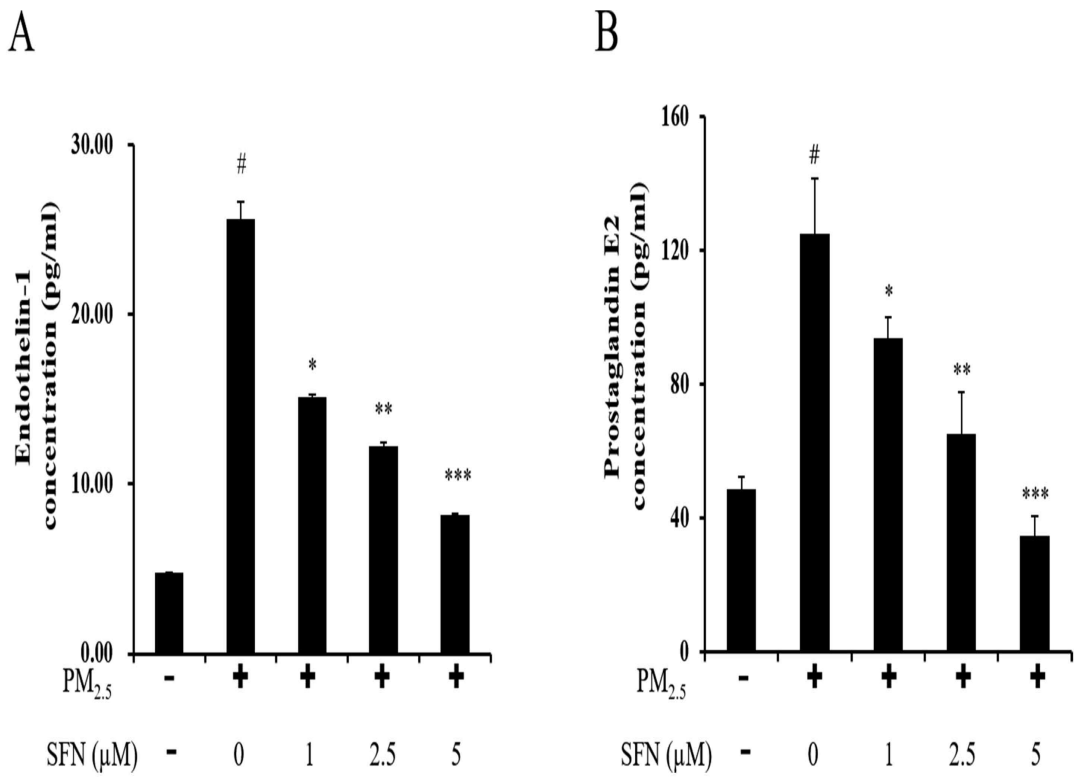

Fig. 8. Effect of SFN on the levels of melanogenic cytokines in culture medium by keratinocytes exposed to PM2.5 in keratinocyte/melanocyte coculture. (A) Level of EDN1 in keratinocyte medium after PM2.5 exposure. (B) Level of PGE2 in keratinocyte medium following PM2.5 exposure. Keratinocytes in the upper chamber were pre-incubated for 24 h with PM2.5 (50 ppm), and SFN was added (1, 2.5, and 5 μM) over the melanocytes. After 72 h, upper-chamber medium was collected for determination of melanogenic cytokine levels by ELISA. Bars are means ± standard deviation (SDs) of data from three independent experiments (n = 3). #$p < 0.01$ vs. negative control; *$p < 0.05$, **$p < 0.01$ vs. PM2.5-treated sample.

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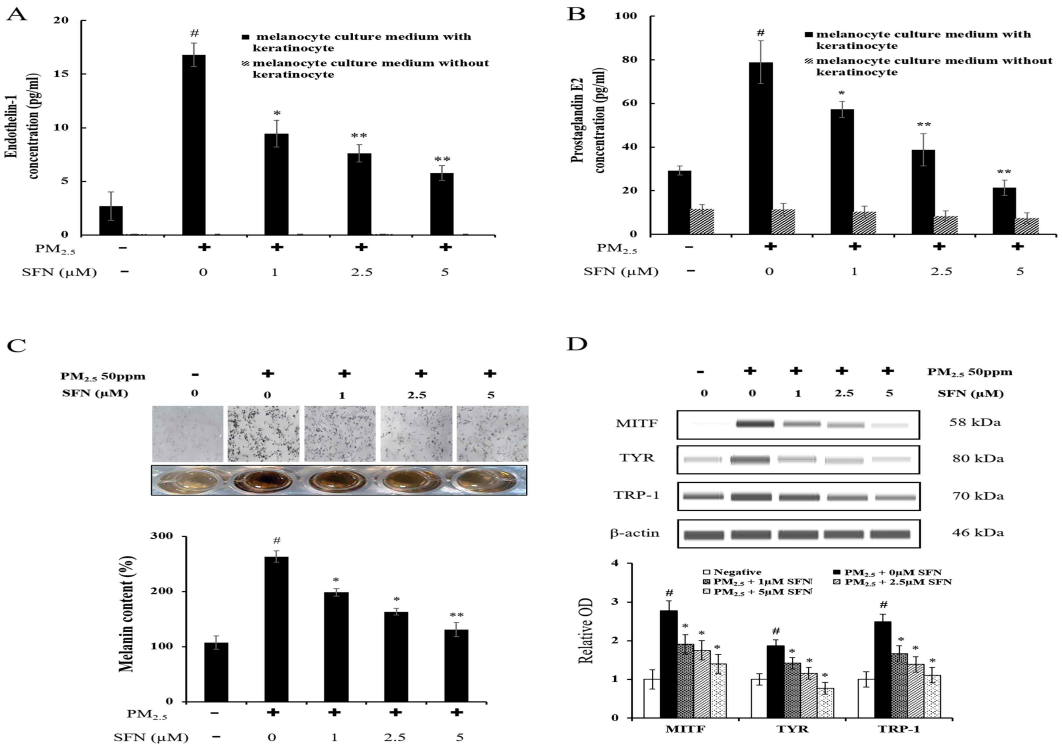

Fig. 9. Effect of SFN treatment of keratinocytes on melanogenesis by melanocytes cocultured with PM2.5-stimulated keratinocytes. (A) Level of EDN1 in medium of melanocytes with or without keratinocytes following indirect PM2.5 exposure. (B) Level of PGE2 in medium of melanocytes with or without keratinocytes following indirect PM2.5 exposure. (C) Effect of SFN on the melanin level in melanocyte coculture. (D) Effect of SFN on the levels of MITF (~ 58 kDa), TYR (~ 80 kDa), and TRP-1 (~ 70 kDa) in melanocyte coculture using the WES system. Results are normalized to β-actin. Keratinocytes or without keratinocytes in the upper chamber were preincubated for 24 h with PM2.5 (50 ppm), and SFN was added (1, 2.5, and 5 μM). After 72 h, lower-chamber melanocytes and medium were collected. Bars are means ± standard deviations (SDs) of data from three independent experiments (n = 3). #p < 0.01 vs. negative control; *p < 0.05, **p < 0.01 vs. PM2.5-treated sample.

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4.4. SFN suppresses keratinocyte inflammationin the keratinocyte/fibroblast coculture system

$PM_{2.5}$ upregulated keratinocyte pNF-κB-p65 levels, but these levels decreased in the presence of SFN (Fig. 10A). The levels of mRNAs encoding cyclo-oxygenase (COX)-2 and pro-inflammatory cytokines including IL-1β, IL-6, and TNF α substantially increased after $PM_{2.5}$ exposure but significantly decreased in the presence of SFN (Fig. 10B).

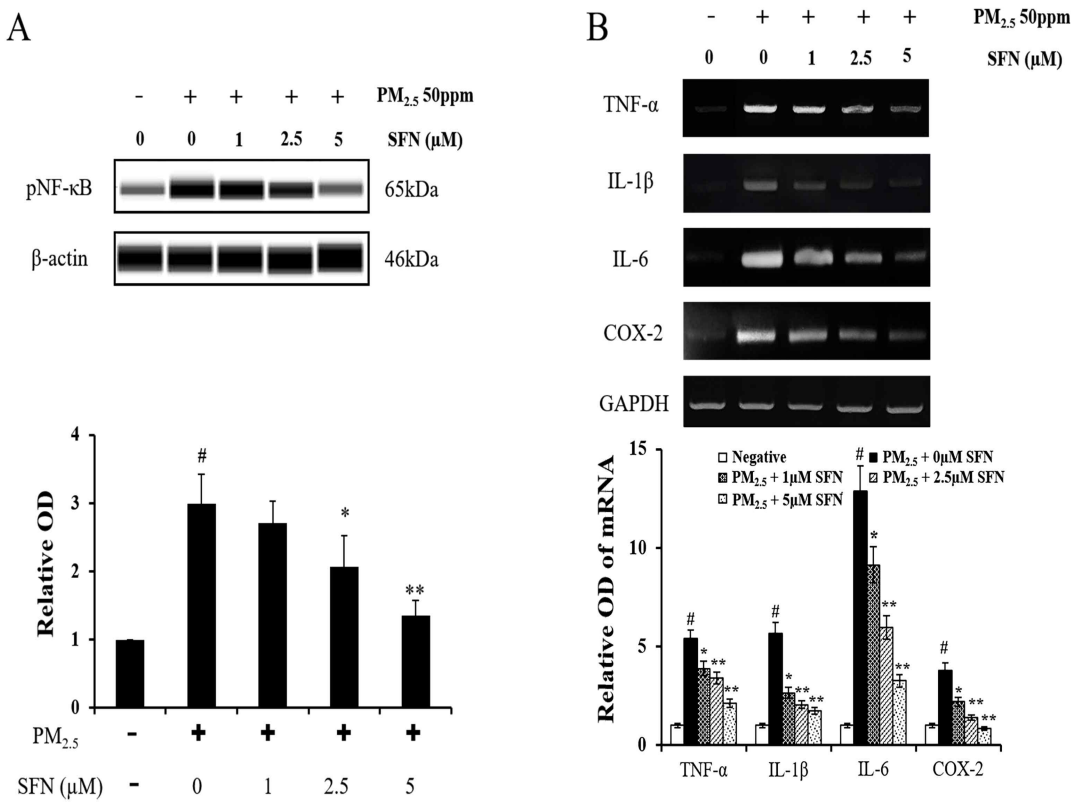

Fig. 10. Effect of SFN on the levels of PM2.5-induced paracrine mediators in keratinocytes in coculture with fibroblasts. (A) Western blot analyses of NF-κB (~ 65 kDa) activation. Results are normalized to β-actin. (B) RT-PCR analysis of the expression levels of COX-2 and proinflammatory cytokines (IL-1β, IL-6, and TNF-α). Results are normalized to GAPDH. Keratinocytes in the upper chamber were preincubated for 24 h with PM2.5 (50 ppm), and SFN was added (1, 2.5, and 5 μ M) above the fibroblasts. After 72 h, upper-chamber keratinocytes were collected. Bars are means ± standard deviation (SD) of data from three independent experiments (n = 3). #p < 0.01 vs. negative control; *p < 0.05, **p < 0.01 vs. PM2.5-treated sample.

Figure is excerpted from "***Phytomedicine*, volume 77, 2020, 153276**"

4.5. SFN maintains fibroblast collagen homeostasis in the keratinocyte/fibroblast coculture system

The levels of TNF-α and IL-6 in fibroblast culture medium with keratinocytes were significantly increased in the $PM_{2.5}$-treated groups compared to the negative controls (both $p < 0.01$ vs. negative controls). By contrast, the levels of TNF-α and IL-6 were decreased in the SFN-treated group ($p < 0.05$ vs. $PM_{2.5}$-treated sample; Fig. 11A and 11B). However, the levels of TNF-α and IL-6 in fibroblast culture medium without keratinocytes were not significantly increased in the $PM_{2.5}$-treated groups compared to the negative controls (Fig. 11A and 11B). Exposure of keratinocytes to $PM_{2.5}$ increased the pNF-κB and CCN1 expression levels in fibroblasts of the lower chamber (both $p < 0.01$ vs. negative controls), but these increases were significantly inhibited by SFN ($p < 0.05$ vs. $PM_{2.5}$ treated sample; Fig. 11C and D). The MMP-1 levels markedly increased in $PM_{2.5}$ treated sample compared to negative controls ($p < 0.01$, Fig. 11E). However, $PM_{2.5}$-induced MMP-1 secretion was significantly decreased by SFN ($p < 0.05$ vs. the figure for the $PM_{2.5}$ treated keratinocyte/fibroblast coculture, Fig. 11E). In addition, the level of secreted procollagen type I from fibroblast of $PM_{2.5}$-treated keratinocytes/fibroblast co culture was significantly reduced in the absence of SFN compared to negative controls ($p < 0.01$, Fig. 11F). SFN eliminated downregulation of procollagen type I secretion ($p < 0.05$ vs. that of the $PM_{2.5}$ treated keratinocyte/fibroblast coculture, Fig. 11F). Thus, SFN maintains fibroblast collagen homeostasis by inhibiting inflammatory cytokine expression by $PM_{2.5}$-exposed keratinocytes (Fig. 12).

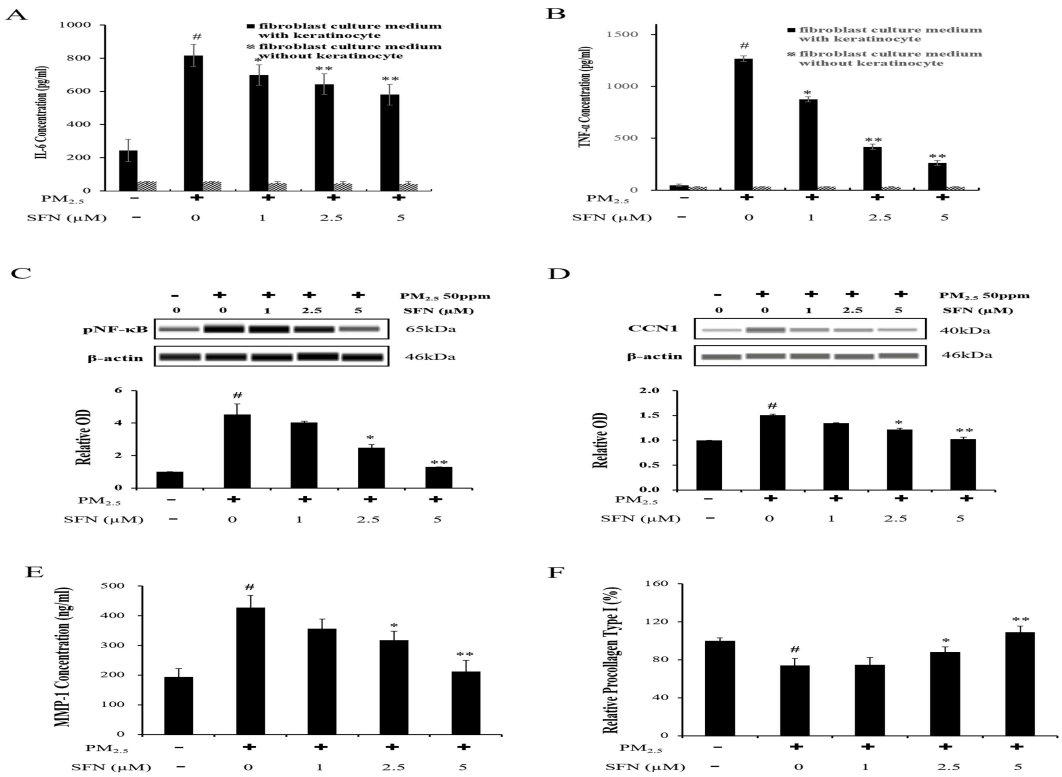

Fig. 11. Effect of SFN treatment of keratinocytes on collagen homeostasis in coculture of fibroblasts and PM2.5-stimulated keratinocytes. (A) IL-6 level in culture medium of fibroblasts with or without keratinocytes following indirect PM2.5 exposure. (B) TNF-α level in culture medium of fibroblasts with or without keratinocytes following indirect PM2.5 exposure. (C) Western blot analyses of NF-κB activation in fibroblasts. Results are normalized to β-actin. (D) Western blot analyses of CCN1 expression in fibroblasts. Results are normalized to β-actin. (E) MMP-1 protein level in fibroblast culture medium. (F) Type I procollagen protein level in fibroblast culture medium. Fibroblasts with or without keratinocytes in the upper chamber were preincubated for 24 h with PM2.5 (50 ppm), and SFN was added (1, 2.5, and 5 μM) above the fibroblasts. After 72 h, lower-chamber medium and cells were collected for IL-6, TNF-α, NF-κB, CCN1, MMP-1, and type I procollagen assay. Bars are means ± standard deviation (SD) of data from three independent experiments (n = 3). #p < 0.01 vs. negative control; *p < 0.05, **p < 0.01 vs. PM2.5-treated sample. Figure is excerpted from "***Phytomedicine*, volume 77, 2020, 153276**"

5. Discussion

This study is the first to show that SFN mitigates both melanocyte melanogenesis and the collagen homeostasis imbalance of fibroblasts cocultured with $PM_{2.5}$-exposed keratinocytes; SFN inhibited secretion of paracrine mediators and inflammatory cytokines by the keratinocytes of keratinocyte/melanocyte or keratinocyte/fibroblast coculture systems (a schematic is shown in Fig. 12).

Keratinocytes are the key source of mitochondrial ROS that play important roles in skin aging. Both UV (Kammeyer and Luiten, 2015) and PM (Lee et al., 2016) strongly induce ROS synthesis in the keratinocytes of mouse skin and keratinocyte monocultures (Lee et al., 2016; Ryu et al., 2019). High ROS levels trigger the release of intrinsic cytokines including IL-1β and IL-6 by human and mouse keratinocytes; the toll-like receptor 5-NADPH oxidase 4-ROS signaling pathway is in play (Ryu et al., 2019). In addition, ROS stimulate MMP synthesis, triggering collagen degradation (Quan et al., 2012) and affect the extracellular matrix of skin tissue (Kammeyer and Luiten, 2015). We found that the antioxidant SFN significantly suppressed intracellular ROS accumulation induced by $PM_{2.5}$. We thus postulate that SFN protects keratinocytes exposed to $PM_{2.5}$ by reducing ROS levels.

$PM_{2.5}$-exposed keratinocytes initiate molecular crosstalk with melanocytes via diffusible signaling molecules such as paracrine factors (Borg et al., 2013; Ryu et al., 2019). We found that $PM_{2.5}$-stimulated keratinocytes secrete soluble melanogenic factors, as evidenced by the increased production of EDN1 and PGE_2 (melanogenic paracrine mediators) in $PM_{2.5}$-exposed keratinocytes and in melanocyte culture medium. By contrast, the production of EDN1 and PGE_2 was not increased in melanocytes cultured without keratinocytes indirectly exposed to $PM_{2.5}$. We found that SFN inhibited EDN1 and PGE_2 release from keratinocytes stimulated by $PM_{2.5}$, and that the levels of melanogenesis regulators including MITF, TYR, and TRP-1 were upregulated in melanocytes cocultured with $PM_{2.5}$-stimulated keratinocytes. SFN

treatment of keratinocytes decreased melanogenesis by melanocytes, consistent with previous work on the soluble intrinsic cytokines involved in UVB-induced pigmentation (Jung et al., 2013). As $PM_{2.5}$ is insoluble, and thus cannot penetrate membranes, $PM_{2.5}$ had no influence on the melanocytes of our membrane-separated coculture model with inserts, indicating that $PM_{2.5}$ has no direct effect on melanocytes. We suggest that EDN1 and PGE_2 expressed by $PM_{2.5}$-exposed keratinocytes play key roles in the paracrine stimulation of melanocytes. We postulate that SFN acts as an anti-melanogenic agent by inhibiting EDN1 and PGE_2 production.

Connell et al. (Connell et al., 1992) used an integrated cell culture model of keratinocytes and fibroblasts to mimic the viable skin layer. In aged skin, collagen homeostasis becomes unbalanced via the reduction of procollagen biosynthesis and increased MMP-1-initiated collagen degradation (Quan et al., 2012). CCN1, a member of the CCN family, negatively regulates collagen homeostasis in primary human skin dermal fibroblasts (Quan et al., 2012). Fibroblasts, the major collagen-producing cell type in human skin, respond to elevated CCN1 levels by downregulating type I collagen production and upregulating MMP-1 synthesis; MMP-1 initiates collagen degradation (Quan et al., 2006; Quan et al., 2012). TNF-α, a 17 kDa inflammatory cytokine, is expressed by various skin cells during both intrinsic and extrinsic aging. TNF-α downregulates collagen expression and upregulates MMP expression, triggering extracellular matrix deposition (Brenner et al., 1989). IL-6 produced by irradiated keratinocytes may play a role in MMP activation in dermal fibroblasts (Leiros et al., 2017). In a previous study, SFN itself had no effect on MMP-1 or -3 in human dermal fibroblasts (Lee and Moon, 2012). Our results suggest that because SFN reduced TNF-α and IL-6 secretion by keratinocytes, the levels of TNF-α and IL-6 in fibroblast culture medium decreased. As a result, fibroblast procollagen type I expression was significantly upregulated, decreasing CCN1 expression and MMP-1 secretion. Therefore, we postulated that SFN maintains fibroblast collagen homeostasis

by inhibiting inflammatory cytokine expression in keratinocytes.

Fernando et al (Fernando et al., 2019) showed that inhibition of PM-induced, NF-κB-mediated cytokine expression by fucosterol was reflected in the MMP1 expression levels of fibroblasts cultured In medium conditioned by PM-/fucosterol-treated keratinocytes; our results are consistent with those findings. We suggest that PM2.5-exposed, keratinocyte-derived, NF-κB-mediated cytokine expression plays a key role in the paracrine stimulation of fibroblasts. The anti-inflammatory effects of agents such as SFN on PM2.5-stimulated keratinocytes restore the balance of fibroblast collagen homeostasis.

Overall, we postulate that PM2.5 induces hyperpigmentation and wrinkle formation in a paracrine manner in a skin model featuring inserts; these effects were reversed by SFN, which exhibited anti-inflammatory and radical-scavenging activity. From both the dermatological and cosmetic viewpoints, the development of anti-pollution agents is very important (Vierkötter et al., 2010). SFN may be a valuable anti-pollution cosmetic preserving skin health by preventing premature skin aging attributable to PM2.5.

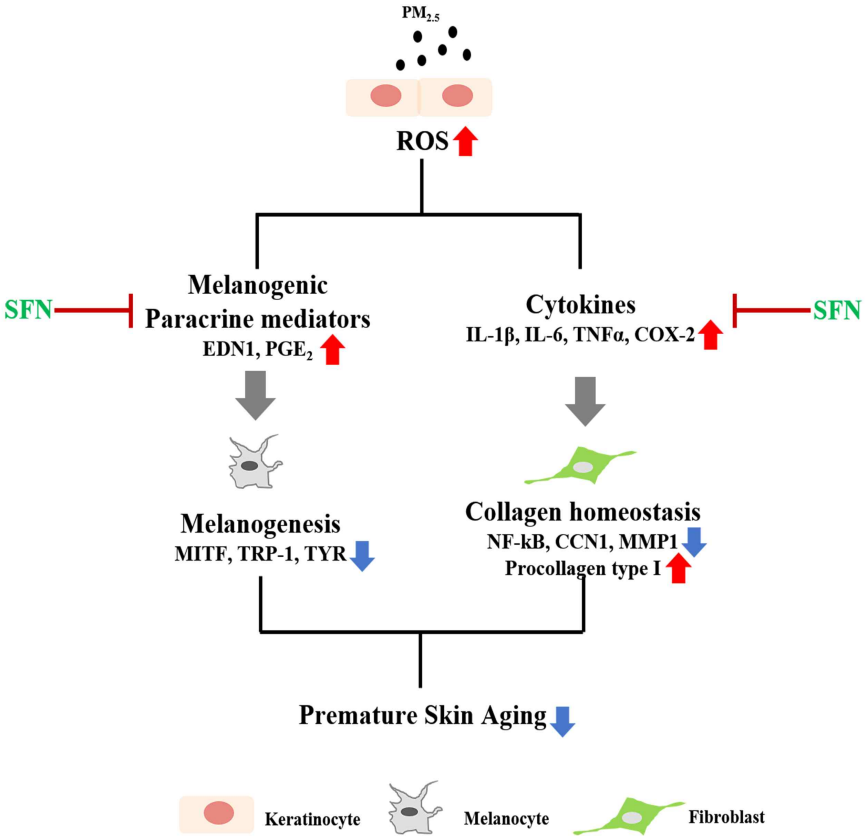

Fig. 12. Schematic illustration of the anti-premature skin aging effects of SFN in keratinocyte/melanocyte or keratinocyte/fibroblast coculture systems. After PM2.5 exposure, keratinocytes produced high levels of ROS, paracrine mediators, and inflammatory cytokines. In the keratinocyte/melanocyte coculture system, SFN inhibited hyperpigmentation by decreasing the production of melanogenic paracrine mediators in keratinocytes and by suppressing melanogenesis in melanocytes. Fibroblast collagen homeostasis was maintained by SFN, which inhibited the release of cytokines by PM2.5-exposed keratinocytes of the keratinocyte/fibroblast coculture system

Figure is excerpted from "***Phytomedicine*, volume 77, 2020, 153276**"

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Abstract in Korean

초미세먼지에 의한 외인적 피부노화모델에서 설포라판의 항노화 기전에 관한 연구

(지도 교수 : 신 태 균)

고 현 주

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대기오염, 그 중에서도 미세먼지 (Particulate matter, PM)는 건강에 악영향을 주는 것으로 잘 알려져 있다. 특히 피부는 미세먼지 (PM)에 가장 쉽게, 가장 직접적으로 노출되며 이후 주름 형성, 비정상적인 색소 침착, 피부 건조를 특징으로 하는 피부 노화가 진행된다.

미세먼지에 의한 노화징후 개선 활성 물질을 찾는 대부분의 연구는 피부의 표피층의 90% 이루고 있는 각질형성세포를 중심으로 연구되어 왔다. 그러나 미세먼지에 의한 피부 노화는 각질형성세포가 주위에 있는 멜라닌 형성세포, 섬유아세포, 면역세포에 신호 전달을 해 피부 노화의 임상징후를 나타낸다. 또한 십자화과 채소에서 흔히 발견되는 설포라판 (4- 메틸 설피 닐 부틸 이소 티오 시아 네이트, SFN)은 여러 연구를 통해 피부 조직에 다양한 생물학적 작용을 갖는 것으로 알려졌다.

이에 본 연구에서는 피부세포 공 배양 방법으로 초미세먼지($PM_{2.5}$)에 의한 피부노화 모델을 확립하여 설포라판이 초미세먼지 ($PM_{2.5}$)에 의해 유도된 노화징후를 완화할 수 있는지 평가하고자 하였다. 이를 위한 기전 연구로 초미세먼지에 의해 각질형성세포에서 생성되는 파라크린 신호전달 물질을 분석하였다.

분석 결과, 각질 세포 / 멜라닌형성세포 공배양 시스템에서 각질형성세포에만 초미세먼지를 노출 시켰을 때 활성산소종 (ROS)의 생성이 유의하게 증가하였으나 설포라판에 의해 유의하게 감소 되었다. 또한 초미세먼지에 의해 각질형성세포에서 멜라닌 형성을 촉진하는 Endothelin 1 (EDN1) 과 Prostaglandin E_2 (PGE_2) 의 생성이 증가하여, 멜라닌 형성 세포에서 멜라닌 형성에 관여하는 단백질들 예를 들어 microphthalmia-associated transcription factor (MITF), tyrosinase-related protein 1(TRP-1) 와 tyrosinase (TYR) 와 같은 단백질의 발현 증가를 통해 멜라닌 형성이 증가했다. 설포라판은 각질형성세포에서 EDN1 과 PGE_2 의 생성을 유의하게 감소시켜 멜라닌형성세포에서 멜라닌 형성을 유의하게 억제함을 확인하였다..

다음으로 각질 세포 / 섬유아세포 공배양 시스템에서 설포라판의 처리는 각질형성세포에서 초미세먼지에 의해 유도된 nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) 매개성 염증인자인 interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) 와 cyclo-oxygenase-2 (COX-2)의 분비를 유의하게 감소시켰다. 이에 따라 섬유아세포의 phospho-NF-κB, cysteine-rich protein 61(CCN1), matrix metalloproteinase-1 (MMP-1)의 발현 수준은 유의하게 감소했으며, procollagen type I 합성은 유의하게 증가했음을 확인하였다.

본 연구에서 설포라판이 초미세먼지에 의해 유도된 멜라닌 형성과 세포 외 기질분해 작용을 억제하였는데 이는 각질형성세포에서 분비하는 파라크린 신호전달 인자인 EDN1, PGE2, Cytokines의 분비 억제를 통해 이루어졌음을 확인하였다. 이러한 결과는 설포라판을 표피에 바르는 것만으로도 미세먼지에 의한 피부 세포의 노화 징후가 개선될 수 있음을 시사한다. 이 결과를 바탕으로 설포라판은 초미세먼지에 의한 노화를 예방하는 활성물질로 이용될 수 있을 것이다.

주요어 : 멜라닌 형성, 설포라판, 세포외 기질 분해, 외인성 피부노화, 초미세먼지, 피부세포 공배양 시스템

감사의 글

박사학위 논문을 마무리하면서 생각해보면, 워킹 맘, 대학원 수업 및 논문 진행에 힘든 점이 많았지만 많은 분들의 격려와 도움 덕분에 졸업을 앞두게 되었습니다. 저를 아껴주시고 도움을 주신 모든 분들께 진심으로 감사의 마음을 전합니다.

몇 번의 박사학위 진학의 기회가 있었지만 여러 가지 이유로 포기하고 직장생활과 육아에 지쳐갈 즈음 대학원에 진학할 수 있도록 기회를 주신 신태균 교수님께 감사드립니다. 교수님의 지도로 다시 한번 학문의 즐거움을 깨달았고, 직장생활 및 삶을 살아가는 방식의 변화를 주셨습니다. 앞으로도 교수님과의 인연을 소중하게 간직하고 살아가도록 하겠습니다.

대학원 진학과 수업을 받을 수 있도록 배려해주시고 응원해주신 잇츠한불 C&I 연구소 이근수 소장님, 피부과학연구팀 김진화 팀장님, 오정영 책임, 권순우 책임, 배수정 선임, 양다운 연구원에게도 깊은 감사드립니다.

힘든시기에 소중한 시간을 내어 논문심사를 위해 먼 길을 달려와 주신 김승준 교수님, 문창종 교수님께 깊이 감사드립니다.

언제나 논문 작성에 함께 힘써주고 조언을 아끼지 않은 안미정 교수님, 김정태 교수님께도 감사드립니다. 그리고 학위 과정 동안 희노애락을 함께한 창남, 유나, 전지윤 선생님에게 고마운 마음을 전합니다.

제가 박사학위를 받는 걸 누구보다 기다리셨던 하늘에 계신 아버지께 감사를 올립니다. 항상 저를 아껴주시고 응원해주시는 어머니, 시어머님과 시아버지께도 감사를 드리며, 건강하시길 기원합니다. 누나의 부탁으로 언제나 그림을 열심히 그려준 내 동생 현석, 현애에게도 감사함을 전합니다. 끝으로 오늘이 있기까지 가장 고생하고 물심양면으로 도와준 남편 태현, 바쁜 엄마 때문에 힘들지만 그래도 밝게 자라고 있는 아들 선우, 시우, 딸 시연이에게

고마움을 전하며 앞으로의 삶에 충실할 것을 다짐하며 모두에게 감사의 말을 다시 한번 남깁니다.